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Analysis of Total Contents of Hydroxytyrosol and Tyrosol in Olive Oils

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ABSTRACT: The most abundant phenolic compounds in olive oils are the phenethyl alcohols hydroxytyrosol and tyrosol. An optimized method to quantify the total concentration of these substances in olive oils has been described. It consists of the acid hydrolysis of the aglycons and the extraction of phenethyl alcohols with a 2 M HCl solution. Recovery of the phenethyl alcohols from oils was very high (<1% remained in the extracted oils), and the limits of quantification (LOQ) were 0.8 and 1.4 mg/kg for hydroxytyrosol and tyrosol, respectively. Precision values, both intraday and interday, remained below 3% for both compounds. The final optimized method allowed for the analysis of several types of commercial olive oils to evaluate their hydroxytyrosol and tyrosol contents. The results show that this method is simple, robust, and reliable for a routine analysis of the total concentration of these substances in olive oils.

KEYWORDS: *hydroxytyrosol, tyrosol, olive oil, analysis, phenolic compounds*

INTRODUCTION

Olive oil has been consumed in the Mediterranean basin for centuries because of its organoleptic and nutritive properties, and many international regulations have been developed to maintain the quality of this commodity and to avoid commercial frauds.[1] Nowadays, consumers all over the world appreciate this healthy fat due to its high content of monounsaturated fatty acids and other minor components such as squalene, tocopherols, sterols, and, particularly, phenolic compounds.[2,3] Among others, olive oil polyphenols have been attributed free radical scavenging, anticarcinogenic, cardiopreventive, and antimicrobial properties.[2] In 2011, the NDA Panel of the European Food Safety Authority (EFSA) concluded that there is evidence of a cause and effect relationship between the consumption of olive oil polyphenols and the protection of low-density lipoprotein (LDL) chlolesterol particles from oxidative damage,[4] and recently the European Commission has authorized this claim in May 2012, although it "may be used only for olive oil which contains at least 5 mg of hydroxytyrosol and its derivatives (e.g. oleuropein complex and tyrosol) per 20 g of olive oil".

Despite the huge number of papers published on olive oil polyphenols, there is no international regulation for the analysis of these substances. There are several reasons for the lack of a robust and reliable method for quantifying phenolic compounds in olive oil. First, the extraction methodology must be exhaustive. It has been reported that liquid−liquid extraction gives rise to higher recovery of phenolic compounds than solid-phase extraction,[5,6] although it also depends on the solvent used. For example, *N*,*N*-dimethylformamide (DFM) achieves better recoveries of polyphenols than methanol/water mixtures.[7]

The phenolic fraction of olive oil consists of a heterogeneous mixture of compounds, with the most abundant being the oleuropein and ligustroside aglycons (HyEA and TyEA), the dialdehydic form of decarboxymethyl elenolic acid linked to hydroxytyrosol and tyrosol (HyEDA and TyEDA), the lignans 1-acetoxypinoresinol and pinoresinol, hydroxytyrosol acetylated, hydroxytyrosol, tyrosol, luteolin, apigenin, and other minor substances.[8,9] There are two drawbacks to quantifying these substances in olive oils, particularly the secoiridoid aglycons: (i) there are no commercial standards available regarding these polyphenols, and (ii) peaks in the HPLC and GC chromatograms are not well-defined. Researchers have isolated these secoiridoid aglycons by semipreparative HPLC, but they are unstable and difficult to obtain in their pure form in large amounts.[10−12] An alternative to the lack of standards has been the quantification of all peaks with only one commercial compound such as tyrosol, 3,4-dihydroxyphenethylacetic acid, oleuropein, or others.[13−15] This alternative has been proposed by the International Olive Council.[16] However, olive oil polyphenols have different responses under UV detection after their HPLC separation. In fact, the response factors of tyrosol and hydroxtyrosol at 280 nm are different.[17]

