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Direct Measurement of Oleocanthal and Oleacein Levels in Olive Oil by Quantitative ^{1}H NMR. Establishment of a New Index for the Characterization of Extra Virgin Olive Oils

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Supporting Information

ABSTRACT: A new method for direct measurement of the oleocanthal and oleacein levels in olive oil by quantitative ^{1}H NMR was developed. The method was applied to the study of 175 monovarietal commercial Greek and California olive oil samples. The main findings were as follows: (1) There was a significant variation concerning the concentrations of oleocanthal and oleacein among the studied samples. Their concentrations ranged from nondetectable to 355 mg/kg and their sum (index D1) from 0 to 501 mg/kg. (2) There are olive varieties that independent of geographic origin and harvest time produce oil that contains both compounds in low levels. (3) There is a positive correlation of a high level of oleocanthal and oleacein in olive oils with the early time of harvest. Although there is a need for more extensive study, a new index for the characterization of extra virgin olive oils, which is a combination of D1 = oleocanthal + oleacein level and D2 = oleocanthal/oleacein ratio, seems to be very useful.

KEYWORDS: *extra virgin olive oil, oleocanthal, oleacein, quantitative NMR*

INTRODUCTION

The traditional Mediterranean diet is rich in fruits, vegetables, cereals, fish, wine, olives, and olive oil. This diet is associated with a lower incidence of atherosclerosis, cardiovascular disease, neurodegenerative diseases, and certain kinds of cancer. These appreciable health-promoting properties have been partially correlated with the regular consumption of virgin olive oil as the principal source of fat.[1,2]

Extra virgin olive oil (EVOO) is unique among other vegetable oils due to its high levels of monounsaturated fatty acids and the presence of minor components, such as phenolic compounds. The latter are responsible for the organoleptic characteristics of EVOO and several of its health-promoting properties. The major phenolic compounds identified and quantified in olive oil belong to three different classes: simple phenols (hydroxytyrosol, tyrosol), secoiridoids (the aglycone of oleuropein, the aglycone of ligstroside, and their respective decarboxylated dialdehyde derivatives), and lignans [(+)-1-acetoxypinoresinol and (+)-pinoresinol].[3−5]

One olive oil phenolic compound of particular interest due to its health-benefiting properties is the dialdehydic form of decarboxymethyl ligstroside aglycone, also known as oleocanthal. This compound was identified for the first time in olive oil by Montedoro et al. through spectroscopic characterization,[6] and it is responsible for the pungency associated with some extra virgin olive oils.[7] Beauchamp et al. isolated and identified oleocanthal as a natural nonsteroidal antiiflammatory drug due to its ibuprofen-like cyclooxygenase (COX-1 and COX-2) inhibiting activity.[8] These properties of oleocanthal are proposed to be responsible for the therapeutic properties of virgin olive oil, because inflammation plays a significant role in the development of numerous chronic diseases, such as cardiovascular disease, and certain kinds of cancers.[9] In addition, recent research has demonstrated that oleocanthal is a promising therapeutic agent for the treatment of inflammatory degenerative joint diseases.[10]

Oleocanthal can be a potentially useful agent for the development of new treatments for neurodegenerative tauopathies, such as Alzheimer's disease, due to its capacity to form an adduct with the lysine via initial Schiff base formation and prevent Aβ oligomerization and tau fibrillization as well, reducing thereby the pathogenicity.[11,12]

Moreover, recent research has shown that oleocanthal is stable in the gastric juice and displays antimicrobial activity against *Helicobacter pylori*, which is linked to a majority of peptic ulcers and to some types of gastric cancer.[13] Another biological property of oleocanthal is that it can control skin aging, so that it could be used for the treatment of damaged skin,[14] or that it can reduce a variety of disorders due to metabolic syndrome.[15,16]

The dialdehydic form of decarboxymethyl oleuropein aglycone, known as oleacein, has shown activities similar to those of oleocanthal, but the former has displayed significant antibreast cancer properties.[9] Oleacein has, also, potent antioxidant activities, even better than those of hydroxytyrosol.[17]

