Oxidized Forms of Olive Oil Secoiridoids: Semisynthesis, Identification and Correlation with Quality Parameters[#]

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ABSTRACT

Secoiridoids is the prominent chemical class of olive oil polar constituents and are characterized by significant biological properties. They are abundant in different chemical forms and relatively high concentrations compared to other components, while prone to oxidation due to their chemical motif. In recent years, oxidized derivatives of secoiridoids have been reported, either as natural constituents of olive oil or as components which are gradually formed in all stages of its production and storage. The mono-oxidized forms of oleocanthal and oleacein named as the respective acids have been recently isolated from olive oil and unambiguously structurally characterized. Other oxidized forms of elenolic acid or more complex secoiridoids, such as those of oleuropein and ligstroside aglycones are also sporadically mentioned in the literature. No further information is provided since they have not been isolated in pure form in order to be accurately identified. Most of the time, they are generally referred as oxidized forms of the parent compounds and commonly identified based on mass spectrometric data. In the current study, the semi-synthesis of the main oxidized olive oil secoiridoids, i.e., oleocanthalic acid, oleaceinic acid, EDA acid, carboxylic form of elenolic acid, carboxylic form of ligstroside aglycon, and carboxylic form of oleuropein aglycon is described starting from the corresponding aldehydic derivatives, using SeO_2/H_2O_2 as oxidative agents. Furthermore, their presence in a number of Greek olive oils was investigated as well, as possible correlation thereof with quality parameters.

[#]   Dedicated to Professor Dr. A. Douglas Kinghorn on the occasion of his 75th birthday.

Introduction

It is highly acknowledged that olive oil (OO), the main product of olive (*Olea europaea* L., Oleaceae) and the key ingredient of Mediterranean diet, is characterized by substantial nutritional and health beneficial value [1]. Its significance is attributed to the high content of monounsaturated and polyunsaturated fatty acids (MUFA and PUFA, respectively) [2], as well as minor polar constituents with strong antioxidant profile, which are abusively termed as polyphenols [3,4]. The sum of these compounds in OO is also referred as Total Polyphenols Fraction (TPF) and is composed of phenolic alcohols, phenolic acids, secoiridoids, lignans, flavonoids and hydroxy-isochromans [5,6]. The secoiridoids together with the two phenyl alcohols tyrosol and hydroxytyrosol constitute approximately the 90 % of the TPF of OO [5].

OO polyphenols have a broad spectrum of health beneficial properties related to positive impact on platelet and cellular function and bone formation as well as protective effect against cell oxidative damage, microbial infections and inflammation [1,3, 4]. Based on this profile, and after assessment, the European Food Safety Agency (EFSA) issued a scientific opinion in 2012. More specifically, they announced a specific health claim related to protection of blood lipids from oxidative damage quoting specific polyphenols and certain concentration levels in OO (Regulation No 432/2012 of EC) [4,7,8]. EFSA announcement triggered more in-depth study of polyphenols regarding their biological properties but also their chemical features as OO components. Thus, many analytical methods have been reported, aiming toward the determination of OO polyphenols qualitatively and/or quantitatively [8–12]. Along these lines, the International Olive Council (IOC) proposed an HPLC-UV-based method [13] for the determination of OO polyphenols following the structural requirements as described in EFSA's opinion and satisfying the health claim. In this IOC-proposed method, 27 single compounds are determined in total, including simple phenolic acids, phenyl alcohols, flavonoids, and secoiridoids. Amongst secoiridoids, several compounds are listed and described generally as oxidized and/or hydroxylic forms i.e., decarboxymethyl oleuropein aglycone, oxidized dialdehyde form; decarboxymethyl ligstroside aglycone, oxidized dialdehyde form; oleuropein aglycone, oxidized aldehyde and hydroxylic form; ligstroside aglycone, oxidized aldehyde, and hydroxylic form. However, their exact structures are not given and remain unclear [13].

There are only a few studies investigating the oxidized secoiridoids of OO and propose structural patterns based only on LC-MS or LC-HRMS/MS data without reference standards for verification purposes [9–12]. This is because they are minor constituents of OO, and their isolation is challenging. For instance, hydroxylated forms of elenolic acid and oleuropein aglycone are frequently mentioned in the literature as minor components of OO and olive oil byproducts, without further information [13–17]. Most probably, these compounds correspond to the carboxylic forms of elenolic acid and monoaldehydic form of oleuropein aglycone, resulting from the oxidation of the aldehydic group in position 1 as shown in ▸ **Fig. 1**.

Apart from the vague description of the oxidized secoiridoids in literature, another issue further complicates their identification

– the presence of several similar structures and isomers. Based on the literature, the oxidized form of ligstroside aglycone is a constitutional isomer of oleuropein aglycone, and further information is needed to elucidate its structure [10]. Indeed, in a recent study by Farre M et al., a compound with pseudomolecular ion [M – H]⁻ identical to oleuropein aglycone at *m/z* 377.1244 was referred, showing though a different MS/MS fragmentation pattern and therefore listed as unknown [18]. Nevertheless, based on the proposed fragmentation pattern, it could possibly correspond to the oxidized form of ligstroside aglycone.

Based on our knowledge, the only oxidized OO secoiridoid compounds that have been reported to date, with their structures unambiguously identified by NMR, are oleocanthalic acid (**1**), oleaceinic acid (**2**), and EDA acid (**3**). These compounds are the oxidized forms of oleocanthal (**4**), oleacein (**5**), and dialdehydic form of decarboxymethyl elenolic acid (EDA) (**6**), and have been isolated and fully characterized in a previous work by our team [19]. These oxidized derivatives are minor constituents of OO, and as recent studies indicate, their levels seems to increase through processing from olive fruits to the storage of the final product [20–22].

Thus, the aim of the present work is the semi-synthesis of the main oxidized components of OO (▸ **Fig. 1**), including oleocanthalic acid (**1**), oleaceinic acid (**2**), EDA acid (**3**), carboxylic form of ligstroside aglycon or CFLA (**7**), carboxylic form of oleuropein aglycon or CFOA (**8**) and carboxylic form of elenolic acid (**9**) starting from the corresponding aldehydic derivatives, **4, 5, 6, 10, 11, 12**, using a simple oxidation protocol. Following their purification and identification, we proceeded with the investigation of their presence in a representative number of OO samples. Finally, the possible correlations between oxidized secoiridoids and regulated quality parameters of extra virgin OO were explored.

Results and Discussion

Secoiridoids constitute a prominent chemical class of OO polyphenols and as already mentioned they can be also found in oxidized forms. However, the lack of reference standards and unavailability of information regarding their accurate structure complicates their investigation. Thus, their semi-synthesis could assist significantly towards this direction. The target compounds of the current study are the oxidized analogues of the major secoiridoids of OO and specifically of oleocanthal (**4**), oleacein (**5**), EDA (**6**), monoaldehydic form of ligstroside aglycon or MFLA (**10**), monoaldehydic form of oleuropein aglycon or MFOA (**11**), and elenolic acid (**12**). Moreover, an orthogonal separation methodology was followed for their isolation and purification combining CPC and preparative HPLC techniques as described in previous works by our team [23,24]. The application of these techniques allows the isolation of the main secoiridoid compounds of OO in large quantity and high purity. Despite the efficacy of the methodology, the two diastereomers in position 8 of monoaldehydic form of secoiridoid aglycones **10** and **11** were obtained as an inseparable but well characterized mixture. Regarding elenolic acid (**12**), its major isomer 5S, 8S, 9S was isolated in high purity.

