

# S-(E)-Elenolide: a new constituent of extra virgin olive oil

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## Abstract

**BACKGROUND:** Extra virgin olive oil is a food with a recognized health claim in the EU related to its phenolic content. Based on nuclear magnetic resonance (NMR) analysis, we observed for the first time that most high-phenolic olive oils also contain significant quantities of another potential beneficial ingredient, S-(E)-elenolide, which is a non-phenolic compound related to oleuropein or ligstroside. Elenolide had only been found in olive leaves and fruits as the Z isomer or had been synthesized and had been recognized as an antihypertensive agent.

**RESULTS:** (E)-Elenolide was isolated from olive oil and its structure was elucidated and completely characterized for the first time using 1D and 2D NMR and gas chromatography–mass spectrometry. In addition, we developed a method of quantitative measurement based on qNMR. Investigation of 2120 olive oil samples showed that elenolide was present in the majority of samples, in quantities ranging from 0 to 2821 mg kg<sup>-1</sup>. Although elenolic acid, which is a hydrated derivative of elenolide, had been reported as an olive oil ingredient, this is the first time that elenolide has proved to be transformed to elenolic acid after reaction with water. Finally, it was found that the quantity of elenolide in olive oil depends on the quantity of water remaining in the olive oil during storage.

**CONCLUSION:** S-(E)-Elenolide is a new important substance of olive oil and could be used as marker of high-quality oils with low water content.

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**Keywords:** olive oil; oleoside; NMR; elenolide; elenolic acid

## INTRODUCTION

Olive oil has been officially recognized, since 2012, by the European Union as a food that under specific conditions can bear a health claim related to the protection of blood lipids from oxidation.<sup>1</sup> If an olive oil contains at least 5 mg hydroxytyrosol and its derivatives (oleuropein complex and tyrosol) per daily dose of 20 g, it can be considered as beneficial for consumers health and specifically for protection against oxidative stress.<sup>2–4</sup> A variety of compounds related to oleuropein (**1**) and ligstroside (**2**), such as oleacein (**3**),<sup>5</sup> oleocanthal (**4**),<sup>5</sup> oleuropein and ligstroside aglycons (**5**, **6**)<sup>6</sup> and their dialdehydic, monoaldehydic and enolic forms (known also as oleuropeindials (**7**), ligstrodials (**8**), oleomissional (**9**) and oleokoronol (**10**)),<sup>7</sup> have been recognized as belonging to the class of phenols that are measured in order to support the above health claim.<sup>8</sup>

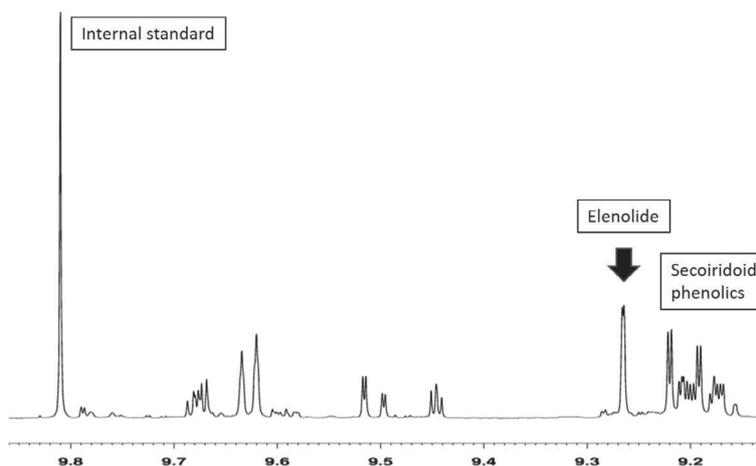
A reliable analytical method based on 1D quantitative nuclear magnetic resonance (qNMR) spectroscopy has been recently developed to measure the concentration of all the compounds mentioned in the regulation with a simple experiment, avoiding the formation of artifacts and providing the necessary data for certification of the health claim.<sup>5,6</sup> More than 5000 samples have been analyzed with this method since 2012 and the corresponding spectra have been included in the olive oil database of the Laboratory of Pharmacognosy of the University

of Athens. One of the main advantages of this method is that any new compound detected in the olive oil can be quantified any time after its identification by reprocessing of the recorded spectra.

