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Pre-print version

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

Running title: Virgin olive oil polar phenol standardized extraction

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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-HRMS, 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. Providing 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 system.

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

Abbreviations: **Htyr**, hydroxytyrosol, **Tyr**, tyrosol, **DHPA**, dihydroxyphenylacetic acid, **MeOH**, methanol, **ACN**, acetonitrile, **IOC**, International olive council, **P'**, polarity index, **Et(30)**, solvent polarity parameter, **F-C**, Folin Ciocalteu, **H-ESI**, heated electrospray ionization, **HRMS**, high resolution mass spectrometer, **TOF**, time of flight, **TQd**, triple quadrupole, **MRM**, multiple reaction monitoring, **DAD**, diode array detection, **FL**, fluorescence, **MeHA**, methyl hemiacetal, **DiMeA**, dimethyl acetal

1. Introduction

Hydroxytyrosol (Htyr), tyrosol (Tyr) and their derivatives, are the most abundant polar phenols of virgin olive oil,^[1] 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] and 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 health, processing parameters and storage length and conditions all of which impact the commercial quality and claimed nutritional and health benefits of the oil. 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 can be expected for methanol. 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 indicate 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. The latter 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 methanol and acetonitrile 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 methanol and it is more expensive than that. The extraction solvent system 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.

2. Materials and methods

2.1. Chemicals

Tyr (98%) and 1,3,5-triazine (97%) were products of Alfa Aesar GmdH & CoKG, (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%), DMSO-d₆, (99.8%) and MeOH-d₄, (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 virgin olive oil 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 systems composition

The composition of aqueous acetonitrile mixtures of equal polarity index values to the aqueous methanol ones was calculated from the equation $P' = \phi a \times Pa + \phi b \times Pb$, where ϕa and ϕb is the volume fraction of each solvent in the binary mixture and Pa , Pb refer 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 acetonitrile mixtures of equal polarity $E_T^{(30)}$ value to the aqueous methanol 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 system. 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 F-C assay.

2.4. Colorimetric assessment of 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 was used as an external standard.^[19] 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 μL) of the polar fraction was mixed with 200 μL of a 1 M H_2SO_4 solution. The mixture was incubated in a water bath at 80 °C for 2 h. The procedure was carried out in triplicate. Each hydrolysate was then diluted with 200 μL of the polar fraction extracting solvent. The three replicates were combined to obtain a representative hydrolysate. The latter was filtered through a 0.45 μm pore size regenerated cellulose membrane (Schleicher and Schuell, MicroScience GmbH, Dassel, Germany) before injection onto the chromatograph.^[19] The intra and interday repeatability of hydrolysis for Htyr and Tyr determination were 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 detector (DAD; Thermo Separation Products) in series with an SSI 502 fluorescence detector (FLD; 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 system consisted of 1% aqueous acetic acid (solvent A) and acetonitrile (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 the separation was carried out using the gradient elution system proposed by IOC.^[16]

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 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 Diode Array Detector (190-600 nm) interfaced with a triple-quadrupole 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 following experimental parameters were followed: 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 absolute amount of phenolic constituent determination in the polar fraction was carried out according to Dais and Christophoridou.^[20] The results were expressed as $\mu\text{mol}/100 \text{ g oil}$.

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 the appropriate aqueous acetonitrile system 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 acetonitrile mixture with regard to the performance of the aqueous methanol mixture proposed by IOC, namely MeOH:H₂O, 80:20 v/v, was, thus, based on different selectivity criteria and not on percentages. The aqueous acetonitrile mixtures tested had a composition of comparable polarity to those of the methanolic 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 overall performance differences between the two organic solvents. The tests were performed on a virgin olive oil (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 total phenol content. Pure acetonitrile and 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 solvent system (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 virgin olive oils (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 $^1\text{H-NMR}$ spectroscopy (Table 3) gave a strong evidence that further examination of the MeOH:H₂O, 60:40, v/v system was not promising for the aim of a standardised protocol. However, the other two systems deserved further examination.

3.2. The potential of MeOH:H₂O, 80:20, v/v, vs ACN:H₂O, 70:30, v/v mixtures as extraction solvents in a standardized protocol

The polar fraction of an olive oil can be used for the determination of total polar phenols by F-C or HPLC or for the determination of the total content of Htyr and Tyr after hydrolysis.^[16, 19] The proposal for which is the most suitable extraction solvent between the two prevailing systems is consequently strongly dependent on the analytical means used for their determination. The expression of results may augment or not the differences, their statistical significance or even the magnitude of the obtained values. Our systematic investigation revealed interesting details that should not be ignored in the process of harmonization of the determination of polar phenolic compounds.

