Antioxidant Effect of Phenolic Compounds, α-Tocopherol, and Other Minor Components in Virgin Olive Oil

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The effect of acidity, squalene, hydroxytyrosol, aldehydic form of oleuropein aglycon, hydroxytyrosyl acetate, tyrosol, homovanillic acid, luteolin, apigenin, α-tocopherol, and the mixtures hydroxytyrosol/ hydroxytyrosyl acetate, hydroxytyrosol/tyrosol, and hydroxytyrosol/α-tocopherol on the oxidative stability of an olive oil matrix was evaluated. A purified olive oil was spiked with several concentrations of these compounds and, then, subjected to an accelerated oxidation in a Rancimat apparatus at 100 °C. Acidity, squalene, homovanillic acid, and apigenin showed negligible effect. At the same millimolar concentrations, the different *o*-diphenolic compounds yielded similar and significant increases of the induction time, α-tocopherol a lesser increase, and tyrosol a scarce one. At low concentrations of *o*-diphenols and α-tocopherol, a linear relationship between induction time and concentration was found, but at high concentrations the induction time tended toward constant values. To explain this behavior, a kinetic model was applied. The effect of the mixtures hydroxytyrosol/hydroxytyrosyl acetate was similar to that of a single *o*-diphenol at millimolar concentration equal to the sum of millimolar concentrations of both compounds. Concentrations of tyrosol >0.3 mmol/kg increase the induction time by 3 h. The mixtures hydroxytyrosol/α-tocopherol showed opposite effects depending on the relative concentrations of both antioxidants; so, at hydroxytyrosol concentrations <0.2 mmol/kg, the addition of α-tocopherol increased the induction time, whereas at higher hydroxytyrosol concentrations, the α-tocopherol diminished the stability.

KEYWORDS: Acidity; squalene; hydroxytyrosol; hydroxytyrosol derivatives; tyrosol; luteolin; apigenin; homovanillic acid; α-tocopherol; antioxidant activity; Rancimat stability; olive oil

INTRODUCTION

Oxidation of vegetable oils during storage modifies their organoleptic properties, affecting the shelf life of this product. The oxidative process depends on illumination, fatty acid composition, availability of oxygen, temperature, and nature and concentration of the antioxidant and prooxidant minor components. However, oil stored in bulk is kept away from light and air, and bottled oil is exposed to light only at the retail outlet. Therefore, the main factors affecting oil shelf life are the minor components, the fatty acid composition of the lipid matrix, and the storage temperature.

In most seed oils, tocopherols are the main antioxidants, whereas in virgin olive oils, a fair correlation has been found between total phenols and oxidative stability, measured both at low temperature (open cup) (*1, 2*) and at high temperature (AOM or Rancimat methods) (*3, 4*). Nevertheless, a low correlation with α-tocopherol concentration has been reported (*5, 6*). A statistical study showed that the contribution of phenolic compounds to Rancimat stability was ~51%, that of the fatty acid composition 24%, and that of α-tocopherol 11% (*7*). Moreover, edible virgin olive oils contain significant amounts of squalene (1000−8000 mg/kg) and free fatty acids (0.2−2.0%). It seems that squalene is involved in the oxidation process because alcoholic derivatives are found in aged virgin olive oils (*8*), and it increases the stability of rapeseed oil heated at 170 °C (*9*); however, it does not affect the Rancimat stability of olive oil matrices (*10*). The addition of stearic acid (1%) to refined soybean oil accelerates the autoxidation process (*11*).

