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Quantitative Measurement of Major Secoiridoid Derivatives in Olive Oil Using qNMR. Proof of the Artificial Formation of Aldehydic Oleuropein and Ligstroside Aglycon Isomers

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Supporting Information

ABSTRACT: A previously developed method for measurement of oleocanthal and oleacein in olive oil by quantitative ^{1}H NMR was expanded to include the measurement of the monoaldehydic forms of oleuropein and ligstroside aglycons. The method was validated and applied to the study of 340 monovarietal Greek and Californian olive oils from 23 varieties and for a 3-year period. A wide variation concerning the concentrations of all four secoiridoids was recorded. The concentration of each one ranged from nondetectable to 711 mg/kg and the sum of the four major secoiridoids (named as D3) ranged from nondetectable to 1534 mg/kg. Examination of the NMR profile of the olive oil extract before and after contact with normal or reversed stationary chromatography phase proved the artificial formation of the 5*S*,8*S*,9*S* aldehydic forms of oleuropein and ligstroside aglycon isomers during chromatography. Finally, methyl elenolate was identified for the first time as a minor constituent of olive oil.

KEYWORDS: *extra virgin olive oil, secoiridoids, oleuropein aglycon, ligstroside aglycon, quantitative NMR*

INTRODUCTION

Olive oil is a fundamental constituent of the traditional Mediterranean diet which is continuously attracting the interest of the scientific community for its health-protecting activities.[1−3] Among the numerous constituents of olive oil, the secoiridoid derivatives present an increasing potential for health protection, which tends to place olive oil in the interface between food and drugs.[4] A very important change in the European Union legislation with potentially very significant impact on the field of olive oil marketing and labeling is the recent recognition by EFSA of specific health claims related to the levels of specific phenolic compounds found in olive oil.[5] More specifically, hydroxytyrosol, tyrosol, and their derivatives have been correlated with protection from LDL oxidation, creating thus a new need for the accurate measurement of the levels of those compounds in olive oil. It is noteworthy that up to today there is no internationally accepted regulation concerning the method for their measurement because of several technical difficulties. It is well-known that the concentration of free hydroxytyrosol and tyrosol in olive oil is very low while their esterified derivatives are much more abundant.[6] Hydroxytyrosol is mainly present in the form of oleacein (3,4-DHPEA-EDA) and the monoaldehydic form of oleuropein aglycon (3,4-DHPEA-EA), while tyrosol is mainly present in the form of oleocanthal (*p*-HPEA-EDA) and the monoaldehydic form of ligstroside aglycon (*p*-HPEA-EA). All those four secoridoid derivatives possess significant biological activities, as previously summarized.[7] Especially oleocanthal and oleuropein aglycon have been very recently correlated with protection against Alzheimer's disease,[8,9] giving a new potential field for medical applications of olive oil. Although there are several works concerning the chromatographic analysis of those compounds (HPLC−UV or LCMS)[10−13] we have recently described[7] the reactivity of oleocanthal and oleacein with methanol and water, which are commonly and officially used for the extraction of polyphenols[14] and as constituents of the mobile phase during their analysis, leading to the formation of several artifacts and making the analysis very difficult. To overcome these problems that make the chromatographic analysis complicated and questionable, we recently developed a simple and rapid method using quantitative NMR (qNMR) and avoiding any interacting solvents as well as the need of chemically pure standards.[7] In the present work we have expanded the previous method to include all four major secoiridoid derivatives of hydroxytyrosol and tyrosol. Interestingly, during this study we recognized the instability of oleuropein and ligstroside aglycon when they come in contact with common normal or reversed stationery phases, leading to the artificial formation of isomers. In addition, during the isolation of olive oil constituents we identified a compound that is for the first time reported from a natural source. The qNMR method was applied to 340 olive oil samples, and the varieties presenting the highest concentrations of the studied compounds were recognized.

MATERIALS AND METHODS

Extra Virgin Olive Oil Samples. The commercial extra virgin olive oil (EVOO) samples used in the study were obtained from olives (*Olea europaea* L.) harvested and extracted in three seasons:

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November 2010−February 2011, October 2011−February 2012, and October 2012−February 2013. A total of 320 monovarietal commercial samples were obtained from Greece and 20 from California. In total, samples of 23 different varieties were included in the study. The olive oil production was performed in either two-phase or three-phase mills. All samples were provided by small-scale producers that could guarantee their monovarietal origin.

Olive Oil Extraction and Sample Preparation for NMR Analysis. Olive oil (5.0 g) was mixed with cyclohexane (20 mL) and acetonitrile (25 mL). The mixture was homogenized using a vortex mixer for 30 s and centrifuged at 4000 rpm for 5 min. A part of the acetonitrile phase (25 mL) was collected, mixed with 1.0 mL of a syringaldehyde (4-hydroxy-3,5-dimethoxybenzaldehyde) solution (0.5 mg/mL) in acetonitrile, and evaporated under reduced pressure using a rotary evaporator (Buchi, Switzerland).

