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# Antioxidant activity evaluation of alkyl hydroxytyrosyl ethers, a new class of hydroxytyrosol derivatives

Gema Pereira-Caro [a], Andrés Madrona [b], Laura Bravo [c], José Luis Espartero [b], Felipe Alcudia [b], Arturo Cert [d], Raquel Mateos [a,*]

[a] IFAPA, Centro Venta de Llano, E-23620 Mengíbar (Jaén), Spain
[b] Department of Organic and Pharmaceutical Chemistry, Faculty of Pharmacy, University of Seville, E-41012 Seville, Spain
[c] Instituto del Frío, CSIC, E-28040 Madrid, Spain
[d] Instituto de la Grasa, CSIC, E-41012 Seville, Spain

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ABSTRACT

The antioxidant activity of a new series of alkyl hydroxytyrosyl ethers was evaluated by the Rancimat test in a lipophilic food matrix and by the FRAP, ABTS and DPPH assays in a hydrophilic medium, and compared to free hydroxytyrosol (HTy), butylhydroxytoluene (BHT) and α-tocopherol. All methods used to assess the antioxidant activity of the new compounds emphasised the importance of the *ortho*-diphenolic structure on the maintenance of the high antioxidant activity associated with free HTy. The results obtained support the 'polar paradox' since the antioxidant activity of the lipophilic hydroxytyrosyl ethers was slightly lower in bulk oils and higher in hydrophilic media in comparison with their reference HTy. Although the length of the alkyl chain did not significantly affect the antioxidant activity in bulk oils, it did have a positive influence in hydrophilic medium for ethers with a short alkyl chain (methyl, ethyl and propyl), while ethers with longer alkyl chain (butyl, hexyl, octyl, dodecyl and octadecyl) maintained or even decreased their antioxidant activity, probably due to the steric effect of the side chains. In conclusion, these results show the strong antioxidant activity of a new group of lipophilic HTy derivatives that satisfies the food industry demands for new antioxidants with potential use as functional ingredients to improve the quality and nutritional properties of foods.



## 1. Introduction

Epidemiological studies have attributed a lower incidence of degenerative pathologies, including coronary heart disease and certain tumours, to the Mediterranean diet where the olive oil is the main fat source (Keys, 1995; Martin-Moreno et al., 1994; Trichopoulou & Critselis, 2004; Trípoli et al., 2005). In the last years the number of reports describing the beneficial properties of olive oil has increased. This olive oil popularity has mainly been attributed to its high content of monounsaturated fatty acids (Gimeno et al., 2002; Ramirez-Tortosa et al., 1999) and its richness in phenolic compounds, which may contribute to the prevention of diseases mentioned above as shown in animal and human studies (Covas et al., 2006; Fitó et al., 2000; Visioli & Galli, 1998).

In this sense, the presence of phenolic compounds in olive oil as the biggest group of natural antioxidants has attracted much attention due to their known and widely-ranged biological activities as well as to their health effects (Bendini, Cerretani, Carrasco-Pancorbo, & Gomez-Caravaca, 2007; Trípoli et al., 2005; Tuck & Hayball, 2002). Particular attention has been focussed on 3,4-dihydroxyphenylethanol (hydroxytyrosol, HTy), an *o*-diphenolic compound that is present in virgin olive oil either as secoiridoid derivatives (Montedoro et al., 1993) or as an acetate ester (Brenes, Garcia, Garcia, Rios, & Garrido, 1999). Several *in vitro* and *in vivo* studies carried out with pure HTy reported its capacity to reduce the risk of coronary heart disease and atherosclerosis (Carluccio et al., 2006; Salami, Galli, De Angelis, & Visioli, 1995).

Other biological properties of the HTy derivatives present in olive oil include antimicrobial (Bisignano et al., 1999; Furneri, Piperno, Sajia, & Bisignano, 2004), antiinflammatory (Bitler, Viale, Damaj, & Crea, 2005), hypotensive and hypoglycaemic activities (Visioli, Bellomo, Montedoro, & Galli, 1995), inhibition of platelet aggregation (Visioli et al., 1995) and several lipoxygenases (De la Puerta, Ruiz-Gutiérrez, & Hoult, 1999), or induction of apoptosis in HL-60 cells (Manna et al., 1997).