3.2.1. F-C assay

Despite the many criticisms over the years that this assay has been received for the analysis of the total polar phenol of olive oil, it must be accepted that it is used both in research and routine analysis as a screening test before further examination of the polar fraction for profiling the phenolic compounds or for the determination of the total Htyr and Tyr content after hydrolysis.^[15, 19, 24-26] Twenty one (21) authentic virgin olive oils (S7-S27) were extracted with the two prevailing systems. The data are shown in Figure 1 and indicate that aqueous acetonitrile 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 systems are of equivalent potency.

3.2.2. RP-HPLC –DA–FL analysis

Since the RP-HPLC separation and determination of the polar phenolic compounds of olive oil is unquestionably the method of choice.^[5] The elution systems applied in the hundreds of published papers vary slightly using either MeOH or ACN as the organic modifier or even mixtures of them whereas acidification of the aqueous phase at a pH value ≤ 3.2 is accomplished with various organic acids. Sample analysis is usually carried out via the introduction of the polar extract after drying and redilution in a volume of MeOH:H₂O or methanol even if in the gradient elution acetonitrile is used.^[19, 22, 24, 25, 27] On the other hand, in the adopted protocol by IOC 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]

Among the samples of Figure 1, five virgin olive oil samples (S10, 13, 17, 19 and 24) of the same cultivar and location with almost comparable total phenol content estimated colorimetrically were analysed by HPLC. 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 without (total phenol content) or after (total Htyr and Tyr content) hydrolysis 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 evident that they were qualitatively similar using either UV 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 total phenol content- the values obtained for the aqueous methanol extracts differed

statistically from those of the aqueous acetonitrile extracts (Table 4). Using Htyr for quantification lower values (1.67-1.70 fold) than those acquired with Tyr 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, statistical significant differences were observed in three of the samples for both constituents. Nevertheless, no clear superiority of one system over the other was observed regardless of the result expression.

3.2.3. LC-MS analysis

To reveal any hidden information in the liquid chromatograms due to the different extraction systems three pairs of sample (S28, 29 and 30) extracts prepared using MeOH:H₂O, 80:20, v/v and ACN:H₂O, 70:30, v/v were also analysed by different LC-MS facilities. In all cases the organic modifier of the elution system was MeOH:ACN, 1:1, v/v (IOC, 2009). The profiles of the polar extracts recorded at 280 nm (Figure S1) were qualitatively similar. Information obtained with HRMS revealed that some classes of the detected Htyr and Tyr derivatives were influenced by the extraction solvent (Figure 3 and 4). In particular, some isomers of Htyr- and Tyr-elenolic acid derivatives eluting at the end of the chromatogram (elemental formula C₁₉H₂₁O₈ and C₁₉H₂₁O₇, respectively) were favoured by the aqueous acetonitrile extraction. These peaks were present in different proportions in both types of extracts, and their mass spectra were the same or very similar to the rest of Htyr or Tyr with the same molecular formula. TOF analysis of the same sample extracts, indicated that two late eluting peaks at retention time 15.054 min ([M-H]⁻ 361.1290) and 16.499 min ([M-H]⁻ 377.1237) respectively, were more pronounced in the extracts obtained with aqueous acetonitrile (Figure S2). Although the use of MeOH in the extraction

system would be expected to contribute to some qualitative differences due to the reaction with the complex forms of Htyr and Tyr bearing aldehyde groups such as oleacein and oleocanthal, analysis with all three MS platforms verified the presence of artifacts in both types of extracts. The artifacts eluted at the same retention time with that of the precursors. In addition, the calculated areas using TQd and MRM mode^[28, 29] showed that their quantity was rather the same or slightly differed in both extracts (TableS.2). All of the above observations suggest 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-MS/MS apure ACN or a MeOH:H₂O 60:40, v/v polar extract reported a similar % of artifacts formed (~16%) in both of them when the elution protocol had MeOH as the organic modifier. Semi-quantitative information for oleacein and oleocanthal, which, being the most abundant complex forms of Htyr and Tyr, respectively, are expected to contribute the most to the total Htyr and Tyr content calculation, showed that MeOH:H₂O, 80:20, v/v provided comparable or slightly higher signal than that of ACN:H₂O, 70:30, v/v (Table S3, S4).

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”.