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It is astonishing that ancient reports by Dioscorides have correlated the health effects of olive oil and especially its anti-inflammatory activity with the use of unripe olives or specific varieties. Ancient recipes included in *Materia Medica* of Dioscorides propose olive oil, especially from unripe olives, "omotrives", against conditions such as headache and toothache, which are clear indications of in vivo anti-inflammatory activity. The modern finding of the strong anti-inflammatory activity of oleocanthal and its potential health effects that confirmed the ancient report prompted us to assume that the several varieties of olive oils could be classified on the basis of their content in oleocanthal and related secoiridoids. For this purpose we investigated the levels of oleocanthal and its related analogue oleacein in Greek and California olive oils of monovarietal origin in relation with the variety, the geographic origin, and the time of harvest. Our main target was first to show the significant variability of the oleocanthal and oleacein contents of commercially available olive oils. To achieve the above target, it was necessary to develop a fast and accurate method of analysis and to show that the new analytical tool could be used to study the main parameters that influence the olive oil content regarding these two compounds.

Until today, there are some chromatographic methods that have been described for that purpose, but the main problem for the oleocanthal and oleacein measurement is that they react rapidly with water or methanol used in the mobile phase, leading to broadened or multiple peaks, otherwise making necessary the use of derivatization reactions.[18]

For this reason we developed a method for olive oil polyphenol extraction without the use of any reacting solvent and a method for direct measurement of the oleocanthal and oleacein levels by quantitative ^{1}H NMR in $CDCl_3$ at 600 and 800 MHz.

The method was applied in the study of 175 monovarietal commercial olive oil samples from Greece and California, and the main findings led to the proposal of a new index for the characterization of extra virgin olive oils.

MATERIALS AND METHODS

Reference Compounds. Pure oleocanthal and oleacein were isolated from an olive oil extract prepared using the extraction method similar to that described below for sample preparation. The obtained extract was first fractionated using column chromatography, and then the fractions containing oleocanthal or oleacein were purified after repeated 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. The ^{1}H NMR purity of both compounds was >98%. The identity of both compounds was undoubtedly defined by extensive 2D NMR study and comparison with literature data.[6] 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 refrigerator. All NMR solvents used throughout the experiments were obtained from Sigma-Aldrich.

Extra Virgin Olive Oil Samples. The commercial EVOO samples used in the study were obtained from olives (*Olea europaea* L.) harvested and extracted in two seasons: November 2010–February 2011 and October 2011–February 2012. In total, 158 monovarietal commercial samples were obtained from Greece and 17 from California; samples of 19 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 solution (0.5 mg/mL) in acetonitrile, and evaporated under reduced pressure using a rotary evaporator (Buchi, Flawil, Switzerland).

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 using a NMR spectrometer (Bruker Avance 600). 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 weighting factor corresponding to a line broadening of 0.3 Hz was applied. The spectra were phased corrected and integrated automatically using TOPSPIN. When necessary, accurate integration was performed manually for the peaks of interest.

Calibration Curves and Quantitation. Calibration curves were prepared by the addition of known quantities of pure oleocanthal or oleacein to a selected olive oil, from the Adramytini variety, naturally free of oleocanthal and oleacein 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 oleocanthal or oleacein appearing at 9.23 and 9.19 ppm, respectively. It should be noted that the olive oil used for the calibration curves should be fresh. We have observed that aged or oxidized olive oil could rapidly lead to the formation of oleocanthal or oleacein derivatives disturbing the accurate measurement.

Standard and Spiked Solutions. Stock standard solutions of oleocanthal and oleacein were prepared in acetonitrile at the 0.5 mg/mL level and were kept in refrigeration. Spiked olive oil samples were prepared to give concentrations of oleocanthal and oleacein of 10, 20, 40, 80, 160, and 320 mg/kg by diluting appropriate volumes of the stock standard solutions in 5.0 g of olive oil of variety Adramytini. A sample of Adramytini variety was selected as a blank because it was found that it did not contain oleocanthal or oleacein 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 linearity, precision [calculated as the relative standard deviation % (RSD %)], accuracy [evaluated as the relative percentage error % (Er %)], and sensitivity [evaluated as the limits of detection (LOD) and quantitation (LOQ)].

