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Antioxidizing Potency of Phenol Compounds in Olive Oil Mill Wastewater

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The antioxidizing potency of phenol compounds contained in olive oil mill wastewater (OOMWW) has been elucidated. Commercially available phenol standards at varying concentrations and the Rancimat oxidation test have been used. Refined purified olive oil was utilized as an oxidation lipid substrate. Synthetic antioxidants, such as 2,3-*tert*-butyl-4-hydroxyanisole (BHA), 3,5-di-*tert*-butyl-4-hydroxytoluene (BHT), L-ascorbic acid, and gallates (commonly used as food preservatives), and other known chemicals endowed with antioxidizing properties have been employed as reference compounds. The OOMWW phenol compounds have been classified into different groups depending on their antioxidizing potency. This was significantly affected by the tested concentrations of the standards. Mixtures of phenol standards and other antioxidants (L-proline, chlorophyll *a*, chlorophyll *b*, and α-, γ-, and δ-tocopherol) have also been tested. Many phenol compounds present in OOMWW showed antioxidizing potency higher compared to that of the less safe synthetic antioxidants and could therefore replace these in the industrial preservation of food items. They could also be used in combination with other natural antioxidants (e.g., tocopherols). In fact, some mixtures of antioxidants, owing also to the synergistic phenomena, showed strong antioxidizing potency.

KEYWORDS: Olive oil mill wastewater; natural phenols; antioxidizing potency; antioxidant mixtures; synergistic effects; synthetic antioxidants

INTRODUCTION

Huge amounts (6−7 million tons/year) of OOMWW are produced in the Mediterranean countries cultivating the olive tree (*Olea europaea* L., Oleacee). Europe produces about 75−80% of the total amount (Spain, Italy, and Greece are the major producers) (*1*). This waste is claimed to be one of the most polluting effluents among those produced by the agrofood industries, owing to its contents (14−15%) of organic substances and phenols (up to 10 g/L). The latter are characterized by high specific COD (chemical oxygen demand) (*2*−*4*).

Phenols accumulate into the fruit and leaf prevalently as complex molecules (glycosides and esters). Among these, oleuropein (heterosidic ester of elenolic acid and 3,4-dihydroxyphenylethanol) is by far the most abundant compound (reaching up to 14% of the dry weight in unripe olives) (*5*−*7*). Other occurring complex phenol structures are demethyloleuropein, ligustroside, oleoside, verbascoside (ramnoside), flavonoids (rutin, apigenin, and luteolin-7-glucoside), anthocyanins (including essentially cyanidin-3-rutinoside), and dihydroxyphenylglycol (a minor C_6-C_2 phenolic) (*8*, *9*). Four glucosides containing hydroxytyrosol were also found (*10*).

During olive processing, simple or less complex phenol compounds (phenyl acids, phenyl alcohols, and neutral phenols) form, owing to enzymatic or chemical degrading reactions (almost 80% of oleuropein is degraded upon crushing), which dissolve preponderantly in the water phase (53%) (*1*).

Several secoiridoid aglycons, including aldehydic, dialdehydic, decarboxylated, and oxidized structures (*11*−*13*) as well as the 4-dihydroxyphenylethyl acetate (hydroxytyrosol acetate) (*5*), and two lignans (pinoresinol and 1-acetoxypinoresinol) have recently been identified in virgin olive oil (*14*, *15*). The new phenol nüzhenide was identified in the kernel of olive fruit (*16*). A dark organic polymerin fraction exploitable as a soil bioamendant was identified in OOMWW (*17*, *18*).

Phenols are able to donate a hydrogen atom to the free radicals (which consequently become no longer reactive), thus stopping the propagation chain during the lipid oxidation process (*19*). In this work the antioxidizing potency of individual phenols naturally occurring in OOMWW (*1*−*4*), along with their classification based on this characteristic, is presented. Such a basic aspect has not been investigated in depth (*20*−*25*). Indeed, it is of considerable importance being connectable with the industrial process kinds to set up to recover the natural phenol antioxidants from OOMWW. The technological procedures capable of recovering mainly the phenol compounds endowed with higher antioxidizing potency compared to that owned by the above-mentioned synthetic antioxidants should be taken into consideration for technical and economical testing and possible future industrial recovery applications. Projects have been presented to the European Union concerning this research area. The antioxidizing potency of individual phenol compounds

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mixed with other antioxidants has also been investigated in this work. To carry out the investigations, commercially available analytical standards were mostly employed.

