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Toward a harmonized and standardized protocol for the determination of total hydroxytyrosol and tyrosol content in virgin olive oil (VOO). Extraction solvent

Running title: Standardizing VOO polar phenol extraction solvent

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Abstract: The determination of the total hydroxytyrosol (Htyr) and tyrosol (Tyr) content of virgin olive oil is of utmost interest for the International Olive Council (IOC), food authorities, producers and distributors after the issuing of a health claim that ‘olive oil polyphenols contribute to the protection of blood lipids from oxidative stress’. To address the need of a harmonized and standardized protocol the present study focuses on the extraction solvent of the polar fraction. Aqueous mixtures of methanol and acetonitrile of equal polarity were prepared and comparatively examined. Different analytical approaches (Folin-Ciocalteu assay, reversed phase chromatography-diode array-fluorescence detection, LC-Orbitrap, LC-TOF, LC-TQd, ¹H-NMR) were applied to highlight the extracting efficiency of the tested mixtures regarding the phenolic content and composition. The use of acetonitrile had not a clear positive effect that could compensate its higher cost, commercial availability and toxicity. The findings justify further why methanol:water, 80:20 v/v should be retained in a future IOC protocol for the accurate and repeatable determination of total Htyr and Tyr content, which is necessary to support the health claim for ‘olive oil polyphenols’.

Practical applications: The development of a harmonized and standardized methodology for the determination of total Htyr and Tyr content in an explicit manner is a request of the olive oil sector. A tool that can be introduced easily in the olive industry and official laboratories for the control of the label that bears a health claim for ‘olive oil polyphenols,’ is also requested by the consumers and the IOC. In this view, the present study contributes to the cornerstone that is the standardization of the extraction solvent.

Keywords: Virgin olive oil phenols/ liquid-liquid extraction/ health claim/ total hydroxytyrosol and tyrosol/ standardization

Abbreviations: ACN, acetonitrile; CA, caffeic acid; DA, diode array; DHPA, dihydroxyphenylacetic acid; DiMeA, dimethyl acetal; ESI, electrospray ionization; Et(30), solvent polarity parameter; F-C, Folin Ciocalteu; FL, fluorescence; FWHM, full width at half maximum; H-ESI, heated electrospray ionization; HRMS, high resolution mass spectrometer; Htyr, hydroxytyrosol; IOC, International Olive Council; MeHA, methyl hemiacetal; MeOH, methanol; MRM, multiple reaction monitoring; P', polarity index; QTOF, quadrupole time of flight; TOF time of flight; TPC, total phenol content; TQd, triple quadrupole; Tyr, tyrosol; VOO, virgin olive oil

1. Introduction

Hydroxytyrosol (Htyr) and tyrosol (Tyr) in their free and bound forms are the most abundant polar phenols of VOO.^[1] They contribute to its high resistance to oxidation, to various health benefits associated with its frequent consumption such as the protection of low-density lipoprotein particles from oxidative damage.^[2-4] The bound forms are responsible for the bitterness and astringency of the fresh product.^[4] The concentration and profile of these compounds depends on the cultivar, agricultural practices, fruit maturity and integrity, processing parameters and storage length and conditions all of which impact the commercial quality and claimed nutritional and health benefits of VOO. Therefore, since the late 60's much effort was devoted to the analysis of these compounds including the step of their extraction from the oil matrix.^[1, 4-7] The polar phenolic compounds are determined in the 'polar fraction' of the oil, which is obtained with the aid of polar solvents. Evidence built up over the years indicates that liquid-liquid extraction is more efficient than solid phase one for this purpose.^[4] Aqueous mixtures of methanol (MeOH), mainly MeOH:H₂O (80:20 v/v and 60:40 v/v), are used in the majority of applications,^[7] whereas, lately, the use of acetonitrile (ACN) is also discussed because - being a non-protic solvent - does not lead to the formation of artifacts as it is expected for MeOH. In addition, its use seems to be beneficial for the recovery of the complex forms of Htyr and Tyr.^[8, 9] Still, literature search among the

numerous papers on the analysis of olive oil polar phenolic compounds indicates the need for standardization of this critical step.

In the frame of OLEUM project, seeking for the harmonization of procedures that can then be adopted in the analysis of olive oil for both regulatory and research purposes^[10] a systematic work to standardize step by step a procedure for the determination of total Htyr and Tyr content is undertaken. In particular, this study addresses the effect of the polar phenol extraction solvent. Such a work is of utmost interest for IOC, European food authorities, producers and distributors^[11, 12] after the issuing of a health claim that ‘olive oil polyphenols contribute to the protection of blood lipids from oxidative stress’.^[13] For the aim of the study, aqueous mixtures of MeOH and ACN were comparatively examined as extraction solvents of the polar fraction. Ethanol, which is sometimes also reported,^[14, 15] was not included as it exerts similar selectivity to MeOH and it is more expensive than that. The extraction solvent mixture used in the protocol that has been adopted by the IOC for the determination of biophenols in olive oil by HPLC^[16] was the reference point for our efforts to standardize this step. Several analytical methods (Folin Ciocalteu, HPLC-DA-FL, ¹H-NMR, LC-MS) have been applied to evaluate the recovery of phenolic compounds using the selected extraction solvent mixtures and to verify the presence/absence of artifacts.

2. Materials and methods

2.1. Chemicals

Tyr (98%) and 1,3,5-triazine (97%) were products of Alfa Aesar GmdH & Co KG, (Karlsruhe, Germany). Caffeic acid (CA, 98%) and 3,4-dihydroxyphenylacetic acid (DHPA, 98%) were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Htyr (98%) was from Extrasynthèse (Genay, France). H₂O-d₂, (99.9%) and DMSO-d₆, (99.8%) were obtained from Deutero GmdH (Kastellaun, Germany). For mass spectrometry analysis, hexane and methanol of

mass spectrometric grade (MS SupraSolv®) and formic acid (Suprapur®) were purchased by Merck (Darmstadt, Germany). Water was of ultrapure Milli-Q grade. Nitrogen (Alphagaz N2, purity 99.999%, Air Liquid) was used in the Orbitrap as nebulization and fragmentation gas. Other reagents and solvents of appropriate grade were purchased from various producers.

2.2. Virgin olive oil samples

The VOO samples examined (S1-S30) were obtained directly from various producers and locations belonged to AUTH-OLEUM collection. Other details are given in the respective Tables and Figures.

2.3. Preparation of the polar fraction

2.3.1. Calculation of the extraction solvent mixture composition

The composition of aqueous ACN mixtures of equal polarity index values to the aqueous MeOH ones was calculated from the equation $P' = \varphi_a \times Pa + \varphi_b \times Pb$, where φ_a and φ_b is the volume fraction of each solvent in the binary mixture and Pa , Pb refers to P' values of the pure solvents. The P' values for MeOH (5.1), ACN (5.8) and H₂O (10.2) were from Snyder et al.^[17] Similarly, the composition of aqueous ACN mixtures of equal polarity $E_T(30)$ value to the aqueous MeOH ones was calculated from the equation $E_T(30) = 63.0412 - 0.1773 \times (\% \text{ ACN}) + 0.0010 \times (\% \text{ ACN})^2$ using the $E_T(30)$ values provided by Dorsey and Johnson.^[18] According to the authors the equation is valid for ACN:H₂O mixtures in the range 0-80% v/v.

2.3.2. Extraction procedure

The extraction procedure was as follows unless otherwise stated in the experimental, discussion, figures or tables: An aliquot of VOO (2.5 g) was dissolved in 5 mL of hexane and the polar fraction was extracted using an equal volume of the tested solvent. The mixture was vortexed for 2 min and centrifuged for 10 min at 3500 rpm.^[19] The extraction was carried out once after examination of the repeatability over the period of the last four years by different analysts ($CV\%=1.1-5.1\%$, $n=5$)

using the Folin-Ciocalteu (F-C) assay.