Reference Compounds. Oleuropein aglycon and ligstroside aglycon were isolated from an olive oil extract prepared using the extraction method similar to that described above for sample preparation. The obtained extract was first fractionated using column chromatography, and then the fractions containing the two aglycons were purified with preparative TLC until maximum purity was achieved. In both cases, the mobile phase was prepared using mixtures of cyclohexane with ethyl acetate and silica gel as stationary phase. Details of the purification procedure are provided as Supporting Information. Each aglycon was obtained as an inseparable mixture of the 5*S*,8*R*,9*S* and 5*S*,8*S*,9*S* diastereomers in a ratio 3:1 and 4:1 for oleuropein and ligstroside aglycons, respectively. The ^{1}H NMR purity of both compounds (as a total of the two diastereomers in each case) was >98%. The identity of both compounds was undoubtedly defined by extensive 2D NMR study and comparison with literature data.[15−19] Syringaldehyde (98% purity) used as internal standard (IS) was purchased from Sigma-Aldrich (Steinheim, Germany). IS solution was prepared in acetonitrile at a concentration of 0.5 mg/mL and kept in a refrigerator. Prior to use the IS solution was left to come to room temperature. All NMR solvents used throughout the experiments were obtained by Sigma-Aldrich.

NMR Spectral Analysis. The residue of the above procedure was dissolved in $CDCl_3$ (750 μL) and an accurately measured volume of the solution (550 μL) was transferred to a 5 mm NMR tube. ^{1}H NMR spectra were recorded at 600 MHz (Bruker Avance600) or 400 MHz (Bruker DRX400). Typically, 50 scans were collected into 32K data points over a spectral width of 0−16 ppm with a relaxation delay of 1 s and an acquisition time of 1.7 s. Prior to Fourier transformation (FT) an exponential weighing factor corresponding to a line broadening of 0.3 Hz was applied. The spectra were phased corrected and integrated automatically using TopSpin software (Bruker). Where necessary, accurate integration was performed manually for the peaks of interest.

Calibration Curves and Quantitation. Calibration curves were prepared by addition of known quantities of isolated oleuropein aglycon or ligstroside aglycon to a selected olive oil, from cv. Adramytini, which is naturally free of secoiridoid derivatives, and following the above-described extraction and measurement method. The quantitation was based on the integration ratio between the aldehydic proton signal of syringaldehyde at 9.81 ppm and the aldehydic protons of oleuropein aglycon at 9.51 and 9.58 ppm and ligstroside aglycon at 9.49 and 9.55 ppm, respectively, for each pair of diastereomers.

Standard and Spiked Solutions. Stock standard solutions of oleuropein aglycon and ligstroside aglycon were prepared in acetonitrile at the 0.5 mg/mL level and were kept under refrigeration. Prior to use the stock solutions were allowed to come to room temperature. Spiked olive oil samples were prepared to give concentrations of both aglycons of 10, 20, 40, 80, 160, and 320 mg/kg by mixing appropriate volumes of the stock standard solutions with 5.0 g of olive oil of the variety Adramytini. The mixture was homogenized using a vortex mixer for 30 s and then extracted with cyclohexane (20 mL) and acetonitrile (as much as needed to become a total of 25 mL) as described above. A sample of Adramytini variety was selected as a blank because it was found that it did not contain oleuropein and ligstroside aglycons in detectable quantities and it did not give any interfering peaks at the chemical shift of the analytes.

Method Validation. The method was checked for the linearity, precision [calculated as the relative percent standard deviation (RSD %)], accuracy [evaluated as the relative percentage error (Er%), defined as (assayed concentration − nominal concentration)/(nominal concentration) × 100], and sensitivity [evaluated as the limits of detection (LOD) and quantitation (LOQ)].

Linearity. Spiked olive oil samples were prepared to give concentrations of oleuropein aglycon and ligstroside aglycon at 10, 20, 40, 80, 160, and 320 mg/kg and were analyzed for the determination of the linearity. The relationship of the integration ratio of the analytes versus the internal standard and the corresponding concentration of the spiked olive standards was determined by linear regression analysis.

Precision. The intraday precision was determined by analyzing five replicates of spiked olive oil samples at two concentration levels of both aglycons (80 and 160 mg/kg). The interday precision was assessed by analyzing spiked olive oil samples at two concentration levels, namely, 80 and 160 mg/kg levels, on five consecutive days.

Accuracy. Spiked olive oil samples at three concentration levels of both aglycons, 10, 80, and 160 mg/kg, were analyzed in order to determine the accuracy of the method.

Recovery. For the calculation of the recovery, spiked olive oil samples with concentrations of both analytes at the 160 mg/kg level (n = 5 for each analyte) were analyzed by employing the proposed extraction procedure. The recovery was calculated as the ratio of the response of both compounds in the spiked olive oil samples against that of the standards at the same levels and was expressed as the mean ± SD (standard deviation).

Limits of Detection and Quantitation. The LOD and LOQ were determined by running six blank samples of Adramytini olive oil and measuring the background response at the chemical shift of each analyte. Signal-to-noise (S/N) ratios of 3:1 and 10:1 were used for the calculation of the LOD and LOQ, respectively.