Another drawback is related to the resolution of the peaks, particularly in HPLC chromatograms. Peaks overlap on many occasions, and the resolution of those corresponding to secoiridoid aglycons is not good. These compounds, for example, the dialdehydic forms, show broad peaks in HPLC-DAD and HPLC-MS chromatograms, which have been attributed to the formation of isomeric forms that limit their quantification.[8,12,18] In addition, during heating and storage of the oil these secoiridoid aglycons originate oxidized products that coelute with nonoxidized products and possess different response factors under UV detection.[14,19−21]

Most of the biochemical and pharmacological effects of olive polyphenols have been attributed to hydroxytyrosol and, to a lesser extent, to tyrosol. Both substances are absorbed in the intestine and can be detected in urine, plasma, and LDL

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particles.[22−24] However, the analysis of their total content in olive oils fails to provide reliable data because of the unreliable quantification of secoiridoid aglycons. In fact, the NDA Panel of the EFSA makes mention of "5 mg of hydroxtyrosol and its derivatives (e.g. oleuropein complex and tyrosol)", which is rather confusing.

The aim of this study was to optimize a simple and reliable method for analyzing the total contents of hydroxytyrosol and tyrosol in olive oils regardless of whether they are free or combined.

MATERIALS AND METHODS

Reagents. Hydroxytyrosol, tyrosol, hydroxytyrosol glycol, and syringic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Analytical grade methanol, DMF, hexane, phosphoric acid, and hydrochloric acid were supplied by Panreac (Barcelona, Spain). Ultrapure water from a Mili-Q system (Millipore, Bedford, MA, USA) was used throughout this research.

Standard Solutions. Stock standard solutions of 5000 mg/L of hydroxytyrosol and tyrosol were prepared by dissolving these substances in a mixture of methanol/water. Calibration samples were prepared in the range of 5−250 mg/L by dilution in the same matrix.

Olive Oil Samples. Olive oils were purchased from supermarkets located in the Seville province (Spain). Extra virgin olive oils of the Arbequina, Picual, Hojiblanca, Manzanilla, and undefined olive variety of six different commercial brands were obtained. Mild-flavored olive oils and intensely flavored olive oils of 6six different commercial brands were also studied. These are a mixture of refined olive oil and virgin olive oil.

Analysis of Phenolic Compounds in Olive Oil. Phenolic extracts of olive oils were obtained following the procedure described elsewhere.[7] Briefly, 0.6 g of olive oil was extracted using 3 × 0.6 mL of DMF; the extract was then washed with hexane, and N_2 was bubbled into the DMF extract to eliminate residual hexane. Finally, the extract was filtered through a 0.22 μm pore size and injected into the chromatograph.

The chromatographic system consisted of a Waters 717 plus autosampler, a Waters 600E pump, and a Waters heater module (Waters Inc., Milford, MA, USA). A Spherisorb ODS-2 (5 μm, 25 cm × 4.6 mm i.d., Waters Inc.) column was used. Separation was achieved using an elution gradient with an initial composition of 90% water (pH adjusted to 3.0 with phosphoric acid) and 10% methanol. The concentration of the later solvent was increased to 30% over 10 min and maintained for 20 min. Subsequently, the methanol percentage was raised to 40% over 10 min, maintained for 5 min, and then increased to 50%. Finally, the methanol percentage was increased to 60, 70, and 100% in 5 min periods. Initial conditions were reached in 15 min. A flow of 1 mL/min and a temperature of 35 °C were used in all of the experiments. A Waters 996 diode array detector and a Jasco FP-920 fluorescence detector (Jasco, Tokyo, Japan) were connected in series. Most of the phenolic compounds used for standards were obtained by semipreparative HPLC as described elsewhere.[7]

Assays for the Extraction and Hydrolysis of Olive Oil Polyphenols. Olive oil (2.5−25 g) and 2 M HCl (25−50 mL) were put into a 100 mL glass bottle that was closed with a polypropylene cap. The mixture was vigorously homogenized by agitation at 400 rpm in an orbital shaking incubator model WY-200 (Comecta, S.A., Barcelona, Spain). Experiments were run at 25 °C. Finally, 1−2 mL of the aqueous phase was removed by a plastic pipet, filtered through a 0.22 μm pore size, and injected into the chromatograph. The chromatographic system and conditions were the same as noted above, except the gradient program of solvents that was modified, the washing period starting at 20 min from injection.