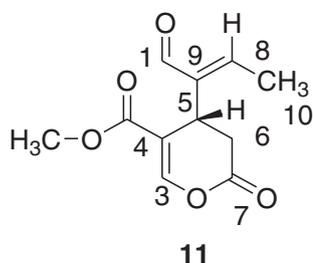
Careful observation of the recorded <sup>1</sup>H-NMR spectra that were included in the database led to the detection of a peak at 9.27 ppm that appeared in most olive oil samples and did not correspond to any known ingredient of olive oil mentioned in the literature (Fig. 1). This observation prompted us to isolate the compound that was responsible for this signal at 9.27 ppm. The isolation of this compound permitted us to elucidate its structure using 1D and 2D NMR and gas chromatography–mass spectrometry (GC-MS) and identify it as a compound known as S-(E)-elenolide (**11**) (Fig. 2). Subsequently, we developed a quantitation method for elenolide allowing the measurement of its concentration in an array of olive oils. A total of 2120 previously recorded olive oil

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**Figure 1.**  $^1\text{H-NMR}$  spectrum of EVOO extract at 700 MHz showing the characteristic peak of elenolide that was used for quantitation.



**Figure 2.** The chemical structure of elenolide (**11**).

spectra were reprocessed, permitting us to define the range of elenolide concentration in olive oil.

Finally, we were able to show that elenolide is transformed to elenolic acid (**12**) when it comes into contact with water, and as a result its concentration should be dependent on the concentration of remaining water during storage.

## MATERIALS AND METHODS

### Chemicals and standards

All solvents were of analytical grade and purchased from Merck (Darmstadt, Germany). Preparative thin-layer chromatography (TLC) with silica gel 60 RP-18 F<sub>254</sub>-Merck and column chromatography with silica gel POLYGOPREP 60-130 C<sub>18</sub> were used for the isolation of elenolide (**11**). 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<sup>-1</sup> and kept in a refrigerator. Prior to use the IS solution was allowed to reach room temperature.

### Instrumentation

GC-MS chromatograms for the determination of elenolide (**11**) were recorded in a Hewlett-Packard 6890-5973 apparatus with a 30 m × 0.25 mm HP-5 MS (DB-5) column, 0.25 μm membrane thickness, 60 °C (5 min) temperature at 280 °C at a rate 3 °C min<sup>-1</sup> and a flow of 0.8 mL min<sup>-1</sup>.

The structure elucidation of the isolated elenolide and its quantitative determination in olive oil were performed using NMR spectroscopy on Bruker Avance 700 MHz (Virginia Commonwealth

University) and DRX 400 MHz (University of Athens) instruments.  $^1\text{H-NMR}$  spectra as well as two-dimensional correlation spectroscopy (COSY), nuclear Overhauser effect spectroscopy (NOESY), heteronuclear single quantum coherence (HSQC) and heteronuclear multiple bond correlation (HMBC) were recorded. CDCl<sub>3</sub> was used as a solvent because of its advantage in not reacting with the studied compound. The spectra were processed using either the MNova (Mestrelab Research) or the TOPSPIN program.

### Origin of olive oil samples

The EVOO samples used in the current study were obtained from olives (*Olea europaea* L.) harvested and extracted in eight consecutive years: November 2010 to February 2018. A total of 2120 monovarietal samples were obtained from Greece, Italy, Spain, Croatia, Cyprus and USA, including samples of 25 different varieties in the study. 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 origin and were analyzed fresh, no later than 2 weeks after their production.

For the isolation procedure, an olive oil with high content of elenolide (**11**), based on the integration at 9.27 ppm, was selected: Milestone (Mammasis Konstantinos) from Achaia, Greece, Koroneiki variety.

### Olive oil extraction for analysis

Olive oil (5.0 g) was mixed with cyclohexane (20 mL) and acetonitrile (25 mL). The mixture was homogenized using a vortex mixer (VXMTAL multi-tube vortex mixer, OHAUS) for 30 s and centrifuged at 4000 rpm for 5 min. Part of the acetonitrile phase (25 mL) was collected, mixed with 1.0 mL of a syringaldehyde solution (0.5 mg mL<sup>-1</sup>) in acetonitrile, and evaporated under vacuum using a rotary evaporator (Buchi, Flawil, Switzerland).