The food industry is demanding new, powerful antioxidants with proven functional properties to improve the quality and nutritional properties of foods and foodstuffs (Evans, 1997; Moure et al., 2001). These compounds must meet basic requirements concerning safety, sensory quality and applicability in industrial

* Corresponding author. Fax: +34 953 366 380.
*E-mail address:* mariar.mateos@juntadeandalucia.es (R. Mateos).

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processes thus offsetting the drawbacks of the presently authorised food antioxidants, such as the cytotoxic effects of BHT and BHA, the particular sensorial characteristics (astringency and/or bitterness) of polyphenolic extracts, or the limitations of lipophilic antioxidants for practical applications in hydrophobic/lipid media (Sarafian, Kouyoumjian, Tashkin, & Roth, 2002; Silveira, Monereo, & Molina, 2003). In this sense, the demonstrated biological activities of HTy and its affordable recovery from olive oil wastewaters (Fernández-Bolaños et al., 2005) have focussed attention on this phenol as a potential functional food ingredient (Fki, Allouche, & Sayadi, 2005), which has already been used as an additive in tomato juice (Larrosa, Espin, & Tomas-Barberan, 2003) and fish products (Pazos, Alonso, Sánchez, & Medina, 2008) with good results. However, the highly polar nature of HTy reduces its solubility in lipophilic preparations, and thus chemically modified HTy derivatives with increased lipophilicity were synthesised by our research team. Lipophilic hydroxytyrosyl esters were obtained from HTy by a patented protocol (Alcudia et al., 2004), showing enhanced functional properties in comparison with free HTy (Trujillo et al., 2006). More recently, a new group of lipophilic hydroxytyrosyl derivatives with linear alkylic side chains of variable length have been synthesised from HTy recovered from olive oil wastewaters (Madrona et al., unpublished results). The aim of the present work was to study the antioxidant activity of these new synthetic alkyl hydroxytyrosyl ethers. Different methods were used to determine their antioxidant activity in lipid matrices (Rancimat test) as well as in hydrophilic media, assessing the radical scavenging capacity by the ABTS and DPPH methods and the reducing capacity by the FRAP method. Free HTy, α-tocopherol and BHT served as reference compounds.

## 2. Materials and methods

### 2.1. Chemicals

All solvent and reagents were of analytical grade unless stated otherwise.

Iron(II) sulphate 7-hydrate was acquired from Panreac (Sevilla, Spain), 2,6-di-*tert*-butyl-4-metylphenol (BHT), α-tocopherol and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (97%) (Trolox) were from Aldrich (Steinheim, Germany), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (98%) (ABTS), 2,4,6-tri-(2-pyridyl)-1,3,5-triazine (98%) (TPTZ), iron(III) chloride hexahydrate (97%), and neutral alumina, type 507C, grade I were all obtained from Sigma (Steinheim, Germany).

Hydroxytyrosol (compound **1** in Fig. 1) was isolated with a 95% purity from olive oil wastewaters by a procedure protected by patent (Fernández-Bolaños et al., 2005), and further purified by column chromatography. Lipophilic hydroxytyrosyl ethers (**2**–**9**) (Fig. 1) were prepared by chemical synthesis from HTy (Madrona et al., unpublished results).

### 2.2. Lipid matrix

The lipid matrix was obtained from commercial sunflower oil by purification through alumina according to the 'free solvent' procedure (Yoshida, Kondo, & Kajimoto, 1992). Briefly, 200 g of oil were poured into a glass chromatographic column (45 × 3 cm id) packed with 100 g of activated alumina at 200 °C during 3 h.

