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## **Pilot continuous centrifugal liquid-liquid extraction of extra virgin olive oil biophenols and gram-scale recovery of pure oleocanthal, oleacein, MFOA, MFLA and hydroxytyrosol**

Apostolis Angelis, Dimitris Michailidis, Lemonia Antoniadis, Panagiotis Stathopoulos, Vasiliki Tsantila, Jean-Marc Nuzillard, Jean-Hugues Renault, Leandros Skaltsounis

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1 **Pilot continuous centrifugal liquid-liquid extraction of extra virgin olive oil**  
2 **biophenols and gram-scale recovery of pure oleocanthal, oleacein, MFOA,**  
3 **MFLA and hydroxytyrosol**

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17

1 Abstract

2 This study provides an efficient pilot-scale liquid-liquid extraction process for the  
3 recovery of total phenolic fraction (TPF) from extra virgin olive oil (EVOO) and the  
4 further purification of the major TPF constituents (oleocanthal, oleacein,  
5 monoaldehydic form of oleuropein aglycone, monoaldehydic form of ligstroside  
6 aglycone and hydroxytyrosol) using successively centrifugal partition chromatography  
7 and reversed phase high pressure liquid chromatography. Special attention was paid for  
8 the selection of the appropriate raw material, which should be rich in the targeted high-  
9 value compounds. For the extraction of the selected EVOOs, a green technique based  
10 on liquid-liquid partitioning using the biphasic system n-Hept/EVOO/EtOH/H<sub>2</sub>O  
11 3:2:3:2 v/v was carried out, first on pioneering laboratory-scale annular centrifugal  
12 extractor (ACE) and then on pilot device. 240 L from three different EVOOs (3x80 L  
13 of each EVOO) were extracted yielding 285 g of TPF in approximately 2 hours. The  
14 second step of this procedure was the purification of the target compounds. A  
15 preparative centrifugal partition chromatography (CPC) process was firstly applied for  
16 the initial TPF fractionation using a step gradient elution extrusion method with a series  
17 of 4 biphasic systems composed of n-Hept/EtOAc/EtOH/H<sub>2</sub>O in ratios 4:1:3:2, 3:2:3:2,  
18 2:3:3:2 and 1:4:3:2 (v/v/v/v). Preparative reversed phase high performance liquid  
19 chromatography (prep-RP-HPLC) was then carried out to polish the target compounds  
20 from the enriched CPC fractions resulting in purity higher than 97%. In parallel, the  
21 above purification analysis led to the isolation of four minor TPF constituents of which  
22 three are new natural products (EDA lactone, (1*R*, 8*E*)-1-ethoxy-ligstroside aglycon  
23 and (1*S*, 8*E*)-1-ethoxy-ligstroside aglycon) and one ((9*E*, 11*E*)-13-oxotrideca-9,11-  
24 dienoic acid) is described for the first time as EVOO constituent.

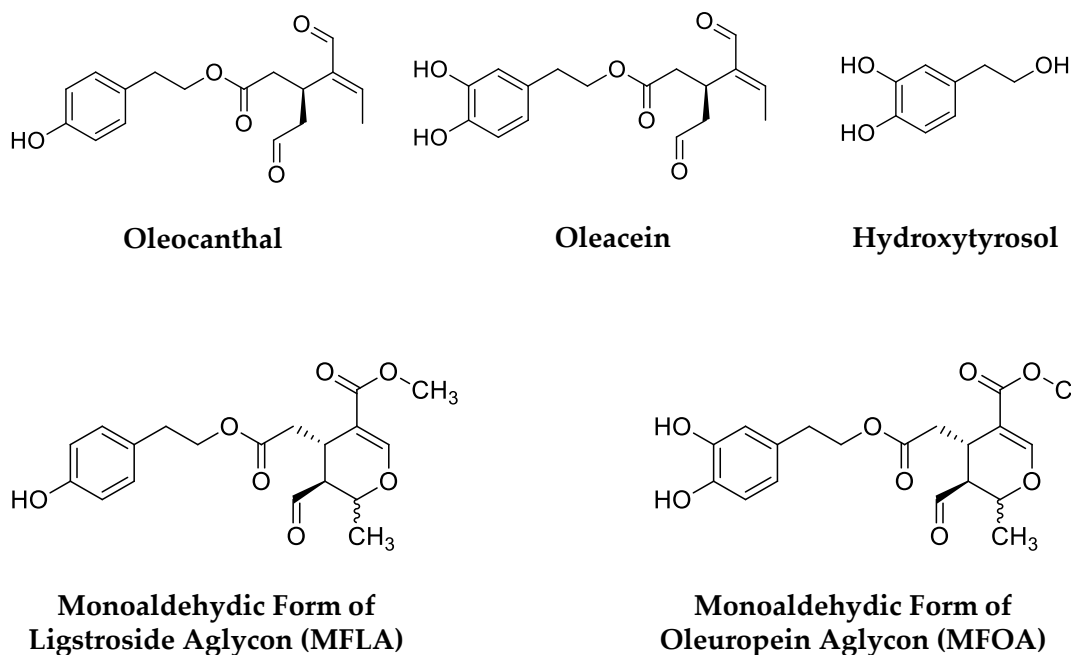
25 **Keywords:** extra virgin olive oil, oleocanthal, oleacein, centrifugal partition  
26 chromatography, continuous liquid-liquid extraction, annular centrifugal extractor.

27 **Abbreviations:** EVOO-Extra Virgin Olive Oil, TPF-Total Phenol/polyphenol  
28 Fraction, ACE- Annular Centrifugal Extractor, CPC- Centrifugal Partition  
29 Chromatography.

30

1 1. Introduction

2 Olive oil (OO), since ancient time has a highly economic importance, especially for the  
3 Mediterranean area [1]. Nowadays, OO has expanded all over the world, from USA  
4 and Chile to Australia, Japan and China [2–4]. OO and especially extra virgin olive oil  
5 (EVOO) is straightly correlated with the Mediterranean diet and the providing health  
6 benefits [5,6]. Most of the beneficial characteristics of EVOO are attributed to minor  
7 compounds, named biophenols which compose the total phenolic fraction (TPF) [7].  
8 Hydroxytyrosol (HT) is the most known phenylethanoid of EVOO phenolic fraction.  
9 Other significant compounds of TPF are the secoiridoid derivatives oleacein (OLEA),  
10 oleocanthal (OLEO), monoaldehydic form of oleuropein aglycone (MFOA) and  
11 monoaldehydic form of ligstroside aglycone (MFLA) (Figure 1) [8]. These molecules  
12 have been widely investigated, providing significant bioactivities. It is worth noting that  
13 the last years, the scientific interest has focused on HT and its derivatives as the most  
14 important non-nutritive components of olive oil, due to EFSA (European Food & Safety  
15 Authority) health claim about their protecting effect on cardiovascular system [9].  
16 Specifically, numerous biological activities were reported for HT, such as strong anti-  
17 oxidant and anti-inflammatory properties [10][11]. Even more, are the studies investi-  
18 gating anti-cancer activity, neuroprotective effects, potency against Alzheimer’s dis-  
19 ease and anti-inflammatory properties of OLEO [12–14]. Additionally OLEA, has  
20 shown significant bioactivities such as anti-oxidant, anti-inflammatory, anti-prolifera-  
21 tive, anti-microbial and anti-cancer properties [14–17]. To the best of our knowledge,  
22 there are only few studies for the activity of MFOA, focus mainly on its antioxidant  
23 properties, while for the bioactivity of MFLA there is no significant information in the  
24 literature [18,19]. It is noteworthy, that there are limited reports related to the biological  
25 activity of the above mentioned secoiridoids in animal models. One critical reason is  
26 the required quantities and purity levels in such studies, thus hindering significantly  
27 their investigation in animal models and their comprehensive biological and pharma-  
28 cological evaluation. [20–22].