Based on the literature, many oxidation protocols have been applied to transform aldehydes to carboxylic acids, using organo-
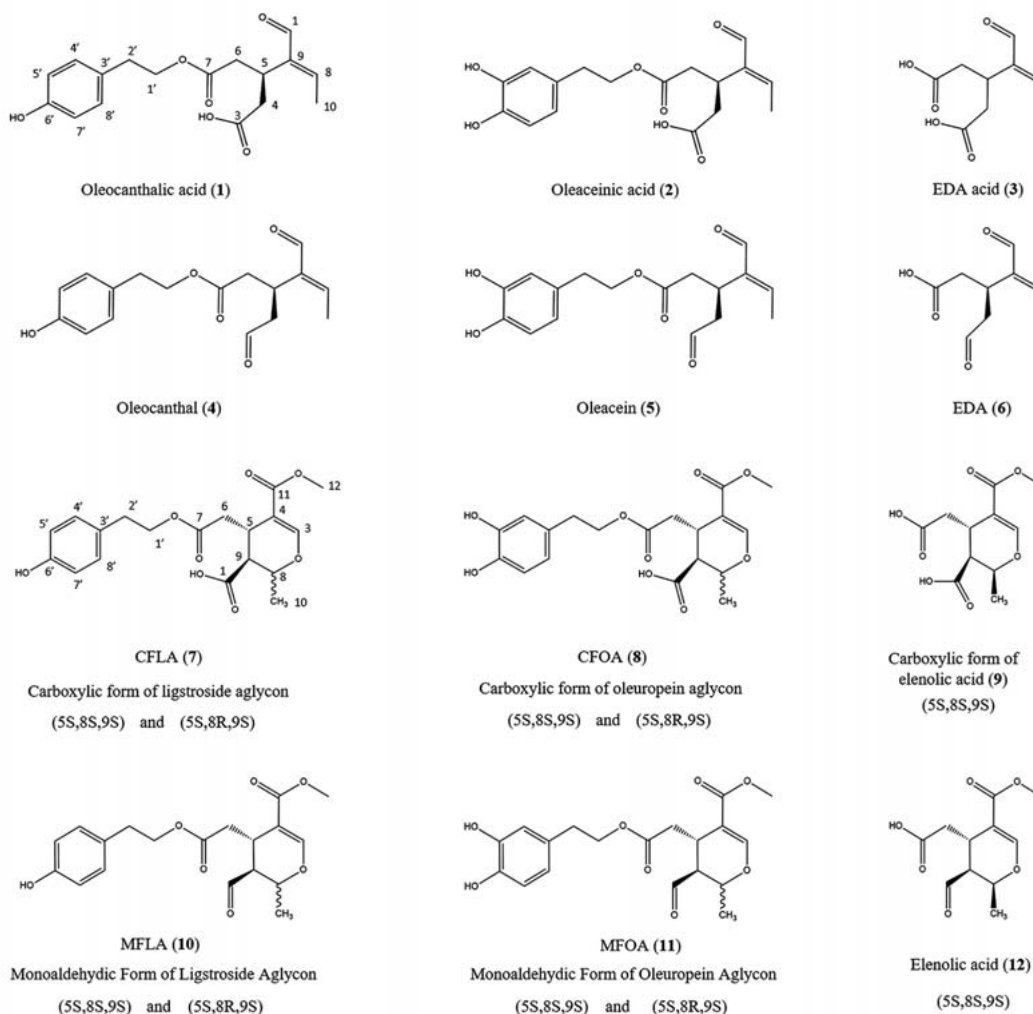
► **Fig. 1** Structures of the main Olive Oil Secoiridoid Compounds (**4**, **5**, **6**, **10**, **11**, and **12**) and corresponding oxidized forms (**1**, **2**, **3**, **7**, **8**, and **9**).

catalyzed aerobic oxidation [25], metal-free direct oxidation using TCCA [26], or pyridinium chlorochromate catalyzed oxidation [27]. To achieve a fast and efficient method, SeO_2/H_2O_2 were used as oxidative reagents [28, 29]. It is worth noting that the re-action proceeds rapidly in one step, without prior protection of chemical groups. The overall yield of this approach was deter-mined between 64 and 71% (► **Table 1**). As shown in ► **Fig. 2**, the key element of this procedure was the regioselective oxidation of the aldehyde in position 3 at the open skeleton **4**, **5**, **6** without affecting the aldehyde in position 1 and the oxidation of aldehyde in position 1 of the closed forms **10**, **11**, **12**. For open forms (► **Fig. 2a**), the transformation of aldehyde to the corresponding carboxylic acid could be easily detected in 1D NMR spectra by the absence of one of the two aldehydic protons (≈ 9.5–9.6 ppm) and carbons (≈ 200 ppm) as well by the HMBC NMR correlations of protons H-4a, H-4b and H-5 to the carboxylic carbon in C-3 (≈ 175 ppm). Similarly, for closed forms (► **Fig. 2b**), the aldehydic proton (9.5–9.6 ppm) and carbon (200 ppm) have disappeared and the allocation of the carboxylic group at C-1 is determined

by the J^2 correlation of H-9 with the carboxylic carbon at ≈ 175 ppm in the corresponding HMBC spectrum. The closed forms **8** and **9** have been obtained as a mixture of two diaster-eomers in position 8. NMR and HRMS spectra of oxidized com-pounds **1**, **2**, **3**, **7**, **8** and **9** are available in Supporting Information (**Fig. 1S–30S**).

The next step of our study was to use the semi-synthetic oxi-dized secoiridoids as reference standards and investigate their presence in different TPF samples. In the context of our continu-ing work in analysis of OO, a UPLC-ESI-HRMS and HRMS/MS methodology was applied in numerous TPF from our in-house library [11, 30].

In general terms, oleocanthalic acid (**1**), EDA acid (**3**), CFLA (**7**) and CFOA (**8**), were identified in a high number of the analyzed samples, while oleaceinic acid (**2**) and carboxylic form of elenolic acid (**9**) were less abundant. In ► **Fig. 3**, a characteristic HRMS full-scan spectrum obtained from the analysis of a representative TPF is presented.

▶ **Table 1** Overall yield of synthesis of open and closed forms of oxidized secoiridoid compounds.