To check the composition of the matrix the following determinations were carried out: total phenolic compounds by solid phase extraction and HPLC analysis with UV detector (Mateos et al., 2001); tocopherols by HPLC analysis on a silica gel column using a UV–vis detector (Paquot & Hautfenne, 1992) and fatty acid composition by capillary GC analysis of the methyl esters obtained by transmethylation of the oil with KOH in methanol (Cert, Moreda, & Pérez-Camino, 2000).

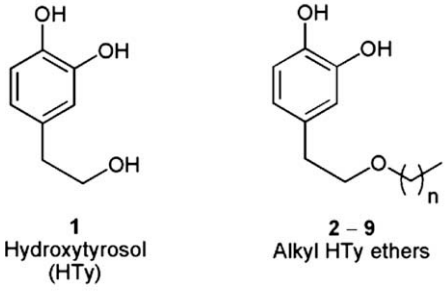
The purified matrix, free of antioxidants, was stored at −18 °C under nitrogen atmosphere. The fatty acid composition of the matrix was: C16:0 (6.1%), C16:1 (0.1%), C18:0 (3.6%), C18:1 (29.3%), C18:2 (59.7%), C18:3 (0.1%) and C22:0 (0.6%).

### 2.3. Evaluation of oxidative stability by the Rancimat method

The oxidative stability was evaluated by an accelerated automated test using Rancimat equipment (Model 743, Metrohm Co. Basel, Switzerland). Aliquots of the glyceridic matrix were spiked with increasing amounts of alkyl hydroxytyrosyl ethers (**2**–**9**), free HTy (**1**) as well as α-tocopherol and BHT, ranging from 0.2 to 3 mM (the maximum concentration to reach a steady state of oxidative stability) and then subjected to accelerated oxidation in a Rancimat apparatus at 90 °C. Results are expressed as induction time (IT) in hours, corresponding to the stability of the lipid matrix evaluated. All determinations were carried out in duplicate.

### 2.4. Ferric reducing antioxidant power (FRAP) assay

The FRAP assay was carried out according to the procedure described by Benzie and Strain (1996) with some modifications (Pulido, Bravo, & Saura-Calixto, 2000). The antioxidant potential of the synthesised compounds was estimated from their ability to reduce the ferric tripyridyltriazine (TPTZ–Fe(III)) complex to its stable ferrous form (TPTZ–Fe(II) complex). FRAP values are expressed as mM TE (Trolox equivalent). All analyses were run in triplicate.




| Compound | n | Name |
|---|---|---|
| 2 | 0 | Methyl_HTy |
| 3 | 1 | Ethyl_HTy |
| 4 | 2 | Propyl_HTy |
| 5 | 3 | Butyl_HTy |
| 6 | 5 | Hexyl_HTy |
| 7 | 7 | Octyl_HTy |
| 8 | 11 | Dodecyl_HTy |
| 9 | 18 | Octadecyl_HTy |

**Fig. 1.** Chemical structures of hydroxytyrosol (**1**) and the newly synthesised alkyl hydroxytyrosyl ethers (**2**–**9**).

### 2.5. ABTS assay

The free radical scavenging capacity was measured using the ABTS decoloration method (Re et al., 1999) with some modifications. The method is based on the capacity of different components to scavenge the ABTS radical cation ($ABTS^{\cdot+}$) compared to a standard antioxidant (Trolox). Results are expressed in mM TE (Trolox equivalent). Each value is the average of three determinations.

### 2.6. DPPH assay

The DPPH method is based on the measurement of the free radical scavenging capacity of the antioxidant against the stable radical $DPPH^{\cdot}$ and was performed according to the procedure described by Sanchez-Moreno, Larrauri, and Saura-Calixto (1998). The radical scavenging capacity of the antioxidants was expressed as an $EC_{50}$ effective concentration (mol antioxidant/mol DPPH). Also, the time needed to reach the steady state (in minutes), as well as the time to reach the steady state for the $EC_{50}$ concentration ($T_{EC_{50}}$ in min) and the antiradical efficiency (AE, $[(\text{mol AO/mol DPPH}) \times \text{min}]^{-1}$) were used for the characterisation of the antioxidant activity of the test compounds. All measurements were performed in triplicate.