MATERIALS AND METHODS

Chemicals. (i) BHT, BHA, β-carotene, trolox (water-soluble analogue of α-tocopherol), quercetin, epicatechin, pirogallol, 4-methylcathecol, 2,6-di-*tert*-butyl-*p*-cresol (BC), lutein, α-tocopherol, γ-tocopherol, δ-tocopherol, propyl gallate, lauryl gallate, octyl gallate, 4-methylcathecol, 2,6-di-*tert*-butyl-4-methylphenol (BMP), cathecol, 2-(4-hydroxyphenyl)ethanol (tyrosol), rutin hydrate, apigenin, and cyanohydrin chloride were purchased from Sigma-Aldrich Chemical (St. Louis, MO); (ii) homovanillic, syringic, protocatecuic, caffeic, homoprotocatechuic, homogentisic, gallic, synapinic, L-ascorbic, *trans*-4-hydroxymethoxycinnamic (HMCA), *p*-coumaric, *o*-coumaric, quinic, shikimic, 3,4-dimethoxybenzoic, 2-methoxycinnamic, ursolic, and oleanolic acids were likewise purchased from Sigma-Aldrich Chemical (St. Louis, MO); (iii) ellagic acid, luteolin-7-glucoside, apigenin-7-glucoside, chlorogenic acid, and oleuropein were obtained from Extrasynthése (Genay, France). The last compound was also extracted from olive leaves according to the procedure described by Gariboldi et al. (*7*); (iv) catechin hydrate was provided by Fluka (Buchs, Switzerland); (v) 2-(3,4-dihydroxyphenyl)ethanol (hydroxytyrosol) was commercially unavailable and therefore synthesized according to the method described by Capasso et al. (*26*), by reducing 3,4-dihydroxyphenyl acetic acid with $LiAlH_4$ (in tetrahydrofuran); (vi) methanol, ethanol, and acetone of analytical grade and activated charcoal were supplied by Carlo Erba (Milan, Italy); (vii) silicic acid and powdered sugar were purchased from Sigma-Aldrich Chemical (St. Louis, MO); and (viii) Celite was obtained from BDH (Poole, United Kingdom).

Preparation of Standard Solutions. For the majority of the standards, methanol was used as the first solvent. Hexane was used for tocopherols, ethanol for chlorophylls, and acetone for β-carotene. For each standard, 100 mg (for chlorophylls 1 mg) was dissolved in 100 mL of solvent, and from the prepared mother solutions, volumes of 0.25, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 mL were taken with a pipet and transferred into Rancimat tubes. The solvent was subsequently removed under a nitrogen flow at 33 °C. Next, 3 mL of refined purified olive oil was used to redissolve the standard. The obtained oil solutions were stirred for 25 min at room temperature. In this way, growing concentrations (83, 167, 333, 500, 667, 833, and 1000 mg/L oil) were obtained for every standard and subjected to the oxidation tests. Refined olive oil substrate was chosen to evaluate the lipid oxidation inhibition activity of the antioxidant standards. These were also almost all tested in combination with other antioxidants (six concentrations), such as L-proline, tocopherols, and chlorophylls. The preparation procedure of the oil solutions was the same as that followed for individual standards.

Purification of Refined Olive Oil. To purify 300 g of refined bleached and deodorized olive oil (from Scibilia Co., Pescara, Italy), the procedure of Lee and Min (*27*) was followed. A chromatographic column (55 × 5 cm) packed with 20 g of activated silicic acid (100 mesh), 10 g of activated charcoal and Celite (2:1), 40 g of powdered sugar and Celite (2:1), and 20 g of activated silicic acid was used. When purifying another aliquot of oil, a new column was prepared. The purified oil was devoid of peroxides and free fatty acids (as checked by titrimetic methods) (*1*) as well as of phenolics, tocopherols, and pigments. For the last controls, an HRGC (high-resolution gas chromatographic), an HPLC (high-performance liquid chromatographic), and a spectrophotometric method were used, respectively (*12*).

Rancimat Test. To measure the antioxidizing power of the standards and their mixtures, the established repetitive Rancimat method, correlating well with the active oxygen method (AOM), was applied (*28*). A Rancimat apparatus (model 679) operated at 120 °C with an air flow rate of 20 L/h was from Metrohom Co. (Basel, Switzerland). Glassware was scrupulously cleaned and oven dried at 180 °C before use. Conductivity cells and electrodes were soaked overnight in a detergent solution and then rinsed with tap water, acetone, and distilled water. The volatile oxidation products (small molecules) stripped from the lipid medium dissolved in cold distilled water with an increase of the electrical conductivity parameter value. The time (h) taken to reach a determined level of conductivity, corresponding to the flex point of the peroxidation curve (recorded at the paper speed of 1 cm/h), was considered as an oxidation mark (induction time) (IT). The higher the induction time value, the higher the antioxidizing potency of the standards. All tests were run in triplicate and averaged. Each standard or mixture was tested in comparison with a blank (refined purified olive oil alone).

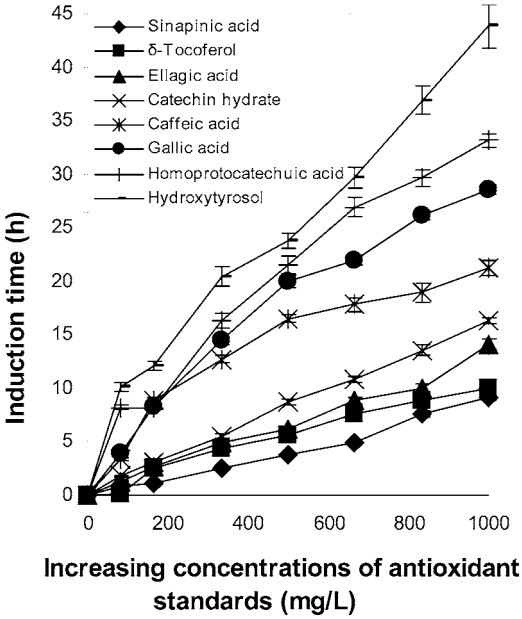

Figure 1. Average induction time values ($n = 3$) of solutions containing an antioxidant standard dissolved in refined purified olive oil and endowed with high antioxidizing power. Concentrations: 0, 83, 167, 333, 500, 667, 833, and 1000 mg/L. Error bars indicate standard deviation.