**Oleocanthal**

**Oleacein**

**Hydroxytyrosol**

**Monoaldehydic Form of  
Ligstroside Aglycon (MFLA)**

**Monoaldehydic Form of  
Oleuropein Aglycon (MFOA)**

1

2

**Figure 1:** Structures of the main phenolic compounds of olive oil.

3

In the current study, an integrated process combining continuous liquid-liquid extraction and chromatographic techniques with scale-up capabilities was developed for the isolation of OO phenolic compounds in high yield. The liquid-liquid extraction step was carried out using liquid-liquid centrifugal extraction devices, which allows the development of continuous processes. Two types of equipment were used: a single stage annular centrifugal extractor (ACE) using two co-axial cylinders in order to ensure phase mixing [23] for the lab scale feasibility step and then a single stage centrifugal extractor using a rotating agitator disc in order to mix the two phases for the pilot-scale extraction. Both of these apparatuses are based on continuous counter-current liquid-liquid extraction principle, the only difference of them being allocated at the phase mixing mechanism. They provide high mass transfer efficiency as well as excellent phase decantation, thus leading to high throughputs in compact contactors [24]. The rapid start-up and shut-down of experimental procedure makes it also very useful for the industrial sector [25]. Centrifugal extraction has already been used in many different industrial fields, including pharmaceutical or food industry [26].

18

Secondly, centrifugal partition chromatography (CPC) [27,28] was used for the fractionation of TPF in order to obtain enriched fractions in compounds of interest [29]

19

CPC is a liquid-liquid solid support free chromatography based on solutes partitioning at least between two immiscible phases in a thermodynamic equilibrium [30]. A CPC

20

21

1 column consists of a series of partition cells linked in cascade by ducts in cascade and  
2 arranged in a centrifuge (one axis, two high pressure rotary seals). The stationary phase  
3 is maintained inside the column by the centrifugal force acceleration generated by  
4 column rotation (50 to 800 times the gravity, depending on the apparatus), while the  
5 mobile phase is pumped through it. CPC technique has numerous advantages mainly  
6 due to the absence of chromatographic solid support. It is characterized by total sample  
7 recovery (irreversible adsorption on solid support are avoided), high repeatability, low  
8 solvent consumption and high capacity [8][31]. Additionally, it is considered as gentle  
9 and versatile technique leading to high selectivity, as a huge range of solvent  
10 combinations are available and reported in literature. [32,33][29][34][35]. Moreover,  
11 the industrial potential of CPC was demonstrated using a scale up methodology based  
12 on both column capacity and mass transfer efficiency as invariants parameters [36].  
13 Using continuous centrifugal extraction and CPC at-line offers advantages concerning  
14 the amounts and the required quality for *in vivo* experiments and human studies. It has  
15 to be highlighted that both of the above techniques, continuous centrifugal liquid-liquid  
16 extraction such as ACE and CPC, could be considered as green methodologies. Indeed,  
17 they are characterized by a low solvent consumption when the intensification step is  
18 correctly carried out. Moreover, the short experimental duration minimizes the energy  
19 expenditure and the liquid-liquid nature of the techniques provides the ability to recycle  
20 the used solvents reducing thus drastically the generated wastes. All these  
21 characteristics have a positive impact on the environmental fingerprint of these liquid-  
22 liquid separation techniques [37,38].

23 Three EVOO from different geographical areas of Greece were selected, based on  
24 HPLC analysis, for their high abundance in the compounds of interest. TPF extraction  
25 was conducted with ACE in the selected OO samples, in both lab- and pilot-scale.  
26 Afterwards, the fractionation of the corresponding TPFs was carried out using CPC in  
27 step gradient elution mode. Selected fractions, enriched in the target compounds, were  
28 finally purified with preparative reversed phase HPLC (prep-RP-HPLC), reaching  
29 purity higher than 97% (HPLC based). Thus, the combination of two liquid-liquid  
30 support-free techniques (i.e. ACE and CPC) with prep-RP-HPLC also based on the  
31 partition mode for the polishing step allowed the extraction of TPF and the isolation of  
32 OLEO, OLEA, MFOA, MFLA and HT in considerably high yields. It should be also  
33 underlined that due to the high TPF availability and consequently the high quantity of

1 CPC fractions, four minor constituents, never been reported before in olive oil were  
2 also isolated and structurally identified by NMR and HRMS analysis.

## 3 2. Materials and Methods

### 4 2.1. Reagents

5 All the reagents were purchased from Sigma-Aldrich (Missouri, USA). The solvents  
6 used for the extraction and separation processes were of analytical grade while those  
7 used for UPLC-HRMS analysis were of LC-MS grade. All solvents were supplied from  
8 Fisher Scientific (Pennsylvania, USA). TLC analysis was performed on Silica gel 60  
9 F254 20cm x 20cm plates purchased from Merck Millipore (Massachusetts, USA). The  
10 standard compounds (purity > 98%) used for the HPLC qualitative and quantitative  
11 analysis were purchased from Chembiotin (Greece) (Hydroxytyrosol, Tyrosol  
12 standards) and Pharmagnose S.A. (Inofyta, Greece) (Oleocanthal, oleacein, MFOA and  
13 MFLA standards).

### 14 2.2. Apparatus

15 The analytical scale extractions were performed on a laboratory scale BXP012  
16 apparatus (Rousselet-Robatel Kromaton, Annonay, France) with 2.2 mL bowl volume  
17 and internal diameter of 12 mm. The two phases of the biphasic system were pumped  
18 into the annular extractor using two Basic Verderflex pumps (Castleford, United  
19 Kingdom) connected to the respective inlets for heavy and light phase. The large-scale  
20 extractions of OO biophenols were performed using the pilot scale ACE BXP190  
21 (mono-stage unit) (Rousselet-Robatel Kromaton, Annonay, France). The apparatus  
22 consists of a casing and a bowl driven by a motor directly coupled to the extractor  
23 pendular shaft. The bowl has an internal diameter of 190 mm with a useful volume of  
24 4.2 liters. The two liquid phases are mixed in the annular space of the extractor and are  
25 separated in the weir system which consists of a stable light phase weir of 90 mm and  
26 an interchangeable heavy phase weir of 100 mm. The motor is fed by a variable  
27 frequency drive to achieve variable speed operation with the maximum acceptable  
28 speed of 2900 rpm. Both heavy and light phases were pumped through the extractor  
29 with two ATEX FLUX eccentric worm-drive pumps with FBM 4000 Ex Motor and  
30 flow rate up to 75 L/min (FLUX-GERÄTE GmbH, Maulbronn, Germany) connected  
31 to the two solvent inlets of the apparatus.