### NMR spectra analysis

The residue of the above procedure was dissolved in CDCl<sub>3</sub> (750 μL) and an accurately measured volume of the solution (550 μL) was transferred to a 5 mm NMR tube.  $^1\text{H NMR}$  spectra were recorded at 400 MHz. Typically, 32 scans were collected into 32 K 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

**Table 1.**  $^1\text{H-NMR}$  and  $^{13}\text{C-NMR}$  data of elenolide in  $\text{CDCl}_3$ 

	$^1\text{H-NMR}$	$^{13}\text{C-NMR}$
1	9.27, d, 0.9	194.0
3	7.65, s	152.0
4		108.8
5	4.05, dd, 10.0, 1.2	27.5
6	2.57, dd, 16.7, 1.2 2.87, dd, 16.7, 10.0	35.1
7	–	164.0
8	6.74, q, 8	153.0
9	–	142.8
10	2.17, d, 8	15.2
—C=O	–	165.8
—OCH <sub>3</sub>	3.74, s	51.7

to Fourier transformation (FT), an exponential weighting factor corresponding to a line broadening of 0.3 Hz was applied. The spectrum was phase corrected and accurate integration was performed manually for the peaks of interest. Elenolide was identified and quantitated by integrating the peak of the aldehydic proton at 9.27 ppm.

### Elenolide isolation

The 'Milestone' olive oil sample (125 g), 2 months after its production, was extracted with acetonitrile (125 g) and the acetonitrile phase was obtained after centrifugation and evaporated under vacuum using a rotary evaporator. Then the residue was submitted to column chromatography using reversed-phase silica gel for the removal of the lipids. The first four fractions (100 mL) were eluted with 100% acetonitrile. Fractions 1–4 containing elenolide (**11**) and other phenols but no lipids were identified with spotting on TLC reversed-phase plates, developed with  $\text{H}_2\text{O}$ –acetonitrile (60:40) v/v.

Subsequently, a portion of the above mixture (28 mg) was purified with reversed-phased preparative TLC developed with 60:40 v/v  $\text{H}_2\text{O}$ –acetonitrile. Pure elenolide (2.8 mg) was isolated from the zone with  $R_f = 0.26$  after extraction with acetonitrile. The final purification step was repeated until a sufficient amount of elenolide was isolated to develop the quantitation method. The elenolide was in the form of colorless needle-shaped crystals in accordance with literature data.<sup>9–11</sup> The purity of the isolated elenolide was checked by  $^1\text{H-NMR}$  spectroscopy and was found to be >95%. The complete NMR data of elenolide are provided in Table 1.

### Quantitative determination of elenolide in olive oil with qNMR

#### Preparation of the concentrated olive oil

Elenolide (16.8 mg) was dissolved in 30 g of a specifically selected olive oil (blank) with zero phenolic and zero elenolide content. This olive oil was selected after NMR analysis among extra virgin olive oils obtained from ripe olives using the above-mentioned protocol. The blank oil presented no signal at the region between 9.0 and 9.8 ppm in the  $^1\text{H-NMR}$  spectrum.

#### Preparation of internal standard

5.0 mg syringaldehyde was dissolved in 10 mL acetonitrile ( $c = 0.5 \text{ mg mL}^{-1}$ ).

### Calibration curves and quantitation

Calibration curves were constructed using olive oils containing 560, 280, 140, 70, 35 and  $17.5 \text{ mg kg}^{-1}$  elenolide (**11**) that were analyzed following the above-described measurement method. The corresponding olive oils were prepared by mixing appropriate amounts of the concentrated olive oil with the blank olive oil.

Quantitation was based on the integration ratio between the proton signal of syringaldehyde at 9.81 ppm and the proton of elenolide at 9.27 ppm. Using the following formula, which includes the relative integration to the internal standard, the final concentrations were expressed as milligrams per kilogram of olive oil:  $C = 618.4 \times \text{Integr} - 4.9$ .

### qNMR 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)  $\times 100$ ), and sensitivity (evaluated as the limits of detection (LOD) and quantitation (LOQ)).

**Linearity.** Olive oil samples were prepared to give concentrations of 560, 280, 140, 70, 35 and  $17.5 \text{ mg kg}^{-1}$  of elenolide and were analyzed for the determination of linearity. The relationship of the integration ratio of the analytes versus the internal standard and the corresponding concentration of elenolide was determined by linear regression analysis (provided as supporting information).

**Precision.** The intraday precision was determined by analyzing three replicates at three concentration levels: 17.5, 140 and  $560 \text{ mg kg}^{-1}$  olive oil.

**Accuracy.** Samples were prepared at three concentration levels – 17.5, 140 and  $560 \text{ mg kg}^{-1}$  olive oil – and were analyzed in order to determine the accuracy of the method. The results were expressed as the relative percentage error, defined as [(assayed concentration – nominal concentration)  $\times 100$ ]/[nominal concentration].

**Recovery.** For the calculation of recovery, spiked olive oil with concentration levels 17.5, 140 and  $560 \text{ mg kg}^{-1}$  olive oil ( $n = 3$ ) were analyzed by employing the proposed extraction procedure. The recovery was calculated as the ratio of the response of elenolide in the spiked olive oil samples against that of the standard at the same levels and was expressed as the mean  $\pm$  standard deviation (SD).