Statistical Analysis. An experimental design with two independent variables (antioxidant standard × concentration) was adopted. Data concerning the antioxidizing potency of the standards at varying concentrations were processed by analysis of variance (ANOVA). When a significant *F* value was found, means were separated by Scheffé's post hoc pairwise test (*29*) at the 5% level.

RESULTS AND DISCUSSION

Phenol standards including either phenols found in OOMWW or phenols potentially present in this waste were essentially tested (*1*−*4*). In addition to the traditional synthetic antioxidants (BHA, BHT, ascorbic acid, and gallates) used for elongating the shelf life of foods, other common chemicals (commercially available) have been tested as reference antioxidants.

Antioxidizing Potency of Individual Standards. From the results achieved, it clearly emerged that the individual phenol compounds present in OOMWW have different antioxidizing potencies. These compounds appeared to be classifiable into four groups: (i) phenols endowed with high antioxidizing potency (IT > 8), (ii) phenols endowed with medium antioxidizing potency (IT > 3), (iii) phenols endowed with weak antioxidizing potency (IT < 3), and (iv) phenols having no antioxidizing ability (IT around 0).

Figures 1 and **2** together indicate the group of phenols having high antioxidizing potency. Within this group, this characteristic was possessed according to the following order: hydroxytyrosol > homoprotocatechuic acid > homogentisic acid > gallic acid > caffeic acid > catechin hydrate > pirogallol > epicatechin > quercetin > sinapinic acid > protocatechuic acid. This group also included ellagic acid and δ-tocopherol (**Figure 1**) and

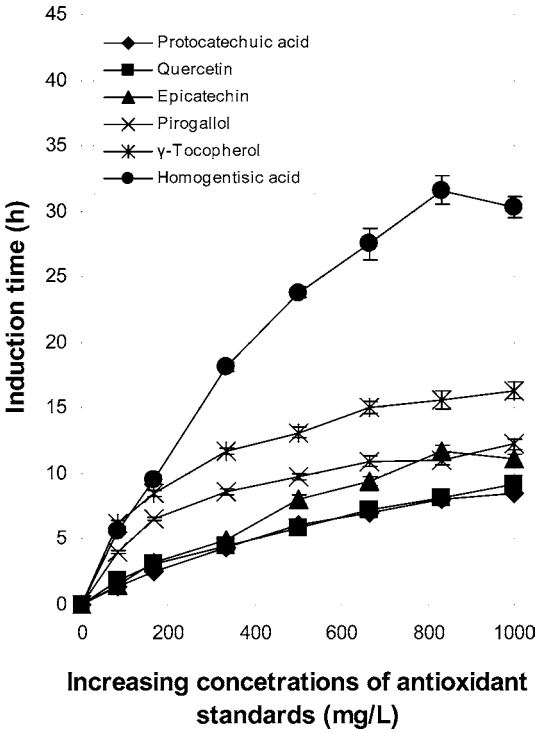

Figure 2. Average induction time values ($n = 3$) of other solutions containing an antioxidant standard dissolved in refined purified olive oil and endowed with high antioxidizing power. Concentrations: 0, 83, 167, 333, 500, 667, 833, and 1000 mg/L. Error bars indicate standard deviation.

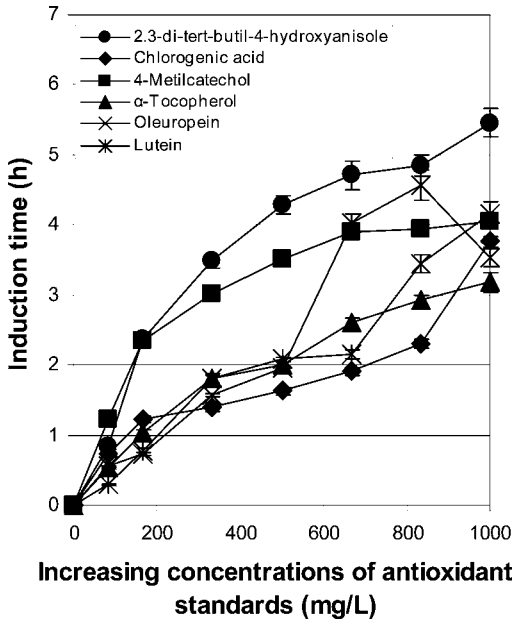

Figure 3. Average induction time values ($n = 3$) of solutions containing an antioxidant standard dissolved in refined purified olive oil and endowed with medium antioxidizing power. Concentrations: 0, 83, 167, 333, 500, 667, 833, and 1000 mg/L. Error bars indicate standard deviation.

