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ARTICLE

Identification of Throuba Thassos, a Traditional Greek Table Olive Variety, as a Nutritional Rich Source of Oleuropein

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The content of polyphenols in table olives is highly influenced by the olive variety and the debittering process applied on the fruits. Nine commercial types of Greek table olives were examined for their content in oleuropein and hydroxytyrosol. A very simple extraction procedure and a chromatographic methodology were applied for the simultaneous quantitation of oleuropein (OE) and hydroxytyrosol (HT) in drupes, using boiling water extraction followed by direct HPLC analysis. Hydroxytyrosol was found in all the types of olives that were studied. Kalamata olives and Green “tsakistes” of the variety Megaritiki contained the highest quantity of hydroxytyrosol (1.8−2.0 mg/fruit) followed by Greek-style “chondrolies” with quantity 1.0 mg/fruit. Oleuropein was found in small quantities in two cases, but in the case of Throuba Thassos which is processed by dry salt in a traditional Greek way, oleuropein was found in important quantities (1.2 mg/fruit) recorded over a 4-year period. This is the most important finding of this study showing that this particular table olive type is a nutritional rich source of oleuropein. Additionally, assuming a usual consumption of 20 olive fruits per day, an approximate quantity of 25 mg of oleuropein per day can be considered as safe for human use, since it can be found in the usual diet.

KEYWORDS: Table olives; oleuropein; hydroxytyrosol; Throuba Thassos

INTRODUCTION

The chemistry of the minor products present in olives and the study of their properties, especially the biological properties, is a field of extensive scientific research (*1*). Although olive products (oil, leafs, fruits, extracts, food supplements) are considered as health protective, a lot of work is still needed to establish a credible basis for the health claims and to evaluate the effect on the human body of all the minor constituents of olive. It is also very possible that the health benefits are due to the simultaneous presence of various active compounds or classes of compounds and to potential synergistic effects. Even though the olive fruit contains several types of antioxidant compounds (*1*, *2*) like simple phenols, caffeic acid derivatives, verbascoside, lignans, luteolin, luteolin glycosides and other flavonoids, our main interest in the present study was focused on oleuropein and secondarily on its hydrolysis derivative, hydroxytyrosol.

Oleuropein, a glucoside ester of hydroxytyrosol and elenolic acid (**Figure 1**), is an important constituent of olive fruits and a widely studied natural product with numerous health related activities and several applications as a constituent of food supplements. Oleuropein is a compound that possesses antioxidant and radical scavenging properties (*3*, *4*), and contributes to the prevention of atherosclerosis by inhibiting the oxidation of LDL and by scavenging several species that react with oxygen in the vascular wall (*5*). Additionally, oleuropein has exhibited interesting antimicrobial (*6*), antihypertensive and anticancer activities (*7*) as well as protective activity against osteoporosis (*8*, *9*). But most interestingly pure oleuropein has been recently found to decrease the total cholesterol and triglyceride levels and to considerably reduce the infarct size *in vivo* (*10*), giving promise for a future use in the treatment of coronary heart disease. The use of olive polyphenols for the prevention of cardiovascular diseases is also supported by epidemiological data which have shown that in the Mediterranean area the cardiovascular diseases were considerably lower than in the rest of Europe (*11*, *12*). This finding has been attributed to the traditional Mediterranean diet (*13*), which among others is characterized by the increased consumption of olive oil and olives.

However, oleuropein is mainly found in the leaves (*14*) and the unprocessed olive drupes of *Olea europaea* and for this reason the nutritional intake of oleuropein is usually considered as questionable. Although the majority of the polyphenols found in olive oil or table olives are derivatives of its hydrolysis, in most cases, the concentration of oleuropein in edible sources like table olives and olive oil has been found to be very low (*15*). Especially in the