**Limits of detection and quantitation.** LOD and LOQ were determined by running five blank samples and measuring the background response at 9.27 ppm. Signal-to-noise (S/N) ratios of 3:1 and 10:1 were used for the calculation of the LOD and LOQ, respectively.

### Quantitative determination of elenolide with GC-MS

The calibration curve for the GC-MS measurements was constructed using the following concentrations: 560, 280, 140, 70, 35 and  $17.5 \text{ mg kg}^{-1}$  (provided as supporting information). The elenolide peak was integrated at 14.4 min, corresponding to a 224 amu molecular ion. Syngaldehyde in acetonitrile ( $0.5 \text{ mg mL}^{-1}$ ) was used as internal standard.

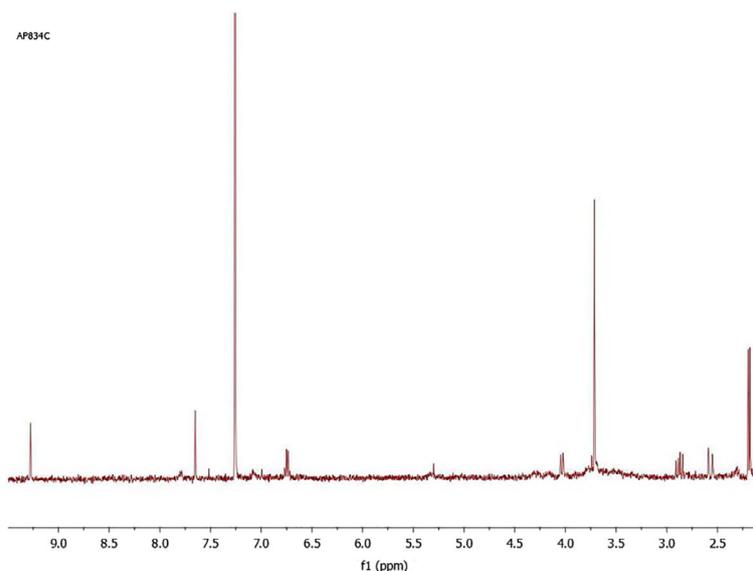


Figure 3. NMR spectrum of elenolide (**11**) at 400 MHz.

### Impact of water content during storage

An olive oil sample of Koroneiki variety from Messinia was dried using sodium sulfate, filtered and analyzed with the above-described NMR method to measure its precise elenolide (**11**) content as well as its phenolic content. The dry olive oil sample was divided into four equal parts of 10 g and different amounts of water (200, 100, 50 and 20 mg) were added to each one, affording olive oil samples containing 2, 1, 0.5 and 0.2% (w/w) of water. The samples were stored in airtight vials without headspace, in a dry and dark place for 14 days at 25 °C. After the end of the storage period, 5 g olive oil was obtained from each vial and the samples were analyzed by the above-described qNMR method. The whole experiment was run in triplicate using the same initial oil.

### Artificial formation of elenolic acid

A part of the olive oil that was treated with the water in the above experiment was submitted to extraction and purification as in the case of the isolation of elenolide. At the final step, reversed-phase preparative TLC with 60:40 v/v H<sub>2</sub>O–acetonitrile led to the appearance of an additional zone with  $R_f = 0.38$  and a large reduction of the zone with  $R_f = 0.26$  (elenolide). Extraction of the new zone with acetonitrile and subsequent analysis by NMR led to the identification of elenolic acid (**12**).<sup>12</sup>

## RESULTS AND DISCUSSION

Investigation of the <sup>1</sup>H-NMR spectra of olive oil samples, recorded following the protocol of analysis developed by Karkoula *et al.*, led to the detection of a major peak at 9.27 ppm that could not be attributed to any known ingredient of olive oil (Fig. 1). Subsequent isolation of the compound presenting the characteristic signal led to the discovery of a previously unknown major ingredient of olive oil, which was identified as (*E*)-elenolide. The structure of elenolide (**11**) was elucidated using 1D and 2D NMR in combination with GC-MS.

