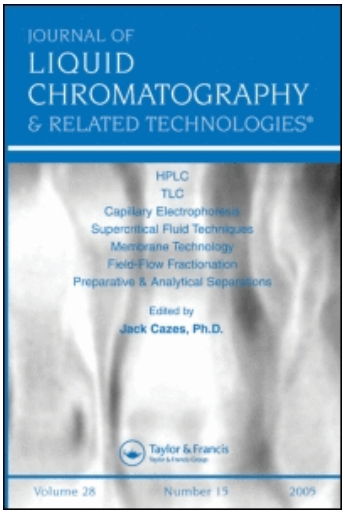


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# Quantitation of Oleuropein and Related Metabolites in Decoctions of *Olea europaea* Leaves from Ten Greek Cultivated Varieties by HPLC with Diode Array Detection (HPLC-DAD)

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# Quantitation of Oleuropein and Related Metabolites in Decoctions of *Olea europaea* Leaves from Ten Greek Cultivated Varieties by HPLC with Diode Array Detection (HPLC-DAD)



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**Abstract:** An extraction procedure and chromatographic methodology for the simultaneous quantitation of four major constituents in the boiling water extracts (decoctions) of *Olea europaea* leaves has been developed. The four studied constituents were oleuropein, elenolic acid, hydroxytyrosol, and tyrosol. The quantitation was performed using HPLC-DAD, whereas qualitative data were acquired using LC-MS. The developed methodology was applied in the study of ten *Olea europaea* varieties commonly cultivated in Greece. The chromatographic analysis revealed important differences among the varieties. The decoction of variety *gaidouroelia* was identified as the best source of oleuropein, but it was completely lacking of elenolic acid. The decoction of variety *koronaiiki* was the best source of hydroxytyrosol, whereas the variety *mastoides* was the best source of tyrosol and elenolic acid. In addition, the methanol and acetone extracts of one of the studied varieties (*koranaiiki*) were investigated, in order to compare the concentration of oleuropein in the extracts and

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the decoction. Interestingly, only a very low percent of the total oleuropein is present in the traditionally prepared decoction, while elenolic acid, which is a minor constituent of the extracts, was found to be one of the major constituents of the decoction.

**Keywords:** *Olea europaea*, Leaves, Decoction, Oleuropein, Elenolic acid, Hydroxytyrosol, Tyrosol



## INTRODUCTION

Traditionally, the boiling water extract (decoction) of *Olea europaea L.* leaves has been used as a folk remedy against several diseases. Among them, the most important are hypertension, arrhythmia, intestinal muscle spasms, and cancer.[1–3] The pharmacological activity of this extract has been attributed to polyphenols and secoiridoids such as oleuropein, hydroxytyrosol, tyrosol, and elenolic acid[4] (Figure 1). Oleuropein and hydroxytyrosol has been found to be active against various diseases, such as Coronary Artery Disease (CAD), by preventing LDL oxidation,[5] cancer,[6,7] osteoporosis.[8] Hydroxytyrosol is considered to be one of the most powerful antioxidants.[9] Furthermore, elenolic acid has been proposed to possess antiviral properties,[10] whereas the olive oil leaf extract has been found to be potent against HIV-1 virus.[11] Olive leaf extract has demonstrated high antimicrobial activity.[12,13] Although, there are many nutrition supplements or cosmetics containing olive leaf extracts, up to date there has been little attention drawn to the chemical differences between the several cultivated varieties[14] and, consequently, their possible therapeutic value. Only in