Synthesized Compound	Starting Material	Yield of Reaction
Oleocanthalic acid	Oleocanthal (100 mg, 0.41 mmol)	64 % (68.67 mg, 0.21 mmol)
Oleaceinic acid	Oleacein (100 mg, 0.41 mmol)	67 % (69.81 mg, 0.21 mmol)
Eda acid	Eda (100 mg, 0.54 mmol)	67 % (72.38 mg, 0.36 mmol)
CFLA	MFLA (100 mg, 0.28 mmol)	71 % (75.17 mg, 0.2 mmol)
CFOA	MFOA (100 mg, 0.26 mmol)	65 % (66.61 mg, 0.17 mmol)
Carboxylic form of elenolic acid	Elenolic acid (100 mg, 0.41 mmol)	66 % (70.89 mg, 0.27 mmol)
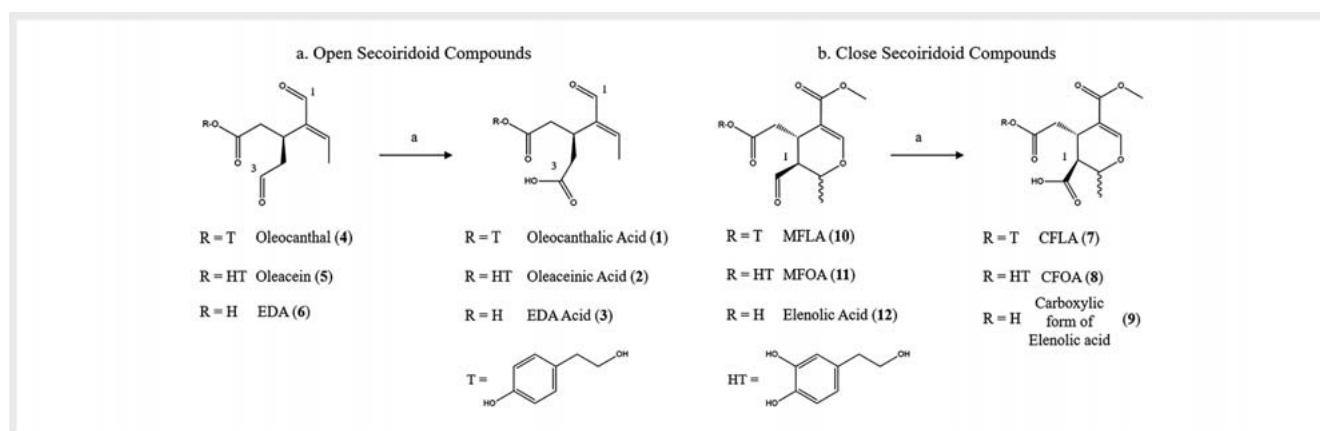
▶ **Fig. 2** Synthesis of Open (**a**) and Closed (**b**) Forms of Oxidized Secoiridoid Compounds. **a** SeO_2/H_2O_2, THF reflux, 2 h.

In more detail, an ion was detected at m/z 199.0613 with proposed EC of $C_9H_{11}O_5$ and RDBeq. value of 4.5, in negative ion mode. In HRMS/MS spectrum, two major fragment ions were detected at m/z 155.0713 and 181.0505 indicating the presence of EDA acid (**3**). Similarly, an ion at m/z 257.0669 was also detected (EC: $C_{11}H_{13}O_7$, RDBeq.: 5.5) accompanied by a characteristic HRMS/MS fragment ion at m/z 137.0606 corresponding to carboxylic form of elenolic acid (**9**). Following the same approach, oleocanthalic acid (**1**) ([M − H]$^-$ at m/z 319.1189; EC: $C_{17}H_{19}O_6$; RDBeq.: 8.5 and HRMS/MS major fragment ions: 181.0507 and 199.0612); oleaceinic acid (**2**) ([M − H]$^-$ at m/z 335.1136; EC: $C_{17}H_{19}O_7$; RDBeq.: 8.5 and HRMS/MS major fragment ion: 199.0612; CFLA (**7**) ([M − H]$^-$ at m/z 377.1239; EC: $C_{19}H_{21}O_8$; RDBeq.: 9.5 and HRMS/MS major fragment ions: 333.1342 and 301.1081); CFOA (**8**) ([M − H]$^-$ at m/z 393.1186; EC: $C_{19}H_{21}O_9$; RDBeq. 317.1032 and 349.1295). Special attention should be given to the identification of CFLA (**7**) and MFOA (**11**). Being isomers, only the difference in MS/MS fragmentation pattern could lead to accurate determination. The pseudomolecular ion of oleuropein aglucone is detected at m/z 377.1246 with proposed EC of $C_{19}H_{21}O_8$ and RDBeq. of 9.5, in negative ion mode and HRMS/MS major fragment ions at m/z 307.0824, 275.0926, and 345.0979. The same motif is observed in the pair of compounds oleocanthalic acid (**1**) and oleacein (**5**). The pseudomolecular ion of **5** is detected at m/z 319.1121 with proposed EC of $C_{17}H_{19}O_6$ and RDBeq. of 8.5, in negative ion mode and HRMS/MS major fragment ions at m/z 195.0664, 301.1078, and 165.0560. Spectrometric data for the abovementioned compounds are given in Supporting Information (**Fig. 31S–32S**).

The acquisition of chromatographic and spectrometric data of the synthesized oxidized secoiridoid compounds also allowed the assessment of published data regarding OO constituents, in light of this new information. We first focused on the elucidation of compounds proposed in the official HPLC-UV method of IOC [13]. Indeed, comparing the chromatographic profile of the IOC method and oxidized secoiridoid compounds, it was possible to clearly identify the compounds listed with vague structural description as follows: oleaceinic acid (**2**) instead of decarboxymethyl oleuropein aglycone, oxidized dialdehyde form (*compound 11*); oleocanthalic acid (**1**) instead of decarboxymethyl ligstroside aglycone, oxidized dialdehyde form (*compound 16*); carboxylic form of oleuropein aglycon (**8**) instead of oleuropein aglycone oxidized aldehyde and hydroxylic form (*compound 21*); carboxylic form of ligstroside aglycone (**7**) instead of ligstroside aglycone oxidized aldehyde and hydroxylic form (*compound 24*). The corresponding compounds according to IOC method are given in Supporting Information (**Table 1S**). In ▶ **Fig. 4**, an HPLC-UV chromatograph obtained from the analysis of a representative TPF using the IOC method is shown and the identified compounds are annotated.
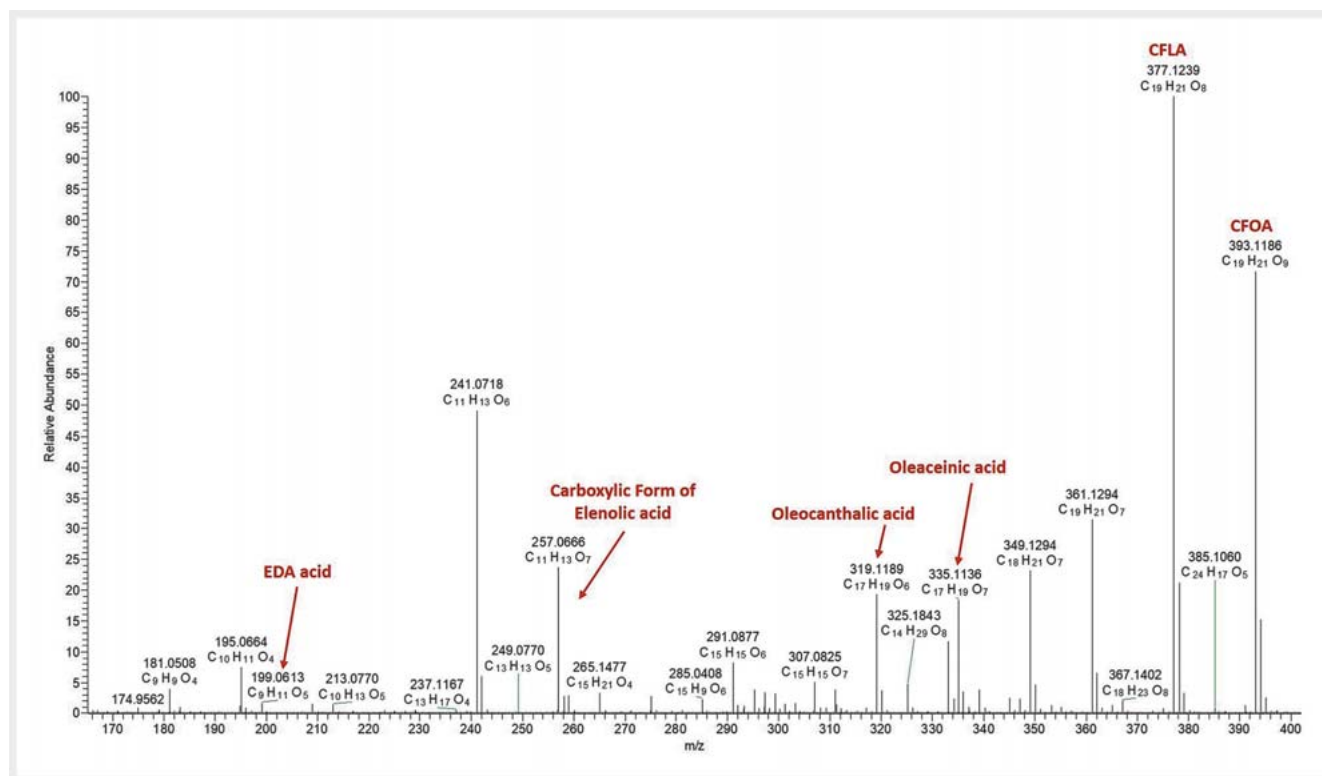
▶ **Fig. 3** Characteristic HRMS full-scan spectrum obtained from the analysis of a representative TPF extract. The oxidized secoiridoids oleocanthalic acid (**1**), oleaceinic acid (**2**), EDA acid (**3**), CFLA (**7**), CFOA (**8**), and carboxylic form of elenolic acid (**9**) are highlighted.