γ-tocopherol (**Figure 2**). The last compound showed an antioxidizing potency higher than that of δ-tocopherol.

α-Tocopherol was included in the group of compounds with medium antioxidizing potency (**Figure 3**) along with chlorogenic acid and oleuropein and two other compounds (lutein and 4-methylcatechol) having relatively more marked antioxidizing power. BHA (a largely used synthetic preservative of foods) was finally assigned to this class of antioxidants (among which

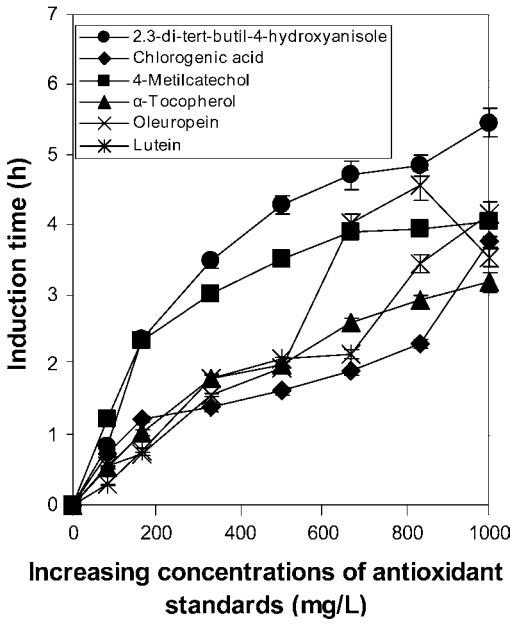

Figure 4. Average induction time values ($n = 3$) of solutions containing an antioxidant standard dissolved in refined purified olive oil and endowed with weak antioxidizing power. Concentrations: 0, 83, 167, 333, 500, 667, 833, and 1000 mg/L. Error bars indicate standard deviation.

was the compound with the higher antioxidizing potency) (**Figure 3**).

The group of phenols endowed with weak antioxidizing power (**Figure 4**) included HMCA, homovanillic acid, and syringic acid as well as catechol (showing a slightly lower antioxidizing power), BHT, BMP, and BC. The last compound was slightly more effective compared to BHT and BMP.

A group of phenol compounds, including *p*-coumaric, *o*-coumaric, quinic, shikimic, dimethoxybenzoic, and 2-methoxycinnamic acids, as well as rutin hydrate, apigenin, apigenin-7-glucoside, luteolin-7-glucoside, 2-(4-hydroxyphenyl)ethanol (tyrosol), and cyanohydrin chloride, showed no antioxidizing power with the corresponding induction time values practically equal to 0 (not shown). Ursolic and oleanolic acids also were assigned to this group.

Some of the above antioxidant standards were also tested by other authors (*20*−*25*), and the results, in general, agree with those achieved by us. Many phenol antioxidants naturally occurring in OOMWW had not been tested yet.

Our results thus fill a gap in this field and may be considered to be very reliable because of the repeatability of the Rancimat test (*28*). In fact, our induction time data, obtained by this procedure, never showed a coefficient of variation (%) exceeding the value of 5%.

The antioxidizing potency of the antioxidant standards, however, was frequently significantly influenced by their concentration. In fact, this characteristic mainly increased either at low, medium, or at high concentrations. With some standards, no increased (or even decreased) antioxidizing potency was observed when increasing their concentration beyond a certain level.

Antioxidizing Potency of Mixtures of Individual Standards. Single antioxidant standards (mostly phenol standards) mixed with either L-proline, chlorophyll *a*, chlorophyll *b*, tocopherols, or chlorophylls + tocopherols were tested for their antioxidizing potency (with the Rancimat method). In fact, in this case more marked antioxidant effects should be exerted even though these can be modified by the positive or negative interactions.

Table 1. Induction Time Values (h) of Solutions Containing an Antioxidant Standard Dissolved in Refined Purified Olive Oil and Mixed with Growing Amounts of L-Proline[a]