2.4. Colorimetric assessment of the total polar phenol content (TPC)

Suitable aliquots of the polar extracts were transferred in a 10 mL volumetric flask and, subsequently, water (5 mL) and the Folin-Ciocalteu reagent (0.5 mL) were added. After 3 min, 1 mL of saturated (37%, w/v) sodium carbonate solution was added to the reaction mixture. The solution was diluted with water to 10 mL and after 1 h the absorbance at 725 nm was measured against a blank solution with a spectrophotometer UV-1601 (Shimadzu Co., Kyoto, Japan). Caffeic acid (CA) was used as an external standard^[19] and results were expressed as mg CA/20 g of oil. The determination was performed in triplicate for each extract (the repeatability over the period of the last four years by different analysts was $CV\%=1.1-2.7\%$, $n=5$).

2.5. Acidic hydrolysis of the polar fraction

An aliquot (200 μ L) of the polar fraction was mixed with 200 μ L of a 1 M H₂SO₄ solution. The mixture was incubated in a water bath at 80 °C for 2 h.^[14] The procedure was carried out in triplicate. Each hydrolysate was then diluted with 200 μ L of the polar fraction extracting solvent. The three replicates were combined to obtain a representative hydrolysate. The latter was filtered through a 0.45 μ m pore size regenerated cellulose membrane (Schleicher and Schuell, MicroScience GmbH, Dassel, Germany) before injection onto the chromatograph.^[19] Analysis was carried out in triplicate. The intra and interday repeatability of hydrolysis for Htyr and Tyr determination were ($CV\%$) 0.9/2.4 and 1.5/2.2 ($n=5$), respectively.

2.6. RP-HPLC analysis of the polar phenolic compounds

The HPLC system at AUTH was consisted of a pump, model P4000 (Thermo Separation Products, San Jose, CA, USA), a Midas autosampler (Spark, Emmen, The Netherlands), and a UV 6000 LP diode array (DA) detector (Thermo Separation Products) in series with an SSI 502 fluorescence (FL)

detector (Scientific Systems Inc., State College, PA, USA). Phenolic compounds in the tested extracts were monitored at 280 nm using DA and at 280 nm excitation and 320 nm emission using FL detection. The data were processed with the aid of Chrom Quest software (version 3.0, Thermo Separation Products). Analysis was carried out on a Nucleosil 100, C18 (250 × 4.6 mm, 5 µm) column (MZ-Analysentechnik GmbH, Mainz, Germany). The elution solvent was consisted of 1% aqueous acetic acid (solvent A) and ACN (solvent B). The gradient used was: 0 – 2 min, 5% B; 2 – 15 min, 25% B; 15 – 22 min, 25% B; 22 – 30 min, 40% B; 30 – 40 min, 60% B; 40 – 50 min, 95% B; 50 – 52 min, 95% B; 52 – 54 min, 100% B; 54 – 60 min, 5% B, at a flow rate of 0.5 mL/min and an injection volume of 10 µL.^[19] In certain experiments (section 3.2) the separation was carried out using the gradient proposed by IOC^[16] or with slight modifications. Quantitative data using various standards were expressed as mg or µmol /20 g oil.

2.7. LC-MS analysis

LC-MS analysis of the same samples was carried out at 3 different laboratories, each one having different MS facilities within a specific timetable (~2 months): (i) an LC system consisted of a Vanquish pump and autosampler (Thermo Scientific – Dionex Softron, Germering, DE), coupled to a Q-Exactive hybrid Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany), which was equipped with an electrospray source (H-ESI II) at UB; (ii) a 1200 Series LC and a 6230 time-of-flight (TOF) mass spectrometer (Agilent, Santa Clara, CA, USA) at FERA and (iii) an Infinity 1260 HPLC (Agilent Technologies, Waldbronn, Germany) consisted of a G1312B binary pump, a G4225 degasser, a G1329B ALS, a G1330B thermostat, a G1316A thermostated column compartment and a G4212B DA detector (190-600 nm) interfaced with a triple-quadrupole (TQd) mass spectrometer (Triple Quad G6420A LC/MS; Agilent Technologies, Singapore) at ZRS. Details on the elution conditions adjusted to each MS instrument are provided as supplementary material (Text S1).

2.8. ¹H-NMR analysis

¹H-NMR experiments were conducted on a 500 MHz NMR (Agilent Technologies Inc., Santa Clara, CA). The probe temperature was 25°C. All chemical shifts were given in ppm from TMS (δ 0.00). For quantitative ¹H-NMR measurements, the experimental parameters were: repetition time (acquisition time + relaxation delay) 12s, 32k data points, number of scans 32 with 4 dummy scans. Baseline correction (applying a polynomial fourth-order function) and phasing were carried out manually prior to the integration process using appropriate software (Mestrelab Research Lab, ver. 6.0.2-5475, 2009, La Coruña, Spain). The preparation of the polar fraction using MeOH:H₂O, 80:20, v/v, MeOH:H₂O, 60:40, v/v or ACN:H₂O, 70:30, v/v, and total Htyr and Tyr determination in the polar fraction was carried out according to Dais and Christophoridou.^[20] The results were expressed as $\mu\text{mol}/20\text{ g}$ of oil. The CV% of the measurement for a VOO sample was 0.6 ($n=5$).

2.9. Statistical analysis

Statistical comparisons of the mean values were performed by one-way ANOVA, followed by the multiple Duncan test ($p<0.05$ confidence level) using the SPSS 14.0 software (SPSS Inc., Chicago, IL, USA). The same software was used for pairwise student's t-test comparison of means at $p<0.05$ where necessary.

3. Results and discussion

3.1. Selection of aqueous acetonitrile composition

MeOH and ACN are two polar solvents of different selectivity characteristics (Table S1)^[17, 21] so that the study of their extracting efficiency at equal percentages in aqueous mixtures^[22] is not the most appropriate one. In the present study selection of the most efficient aqueous ACN mixture with regard to the performance of the aqueous MeOH mixture proposed by IOC, namely MeOH:H₂O, 80:20 v/v, was, thus, based on different selectivity criteria and not on percentages.

The aqueous ACN mixtures tested had a composition of comparable polarity to those of the MeOH one using either the Snyder polarity index (P')^[17] or the solvent polarity parameter $E_T(30)$.^[18] The P' is a measure of solvent ability to interact with solutes of different functionality,^[17] whereas $E_T(30)$ is a descriptor of both hydrogen bond and electrostatic interactions of the solvent.^[23] The percentages were calculated as described in section 2.3.1. The mixtures examined were, consequently, ACN: H₂O 93:7, v/v (P' index) and 51:49, v/v ($E_T(30)$ parameter). Moreover, the mixtures 70:30 v/v (P' index) and 39:61, v/v ($E_T(30)$ parameter) corresponding to the polarity of the MeOH:H₂O, 60:40, v/v were tested to highlight further the overall performance differences between the two organic solvents. The tests were performed on a VOO (S1) containing both free and bound forms of Htyr and Tyr because it was a blend of freshly produced oil and of an oil stored at room temperature for about one year in the dark. Estimation of the solvent efficiency was evaluated in terms of F-C assay and HPLC determination of the TPC. Pure ACN and its 93% aqueous mixture extracted lipids and pigments that are expected to interfere in subsequent analyses, colorimetric or liquid chromatographic, so that they were rejected as options. From the rest of the combinations of ACN:H₂O mixtures the most efficient was the 70:30, v/v one with reference to the IOC proposed solvent (Table 1). The latter was in turn more efficient than the MeOH:H₂O, 60:40, v/v ($p < 0.05$). The same trend was observed by both the colorimetric and chromatographic analysis. These observations were verified for 5 more VOOs (S2-S6) randomly picked up from the AUTH OLEUM collection (Table 2).