Finally, we investigated the possibility of any correlation between the presence of these oxidized secoiridoids and parameters applied in the assessment of OO quality, which are officially regulated. To this end, a representative number of 40 OO samples collected from various regions of Greece during the olive growing period 2018–2019 (**Table 2S**, Supporting Information) were analyzed by the IOC method and the regulated parameters free acidity, peroxide values, and constants K were determined. Moreover, these parameters were used for the classification of OO as extra virgin, virgin, or lamp [31, 32]. In parallel, the same samples were analyzed by the already developed UPLC-ESI(−)-HRMS & HRMS/MS methodology for the determination of the oxidized secoiridoid compounds (**1**), (**2**), (**3**), (**7**), (**8**), and (**9**) [11].

Evaluating the results of the determination of the quality parameters as well as of the UPLC-HRMS/MS analysis, it wasn't possible to obtain any clear linear correlation. Nevertheless, a useful observation was made. Specifically, from the 40 fresh OO samples studied in total, in 20 of them, at least one of the oxidized secoiridoids, oleocanthalic acid (**1**), oleaceinic acid (**2**), EDA acid (**3**), CFLA (**7**), CFOA (**8**), or carboxylic form of elenolic acid (**9**) was detected in high levels. At the same time, another common feature of all these 20 samples was the fact that at least one of the quality parameters values i.e., free acidity, peroxide values, coefficients K232, K270, and ΔK constant were outside the EU limits for the classification of OO as extra virgin, and therefore fell in the lower category of virgin OO (**Table 3S**, Supporting Information) [31, 32]. The features of the 40 fresh OO samples are given in Supporting

Information (**Table 3S**). This observation leads to the hypothesis that these oxidized secoiridoids could contribute as chemical markers for the quality categorization of OO as extra virgin, virgin, or lamp and therefore assist in tackling adulteration. Nevertheless, further studies are needed to also assess the quantitative aspects implicated by these correlations.

Material and Methods

General experimental procedures

Dichloromethane (DCM), Tetrahydrofuran (THF), Ethanol (EtOH), Ethyl-Acetate (EtOAc), Methanol (MeOH), n-Heptane, and n-Hexane were obtained from Fisher Scientific. All solvents were of analytical grade, and deionized water was used to prepare all aqueous solutions. For HPLC and LC-MS analyses the solvents were of respective grade. Vanillin standard, Selenium Dioxide reagent (Se$_2$O$_2$) (98% purity), and Hydrogen Peroxide (H$_2$O$_2$) were purchased from Sigma-Aldrich. For 1D and 2D NMR experiments, all samples were dissolved in 600 µL of deuterated chloroform (CDCl$_3$) and 1D (^{1}H NMR, ^{13}C-NMR) and 2D (COSY, NOESY, HMBC, HSQC -DEPT135) spectrums were acquired on a Bruker Avance III-600 spectrometer. Chemical shifts (δ) are expressed in ppm while coupling constants (J) in Hz. The multiplicity of vertices is expressed as s (singlet), brs (broad singlet), d (doublet), t (triplet), q (quartet), dd (doublet of doublets), qd (quartet double), and m (multiple). NOESY experiment was used to determine the R or
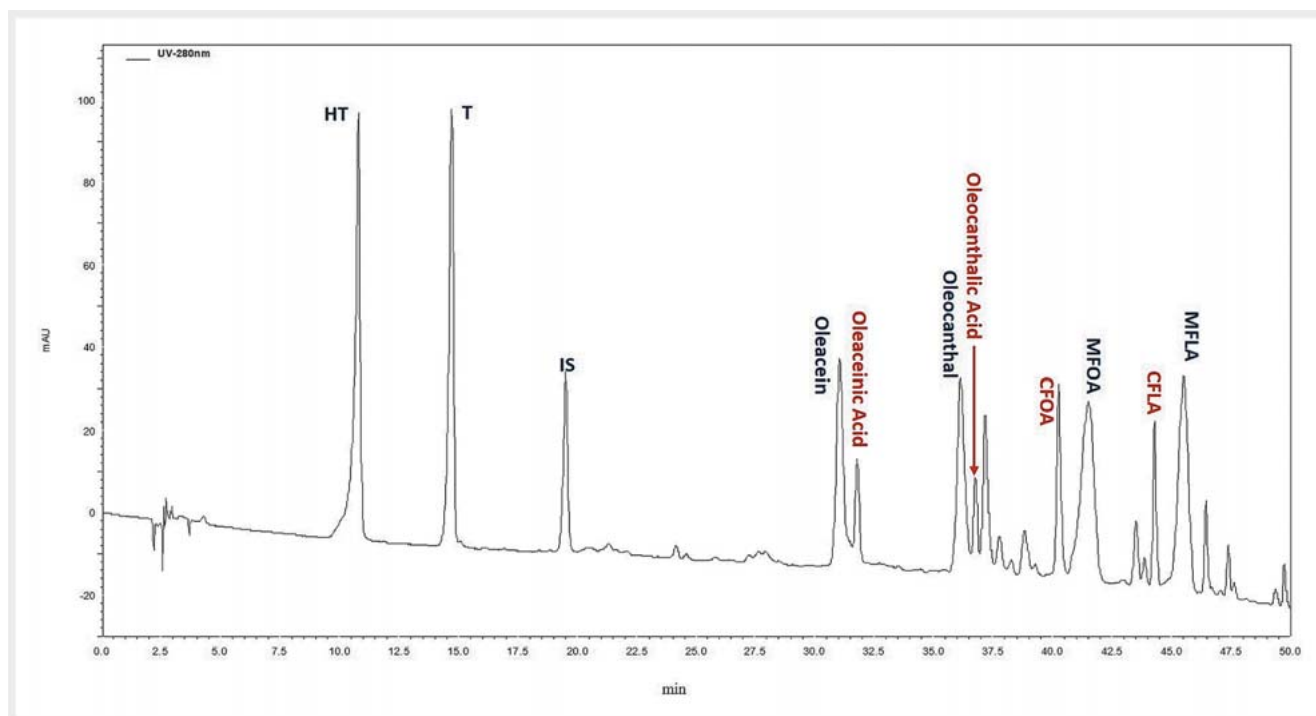
► **Fig. 4** Characteristic HPLC-DAD chromatograph obtained from the analysis of a representative TPF extract, applying the IOC proposed method. The oxidized secoiridoids oleocanthalic acid (**1**), oleaceinic acid (**2**), EDA acid (**3**), CFLA (**7**), CFOA (**8**), and carboxylic form of elenolic acid (**9**) are highlighted with red, while the natives (Hydroxytyrosol (HT), Tyrosol (T), Oleacein (**5**), Oleocanthal (**4**), MFOA (**10**) and MFLA (**11**)) are with blue. EDA acid and carboxylic form of elenolic acid are not annotated from chromatogram is due to the fact that these molecules do not absorb at 280 nm.