RESULTS AND DISCUSSION

Figure 1 shows a representative HPLC chromatogram of olive oil polyphenols monitored by ultraviolet (UV) and fluorescence detection (FL). Phenethyl alcohols, hydroxytyrosol and tyrosol, exhibited sharp peaks in both UV and FL detection, although the heights of their peaks were much higher with the latter technology. Precisely, FL detection is recommended for lignans, hydroxytyrosol acetate, and hydroxytyrosol glycol analysis.[25] Oleuropein and ligustroside aglycons (HyEA and TyEA) can also be detected with FL detection, although broad peaks are currently formed. Peak splitting was observed for these substances in both UV and FL detection due to isomeric forms. On the contrary, poor resolution of the peaks corresponding to the dialdehydic forms of decarboxymethyl elenolic acid linked to hydroxytyrosol (HyEDA) and tyrosol (TyEDA) was obtained using fluorometric evaluation; therefore, UV detection was required.[26] However, the peaks of lignans and TyEDA were not well-defined using UV detection. Again, the presence of isoforms of TyEDA and particularly HyEDA made the analysis of these substances difficult .[4,26]

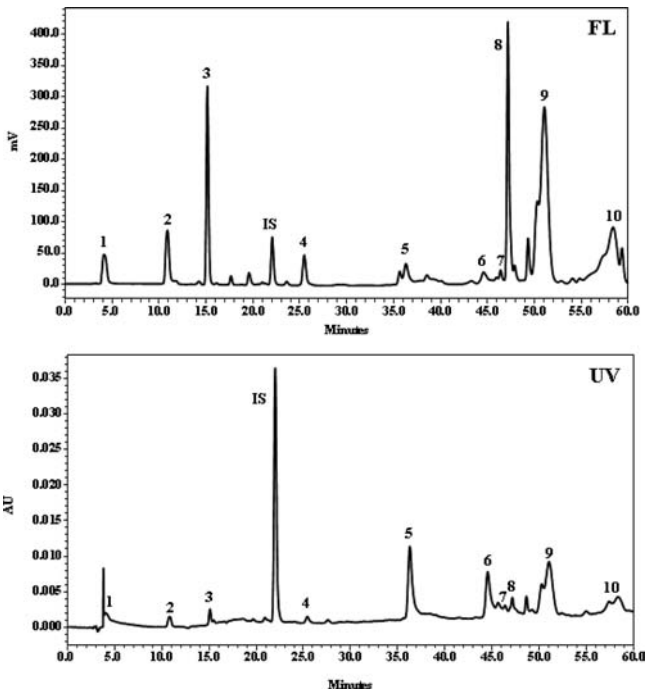

Figure 1. HPLC chromatogram of phenolic compounds isolated from an extra virgin olive oil of the Picual variety. They were monitored by ultraviolet (UV) and fluorescence (FL) detection. Peaks: (1) hydroxytyrosol glycol; (2) hydroxytyrosol; (3) tyrosol; (4) hydroxytyrosol acetate; (IS) internal standard, syringic acid; (5) dialdehydic form of decarboxymethyl elenolic acid linked to hydroxytyrosol; (6) dialdehydic form of decarboxymethyl elenolic acid linked to tyrosol; (7) 1-acetoxypinoresinol; (8) pinoresinol; (9) oleuropein aglycon; (10) ligustroside aglycon.