### 2.7. Statistical analysis

Results are expressed as means ± standard deviation (SD) of three measurements. Data were subjected to a one-way analysis of variance (ANOVA) using Statistix 8.0. Differences were considered significant when $p < 0.05$.

## 3. Results

### 3.1. General

The potential antioxidant activity of the alkyl hydroxytyrosyl ethers (**2**–**9**) has been evaluated by different methods and compared to free HTy (**1**), and two other well-known antioxidants traditionally used in the food industry (*tert*-butylhydroxytoluene (BHT) and α-tocopherol). The Rancimat test is a method commonly used to evaluate the potency of antioxidants in lipophilic food matrices, such as oils and fats, while the FRAP, ABTS and DPPH assays are three characteristic methods used for the evaluation of antioxidant activity in hydrophilic media.

### 3.2. Antioxidant activity in lipid matrix – Rancimat test

The efficacy of the new synthesised compounds as food antioxidants was evaluated in a lipid matrix consisting of antioxidant-free commercial sunflower oil using the accelerated Rancimat method at 90 °C. The induction time (IT) values corresponding to purified matrices of sunflower oil spiked with increasing concentrations of HTy (**1**), hydroxytyrosyl ethers (**2**–**9**), BHT and α-tocopherol are plotted in Fig. 2. All the new hydroxytyrosyl ethers showed similar antioxidant activities per millimole of substance, with IT values slightly lower than those obtained in matrices spiked with free HTy (**1**). Nevertheless, these newly synthesised compounds showed substantially higher antioxidant activity than α-tocopherol or BHT in a lipid matrix.

### 3.3. Ferric reducing antioxidant power (FRAP) assay

The reducing capacities of the new alkyl hydroxytyrosyl ethers (**2**–**9**) were determined by the FRAP assay and are summarised in Table 1. Results are expressed as mM TE (Trolox equivalent).

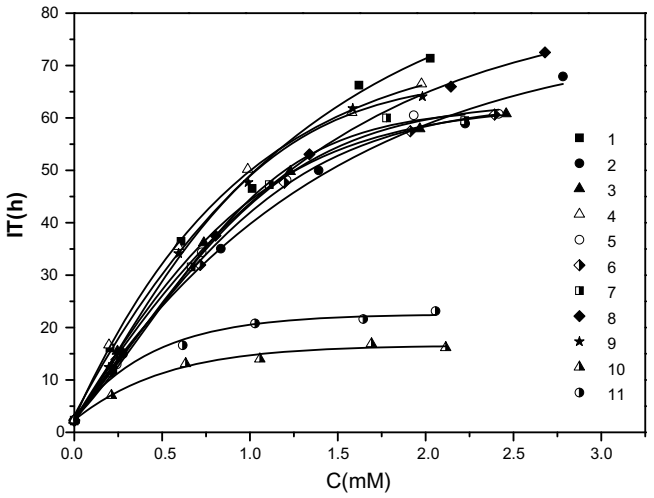



**Fig. 2.** Induction times (ITs) of lipid matrices spiked with hydroxytyrosol (HTy, **1**); alkyl hydroxytyrosyl ethers such as methyl (**2**), ethyl (**3**), propyl (**4**), butyl (**5**), hexyl (**6**), octyl (**7**), dodecyl (**8**), octadecyl (**9**); and food antioxidants as BHT (**10**) and α-tocopherol (**11**).