The name 'elenolide' had already been given in 1957 to a lactone obtained after acidification of olive leaves and fruit extracts.<sup>9</sup> The structure corresponding to that lactone was elucidated in 1961

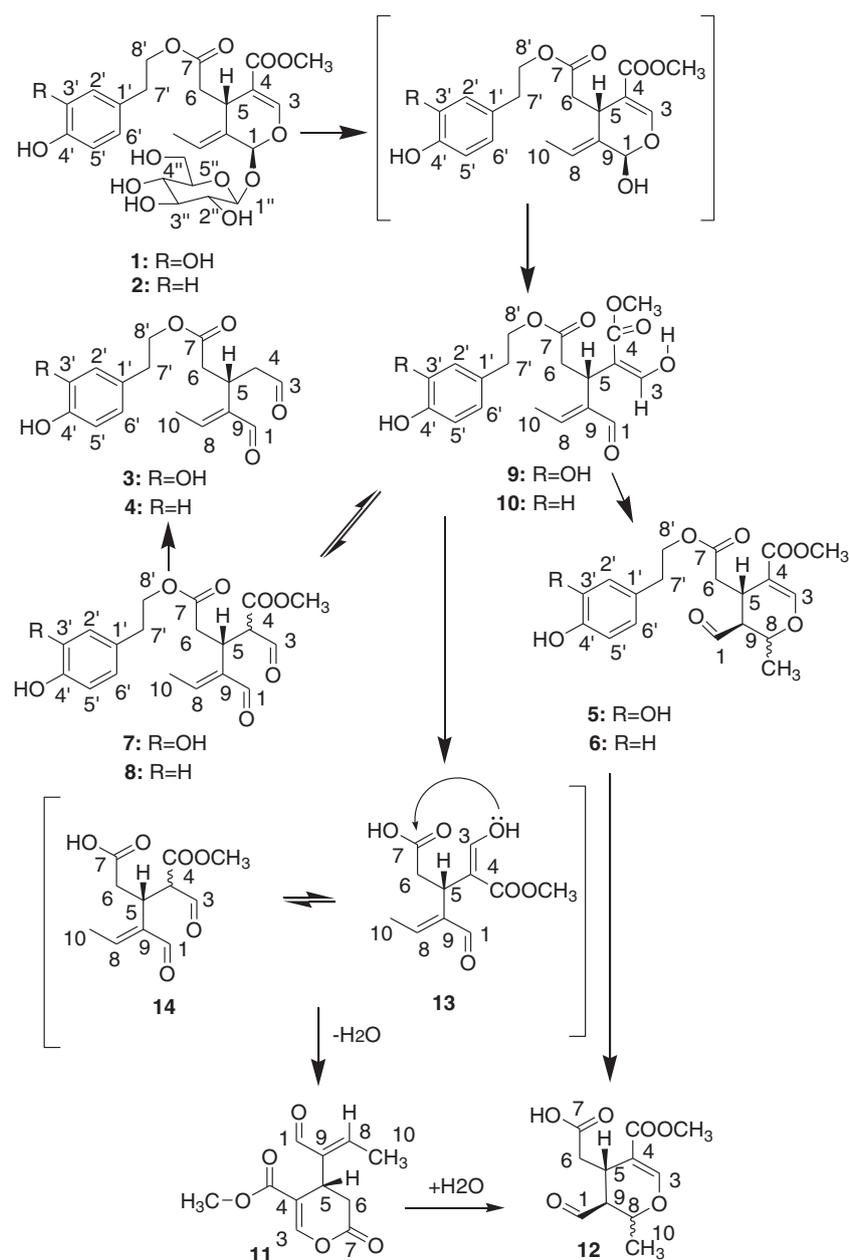
and the geometry of the double bond was proposed as *Z*.<sup>10,11,13</sup> The *E* isomer described herein had been chemically synthesized<sup>10</sup> without substantial NMR description and, interestingly, it had never been recorded as an ingredient of olive oil and it had never been completely characterized by NMR spectroscopy.

The new ingredient can be considered as a secoiridoid derivative, and below we present a possible biosynthetic pathway. High concentrations of elenolide were mainly found in fresh oils coming from unripe olives processed with very low use of water during malaxation. During the isolation procedure we observed that it was difficult to purify with normal-phase chromatography and also we observed its sensitivity to water during reversed-phase chromatography; for this reason we performed a more thorough investigation of this behavior.

### Structure elucidation of elenolide

Observation of the <sup>1</sup>H-NMR spectrum of the isolated compound revealed that in the aldehyde region there was a double peak at 9.27 ppm, with a small coupling constant of 0.9 Hz. A methyl group attached to a double bond at 2.17 ppm (H-10) was also apparent. This signal appeared as a double peak owing to its correlation with an olefinic proton, which appeared as a quadruplet peak at 6.74 ppm (H-8). The characteristic singlet peak integrating for three protons at 3.74 ppm revealed the presence of a carbomethoxy group, and also a singlet peak at 7.65 ppm revealed a proton of a double bond near oxygen. Finally, two dd peaks at 2.57 and 2.87 ppm corresponding to geminal protons (H-6a and H-6b) and a third dd slightly deshielded at 4.05 ppm (H-5) were also observed (Fig. 3).