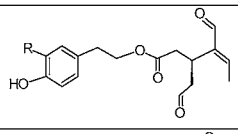
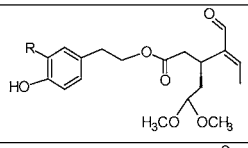
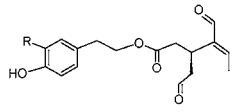
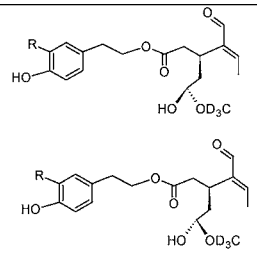
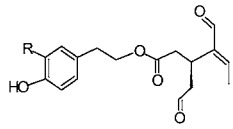
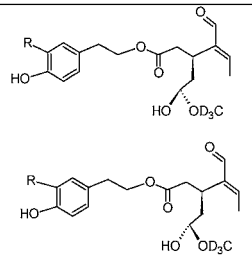
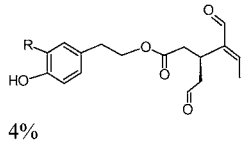
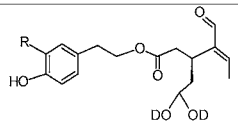
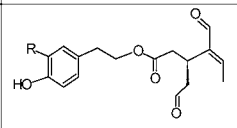
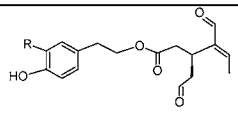
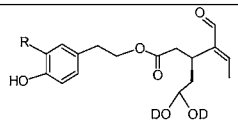
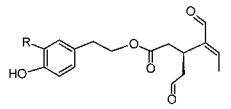
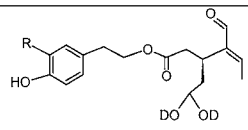
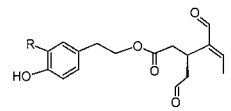
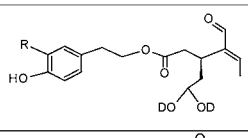
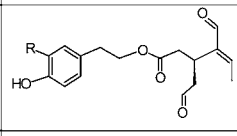
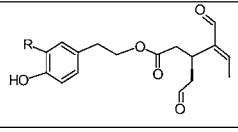
Linearity. Spiked olive oil samples were prepared to give concentrations of oleocanthal and oleacein at 10, 20, 40, 80, 160, and 320 mg/kg and were analyzed for the determination of the linearity. The relationship of the integration ratio of the analytes versus the IS and the corresponding concentration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

Precision. The intraday precision was determined by analyzing five replicates of spiked olive oil samples at two concentration levels (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, prepared on five different days.

Accuracy. Spiked olive oil samples at three concentration levels, 10, 80, and 160 mg/kg, were analyzed to determine the accuracy of the method. The results were expressed as the relative percentage error Er %, defined as [assayed concentration − nominal concentration]/[nominal concentration] × 100.

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 ± standard deviation (SD).

Table 1. Oleocanthal (R = H) and Oleacein (R = OH) Derivatives Observed Using Several NMR Solvents

NMR Solvent	Observed molecules (R=OH, R=H)	
$CDCl_3$	 100%	
$CDCl_3$ +10μL CH_3OH	 95%	 5%
CD_3OD	 48 % + 48%	 4%
CD_3OD : D_2O (1:1)	 48 % + 48%	 4%
D_2O	 80%	 20%
d-ACN	 100%	
d-ACN -D_2O (9 : 1)	 20%	 80%
d-ACN -D_2O (1 : 1)	 25% (after 24 h, 60%)	 75% (after 24 h, 40%)
d-ACN -D_2O (1 : 9)	 66%	 34%
DMSO	 100%	

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.

RESULTS AND DISCUSSION

NMR Study of the Reaction of Oleocanthal and Oleacein with Different Solvents. Our first attempt to quantify oleocanthal in olive oil extracts was performed