32 The TPF fractionations were performed on a FCPC1000® apparatus (Kromaton,  
33 Anonay, France) equipped with rotor made of 45 circular partition disks engraved with  
34 32 partition cells (555 µL per cell). The total column volume capacity is 955 mL. The

1 partition cells are interconnected by capillary ducts, the latter representing a volume of  
2 156 mL (dead volume). Rotation speed could be adjusted from 200 to 1000 rpm,  
3 producing a stable centrifugal force field in the partition cells up to 161 g at 1000 rpm.  
4 The solvents were pumped through two preparative ECP2000 pumps (Ecom, Prague,  
5 Czech Republic), and the effluent was monitored online by a Flash 14 UV-DAD  
6 detector (Ecom, Prague, Czech Republic). Fractions were collected by a C6-60  
7 collector (Büchi, Flawil, Switzerland). The LC system (pumps and UV detector) was  
8 controlled by Clarity™ 5.4 (DataApex, Prague, Czech Republic).

9 The purification of target compounds from enriched CPC fractions was performed on  
10 a preparative HPLC chain consisted of two Lab Alliance preparative 36 Pumps (State  
11 College, USA), a UV Flash 06S DAD 800 Detector of ECOM (Prague, Czech  
12 Republic) and injection valve Rheodyne 7755-027 equipped with 1 mL loop (Target  
13 Analysis, Thessaloniki, Greece). The separation was run on a Supelco supelcosil™ LC-  
14 18, 25 cm x 21.2 mm, 5µm (Pennsylvania, USA) preparative HPLC column while the  
15 fractions were collected manually based on UV chromatograms. The HPLC system was  
16 controlled by Clarity™ 5.4 (DataApex, Prague, Czech Republic).

17 HPLC qualitative and quantitative analysis was performed on a Thermo Finnigan  
18 HPLC system (Ontario, Canada) equipped with a SpectraSystem 1000 degasser, a  
19 SpectraSystem P4000 pump, a SpectraSystem AS3000 automated injector and an UV  
20 SpectraSystem UV6000LP detector. Data acquisition was monitored by the  
21 ChromQuest™ 5.0 software (ThermoScientific™).

22 The pure compounds were analyzed by UPLC-ESI-HRMS using an Acquity H-Class  
23 UPLC system (Waters Corp., Milford, USA), equipped with a quaternary pump, an  
24 autosampler, an online vacuum degasser, and a temperature-controlled column and  
25 sample compartment and hyphenated to a hybrid LTQ-Orbitrap Discovery XL (Thermo  
26 Scientific, Brehmen, Germany) mass spectrometer with an electrospray ionization  
27 (ESI) source. Xcalibur 2.0.7 (Thermo Scientific) software was used for data acquisition  
28 and processing.

29 NMR spectra (<sup>1</sup>H, <sup>13</sup>C, COSY, NOESY, HSQC-DEPT, HMBC) were recorded on a  
30 Bruker Avance III 600 spectrometer (Bruker Biospin GmbH, Rheinstetten, Germany),  
31 equipped with a 5-mm PABBI 1H/D-BB inverse detection probe with a z-gradient. <sup>1</sup>H  
32 and <sup>13</sup>C NMR spectra were acquired at 600.15 MHz and 150.91 MHz, respectively.



1 2.3. HPLC-DAD analysis for olive oil selection

2 A HPLC-DAD method was employed according to the COI / T.20 / Doc No 29 method  
3 (International Olive Council (IOC) 2009) for the screening of in-house olive oil library  
4 [39]. For sample preparation, 2.0 g of olive oil and 1 mL of the internal standard  
5 solution (syringic acid) were mixed and vortexed for 30 s. Then, 5 mL of the  
6 methanol/water (80:20 v/v) were added and vortexed for 1 min more. The mixture was  
7 placed in an ultrasonic bath for 15 min at room temperature and then centrifuged for 25  
8 min. An aliquot of the supernatant phase was forwarded for HPLC analysis after  
9 filtration. HPLC quantification was achieved on a reversed-phase Spherisorb Discovery  
10 HS C18 column (250 × 4.6 mm, 5µm, Supelco) using a mobile phase consisted of 0.2%  
11 aqueous orthophosphoric acid (A) and methanol/acetonitrile (50:50 v/v) (B). The  
12 applied gradient elution was as follows: 0 min, 96% A and 4% B; 40 min, 50% A and  
13 50% B; 45 min, 40% A and 60% B; 60 min, 0% A and 100% B; 70 min, 0% A and  
14 100% B; 72 min, 96% A and 4% B; 82 min, 96% A and 4% B. A flow rate of 1.0  
15 mL/min was used and the injection volume was set to 20µl. Chromatograms were  
16 monitored at 280 nm.

17 In total 10 EVOO samples were analyzed and the levels of HT, OLEA, OLEO, MFOA  
18 and MFLA were determined (Figure S1: supplementary material). The same analysis  
19 method was used to determine the levels of the target compounds in TPFs obtained  
20 from the pilot-scale extraction as well as the purity of the isolated compounds. The  
21 purity of the OLEO, OLEA, MFOA, MFLA and HT was also supported by UPLC-ESI-  
22 HRMS/MS and NMR analysis.

23 2.4. Liquid-liquid extraction of EVOO

24 The continuous centrifugal liquid-liquid extraction processes were started with parallel  
25 pumping of both feed oil phase (light) and extraction phase (heavy) into the extractor  
26 bowl. The biphasic system was mixed (at the mixing zone) and then was separated (by  
27 centrifugal forces) to treated oil phase and enriched aqueous phase. The biphasic system  
28 used for the extractions previously described by *Angelis et al* [8], was the following: n-  
29 Hept/EVOO/EtOH/H<sub>2</sub>O in ratio 3:2:3:2 (v/v/v/v). The extraction procedure was ini-  
30 tially developed in a laboratory scale extractor (BXP012) using *EVOO-I* as study ma-  
31 terial and then the method was transferred in pilot-scale extractor (BXP190) where all  
32 three chosen OOs were treated.

1 2.4.1. Extraction of TPF using the laboratory scale annular centrifugal extractor  
2 BXP012

3 The feed oil phase was prepared by mixing 80 mL of *EVOO-I* and 120 mL of *n*-heptane  
4 while the extraction phase was prepared by mixing 120 mL ethanol and 80 mL water.  
5 The rotation speed of laboratory scale ACE BXP012 was set at 3950 rpm and the flow  
6 rate at 8 mL/min for both phases (Table 1). Subsequently, the biophenol-enriched aque-  
7 ous phase was evaporated under vacuum. The experiment was repeated two more times.  
8 The repeatability of the process was evaluated by comparing the yields standard devi-  
9 ation, as well as using TLC and RP-HPLC-DAD analysis of the three obtained TPFs.

10 2.4.2. Extraction of TPF using pilot scale extractor BXP190

11 The pilot-scale extraction of OO TPF was performed using a mono-stage pilot scale  
12 ACE BXP190. The first step of the procedure was the separately preparation of the two  
13 phases of solvent system: 200 L of the feed oil phase composed of *n*-Hept/*EVOO* (in  
14 ratio 3:2 v/v) and 200 L of the aqueous extraction phase composed of EtOH/ $H_2O$  (in  
15 ratio 3:2 v/v). The extraction procedure started by pumping the heavy phase at a flow  
16 rate of 10 L/min and at the rotation speed of 1050 rpm. When the heavy phase came  
17 out from the extractor, its flow rate decreased at 5 L/min and the light phase was  
18 pumped with the same flow (5 L/min), while the rotation speed was maintained at 1050  
19 rpm (Table 1). The extraction procedure lasted 40 min and finally 200 L of treated oil  
20 phase and the same volume of enriched in biophenols aqueous phase were separately  
21 collected into two different barrels.