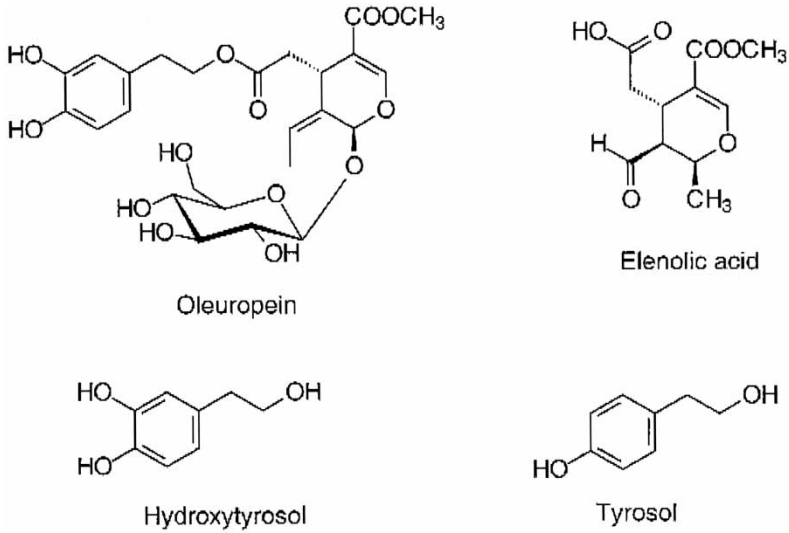


***Figure 1.*** Structures of the major bioactive metabolites of *Olea europaea*.

Greece are there more than twenty usually cultivated varieties, but there is not yet any evidence about the influence of the variety on the quality of the final product.

The decoction (boiling water extract) has been used in the traditional medicine due to its fever lowering properties, especially against the malaria induced fever, where it had been frequently used during the Spanish War of 1808 for severe cases of fever. It has also been used in traditional Greek therapeutics, and it has been reported that it was extensively used in 1865 on the Greek Island of Mytelene for its fever lowering activity against a fatal fever during that year. It has also been reported that the leaf extract is even more effective than quinine itself. Furthermore, today there is a variety of olive leaf extracts commercially available but little or no information is given about the origin (especially about the botanical variety) of this extract. It is, therefore, important to determine if there are any differences in the chemical content of the decoctions resulting from the variation due to the specific cultivar.



The major constituents of this decoction having therapeutic applications are oleuropein (OE), elenolic acid (EA), hydroxytyrosol (HT), and tyrosol (T). The goal of this work was to investigate the major differences between decoctions prepared from ten different varieties of Greek flora and to quantify their content regarding the four aforementioned bioactive constituents. The analytical methodology developed, which incorporates the simultaneous determination of four olive tree metabolites, is also important for the quality control of the manufacturing process of decoctions already present in the market, as well as for manufacturing new food supplements in the future. For this purpose, an extraction procedure and a chromatographic methodology have been developed for the simultaneous quantitation of oleuropein, elenolic acid, hydroxytyrosol, and tyrosol in the decoctions of *Olea europaea* leaves. In addition, in order to compare the final concentration of OE in the decoction and the common organic solvent extracts, one of the studied varieties was investigated using methanol and acetone.

## EXPERIMENTAL

### Reagents and Chemicals

All solvents (including water) used throughout the experiments, were of HPLC grade and were obtained from Merck (Darmstad, Germany). The solvents were vacuum filtered through a 0.2 μm Titan Membrane Filter and sonicated for 5 min prior to their use. Ammonium acetate was obtained from Riedel de-Haen GmbH (Seelze, Germany). The purity of all organic reagents was checked by HPLC prior to their use. Pure samples of OE, HT, and T were isolated from olive leaves according to a previously described procedure.[15] Elenolic acid was prepared by hydrolysis of oleuropein.[15]

## Plant Material

Plant material was collected from Attica, Arcadia, and Crete (Greece) as described in Table 1, in December 2002. A voucher specimen of each variety is deposited in the herbarium of the laboratory of Pharmacognosy, University of Athens, Greece.

## Decoction Preparation

For the preparation of the decoction the traditional Greek recipe was used, which involves the boiling of the leaves for a short time, i.e., about three minutes. This procedure minimizes the hydrolysis of the aforementioned substances. Thus, fresh leaves randomly collected from three different trees for each variety were blended using a general-purpose electric blender. A portion of the blended material (50.0 g) was added to water (250 mL) and boiled for 3 min. Then, the hot mixture was filtered through a filter paper and the filtrate was extracted once with EtOAc (250 mL). A portion of the EtOAc layer (50.0 mL) was obtained and evaporated to dryness under reduced pressure to give a solid residue.