	increasing L-proline concentrations (mg/L oil)					
standards	0	83	167	333	667	1000
oleuropein	0.78 ± 0.03[a]	1.75 ± 0.06[b]	1.47 ± 0.04[b]	1.52 ± 0.05[b]	1.98 ± 0.07[c]	1.77 ± 0.05[d]
homovanillic acid	1.34 ± 0.04[a]	1.45 ± 0.05[a]	1.62 ± 0.04[b]	1.89 ± 0.09[c]	2.92 ± 0.09[d]	3.20 ± 0.11[e]
syringic acid	2.72 ± 0.07[a]	2.88 ± 0.11[a]	3.09 ± 0.15[b]	3.50 ± 0.12[c]	4.67 ± 0.21[d]	5.50 ± 0.18[e]
protocatechuic acid	9.14 ± 0.17[a]	9.72 ± 0.17[a]	11.12 ± 0.58[b]	12.82 ± 0.33[c]	13.92 ± 0.47[d]	14.92 ± 0.54[e]
caffeic acid	10.57 ± 0.34[a]	12.67 ± 0.46[b]	13.57 ± 0.47[c]	13.77 ± 0.40[c]	16.57 ± 0.60[d]	24.87 ± 0.82[e]
homoprotocatechuic acid	45.87 ± 2.11[a]	45.77 ± 1.56[a]	47.17 ± 1.70[b]	46.87 ± 1.27[c]	44.97 ± 1.71[d]	48.07 ± 1.54[e]
homogentisic acid	12.42 ± 0.50[a]	19.32 ± 0.50[b]	23.42 ± 0.77[c]	27.72 ± 1.05[d]	27.82 ± 1.00[d]	42.12 ± 1.22[e]
3,5-di-*tert*-butyl-4-hydroxytoluene (BHT)	3.17 ± 0.08[a]	3.20 ± 0.08[a]	2.83 ± 0.10[b]	2.87 ± 0.04[b]	3.40 ± 0.11[c]	5.19 ± 0.18[d]
β-carotene	0.25 ± 0.01[a]	0.27 ± 0.01[a]	0.25 ± 0.01[a]	1.64 ± 0.05[b]	2.62 ± 0.09[c]	3.04 ± 0.08[d]
trolox	0.12 ± 0.00[a]	6.27 ± 0.19[b]	5.69 ± 0.19[c]	4.03 ± 0.11[d]	7.10 ± 0.18[e]	3.72 ± 0.12[f]
quercetin	12.47 ± 0.45[a]	14.07 ± 0.37[b]	16.27 ± 0.50[c]	18.87 ± 0.68[d]	19.57 ± 0.39[e]	24.27 ± 0.66[f]
epicatechin	9.77 ± 0.35[a]	6.00 ± 0.19[b]	5.87 ± 0.18[b]	6.45 ± 0.21[c]	5.22 ± 0.20[d]	5.85 ± 0.18[b]
pirogallol	15.12 ± 0.71[a]	20.32 ± 0.79[b]	20.72 ± 1.06[b]	20.32 ± 0.69[b]	30.82 ± 1.02[c]	36.02 ± 1.44[d]
gallic acid	25.14 ± 0.43[a]	26.47 ± 0.79[b]	31.07 ± 0.84[c]	40.87 ± 1.31[d]	51.17 ± 2.30[e]	60.97 ± 2.07[f]
trans-4-hydroxy-3-methoxycinnamic acid (HMCA)	9.57 ± 0.34[a]	14.37 ± 0.47[b]	15.47 ± 0.56[c]	15.77 ± 0.46[c]	15.87 ± 1.01[c]	16.07 ± 0.63[d]
2,6-di-*tert*-butyl-4-methylphenol (BMP)	3.49 ± 0.18[a]	3.40 ± 0.14[a]	3.42 ± 0.13[a]	3.12 ± 0.12[a]	4.47 ± 0.16[b]	4.37 ± 0.16[b]
ellagic acid	1.79 ± 0.08[a]	1.88 ± 0.07[a]	2.07 ± 0.06[b]	2.69 ± 0.09[c]	4.45 ± 0.12[d]	6.17 ± 0.11[e]
4-methylcatechol	5.00 ± 0.17[a]	6.25 ± 0.16[b]	9.07 ± 0.27[c]	10.77 ± 0.29[c]	13.27 ± 0.46[d]	14.67 ± 0.38[e]
catechol	2.70 ± 0.09[a]	4.42 ± 0.18[b]	6.47 ± 0.10[c]	7.02 ± 0.13[d]	7.52 ± 0.12[e]	9.17 ± 0.17[f]
2,6-di-*tert*-butyl-*p*-cresol (BC)	3.19 ± 0.07[a]	3.20 ± 0.08[a]	2.80 ± 0.08[b]	3.22 ± 0.07[a]	3.40 ± 0.09[a]	5.19 ± 0.13[c]
2,3-di-*tert*-butyl-4-hydroxyanisole (BHA)	3.12 ± 0.08[a]	4.37 ± 0.14[b]	6.17 ± 0.28[c]	6.77 ± 0.26[c]	8.09 ± 0.32[d]	8.77 ± 0.32[e]
lutein	2.27 ± 0.10[a]	2.37 ± 0.09[a]	3.77 ± 0.11[b]	3.97 ± 0.10[b]	6.90 ± 0.14[c]	9.67 ± 0.21[d]
α-tocopherol	0.87 ± 0.01[a]	0.29 ± 0.01[b]	0.42 ± 0.01[b]	0.97 ± 0.03[a]	2.50 ± 0.08[c]	3.69 ± 0.10[d]
γ-tocopherol	2.62 ± 0.12[a]	1.87 ± 0.03[b]	2.87 ± 0.04[a,b]	3.00 ± 0.06[b]	5.60 ± 0.17[c]	5.78 ± 0.20[c]
δ-tocopherol	2.49 ± 0.08[a]	4.75 ± 0.12[b]	10.67 ± 0.46[c]	12.57 ± 0.34[d]	15.27 ± 0.50[e]	16.77 ± 0.42[f]
sinapinic acid	3.32 ± 0.06[a]	3.75 ± 0.09[a]	4.65 ± 0.20[b]	5.00 ± 0.16[b]	6.32 ± 0.18[c]	7.69 ± 0.25[d]
propyl gallate	24.32 ± 1.06[a]	24.62 ± 1.11[a]	25.12 ± 0.90[b]	29.32 ± 0.10[c]	35.72 ± 0.79[d]	41.42 ± 1.04[e]
lauryl gallate	19.25 ± 0.69[a]	21.07 ± 0.65[b]	21.59 ± 0.71[b]	26.17 ± 0.71[c]	30.77 ± 0.89[d]	36.77 ± 1.47[e]
octyl gallate	16.27 ± 0.60[a]	19.27 ± 0.75[b]	19.47 ± 0.56[b]	21.87 ± 0.68[c]	37.17 ± 1.23[d]	41.57 ± 0.91[e]
L-ascorbic acid	1.29 ± 0.05[a]	2.09 ± 0.05[b]	3.55 ± 0.07[c]	6.15 ± 0.14[d]	7.84 ± 0.20[e]	10.62 ± 0.28[f]
chlorogenic acid	0.78 ± 0.02[a]	0.98 ± 0.02[a,b]	1.07 ± 0.02[b]	1.35 ± 0.02[c]	1.95 ± 0.03[d,e]	1.63 ± 0.02[c,e]
catechin hydrate	10.92 ± 0.36[a]	15.22 ± 0.33[b]	17.62 ± 0.65[c]	17.32 ± 0.57[c]	14.72 ± 0.41[b]	15.22 ± 0.55[b]