Isolation and Spectroscopic Identification of Methyl Elenolate. Methyl elenolate was isolated using column chromatography during the purification of the reference compounds as described above and in more details in the Supporting Information. The complete NMR data of methyl elenolate (^{1}H NMR, ^{13}C NMR) as well as the original 1D and 2D NMR spectra are provided in Supporting Information and were in accordance with literature data.[19,20]

RESULTS AND DISCUSSION

Purification of Oleuropein and Ligstroside Aglycons. In order to set up the qNMR method for the measurement of oleuropein and ligstroside aglycons it was necessary to obtain the target compounds in the maximum possible purity to be used as standards for the construction of the calibration curve. For this purpose usual chromatographic methodologies were followed either using normal or reversed stationary phases. In a first step the olive oil was extracted with acetonitrile and the ^{1}H NMR spectrum of the extract was recorded to ensure that the target compounds had been successfully extracted. Then the extract was submitted to column chromatography using silica gel as stationary phase and mixtures of cyclohexane with ethyl acetate as mobile phase. When both compounds were isolated, the ^{1}H NMR spectrum showed that each one contained 20−25% of the 5*S*,8*S*,9*S* isomer and 75−80% of the 5*S*,8*R*,9*S* isomer, identified by comparison with literature data.[17−19] However, when the spectra of the purified compounds were compared with the spectrum of the initial extract, surprisingly, we observed that the peaks corresponding to the 5*S*,8*S*,9*S* isomers were almost absent (Figure 1). The 5*S*,8*R*,9*R* isomer reported by Perez-Trujillo et al.[17] was not observed neither before nor after purification (and probably this is related with differences in olive variety or the followed extraction

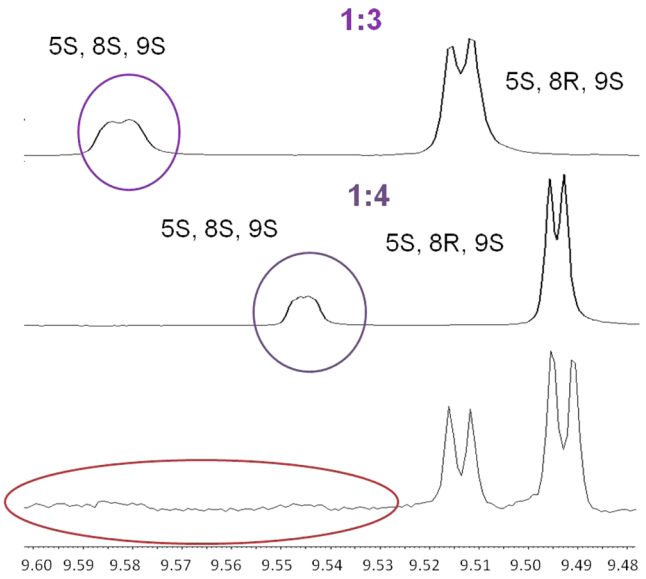

Figure 1. NMR region from 9.48 to 9.60 ppm showing the aldehyde protons of oleuropein and ligstroside aglycons. Top: Oleuropein aglycon after purification with normal-phase silica gel. Middle: Ligstroside aglycon after purification with normal phase silica gel. Bottom: Olive oil extract before submission to purification. The peaks of the 5*S*,8*S*,9*S* isomers of both aglycons are almost absent.

procedure). This observation led us to suppose the artificial formation of the minor isomers. Interestingly, the same was observed when the extract from another olive oil sample was submitted to reversed chromatography using RP-18 silica and a mixture of acetonitrile and water as mobile phase. In that case, the 5*S*,8*S*,9*S* isomers were observable before purification, but after purification, the ratio between the two isomers changed to 1:2 or 1:1 for ligstroside and oleuropein aglycons, respectively (Figure 2a,b).

To further study the role of the stationary phase in this isomerization, the following experiment was performed: A quantity of olive oil extract was diluted in a solvent (dichloromethane, cyclohexane, ethyl acetate, or mixtures of them) and added to a quantity of normal stationary phase and allowed to stand for 2 h. The same was performed with acetonitrile and water and reversed stationary phase. At the end, the mixture was filtered and evaporated and the residue was studied by NMR using $CDCl_3$. The comparison of the NMR profile of the treated and untreated olive oil extract showed that in all cases the percentage of the 5*S*,8*S*,9*S* isomers was significantly increased (Figure 3). The only combination that left the ratio unchanged was acetonitrile 100% with RP-18 silica. However, this combination was not useful for the purification of the target compounds. Although one previous work reports the purification of each isomer using Si 60 stationary phase,[19] in our hands it was obvious that it was not possible to purify oleuropein and ligstroside aglycons without isomerization using silica-based chromatography. Moreover, as it has been previously shown by LC−NMR, the two compounds are inseparable by HPLC.[17,18]

On the basis of the above observations, it is obvious that when oleuropein and ligstroside aglycons are needed for biological testing their diastereomeric purity cannot be safely determined by liquid chromatography methods. Any characterization of bioactivity should be accompanied by the isomer ratio as measured by NMR.