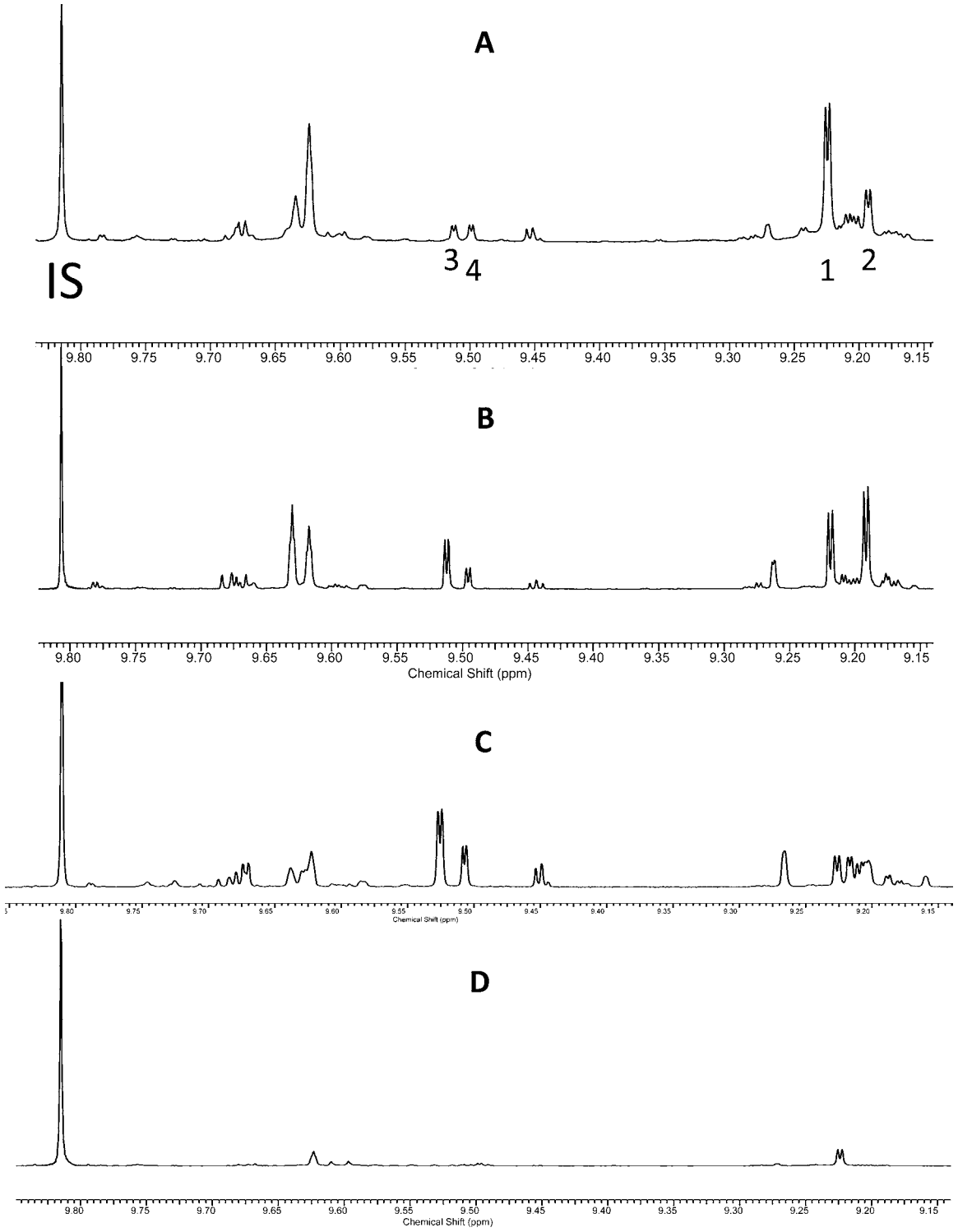


Figure 1. Each variety presents a unique profile in the 9.1−9.8 ppm region in the ^{1}H NMR spectrum: (A) Koroneiki variety (with typical oleocanthal/oleacein ratio); (B) Koroneiki variety (with unusual oleocanthal/oleacein ratio); (C) Mission variety; (D) Megaritiki variety. The signals for quantitation of oleocanthal and oleacein are noted as 1 and 2, respectively. Peaks 3 and 4 correspond to 3,4-DHPEA-EA (oleuropein aglycon) and p-HPEA-EA (ligstroside aglycon), respectively. Mission was the only variety among the 19 studied varieties in which the oleacein peak could not be accurately integrated. IS, internal standard.

following previous works[8,19] using HPLC-UV with reversed phase columns and aqueous mobile phase, and in that case we observed that pure oleocanthal did not give a single sharp peak. This problem, which had been previously observed,[18] prompted us to investigate in more detail the reaction of oleocanthal and oleacein with water, methanol, acetonitrile, DMSO, or their mixtures. The study was performed by NMR using deuterated solvents and monitoring in situ the formation of the corresponding derivatives. We found that both compounds react spontaneously with water or methanol to give mixtures of hemiacetals or acetals that were characterized using 1D and 2D NMR spectra (Table 1). The proportion of each form

presented in Table 1 was determined by integration of the aldehyde protons of the dialdehyde form in comparison with the integration of the hemiacetal or acetal proton of the produced monoaldehydes. Interestingly, oleocanthal and oleacein gave a NMR spectrum corresponding each to a single molecule only in the case of pure $CDCl_3$, *d*-ACN, and DMSO.