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Conflict of interest

The authors state that there is no conflict of interest

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

% MeOH (v/v)	% ACN (v/v)		
	70	51	39
TPC (mean value \pmSD, $n= 3$, mg CA /kg oil)^{*,**}			
159 \pm 5.8 ^a	177 \pm 1.0 ^b	129 \pm 4.0 ^c	125 \pm 5.4 ^c
TPC (mean value \pmSD, $n= 3$, mg Tyr /kg oil)^{***,**}			
107.4 \pm 1.0 ^{b/}	112.9 \pm 0.5 ^{a/}	100.2 \pm 0.4 ^{c/}	92.9 \pm 1.1 ^{d/}
66.7 \pm 0.3 ^a	68.3 \pm 0.4 ^a	64.5 \pm 0.0 ^{b,c}	59.8 \pm 0.3 ^d

* Total phenol content estimated by Folin Ciocalteu assay; ** Values in the same row bearing different lower case letters as superscripts are statistically different ($p < 0.05$) ***Total phenol content estimated by HPLC DA_{280 nm}/FL exc 280 nm/em 320 nm

Table 2. Total phenol content of virgin olive oils (S2-S6) estimated by the F-C assay and RP-HPLC- DA (280 nm) or FL (exc 280 nm/em 320 nm) after extraction with selected MeOH and ACN aqueous systems

	S2	S3	S4	S5	S6
F-C assay					
TPC (mean value \pmSD, $n=3$, mg CA /kg oil)					
60:40 [*]	185.5 \pm 4.1 ^a	211.3 \pm 3.4 ^a	158.0 \pm 3.2 ^a	177.1 \pm 4.1 ^a	106.8 \pm 1.5 ^a
80:20 ^{**}	211.6 \pm 3.7 ^b	226.5 \pm 6.1 ^b	178.5 \pm 2.9 ^b	208.4 \pm 2.8 ^b	131.2 \pm 2.1 ^b
70:30 ^{***}	203.9 \pm 1.7 ^{b,c}	221.2 \pm 2.4 ^b	171.9 \pm 3.3 ^{b,c}	198.6 \pm 3.3 ^c	138.7 \pm 2.4 ^c
RP-HPLC DA/FL					
TPC (mean value \pmSD, $n=3$, mg Tyr /kg oil)					
60:40 [*]	120.4 \pm 3.1 ^{a/}	116.6 \pm 4.1 ^{a/}	88.9 \pm 1.4 ^{a/}	100.7 \pm 1.5 ^{a/}	59.9 \pm 1.0 ^{a/}
	38.7 \pm 0.4 ^a	73.5 \pm 2.7 ^a	36.5 \pm 0.3 ^a	49.9 \pm 0.1 ^a	28.5 \pm 0.3 ^a
80:20 ^{**}	122.3 \pm 1.8 ^{a/}	140.3 \pm 4.8 ^{b/}	90.6 \pm 1.5 ^{b/}	108.2 \pm 1.6 ^{b/}	67.7 \pm 0.5 ^{b/}
	40.1 \pm 0.1 ^b	86.4 \pm 1.9 ^b	40.9 \pm 0.9 ^b	53.5 \pm 0.5 ^b	28.8 \pm 0.0 ^a
70:30 ^{***}	123.1 \pm 2.1 ^{a/}	142.4 \pm 0.6 ^{b/}	95.9 \pm 1.8 ^{c/}	116 \pm 1.4 ^{c/}	70.5 \pm 1.1 ^{c/}
	42.5 \pm 0.7 ^c	87.7 \pm 0.8 ^b	44.9 \pm 0.4 ^c	63.2 \pm 0.3 ^c	31.1 \pm 0.1 ^b

Values in the same row bearing different lower case letters as superscripts are statistically different ($p < 0.05$); *. ** aqueous methanol; *** aqueous acetonitrile

Table 3. Total Htyr and Tyr content of virgin olive oils (S1-S6) olive oils determined by ¹H-NMR after extraction with different solvent mixtures

	S1	S2	S3	S4	S5	S6
	Total Htyr and Tyr (μmol/100 g of oil)					
60:40*	152.5	129.9	95.6	94.4	164.3	152.0
80:20**	165.6	135.1	136.6	119.8	210.1	156.5
70:30***	172.2	139.8	140.7	128.8	188.9	163.1

*** aqueous methanol; ** aqueous acetonitrile

Table 4. Total phenol content and Total Htyr and Tyr content values obtained for 5 monovarietal oils after extraction with MeOH:H₂O, 80:20, v/v or ACN:H₂O, 70:30, v/v (IOC elution system, detection at 280 nm)