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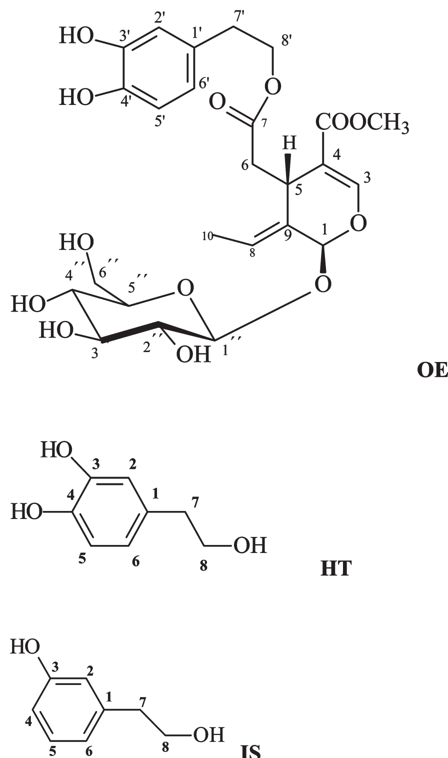

Figure 1. Chemical structures of oleuropein (OE), hydroxytyrosol (HT), and 3-hydroxyphenylethanol used as internal standard (IS).

case of table olives, it should be noted that the main purpose of processing is the removal of bitterness related to oleuropein (*16*). For this purpose, several processes mainly based on alkaline hydrolysis or diffusion in brine are employed leading to oleuropein decrease and hydroxytyrosol increase (*17*).

Despite the presence of both compounds in all unprocessed olive drupes, each cultivated variety has its own polyphenolic profile (*18*–*20*). In addition, other factors highly influencing the level of polyphenolic constituents and especially of oleuropein are the fruit maturation stage, the process type and the type of fermentation (*21*, *22*).

The phenolic content of nonedible unprocessed drupes has been under investigation for many years (*23*). Despite the great importance of phenolic compounds from the health point of view, commercial market table olives have not been equally studied. In Greece, although table olives are a reputable traditional product with overall production of 200,000 tons, very few data are available (*24*, *25*). In these studies hydroxytyrosol and tyrosol are referred to as the major phenolic compounds, while oleuropein has not been found in any case.

Based on the established bioactivity of oleuropein and hydroxytyrosol and the commercial interest of Greek table olives and their potential health effect we set as aim of this work the identification of the best source of oleuropein (OE) and hydroxytyrosol (HT) among table olives. For this purpose, an extraction procedure, with water at boiling temperature offering the best recovery of the analytes, and an HPLC-UV method were used for the simultaneous quantitation of OE and HT. The combined methodology was applied for the study of nine commercial types of Greek table olives, leading to the identification of Throuba Thassos as the richest source of oleuropein.

MATERIALS AND METHODS

Samples. Nine commercial types of Greek table olives packed in plastic bags, in cans or bulk, were purchased from the local market.

Reference Compounds. Pure hydroxytyrosol and oleuropein were isolated as previously described (*10*). Their purity (95%) was checked by HPLC analysis. The chemical structure was verified with ^{1}H and ^{13}C NMR. *m*-Hydroxyphenylethanol (3-hydroxyphenylethanol) used as internal standard (IS) was purchased from Sigma–Aldrich (Steinheim, Germany). Ammonium acetate was obtained from Riedel de-Haen (Germany). All solvents used throughout the experiments were obtained by Merck (Darmstadt, Germany) and were HPLC grade.

Internal Standard. IS was prepared in methanol and kept in refrigerator. Daily an IS aqueous solution (100 μg/mL) was prepared by diluting the stock standard 10-fold with water, and a standard volume of 100 μL was used in every case.

Separation, Identification, and Quantification of Phenolic Compounds by RP-HPLC-Diode Array Detection. *Sample Preparation.* 50 g of table olives from each type was obtained, and the flesh was separated from the kernel and weighed. A portion of the flesh was lyophilized in order to determine the dry weight.

Extraction Procedure. Wet olive flesh (1 g) was added to 5 mL of boiling water, stirred for 1 min, and left to stand for 2 min, and the mixture was filtered while hot. The filtrated volume was measured and consequently centrifuged at 14000 rpm for 2 min. A portion of the supernatant (100 μL) was diluted 10-fold with water and was subjected to HPLC analysis.

Apparatus and Chromatographic Methodology. High-perfomance liquid chromatographic analysis of the individual phenols was performed with a system consisting of a Finnigan Spectra system P4000 quaternary pump coupled to a Finnigan Spectra system UV6000LP diode array detector. Chromatographic separation of OE and HT was performed on a Altech C8 reversed-phase column (250 × 4.0 mm, ID 5 μm) equipped with a C8 Altech precolumn (10 × 4.0 mm, ID 5 μm).