22 The next step of the procedure was the evaporation of the collected extract and raffinate  
23 in order to recover the biophenolic fraction as well as the treated olive oil, respectively.

24 The solvents (*n*-heptane and the mixture of EtOH/ $H_2O$ ) obtained from the evaporation  
25 were recycled and reused in further extraction processes. The evaporation was per-  
26 formed in a QVF thermal evaporation system (200 L evaporation vessel) under 15-100  
27 mbar vacuum at 39°C, while the condensation of the produced solvents vapors was  
28 facilitated using coolant (glycol) at -7°C. The TPF obtained after processing of 80 L  
29 *EVOO* was 85.8 g (*EVOO-I* → *TPF-I*).

30 The procedure was repeated for the extraction of *EVOO-II* and *EVOO-III*. Each  
31 extraction was performed by using the recycled solvents. The TPFs obtained after  
32 extraction of 80 L of *EVOO-II* and 80 L of *EVOO-III* were 109.4 g (*TPF-II*) and 89.5  
33 g (*TPF-III*), respectively.

1 **Table 1:** Experimental parameters of EVOO extractions and the corresponding yields,  
 2 using analytical and pilot scale ACE.

<b>Biphasic system</b>	<i>n</i> -Hept/EVOO/ethanol/water 3:2:3:2 (v/v/v/v)			
<b>Aparatus</b>	BXP012 (Analytical scale)	BXP190** (Pilot scale)		
<b>EVOO</b>	<i>EVOO-I</i>	<i>EVOO-I</i>	<i>EVOO-II</i>	<i>EVOO-III</i>
<b>Flow rate of feed oil phase</b>	8 mL/min	5 L/h	5 L/h	5 L/h
<b>Flow rate of extraction phase</b>	8 mL/min	5 L/h	5 L/h	5 L/h
<b>Volume of treated EVOO</b>	80 mL	80 L	80 L	80 L
<b>Volume of feed oil phase</b>	200 mL	200 L	200 L	200 L
<b>Volume of extraction phase</b>	200 mL	200 L	200 L	200 L
<b>Rotation speed</b>	3950 rpm	1050	1050	1050
<b>Experimental Time</b>	20 min	~40 min	~40 min	~40 min
<b>Yield of TPFs</b>	<b>83.3 mg* (±0.36)</b>	<b>85,8 g</b>	<b>109.4g</b>	<b>89.5g</b>

3 \* The average of three repetitions (standard variation)

4 \*\*The heavy phase weir used for both three pilot analysis was of 100 mm.

## 5 2.5. Isolation procedure

### 6 2.5.1. Preparative CPC fractionation of TPFs using step-gradient elution extrusion 7 method.

8 Preparative CPC fractionations of the produced TPFs (*TPF-I*, *TPF-II* and *TPF-III*) were  
 9 achieved by following a previously described method with slight modifications [8]. All  
 10 experiments were run in a step gradient elution extrusion mode using the series of four  
 11 biphasic systems (composed of the solvents *n*-Hept/EtOAc/EtOH/H<sub>2</sub>O in ratio 4/1/3/2  
 12 (S1), 3/2/3/2 S2), 2/3/3/2 (S3) and 2/4/3/2 (S4), v/v/v/v) and the same experimental  
 13 parameters. The aqueous phase of S1 was used as stationary phase while the gradient  
 14 elution was achieved by pumping successively the upper phases (mobile phases, MP)  
 15 of the four systems in ascending mode (500 mL of MP1, 1100 mL of MP2, 1400 mL  
 16 of MP3 and 1000 mL of MP4). The experiments were ended by extruding the column  
 17 content using the lower phase of S4 as mobile phase in descending mode. The injected  
 18 sample mass of each TPF was 10 g, while rotation speed and flow rate were remained  
 19 constant during all experiment at 900 rpm and 15 mL/min, respectively. All collected  
 20 fractions (190 fractions of 25 mL) were initially off-line analyzed by TLC and  
 21 combined based on their chemical profile similarity (Figure S2: supplementary  
 22 material).

### 23 2.5.2. Preparative HPLC purification

24 Different preparative HPLC methods were developed for each target compound using  
 25 H<sub>2</sub>O (solvent A)-CH<sub>3</sub>CN (solvent B) gradients at flow rate of 15 mL/min. The gradient  
 26 profiles of methods A-E were as described below. Method A (for OLEO enriched  
 27 fractions): 0 min – 80% A, 20 min- 70% A, 25 min– 10% A and 30 – 80% A; Method  
 28 B ( for OLEA enriched fractions): 0 min – 80% A, 20 min- 70% A, 25 min– 70% A,

1 30 min– 10% A and 35 – 80% A; Method C (for MFLA enriched fractions): 0 min –  
2 80% A, 10 min – 70% A, 22 min- 50% A, 25 min- 50% A, 30 min– 10% A and 35 min  
3 – 80% A; Method D (for MFOA enriched fractions): 0 min – 800% A, 20 min – 70%  
4 A, 25 min- 50% A, 30 min– 10% A and 35 min – 80% A; and Method E (for HT  
5 enriched fractions): 0 min – 100% A, 5 min – 99% A, 10 min- 95% A, 20 min- 50% A,  
6 25 min– 10% A and 30 min – 100% A. All the samples were dissolved in CH<sub>3</sub>CN in  
7 order to reach a concentration of 150 mg/mL and were injected with 1 mL volume  
8 capacity loop.

### 9 2.5.3. TLC, UHPLC-HRMS/MS and NMR analysis

10 TLC analysis was performed on normal phase plates. The chromatograms were  
11 development with a mixture of dichloromethane (CH<sub>2</sub>Cl<sub>2</sub>) and methanol (MeOH) in  
12 ratio CH<sub>2</sub>Cl<sub>2</sub>/MeOH 95:5 v/v and observed at 254 nm, 366 nm and at visible after  
13 treatment with a sulfuric vanillin solution (5% w/v in methanol) – H<sub>2</sub>SO<sub>4</sub> (5% v/v in  
14 methanol) and heated at 100–120°C for 1 minute.

15 For UHPLC-HRMS/MS analysis all the samples were diluted in MeOH/H<sub>2</sub>O 1:1 v/v  
16 and injected (10µL) on a Fortis C-18 (1.7 µm, 150 x 2.1 mm) column at 40°C. The flow  
17 rate was set at 400 µL/min while acidified water with 0.1% formic acid (A) and CH<sub>3</sub>CN  
18 (B) were used as elution system, in the following gradient mode: 0-3 min 95% A, 3-21  
19 min from 95% to 5% A, 21-23 min 5% A, 23-24 min from 5% to 95% B and 24-30 min  
20 95% A. Ionization was achieved in negative ion mode (ESI-) at 350°C. The mass  
21 spectrometric parameters were: capillary voltage -20V and tube lens -80V for the  
22 negative mode; sheath gas and aux gas flow rate 40 and 10 units, respectively. The mass  
23 range was 113 - 1000 *m/z*.

24 NMR samples were dissolved in 600 µL of deuterated chloroform (CDCl<sub>3</sub>) and  
25 experiments were recorded using standard Bruker microprograms. Chemical shifts (δ)  
26 are expressed in ppm, while coupling constants (*J*) in Hz. The multiplicity of vertices  
27 is expressed as s (singlet), brs (broad singlet), d (doublet), t (triplet), q (quartet), dd  
28 (doublet of doublets) and m (multiplet).