From the 2D NMR HSQC spectrum (provided as supporting information), it was obvious that the aldehydic proton H-1 was connected with a carbon at 194 ppm, the deshielded proton H-3 was attached to an olefinic carbon at 152 ppm, and the other olefinic proton H-8 was attached to a carbon at 153 ppm. From the same spectrum, the proton at 4.05 ppm (H-5) was connected with a carbon at 27.5 ppm, while the carbon C-6 observed at 35.1 ppm was connected with two geminal protons. The characteristic signal of the carbomethoxy group appeared at 51 ppm.



**Figure 4.** Proposed biosynthetic pathway of elenolide (11) and elenolic acid (12) from oleuropein (1) or ligstroside (2) via oleomissional or oleokoronal (9, 10).

All the above-mentioned signals led to the hypothesis that the structure of this compound was similar to the already known elenolide,<sup>10,11,13</sup> which had never been characterized by 2D NMR. This hypothesis was confirmed by the HMBC spectrum (provided as supporting information), which revealed that the aldehydic proton H-1 was also correlated with the carbons C-9 and C-5. Respectively, C-1 was correlated with the protons H-8, H-5 and H-10. From the COSY spectrum (provided as supporting information), H-5 apart from H-1 was also correlated with H-6a and H-6b. From the HMBC spectrum, it was apparent that these two protons (H-6a and H-6b) were correlated with the carbonyl group of C-7. This carbon also had a J<sub>3</sub> coupling with H-3. The correlation of the carbomethoxy group with the carbonyl group at 165.8 ppm discriminated this carbon from the carbonyl of C-7 at 164 ppm.

The structure of elenolide was confirmed by the electron impact mass spectrum (provided as supporting information), which showed the molecular ion at 224 amu and a major fragment of 164 amu corresponding to the loss of the COOCH<sub>3</sub> group.

Finally, the stereochemistry of the double bond was studied by NOESY (provided as supporting information). A correlation between the aldehydic proton at 9.27 ppm and the olefinic proton H-8 at 6.74 ppm proved unequivocally the *E* geometry of the double bond.

In our hands, the isolated *E*-elenolide showed similar but significantly different H-NMR chemical shifts in comparison to the *Z* isomer described in 1961.<sup>10</sup> More specifically, there was a significant difference in the chemical shift of the aldehydic proton (9.20 vs. 9.27 ppm) and the olefinic proton H-8 (6.85 vs. 6.74 ppm), both influenced by the geometry of the double bond. Small differences

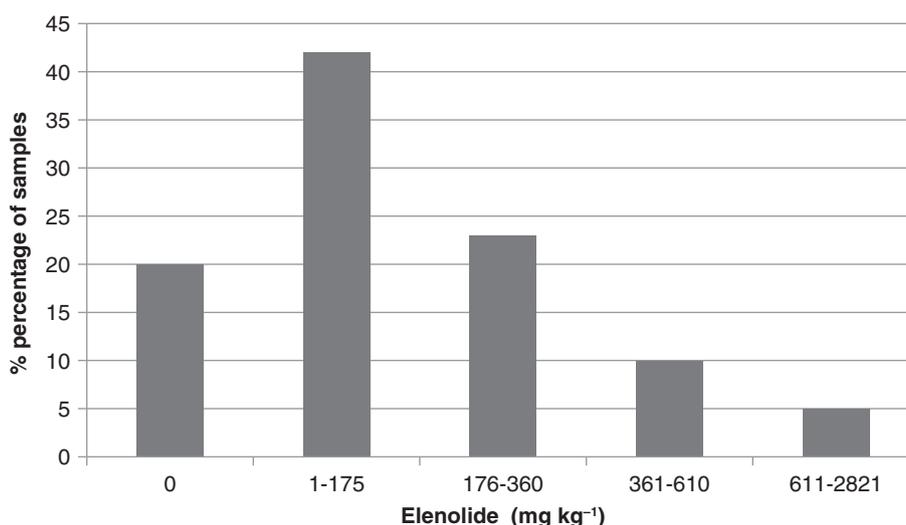


Figure 5. Variability of elenolide concentration in 2120 olive oil samples.

also occurred for all other signals. Although both compounds originated from the same plant, we cannot be sure if the previously described *Z*-isomer is a natural ingredient or an artifact produced during the chemical procedures followed at that time for the extraction and purification. In addition, due to the fact that the older spectrum had been recorded at 60 MHz, we cannot exclude the possibility that the *Z* isomer had not been correctly described and in fact could be the same compound as the *E* isomer unequivocally described herein. The *E* geometry of elenolide had also been proposed by Brown and Jones through a biomimetic chemical synthesis,<sup>13</sup> although again the spectroscopic data were incomplete. In any case, this is the first report of (*E*)-elenolide as an ingredient of olive oil with complete NMR data.