Gradient elution was performed for the separation of the analytes. The mobile phase used consisted of A, acetonitrile, and B, 0.05 M ammonium acetate buffer adjusted to pH 5.0 with glacial acetic acid. The flow rate was 1 mL/min. The following elution program was employed: 0–10 min linear to 10% A; 10–25 min linear gradient to 40% A; 25–28 min linear gradient to 95% A; 28–30 min linear gradient to 10% A, whereas a 5 min equilibration time was used after each run. The injection volume was 20 μL. The λ_{max} employed for the detection of OE was at 240 nm whereas for both HT and the IS was at 280 nm. The corresponding retention times were 14.5 min for OE (**Figure 2**), 7.0 min for HT and 12.2 min for the IS. The area ratio of each substance against that of the IS was used for the quantification. The areas of the isolated peaks were estimated by Qhromquest V.2.51. software. The assignment of OE and HT chromatographic peaks was achieved by comparing the retention time and the corresponding spectra to those of the original standards. The OE and HT contents of the extracts, were estimated from the calibration curve of the isolated standards.

Standard Solutions. Stock standard solutions of OE, HT and IS were prepared in methanol at the 1 mg·mL^{-1} level. An IS aqueous solution (100 μg·mL^{-1}) was prepared by diluting the stock standard 10-fold with water. Standard solutions were prepared at concentrations of OE/HT 1, 2, 5, 10, 50, and 100 μg·mL^{-1} by diluting appropriate volumes of the stock standard solutions in water, whereas a fixed volume of 100 μL of IS aqueous solution was added in each solution. Stock standard solutions were kept refrigerated. All standard solutions and olive standards were prepared daily, and a portion of 100 μL was injected for HPLC analysis. Spiked olive samples were prepared to give concentrations of OE and HT at the 1, 2, 5, 10, 50, and 100 μg·mL^{-1} levels by diluting appropriate volumes of the stock standard solutions in water, whereas fixed volumes, 100 μL of IS aqueous solution and 50 μL of the extract of variety Megaritiki black, diluted 20-fold with water, were added. Variety Megaritiki black was selected as a blank because its phenolic profile was poor and as a result it did not give any interfering peaks at the analyte's t_R's.

Method Validation. The method was checked for the linearity, precision (calculated as the relative standard deviation % (RSD %)), accuracy (evaluated as the relative percentage error % (Er %)), sensitivity (evaluated as the limits of detection and quantitation LOD, LOQ).

Linearity. Spiked olive standards at six concentration levels, namely, 1, 2, 5, 10, 50, and 100 μg·mL^{-1} for both OE and HT, and 10 μg·mL^{-1} for

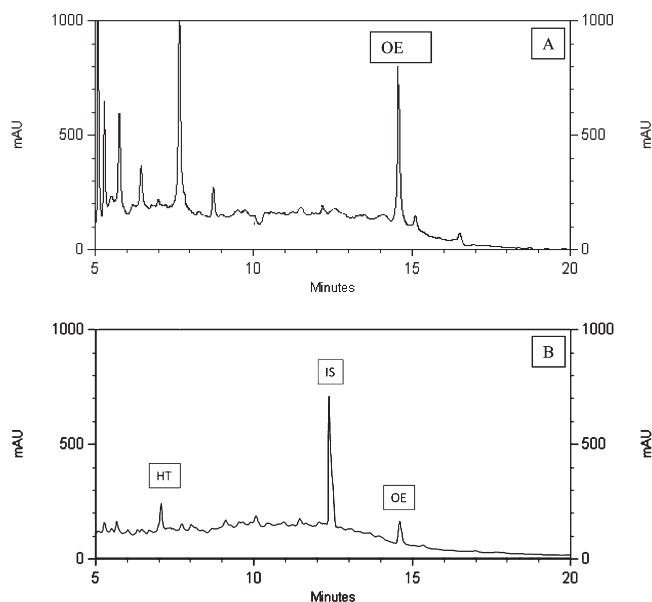

Figure 2. HPLC chromatograms of Throuba Thassos. **A**: UV detection at 240 nm, showing the peak of oleuropein (OE). **B**: UV detection at 280 nm showing the peaks of hydroxytyrosol (HT), internal standard (IS) and oleuropein (OE).

the IS were analyzed for the determination of the linearity. The relationship of the peak area ratio of the analyte 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 standards at two concentration levels (1 and 50 $\mu g \cdot mL^{-1}$). The interday precision was assessed by analyzing spiked olive standards of an olive standard at two concentration levels, namely, the 1 and 50 $\mu g \cdot mL^{-1}$ levels, prepared on five different days.