[a] Data are means of three replicates ±SD (standard deviation). Means within the same row with different superscripts are significantly different (Scheffé's test, $p \leq 0.05$).

Table 1 shows the induction times of refined purified olive oil solutions containing an individual standard in combination with growing concentrations of L-proline (0, 83, 167, 333, 667, and 1000 mg/L). This compound increased the inhibitory potency of almost all the antioxidants (including the synthetic ones), independently of their initial antioxidation power. Protocatechuic acid, caffeic acid, homogentisic acid, quercetin, pirogallol, gallic acid, ellagic acid, 4-methylcatechol, catechol, BHA, lutein, δ-tocopherol, gallates, and L-ascorbic acid were the standards showing the greater antioxidizing potency increases. HMCA was significantly affected only at a low concentration of L-proline (83 mg/L). Epicatechin was the only standard that showed a decrease in antioxidant potency. On the basis of the initial antioxidizing potency of both standards and L-proline, it was evident that the increases in antioxidizing potency of the standards were also connectable with synergistic phenomena (which in some cases appeared to be rather marked).

When using chlorophyll *a* (at the growing concentrations of 0, 0.8, 1.7, 3.3, 6.7, and 10.0 mg/L oil) in place of L-proline, the antioxidizing potency of the standards was by far less affected (data not shown). In this case, the greater increases in antioxidizing potency were recorded for protocatechuic acid, caffeic acid, homoprotocatechuic acid, quercetin, epicatechin, pirogallol, and gallic acid. BC, BHA, BHT, BMP, homogentisic acid, oleuropein, sinapinic acid, lutein, α-tocopherol, δ-tocopherol, and catechin hydrate were practically not or even negatively affected.

Chlorophyll *b* (used at the same concentrations as chlorophyll *a*) seemed to have a slightly higher antioxidizing potency and exerted its positive effects also with the standards homogentisic acid, α-, γ-, and δ-tocopherol, and propyl gallate. With several standards its effects were either not significant or slightly negative. The positivity or negativity of these effects and their magnitude were connectable with its concentration.

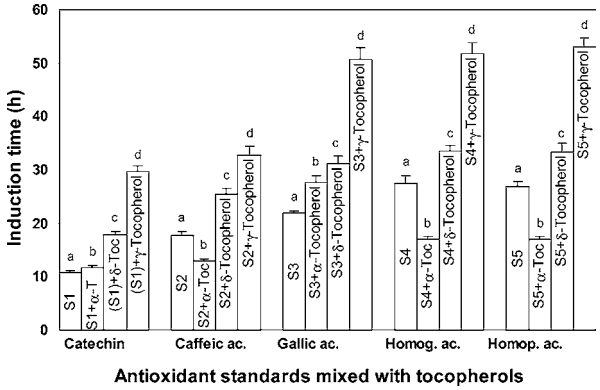

Figure 5. Average induction time values ($n = 3$) of solutions containing an antioxidant standard (S1–S5) dissolved in refined purified olive oil and mixed with an equal amount (1000 mg/L) of either α-, γ-, or δ-tocopherol. Within each antioxidant standard, rectangles with different superscripts are significantly different (Scheffé's test, $p \leq 0.05$). Error bars indicate standard deviation.