The phenolic fraction of olive oils contains several kinds of chemical compounds. The main components are secoiridoid derivatives of 2-(4′-hydroxyphenyl)ethanol (tyrosol) and of 2-(3′,4′-dihydroxyphenyl)ethanol (hydroxytyrosol) (HTy) and 2-(3′,4′-dihydroxyphenyl)ethyl acetate (hydroxytyrosyl acetate) (HTyAc). Minor amounts of hydroxytyrosol, tyrosol, tyrosyl acetate, vanillic, *p*-coumaric and ferulic acids, vanillin, the flavonoids apigenin and luteolin, and the lignans pinoresinol

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and 1-acetoxypinoresinol are found (*12−15*). Comparative studies on the antioxidant activity of some of these compounds in glyceridic matrices have been reported. At low temperatures, no or very low antioxidant activity was observed for tyrosol and *p*-coumaric and ferulic acids, whereas high antioxidant activity was found for hydroxytyrosol (*16−18*). No effect on the Rancimat stability at 120 °C was observed for tyrosol, whereas the same linear relationship was found between induction time and millimolar concentration of hydroxytyrosol and its secoiridoid derivatives (*5*). At low concentrations of these compounds, a positive synergistic effect with α-tocopherol has been reported, but it has not been studied in detail. Luteolin is a strong radical scavenger (*19*), but its effect on oil stability is unknown.

This work evaluates the antioxidant or prooxidant activities of squalene, oleic acid, α-tocopherol, tyrosol, hydroxytyrosol, hydroxytyrosyl acetate, aldehydic form of oleuropein aglycon (AOA), luteolin, and apigenin at concentration ranges similar to those found in edible virgin olive oils. Homovanillic acid—a 4-hydroxy-3-methoxyphenolic compound—is taken as a model to test the activity of lignans. The possible synergistic effects of hydroxytyrosol/hydroxytyrosyl acetate, tyrosol/hydroxytyrosyl acetate, homovanillic acid/hydroxytyrosyl acetate, and α-tocopherol/hydroxytyrosol mixtures, at various ratios, are investigated. The antioxidant activity is evaluated by determination of the Rancimat stability of lipid matrices obtained from olive oils spiked with the antioxidants and their mixtures. A kinetic approach to the role of these antioxidants in the oxidation process at high temperature is proposed. A relationship between minor components content and stability of the virgin olive oil is found.

MATERIALS AND METHODS

Analytical Materials and Reagents. All solvents and reagents were of analytical grade unless otherwise stated. Neutral alumina, type 507C, grade I, from Fluka AG (Buchs, Switzerland) and silica gel 60 for column chromatography were purchased from Merck, KgaA (Darmstadt, Germany). 2-(4′-Hydroxyphenyl)ethanol was obtained from Lancaster Synthesis (Morecambe, U.K.). Squalene, oleic acid, and luteolin were from Sigma Chemical Co. (St. Louis, MO). Apigenin, homovanillic acid, and α-tocopherol were from Fluka AG.

2-(3′,4′-Dihydroxyphenyl)ethanol was synthesized from 3,4-dihydroxyphenylacetic acid (Sigma Chemical Co.) by reduction with LiAlH$_4$ (*20*). 2-(3′,4′-Dihydroxyphenyl)ethyl acetate was obtained from hydroxytyrosol by transesterification with ethyl acetate (*21*).

AOA—a secoiridoid derivative of hydroxytyrosol—was obtained by enzymatic hydrolysis of oleuropein with β-glucosidase from almonds (Sigma Chemical Co.) (*22*). This compound was purified by fractionation on a silica gel column using dichloromethane/acetone/hexane (3:2:5) as mobile phase. NMR data were in accordance with those reported by Montedoro et al. (*12*).

Lipid Matrices. The lipid matrix was obtained from virgin olive oil of Picual variety (VOO) by purification through alumina and silica gel. A glass chromatographic column (1.2 × 22 cm) was packed sequentially with two slurries of adsorbents in hexane/silica gel (3 g) at the bottom and neutral alumina (15 g) at the upper layer. A solution of 7.5 g of the oil in 50 mL of *n*-hexane was poured into the column, and when the oil solution was drawn in, a portion of 10 mL of hexane was passed. The combined fractions were evaporated in a rotary evaporator at room temperature under vacuum, and the oily residue was bubbled with a nitrogen stream. The purified matrix (PVOO) was stored at −18 °C under nitrogen atmosphere. High-oleic sunflower oil (HOSO) and the purified oil (PHOSO) were also used as glyceridic matrices. Olive oil with low phenolic content (LPOO) was obtained from VOO by extraction with 2 volumes of methanol/water (80:20). The extracted oil was dried on anhydrous sodium sulfate and filtered through a filter paper, and the residual methanol was eliminated in a rotary evaporator at room temperature under vacuum.