The above findings confirm that the classic chromatographic measurement of these compounds in aqueous media is problematic and that many of the previous measurements reported in the literature are more or less questionable. For example, as shown in Table 1, the proportion between the aldehydic and the hydrated form in water/acetonitrile mixtures is time and solvent ratio dependent, making very difficult the accurate measurement.

To override the above-described problem, we applied a method for olive oil extraction without the use of any reacting solvent and developed a method for direct measurement of the oleocanthal and oleacin levels by quantitative ^{1}H NMR in $CDCl_3$ at 600 MHz.

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 analytes. In contrast, methanol, which is commonly used for the extraction of phenolics from olive oil, reacts immediately with the aldehydic form oleocanthal or oleacein, leading to the corresponding acetals or hemiacetals. Evaporation of methanol and redilution in chloroform afford again the aldehydic form, but we have not studied if this reaction is quantitative, and for this reason we avoided the use of methanol as extraction solvent. Moreover, one extraction with acetonitrile was sufficient for >85% recovery of both 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 of pure compounds added in olive oil free of both compounds.

NMR Spectral Analysis of Oleocanthal and Oleacein in Extra Virgin Olive Oil. ^{1}H NMR spectroscopy was envisaged as a simple and reliable alternative methodology for monitoring oleocanthal and oleacein in olive oil and has been recently applied for the quantification of other olive oil phenolics[20] as well as in chemometric studies.[21,22]

The method was based on the observation that the ^{1}H NMR spectrum of olive oil acetonitrile extracts when recorded in $CDCl_3$ and in magnetic fields of 600 or 800 MHz 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 1). This spectrum region in all of the studied samples was clearly resolved, making feasible the integration of the corresponding peaks and their comparison with the peak of the internal standard. Oleocanthal and oleacein were quantified by integrating doublets at 9.23 and 9.19 ppm, respectively. In addition, oleuropein aglycon (3,4-DHPEA-EA) at 9.50 ppm and ligstroside aglycon (p-HPEA-EA) at 9.52 ppm could also be quantified, but their study has not been included in this work. It should be stated that in a very few cases (<3%) the aldehydic proton of oleacein partially overlapped with other aldehydic protons of yet unknown compounds, making difficult their exact integration. However, we never observed a similar problem for oleocanthal, and for the partially overlapped oleacein we could still have a good estimation of its quantity. In those cases, even the measurement of the ^{1}H NMR spectrum at 800 MHz did not offer significant amelioration. On the contrary, measurement of the ^{1}H NMR spectrum at 400 MHz was problematic for both oleacein and oleocanthal.

Selection of Internal Standard. The choice of syringaldehyde as internal standard was based on the following reasons: (1) None of the studied olive oil extracts contained syringaldehyde or any other peak overlapping with the aldehydic proton of syringaldehyde. (2) It is a cheap, stable, and easily affordable compound, well soluble in $CDCl_3$. (3) The ^{1}H NMR spectrum of syringaldehyde is very simple, consisting only in three singlets, minimizing the possibility of overlap with other interesting spectrum regions.

Selection of NMR Solvent. The selection of $CDCl_3$ as solvent for the NMR measurement was based on the observation that it was one of the three common solvents that does not react with the studied compounds, in contrast to methanol or water. The advantage of $CDCl_3$ when compared with *d*-ACN or d_6-DMSO is that in the two latter solvents the aldehydic protons of the studied compounds overlap and cannot be integrated,[20] whereas in the case of $CDCl_3$ all of the measured peaks could be clearly observed.

Linearity. Good linearity was achieved for both analytes, as indicated by the equations listed in Table 2, for concentration ranging from 10 to 320 mg/kg, with satisfactory correlation coefficients, r^2 (0.999 and 0.994 for oleocanthal and oleacein, respectively).