Accuracy. Spiked olive standards at three concentration levels, 1, 10, and 50 $\mu g \cdot mL^{-1}$, were analyzed in order 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 standards with concentrations of both OE and HT at the 1, 5, 10, and 100 $\mu g \cdot mL^{-1}$ levels ($n = 5$ for each level), and the IS at 10 $\mu g \cdot mL^{-1}$ were analyzed employing the proposed extraction procedure. The recovery was calculated as the ratio of the response of OE or HT in the spiked olive standards against that of the standards at the same levels and was expressed as the mean (± standard deviation) for each level.

Limits of Detection and Quantitation. The LOD and LOQ were determined running six blank samples of Megaritiki olive and measuring the background response at the t_R 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

Extraction Procedure. Usually olive drupes are treated using a rather complex protocol needing several steps in order to extract the contained polyphenols (*24*, *25*). In most cases methanol or methanol−water mixtures are used. Interestingly, a previous work by Sousa (*26*) has shown that the most appropriate solvent for the efficient extraction of total polyphenols from table olives is water at boiling temperature, but this method has not been validated for the case of oleuropein and hydroxytyrosol. To identify the solvent with the best recovery and the simplest chromatograms, three different polar solvents, methanol at room temperature, methanol:water 50−50 at room temperature and boiling water, were used. The above solvents were tested for the extraction of known amounts of OE and HT from an olive sample. For the aforementioned recovery experiments five samples have been processed and measured in each level and the result has been expressed as the arithmetic mean (± standard deviation). Methanol gave more complex extracts needing further purification and low recovery. The use of boiling water was excellent for the extraction of OE and HT and gave adequate recovery and less complex chromatograms than the mixture of methanol:water. Thus, the most suitable solvent for the extraction of OE and HT from the flesh of the olive fruit was proved to be the boiling water.

Table 1. Linearity of the OE, HT Determination in Olive Samples

ratio	regression equation	correlation coefficient, r^2
OE/IS	$y = 0.1604x + 0.3178$	0.993
HT/IS	$y = 0.168x + 0.098$	0.998

Table 2. Precision Data of the OE, HT for Olive Samples

	precision (RSD, %)			
	intraday		interday	
ratio	1 μg/mL	50 μg/mL	1 μg/mL	50 μg/mL
OE/IS	10.8	10.7	1.5	3.8
HT/IS	11.3	7.3	4.3	7.7

Table 3. Accuracy Data of the OE, HT Determination in Olive Samples

	accuracy (Er %)		
ratio	1 μg/mL	10 μg/mL	50 μg/mL
OE/IS	−4.3	10.1	8.8
HT/IS	5.7	11.9	−0.4

Method Validation. *Internal Standard*. The choice of 3-hydroxyphenylethanol as the IS was based on its chemical structure similarity to the substances under analysis. Furthermore it was baseline separated from OE and HT and presented no interference with other olive extract substances. Additionally, as it is not a naturally occurring substance in the olive tree, there are no basal levels that could interfere with the analysis.

Linearity. Good linearity was achieved for OE and HT, as indicated by the equations listed in **Table 1**.

Precision. The intraday precision, expressed as the relative standard deviation (RSD), ranged from 7.3 to 11.3% for the two analytes, as shown in **Table 2**. The interday precision ranged from 1.5% to 3.8 for OE, 4.3 to 7.7% for HT (**Table 2**). The RSD values are adequate and indicate the suitability of the method.

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 phenolic components. The obtained data indicate that the method could be considered as accurate.

Recovery. The recoveries were found to be 82.4%(±6.1) at the 1 $\mu g \cdot mL^{-1}$, 84.8%(±5.4) for the 5 $\mu g \cdot mL^{-1}$, 89.0%(±6.5) for the 10 $\mu g \cdot mL^{-1}$ and 90.8%(±5.1) for the 100 $\mu g \cdot mL^{-1}$ levels for OE and 70.3(±6.9) at the 1 $\mu g \cdot mL^{-1}$, 72.3(±6.2) at the 5 $\mu g \cdot mL^{-1}$, 75.0(±3.9) at the 10 $\mu g \cdot mL^{-1}$ and 78.5(±5.9) at the 100 $\mu g \cdot mL^{-1}$ for HT, indicating acceptable recovery. Moreover it seems that the background of the olive fruit extract does not interfere with the analysis.