Solvent	Sample	Total polar phenol content ^{†,§} as mg/kg oil			Htyr [§]	Tyr [§]	Sum of Htyr and Tyr	Htyr [§]	Tyr [§]	Sum of Htyr and Tyr [‡]	Htyr [§]	Tyr [§]	Sum of Htyr and Tyr
		Htyr	Tyr	DHPA 1									
mean values ±SD (n= 3)													
80:20*	S10	134.5 ± 17.3 ^a	227.6± 27.9 ^a	129.3± 14.6 ^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
70:30**		190.4 ± 0.4 ^b	317.5± 0.7 ^b	176.5± 0.4 ^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
80:20*	S13	149.4 ± 5.4 ^b	251.5± 8.8 ^b	141.9± 4.6 ^b	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
70:30**		119.5 ± 11.1 ^a	203.5± 17.9 ^a	116.7± 9.4 ^a	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
80:20*	S17	152.6 ± 0.8 ^a	256.7± 1.3 ^a	144.6± 0.7 ^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
70:30**		182.7± 5.4 ^b	305.2± 8.7 ^b	170.0± 4.6 ^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
80:20*	S19	152.7± 3.2 ^b	256.9± 5.2 ^b	144.7± 2.7 ^b	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
70:30**		130.8± 3.5 ^a	221.7± 5.6 ^a	126.2± 3.0 ^a	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
80:20*	S24	190.0± 29.1 ^a	316.8± 46.8 ^a	176.1± 24.6 ^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
70:30**		131.6± 16.5 ^b	222.9± 26.6 ^b	126.9± 13.9 ^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

*aqueous methanol; ** aqueous acetonitrile; †TPC: Total phenol content; ‡According to the correction proposed by Mastralexi et al.^[19]; §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 ($p < 0.05$)