Mixtures containing an individual standard and a single tocopherol compound (either α-, γ-, or δ-tocopherol) (1000 mg/L) were also prepared and tested for their antioxidizing potency. **Figure 5** shows that both δ-tocopherol and γ-tocopherol significantly increased the antioxidizing potency of the standards catechin, caffeic acid, gallic acid, homogentisic acid, and

Table 2. Induction Time Values (h) of Solutions Containing an Antioxidant Standard Dissolved in Refined Purified Olive Oil and Mixed with a Growing Amount of α-, γ-, and δ-Tocopherol and of Chlorophylls a and b[a]

standards	increasing concentations (mg/L oil)						
	tocopherols	0	83	167	333	667	1000
	chlorophylls	0	0.8	1.7	3.3	6.7	10.0
oleuropein		4.03 ± 0.14[a]	5.97 ± 0.17[b]	9.52 ± 0.31[c]	13.92 ± 0.39[d]	17.42 ± 0.45[e]	20.72 ± 0.52[f]
homovanillic acid		2.47 ± 0.05[a]	3.94 ± 0.14[b]	6.04 ± 0.17[c]	8.73 ± 0.23[d]	12.42 ± 0.31[e]	15.22 ± 0.41[f]
syringic acid		2.67 ± 0.12[a]	4.60 ± 0.16[b]	8.07 ± 0.22[c]	11.72 ± 0.29[d]	15.22 ± 0.40[e]	17.42 ± 0.54[f]
protocatechuic acid		7.60 ± 0.21[a]	9.52 ± 0.32[b]	12.32 ± 0.32[c]	15.72 ± 0.28[d]	19.72 ± 0.49[e]	23.02 ± 0.39[f]
caffeic acid		17.92 ± 0.47[a]	21.82 ± 0.55[b]	25.02 ± 0.83[c]	26.72 ± 1.10[d]	30.72 ± 1.08[e]	34.72 ± 0.90[f]
homoprotocatechuic acid		21.32 ± 0.41[a]	24.92 ± 0.52[b]	29.02 ± 0.75[c]	31.72 ± 1.05[d]	34.62 ± 1.32[e]	36.02 ± 1.33[f]
homogentisic acid		9.32 ± 0.24[a]	15.72 ± 0.24[b]	24.32 ± 0.61[c]	27.52 ± 0.77[d]	29.62 ± 1.04[e]	33.92 ± 0.98[f]
3,5-di-*tert*-butyl-4-hydroxytoluene (BHT)		1.95 ± 0.05[a]	3.94 ± 0.13[b]	6.73 ± 0.18[c]	8.92 ± 0.32[d]	13.72 ± 0.60[e]	16.92 ± 0.68[f]
quercetin		7.38 ± 0.27[a]	10.62 ± 0.42[b]	14.72 ± 0.52[c]	16.92 ± 0.71[d]	20.72 ± 0.91[e]	23.52 ± 0.89[f]
epicatechin		9.42 ± 0.33[a]	12.92 ± 0.52[b]	15.42 ± 0.57[c]	17.72 ± 0.53[d]	20.92 ± 0.94[e]	24.22 ± 1.14[f]
pirogallol		11.32 ± 0.41[a]	18.02 ± 0.63[b]	19.42 ± 0.66[c]	22.52 ± 0.88[d]	26.62 ± 1.09[e]	30.92 ± 1.14[f]
gallic acid		13.82 ± 0.61[a]	23.12 ± 0.83[b]	32.72 ± 0.95[c]	41.12 ± 1.03[d]	49.02 ± 1.86[e]	49.52 ± 1.68[f]
trans-4-hydroxy-3-methoxycinnamic acid (HMCA)		2.03 ± 0.06[a]	5.05 ± 0.17[b]	7.93 ± 0.25[c]	12.42 ± 0.32[d]	14.72 ± 0.59[e]	16.92 ± 0.61[f]
2,6-di-*tert*-butyl-4-methylphenol (BMP)		1.95 ± 0.05[a]	3.94 ± 0.10[b]	6.73 ± 0.24[c]	8.92 ± 0.34[d]	12.62 ± 0.45[e]	15.72 ± 0.53[f]
ellagic acid		0.79 ± 0.02[a]	3.05 ± 0.10[b]	6.34 ± 0.23[c]	9.52 ± 0.32[d]	13.72 ± 0.41[e]	17.92 ± 0.68[f]
4-methylcatechol		1.61 ± 0.06[a]	3.67 ± 0.12[b]	6.73 ± 0.20[c]	9.22 ± 0.38[d]	13.42 ± 0.39[e]	16.92 ± 0.58[f]
catechol		1.58 ± 0.03[a]	3.03 ± 0.06[b]	5.69 ± 0.13[c]	8.77 ± 0.23[d]	11.62 ± 0.34[e]	15.22 ± 0.50[f]
2,6-di-*tert*-butyl-*p*-cresol (BC)		2.77 ± 0.12[a]	3.93 ± 0.16[b]	7.68 ± 0.25[c]	11.32 ± 0.32[d]	15.22 ± 0.46[e]	18.62 ± 0.63[f]
2,3-di-*tert*-butyl-4-hydroxyanisole (BHA)		3.53 ± 0.14[a]	5.78 ± 0.16[b]	10.03 ± 0.30[c]	12.62 ± 0.33[d]	15.02 ± 0.38[e]	18.92 ± 0.49[f]
lutein		1.69 ± 0.06[a]	4.23 ± 0.13[b]	7.47 ± 0.21[c]	11.02 ± 0.39[d]	15.52 ± 0.56[e]	18.12 ± 0.72[f]
sinapinic acid		5.93 ± 0.21[a]	10.62 ± 0.28[b]	14.72 ± 0.29[c]	17.52 ± 0.67[d]	18.92 ± 0.59[e]	21.22 ± 0.89[f]
propyl gallate		20.72 ± 0.35[a]	22.12 ± 0.46[b]	22.92 ± 0.62[b]	26.72 ± 0.91[c]	30.22 ± 0.79[d]	34.42 ± 1.07[e]
lauryl gallate		9.02 ± 0.25[a]	13.12 ± 0.18[b]	17.72 ± 0.60[c]	22.22 ± 0.67[d]	29.82 ± 0.95[e]	31.32 ± 0.81[f]
octyl gallate		8.47 ± 0.25[a]	14.52 ± 0.26[b]	19.72 ± 0.67[c]	25.12 ± 0.38[d]	25.72 ± 0.87[d]	31.72 ± 0.89[e]
L-ascorbic acid		0.25 ± 0.01[a]	4.44 ± 0.12[b]	8.71 ± 0.25[c]	12.02 ± 0.43[d]	17.12 ± 0.51[e]	20.92 ± 0.52[f]
chlorogenic acid		3.03 ± 0.05[a]	4.24 ± 0.11[b]	6.21 ± 0.22[c]	9.02 ± 0.15[d]	11.42 ± 0.50[e]	14.72 ± 0.56[f]
lycopene		0.81 ± 0.02[a]	1.94 ± 0.06[b]	5.25 ± 0.08[c]	9.12 ± 0.32[d]	14.22 ± 0.51[e]	16.92 ± 0.64[f]
catechin hydrate		8.04 ± 0.33[a]	9.72 ± 0.34[b]	11.42 ± 0.30[c]	12.92 ± 0.23[d]	24.42 ± 0.76[e]	26.82 ± 0.51[f]