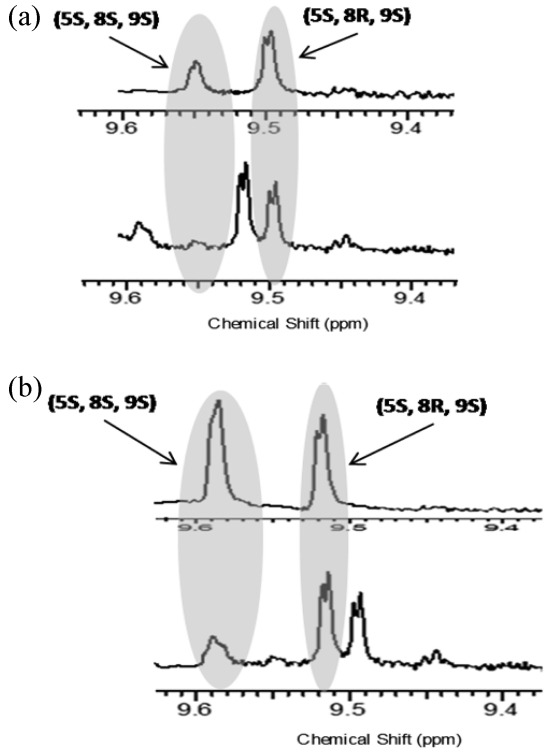

Figure 2. (a) Top: Ligstroside aglycon after purification with RP-18 and acetonitrile/water. Bottom: Olive oil extract before purification. (b) Top: Oleuropein aglycon after purification with RP-18 and acetonitrile/water. Bottom: Olive oil extract before purification.

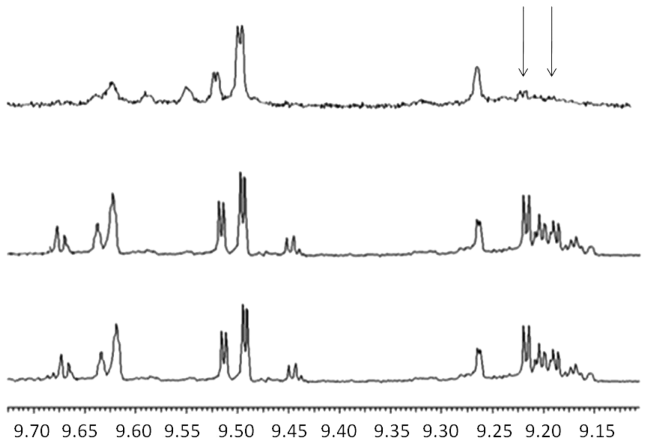

Figure 3. Oleocanthal and oleacein decomposition after contact with normal-phase silica gel. Top: NMR profile of the aldehydic region after contact with normal-phase silica gel. Middle: NMR profile of the aldehydic region after contact with reversed-phase silica gel and acetonitrile 100%. Bottom: NMR profile of the aldehydic region before contact with any stationary phase. The arrows show the strong reduction of the signals of oleocanthal and oleacein after contact with normal-phase silica gel. The signals of oleuropein and ligstroside aglycons are also reduced and new peaks corresponding to their isomers appear.

One additional observation based on the comparison of the NMR spectra of the olive oil extract before and after contact with normal phase silica (Figure 3) is that there is a significant percentage of decomposition of both oleocanthal and oleacein. This is a reasonable explanation for the low reported yield of both compounds when they are isolated by column

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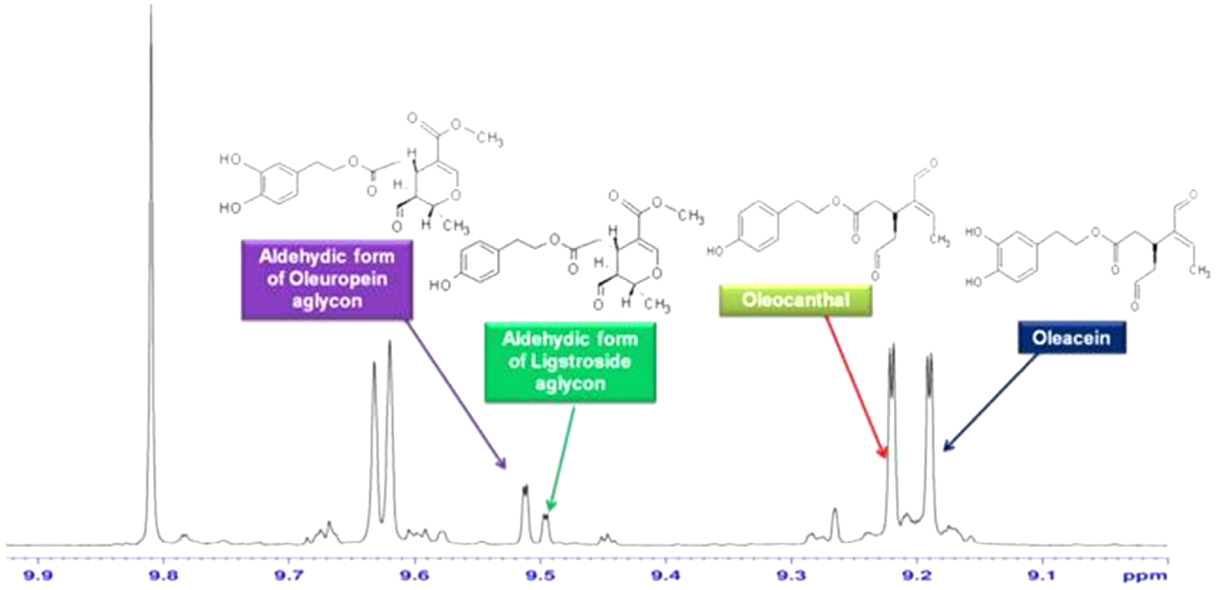

Figure 4. Typical NMR spectrum showing the peaks used for integration for each analyte.

chromatography. The mechanism of decomposition and the decomposition products were not studied.