Consequently, we explored the analysis of the total contents of hydroxytyrosol and tyrosol in the oils using acid hydrolysis. This methodology has been proposed for the analysis of ellagic acid in berries and strawberries[27,28] and flavonoids in foods.[29] With regard to olive oil polyphenols, Miró-Casas et al.[30] used acid hydrolysis to quantify hydroxytyrosol and tyrosol in virgin olive oil, but after extraction of polyphenols from the oil with a mixture of methanol/water. A strong acid hydrolysis has also been reported to release hydroxytyrosol from olive leaves.[31] A treatment with HCl of olive oils was used to test the exhaustiveness of the solvent extraction of olive oil polyphenols.[7] Therefore, we studied the acid hydrolysis of aglycons

with 2 M HCl. Figure 2 displays a chromatogram of the phenolic compounds present in the acidic phase of a mixture

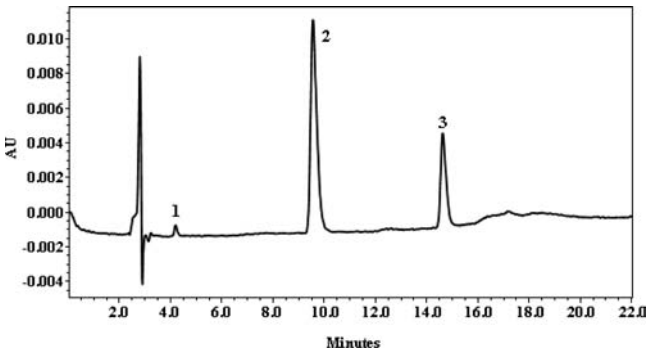

Figure 2. HPLC chromatogram of phenolic compounds obtained from extra virgin olive oil treated with 2 M HCl. They were monitored by UV detection at 280 nm. Peaks: (1) hydroxytyrosol glycol; (2) hydroxytyrosol; (3) tyrosol.

oil/HCl maintained at 25 °C for 6 h. Hydroxytyrosol and tyrosol were the main polyphenols detected at 280 nm together with a small peak corresponding to hydroxytyrosol glycol. No other phenolic compound was observed in the chromatogram. It must be said that the acidic solution was directly injected into the chromatograph without any previous treatment except filtration through a 0.22 μm nylon filter, and no interference peaks appeared at the retention times of hydroxytyrosol or tyrosol.

Two phenomena occurred during the contact of oil with 2 M HCl: (i) the acid hydrolysis of the secoiridoid aglycons and (ii) the diffusion of phenethyl alcohols from the oily to the aqueous phase. The hydrolysis of secoiridoid aglycons was very fast (Figure 3), and their concentration was reduced almost completely during the first 2 h of treatment. Moreover, hydroxytyrosol acetate was rapidly hydrolyzed. At the same time, the diffusion of hydroxytyrosol and tyrosol to the acidic phase was fast, although it depended to a large extent on the ratio of grams of oil per milliliters of acidic solution (Figure 4). This was a crucial point to optimize the methodology. It was observed that at least a ratio of 1:10 (2.5 g of oil and 25 mL of 2 M HCl solution) was necessary to reach high concentrations of hydroxytyrosol and tyrosol in the acidic phase. Finally, a ratio of 1:20 (2.5 g of oil and 50 mL 2 M HCl) was chosen as appropriate to evaluate the effect of agitation time on the exhaustiveness of the method. It can be deduced from the data reflected in Figure 5 that 4−6 h was necessary to complete the hydrolysis of secoiridoid aglycons and the diffusion of phenethyl alcohols from the oil to the acidic solution. It must also be noted that assays were run at 25 °C and under intensive agitation (400 rpm) of the mixture.

With the aim of evaluating the recovery of the method, 2.5 g of three virgin olive oils was mixed with 50 mL of 2 M HCl, and the mixture was thoroughly agitated for 6 h. The contents of hydroxytyrosol and tyrosol of the aqueous phase were analyzed, and subsequently 1 g of the extracted oil was again put into contact with 1 mL of 2 M HCl for another 2 h. The results of this assay are presented in Table 1. It can be observed that a very high recovery of hydroxytyrosol and tyrosol from oils was achieved and that the amount of these substances remaining in the first extracted oil was below 1%. Hence, <1 or 2 mg/kg of hydroxytyrosol and tyrosol, respectively, was not extracted from