Information from Table 2 together with that obtained for the total Htyr and Tyr content determined using ¹H-NMR spectroscopy (Table 3) gave a strong evidence that further examination of the MeOH:H₂O, 60:40, v/v mixture was not promising for the aim of a standardised protocol. The other two solvent systems deserved further examination.

3.2. MeOH:H₂O, 80:20, v/v, vs ACN:H₂O, 70:30, v/v mixtures as extraction solvents

The VOO polar fraction is used for the estimation of the total content or individual phenol before or after hydrolysis, mainly using RP-HPLC.^[5, 15, 16, 19, 24-26] Data shown in Figure 1 for the TPC (mg

CA /20 g oil) for twenty-one (21) authentic VOOs (S7-S27) extracted with the two prevailing solvent mixtures indicated that aqueous ACN gave statistically significant higher values only in five samples. In particular, these values were only 1.1-1.3-fold higher, suggesting that the two solvent mixtures are of equivalent potency. Further insight in quantitative and qualitative differences was sought using RP-HPLC-DA-FL and LC-MS analyses.

3.2.1. RP-HPLC-DA-FL analysis

Introduction of the polar extract to the liquid chromatograph is usually carried out after drying and redilution in a volume of MeOH:H₂O or MeOH even if in the gradient elution ACN is used.^[19, 22, 24, 25, 27] In the IOC recommended protocol, the polar fraction is injected directly onto the HPLC system probably to avoid further sample manipulation, which may increase the labour and time of analysis. There is no published paper that details all the steps of the development of this protocol which has been accepted after a collaborative study and data evaluation according to ISO 5725.^[16] Seeking for differences between the two extraction solvent mixtures, the VOOs S10, S13, S17, S19 and S24 were also examined using this protocol. The samples were of the same cultivar and location and contained almost comparable TPC by F-C. The choice aimed at reducing the effect of cultivar, geographical origin and magnitude of content among findings. The profiles recorded using DA and FL detectors are given in Figure 2. Quantification was carried out before (TPC) and after hydrolysis (total Htyr and Tyr content) at 280 nm. Different standard curves or result expression were used. The quantitative results are presented in Table 4.

From the recorded profiles of the two extracts it was observed that they were qualitatively similar using either DA or FL detection. The F-C findings (see Figure 1) were not reflected in the chromatograms but for all pairs of samples -regardless of the standard used for quantification of the TPC- the values obtained for the aqueous MeOH extracts differed statistically from those of the aqueous ACN ones (Table 4). Using Htyr for quantification, lower values (1.67-1.70 fold) than

those acquired with Tyr as a standard were obtained. These values were only 1.02-1.07 fold higher than those expressed as DHPA equivalents. The latter phenolic acid was selected as a possible alternative to Htyr considering the structural similarity and its lower cost. Despite the fluctuation in the absolute values, the use of different standards did not augment or suppress the relative differences. As a consequence, the findings led to similar judgments. Focusing on total Htyr and Tyr content by hydrolysis of the polar fraction, statistical significant differences were observed in three of the samples for both constituents. Nevertheless, no clear superiority of one solvent mixture over the other was observed regardless of the result expression.

3.2.2. LC-MS analysis

Three pairs of polar phenol extracts from samples S28, S29 and S30 were then prepared using MeOH:H₂O, 80:20, v/v and ACN:H₂O, 70:30, v/v. Their profiles recorded at 280 nm (Figure S1) were qualitatively similar. Then, they were further studied by different LC-MS facilities, to unravel, mainly, any qualitative differences. In all cases the organic solvent of the gradient was MeOH:ACN, 1:1, v/v as in the IOC protocol.^[16] The ESI⁺ and ESI⁻ full scan chromatograms of the extracts in the mass range m/z 315-395 (Figures 3 and 4, respectively) obtained by the LC-Orbitrap mass spectrometer showed no qualitative differences. This was further verified for the main bound forms on the basis of the extracted ion chromatograms using their exact mass as presented in the same Figures. Only quantitative differences were observed. Thus, aqueous ACN extraction favored some compounds eluting at the end of the chromatogram (e.g. $t_R=18.10$, m/z 377.1242 [M-H]⁻, C₁₉H₂₁O₈; $t_R=20.23$, m/z 361.1293 [M-H]⁻, C₁₉H₂₁O₇). On the other hand, aqueous MeOH extraction favored some more polar derivatives (e.g. $t_R=12.82$, 13.33 m/z 377.1242 [M-H]⁻, C₁₉H₂₁O₈; $t_R=14.92$, 15.49, m/z 361.1293 [M-H]⁻, C₁₉H₂₁O₇). The evidence that aqueous ACN favored the extraction of less polar compounds was verified by LC-TOF ($t_R=15.054$ min, m/z 361.1290 [M-H]⁻; $t_R=16.499$ min, m/z 377.1237 [M-H]⁻) (Figure S2). The hypothesis that artifacts due to the presence of MeOH in the extract could be detected was examined for oleacein and

oleocanthal ones using also TQd at the MRM mode.^[28, 29] Using all LC-MS facilities the methyl hemiacetal (MeHA) of the two phenols was detected in both type of extracts. Only with the aid of TQd at the MRM mode, which is the most sensitive type of experiment, the dimethylacetal (DiMeA) of oleocanthal was found in all the extracts, whereas DiMeA of oleacein in the two extracts only of S28. The artifacts eluted at the same retention time with that of the precursors. Furthermore, calculation of the relative concentration of the artifacts detected (Table S.2) on basis of the peak area recorded by the three MS platforms showed that their levels in both type of extracts per sample were always comparable (maximum difference $\leq 2\%$). Such an observation indicates an effect of the mobile phase composition and not of the extraction system. Present findings are in agreement with those of Sánchez-de Medina et al.^[29] who analysing with LC-QTOF at MRM mode a pure ACN or a MeOH:H₂O, 60:40, v/v polar extract: (i) reported a similar % formation of oleacein and oleocantal artifacts (~16%)- the MeHAs were the main artifacts-in both of them when the elution protocol had MeOH in the gradient composition; (ii) when the same extracts were analyzed using a gradient containing only ACN, the corresponding artifacts were not detected. Thus, using the IOC proposed HPLC protocol the presence of MeOH artifacts is unavoidable regardless of the extract solvent.

4. Conclusion

The systematic work proved to be useful toward harmonization and standardization of an extraction protocol for the polar fraction of olive oil, which then can be used for the determination of total polar phenols or of the total content of Htyr and Tyr. The use of ACN had not a clear positive effect that can compensate its higher cost, commercial availability and toxicity that could support its adoption in a standardized protocol. The findings of this work justify further why MeOH:H₂O should be retained in an IOC protocol for the accurate and repeatable determination of total Htyr and Tyr content necessary to support the health claim for 'olive oil polyphenols'.

Artifacts are rather related to the presence of MeOH in the elution gradient but if the total Htyr and Tyr content is determined after hydrolysis of the polar fraction no effect in the quantitation should be expected.