## 29 3. Results and Discussion

### 30 3.1. EVOOs selection

31 In a previous study of our research group the TPFs of 130 EVOO's - constituting our  
32 in-house library of EVOOs - from all over Greece, were analyzed [22]. As it was  
33 expected, the results showed a significant difference in the yields of both TPF and its

1 major components *i.e.* hydroxytyrosol, tyrosol, oleacein and oleocanthal, depending on  
2 the olive variety, geographical origin, production procedure and cultivation practice.  
3 Thus, 10 EVOO samples with high levels of phenolic compounds were re-collected  
4 during the 2018-2019 harvesting period and were re-analyzed. All samples showed  
5 different levels of the target compounds after HPLC-DAD analysis (Figure S1:  
6 supplementary material). The best candidate of EVOOs for the designed pilot-scale  
7 isolation workflow were OLE\_1474 obtained from Lakonia-Peloponnese (code name  
8 *EVOO-I*), OLE\_1464 from Heraklion-Crete (code name *EVOO-II*) and OLE\_1437  
9 from Lasithi-Crete (code name *EVOO-III*). The three EVOOs contained high levels of  
10 two or more target compounds. More precisely, *EVOO-I* showed high levels for OLEA  
11 and OLEO, *EVOO-II* high levels for OLEO, OLEA and MFOA, while the *EVOO-III*  
12 was rich in HT, OLEO and MFLA. Therefore, the three EVOOs were re-procured in  
13 higher quantities for the following steps of this study.

### 14 3.2. Continuous liquid-liquid centrifugal extraction of selected EVOOs

15 Two different extraction methods were previously developed [8] on a laboratory-scale  
16 Centrifugal Partition Extractor equipped with 231 partition cells for a column volume  
17 of 303 mL: a sequential strategy consisted of several “extraction-recovery” cycles and  
18 a continuous strategy based on stationary phase co-current elution. In both cases,  
19 EVOO was used as mobile phase diluted in food grade n-heptane (feed mobile phase)  
20 and the required biphasic system was obtained by adding ethanol and water as polar  
21 solvents [8]. Considering its efficacy, the same biphasic system was used in the present  
22 work. Therefore, the liquid-liquid extraction feasibility using the biphasic system n-  
23 Hept/EVOO/EtOH/H<sub>2</sub>O in ratio 3:2:3:2 (v/v/v/v) was firstly evaluated on a lab-scale  
24 single stage ACE with a bowl diameter of 12 mm and a bowl volume of 2.2 mL  
25 (BXP012). ACE technique have been previously used for the lab-scale extraction of  
26 lignans from sesame oil by *Michailidis et al* [40] with significant results and the current  
27 study reports the use of this technique for EVOO biophenols extraction for the first  
28 time. Considering the density ratio between the two phases of 1.22 (feed oil phase  
29 density = 704,4 kg/m<sup>3</sup>, extraction phase density = 853.8 kg/m<sup>3</sup>), and the quite low  
30 viscosity of the EVOO-rich upper phase, the rotation speed was set at 3950 rpm  
31 corresponding to a centrifuge acceleration of 1046 g (up to 6700 g can be reached with  
32 this equipment). The flow rate was fixed at 8 mL/min for each phase, corresponding to  
33 50% of the maximum flow rate for this lab-scale extractor and an average residence

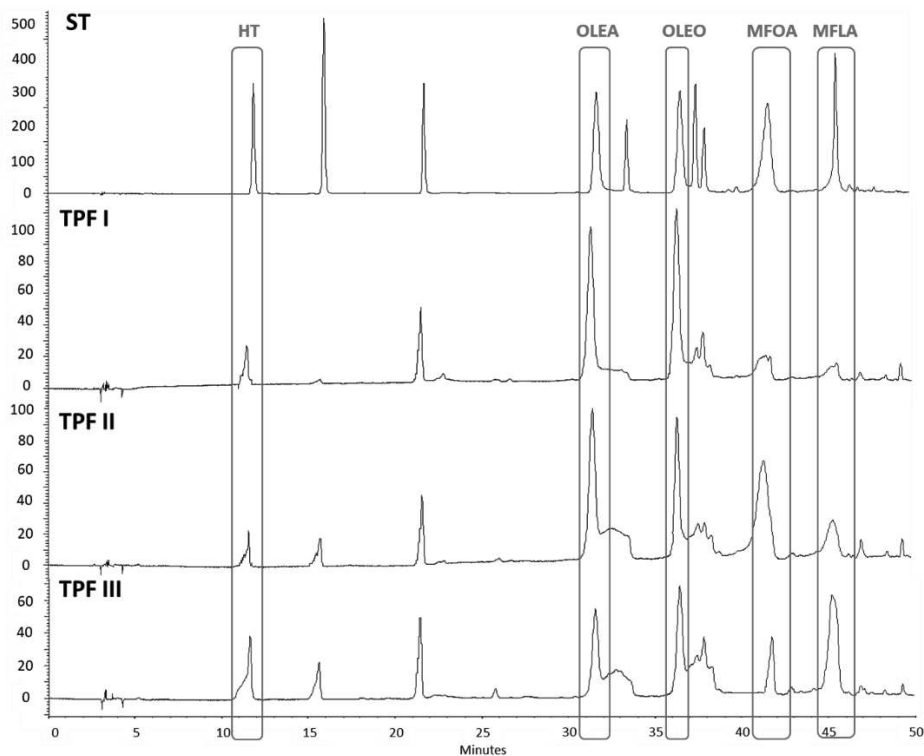
1 time of 8 s for each phase. The 80 mL of *EVOO-I* were successfully extracted in 20  
2 min, providing 83.23 mg of TPF, (done in triplicate, standard deviation: 0.36), without  
3 the presence of persistent emulsion at the outlet of the extractor, corresponding to a  
4 productivity of 113 g/(h.L) of extractor capacity. It is important to note that in the  
5 recently developed CPE extraction methods [8], the productivity was calculated at 8.9  
6 g/(h.L) of CPE column using the co-current CPE process, even though the analyzed  
7 olive oil was slightly more concentrated in TPF (1.3 g TPF/L of olive oil) compared to  
8 *EVOO-I* (1.04 g TPF / L of olive oil). This first results highlighted the efficiency of the  
9 single stage continuous liquid-liquid centrifugal extraction process for TPF recovery  
10 from EVOO.

11 The scale-up was directly achieved on a pilot-scale single stage liquid-liquid centrifugal  
12 contactor with the following bowl dimensions: 190 mm and 4.2 L for diameter and  
13 capacity, respectively. The phase mixing take place in the annular space and the  
14 separation occurs in the weir system (light phase weir of 90 mm and heavy phase weir  
15 of 100 mm). The centrifuge acceleration was chosen as scale change invariant in order  
16 to provide nearly the same mixing state and so mass transfer of polyphenolic  
17 compounds [41]. Considering the diameter of the bowl, the rotation speed was set at  
18 1000 rpm, reaching approximately 1060 g. The flow rate of each phase was set at 5  
19 L/min, corresponding to a residence time of 25 s in order to allow an efficient  
20 dispersion-coalescence of the dispersed phase together with a complete mass transfer  
21 of the compounds of interest. The above flow rates resulted in high quality of both  
22 separated phases by allowing more time to achieve efficient separation. Nevertheless,  
23 this parameter should be further optimized for an industrial application in order to  
24 increase productivity.