S arrangement of the asymmetric carbon C-8 of the closed forms of acids. Acquity UPLC system (Waters) coupled to a LTQ-Orbitrap XL hybrid mass spectrometer (Thermo Scientific) was used. Column Chromatography was performed over silica gel (por.2) for Chromatography ultrapure 40–60 µm, 60A. Liquid-liquid extraction of EVOO was developed in a laboratory scale centrifugal partition extractor FCPE300 (Rousselet-Robatel Kromaton), while TPF fractionation was carried out on a Fast Centrifugal Partition Chromatograph FCPC1000 (Rousselet-Robatel Kromaton), equipped with a rotor of 955 mL total volume capacity (45 circular partition disks engraved with 32 partition cells), and rotation speed from 200 to 1000 rpm, producing a stable centrifugal force up to 161 g at 1000 rpm. The systems were pumped through the CPC column with a Prep36 LabAlliance dual piston pump. The sample was injected via a 50 mL sample loop. Fractions were collected with a Büchi B – 684 fraction collector. The purification of target compounds was performed on a Preparative HPLC system equipped with two Prep Lab Alliance pumps, a SUPERCOSIL LC-18 (5 µm; SUPELCO) column (i. d. 21.2 × 250 mm), and a FLASH 06S DAD 600 detector (ECOM). TLC analysis was performed on normal phase plates (Merck), and the chromatograms were developed with a mixture of DCM and MeOH in the proportion of 92:8 v/v and observed at 254 nm, 366 nm and at visible with a sulfuric vanillin solution (5% w/v in MeOH) – H_2SO_4 (5% v/v in MeOH) and heated until the pigments' appearance.

Olive Oil Samples

Olive oil samples were collected during the 2018–2019 harvesting period from all over Greece. Samples were collected from different geographical regions and were kindly donated by olive oil producers and olive oil co-operatives. Immediately upon arrival, they were subjected to centrifugation to avoid sediment accumulation which results in rapid polyphenols decomposition. Then, samples were stored in dark, glassy vials, at room temperature and nitrogen conditions to keep matrixes stable.

Recovery of the major EVOO secoiridoids in pure form

The purification of the major secoiridoids from EVOO has occurred by following a well-established isolation process of our group with some modifications [23, 24]. For this procedure, an EVOO rich in all target compounds was selected. The first step of the process was the liquid-liquid extraction of TPF from EVOO using a laboratory scale centrifugal partition extractor FCPE300. equipped with a rotor of 300 mL total volume. The experiment consisted of several "Extraction-Recovery" cycles (multi dual-mode method) using the mixture of n-hexane:EVOO in ratio 3:2 (v/v) as "mobile phase" and EtOH:H_2O in ratio 3:2 (v/v) as "stationary phase". The liquid-liquid extraction was run in ascending mode with a flow rate of 50 mL/min, and after passing 2.5 L of oil containing mobile phase (corresponds to 1 L of EVOO) the operation mode was changed to descending and the enriched in polyphenols stationary phase was collected. This procedure was re-

peated 6 times treating in total 6 L of EVOO and resulting in the recovery of 6.8 g of TPF. The next step was the fractionation of TPF carried out on a Fast-Centrifugal Partition Chromatograph FCPC1000. The received TPF was treated by a step gradient elution extrusion method using the series of four biphasic systems (composed of the solvents n-Hept:EtOAc:EtOH:H$_2$O in ratio 4:1:3:2 (S1), 3:2:3:2 (S2), 2:3:3:2 (S3) and 1:4:3:2 (S4), v/v/v/v) in ascending mode and setting the flow rate at 15 mL/min, and rotation speed at 900 rpm. All collected fractions (190 fractions of 25 mL) were analyzed by TLC and combined based on their chemical similarity. From this procedure, five enriched fractions containing oleocanthal (**4**), oleacein (**5**), EDA (**6**), monoaldehydic form of ligstroside aglycon, MFLA (**10**), monoaldehydic form of oleuropein aglycon, or MFOA (**11**), and elenolic acid (**12**) were obtained in high quantity. The enriched fractions were subjected to Prep-HPLC for further purification of target compound. Each of the five enriched CPC fractions was analyzed with a slightly modified elution method using H$_2$O and ACN [23, 24]. All analyses were run on the same chromatographic column at flow rate of 15 mL/min. 150 mg was set as the initial quantity of each sample, in 1 mL ACN. The target compounds were collected manually based on their UV chromatogram and after solvent evaporation the corresponding compounds were obtained in purity > 95%.

Semi-synthesis

For the semisynthetic procedure [29], the same protocol was applied for all the oxidized compounds, keeping all the parameters stable. SeO$_2$ (0.5 mg) and H$_2$O$_2$ (in equivalent ratio 1:2) were added to 100 mg of each pure compound diluted in 5 mL THF and the reaction mixture was stirred at 70 °C, for 2 hours, under reflux, and its progress was monitored by TLC (DCM:MeOH 92:8 v/v, as eluent). At the completion of the reaction, THF solvent was evaporated under vacuum, the residue was diluted with CHCl$_3$ and H$_2$O, and the lower phase was received, a procedure that was repeated three times. After workup and solvent removal, the crude product was purified by column chromatography using the modified gradient system DCM: MeOH as follows: 0–100 mL 100%, 100–200 mL 99,5–0,5, 200–300 mL 99:1, 300–400 mL 98:2, 400–500 mL 96:4, 500–600 mL 92:8 to afford the corresponding oxidized form.

Oleocanthalic acid (**1**): Yield: 64% (68.67 mg, 0.21 mmol) from Oleocanthal (100 mg, 0.33 mmol, 1eq) using H$_2$O$_2$ (68.11 µL, 0.66 mmol, 2eq); UV (MeOH/H$_2$O) λ_{max}: 234, 276 (sh) nm, ESI (−)-HRMS m/z 319.1185 [M – H]$^-$ (calcd. for C$_{17}$H$_{19}$O$_6$, 319.1189); This was verified by comparison of the spectral data based on our previous work [19].

Oleaceinic acid (**2**): Yield: 67% (69.81 mg, 0.21 mmol) from Oleacein (100 mg, 0.31 mmol, 1eq) using H$_2$O$_2$ (63.98 µL, 0.62 mmol, 2eq); UV (MeOH/H$_2$O) λ_{max}: 235, 280 (sh) nm; ESI (−)-HRMS m/z 335.1132 [M – H]$^-$ (calcd. for C$_{17}$H$_{19}$O$_7$, 335.1136); this was verified by comparison of the spectral data based on our previous work [19].

EDA acid (**3**): Yield: 67% (72.38 mg, 0.36 mmol) from EDA (100 mg, 0.54 mmol, 1eq) using H$_2$O$_2$ (111.45 µL, 1.08 mmol, 2eq); UV (MeOH/H$_2$O) λ_{max}: 229 nm; ESI (−)-HRMS m/z 199.0613 [M – H]$^-$ (calcd. for C$_9$H$_{11}$O$_5$, 199.0613); this was verified by comparison of the spectral data based on our previous work [19].