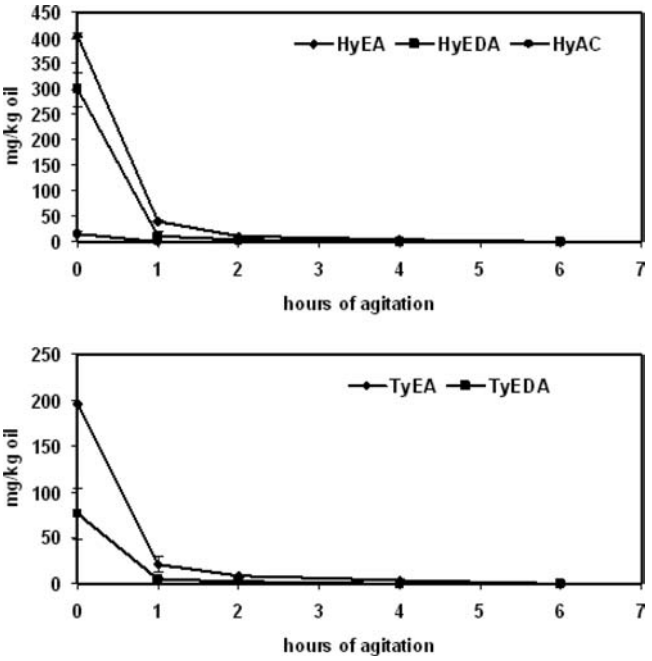

Figure 3. Evolution of the main phenolic compounds in an extra virgin olive oil (2.5 g) during its contact with 2 M HCl (50 mL). HyEDA, didaldehydic form of decarboxymethyl elenolic acid linked to hydroxytyrosol; TyEDA, dialdehydic form of decarboxymethyl elenolic acid linked to tyrosol; HyEA, oleuropein aglycon; TyEA, ligustroside aglycon; HyAC, acetylated hydroxytyrosol.

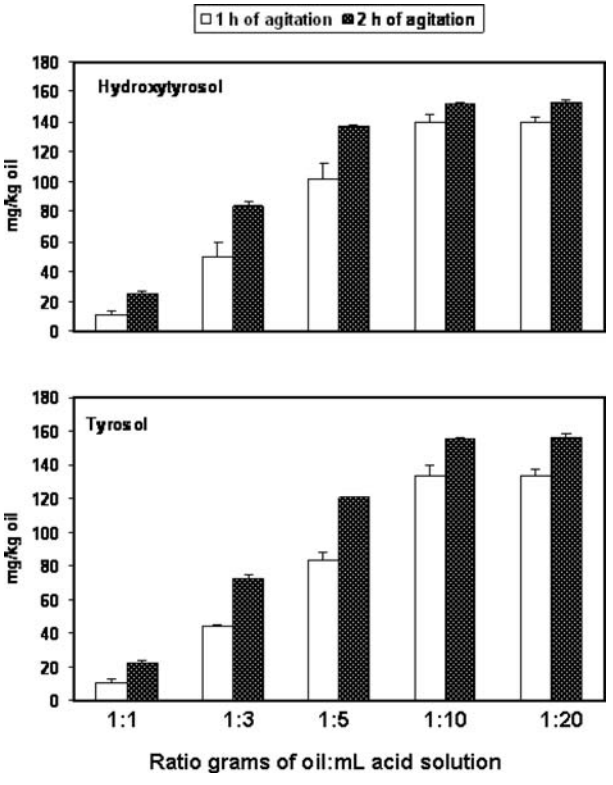

Figure 4. Effect of the ratio of grams of oil to milliliters of 2 M HCl on the estimated concentration of hydroxytyrosol and tyrosol in an extra virgin olive oil. Error bars indicate standard deviation.

the oils. The slightly lower recovery found for tyrosol must be related to its lower polarity relative to that of hydroxytyrosol.