## Organic Solvent Extraction

Air-dried leaves collected from trees of variety *Koronaiiki*, exactly as in the case of decoctions, were pulverized and a portion of the powder (50.0 g) was exhaustively extracted either with methanol (250 mL) or acetone (250 mL) three times for 48 h. Subsequently, the mixture was filtered through a filter paper and the filtrate was evaporated to dryness under reduced pressure to give a solid residue.

***Table 1.*** List of the studied cultivars and their geographic origin

| Cultivar name | Origin |
|---|---|
| *Throumba* | Crete |
| *Gaidouroelia* | Attica |
| *Agrielia* (or var. *sylvestris*) | Attica |
| *Chryssoelia* | Attica |
| *Kalamon* | Peloponnese |
| *Mastoeides* | Crete |
| *Koronaiiki* | Attica |
| *Amfissis* | Peloponnese |
| *Agouromanaki* | Peloponnese |
| *Megaritiki* | Peloponnese |

## Sample Preparation

A portion (5.0 mg) of the described solid residues obtained, was added under sonication to a mixture of MeOH (500 μL) and ammonium acetate 0.05 M (500 μL). The mixture was centrifuged at 10,000 rpm for 10 min and a 100 μL portion of the supernatant was injected for HPLC analysis.

## Instrumentation

### HPLC-Diode Array

The chromatographic separation was conducted on a Finnigan SPECTRA HPLC system comprised of a Finnigan SPECTRA P4000 quaternary pump, coupled to a Finnigan SPECTRA system UV6000LP diode array detector. Degassing of the mobile phase was achieved using a Finnigan on line membrane degasser. Introduction of the sample to the chromatographic column was done through a Rheodyne 7725*i* injector equipped with a 100 μL loop. HPLC controlling, data acquisition, and processing was performed by the ChromQuest v.2.51 software connected to the HPLC by a Finnigan SN4000 controller. The extracts of the *Olea europaea* varieties were chromatographed on an Alltech C8 reversed phase column (250 × 4.0 mm, ID 5 μm) preceded by a C8 Alltech precolumn. The column temperature was maintained at 40°C throughout all experiments, with the aid of an electronically controlled oven.



### Liquid Chromatography-Mass Spectrometry (LC-MS)

The LC-MS experiments were performed with a Finnigan LC system with the same type of pump, injector, and degasser. Two modes of detection were employed, ultra violet using a dual wavelength SPECTRA SYSTEM UV 2000 detector instead of the diode array, and mass spectral (MS) using electrospray ionization. The mass spectra were acquired on a TSQ triple quadrupole mass spectrometer equipped with an API 2 source. The capillary temperature was maintained at 350°C and all experiments were conducted in both positive and negative ion modes. The column used for the LC-MS experiments was a Waters X-Terra MS $C_{18}$ column (2.1 × 150 mm, 3.5 μm particle size) preceded by a Waters X-Terra MS $C_{18}$ precolumn and a particle filter. A 20 μL loop was used in this instance.

### Stock Standard Solutions

For the preparation of stock standard solutions, (1 mg/mL level) appropriate amounts of OE, HT, T, and EL were dissolved in methanol. Mixed standard solutions (100 μg/mL level) were prepared by diluting appropriate volumes

of the aforementioned solutions in HPLC-grade water. The corresponding mixed working solutions were prepared every week by diluting appropriate volumes of the mixed standard solutions (in HPLC grade water) of all the four substances, at the levels determined by the calibration curve. All solutions remained refrigerated at 4°C.