#### Biosynthetic pathway of elenolide

The structure of elenolide is possibly derived biosynthetically from precursors such as oleomissional (**9**) and oleokoronal (**10**),<sup>7</sup> coming respectively from oleuropein (**1**) and ligstroside (**2**). Hydrolytic or enzymatic removal of the hydroxytyrosol or tyrosol moiety, respectively, can lead to structure **13**, which is in equilibrium with structure **14**. Internal lactonization between the carboxyl at position 7 and the enol hydroxyl at position 3 leads to the structure of elenolide (**11**) (Fig. 4). Through this biosynthetic pathway, both the absolute stereochemistry of carbon 5 and the geometry of the double bond between positions 3 and 4 remain the same as in the case of oleomissional (**9**) and oleokoronal (**10**). Based on the above biosynthetic pathway, we confirm that the isolated elenolide is in the form of the (*S*)-*E*-elenolide isomer.

#### Development of a quantitative method of measurement for elenolide

A qNMR method for the measurement of elenolide (**11**) was developed and validated. In addition, a GC-MS method was also used for comparison purposes. It should be noted that common liquid chromatographic methods may present significant problems due to the sensitivity of elenolide to water, as explained below. The qNMR method showed excellent linearity, precision, accuracy (RSD < 10%) and recovery of 99% with LOQ at 17.5 mg kg<sup>-1</sup>.

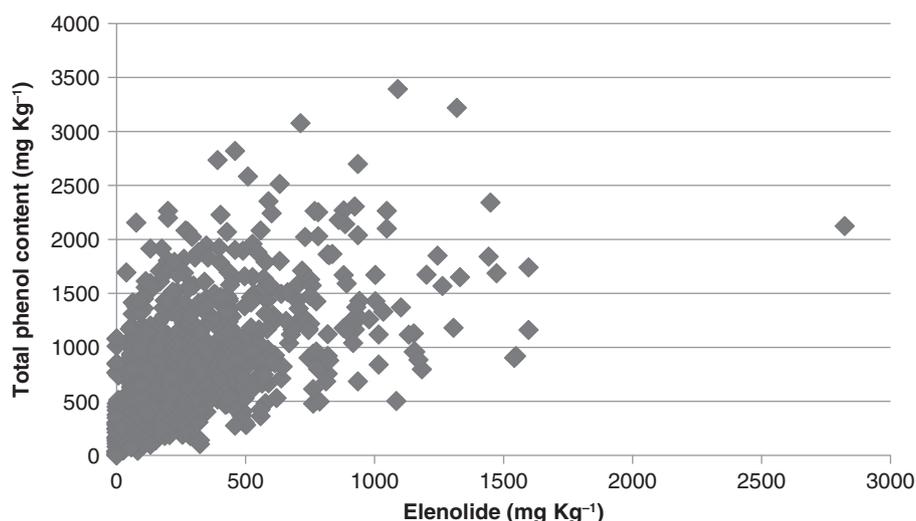
#### Quantitative variation among olive oil samples

Among the 2120 different EVOO samples that were analyzed, belonging to the most well-known varieties, 80% were found to contain elenolide (**11**). We observed a wide variation in the measured concentrations ranging from 0 to 2821 mg kg<sup>-1</sup>. Distribution of the concentrations is presented in Fig. 5. It should be noted that the highest concentrations were observed in olive oils with high phenolic content produced from early-harvest unripe olives that were processed in a two-phase olive mill<sup>14</sup> with minimal use of water at any step of the process. However, we found olive oils with high phenolic content but low elenolide content, but in all cases the samples originated from three-phase mills working with excess of water (data not shown). The correlation between total phenolic content measured by qNMR and the concentration of elenolide is presented in Fig. 6.