CFLA: Yield: 71% (75.17 mg, 0.2 mmol) from MFLA (100 mg, 0.28 mmol, 1eq) using H$_2$O$_2$ (57.79 µL, 0.56 mmol, 2eq); UV (MeOH/H$_2$O) λ_{max}: 248, 271 (sh) nm; ESI (−)-HRMS m/z 377.1248 [M – H]$^-$ (calcd for C$_{19}$H$_{21}$O$_8$, 377.1239);

5S, 8R, 9S CFLA: ^{1}H NMR (600 MHz, CDCl$_3$) δ: 7.58 (brs, 1H, H-3), 7.04 (d, 2H, J$_{4',5'}$ = 8.5 Hz, H-4', J$_{8',7'}$ = 8.5 Hz, H-8'), 6.75 (d, 2H, J$_{5',4'}$ = 8.3 Hz, H-5', J$_{7',8'}$ = 8.3 Hz, H-7'), 4.24 (t, 2H, J$_{1',2'}$ = 6.6 Hz, H-1'), 4.13 (m, 1H, H-8), 3.69 (s, 3H, CH$_3$-12), 3.26 (brs, 1H, H-5), 2.83 (t, 2H, J$_{2',1'}$ = 6.6 Hz, H-2'), 2.79 (dd, 1H, J$_{6a,6b}$ = 16.1 Hz, J$_{6a,5}$ = 6.7 Hz, H-6a), 2.68 (dd, 1H, J$_{6b,6a}$ = 16.1 Hz, J$_{6b,5}$ = 3.9 Hz, H-6b), 2.56 (t, 1H, J = 8.8 Hz, H-9), 1.37 (d, 3H, J$_{10,8}$ = 6.4 Hz, CH3–10); ^{13}C NMR (600 MHz, CDCl$_3$) δ: 175.5 (C-1), 172.2 (C-7), 167.3 (C-11), 155.3 (C-3), 154 (C-6'), 129.9 (C-4', C-8'), 129.8 (C-3'), 115.1 (C-5', C-7'), 108.1 (C-4), 73.3 (C-8), 64.7 (C-1'), 50.8 (C-12), 50.3 (C-9), 36 (C-6), 33.9 (C-2'), 31.3 (C-5), 18.7 (C-10).

5S, 8S, 9S CFLA: ^{1}H NMR (600 MHz, CDCl3) δ: 7.60 (brs, 1H, H-3), 7.06 (d, 2H, J$_{4',5'}$ = 8.5 Hz, H-4', J$_{8',7'}$ = 8.5 Hz, H-8'), 6.75 (d, 2H, J$_{5',4'}$ = 8.3 Hz, H-5', J$_{7',8'}$ = 8.3 Hz, H-7'), 4.31 (t, 2H, J$_{1',2'}$ = 6.7 Hz, H-1'), 4.08 (qd, 1H, J$_{8,9}$ = 6.6 Hz, J$_{8,10}$ = 2.5 Hz, H-8), 3.72 (s, 3H, CH$_3$-12), 3.28 (brs, 1H, H-5), 2.89 (m, 1H, H-6a), 2.87 (t, 2H, J$_{2',1'}$ = 6.6 Hz, H-2'), 2.64 (brs, 1H, H-9), 2.19 (dd, 1H, J$_{6b,6a}$ = 16.1 Hz, J$_{6b,5}$ = 11.1 Hz, H-6b), 1.46 (d, 3H, J$_{10,8}$ = 6.6 Hz, CH3–10); ^{13}C NMR (600 MHz, CDCl$_3$) δ: 173.7 (C-1), 171.8 (C-7), 167.3 (C-11), 156.1 (C-3), 154 (C-6'), 129.9 (C-4', C-8'), 129.8 (C-3'), 115.1 (C-5' C-7'), 106.3 (C-4), 68.8 (C-8), 64.7 (C-1'), 50.8 (C-12), 44.7 (C-9), 39 (C-6), 33.9 (C-2'), 29.9 (C-5), 17.8 (C-10).

CFOA (**8**): Yield: 65% (66.61 mg, 0.17 mmol) from MFOA (100 mg, 0.26 mmol, 1eq) using H$_2$O$_2$ (53.66 µL, 0.52 mmol, 2eq); UV (MeOH/H$_2$O) λ_{max}: 231, 280 (sh) nm; ESI (−)-HRMS m/z 393.1193 [M – H]$^-$ (calcd. for C$_{19}$H$_{21}$O$_9$, 393.1186);

5S, 8R, 9S CFOA: ^{1}H NMR (600 MHz, CDCl$_3$) δ: 7.62 (brs, 1H, H-3), 6.79 (d, 1H, J$_{7',8'}$ = 8.1 Hz, H-7'), 6.75 (d, 1H, J$_{4',8'}$ = 1.8 Hz, H-8'), 6.65 (dd, 1H, J$_{8',7'}$ = 8.1 Hz, J$_{8',4'}$ = 1.8 Hz, H-8'), 4.24 (t, 2H, J$_{2',1'}$ = 6.7 Hz, H-1'), 4.17 (m, 1H, H-8), 3.72 (s, 3H, CH$_3$-12), 3.29 (brs, 1H, H-5), 2.81 (m, 2H, H-2'), 2.73 (m, 1H, H-6b), 2.70 (m, 1H, H-6a), 2.59 (m, 1H, H-9), 1.39 (d, 3H, J$_{10,8}$ = 6.6 Hz, CH$_3$-10); ^{13}C NMR (600 MHz, CDCl$_3$) δ: 175.3 (C-1), 171.9 (C-7), 167.5 (C-11), 156.4 (C-3), 143.1 (C-5'), 142.8 (C-6'), 130.2 (C-3'), 121.6 (C-8'), 116.1 (C-4'), 115.4 (C-7'), 107.8 (C-4), 73.7 (C-8), 65.2 (C-1'), 50.9 (C-9), 51.3 (C-12), 36.9 (C-6), 34.5 (C-2'), 30.5 (C-5), 19.2 (C-10).

5S, 8S, 9S CFOA: ^{1}H NMR (600 MHz, CDCl$_3$) δ: 7.64 (brs, 1H, H-3), 6.85 (d, 1H, J$_{4',8'}$ = 2 Hz, H-4'), 6.78 (d, 1H, J$_{7',8'}$ = 8 Hz, H-7'), 6.61 (dd, 1H, J$_{8',7'}$ = 8 Hz, J$_{8',4'}$ = 2 Hz, H-8'), 4.37/4.20 (m, 2H, H-1'), 4.10 (qd, 1H, J$_{8,10}$ = 6.6 Hz, J$_{8,9}$ = 2.7 Hz, H-8), 3.76 (s, 3H, CH$_3$-12), 3.31 (brs, 1H, H-5), 2.89 (m, 1H, H-6a), 2.81 (m, 2H, H-2'), 2.82 (m, 1H, H-6b), 2.75 (m, 1H, H-9), 2.18 (dd, 1H, J$_{6b,6a}$ = 15.9 Hz, J$_{5,6a}$ = 11.1 Hz, H-6a), 1.48 (d, 3H, J$_{10,8}$ = 6.6 Hz, CH$_3$-10); ^{13}C NMR (600 MHz, CDCl$_3$) δ: 173.3 (C-1), 171.2 (C-7), 168.4 (C-11), 157.1 (C-3), 143.1 (C-5'), 142.9 (C-6'), 130.2 (C-3'), 121.6 (C-8'), 116.7 (C-4'), 115.3 (C-7'), 106.7 (C-4), 69.1 (C-8), 65.2 (C-1'), 51.5 (C-12), 45.1 (C-9), 39.7 (C-6), 34.5 (C-2'), 31.4 (C-5), 18.3 (C-10).