Method Development. *Selection of Extraction Solvent.* The selection of acetonitrile as solvent for extraction of olive oil was based on the observation that it does not react with the two aglycons, as also described previously for the case of oleocanthal and oleacein.[7] It should be emphasized that methanol and/or water, which are commonly[17,18] and officialy[14] used for the extraction of phenolics from olive oil, react immediately with the dialdehydic form of oleocanthal or oleacein, leading to the corresponding acetals or hemiacetals.[7] A large number of compounds identified in HPLC−UV or LCMS chromatograms of olive oil extracts are obviously artifacts produced by that type of reaction. All those artifacts make the chromatograms much more complex and less reproducible and need to be reconsidered. For example, a recent study[13] quantified oleocanthal and oleacein with LC−MS/MS using external calibration curves prepared by dilution of standards in acetonitrile, although the analyzed olive oil extract was prepared using methanol. The proved reactivity of both compounds with methanol poses serious question about the reliability of such measurements. All hemiacetals or acetals produced during the extraction with methanol (and water) are not measured if the calibration is based on the MS/MS fragmentation of the dialdehydic forms and thus only a small part of unreacted oleocanthal or oleacein is finally measured, leading to questionable conclusions.

The two aglycons do not present such reactivity and this is possibly an explanation why they are often measured in higher levels than oleocanthal and oleacein. Moreover, one extraction with acetonitrile was sufficient for more than 85% recovery of all studied compounds. A second extraction achieved quantitative recovery but it was avoided to simplify the procedure. However, it should be noted that the exact percentage of recovery was not used for the calculations, because the final concentrations of the studied compounds in olive oil were calculated using calibration curves prepared by addition of pure compounds in olive oil originally free of both compounds.

Selection of Internal Standard and NMR Solvent. The choice of syringaldehyde as internal standard and of $CDCl_3$ as solvent for the NMR measurement was based on reasons explained previously.[7]

NMR Spectral Analysis of Target Compounds in Extra Virgin Olive Oil. The qNMR method was based on the observation that the ^1H NMR spectrum of olive oil acetonitrile extracts when recorded in $CDCl_3$ presented a very well resolved set of peaks corresponding to the aldehydic protons of the studied compounds between 9.1 and 9.8 ppm (Figure 4). This spectrum region in all the studied samples was clearly resolved, making feasible the integration of the corresponding peaks and their comparison with the peak of the internal standard. Oleuropein aglycon (3,4-DHPEA-EA) and ligstroside aglycon (*p*-HPEA-EA) were quantified by integrating doublets at 9.51and 9.49 ppm, respectively. Oleocanthal and oleacein were measured at 9.23 and 9.19 ppm, respectively, as previously described.[7]

The peaks of both aglycons did not present any overlapping in any studied sample recorded at 600 MHz or even at 400 MHz. The target peak of oleocanthal also did not present any overlapping at 600 MHz, but in some cases there was some overlapping observed at 400 MHz. The problem of overlapping was more significant for oleacein at 400 MHz but only in a few cases at 600 MHz (<3%). Even in those cases for the partially overlapped oleacein a good estimation of its quantity could be obtained. The overlapping problem can be overridden by 2D qNMR, which is currently under investigation.

Development of Calibration Curve. Since in our hands it was not possible to isolate oleuropein and ligstroside aglycons without the 5*S*,8*S*,9*S* isomer, it was obligatory to construct the calibration curve using the mixture of both isomers at a standard ratio. Since their difference is only one asymmetry center, it is reasonable to expect that their behavior during extraction will be similar and that the integration of the aldehyde proton of each isomer in the NMR spectrum in comparison with the internal standard will be the same. Under these conditions, the calibration curve was constructed by the addition of known quantities in a specifically selected olive oil sample which did not contain oleuropein or ligstroside

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aglycons. The sum of the integration of the two peaks corresponding to the aldehyde protons of each isomer of each compound was used as the response. The method was validated for accuracy, precision, and sensititvity.

Linearity. Good linearity was achieved for both analytes, as indicated by the equations listed in Table 1, for a concentration ranging from 10 to 320 mg/kg, with satisfactory correlation coefficients, r^2 (0.9991 and 0.991 for oleuropein and ligstroside aglycons, respectively.

Table 1. Linearity of the Oleuropein and Ligstroside Aglycons Determination in Olive Oil Samples

ratio	regression eq	correlation coeff, r^2
oleuropein aglycon/IS	$y = 336.5x - 8.66$	0.9991
ligstroside aglycon/IS	$y = 324.0x - 0.78$	0.991

Precision. The intraday precision, expressed as the relative standard deviation (RSD) ranged from 3.3 to 4.9% for the two analytes, as shown in Table 2. The interday precision ranged between 3.7 and 4.8% (Table 2). The RSD values are adequate and indicate the suitability of the method.