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Conflict of interest

The authors state that there is no conflict of interest

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Table 1. TPC of sample S1 estimated by F-C assay and RP-HPLC- DA (280 nm) or FL (exc 280 nm/em 320 nm) detection after extraction with different MeOH and ACN aqueous mixtures

	F-C assay	HPLC-DA	HPLC-FL
Extraction solvent (v/v)	TPC (mean value \pm SD, $n=3$)*,**		
MeOH:H ₂ O, 80:20	3.18 \pm 0.12 ^a	2.15 \pm 0.02 ^a	1.33 \pm 0.01 ^a
MeOH:H ₂ O, 60:40	2.46 \pm 0.03 ^b	1.86 \pm 0.01 ^b	1.13 \pm 0.01 ^b
ACN:H ₂ O, 70:30	3.54 \pm 0.02 ^c	2.26 \pm 0.02 ^c	1.37 \pm 0.01 ^c
ACN:H ₂ O, 51:49	2.58 \pm 0.08 ^{b,d}	2.00 \pm 0.01 ^d	1.29 \pm 0.00 ^d
ACN:H ₂ O, 39:61	2.50 \pm 0.11 ^{b,d}	1.86 \pm 0.02 ^b	1.20 \pm 0.01 ^e

*TPC values expressed as mg CA /20 g oil (F-C); mg Tyr /20 g oil (HPLC- DA_{280 nm} and FL_{exc 280 nm/em 320 nm});

**Values in the same column bearing different lower case letters as superscripts are statistically different ($p < 0.05$)

Table 2. TPC of VOOs (S2-S6) estimated by the F-C assay and RP-HPLC- DA (280 nm) or FL (exc. 280 nm/em. 320 nm) detection after extraction with different MeOH and ACN aqueous mixtures

Extraction solvent (v/v)	VOOs				
	S2	S3	S4	S5	S6
	F-C assay				
	TPC				
	mean value \pm SD, $n=3$, mg CA /20 g oil*				
MeOH:H ₂ O, 60:40	3.71 \pm 0.08 ^a	4.23 \pm 0.07 ^a	3.16 \pm 0.06 ^a	3.54 \pm 0.09 ^a	2.14 \pm 0.03 ^a
MeOH:H ₂ O, 80:20	4.23 \pm 0.07 ^b	4.53 \pm 0.12 ^b	3.57 \pm 0.06 ^b	4.17 \pm 0.06 ^b	2.62 \pm 0.04 ^b
ACN:H ₂ O, 70:30	4.08 \pm 0.03 ^c	4.42 \pm 0.05 ^b	3.44 \pm 0.07 ^{b,c}	3.97 \pm 0.07 ^c	2.77 \pm 0.05 ^c
	RP-HPLC DA/(FL)				
	TPC				
	mean value \pm SD, $n=3$, mg Tyr /20 g oil*				
MeOH:H ₂ O, 60:40	2.41 \pm 0.06 ^a	2.33 \pm 0.08 ^a	1.78 \pm 0.03 ^a	2.01 \pm 0.03 ^a	1.20 \pm 0.02 ^a
MeOH:H ₂ O, 80:20	(0.77 \pm 0.01 ^a)	(1.47 \pm 0.05 ^a)	(0.73 \pm 0.01 ^a)	(1.0 \pm 0.00 ^a)	(0.57 \pm 0.01 ^a)
MeOH:H ₂ O, 80:20	2.45 \pm 0.04 ^a	2.81 \pm 0.10 ^b	1.81 \pm 0.03 ^b	2.16 \pm 0.03 ^b	1.35 \pm 0.01 ^b
MeOH:H ₂ O, 80:20	(0.80 \pm 0.00 ^b)	(1.73 \pm 0.05 ^b)	(0.82 \pm 0.02 ^b)	(1.07 \pm 0.01 ^b)	(0.58 \pm 0.00 ^a)
ACN:H ₂ O, 70:30	2.46 \pm 0.04 ^a	2.85 \pm 0.01 ^b	1.92 \pm 0.04 ^c	2.32 \pm 0.03 ^c	1.41 \pm 0.02 ^c
ACN:H ₂ O, 70:30	(0.85 \pm 0.01 ^c)	(1.75 \pm 0.02 ^b)	(0.90 \pm 0.01 ^c)	(1.26 \pm 0.01 ^c)	(0.62 \pm 0.00 ^b)

*Values in the same column, within each technique, bearing different lower case letters as superscripts are statistically different ($p < 0.05$)

Table 3. Total Htyr and Try content of VOOs (S1-S6) determined by ¹H-NMR after extraction with different MeOH and ACN aqueous mixtures

Extraction solvent (v/v)	VOOs					
	S1	S2	S3	S4	S5	S6
	Total Htyr and Tyr μmol /20 g oil					
MeOH:H ₂ O, 60:40	30.5	26.0	19.1	18.9	32.9	30.4
MeOH:H ₂ O, 80:20	33.1	27.0	27.3	24.0	42.0	31.3
ACN:H ₂ O, 70:30	34.4	28.0	28.1	25.8	37.8	32.6

Table 4. TPC, total Htyr and Tyr content of 5 monovarietal VOOs determined by HPLC-DA (280 nm) after extraction with MeOH:H₂O, 80:20, v/v or ACN:H₂O, 70:30, v/v

Extraction solvent (v/v)	Sample	TPC*			Htyr*	Tyr*	Sum of Htyr and Tyr	Htyr ^{*,**}	Tyr ^{*,**}	Sum of Htyr and Tyr ^{**}	Htyr*	Tyr*	Sum of Htyr and Tyr
		Htyr	Tyr	DHPA									
External standards for quantification used		mg /20 g oil						μmol /20 g oil					
mean values ± SD (n= 3)													
		Before hydrolysis			After hydrolysis								
MeOH:H ₂ O, 80:20	S10	2.69 ± 0.35 ^a	4.55 ± 0.56 ^a	2.59 ± 0.29 ^a	1.48 ± 0.08 ^a	1.74 ± 0.10 ^a	3.22	3.26 ± 0.18 ^a	4.35 ± 0.26 ^a	7.61	9.6± 0.5 ^a	12.6 ±0.7 ^a	22.2
ACN:H ₂ O, 70:30		3.81 ± 0.01 ^b	6.35 ± 0.01 ^b	3.53 ± 0.01 ^b	1.35 ± 0.00 ^a	1.60 ± 0.01 ^a	2.95	2.97 ± 0.01 ^a	3.99 ± 0.02 ^a	6.96	8.8± 0.0 ^a	11.6 ± 0.1 ^a	20.3
MeOH:H ₂ O, 80:20	S13	2.99 ± 0.11 ^a	5.03 ± 0.18 ^a	2.84 ± 0.09 ^a	1.56 ± 0.03 ^a	2.41 ± 0.06 ^a	3.97	3.42 ± 0.06 ^a	6.03 ± 0.14 ^a	9.45	10.1± 0.2 ^a	17.4 ± 0.4 ^a	27.6
ACN:H ₂ O, 70:30		2.39 ± 0.22 ^b	4.07 ± 0.36 ^b	2.33 ± 0.19 ^b	2.02 ± 0.03 ^b	2.88 ± 0.03 ^b	4.90	4.45 ± 0.06 ^b	7.19 ± 0.07 ^b	11.64	13.1± 0.2 ^b	20.8 ± 0.2 ^b	33.9
MeOH:H ₂ O, 80:20	S17	3.05 ± 0.02 ^a	5.13 ± 0.03 ^a	2.89 ± 0.01 ^a	1.64 ± 0.00 ^a	3.18 ± 0.02 ^a	4.83	3.62 ± 0.01 ^a	7.96 ± 0.04 ^a	11.57	10.6± 0.0 ^a	23.0 ± 0.1 ^a	33.7
ACN:H ₂ O, 70:30		3.65 ± 0.11 ^b	6.10 ± 0.17 ^b	3.40 ± 0.09 ^b	1.70 ± 0.02 ^b	2.96 ± 0.03 ^b	4.65	3.73 ± 0.04 ^b	7.40 ± 0.08 ^b	11.13	11.0± 0.0 ^b	21.4 ± 0.2 ^b	32.5
MeOH:H ₂ O, 80:20	S19	3.05 ± 0.06 ^a	5.13 ± 0.10 ^a	2.89 ± 0.05 ^a	1.11 ± 0.02 ^a	1.97 ± 0.10 ^a	3.08	2.44 ± 0.04 ^a	4.77 ± 0.23 ^a	7.21	7.2± 0.1 ^a	14.3 ±0.7 ^a	21.5
ACN:H ₂ O, 70:30		2.62 ± 0.07 ^b	4.43 ± 0.11 ^b	2.56 ± 0.42 ^b	1.53 ± 0.02 ^b	2.19 ± 0.02 ^b	3.73	3.37 ± 0.04 ^b	5.33 ± 0.06 ^b	8.70	9.9± 0.1 ^b	15.9 ±0.1 ^a	25.8
MeOH:H ₂ O, 80:20	S24	3.80 ± 0.58 ^a	6.34 ± 0.94 ^a	3.52 ± 0.49 ^a	2.32 ± 0.06 ^b	3.15 ± 0.06 ^a	5.47	5.10 ± 0.13 ^a	7.71 ± 0.18 ^a	12.81	15.0± 0.4 ^a	22.8 ±0.4 ^a	37.8
ACN:H ₂ O, 70:30		2.63 ± 0.33 ^b	4.46 ± 0.53 ^b	2.54 ± 0.28 ^b	2.49 ± 0.04 ^b	3.16 ± 0.05 ^a	5.65	5.48 ±0.09 ^b	7.76 ± 0.12 ^a	13.23	16.2± 0.3 ^a	22.9 ±0.4 ^a	39.0