Carboxylic form of elenolic acid (**9**): Yield: 66% (70.89 mg, 0.27 mmol) from EA (100 mg, 0.41 mmol, 1eq) using H$_2$O$_2$ (84.62 µL, 0.82 mmol, 2eq); UV (MeOH/H$_2$O) λ_{max}: 239 (sh) nm;

ESI (−)-HRMS *m/z* 257.0671 [M − H]⁻ (calcd. for $C_{11}H_{13}O_7$, 257.0669);

5S, 8S, 9S Carboxylic form of elenolic acid: ¹H NMR (600 MHz, $CDCl_3$) δ: 7.62 (brs, 1H, H-3), 4.17 (qd, 1H, $J_{10,8} = 6.2$ Hz, $J_{9,8} = 2.9$ Hz, H-8), 3.73 (s, 3H, CH_3-12), 3.34 (ddd, 1H, $J_{6b,5} = 11.0$ Hz, $J_{6a,5} = 2.5$ Hz, $J_{9,5} = 2$ Hz, H-5), 2.96 (dd, 1H, $J_{6a,6b} = 16$ Hz, $J_{6a,5} = 2.5$ Hz, H-6a), 2.83 (dd, 1H, $J_{9,8} = 2.9$ Hz, $J_{9,5} = 2.0$ Hz, H-9), 2.25 (dd, 1H, $J_{6b,6a} = 16.0$ Hz, $J_{6b,5} = 11.0$ Hz, H-6b), 1.49 (d, $J_{10,8} = 6.2$ Hz, CH_3-10); ¹³C NMR (600 MHz, $CDCl_3$) δ: 176.1 (C-7), 174.8 (C-1), 167.5 (C-11), 156.1 (C-3), 106.1 (C-4), 69.1 (C-8), 50.1 (C-12), 45.5 (C-9), 38.7 (C-6), 34.5 (C-2′), 29.6 (C-5), 17.8 (C-10).

Extraction of OO samples

In a 10 mL test tube, 2 g of OO and 1 mL of the internal standard solution (syringic acid) were mixed and vortexed for 30 sec. Next, 5 mL of the MeOH : H_2O (80 : 20, v/v) were added and vortexed for 1 min more, after which the mixture was placed in an ultrasonic bath for 15 min at room temperature, and then centrifuged for 25 min at 4000 rpm (centrifugal force = 3452.384 g). An aliquot of the supernatant phase (TPF) was taken, filtered, and forwarded to HPLC-DAD analysis.

HPLC-DAD analysis

The IOC proposed method was performed according to analytical conditions referred to IOC/T.20/Doc No 29 method (International Olive Council, 2009) [13]. Specifically, the separation was achieved on a reversed-phase Spherisorb Discovery HS C18 column (250 × 4.6 mm, 5 μm; Supelco) using a mobile phase consisting of 0.2% aqueous orthophosphoric acid (A) and MeOH : ACN (50 : 50 v/v) (B), at a flow rate of 1.0 mL/min and ambient temperature. The injection volume was held constant at 20 μL. The applied gradient elution was as follows: 0 min, 96% A and 4% B; 40 min, 50% A and 50% B; 45 min, 40% A and 60% B; 60 min, 0% A and 100% B; 70 min, 0% A and 100% B; 72 min, 96% A and 4% B; 82 min, 96% A; and 4% B. Chromatograms were monitored at 280 nm.

UPLC-ESI (−)-HRMS & HRMS/MS analysis

All native and oxidized secoiridoids as well as the TPF samples were analyzed using UPLC-ESI (−)- HRMS as well as HRMS/MS analysis. Single compounds were prepared in s final concentration of 50 μg/mL diluted in MeOH : H_2O (1 : 1, v/v) while the TPF in a concentration of 500 μg/mL in the same solvent solution. For the separation, H_2O with 0.1% formic acid (FA) was used as solvent A, and acetonitrile (ACN) as solvent B. The elution method started with 2% of B and in 2 min reached 21%. In the next 4 min the percentage of B increased to 44.5%. Finally, at 9 min, B reached 100% and was maintained for 2 min. The next minute, the system returned to the initial conditions. remaining 3 min for system equilibration. A Thermo Hypersil Gold C-18 (50 mm × 2.1 mm, 1.9 μm) column was used, with a stable temperature of 40 °C. The total acquisition time was 15 min and a flow rate set at 400 μL/min. The injection volume was 10 μL and the autosampler temperature was set at 7 °C. Mass spectra were obtained using ES, in negative ion mode. The capillary temperature was set at 350 °C, capillary voltage at − 10 V, and tube lens at − 40 V. Sheath and auxiliary gas were adjusted at 40 and 10 arb, respectively. Mass spectra were recorded in full scan mode in the range of *m/z* 115–1000 with resolving power 30 000, at *m/z* 500 and scan rate 1 microscan/sec. HRMS/MS experiments were obtained in data-depending method with collision energy 35.0% (q = 0.25). The system was calibrated externally every 50 injections. Two repetitive measurements were performed for each sample.

Determination of quality characteristics of olive oil samples

Free acidity, peroxide values, coefficients K232, K270, and ΔK constant belong to the quality parameters of olive oils, and their values determine their classification into extra virgin, virgin, and lamp OO. If at least one quality parameter of OO is outside of the limits set by the EU for its classification, then olive oil is classified as non-EVOO. These parameters of the OO samples were measured using European Official Method of Analysis [32].

Supporting Information

The following are available as supporting information: NMR and HRMS spectra of compounds **1–3** and **7–9** (Fig. 1S–30S), HRMS/MS spectra of MFOA, CFLA Oleacein and Oleocanthalic acid (**Fig. 31S–32S**), identification of biophenols peaks according to T.20/Doc No 29 method of IOC (**Table 1S**), meta data of analyzed olive oil samples (**Table 2S**), and the comparison data on OO quality parameters and identification levels of oxidized secoiridoids (**Table 3S**).

Contributors' Statement

Conception and Design of study: L. Antoniadi, A. Angelis, P. Stathopoulos, M. Halabalaki, L. A. Skaltsounis; data collection: L. Antoniadi, E. M. Bata, Z. Papoutsaki; analysis and interpretation of data: A. Angelis, P. Stathopoulos, M. Halabalaki, L. A. Skaltsounis; data visualization: L. Antoniadi, A. Angelis, P. Stathopoulos, M. Halabalaki; drafting the manuscript: L. Antoniadi, P. Stathopoulos, L. A. Skaltsounis; critical revision of the manuscript: A. Angelis, P. Stathopoulos, M. Halabalaki, L. A. Skaltsounis.

Conflict of Interest

The authors declare that they have no conflict of interest.