Table 2. Precision Data of the Oleuropein and Ligstroside Aglycons for Olive Oil Samples

	intraday precision (RSD%)		interday precision (RSD%)	
ratio	80 mg/L	160 mg/kg	80 mg/kg	160 mg/kg
oleuropein aglycon/IS	4.7	3.5	4.8	4.3
ligstroside aglycon/IS	4.9	3.3	4.8	3.7

Accuracy. The results for the accuracy are listed in Table 3 and are expressed as the relative percentage error (Er%). The estimated accuracy values with the proposed method are within acceptable levels for the two analytes. The obtained data indicate that the method could be considered as accurate.

Table 3. Accuracy Data of the Oleuropein and Ligstroside Aglycons Determination in Olive Oil Samples

	accuracy (Er%)		
ratio	10 mg/kg	80 mg/kg	160 mg/kg
oleuropein aglycon/IS	3.3	−2.9	0.70
ligstroside aglycon/IS	−9.3	−6.9	4.9

Recovery. The recoveries were found to be 85.5% (±3.5) and 85.8% (±3.1) for the 160 mg/kg levels for oleuropein and ligstroside aglycon, respectively, indicating acceptable recovery.

Sensitivity. The sensitivity of the method as presented by its limit of detection (LOD) and the limit of quantification (LOQ) were found to be 1 and 10 mg/kg, respectively, for both compounds, similarly with oleocanthal and oleacein as previously described.[7]

Isolation of Methyl Elenolate. During the isolation procedure of the four major secoiridoids, one additional minor constituent was also isolated and characterized. The ^{1}H NMR spectrum showed that it was a secoridoid derivative without a phenolic moiety. Examination of 1D and 2D NMR and MS data and comparison with literature data showed that it was the methyl ester of elenolic acid.[19,20] As in the case of oleuropein and ligstroside aglycons, methyl elenolate was isolated as a mixture of two diastereomers in a 1:4 ratio. The major one was identified as the 5*S*,8*R*,9*S* isomer by comparison with a synthetic methylated derivative of *epi*-elenolic acid.[20] The coupling constants of the major isomer were also in accordance with those described for the 5*S*,8*R*,9*S* isomer of oleuropein aglycon.[17] The main difference was the absence of the hydroxytyrosol moiety and its replacement by the methyl group. The minor diastereomer was identified as the 5*S*,8*S*,9*S* diastereomer of methyl elenolate described by Gariboldi et al.[19] as a synthetic product. As in the case of oleuropein and ligstroside aglycons, the formation of the minor isomer is most probably artificial (or artificially enhanced) due to the contact with the stationary phase during the chromatographic purification. This compound has been reported only as a synthetic derivative[19,20] and herein is reported for the first time as a constituent of olive oil.

Method Application. The developed method was applied to 340 samples of olive oil. The quantification results for each compound together with data about variety, geographic origin, and harvest time are provided as Supporting Information. The sum of the concentrations of the four compounds (named as D3) showed wide variation ranging from <1 to 1534 mg/kg (Figure 5). The highest recorded concentration of oleocanthal reached 711 mg/kg from cv. Koroneiki, while the concentration of oleacein was in almost all studied cases lower than that of oleocanthal (the highest recorded concentration for oleacein was 588 mg/kg). In total, among the Greek samples the highest concentration of the four secoiridoids were recorded in cv. Koroneiki from Aegean Islands (Paros, Antiparos), Messinia, Zakynthos, and Crete, and in cv. Throuba from Thassos Island (Table 4 and Supplementary Table 1, Supporting Information). Very interesting seems to be the case of olive oil samples from wild trees (Magnesia, Crete, Lakonia) showing very high oleocanthal content (Table 4 and Supplementary Table 1, Supporting Information). A map showing the main olive producing regions of Greece with the major variety in each region and the mean values of D3 has been constructed (Figure 6).

Concerning the two aglycons, their concentration in most studied cases was lower than that of oleocanthal and oleacein, and the previously described D1 index (sum of oleocanthal and oleacein) seems to have a dominating role in the total concentration of the secoiridoid derivatives. The concentration of the two aglycons in the Greek olive oil samples ranged from not detectable to 257 and 228 mg/kg for oleuropein and ligstroside aglycons, respectively. As shown in Figure 6, the sum of the four compounds is very well correlated with D1 as well as oleocanthal alone (supplementary Figure 9, Supporting Information), revealing the role of oleocanthal as the dominating factor at least among the Greek studied varieties. The highest recorded concentration of oleuropein aglycon (342 mg/kg) was recorded in a sample from cv. Mission from California, produced from green olives under stressed irrigation and low-temperature milling. The cv. Mission from California seems to be highly interesting, since in all studied samples the major secoiridoid was oleuropein aglycon. Moreover, in all Mission samples the concentration of oleocanthal and oleacein was lower than that of oleuropein and ligstroside aglycons, revealing that there are two distinct biosynthetic pathways leading to the domination of each group of compounds. It is apparent that regarding the phenolic secoiridoids of olive oil there are at least three different chemotypes: one with the domination of decarboxymethyl dialdehydic forms (oleocanthal,oleacein) (e.g., cv. Koroneiki, cv. Throuba, cv. Athenolia),