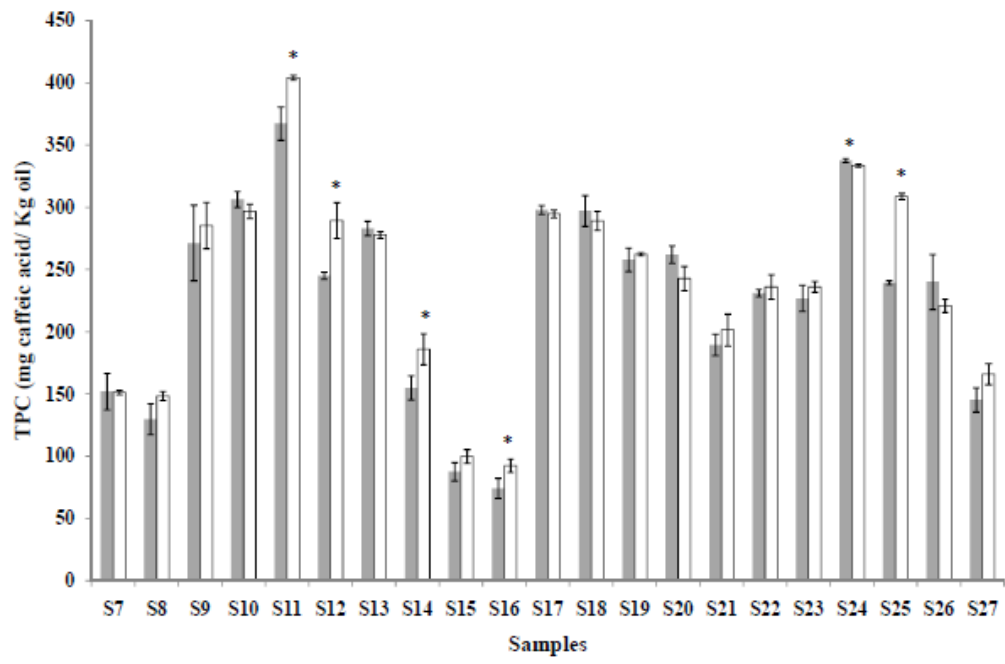


Figure 1

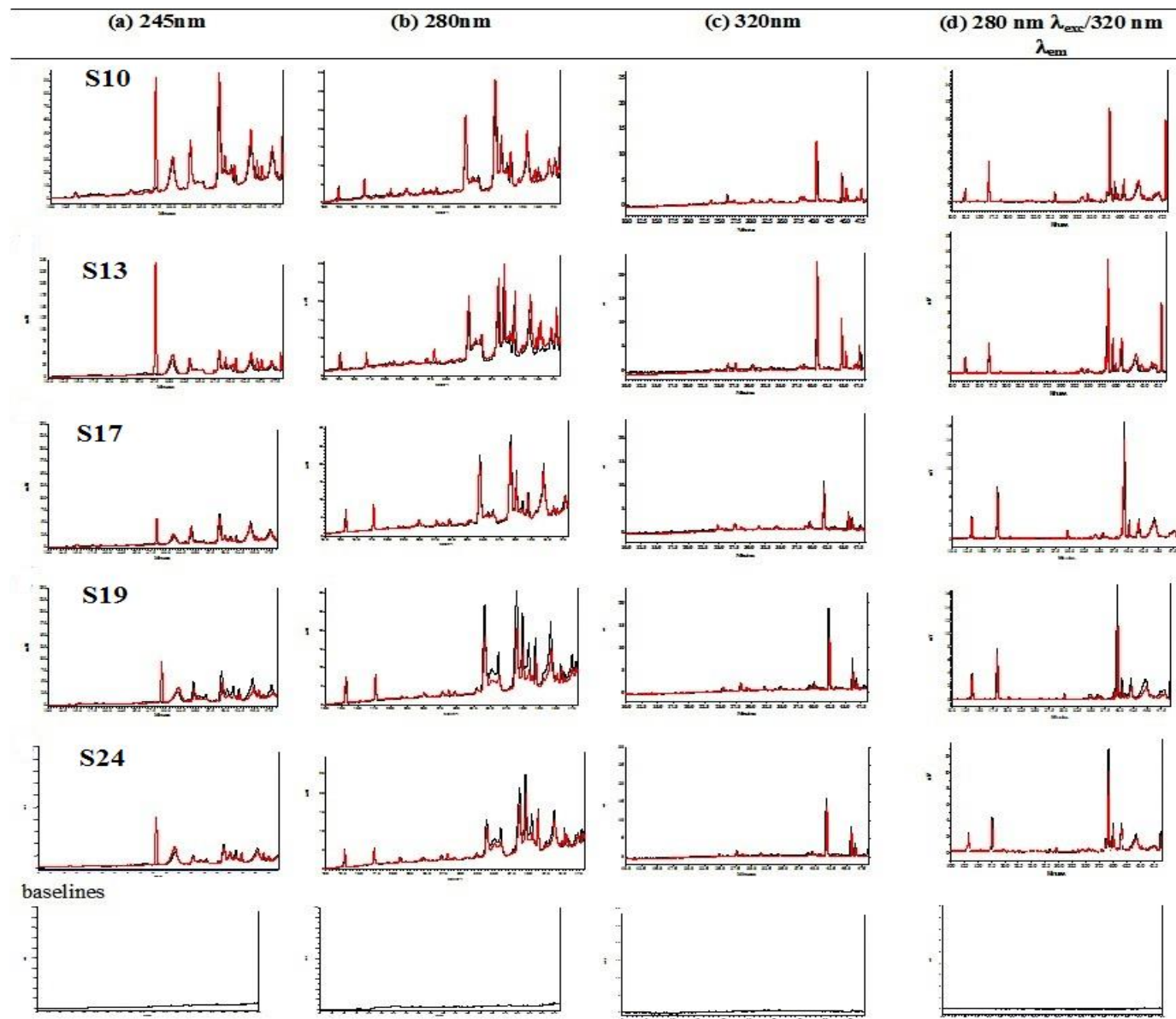


Figure 2

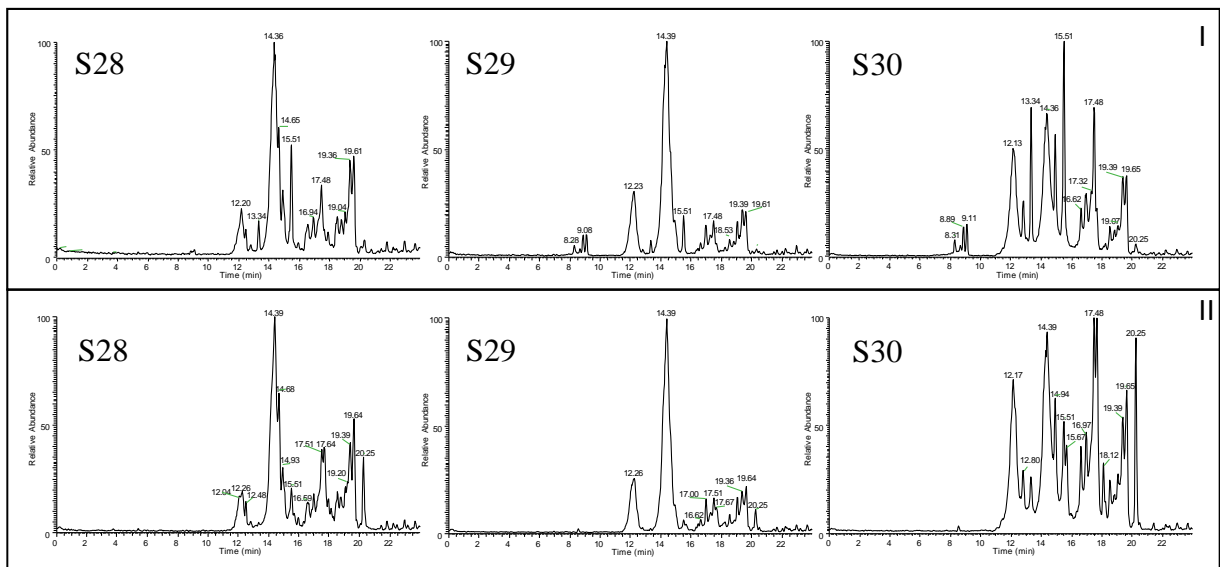


Figure 3

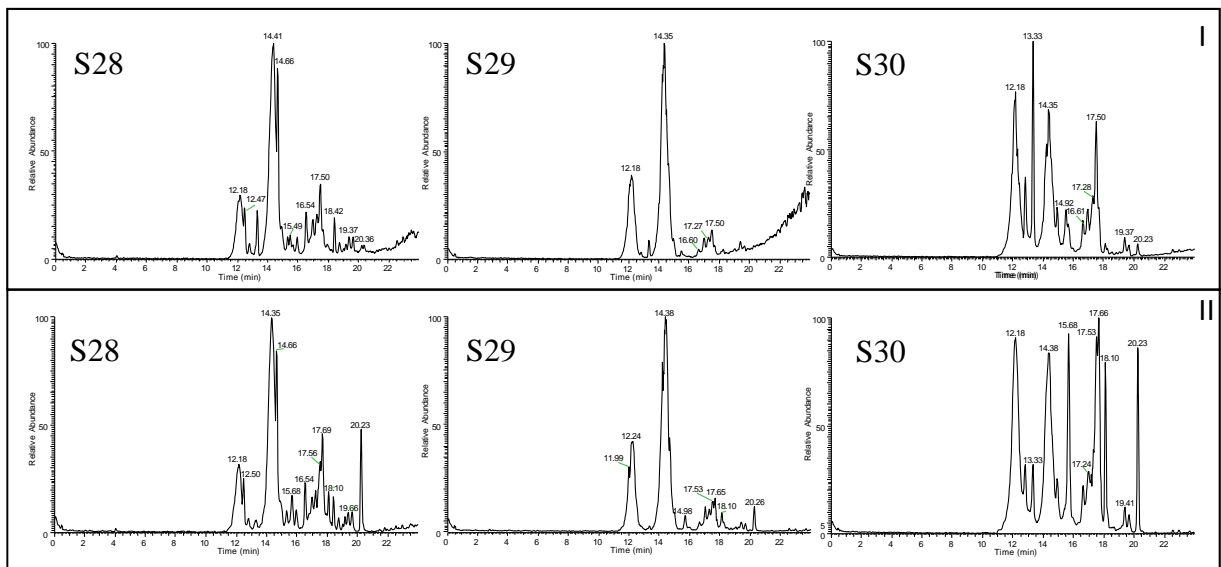


Figure 4

Figure legends

Figure 1. TPC 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-DAD-FLD_{280/320} 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 λ_{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 chromatograms (mass range *m/z* 315-395) of polar fractions of tested oils obtained with (I) MeOH:H₂O, 80:20, v/v; (II) ACN:H₂O, 70:30, v/v. R: 70,000 (*m/z* 200, FWHM).

Figure 4. ESI- full scan chromatograms (mass range *m/z* 315-395) of polar fractions of tested oils obtained with (I) MeOH:H₂O, 80:20, v/v; (II) ACN:H₂O, 70:30, v/v. R: 70,000 (*m/z* 200, FWHM).