## Chemical Analysis

### HPLC-DAD

The chromatographic separation of the substances was performed using a gradient elution program described in Table 2a. Solvent A was 0.05 M ammonium acetate buffer adjusted to pH 5.0 with glacial acetic acid and Solvent B was gradient grade acetonitrile. The flow rate was kept constant at 1 mL/min. After the end of each run, a delay time of 3 minutes before the next injection was necessary in order to equilibrate the column (mobile phase composition 90% A and 10% B). Moreover, the HPLC system was left to equilibrate for at least 30 min before the sample injection, in order to assure that a stable baseline has been attained. The separation achieved under these conditions is depicted in Figure 2 (A and B).

### LC-MS

The LC-MS experiments were performed with a slightly different elution program (because a different column and also lower flow rates had to be used). The gradient program used is shown in Table 2b. The two solvents,



***Table 2.*** (a) Mobile phase programming used at the separation of OE, EL, HT, and T on a $C_8$ column using DAD detection, flow 1 mL/min; (b) Mobile phase programming used at the separation of OE and EL on a $C_{18}$ narrow bore column using electrospray MS detection, flow 0.1 mL/min

| Time (min) | Solvent A (%) | Solvent B (%) |
|---|---|---|
| (a) | | |
| 0 | 90 | 10 |
| 10 | 70 | 30 |
| 25 | 60 | 40 |
| 27 | 90 | 10 |
| (b) | | |
| 0 | 90 | 10 |
| 15 | 20 | 80 |
| 17 | 90 | 10 |