25 80 L of each EVOO (*EVOO-I*, *EVOO-II* and *EVOO-III*) were extracted, tending to  
26 recycle the solvents between the successive extractions. The three final TPFs i.e. *TPF-*  
27 *I* from *EVOO-I*, *TPF-II* from *EVOO-II* and *TPF-III* from *EVOO-III* were evaporated to  
28 dryness yielding 85.9, 109.4 and 89.5 g respectively (Table 1). The corresponding  
29 productivities were 30.6 g/(h.L), 39.7 g/(h.L) and 31.9 g/(h.L).

30 The liquid-liquid extraction process developed here being only governed by single stage  
31 phase transfer phenomena, we can assume that the productivity decrease (113 g/(h.L)  
32 vs 34 g/(h.L), ratio 3.3) observed between the lab-scale and the pilot-scale, respectively  
33 is mainly due to the selected flow rate on the pilot extractor that increased the residence  
34 time (8 s vs 25 s, ratio 3.1).

1 Each TPF obtained after the continuous liquid-liquid extraction step was analyzed by  
2 HPLC in order to qualitatively visualize their chemical profiles (figure 2). As expected,  
3 *TPF-I* was rich in OLEO and OLEA, *TPF-II* in MFOA, OLEO and OLEA and *TPF-III*  
4 in MFLA, OLEO and HT.



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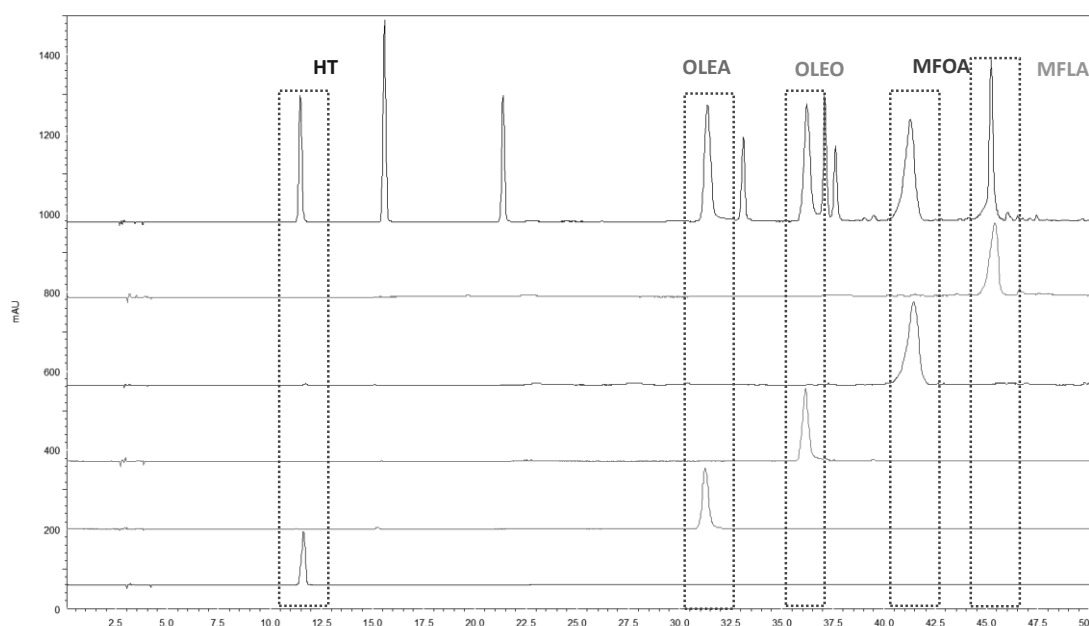
6 **Figure 2:** HPLC chromatograms of standard compounds, *TPF-I* (rich in OLEA and  
7 OLEO), *TPF-II* (rich in OLEA, OLEO and MFOA) and *TPF-III* (rich in HT, OLEO  
8 and MFLA) at 280 nm.

### 9 3.3. CPC fractionation of TPFs and purification of target compounds

10 10 g of each TPF were fractionated by CPC using a previously described step-gradient  
11 elution extrusion method [8]. The different biphasic solvent systems were all composed  
12 of n-heptane, ethyl acetate, ethanol and water (4/1/2/3, 3/2/2/3, 2/3/2/3 and 1/4/2/3  
13 v/v/v/v for S1, S2, S3, S4, respectively). Previous work demonstrated that the  
14 compositions of the aqueous phases of these systems are very close, making it possible  
15 to use the gradient mode without dramatic disturbing of the hydrodynamic equilibrium  
16 during the process [8]. The fractions were pooled based on their chemical profile  
17 similarities, after TLC off-line analysis (Figure S2: supplementary materials).

18 The CPC analysis of *TPF-I* resulted in the recovery of 1135.3 mg of the enriched OLEO  
19 fraction and 1009.1 mg of the enriched OLEA fraction as well as in the recovery of  
20 143.5 mg, 102.3 mg and 38.2 mg of fractions enriched in HT, MFLA and MFOA,  
21 respectively. The same analysis of *TPF-II* led to the recovery of 1045.2 mg, 983.6 mg,

1 845.8 mg, 112.6 mg and 93.2 mg of CPC fractions enriched in OLEO, OLEA, MFOA,  
2 HT and MFLA, respectively. The CPC analysis of *TPF-III* resulted enriched fractions  
3 in 1348.5 mg, 845.3 mg, 372.9 mg, 148.1 mg and 83.2 mg of MFLA, OLEO, HT,  
4 OLEA and MFOA, respectively. Finally, certain amounts of enriched fractions in  
5 MFLA (1544 mg), MFOA (967.2 mg), OLEO (3025.8 mg), OLEA (2140.8 mg) and  
6 HT (629 mg) were recovered from the three CPC analysis (Table S1: supplementary  
7 material). The purity of the target compounds in the enriched CPC fractions was  
8 determined based on HPLC and NMR analysis and found to be more than 80%.  
9 In order to succeed high purity of the target compounds, all the enriched CPC fractions  
10 were purified with prep-RP-HPLC (Figure S3: supplementary material). Aliquots of  
11 150 mg/mL were injected via a 1 mL injection loop, while the chromatographic  
12 separations were run using different elution method for each target compound (as  
13 described at experimental part). This purification step led to the isolation of 1030.4 mg  
14 of MFLA (96% purity), 683.4 mg of MFOA (97% purity), 2020.6 mg of OLEO (98%  
15 purity), 1463.7 mg of OLEA (98% purity) and 407.4 mg of HT (97% purity) (Figure  
16 3). The purity of the isolated compounds was determined by quantitative HPLC analysis  
17 using standard compounds for calibration curves plotting. The applied procedure,  
18 including the CPC fractionation of the EVOO extract and followed by the prep-RP-  
19 HPLC polishing step of the enriched fractions, is an efficient method for the gram-scale  
20 recovery of purified TPF high-value compounds.