Supplementary material

Toward a harmonized and standardized protocol for the determination of total hydroxytyrosol (Htyr) and tyrosol (Tyr) content in virgin olive oil. 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 uL/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 volumen: 1 uL

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) TOF (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 acetonitrile:methanol (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 mins before being held at 100% for

5 minutes. The flow rate was 0.5 mL/minute 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 psig, and fragmentor voltage 150 V. LC-TOFMS 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 Oliveculture):

Chromatographic column: Synergi 4u 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 COI/T.20/Doc. no. 29, November 2009, 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, 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.** Average ($n=2$) peak areas of methyl and dimethyl oleacein and oleocanthal derivatives using TQd-MRM for the analysis of the polar fraction of tested oils obtained with MeOH:H₂O, 80:20, v/v or ACN:H₂O, 70:30, v/v

	S28		S29		S30	
	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
MeHAOleacein	552	495	1705	1710	2478.5	2763
DiMeAOleacein	657	470.5	-	-	-	-
MeHAOleocanthal	4568.5	6286	15810	15291.5	8727	11589
DiMeAOleocanthal	8536.5	10721	23501.5	18807.5	14169.5	15936.5

MeHA: methyl hemiacetal; DiMeA: dimethyl acetal

Table S3. Average ($n=3$) peak areas of oleacein and oleocanthal extracted from TOF ESI TICs of the polar fraction of tested oils obtained with MeOH:H₂O, 80:20, v/v or ACN:H₂O, 70:30, v/v

	S28		S29		S30	
	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
Oleacein	1989372	1966709	4013786	3944416	5937125	5645346
	$p=0.98$		$p=0.59$		$p=0.01$	
Oleocanthal	1661351	1775142	2872390	3036383	1776450	1686786
	$p=0.70$		$p=0.21$		$p=0.02$	

Pair of samples with $p < 0.05$ are significantly different according to t-test

Table S4. Average ($n=2$) peak areas of oleacein and oleocanthal using TQd-MRM from the analysis of the polar fraction of tested oils obtained with MeOH:H₂O, 80:20, v/v or ACN:H₂O, 70:30, v/v

	S28		S29		S30	
	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
Oleacein	4062	5565.5	17117.5	16197.5	19327.5	23650.5
Oleocanthal	20220	27816.5	79475.5	76655.5	37827	49767.5