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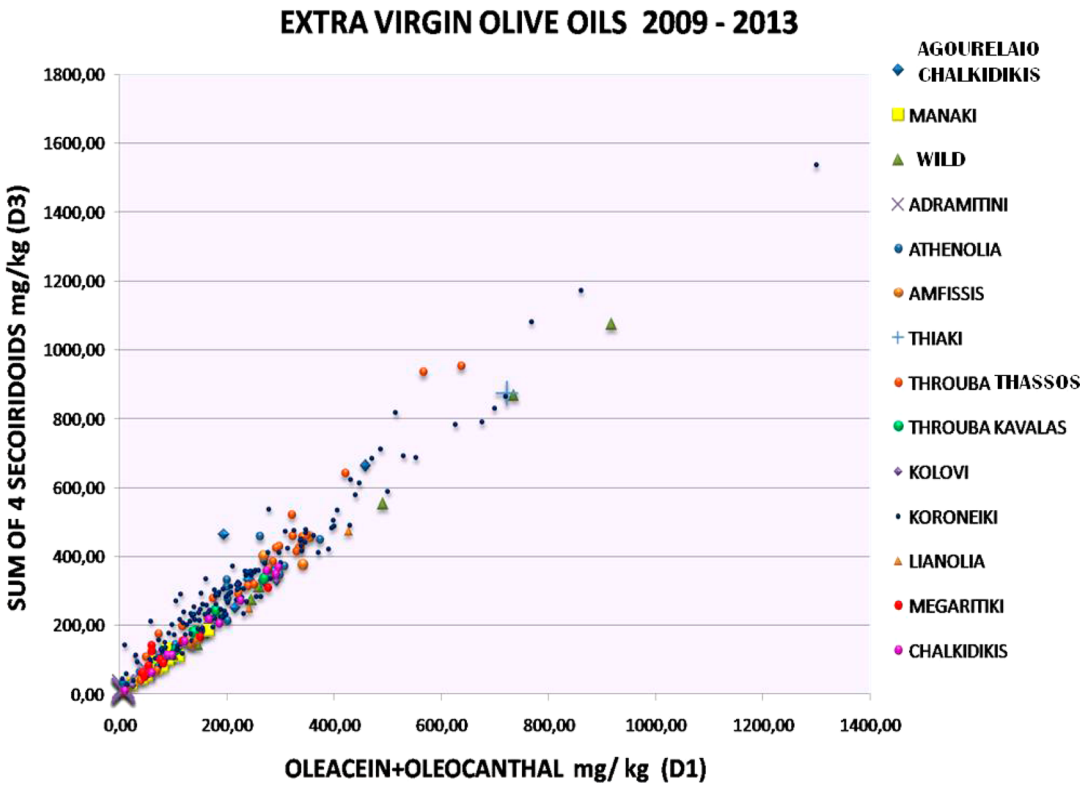

Figure 5. Distribution of oleocanthal, oleacein, oleuropein, and ligstroside aglycons concentrations among the studied Greek varieties.

Table 4. Top 15 Highest Concentration Greek Samples

cultivar	origin	harvest	oleocanthal, mg/kg	oleacein, mg/kg	oleuropein aglycon, mg/kg	ligstroside aglycon, mg/kg	D1,[a] mg/kg	D2[b]	D3,[c] mg/kg
Koroneiki	Paros	10/2012	711	588	120	114	1300	0.79	1534
Koroneiki	Antiparos[d]	10/2012	357	410	180	131	768	1.11	1080
Wild	Crete	10/2012	528	389	83	73	917	0.71	1075
Throuba	Thassos	10/2011	422	214	172	142	637	0.48	951
Throuba	Thassos	11/2011	515	51	139	228	566	0.08	933
Local	Zakynthos[d]	11/2012	559	185	80	93	745	0.31	919
Thiaki	Ithaki	11/2012	403	320	77	71	723	0.76	872
Wild	Lakonia	11/2012	631	103	108	25	735	0.15	868
Koroneiki	Zakynthos[d]	11/2012	423	297	68	74	720	0.67	863
Koroneiki	Zakynthos[d]	11/2012	415	284	64	64	699	0.65	828
Koroneiki	Paros	10/2012	417	98	257	40	516	0.22	814
Koroneiki	Messinia[d]	10/2012	393	283	52	58	676	0.69	788
Koroneiki	Antiparos[d]	11/2012	348	279	90	63	627	0.77	781
Koroneiki	Zakynthos[d]	11/2012	341	189	76	83	530	0.52	690
Koroneiki	Crete[d]	11/2012	284	268	78	52	553	0.90	684

[a]Oleacein + oleocanthal. [b]Oleacein/oleocanthal. [c]Sum of four secoiridoids. [d]Bottled samples.

one with the domination of monoaldehyde aglycons (e.g., cv. Mission), and one with the absence (or significantly reduced content) of both categories (e.g., cv. Adramytini, cv. Megaritiki) (supplementary Figure S1A–C, Supporting Information). However, we have also observed a number of samples with a different pattern presenting aldehyde peaks with significant intensity corresponding to not yet measured derivatives like oleuropein and ligstroside aglycon dialdehyde forms (supplementary Figure S1D, Supporting Information). The existence of varieties with other major secoiridoid derivatives makes necessary their exact identification and measurement. This is currently in progress as well as the expansion of the chemical database to other important varieties.