*Values within the same column and for each pair of extracts of the same sample bearing different lower case letters as superscripts are statistically different ($p < 0.05$); ** According to the correction proposed by Mastralexi et al.^[19]; HPLC conditions according to IOC^[16]

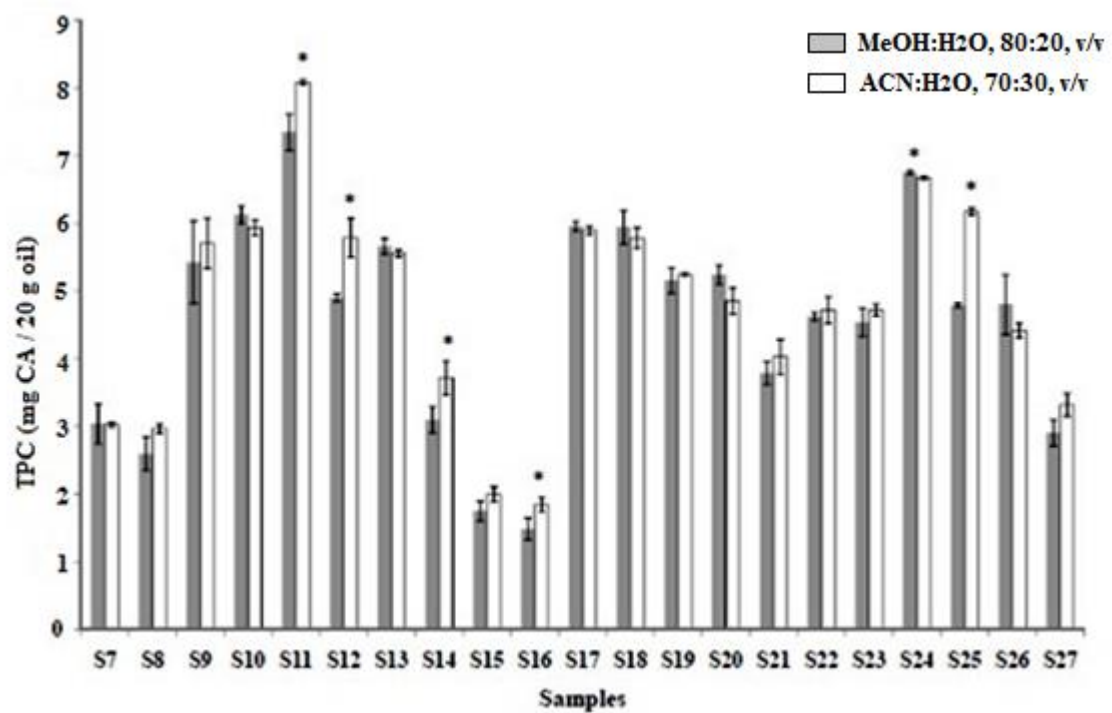


Figure 1

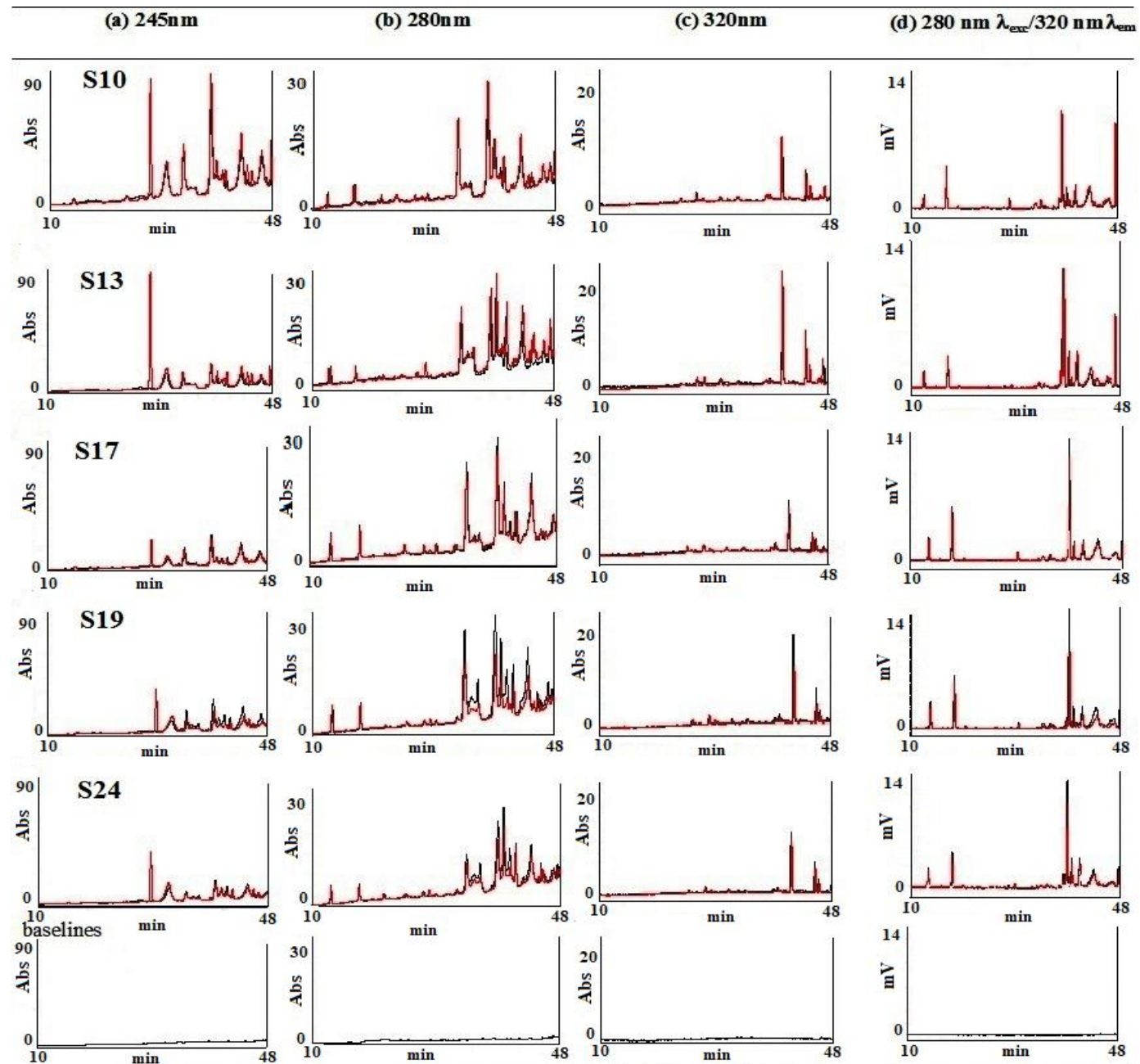


Figure 2

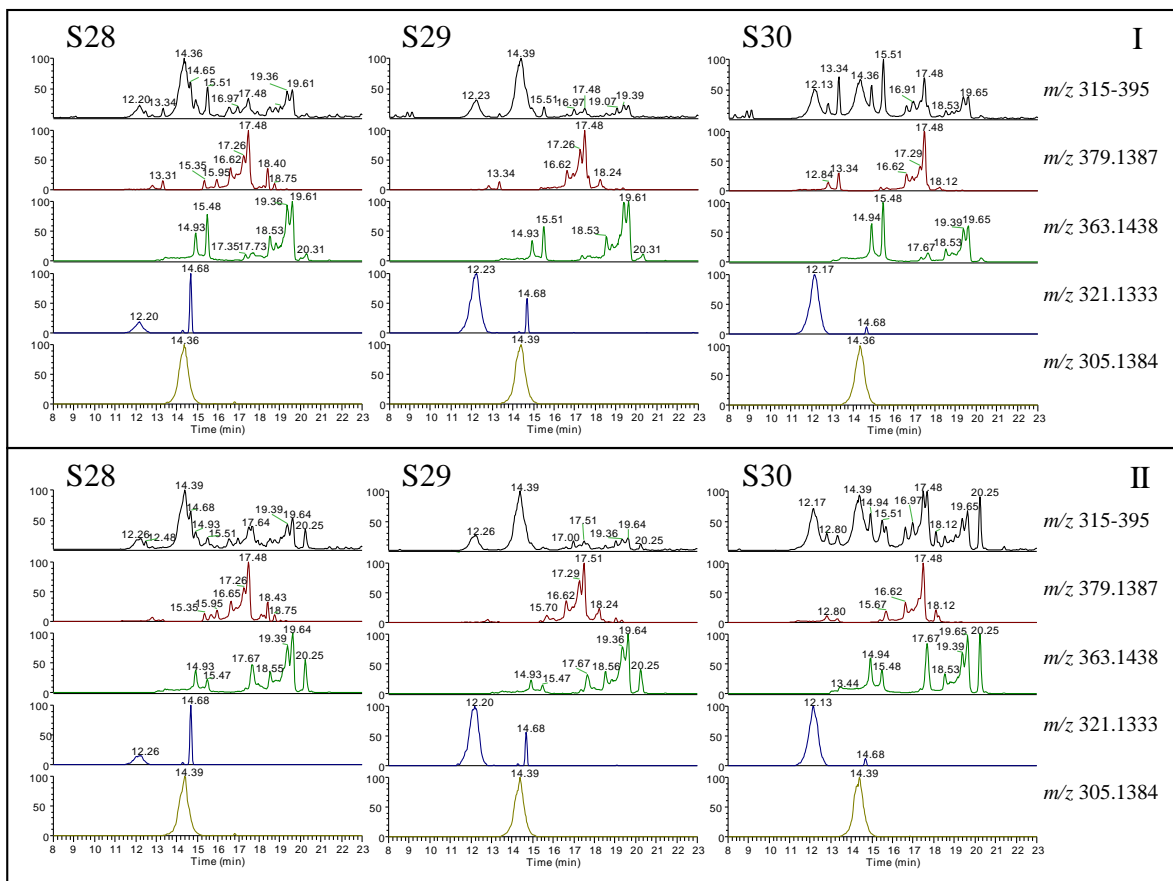


Figure 3

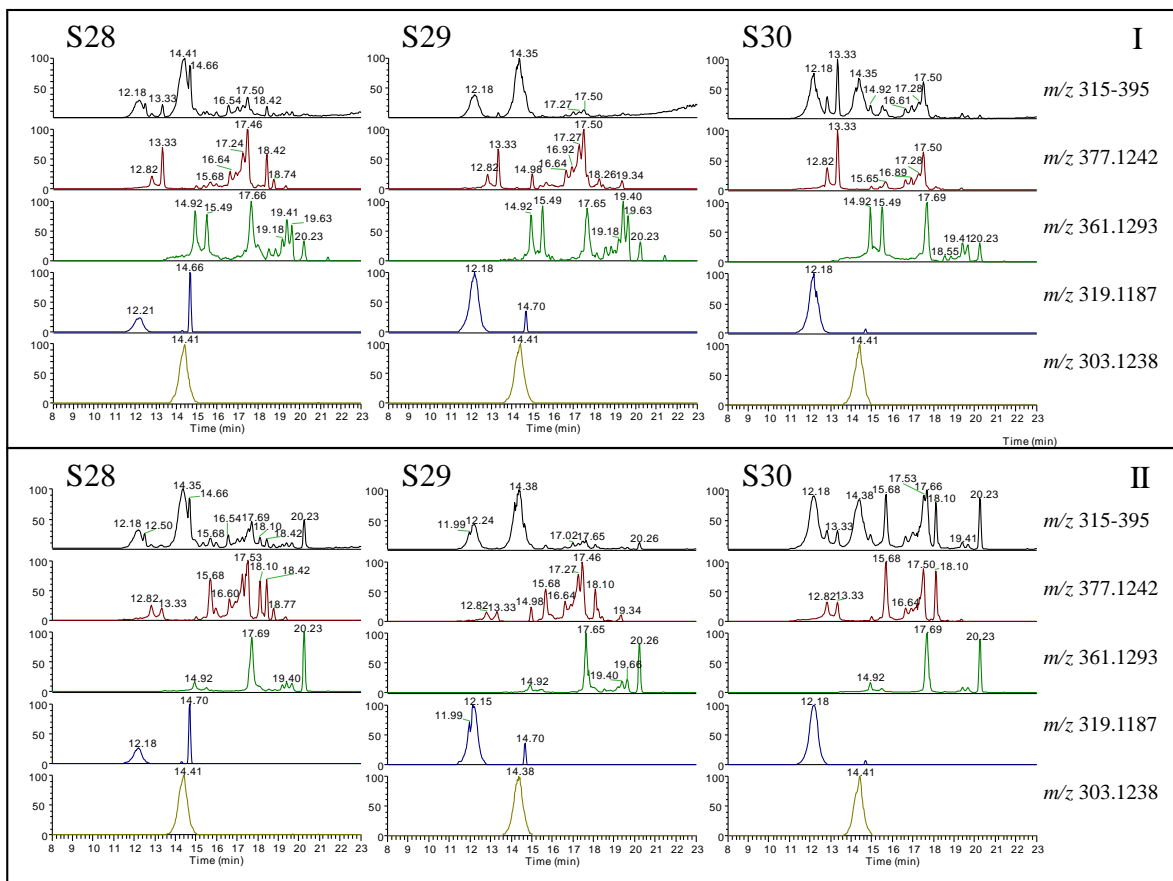


Figure 4

Figure legends

Figure 1. TPC (mg CA/20 g oil) of samples S7-S27 estimated with the F-C assay after extraction with MeOH:H₂O, 80:20 v/v (■) or ACN:H₂O, 70:30 v/v (□) (mean value ± SD, *n*=3; *denotes statistical significant differences at *p*<0.05)

Figure 2. HPLC-DA-FL detection profile of the polar fractions of tested oils and the baselines recorded at (a) 245 nm, (b) 280 nm, (c) 320 nm, (d) 280 nm $\lambda_{exc}/320$ nm λ_{em} (red line: aqueous acetonitrile; black line aqueous methanol; IOC elution system containing MeOH:ACN, 1:1, v/v)

Figure 3. ESI⁺ full scan HRMS (orbitrap) chromatograms of polar fractions of tested oils obtained with (I) MeOH:H₂O, 80:20, v/v; (II) ACN:H₂O, 70:30, v/v. Chromatograms were obtained by selecting the mass range *m/z* 315-395 or the exact mass of the main secoiridoids: oleuropein aglycon (*m/z* 379.1387, C₁₉H₂₃O₈), ligstroside aglycon (*m/z* 363.1438, C₁₉H₂₃O₇), deacetoxy oleuropein aglycon (*m/z* 321.1333, C₁₇H₂₁O₆) and deacetoxy ligstroside aglycon (*m/z* 305.1384, C₁₇H₂₁O₅). R: 70,000 (*m/z* 200, FWHM).