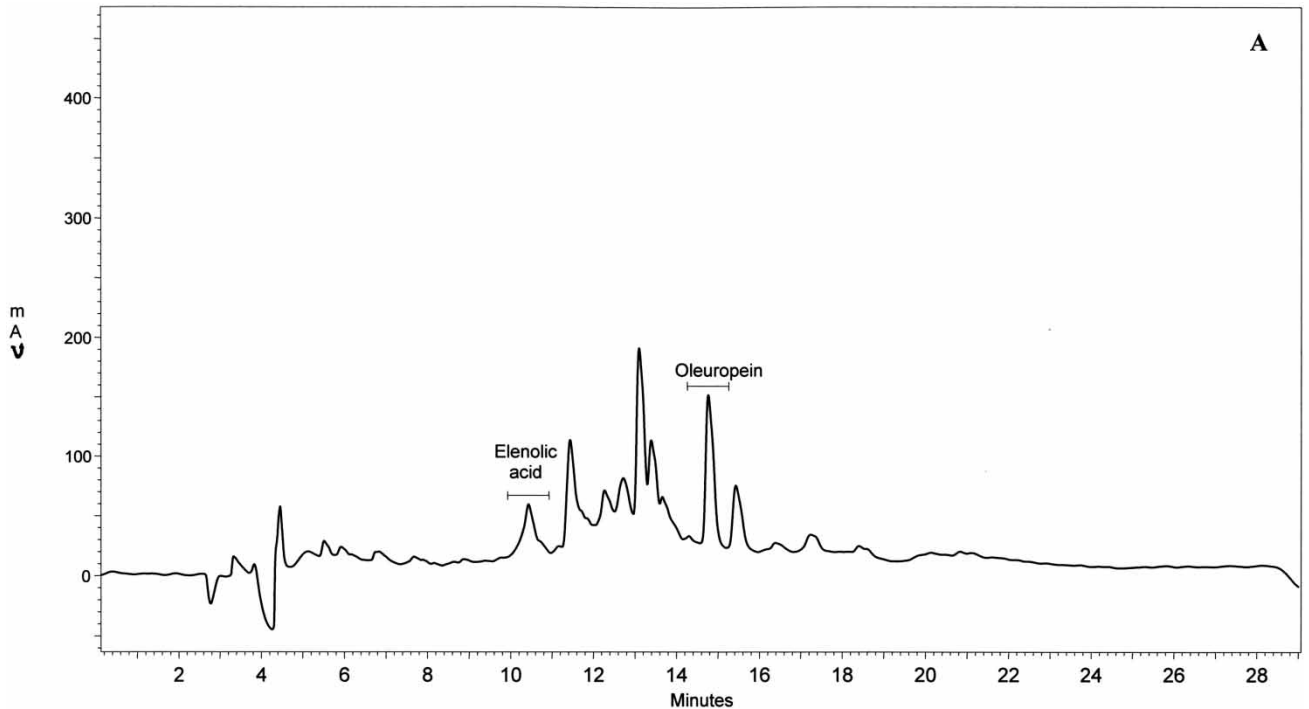


***Figure 2.*** HPLC-DAD chromatogram of the *Olea europaea* var. *Koronaiiki* decoction at (A) 240 nm and (B) 280 nm.

*(continued)*

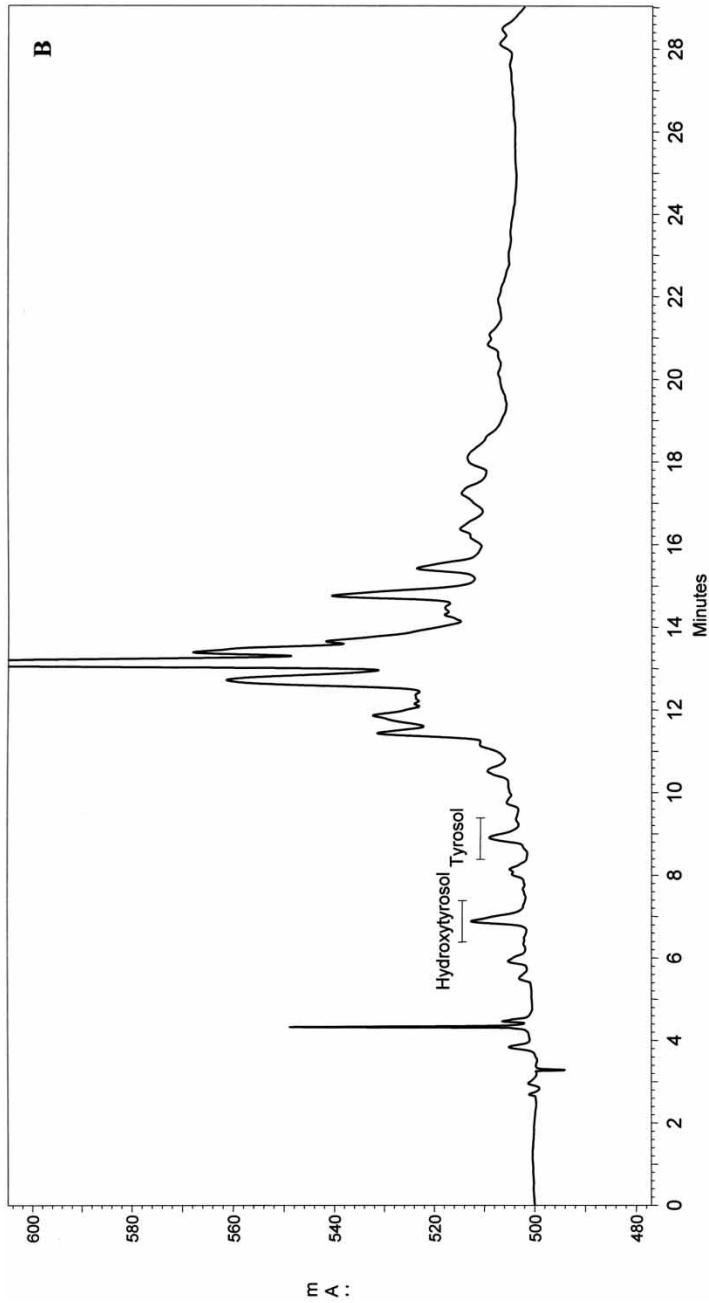


*Figure 2.* Continued.

A and B were the same as for the HPLC-DAD chromatography and the flow rate was set to 0.1 mL/min throughout all LC-MS experiments.