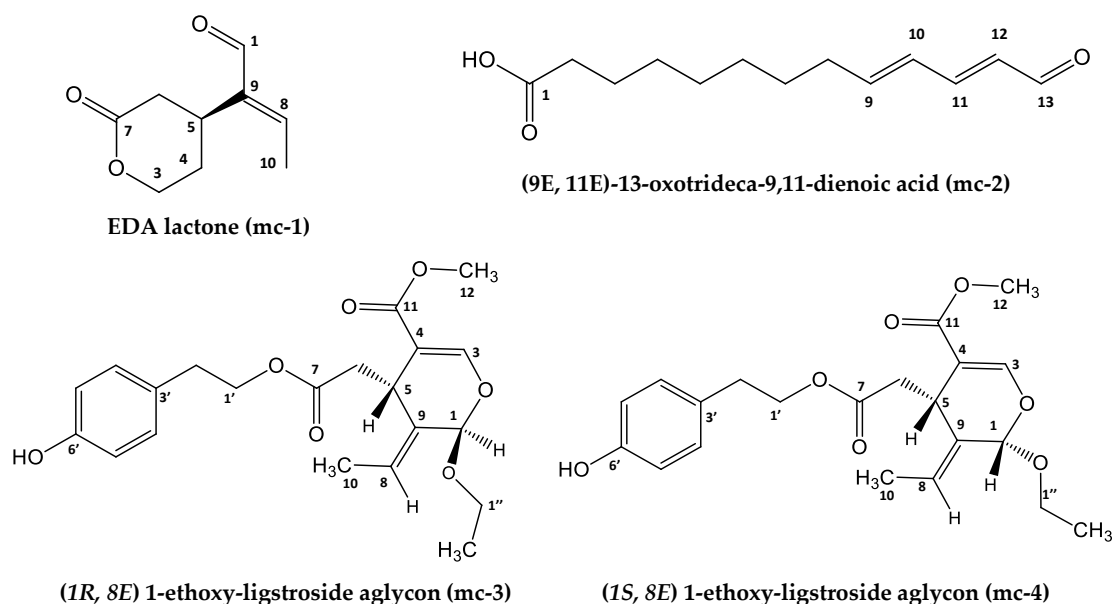
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**Figure 3:** HPLC chromatograms of purified major EVOO compounds.



### 3.4. Isolation and structure elucidation of new EVOO metabolites

The high complexity of TPF in combination with the alteration of OO phenolic composition depending on olive variety and differentiation on harvesting period or cultivation area, make this raw material a continuous source of original natural products. To date, over than 100 metabolites have been reported as EVOO constituents apart from lipids [42] [43] [8,44]. Nevertheless, the use of solid support-free liquid-liquid techniques for the upstream steps of the fractionation process (*i.e.* continuous liquid-liquid centrifuge extraction and CPC) prevented irreversible adsorption phenomena and chemical degradation and/or artifact formation. This extraction-fractionation process combined with the pilot-scale strategy (80 L of each EVOO were used as starting material) and the prep-RP-HPLC allowed the purification, apart from the major metabolites, of four minor compounds.



**Figure 4:** Structures of isolated minor compounds.

In more detail, the purification process of OLEO resulted in the isolation of one more minor secoiridoid (18 mg) which was identified as the lactonic form of EDA (**mc-1**: Fig. 4). The structure of **mc-1** was elucidated by means of 1D and 2D NMR experiments as well as HRMS analysis. The ESI (-)-HRMS spectrum of this compound showed a pseudomolecular ion  $[M-H]^-$  at  $m/z$  167.0718 with a suggested EC of  $C_9H_{11}O_3$  and RDBEq of 4 (Figure S4e: supplementary material). The  $^1H$  NMR spectrum showed the presence of a total of ten peaks of which three correspond to the protons of an 1-Oxobut-2-en-2-yl moiety [a doublet at  $\delta$  9.36 ( $J = 1.4$  Hz) (H-10), a quartet at  $\delta$  6.68 ( $J = 7.2$

1 Hz) (H-8) and a doublet at  $\delta$  2.02 (3H,  $J = 7.2$  Hz) (H-10)] and seven peaks correspond  
2 to the protons of a  $\delta$ -valerolactonic moiety [two peaks at  $\delta$  4.57 and 4.31 (H-3a/3b), one  
3 multiplet at  $\delta$  3.51 (H-5), two doublet of duplet at  $\delta$  2.96 and 2.43 (H-6a/6b) and two  
4 multiplets at  $\delta$  2.14 and 1.88 (H-4a/4b)]. Further study of 2D-NMR spectra (COSY,  
5 HSQC-DEPT, HMBC) verified the structure of the isolated compound. The complete  
6  $^1\text{H}$  and  $^{13}\text{C}$  NMR data of **mc-1** are presented in table 2 while 1D and 2D spectra are  
7 shown in Figures S4a-S4d (supplementary material). Due to the formation of  $\delta$ -  
8 valerolactonic ring through the reaction of aldehydic group at C-3 with the carboxyl  
9 group at C-7 of EDA, the asymmetric carbon C-5 of **mc-1** maintains the same  
10 stereochemical configuration as in EDA skeleton i.e 5S- configuration. To the best of  
11 our knowledge, there are not previous references regarding this metabolite and thus is  
12 characterized as new natural product with the given name EDA lactone.

13 Moreover, two minor picks were collected during the preparative HPLC purification of  
14 MFLA from the corresponding enriched CPC fraction. The NMR and HRMS analysis  
15 (Figures S5a-S5d: supplementary material) of the first peak (12.5 mg) revealed the  
16 presence of an unsaturated fatty acid which was identified as (*9E, 11E*)-13-oxotrideca-  
17 9,11-dienoic acid (**mc-2**: fig. 4). The ESI (-)-HRMS spectrum determined the length of  
18 the fatty chain showing a pseudomolecular ion  $[\text{M-H}]^-$  at  $m/z$  223.1341 with suggested  
19 EC of  $\text{C}_{13}\text{H}_{19}\text{O}_3$  and ERDeq of 4 (Figure S5d: supplementary material). The complete  
20  $^1\text{H}$  and  $^{13}\text{C}$  NMR data of **mc-2** are presented in table 2. This compound was initially  
21 identified as product of hydroperoxide lyase reaction of *Chlorella pyrenoidosa* with 13-  
22 hydroperoxylinolenic acid as a substrate [45] and subsequently was isolated as  
23 antifungal substance from the leaves of *Chenopodium album* L. var. *album* (pigweed)  
24 treated with a cupric chloride solution [46]. The direct isolation from natural sources is  
25 presented here for the first time.