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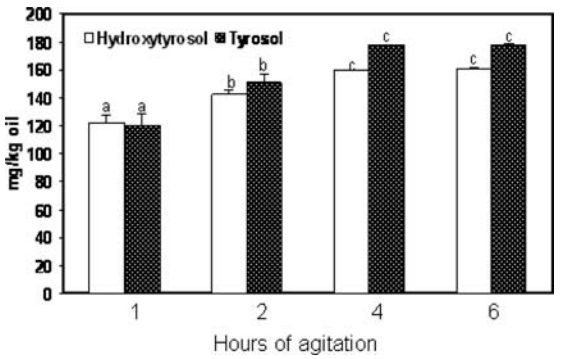

Figure 5. Effect of time of agitation on the estimated concentration of hydroxytyrosol and tyrosol in an extra virgin olive oil. A ratio of 2.5 g of oil to 50 mL of 2 M HCl was used. Different letters on the bars indicate significant differences according to a Duncan's multiple-range test ($P < 0.05$).

Table 1. Assessment of the Contents of Total Hydroxytyrosol and Tyrosol of Three Extra Virgin Olive Oils by Analyzing First the Acidic Phase of a Mixture of Oil (2.5 g) and 2 M HCl (50 mL) Agitated for 6 h and Subsequently the Acidic Phase of a New Mixture of Treated Oil (1 g) and 2 M HCl (1 mL) Agitated for 2 h

	hydroxytyrosol (mg/kg)		tyrosol (mg/kg)	
oil	first extraction	second extraction	first extraction	second extraction
A	160.5	0.4	177.9	1.4
B	201.1	0.6	162.4	1.1
C	63.3	0.1	106.9	0.7

Validation of the quantitative analytical method for simultaneous total hydroxytyrosol and tyrosol determination in olive oil followed Horwitz's recommendations.[32] With regard to sensitivity of the method, the limit of detection (LOD) and the limit of quantification (LOQ) were calculated by measuring the analytical background response at 280 nm. LOD and LOQ were considered to be 3 and 10 times, respectively, the standard deviation of six blank samples analyzed. Table 2 shows that the

Table 2. Sensitivity and Precision of the Method

			RSD (%) (n = 5)	
compound	LOD (mg/L)	LOQ (mg/L)	intraday precision[a]	interday precision
hydroxytyrosol	0.3	0.8	1.2–2.9	0.9–3.0
tyrosol	0.4	1.4	1.4–3.2	1.3–2.3

[a]Determined by analyzing two different olive oil extracts with low and high concentrations of hydroxytyrosol and tyrosol.

LOD was <0.5 mg/L for both hydroxytyrosol and tyrosol, whereas the LOQs were 0.8 and 1.4 mg/L for hydroxytyrosol and tyrosol, respectively. It must be noted that these limits can be lower when using fluorescence detection if necessary.

Method precision was studied as intra- and interday assays (n = 5) for each compound. The intraday precision of the procedure was determined by analyzing replicates (n = 5) of two olive oil extracts, and interday precision by analyzing these two olive extracts on five different days. Precision was calculated as relative standard deviation (RSD). Table 2 shows that intraday and interday precisions expressed as RSD % for both compounds, hydroxytyrosol and tyrosol, were <3%, which is an acceptable level.

Briefly, the optimized method consists of agitating a mixture of oil (2.5 g) and 2 M HCl (50 mL) for 4–6 h at 25 °C. Subsequently, the contents of hydroxytyrosol and tyrosol of the acidic phase are directly analyzed by HPLC and expressed as milligrams of polyphenol per kilogram of oil.