Figure 4. ESI⁺ full scan HRMS (orbitrap) chromatograms of polar fractions of tested oils obtained with (I) MeOH:H₂O, 80:20, v/v; (II) ACN:H₂O, 70:30, v/v. Chromatograms were obtained by selecting the mass range *m/z* 315-395 or the exact mass of the main secoiridoids: oleuropein aglycon (*m/z* 377.1242, C₁₉H₂₁O₈), ligstroside aglycon (*m/z* 361.1293, C₁₉H₂₁O₇), deacetoxy oleuropein aglycon (*m/z* 319.1187, C₁₇H₁₉O₆) and deacetoxy ligstroside aglycon (*m/z* 303.1238, C₁₇H₁₉O₅). R: 70,000 (*m/z* 200, FWHM).

Supplementary material

Toward a harmonized and standardized protocol for the determination of total hydroxytyrosol and tyrosol content in VOO. Extraction solvent

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Text S1

LC-MS analysis

(1) HRMS (UB): Chromatographic column: Halo C18 Fused-Core column 2.1 mm×100 mm, 2.7 μm (Advanced Materials Technology, Wilmington, DE, USA).

Gradient: Solvent A: water 0,2% formic acid Solvent B: MeOH:ACN 50:50 flow rate: of 400 μL/min. Initial: 96% (A)–4% (B), to 50% (B) at 20 min; to 60% (B) at 22.5 min, to 100%(B) at 30 min, 5 min maintenance until 35 min, then 96% (A)–4% (B) at 36 min, followed by 5 min of equilibration. Injection volume: 1 μL.

Mass spectrometric analysis was carried out with a Q-Exactive hybrid Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) equipped with an electrospray source (H-ESI II). The conditions in negative ionization mode were: spray voltage 2.5 kV, capillary voltage –25 V, skimmer voltage –18 V, tube lens voltage –110 V. Optimized conditions in positive mode were: spray voltage 3.00 kV, capillary voltage 32.5 V, skimmer voltage 18 V, tube lens voltage 110 V. In both cases, sheath gas flow rate was set at 55 arbitrary units (au), auxiliary gas flow rate was 25 au, capillary temperature was 250 °C, and heater temperature was 25 °C. Elemental composition and structural information were in a single injection with the Orbitrap mass analyzer alternating full scan mode and all ions fragmentation (AIF) mode, in negative and positive ionization modes at a resolution power of 70,000 (m/z 200, FWHM).

(2) HRMS (FERA): Chromatographic column: Hypersil AA-ODS 2.1 x 200 mm 5 μm column (Agilent Technologies, Santa Clara, CA, USA)

Gradient: Formic acid (0.1%) in water (aqueous) and ACN:MeOH (1:1, v/v organic) were used as the mobile phases. The gradient was initially held at 10% organic for 5 minutes with a linear gradient to 100% organic by 25 min before being held at 100% for 5 minutes.

The flow rate was 0.5 mL/min and the injection volume was 2.5 μ L.

Mass spectrometric analysis was carried out with an Agilent Series 1200 HPLC system (Agilent Technologies, Santa Clara, CA, USA) connected to the time-of-flight mass spectrometer Agilent (TOF) 6230 (Agilent Technologies, Santa Clara, CA, USA) equipped with an electrospray interface operating in the negative ionization mode. The following operation parameters were used: capillary voltage 2,500 V, drying gas 9 L min⁻¹, gas temperature 350 C, nebulizer pressure 40 psi, and fragmentor voltage 150 V. LC–TOF-MS accurate mass spectra were recorded across the range of m/z 50–1,100.

(3) TQd (ZRS, Science and Research Centre Koper, Laboratory of the Institute for Olive culture):

Chromatographic column: Synergi 4 μ m Hydro – RP 80 A (250 \times 4.6 mm) protected with Security Guard Cartridge AQC18 4 \times 3.0 mm (both Phenomenex, Torrance, USA).

Gradient: The one proposed by IOC ^[16], phosphoric acid substituted with 0.1% formic acid in aqueous phase.

Mass spectrometric analysis was carried out with Agilent Technologies Infinity 1260 HPLC equipped with 6420 Triple Quad ESI instrument operating in negative ion mode as follows: gas temperature 300 °C, gas flow 10 L min⁻¹, nebulizer pressure 50 psi and capillary voltage 3 kV.

Detection: The main parameters for MS/MS using the MRM mode are the following:

Species	Precursor	Product	Frag (V)	CE (V)	CACC (V)	Polarity
Oleacein	319	123	110	18	7	Negative
MeHAOleacein	351	123	110	18	7	Negative
DiMeAOleacein	365	123	110	18	7	Negative
Oleochantal	303	137	110	18	7	Negative
MeHAOleochantal	335	137	110	18	7	Negative
DiMeAOleochantal	349	137	110	18	7	Negative
Htyr	153	123	100	20	7	Negative
Tyr	137	108	135	15	7	Negative

MeHA:methyl hemiacetal; DiMeA: dimethyl acetal; Htyr:hydroxytyrosol; Tyr: Tyrosol

Table S1. Solvent properties with regard to their chromatographic performance

Solvent	$E_T(30)$	π^*	A	β	ϵ	P'
H ₂ O	63.1	0.45	0.43	0.18	80	10.2
MeOH	50.4	0.28	0.43	0.29	32.7	5.1
ACN	45.6	0.60	0.15	0.54	37.5	5.8

Snyder et al. ^[17]**Table S2.** Peak areas of oleacein, oleocanthal and their methyl and dimethyl acetals (relative concentration expressed as %) in the polar fraction of tested VOOs obtained with ACN:H₂O, 70:30, v/v or MeOH:H₂O, 80:20, v/v using TQd-MRM, TOF and Orbitrap for the analysis

	VOOs					
	S28		S29		S30	
	Solvent mixture					
	ACN:H ₂ O, 70:30, v/v	MeOH:H ₂ O, 80:20, v/v	ACN:H ₂ O, 70:30, v/v	MeOH:H ₂ O, 80:20, v/v	ACN:H ₂ O, 70:30, v/v	MeOH:H ₂ O, 80:20, v/v
LC-MS facility						
Orbitrap						
Oleacein	85835241	67561676	143661337	141667702	365383870	260374323
Oleocanthal	62377440	55477011	81823864	72422248	67873804	58519602
MeHAOleacein	79665217	70122635	132239975	134349456	333036269	246328731
DiMeAOleacein	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
MeHAOleocanthal	1459851	1237599	2116156	2505889	1757523	1496218
DiMeAOleocanthal	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Sum of artifacts as %	35.4	36.7	37.3	39.0	43.6	43.7
TOF						
Oleacein	1989372	1966709	4013786	3944416	5937125	5645346
Oleocanthal	1661351	1775142	2872390	3036383	1776450	1686786

MeHAOleacein	15102	9340	12285	9433	21889	22861
DiMeAOleacein	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
MeHAOleocanthal	37109	47615	60867	64885	40907	40127
DiMeAOleocanthal	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
<i>Sum of artifacts as %</i>	1.41	1.50	1.05	1.05	0.81	0.85
<i>TQd</i>						
Oleacein	4062	5565.5	17117.5	16197.5	19327.5	23650.5
Oleocanthal	20220	27816.5	79475.5	76655.5	37827	49767.5
MeHAOleacein	552	495	1705	1710	2478.5	2763
DiMeAOleacein	657	470.5	n.d.	n.d.	n.d.	n.d.
MeHAOleocanthal	4568.5	6286	15810	15291.5	8727	11589
DiMeAOleocanthal	8536.5	10721	23501.5	18807.5	14169.5	15936.5
<i>Sum of artifacts as %</i>	37.1	35.0	29.8	27.8	30.7	29.2