Table 2. Oleocanthal and Oleacein Determination in Olive Oil Samples

(A) Linearity		
ratio	regression eq	correl coeff, r^2
oleocanthal/IS	$y = 270.1x + 4.2$	0.999
oleacein/IS	$y = 279.7x + 6.5$	0.994

(B) Precision Data				
	intraday precision (RSD %)		interday precision (RSD %)	
ratio	80 mg/L	160 mg/kg	80 mg/kg	160 mg/kg
oleocanthal/IS	4.9	3.7	5.1	4.5
oleacein/IS	5.4	3.3	4.8	3.8

(C) Accuracy Data			
	accuracy (Er %)		
ratio	10 mg/kg	80 mg/kg	160 mg/kg
oleocanthal/IS	5.8	6.5	−0.9
oleacein/IS	15.6	−0.1	−0.1

Precision. The intraday precision, expressed as the relative standard deviation (RSD), ranged from 3.3 to 5.4% for the two analytes, as shown in Table 2. The interday precision ranged between 3.8 and 5.1% (Table 2). The RSD values are adequate and indicate the suitability of the method.

Accuracy. The results for the accuracy are listed in Table 2 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.

Recovery. The recoveries were found to be 85.2% (±3.6) and 85.9% (±3.0) for the 160 mg/kg levels for oleocanthal and oleacein, respectively, indicating acceptable recovery.

Sensitivity. The sensitivity of the method was represented by its LOD and LOQ, which were found to be 1 and 10 mg/kg, respectively, for both compounds.

Table 3. Top 10 Highest Concentration Greek Samples (All of the Koroneiki Variety)

origin	harvest	oleocanthal integration	oleocanthal (mg/kg)	oleacein integration	oleacein mg/kg	D1	D2
Paros	October 2011	1.30	355.0 ± 12.1	0.50	146.5 ± 4.6	501.4	0.39
Messinia	November 2011	0.71	196.5 ± 5.1	1.02	291.7 ± 10.2	488.2	1.42
Messinia	November 2011	0.77	212.1 ± 4.5	0.90	259.4 ± 9.9	471.5	1.17
Messinia	November 2011	0.78	215.0 ± 3.9	0.75	217.2 ± 7.5	432.2	0.96
Messinia	November 2011	1.02	281.0 ± 8.3	0.43	126.4 ± 2.1	407.4	0.42
Messinia	November 2011	0.86	236.2 ± 6.1	0.56	164.0 ± 2.6	400.2	0.66
Zakynthos	November 2010	0.72	198.3 ± 5.2	0.69	197.2 ± 4.8	395.4	0.95
Lakonia	November 2011	0.94	258.0 ± 7.6	0.36	104.8 ± 2.0	362.8	0.38
Messinia	November 2011	0.66	183.9 ± 5.0	0.56	164.1 ± 5.1	348.0	0.85
Messinia	November 2011	0.72	199.7 ± 4.9	0.51	148.3 ± 5.0	348.0	0.70

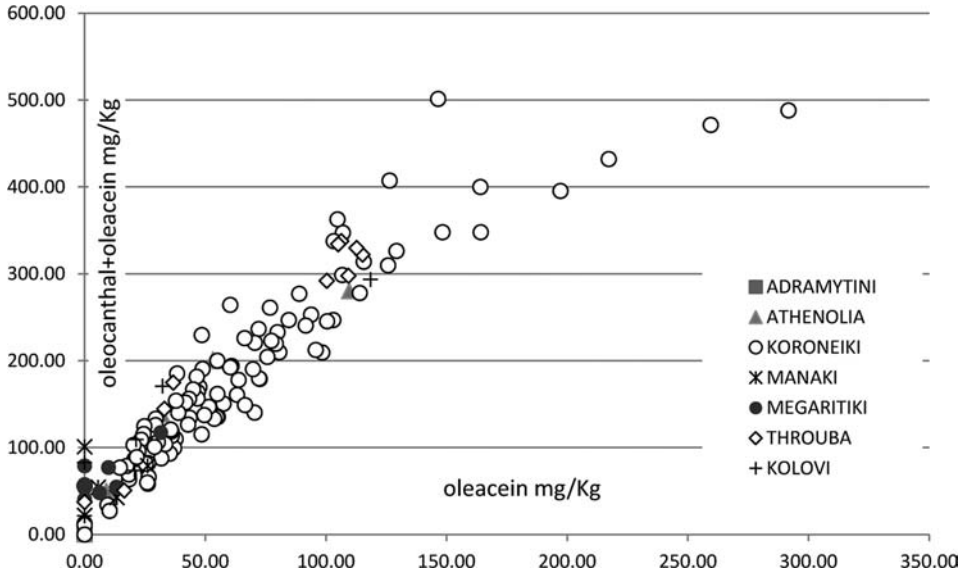


Figure 2. Distribution of oleocanthal and oleacein concentrations among the studied varieties.