**Table 1**
Reducing antioxidant power evaluated by the FRAP assay and radical scavenging capacity evaluated by ABTS assay of hydroxytyrosyl ethers (**2**–**9**), HTy (**1**), α-tocopherol and BHT. Each value is the mean of triplicate measurements ± standard deviations. Results are expressed as mM TE (Trolox equivalents). All values within a column with different superscript letters are significantly different, $p < 0.05$.

| Compound | FRAP mM TE | ABTS mM TE |
|---|---|---|
| HTy (**1**) | $1.39 \pm 0.05^{b,c}$ | $0.84 \pm 0.02^{c}$ |
| **2** | $2.14 \pm 0.07^{f}$ | $1.22 \pm 0.02^{f}$ |
| **3** | $2.40 \pm 0.08^{g}$ | $1.03 \pm 0.02^{e}$ |
| **4** | $2.03 \pm 0.07^{e,f}$ | $0.95 \pm 0.02^{d}$ |
| **5** | $1.90 \pm 0.06^{d}$ | $0.95 \pm 0.02^{d}$ |
| **6** | $1.95 \pm 0.06^{d,e}$ | $1.22 \pm 0.02^{f}$ |
| **7** | $1.50 \pm 0.06^{c}$ | $0.81 \pm 0.02^{c}$ |
| **8** | $1.49 \pm 0.05^{c}$ | $0.77 \pm 0.02^{b}$ |
| **9** | $1.45 \pm 0.05^{c}$ | $0.82 \pm 0.02^{c}$ |
| BHT | $1.32 \pm 0.05^{b}$ | $0.27 \pm 0.01^{a}$ |
| α-Tocopherol | $0.80 \pm 0.04^{a}$ | $1.01 \pm 0.02^{e}$ |

The results show that HTy (**1**) and BHT had similar ferric reducing powers that were significantly higher than that of α-tocopherol. More importantly, the alkyl hydroxytyrosyl ethers of lower side chain length, namely methyl (**2**), ethyl (**3**), propyl (**4**), butyl (**5**) and hexyl (**6**) derivatives, had a ferric reducing ability higher than that of HTy (**1**), and also higher than the antioxidant activity of the widely used antioxidants BHT and α-tocopherol. The compounds with longer side chains such as octyl (**7**), dodecyl (**8**) and octadecyl (**9**) hydroxytyrosyl ethers had a reducing activity similar to HTy (**1**), but significantly higher than that of BHT and α-tocopherol.

### 3.4. ABTS assay

A widely used method for measuring the radical scavenging activity of antioxidants is the ABTS assay, where the scavenging of the stable free radical 2,2-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid ($ABTS^{\cdot+}$) is evaluated. The radical scavenging activity of the test antioxidants is shown in Table 1. The relative order of the ABTS scavenging activities was as follows: methyl (**2**) ∼ hexyl (**6**) ⩾ ethyl (**3**) ∼ α-tocopherol > propyl (**4**) ∼ butyl (**5**) > octyl (**7**) ∼ octadecyl (**9**) ∼ HTy (**1**) > dodecyl (**8**) > BHT. In agreement with the results obtained by the FRAP assay, the length

of the alkyl side chain affects the antioxidant activity, with the compounds with the shorter linear chain ($n \leqslant 5$ hexyl hydroxytyrosyl ether (**6**)) showing an scavenging activity higher than the free parent HTy (**1**), while compounds with longer linear chains (**7**–**9**) steadied or even decreased the scavenger activity shown by the reference HTy (**1**).

The average activity of the alkyl hydroxytyrosyl ethers was similar than that of α-tocopherol and significantly higher than the scavenging capacity of BHT.

### 3.5. DPPH assay

This method determines the efficiency of an antioxidant as a radical scavenger against the stable free radical DPPH·. The radical scavenging activities of the evaluated antioxidants are summarised in Table 2. Results are expressed as $EC_{50}$ (mol AO/mol DPPH), representing the concentration needed to decrease by 50% the initial DPPH· concentration. All the tested alkyl hydroxytyrosyl ethers had a higher DPPH· scavenging capacity than HTy (**1**) and α-tocopherol, except for the butyl ether (**5**) that showed an $EC_{50}$ value similar to that of the vitamin E. BHT, with an $EC_{50}$ concentration double that of the other test and control compounds, was the molecule with the lowest DPPH· scavenging activity.

Hence, in general the alkyl hydroxytyrosyl ethers showed a better scavenger activity in comparison with their reference HTy (**1**), although the results thus expressed did not reflect the influence of the side chain length.