The recently authorized claim by the European Commission has established that "20 g of oil must contain at least 5 mg of hydroxytyrosol and its derivatives", which means a minimum content of 250 mg/kg oil in these substances. Supposedly, these substances must account for free hydroxytyrosol and tyrosol as well as their derivatives HyEDA, TyEDA, HyEA, and TyEA.[4] However, as mentioned above, the analysis of these substances presents some drawbacks, particularly the lack of commercial standards, formation of oxidized forms during oil storage, and appearance of broad peaks in HPLC chromatograms. In addition, secoiridoid aglycons are hydrolyzed during oil storage, giving rise to simple hydroxytyrosol and tyrosol.[14,34,35] Taking into consideration the molecular weight ratio between these phenethyl alcohols and their derivatives (1:2–2.5), a great decrease in total polyphenol concentration will be quantified in oils during their commercial shelf life. The molecular weights of hydroxytyrosol and tyrosol are 154 and 138 uma, respectively, whereas those of the secoiridoid aglycons range between 304 and 378 uma.

The optimized procedure was applied to the determination of total hydroxytyrosol and tyrosol levels in several types of commercial olive oils, and results are reflected in Figures 6 and

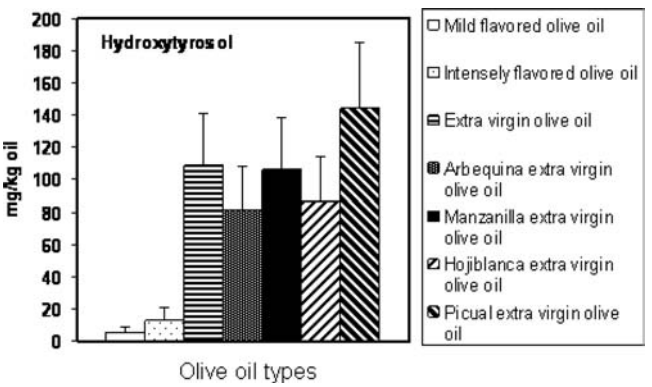

Figure 6. Concentration of total hydroxytyrosol of several types of commercial olive oils. Mean and standard deviation (error bars) of six analyses.

7. Among the oils analyzed, the mild-flavored and intensely flavored olive oils had the lowest contents in both hydroxytyrosol and tyrosol, which is in agreement with the low content in total polyphenols reported for these oils.[33] In fact, commercial olive oil is a mixture of refined olive oil with virgin olive oil, and the results obtained in this study point out that the percentage of virgin olive oil in the mixture is low, probably because of the low content in phenethyl alcohols. With regard to extra virgin olive oils, the content in hydroxytyrosol ranged from 50 to 200 mg/kg oil. A great variability was found among olive varieties and within each monovarietal oil. Similar findings were obtained from the tyrosol analyses (Figure 7), with the content of this polyphenols in virgin olive oils ranging from 40 to 180 mg/kg oil. Therefore, the concentration of total hydroxytyrosol and tyrosol in virgin olive oils ranged from 100 to 400 mg/kg oil. Taking these data into consideration, the total amount of

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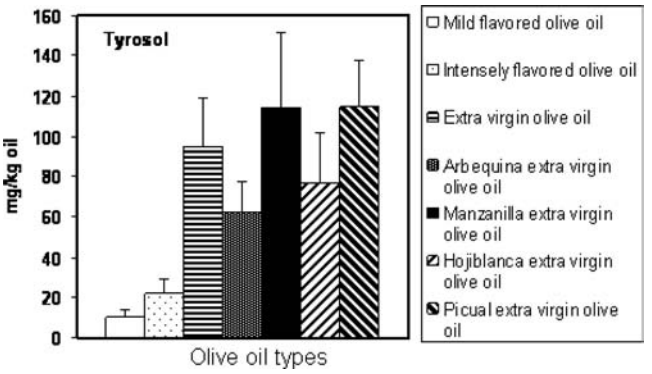

Figure 7. Concentration of total tyrosol of several types of commercial olive oils. Mean and standard deviation (error bars) of six analyses.

hydroxytyrosol and tyrosol required in 20 g of oil should be as low as 2−2.5 mg because of the differences between the molecular weights of these phenethyl alcohols and their derivatives.

In conclusion, a simple, robust, and reliable method has been optimized to measure total hydroxytyrosol and tyrosol in olive oil. It will be useful for studies on the bioavailability of these substances in humans and the nutritional quantification of these polyphenols in olive oils.

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Notes

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