Sensitivity. The sensitivity of the method as presented by its limit of detection (LOD) and the limit of quantification (LOQ) (the lower concentration of the calibration curve) were found to be 0.33 $\mu g \cdot mL^{-1}$ and 1 $\mu g \cdot mL^{-1}$ respectively for both OE and HT.

Table 4. Commercial Table Olive Samples Studied: Hydroxytyrosol (HT) and Oleuropein (OE) Quantities[a]

			HT		OE	
name	process type; characteristics	flesh wet wt (g) per 1 fruit	μg/g olive flesh	μg/fruit	μg/g olive flesh	μg/fruit
Mavrolies	Greek style; naturally black olives in brine, black color in oil (bags)	3.28	70.0 ± 5.5	229.6	18.2 ± 1.9	59.8
Chondrolies	Greek style; naturally black olives in brine, black color in oil, vinegar (bags)	5.09	200.9 ± 16.4	1022.5	8.9 ± 0.4	45.4
Green olives	Spanish style; green olives in brine (cans)	2.83	195.9 ± 14.0	554.4	nd[b]	nd
Megaritiki tsakistes	green olives in brine, "tsakistes" (bulk)	3.70	**505.1** ± 22.5	**1868.9**	nd	nd
Kalamon	Kalamata olives in brine black color in oil (bags)	3.70	**555.1** ± 44.4	**2053.9**	nd	nd
Throuba Thassos	naturally black olives in dry salt, wrinkled, in oil (bags)	2.25	80.0 ± 7.0	180.0	**550.0** ± 39.6	**1237.5**
Megaritiki	naturally black olives in dry salt, wrinkled black color (bulk)	1.80	13.5 ± 1.5	24.3	nd	nd
Agouromanaki	Greek style; green olives in brine (bulk)	1.80	490.2 ± 31.3	882.4	nd	nd
Amfissa	Greek style; naturally black olives in brine, in vinegar (bulk)	5.10	90.1 ± 6.2	459.5	nd	nd

[a] Mean values from 3 samples, except Throuba Thassos in which 20 samples were studied. [b] Not detected.

Method Application. The developed methodology was applied to the analysis of nine widely consumed commercial table olives. The names of the varieties are listed in **Table 4** along with their process type and their characteristics. Among them, there are three of the most widely cultivated varieties in Greece, Chondrolia, Kalamata and Amfissa, and one very preferable by the Greek consumers, Throuba Thassos, which is a Protected Designation of Origin (PDO) product. The percentage of flesh in olive fruits varied and was between 74.7% and 85.66%. The highest appeared in Throuba Thassos while the lowest one appeared in green olives. The dry weight ranged from 47.4% to 24.1% in all the types, except the dry salted olives Throuba Thassos and Megaritiki (75.55% or 71.8% respectively) that had low water content.

Phenolic Profile and Levels of OE and HT in Olive Samples. Chondrolia and Mavrolia fruits are usually used for Greek-style naturally black olives in brine. In this case the olives are harvested when they are black and fully ripe and put directly into the brine for fermentation for 3 to 6 months.

Kalamata olives are mainly used for the production of table olives known as Kalamon, which are very popular in Greece. They are produced by brining and fermentation for a few days (5 to 8 days), and the liquid is changed two or three times each day (*27*). Because of the little time of staying in brine oleuropein is not completely hydrolyzed, but is mainly extracted from the fruit and eliminated with the washing liquids.

Thassos table olives are naturally black olives grown mainly on the island of Thassos and are used to prepare a traditional type of olives known as "naturally black dry salted olives" cv. Throuba Thassos. The fruits are harvested when they are black at the stage of full ripeness, layered with salt 40%, lose water and other solutes including much of oleuropein and become gradually debittered and finally eatable in 40−60 days (*28*). Although the fruits retain a characteristic pleasant bitter taste they are appreciated by a wide range of consumers and constitute about 5% of the Greek olives market.