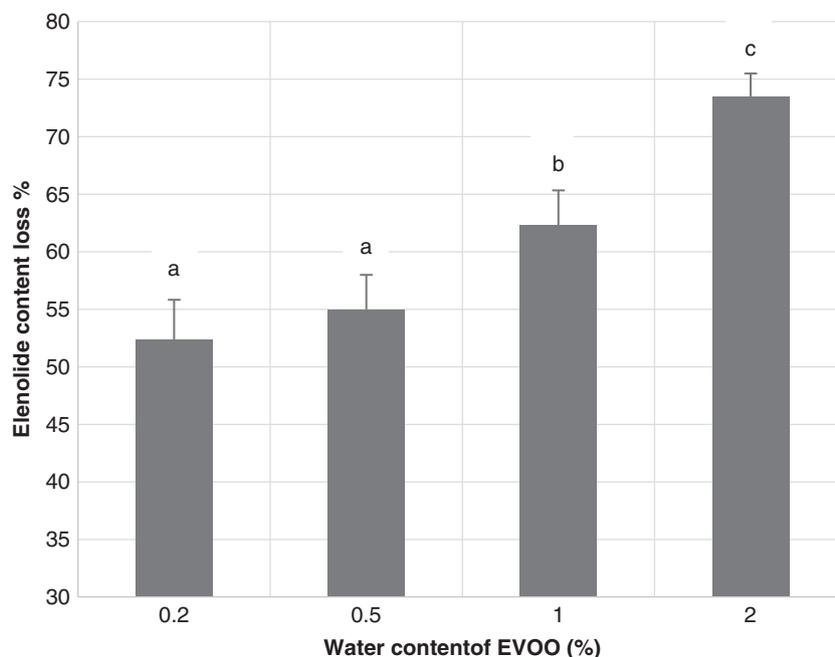
#### Impact of the water content of olive oil on elenolide during storage

To study the sensitivity of elenolide (**11**) to water inside the olive oil matrix, we performed an experiment with addition of a known quantity of water in an olive oil rich in elenolide and we monitored its concentration for a 2-week period. More specifically, we selected an olive oil containing 663 mg kg<sup>-1</sup> elenolide, as measured by qNMR methodology, and we added water corresponding to a concentration of 2%, 1%, 0.5% and 0.2% w/w.

After 2 weeks of storage in airtight vials at room temperature, the olive oil samples were analyzed by the developed qNMR method and the results are presented in Fig. 7. Elenolide loss was clearly connected with the water content, ranging from 50% to 75%, with water content ranging from 0.2% to 2% respectively. It is noteworthy that during storage we observed the increase of a set of peaks that after isolation were found to correspond to elenolic acid (**12**).<sup>12</sup> The concentration of elenolic acid followed an opposite trend in comparison to elenolide, while the phenolic content remained stable during this period (NMR spectra are provided as supporting information). Based on this observation we can propose that high concentration of elenolide and low concentration of elenolic acid (**12**) is a marker of low water content during storage and presumably a marker of low water contact during the production of olive oil. However, elenolic acid is most probably only one of the main degradation products of elenolide. It is possible that



**Figure 6.** Correlation between total secoiridoid-phenolic content measured by qNMR and the concentration of elenolide.



**Figure 7.** Elenolide content loss with water after 2 weeks of storage. Columns with different letters show statistically significant difference ( $P < 0.05$ ).

other, as yet unidentified, pathways of degradation of elenolide could explain why the reduction of elenolide is higher than the increase of elenolic acid.

Although the presence of elenolic acid, which is a hydrated derivative of elenolide, had been reported in olive oil,<sup>15</sup> this is the first time that elenolide has proved to be artificially transformed to elenolic acid due to the presence of water.

### Biological significance

Elenolide (**11**) and its derivatives have been recognized as hypotensive compounds, although not with completely presented experimental results.<sup>11</sup> On the other hand, several clinical studies have shown that the high phenolic olive oils have a significant impact in lowering the blood pressure in comparison with the low phenolic ones, although there is no mechanism connecting

the phenolic compounds with blood pressure.<sup>16–20</sup> In the current study we were able to observe that the high phenolic olive oils also contained high amounts of elenolide (Fig. 6) and this could offer a possible additional explanation for the antihypertensive activity of this type of olive oil. Further studies with high-elenolide olive oils are required to establish a stronger connection between the antihypertensive activity and the role of elenolide.

### CONCLUSION

The structure of (*E*)-elenolide, a previously unreported ingredient of olive oil, was determined after isolation and completely characterized by 1D and 2D NMR and mass spectrometry. The new olive oil ingredient has potential antihypertensive activity and needs further investigation in human studies. Its sensitivity to water can be used as a tool to study the water impact to an olive oil sample and as a marker of fresh oils with low water content.

## ACKNOWLEDGEMENTS

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## SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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