It is worth noting that the scavenging of the DPPH· radical by HTy (**1**) and its alkyl ethers derivatives (**2**–**9**) was completed within a few minutes, reaching a steady state in less than 5 min for the higher concentrations assayed in comparison with the 10 min required by α-tocopherol or the 40 min of BHT. Thus, considering the kinetic behaviour of these antioxidants they could be classified as rapid (HTy (**1**) and its alkyl hydroxytyrosyl ethers (**2**–**9**)), intermediate (α-tocopherol) and slow (BHT) scavengers.

When the time needed to reach the steady state for the $EC_{50}$ concentration ($T_{EC_{50}}$) was taken into account for the characterisation of these new compounds, the differences between the assayed compounds were more pronounced, with the alkyl hydroxytyrosyl ethers (**2**–**9**) having lower $T_{EC_{50}}$ values in comparison with free HTy (**1**), followed by α-tocopherol and BHT.

Finally, the antiradical efficiency (AE) index confirmed the stronger activity of HTy (**1**) and its derivatives in comparison with the selected references BHT and α-tocopherol. This index made apparent the influence of the length of the alkyl chain on the antioxidant activity of the hydroxytyrosyl ethers, with higher AE values for the compounds with short side chains (**3**–**6**) (Table 2) in agreement with the results obtained in the FRAP and ABTS assays.

**Table 2**
Radical scavenging capacity of hydroxytyrosyl ethers (**2**–**9**), HTy (**1**) α-tocopherol and BHT evaluated by DPPH assay. Each value is the mean of triplicate measurements ± standard deviations.[1] Results are expressed as $EC_{50}$ (mol AO/mol DPPH), time to reach steady state (min), $T_{EC_{50}}$ (min) and AE [(mol AO/mol DPPH) × min]$^{-1}$. All values within a column with different superscript letters are significantly different, $p < 0.05$.

| Compound | $EC_{50}$ (mol AO/mol DPPH) | Time to reach steady state (min)[2] | $T_{EC_{50}}$ (min) | AE [(mol AO/mol DPPH) × min]$^{-1}$ |
|---|---|---|---|---|
| HTy (**1**) | 0.23 ± 0.01$^{b}$ | 0.8–4.0 | 3.51 ± 0.01$^{c}$ | 1.23 ± 0.01$^{c}$ |
| **2** | 0.20 ± 0.01$^{a}$ | 0.5–3.5 | 2.80 ± 0.01$^{e}$ | 1.75 ± 0.02$^{e}$ |
| **3** | 0.22 ± 0.01$^{a,b}$ | 0.5–3.0 | 1.60 ± 0.01$^{g}$ | 2.78 ± 0.03$^{f}$ |
| **4** | 0.20 ± 0.01$^{a}$ | 0.4–3.0 | 1.52 ± 0.01$^{h}$ | 3.45 ± 0.03$^{h}$ |
| **5** | 0.25 ± 0.01$^{b}$ | 0.5–2.0 | 1.28 ± 0.01$^{i}$ | 3.16 ± 0.03$^{g}$ |
| **6** | 0.22 ± 0.01$^{a,b}$ | 0.5–3.0 | 1.65 ± 0.01$^{g}$ | 2.76 ± 0.03$^{f}$ |
| **7** | 0.20 ± 0.01$^{a}$ | 1.0–4.0 | 2.96 ± 0.01$^{d}$ | 1.68 ± 0.02$^{d}$ |
| **8** | 0.21 ± 0.01$^{a}$ | 1.5–5.0 | 2.70 ± 0.01$^{f}$ | 1.76 ± 0.02$^{e}$ |
| **9** | 0.20 ± 0.01$^{a}$ | 1.5–5.0 | 2.80 ± 0.01$^{e}$ | 1.79 ± 0.02$^{e}$ |
| BHT | 0.57 ± 0.01$^{c}$ | 6.0–40.0 | 58.65 ± 0.01$^{a}$ | 0.030 ± 0.001$^{a}$ |
| α-Tocopherol | 0.24 ± 0.01$^{b}$ | 2.5–10.0 | 4.78 ± 0.01$^{b}$ | 0.86 ± 0.01$^{b}$ |

[1] $EC_{50}$ the concentration needed to decrease by 50% the initial DPPH concentration (mol AO/mol DPPH); time to reach steady state (min); $T_{EC_{50}}$ as the time needed to reach the steady state to the concentration corresponding to $EC_{50}$ (min); efficiency antioxidant (AE) [(mol AO/mol DPPH) × min]$^{-1}$.