References

[1] Owen RW, Giacosa A, Hull WE, Haubner R, Würtele G, Spiegelhalder B, Bartsch H. Olive-oil consumption and health: the possible role of antioxidants. Lancet Oncol 2000; 1: 107–112

[2] Dais P, Hatzakis E. Quality assessment and authentication of virgin olive oil by NMR spectroscopy: A critical review. Anal Chim Acta 2013; 765: 1–27. doi:10.1016/j.aca.2012.12.003

[3] Ray NB, Hilsabeck KD, Karagiannis TC, McCord DE. Bioactive Olive Oil Polyphenols in the Promotion of Health. In: Singh RB, Watson RR, Takahashi T, eds. The Role of Functional Food Security in Global Health. Oxford: Elsevier Inc.; 2019: 623–637

[4] Covas MI, Fitó M, De La Torre R. Minor bioactive olive oil components and health: Key data for their role in providing health benefits in humans. AOCS Press 2015: 2015: 31–52. doi:10.1016/B978-1-63067-041-2.50008-2

[5] Boskou D, Blekas G, Tsimidou M. Olive Oil Composition. Boskou D, ed. Olive Oil (Second Edition). Oxford: Elsevier Inc.; 2006: 41–72

[6] Bianco A, Coccioli F, Guiso M, Marra C. The occurrence in olive oil of a new class of phenolic compounds: hydroxy-isochromans. Food Chem 2002; 77: 405–411

[7] European Food Safety Authority (EFSA). Scientific Opinion. ESFA J 2011; 9: 2033–2058

[8] De La Torre-Carbot K, Jauregui O, Gimeno E, Castellote AI, Lamuela-Raventós RM, López-Sabater MC. Characterization and quantification of phenolic compounds in olive oils by solid-phase extraction, HPLC-DAD, and HPLC-MS/MS. J Agric Food Chem 2005; 53: 4331–4340

[9] Vichi S, Cortés-Francisco N, Caixach J. Insight into virgin olive oil secoiridoids characterization by high-resolution mass spectrometry and accurate mass measurements. J Chromatogr A 2013; 1301: 48–59. doi:10.1016/j.chroma.2013.05.047

[10] Bongiorno D, Di Stefano V, Indelicato S, Avellone G, Ceraulo L. Bio-phenols determination in olive oils: Recent mass spectrometry approaches. Mass Spectrom Rev 2021; n/a: e21744. doi:10.1002/mas.21744

[11] Nikou T, Witt M, Stathopoulos P, Barsch A, Halabalaki M. Olive oil quality and authenticity assessment aspects employing FIA-MRMS and LC-Orbitrap MS Metabolomic Approaches. Front Public Health 2020; 8: 558226

[12] Kotsiou K, Tasioula-Margari M. Monitoring the phenolic compounds of Greek extra-virgin olive oils during storage. Food Chem 2016; 200: 255–262

[13] International Olive Council. Determination of Biophenols in Olive Oils by HPLC. COI/T20/Doc No 29/Rev1 2017; 29: 1–8

[14] García-Villalba R, Carrasco-Pancorbo A, Oliveras-Ferraros C, Vázquez-Martín A, Menéndez JA, Segura-Carretero A, Fernández-Gutiérrez A. Characterization and quantification of phenolic compounds of extra-virgin olive oils with anticancer properties by a rapid and resolutive LC-ESI-TOF MS method. J Pharm Biomed Anal 2010; 51: 416–429

[15] Daskalaki D, Kefi G, Kotsiou K, Tasioula-Margari M. Evaluation of phenolic compounds degradation in virgin olive oil during storage and heating. J Food Nutr Res (Slovak Republic) 2009; 48: 31–41

[16] Lozano-Sánchez J, Castro-Puyana M, Mendiola JA, Segura-Carretero A, Cifuentes A, Ibáñez E. Recovering bioactive compounds from olive oil filter cake by advanced extraction techniques. Int J Mol Sci 2014; 15: 16270–16283

[17] Lozano-Sánchez J, Giambanelli E, Quirantes-Piné R, Cerretani L, Bendini A, Segura-Carretero A, Fernández-Gutiérrez A. Wastes generated during the storage of extra virgin olive oil as a natural source of phenolic compounds. J Agric Food Chem 2011; 59: 11491–11500

[18] Farré M, Picó Y, Barceló D. Direct analysis in real-time high-resolution mass spectrometry as a valuable tool for polyphenols profiling in olive oil. Anal Methods 2019; 11: 472–482. doi:10.1039/C8AY01865K

[19] Angelis A, Antoniadi L, Stathopoulos P, Halabalaki M, Skaltsounis LA. Oleocanthalic and Oleaceinic acids: New compounds from Extra Virgin Olive Oil (EVOO). Phytochem Lett 2018; 26: 190–194. doi:10.1016/j.phytol.2018.06.020

[20] De Ceglie C, Abbattista R, Losito I, Castellaneta A, Calvano CD, Bianco G, Palmisano F, Cataldi TRI. Influence of horizontal centrifugation processes on the content of phenolic secoiridoids and their oxidized derivatives in commercial olive oils: An insight by liquid chromatography-high-resolution mass spectrometry and chemometrics. J Agric Food Chem 2020; 68: 3171–3183

[21] Abbattista R, Losito I, Castellaneta A, De Ceglie C, Calvano CD, Cataldi TRI. Insight into the storage-related oxidative/hydrolytic degradation of olive oil secoiridoids by liquid chromatography and high-resolution fourier transform mass spectrometry. J Agric Food Chem 2020; 68: 12310–12325

[22] Castillo-Luna A, Criado-Navarro I, Ledesma-Escobar CA, López-Bascón MA, Priego-Capote F. The decrease in the health benefits of extra virgin olive oil during storage is conditioned by the initial phenolic profile. Food Chem 2021; 336: 127730. doi:10.1016/j.foodchem.2020.127730

[23] Angelis A, Michailidis D, Antoniadi L, Stathopoulos P, Tsantila V, Nuzillard JM, Renault JH, Skaltsounis LA. Pilot continuous centrifugal liquid-liquid extraction of extra virgin olive oil biophenols and gram-scale recovery of pure oleocanthal, oleacein, MFOA, MFLA and hydroxytyrosol. Sep Purif Technol 2021; 255: 117692

[24] Angelis A, Hamzaoui M, Aligiannis N, Nikou T, Michailidis D, Gerolimatos P, Termentzi A, Hubert J, Halabalaki M, Renault JH, Skaltsounis AL. An integrated process for the recovery of high added-value compounds from olive oil using solid support free liquid-liquid extraction and chromatography techniques. J Chromatogr A 2017; 1491: 126–136

[25] Dai PF, Qu JP, Kang YB. Organocatalyzed aerobic oxidation of aldehydes to acids. Org Lett 2019; 21: 1393–1396

[26] Gaspa S, Porcheddu A, De Luca L. Metal-free direct oxidation of aldehydes to esters using TCCA. Org Lett 2015; 17: 3666–3669. doi:10.1021/acs.orglett.5b01579

[27] Hunsen M. Carboxylic Acids from Primary Alcohols and Aldehydes by a Pyridinium Chlorochromate Catalyzed Oxidation. In: Synthesis. Stuttgart: Thieme; 2005: 2487–2490

[28] Sancineto L, Tidei C, Bagnoli L, Marini F, Lenardão EJ, Santi C. Selenium catalyzed oxidation of aldehydes: Green synthesis of carboxylic acids and esters. Molecules 2015; 20: 10496–10510

[29] Brzaszcz M, Kloc K, Maposah M. Selenium (IV) oxide catalyzed oxidation of aldehydes to carboxylic acids with hydrogen peroxide. Synthetic Communications 2000; 30: 4425–4434

[30] Nikou T, Liaki V, Stathopoulos P, Sklirou AD, Tsakiri EN, Jakschitz T, Bonn G, Trougakos IP, Halabalaki M, Skaltsounis LA. Comparison survey of EVOO polyphenols and exploration of healthy aging-promoting properties of oleocanthal and oleacein. Food Chem Toxicol 2019; 125: 403–412

[31] International Olive Council. Trade standard applying to olive oils and olive pomace oils. COI/T15/NC 3/Rev2 2006

[32] EEC EU. Regulation No 2568/91 on the characteristics of olive oil and olive-residue and on the relevant methods of analysis. Off J Eur Communities 1991; 34: L248