26 The second collected minor peak of preparative HPLC analysis (9.5 mg) contained a  
27 mixture of two secoiridoid isomers which were identified as (*1R,8E*) 1-ethoxy-  
28 ligstroside aglycon (**mc-3**: fig. 4) and (*1S, 8E*) 1-ethoxy-ligstroside aglycon (**mc-4**: fig.  
29 4). The NMR data of the above isomers (Figures S6a-S6e: supplementary material) are  
30 very close to aglycon moiety of ligstroside. The only spectroscopic difference concerns  
31 the spin system of the ethoxy moiety which replace the sugar moiety in C-1 of  
32 ligstroside (table 2). The stereochemistry of those isomers was based on 2D NOESY  
33 NMR spectrum. The off-diagonal cross-peak between H-5 and H-10, for both isomers,  
34 suggests close proximity of the involved hydrogen atoms indicating thus *E*

1 configuration of C8-C9 double bond. On the other hand, in the NOESY spectrum a  
2 cross-peak between the H-1 and H-6b was appeared for 1*R* isomer and a correlation  
3 peak between H-1 and H-8 for 1*S* isomer verifying thus the suggested structures  
4 (Figures S6c: supplementary material). The complete <sup>1</sup>H-NMR and <sup>13</sup>C-NMR  
5 spectroscopic data of the above compounds are presented in table 2. To the best of our  
6 knowledge, there are not references data regarding both (1*R*, 8*E*)- and (1*S*, 8*E*)- 1-  
7 ethoxy-ligstroside aglycons. Recently, Cavaca *et al.*, published the formation of both  
8 (1*S*)- and (1*R*)- 1-methoxy – oleuropein aglycon after acid-promote methanolysis of  
9 oleuropein[47]. These isomers have the same secoiridoid subunit with the isolated 1-  
10 ethoxy-ligstroside aglycons. The spectroscopic data referring to monoterpen moieties  
11 of (1*S*)- and (1*R*)-1-methoxy – oleuropein aglycons are similar to experimental data of  
12 the corresponding subunits of (1*S*)- and (1*R*)-1-ethoxy-ligstroside aglycons verifying  
13 thus the suggested structures [47].

EDA lactone (mc-1)		(9E, 11E)-13-oxotrideca-9,11-dienoic acid (mc-2)		(1S, 8E) 1-ethoxy-ligstroside aglycon (mc-3)		(1R, 8E) 1-ethoxy-ligstroside aglycon (mc-4)					
<sup>1</sup> H δ=ppm, (J=Hz)	<sup>13</sup> C (ppm)	<sup>1</sup> H δ=ppm, (J=Hz)	<sup>13</sup> C (ppm)	<sup>1</sup> H δ=ppm, (J=Hz)	<sup>13</sup> C (ppm)	<sup>1</sup> H δ=ppm, (J=Hz)	<sup>13</sup> C (ppm)				
<b>1</b>	9,36 d (1.4)	195	<b>1</b>	-	178.4	<b>1</b>	<b>5,19</b> brs	<b>103,5</b>	<b>1</b>	<b>5,37</b> brs	<b>98,3</b>
<b>3</b>	4,57 ddd (11.3/6.5/4.5) 4,31 ddd (11.3/7.8/4.5)	68,1	<b>2</b>	2.35 t (7.4)	33.9	<b>3</b>	7,46 brs	152,9	<b>3</b>	7,50 brs	154,5
<b>4</b>	2,14 m 1,88 m	26,1	<b>3</b>	1.63 m	24.5	<b>4</b>	-	108,8	<b>4</b>	-	107,8
<b>5</b>	3,14 m	28,6	<b>4-</b> <b>6</b>	1.33 m	28.8	<b>5</b>	<b>3,88</b> dd (9.4/4.6)	<b>28,8</b>	<b>5</b>	<b>3,98</b> dd (9.6/4.3)	<b>30,8</b>
<b>6</b>	2,96 dd (16.5/11.3) 2,43 dd (16.5/6.4)	32,9	<b>7</b>	1.45 m	28.5	<b>6</b>	<b>2,84</b> dd (14.8/9.4) <b>2,73</b> dd (14.8/4.6)	39,1	<b>6</b>	<b>2,78</b> dd (14.8/4.3) <b>2,34</b> dd (14.8/9.6)	40,7
<b>7</b>	-	172	<b>8</b>	2.21 m	32.9	<b>7</b>	-	172,3	<b>7</b>	-	171,4
<b>8</b>	6,68 q (7.2)	152,2	<b>9</b>	6.27 m	147.2	<b>8</b>	<b>5,70</b> q (7.1)	<b>128,4</b>	<b>8</b>	<b>6,04</b> q (7.1)	<b>124,1</b>
<b>9</b>	-	144,3	<b>10</b>	6.29 m	128.6	<b>9</b>	-	131,5	<b>9</b>	-	129,5
<b>10</b>	2,02 d (7.2)	14,8	<b>11</b>	7.07 dd (15.2/7.9)	152.8	<b>10</b>	1,71 d (7.0)	13,8	<b>10</b>	1,68 dd (7.1/1.5)	13,8
			<b>12</b>	6.08 dd (15.2/10.0)	130.0	<b>11</b>	-	167,2	<b>11</b>	-	167,2
			<b>13</b>	9.53 d (7.9)	194.1	<b>12</b>	3,71s	51,5	<b>12</b>	3,71s	51,5
						<b>1'</b>	4,25 m 4,16 m	65,5	<b>1'</b>	4,25 m 4,16 m	65,5
						<b>2'</b>	2,85 t (7.2)	34,6	<b>2'</b>	2,85 t (7.2)	34,6
						<b>3'</b>	-	130,1	<b>3'</b>	-	130,1
						<b>4'/8</b>	7,08 d (8.5)	130,6	<b>4'/8</b>	7,07 d (8.5)	130,6
						<b>5'/7</b>	6,75 d (8.5)	115,8	<b>5'/7</b>	6,76 d (8.5)	115,8
						<b>6'</b>	-	154,4	<b>6'</b>	-	154,4
						<b>1''</b>	<b>3,78</b> dq (9.4/7.0) <b>3,55</b> dq (9.4/7.0)	<b>64,7</b>	<b>1''</b>	<b>4,04</b> dq (9.4/7.0) <b>3,68</b> dq (9.4/7.0)	<b>66,3</b>
						<b>2''</b>	1.21 t (7.0)	15,4	<b>2''</b>	1.29 t (7.0)	15,3

**Table 2:** <sup>1</sup>H- and <sup>13</sup>C NMR data of the new EVOO constituents.

#### 4. Conclusion

A pioneering ACE-based extraction was developed from laboratory to pilot scale, leading for the first time to the recovery of EVOO phenolic fraction in large quantities. Prep-CPC analysis was conducted on TPF providing certain amounts of enriched fractions with EVOO biophenols. Furthermore, prep-HPLC was applied for the gram-scale isolation of OLEA, OLEO, HT, MFOA and MFLA in pure form. In parallel, the availability of considerable amounts of enriched CPC fractions provided the opportunity for in depth analysis and made possible the isolation of three novel natural products (EDA lactone, (*1R*, *8E*)-1-ethoxy-ligstroside aglycon, (*1S*, *8E*)-1-ethoxy-ligstroside aglycon) and a fatty acid that was isolated for the first time from EVOO. This highly productive methodology, with the advantage of industrial scale potentials, provide in short period of time, huge amounts of EVOO TPF and high-added value compounds in pure form. It should be underlined that is the first time where OLEA, OLEO, MFOA and MFLA are available in quantities able to meet the needs of *in vivo* experiments.

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#### Supplementary material

**Table S1:** The yields of the enriched in the target compounds CPC fraction recovered from the CPC fractionation of the three TPF extracts obtained after continuous liquid-liquid extraction of the three selected EVOO.

Analyzed TPF	Enriched CPC fractions				
	MFLA	MFOA	OLEO	OLEA	HT
<i>TPF-I</i>	102.3 mg	38.2 mg	1135.3 mg	1009.1 mg	143.5 mg
<i>TPF-II</i>	93.2 mg	845.8 mg	1045.2 mg	983.6 mg	112.6 mg
<i>TPF-III</i>	1348.5 mg	83.2 mg	845.3 mg	148.1 mg	372.9 mg
<b>Total</b>	1544 mg	967.2 mg	3025.8 mg	2140.8 mg	629 mg