Green olives—Spanish style are debittered under alkaline conditions by which oleuropein is hydrolyzed into hydroxytyrosol and elenolic acid glycoside. Then a lactic acid fermentation in brine takes place for 3 to 4 weeks (*29*).

A comparative phytochemical screening of the studied table olives revealed that the naturally black dry salted olives cv. Throuba Thassos presented a very rich phenolic profile followed next by Kalamata olives. This may be attributed mainly to the mild process of Kalamata olives that are not submitted to a long fermentation and to the special treatment in dry salt of Throuba Thassos. The latter processing system does not include debittering in lye or brine as occurs in other types of table olives, so these fruits cannot be considered fermentable products. As a result, an increased number of phenolic compounds, especially in their bound forms, remain in the flesh.

The chromatographic analysis confirmed the presence of hydroxytyrosol in all the studied samples (**Table 4**). Kalamata olives followed by green "tsakistes" of the variety Megaritiki contained the highest quantity of hydroxytyrosol (2.0 and 1.8 mg per fruit). Greek-style "chondrolies" and "mavrolies" possessed quantities 1.0 mg/fruit and 0.2 mg/fruit respectively. Naturally black dry salted olives cv.Throuba Thassou contained 0.18 mg/fruit.

On the other hand, oleuropein was found in Chondrolies and Mavrolies in very low levels (45 and 60 μg/fruit respectively). However, the most important finding was that Throuba Thassos olives were very rich in oleuropein (1.2 mg/fruit), while in the rest of the samples oleuropein could not be detected. These significant differences can be explained by the cultivated variety and the specific process applied on the fruits and especially the use of brine, lye or dry salt.

Sodium hydroxide treatment hydrolyzes ester bonds turning oleuropein to hydroxytyrosol (*30*). Simultaneously, diffusion of polar phenolic compounds like hydroxytyrosol occurs from the flesh to the aqueous medium (*16*). This explains the profile of Green "tsakistes" and Greek-style naturally black olives, as well as the presence of high concentration of HT and very low amounts of oleuropein in these types of processed olives.

During the long fermentation period of Greek-style olives diffusion of constituents and lactic acid hydrolysis of oleuropein to hydroxytyrosol and elenolic acid takes place (*17*). Bound forms of hydroxytyrosol are absent in the flesh of the fruits (*24*). Olives fermented in brine are poor in phenolic substances, when compared with the table olives obtained by other methods (*31*). Thus Greek-style olives Chondrolies, Mavrolies and Amfissa had poor phenolic profile, HT was found in sufficient quantities, while oleuropein existed in traces or not at all.

The special processing of naturally black dry salted olives Throuba Thassos leads to dehydration rather than fermentation, and as expected a part of oleuropein remains not hydrolyzed. This explains the fact that low levels of hydroxytyrosol and a prominent quantity of oleuropein were found in these olives. However, a special comment should be done for the case of dry salted olive variety Megaritiki, which did not contain any oleuropein at all. Despite the use of dry salt for debittering, the lack of oleuropein can be explained either by the different olive variety (*18*, *20*) or by the use of extended water washing before commercialization. It should be noted that the dry-salting process is in many cases empirical (*28*), and if it not appropriately followed can lead to low quality products. For this reason the concentration of oleuropein in Throuba Thassos was recorded for a 4-year period (5 different samples each year) and was consistently found to be very high.

During this period of monitoring two cases of commercial samples with low concentration of oleuropein were recorded, but in both cases the producers mentioned the use of inappropriate extensive contact with aqueous media.

In conclusion, although oleuropein and hydroxytyrosol are major polyphenols of unprocessed olive fruits, their concentration in the processed edible olives is highly variable. The presence of hydroxytyrosol was confirmed in all the types of edible olives examined. Oleuropein was found in important quantities only in one variety (Throuba Thassos) which is processed by dry salt in a traditional Greek way. This finding gives important evidence for the determination of a safe dosology of oleuropein in humans through food supplements. Assuming a usual consumption of 20 olive drupes during a meal, a daily intake of 25 mg of oleuropein can be considered as of nutritional level. Consequently an oral dose of 25 mg per day can be considered as safe for human, since this quantity can be obtained through a usual diet. The addition of edible olives in our diet is necessary, and in combination with olive oil, they can provide important quantities of natural antioxidants, but this is highly dependent on the type of olives consumed.

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