Method Application. The method was applied in the study of 175 commercial olive oil samples and the main findings were the following:

There was a highly significant variation concerning the concentrations of oleocanthal and oleacein among the studied commercial extra virgin olive oil samples (in both Greek and California samples), in accordance with previous studies recently reviewed.[23] The concentrations of both compounds ranged from nondetectable to 354 and 292 mg/kg, respectively, and their sum (index D1) from 0 to 501 mg/kg. Although according to European Union legislation[24] all of the studied samples were considered as extra virgin olive oils, the observed significant variation of the concentration of the bioactive polyphenolic secoiridoids makes more intense the need of a new type of classification especially related to possible health claims of those compounds.

The highest concentrations of oleocanthal and oleacein among the Greek samples were recorded in olive oil samples produced from the cv. Koroneiki, reaching maxima of 355 and 292 mg/kg. respectively (Table 3). The majority (101 of 158) of Greek samples were of cv. Koroneiki because this is the most abundant olive variety (for olive oil production) in Greece. The mean values for oleocanthal, oleacein, and D1 in the Koroneiki group were 117, 65, and 182 mg/kg, respectively. The majority (70%) of the samples from cv. Koroneiki gave values for oleacein of >30 mg/kg and for oleocanthal + oleacein of >100 mg/kg (Figure 2). However, a portion of olive oil samples from cv. Koroneiki showed significantly lower concentrations, and this result in most cases could be attributed either to very late harvest (full ripe fruit) or to high temperature during malaxation or both.

There was also a group of Greek olive varieties (25 samples belonging to cv. Megaritiki, cv. Manaki, and cv. Adramytini) that, independent of geographic origin, harvest time (early or late), or olive mill related parameters, produced olive oil that contains both compounds in lower levels (mean values of 54 and 10 mg/kg, respectively, D1 = 49.3, D2 = 0.13). The olive oil produced by those varieties is traditionally preferred for confectionary due to its lower sensation of bitterness and pungency, and our findings confirmed their lower content of oleocanthal and oleacein, which are related with those specific organoleptic properties.

Another observation was 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. Interestingly, when we focused on a group of 30 samples from only nonirrigated cv. Koroneiki from a very narrow geographic region (Messini), 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 = 0.78) and the mean D2 value was 0.56 ± 0.19 (Figure 3). The mean D2 value for the samples from all other Greek varieties except Koroneiki (57