[a] Data are means of three replicates ±SD (standard deviation). Means within the same row with different superscripts are significantly different (Scheffé's test, $p \leq 0.05$).

homoprotocatechuic acid, while α-tocopherol exerted a positive effect only with catechin and gallic acid but a negative one with caffeic, homogentisic, and homoprotocatechuic acids.

With the standards oleuropein, chlorogenic acid, 4-methylcatechol, lutein, and BHA, each tocopherol compound exerted a regular positive effect, even thought that of α-tocopherol, with the standards oleuropein and 4-methylcatechol, was not statistically significant ($p \leq 0.05$). With the standards BMP, BHT, BC, HMCA, homovanillic acid, catechol, and syringic acid, the three tocopherol compounds exerted a steady significant positive effect (data not shown).

In summary, γ- and δ-tocopherol exerted a constant significant positive effect on the antioxidizing potency of the standards examined. γ-Tocopherol was in general more effective than δ-tocopherol. The latter gave better results only in combination with HMCA, BMF, and catechol. With BC, homovanillic acid, and BHA, no statistically significant differences between their effects were recorded ($p \leq 0.05$). These findings confirm once again that the antioxidative effectiveness is showed by the three tocopherol compounds according to this sequence: γ-tocopherol > δ-tocopherol > α-tocopherol. Our results seem to be, in general, in good agreement with some data found in the literature (*20, 30*).

In addition, complex mixtures of an individual standard with growing amounts of each tocopherol (0, 83, 167, 333, 667, and 1000 mg/L oil) and each chlorophyll (0, 0.8, 1.7, 3.3, 6.7, and 10.0 mg/L oil) were tested for their antioxidizing potency. It was observed that in this case the inhibitory effectiveness of all the antioxidant standards increased sensibly at almost all tocopherol and chlorophyll concentrations (**Table 2**), suggesting that more complex and marked synergistic phenomena take place when increasing the complexity of the mixture.

The results achieved indicate clearly that a large number of phenol antioxidants present in OOMWW (especially those characterized by an *o*-diphenolic structure) are endowed with an antioxidizing potency by far higher than that owned by the synthetic antioxidants (commonly used as preservatives of agrofood items). Another advantage even more important ascribable to them relates to their natural origin, so that their use implies no risks for human health. Thus, industrial technological systems may be set up to exploit OOMWW, which should indeed be considered not a discard but a precious source of natural antioxidants (high added value products) to be employed for replacing the less safe synthetic food preservatives. Our study also led us to identify the most effective natural phenol antioxidants present in OOMWW, stressing the necessity to study adequate selective technologies to recover prevalently them. Such compounds might even be used in combination with other natural antioxidants (e.g., tocopherols) for the preparation of mixtures endowed with strong antioxizing activity.

ABBREVIATIONS USED

OOMWW, olive oil mill wastewater; BHA, 2,3-di-*tert*-butyl-4-hydroxyanisole; BHT, 3,5-di-*tert*-butyl-4-hydroxytoluene; COD, chemical oxygen demand; BMP, 2,6-di-*tert*-butyl-4-methylphenol; BC, 2,6-di-*tert*-butyl-*p*-cresol; HMCA, *trans*-4-hydroxy-3-methoxycinnamic acid; ANOVA, analysis of variance; IT, induction time; AOM, active oxygen method.

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