[2] Time to reach steady state corresponding to the minimum and maximum concentration of the evaluated antioxidants.

## 4. Discussion

The present work evaluated the antioxidant activity in lipophilic and hydrophilic media of a set of lipophilic antioxidants obtained from HTy (**1**) by chemical synthesis, namely alkyl hydroxytyrosyl ethers of different side chain lengths (**2**–**9**). The antioxidant activity of these synthetic compounds was compared with that of HTy (**1**) and two well-known antioxidants, BHT and α-tocopherol.

It is a well-established fact that the *ortho*-diphenolic group is one of the main contributors to the antioxidant activity of phenolic compounds, providing the antioxidant molecule with redox potential, and radical scavenging and metal-chelating capacities (Cao, Sofic, & Prior, 1997; Jovanovic, Steenken, Hara, & Simic, 1996; Rice-Evans, Miller, & Paganga, 1996). Alkylation of the non-phenolic alcohol residue of HTy (**1**) preserved the *ortho*-diphenolic group, and thus the differences in the antioxidant activity between this parent compound and its ether derivatives can be ascribed to the influence of the alkyl residues. Results in lipophilic (Rancimat test) and hydrophilic media (FRAP, ABTS and DPPH assays) indicated that etherification of HTy (**1**) did not negatively influence the inherent potency of free HTy (**1**). Indeed an increase in antioxidant activity was observed for derivatives with short to medium length alkyl chains.

In general, HTy (**1**) and its derivatives (**2**–**9**) showed high antioxidant activities in all the tested methods with some variations depending on the reaction medium and in agreement with the 'polar paradox' described by Porter, Black, and Drolet (1989), that predicts a higher antioxidant activity of non-polar, hydrophobic phenols in polar, hydrophilic media. Thus, the hydrophobic alkyl hydroxytyrosyl ethers (**2**–**9**) were slightly poorer antioxidants in lipid matrices in comparison with the relatively hydrophilic HTy (**1**), whereas they showed higher activity in hydrophilic media as evaluated by the FRAP, ABTS and DPPH assays. These results are in agreement with previous work carried out in our group, where the oxidative stability of lipid matrices spiked with a different set of lipophilic HTy derivatives, namely hydroxytyrosyl esters, was evaluated by the Rancimat test. Hydroxytyrosyl esters were slightly less effective antioxidants in bulk oils than HTy, although more potent than BHT and α-tocopherol (Trujillo et al., 2006). The potential of these ester derivatives of HTy have also been tested in biological matrices by assessing their capacity to protect proteins and lipids against oxidation caused by peroxyl radicals, using a brain homogenate as an ex vivo model. All tested compounds showed a protective effect in this biological model system, with the inclusion of a lipophilic chain in the hydroxytyrosol molecule enhancing the antioxidant capacity of the parent molecule (Trujillo et al., 2006).

The nature of the alkyl chain did not show any apparent effect on the antioxidant activity in bulk oil, where no significant differences were observed in the induction times of lipid matrices spiked with different alkyl hydroxytyrosyl ethers (**2**–**9**) (Fig. 2). On the contrary, the results obtained in hydrophilic media by the ABTS, FRAP and DPPH assays showed important differences between compounds with short alkyl residues such as methyl (**2**), ethyl (**3**), propyl (**4**), butyl (**5**) and hexyl ethers (**6**) and compounds of