Although according to the European union legislation[21] all the studied samples were considered as extra virgin olive oils, the observed significant variation of the concentration of the bioactive polyphenolic secoiridoids confirms our previous conclusion[7] that there is need of a new type of classification of EVOO, especially related to possible health claims of those compounds.

Another observation that was further confirmed is that the ratio between oleocanthal and oleacein (index D2 = oleacein/oleocanthal) seems to be dependent on the olive tree variety, probably due to genetic reasons and independent of the olive mill procedure. Expanding our database[7] and including more samples (from 30 to 56 samples) coming only from cv.

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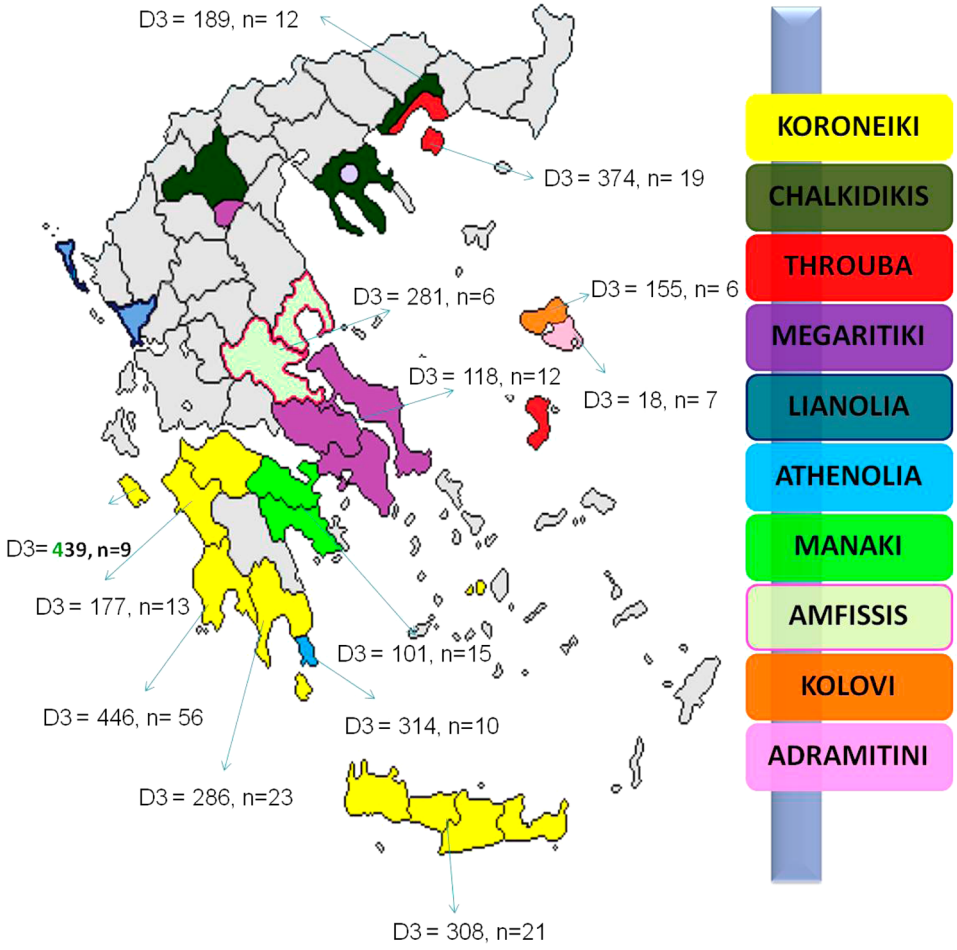

Figure 6. Map of the major olive oil producing regions of Greece showing the main variety cultivated/studied in each region and the mean D3 index for each region, *n* = number of studied samples.

Koroneiki from a narrow geographic region (Messinia) we observed that despite the variability of D1 (due to differences in harvest time or milling procedures), the correlation between oleocanthal and oleacein concentration was almost linear (R^2 = 0.71) and the mean D2 value was 0.50 ± 0.22 (supplementary Figure S8, Supporting Information). In contrast, in other varieties, for example Throuba Thassos (25 samples), the mean D2 value was significantly lower (0.29 ± 0.15) or, for Athenolia (20 samples, D2 = 0.31 ± 0.20), showed that the ratio between olacein and oleocanthal is mainly influenced by the tree variety.

Moreover, as previously reported for oleocanthal and oleacein, the highest levels of the two aglycons were also recorded in samples coming from early harvest showing again the positive correlation of the secoiridoid levels in olive oil with the low maturity index of the olive fruit.

As a final remark and based on the above observations, we would like to propose one more supplementary index, D3, as an addition to the previously described D1 and D2 indexes. Those indexes can be used for the support of health claims, since they are directly related with the European Union regulation[5] about the derivatives of hydroxytyrosol and tyrosol in olive oil. D1 and D3 are more accurate and specific than the commonly used total polyphenols index (expressed as gallic acid equivalent) and could become a new standard for the characterization of olive oil healthfulness.

■ ASSOCIATED CONTENT

Supporting Information

Isolation details for oleuropein and ligstroside aglycons; tables with the complete analysis of all the studied samples, including variety, geographic origin, harvest time, and analysis time; and methyl elenolate NMR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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