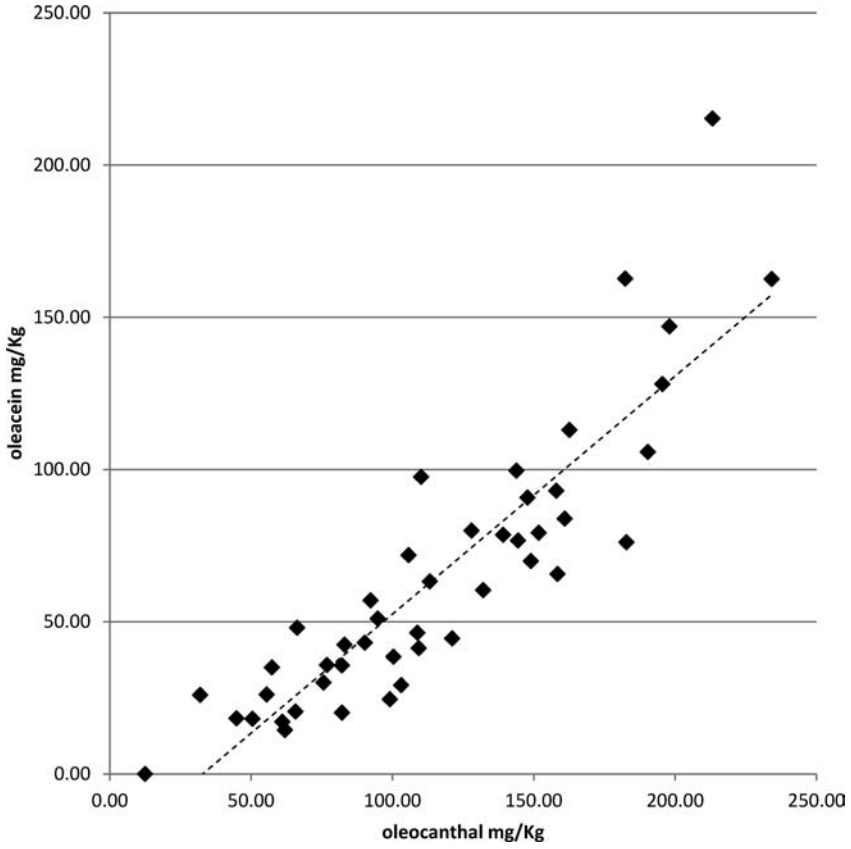


Figure 3. Oleocanthal and oleacein contents of a selected group of the Koroneiki variety from a narrow geographic origin (Messinia, Greece) showing that the ratio between both compounds is relatively stable independent of harvest time and olive mill conditions.

samples), excluding the group of varieties with nondetectable oleacein, was significantly lower (0.35 ± 0.14), showing that the ratio between oleacein and oleocanthal is mainly influenced by the tree variety. However, the relatively small number of each of the other varieties except Koroneiki and the complexity of the factors influencing olive oil productions make necessary the continuation of the study to obtain more reliable results.

Another observation that is in accordance with previously published data[25] is that there is a positive correlation of oleocanthal and oleacein concentration with the early time of harvest. It is noteworthy that the highest concentration of oleocanthal (355 mg/kg) was recorded for a sample from Koroneiki variety (Paros Island) produced using only green olives. Similarly, the second and third highest D1 indices (488 and 471 mg/kg) were recorded for early harvested (early November) Koroneiki variety from Messinia. The same variety from the same olive grove collected after 2 months (early January) and processed in the same olive mill under the same conditions afforded an olive oil with a D1 index of 87 mg/kg.

In total, among the Greek samples the highest concentrations were recorded in Koroneiki from Messinia, Paros, eastern Lakonia, central Crete, Throuba from Thassos Island, and Conservolia from Thesprotia (Supporting Information, Supplementary Table 1).

Similarly to the Greek samples, significant differences were also observed for the California olive oil samples. Samples from seven different varieties were studied, and large differences were recorded. For example, the highest variability in D1 index was found for Tagiasca, Leccino, and Barouni, ranging from 45.5 to 274.8 and 406.0 mg/kg, respectively (Supporting Information, Supplementary Table 2).

Although we measured only two compounds bearing aldehyde groups, the NMR spectrum in the range 9.1−9.8 ppm seems to present a unique recognition pattern especially characteristic for some olive oil varieties. For example, Mission olive oil from several different places, different harvest times, and two different years presented a set of peaks that was clearly different from all other studied varieties (Figure 1). The ^{1}H NMR spectrum except of offering a method for quantitation likely offers a tool for variety recognition, but this finding needs further elaboration.

Although there is need for more extensive study, on the basis of the above observations we propose a new index for the characterization of extra virgin olive oils, which is a combination of D1 = oleocanthal + oleacin level with the D2 = oleocanthal/oleacin molar ratio.

On the basis of these results we were able to categorize the studied olive oils in high and low levels. Although all of the studied samples belong to the category of extra virgin olive oil, there is such a significant difference between them concerning the D1 and D2 indices that the use of those indices could be used to characterize the quality of olive oil. Those indices mainly concern the support of health claims (especially those related to oleocanthal) and are more specific than the gallic acid equivalent related to antioxidant activity.

Effect of Storage on Oleocanthal and Oleacein Levels. A few olive oil samples were measured for their oleocanthal and oleacein levels over a 12 month period. The samples were produced in December 2010 and measured during the first month after production and 12 months later. In the meantime, the samples were kept in amber glass bottles in a dry and cool place. For olive oils with D1 > 200, an average reduction by

10−15% was measured, with oleacein showing in all cases a higher percentage of reduction. However, in the case of olive oils with D1 < 100, the reduction reached 50% at 12 months (Supporting Information, Supplementary Table 3). Although the stability of both compounds in olive oil has been recently reported,[26] we show herein that the stability is not similar in all EVOOs.

■ ASSOCIATED CONTENT

Supporting Information

Oleocanthal and oleacein isolation details, tables with the complete analysis of all the studied samples, including variety, geographic origin, harvest time and analysis time. 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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