longer alkyl side chains such as octyl (**7**), dodecyl (**8**) and octadecyl (**9**) ethers. Compounds with short linear chains ($n \leqslant 5$ hexyl hydroxytyrosyl ether (**2**–**6**)) had an increased antioxidant activity in comparison with free HTy (**1**), while alkyl hydroxytyrosyl ethers with side chain lengths longer than six carbon atoms showed similar or lower antioxidant activities in comparison with the reference HTy (**1**). These differences could be attributed to the steric effect of the elongation of the alkyl chain group present in the hydroxytyrosyl ethers such as octyl (**7**), dodecyl (**8**) and octadecyl (**9**) decreasing the antioxidant capacity, which is in agreement with the results reported by Lu, Nie, Belton, Tang, and Zhao (2006) with gallic acid and its derivatives.

When comparing the results obtained with HTy (**1**) and the alkyl hydroxytyrosyl ethers (**2**–**9**) with those of the commercial antioxidants, BHT and α-tocopherol, HTy and its ethers proved to be more effective than these commonly used antioxidants, both in lipophilic and hydrophilic media. In bulk oils, the observed differences were greater than in hydrophilic media, where BHT showed to be a poor radical scavenger and α-tocopherol was the worst reducing agent.

The antioxidant activity of HTy (**1**) has already been compared with other commonly used food antioxidants (Furneri et al., 2004; Pazos et al., 2008; Perez-Bonilla et al., 2006; Trujillo et al., 2006). Thus, Perez-Bonilla et al. (2006) reported a higher DPPH· scavenging activity of free HTy (**1**) in comparison to rosmarinic acid and BHT. These results were in agreement with those published by Fki et al. (2005), who showed a higher DPPH· free radical scavenging capacity for HTy (**1**) in comparison to BHT and BHA, as well as with other antioxidant phenolic compounds such as ferulic, caffeic and *p*-coumaric acids. Recently, the antioxidant activity of an effluent obtained from olive oil wastes with high HTy (**1**) concentration was evaluated, showing a higher reducing power of this product in comparison to equimolecular quantities of vitamins E and C (Rodriguez, Rodriguez, Fernández-Bolaños, Guillen, & Jiménez, 2007).

The fact that the antioxidant activity of most of the studied hydroxytyrosyl ethers (**2**–**9**) was even higher than that of HTy (**1**) highlights the potential interest of these new antioxidants as functional ingredients. In this sense, HTy (**1**) has been used with excellent results as a food ingredient to functionalize tomato juice, using HTy (**1**) concentrations as high as 1 mg/ml of juice (Larrosa et al., 2003). A good stability of HTy (**1**) was reported, together with a significant increase of the antioxidant capacity of tomato juice, thus adding to the potential health-beneficial properties of the beverage. Similarly, HTy (**1**) proved to be a good antioxidant preventing deterioration of fish lipids rich in polyunsaturated fatty acids, although differences were observed depending on the fish oil matrix studied (i.e., fish bulk oil, fish oil-in-water emulsion or frozen fish muscle) (Pazos et al., 2008). Such protective effects are of great importance in increasing shelf life of fatty fish and the functional properties of ω-3 polyunsaturated fatty acids, which are highly susceptible to oxidation. These two applications of HTy (**1**) might be extended to the new hydroxytyrosyl ethers (**2**–**9**), considering their excellent antioxidant capacity as well as their different lipophilicity, determined by their variable alkyl chain length. This new set of antioxidant compounds might have potential applications as functional ingredients, of special relevance for application in lipophilic foodstuffs. Further studies to determine the safety of these compounds, as well as their bioavailability and potential biological effects are in progress.

In summary, the newly synthesised hydroxytyrosyl ethers showed an antioxidant activity comparable to or even higher than that of free HTy, depending on the length of the alkyl side chain and the nature of the reaction media. These lipophilic derivatives of HTy are consistent with the food industry requirements for new antioxidants to be used as ingredients to stabilize foodstuffs, as well as to improve the functional properties of foods. Then extraction of HTy from olive oil waste waters adds value to this industrial by-product, abundant in Mediterranean countries, and would contribute to diminish the environmental impact caused by such wastes.

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