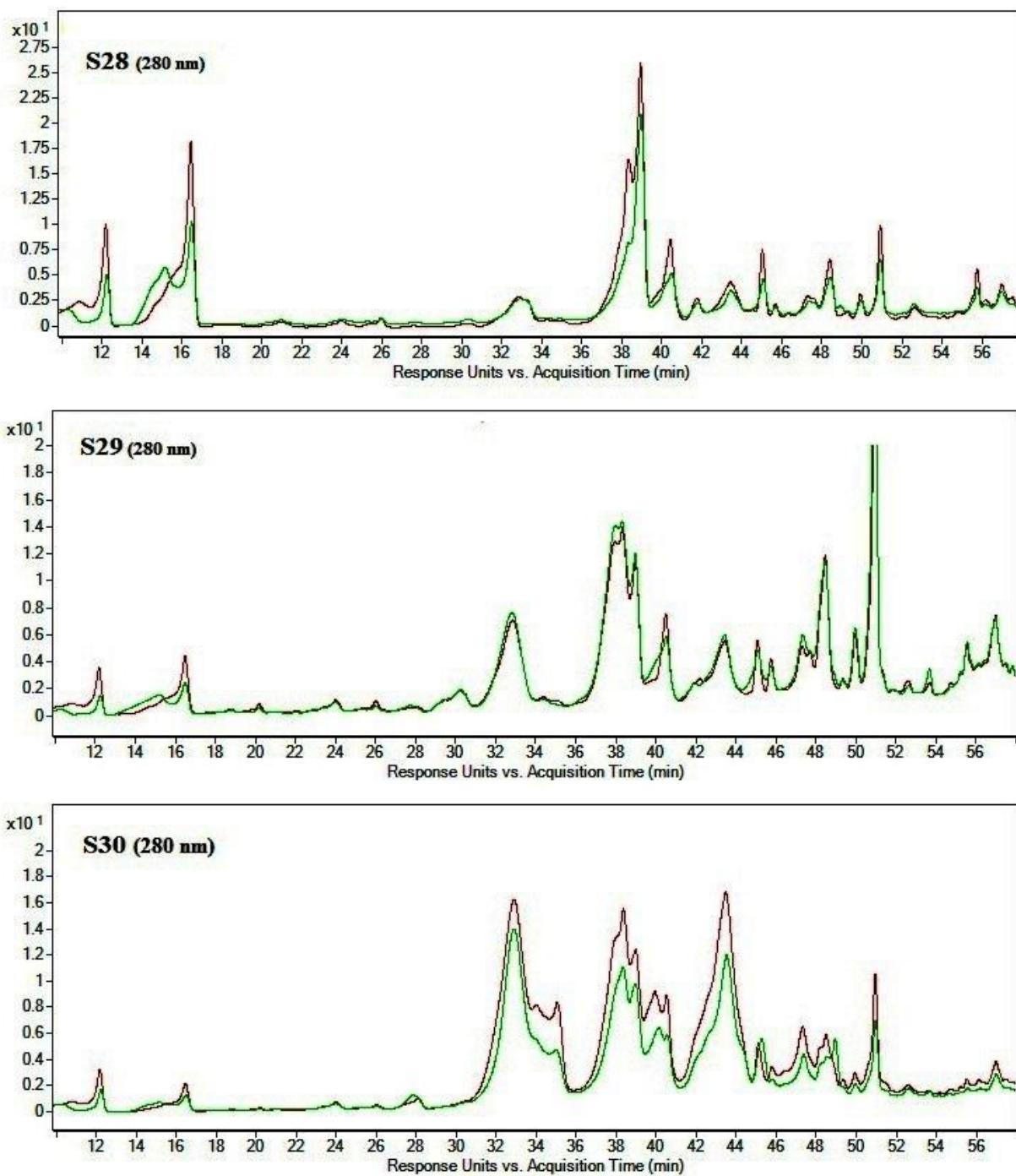


Figure S1. Chromatograms (280 nm) of polar fractions of tested oils obtained with (—) MeOH:H₂O, 80:20, v/v; (—) ACN:H₂O, 70:30, v/v.

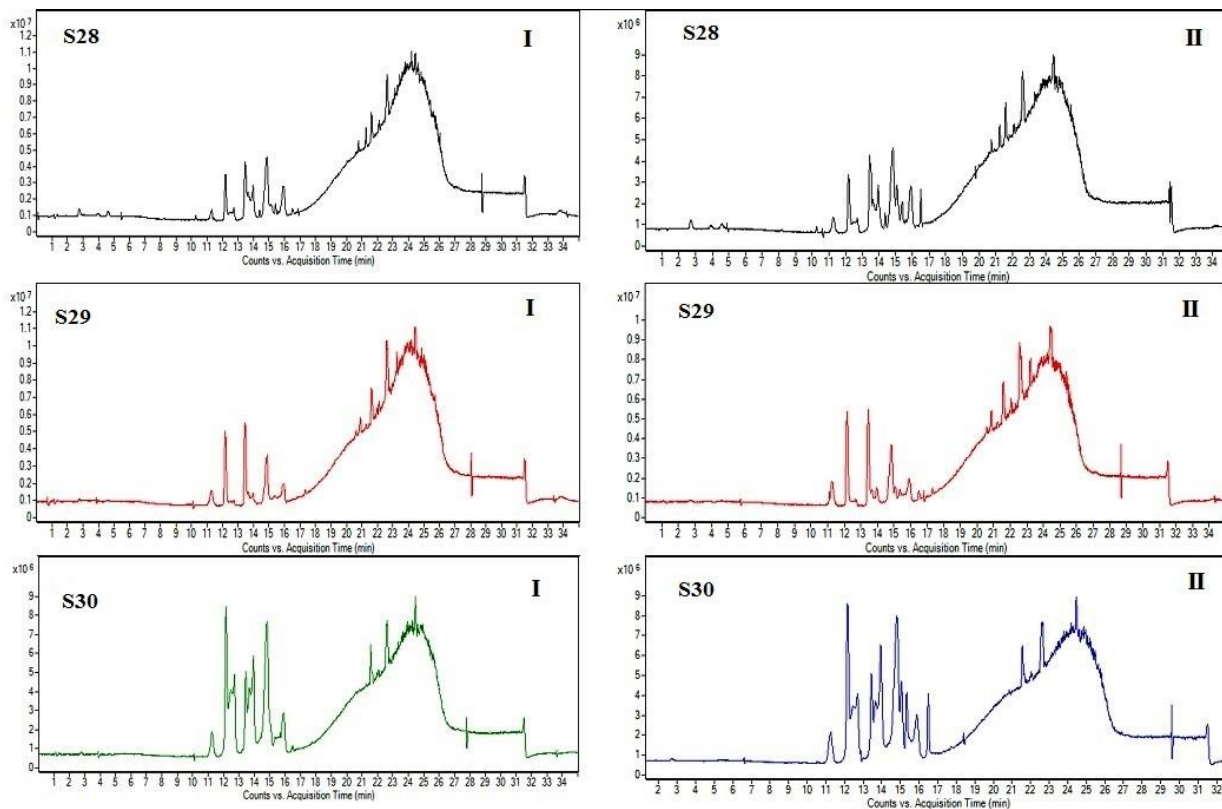


Figure S2. TOF ESI TICs of polar fractions of tested oils obtained with (I) MeOH:H₂O, 80:20, v/v; (II) ACN:H₂O, 70:30, v/v.