### Detection

During the acquisition of the standard solution chromatograms of each substance, the UV spectra were recorded with the aid of the diode array detector. The $\lambda_{max}$ was determined to be 240 nm for oleuropein and elenolic acid and 280 nm for tyrosol and hydroxytyrosol. Both wavelengths were employed for data acquisition in all the UV-DAD chromatographic experiments. The spectra of each substance were also acquired using a Unicam UV-300 UV-Vis spectrophotometer with 10-mm optical path length quartz cuvettes.

In the MS mode, full scan mass spectra were recorded for each chromatogram, both at the positive and negative ion modes. Two of the substances from the extracts, namely OE and EL, when monitored at the negative ion mode as ion extracted chromatograms (XIC – m/z 539 for OE and m/z 241 for EL), eluted at the given retention times as the corresponding standards. Further identification of the substances was achieved by comparing the MS spectra of the extracts versus the acquired ones of the standards and was in agreement with previously reported data.[16]

For the HPLC-DAD analysis, peak assignment was based on retention time $R_t$ (peak window 10% of $R_t$) for every substance. On a second level, the peaks of the analytes were identified by their UV spectrum based on diode array data. Furthermore, the identification of the peaks was accomplished by spiking the decoction with authentic standards and monitoring the expected retention times and UV spectra of the substances.



## Statistical Evaluation

All statistical evaluation and quantification was accomplished by the HPLC-DAD method, whereas the LC-MS analysis was mainly developed for qualitative work and, therefore, no further validation was attempted for the latter.

### Linearity

The linearity relationship between concentration and response for all four analytes was accomplished by analyzing six samples containing 0.5, 1, 2, 5, 10, 15, 20 μg/mL of each substance. The acquired data were evaluated following unweighted linear regression analysis. The 0,0 point was neither included to the calibration curve nor forced as the origin.

Precision and Accuracy

In order to assess the precision of the proposed method, five replicates of the four substances mixture at two different levels (5 and 20 μg/mL) were analyzed. The results were evaluated by calculating the %RSD. The inter day reproducibility was estimated by injecting a sample at the 20 μg/mL level at the beginning of three separate laboratory weeks and calculating the %RSD. The accuracy was evaluated as the %Er (% Relative Error) of the method at each level of the calibration curve.

LOD and LOQ

The assessment of the limit of quantitation (LOQ) and the limit of detection (LOD) for the proposed method was carried out by injecting the lowest concentration exhibiting 3 and 10 times the S/N ratio, respectively.



Recovery

The recovery experiments were carried out by adding a known quantity of the authentic standards to the decoction of a Koronaiiki cultivar. The last was extracted as described and the recovery was calculated as the ratio

$$\text{Recovery} = \frac{\text{quantity found after extraction} - \text{quantity found before extraction}}{\text{quantity added}}$$

for each substance.

## RESULTS AND DISCUSSION

The analysis of the decoctions was performed for cultivars representing three of the major oil-producing regions of Greece namely Peloponnese, Crete, and Attica. The names of the studied cultivars are listed in Table 1 along with their origin. Among them, there are three of the most widely cultivated varieties in Greece namely *Koronaiiki*, *Throuba*, and *Kalamon.* The decoctions were prepared from blended fresh leaves according to the traditional procedure, in order to assure the same content of substances as the ones used in the traditional medicine. As we were interested mainly in the phenolic content of the leaves under the conditions used in the traditional medicine, we did not evaluate the boiling time parameter, which could lead to different results, as the evaluated substances could be thermo labile. The water extracts were filtered while hot in order to remove insoluble material and leaves' particle, the filtrate was left to cool at room temperature and subsequently extracted with EtOAc. This solvent has been proven to exhibit good selectivity for

the extraction of phenolic compounds, and was found to be excellent for the extraction of HT and EL and quite efficient for the extraction of T. The recovery rates obtained for the substances under study were 85.9% for HT, 87.9% for EL, 68.7% for T, and 34.8% for OE. The extraction step was necessary in order to remove the large quantities of water-soluble constituents (such as sugars), which hindered the chromatographic analysis, especially of HT. The EtOAc extract was evaporated and the solid residue, after the appropriate reconstitution, was submitted to HPLC-DAD and LC-MS analysis. The reconstitution solvent was chosen to be water, which has lower strength than the starting percentage of the mobile phase (10% organic modifier). Such a solvent should lead to more symmetric peaks due to solvent focusing.