MeHA: methyl hemiacetal; DiMeA: dimethyl acetal; n.d.: not detected

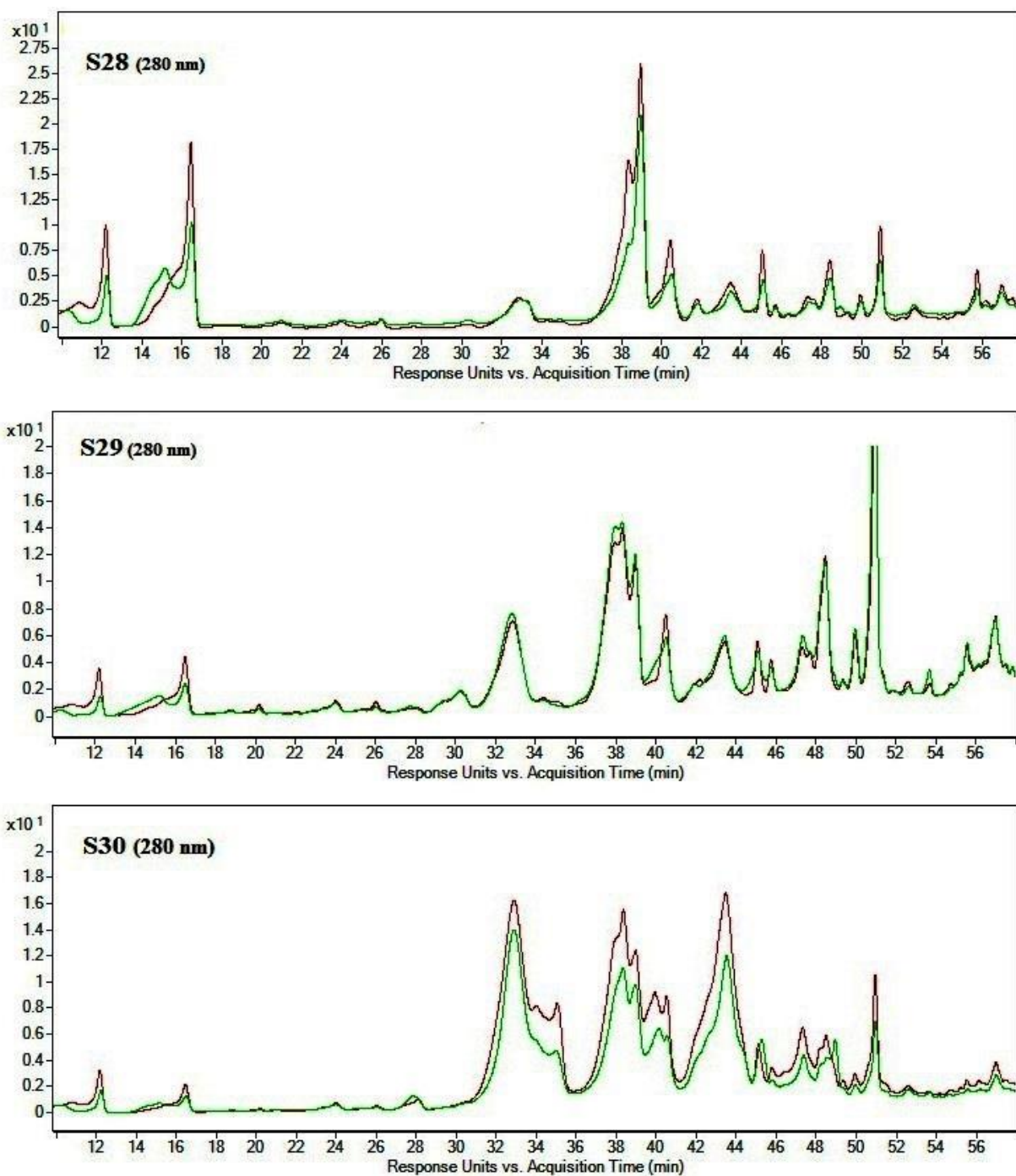


Figure S1. Chromatograms (280 nm) of polar fractions of tested oils obtained with (—) MeOH:H₂O, 80:20, v/v; (—) ACN:H₂O, 70:30, v/v.

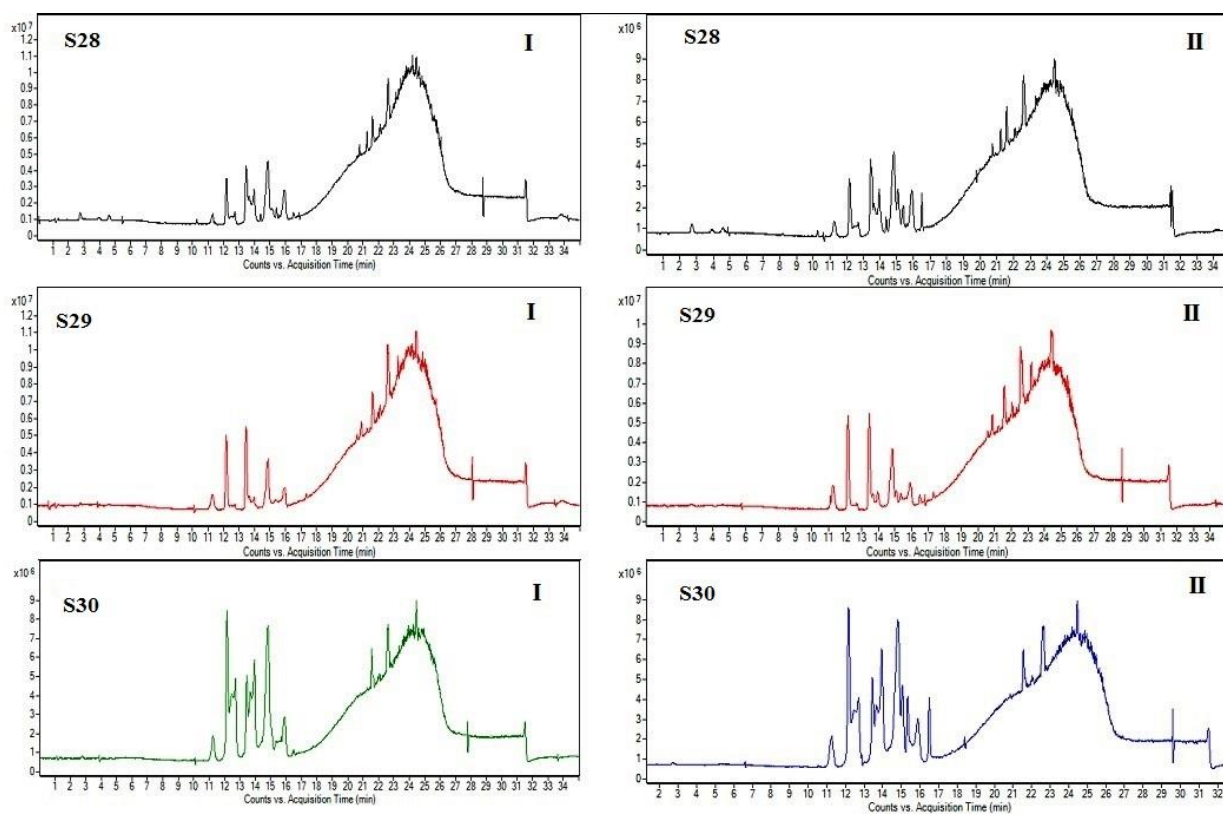


Figure S2. TOF ESI TICs of polar fractions of tested oils obtained with (I) MeOH:H₂O, 80:20, v/v; (II) ACN:H₂O, 70:30, v/v.