The identification of the four studied compounds in the extracts was performed by comparison of their retention times, as well as their UV spectra, with the corresponding spectra of authentic samples previously isolated from olive leaves. In addition, identification OE and EL was also based on their electrospray mass spectra. The LC-MS analysis could not be performed for hydroxytyrosol and tyrosol (due to their weak signal) and, thus, quantification was based only on HPLC-DAD analysis.



## Linearity, Reproducibility, and Accuracy

The relationship between response (measured as peak area) for each of the analytes and the corresponding concentration at the levels refereed above was found to be linear. The equations describing this correlation (as determined by the linear regression analysis) are listed in Table 3, along with the corresponding correlation coefficients and the error of estimation for each substance.

The method was also evaluated by means of the intra-day precision, by injecting multiple samples ($n = 5$) at two different concentration levels, namely 5 and 20 $\mu g/mL$. The % relative standard deviation (%RSD) values are presented in Table 4. The inter-day precision was calculated by injecting a 20 $\mu g/mL$ sample at three different laboratory weeks and the corresponding

***Table 3.*** Statistical evaluation of the OE, HT, T, and EL calibration curves

| Substance | Regression equation | Correlation coefficient (r) | SD[a] | |
|---|---|---|---|---|
| | | | Intercept | Slope |
| HT | $R_{HT} = 234{,}413\ C_{HT} + 6{,}617$ | 0.9993 | 4,144 | 38,964 |
| T | $R_T = 212{,}237\ C_T - 12{,}646$ | 0.998 | 7,262 | 37,069 |
| EL | $R_{EL} = 542{,}707\ C_{EL} + 23{,}403$ | 0.998 | 13,654 | 128,365 |
| OE | $R_{OE} = 269{,}458\ C_{OE} + 41{,}522$ | 0.997 | 6,399 | 38,219 |

[a]Standard deviation of slope and intercept.

*Table 4.* Intra day precision data of the determination

| Substance | Concentration (μg/mL) | RSD (%) | Error (%) |
|---|---|---|---|
| HT | 20 | 5.01 | 1.1 |
| | 5 | 4.31 | −0.9 |
| T | 20 | 4.08 | 0.4 |
| | 5 | 3.79 | −5.4 |
| EL | 20 | 4.48 | −0.6 |
| | 5 | 4.52 | −0.8 |
| OE | 20 | 3.38 | 4.3 |
| | 5 | 4.01 | −6.7 |

%RSD value was found to be 4.64%. The data indicate that the proposed method can be considered as adequate precision for monitoring these substances at the decoction of *Olea europea.* The accuracy of the method was determined by calculating the Er% (% relative percentage error) for the two concentration levels as mentioned above (Table 3). The maximum %Er value did not exceed 7%, which is acceptable for the goals of the present analysis.



## LOQ and LOD

The LOD and LOQ values were determined as three and ten times the S/N ratio for each of the four substances, and they were found to be 0.15 μg/mL and 0.5 μg/mL, respectively. All compounds exhibit the same LOD and LOQ values, because the substances under analysis have approximately the same molecular absorptivity values.

## Specificity

All four substances were baseline resolved under the described experimental conditions. Nevertheless, it has not been possible to isolate a matrix of the same nature not containing the aforementioned substances, so we couldn't obtain a reliable blank sample. Nevertheless, the diode array data at the peak purity mode, indicate that the eluted substances were pure in terms of their UV peak shape and $\lambda_{max}$, denoting that the method can be considered specific.

## Quantitation

The results for the quantitation of the four compounds under study are presented in Table 5. It is obvious that there is an important variation for all the compounds among the ten examined cultivars. For prepared decoction

***Table 5.*** Quantity (mg) of oleuropein (OE), elenolic acid (EL), hydroxytyrosol (HT), and tyrosol (T) contained in decoctions of each variety, prepared from 50.0 g fresh leaves and 250 mL water

| Cultivar name | HT | OE | T | EL |
|---|---|---|---|---|
| *Throumba* | 1.189 | 49.649 | 0.410 | 13.170 |
| *Gaidouroelia* | 13.344 | 121.934 | 4.038 | 0.000 |
| *Agrielia* (or var. *sylvestris*) | 2.364 | 28.517 | 1.251 | 8.715 |
| *Chryssoelia* | 0.597 | 11.807 | 0.877 | 8.217 |
| *Kalamon* | 1.534 | 40.891 | 1.158 | 4.980 |
| *Mastoeides* | 4.074 | 50.043 | 20.537 | 33.146 |
| *Koronaiiki* | 23.964 | 40.448 | 1.268 | 9.519 |
| *Amfissis* | 1.760 | 32.626 | 1.429 | 10.329 |
| *Agouromanaki* | 1.833 | 32.963 | 1.473 | 12.981 |
| *Megaritiki* | 2.575 | 29.882 | 1.490 | 14.231 |



of 50.0 g fresh leaves boiled with 250 mL water, OE ranges between 11.8 and 121.9 mg, HT between 0.6 and 23.9 mg, T between 0.4 and 20.5 mg, and elenolic acid between 0.0 and 33.1 mg. Variety *Koronaiiki*, which is widely cultivated for the production of excellent quality olive oil, was found to be the best source of HT, with a yield of 2-40 fold higher than all the other varieties. Variety *Mastoides*, which is mainly cultivated in western Crete for the production of fine quality olive oil, was found to be the best source of elenolic acid (3–5 fold higher yield) and tyrosol (5–40 fold higher yield), and also the second best source of OE. The best source of OE (2–10 fold higher yield) was *Gaidouroelia* variety, which is occasionally cultivated for its edible olive fruits. That variety presents special interest because its fruits are characteristically larger than all the other studied varieties, and this morphological difference is obviously related with big differences in the chemical consistency. This variety is the only one that does not contain EL, but it the second best source of HT and T.

The low concentration of OE in the prepared decoctions compared with the previously reported literature data[14,15] on its concentration in common organic solvent extracts, prompted us to investigate the methanol and acetone extract of one of the studied varieties (*Koronaiiki*). Using the same quantitation methodology as for the decoctions, the methanol extract (8.48 g from 50.0 g dried leaves) was found to contain 2.56 g (30.2%) of OE, while the acetone extract (4.81 g from 50.0 g dried leaves) was found to contain 1.8 g (37.7%).

Thus, it is interesting that only a very low percent of the total OE is present in the traditionally prepared decoction, and this is a very important factor when considering the dosology of the formulations of food supplements based on extracts from olive leaves. In contrast, EL, which is considered as a pharmacologically active metabolite of OE,[17] exhibits elevated

concentration in the prepared decoctions. Thus, the antiviral and anti-inflammatory properties of the above described preparations could be attributed, at least partially, to the high concentration of this metabolite.

The results obtained demonstrate the need of strict quality control of the decoctions already present in the market. A decoction of the same species (*Olea europea*) may not be equipotent in terms of variety (consider the case of Gaidouroelia with no EL and Mastoides with 33.146 mg/50 g fresh leaves, respectively). The variety factor must also be taken into consideration in the design process of new functional food or herbal extracts. Presumably, decoctions or other extracts with high levels of EL would exhibit fever lowering and antiviral properties, whereas OE and HT rich extracts will demonstrate stronger antioxidant and antiatherogenic properties. In order to establish these results, preliminary pharmacological screening of the obtained extracts is underway.



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