Oily matrices containing minor components were prepared by the addition of 0.2−1.0% solutions of phenols and α-tocopherol in methanol and of squalene and oleic acid in hexane. The concentration ranges of minor components in the spiked matrices—2000−8000 mg/kg of squalene, 0.2−1.0% of oleic acid, 0.05−1.0 mmol/kg of HTy, HtyAc, Ty, AOA, and α-tocopherol, and 0.007−0.0350 mmol/kg of luteolin and apigenin—cover the concentration ranges usually found in virgin olive oils.

To check the composition of the matrices, the following determinations were carried out: total phenolic compounds by solid phase extraction and HPLC analysis with a UV detector (*15*); acidity and peroxide value (PV) according to Annex II and III in Economic European Regulation EEC/2568/91 (*23*), respectively; tocopherols by HPLC analysis on silica gel column using a fluorescence detector (*24*); chlorophylls and carotenoids were isolated by solid phase extraction and then analyzed by RP-HPLC with a UV−vis detector at 670 and 472 nm, respectively (*25*); fatty acid composition was determined by capillary GC analysis of the methyl esters obtained by transmethylation of the oil with KOH in methanol (*26*); the wax fraction was isolated by column chromatography on silica gel eluting with hexane/diethyl ether (98.5:1.5) and then analyzed by GC according to EEC/183/93 (*27*); squalene was isolated from olive oil by solid phase extraction on silica gel cartridges, and the fraction was analyzed by GC (*28*).

Evaluation of Oxidative Stability. The oxidative stability was evaluated according to an accelerated automated test using the Rancimat apparatus, model CH 9100 (Metrohm Co., Basel, Switzerland). A flow of air (20 L/h) was bubbled successively through the oil heated at 100 °C and cold water. In this process, the volatile oxidation products were stripped from the oil and dissolved in the water, increasing the water conductivity. The time taken until there is a sharp increase of conductivity is termed the induction time (IT), and it is expressed in hours. IT is determined by the intersection of the baseline with the tangent to the conductivity curve. Solutions of antioxidants and 0.5 mL of acetone were added to each Rancimat vessel containing 2.5 g of the oily matrix, and then the mixtures were homogenized. The vessels were covered with the heads, placed into the Rancimat apparatus at room temperature, and then heated under an air flow rate of 4 L/h. When the temperature reached 100 °C (~35 min), the vessel head outlets were connected to the conductivity cells, the air flow rate was increased to 15 L/h, and the time measurement was started. Using this procedure, the solvents were evaporated from the oil before the measurement of stability, because no differences in behavior were found between oils and oils with solvents added.

Statistical Analysis. Variance analysis between sets of IT results was carried out by SPSS software, version 11.5 (SPSS Inc., Chicago, IL). Differences were considered to be statistically significant if the confidence level was >95%.

RESULTS AND DISCUSSION

The lipid matrices obtained by purification of virgin olive and high-oleic sunflower oils did not contain peroxides, free fatty acids, phenols, chlorophylls, tocopherols, and other minor polar compounds. The fatty acid compositions of the two matrices are shown in **Table 1**. The purified olive oil batches (PVOO) contained squalene (6520 mg/kg), aliphatic waxes (91 mg/kg), steroidal waxes (853 mg/kg), and β-carotene (0.1 mg/kg).

Effect of Squalene. High-oleic sunflower oil (HOSO), with a fatty acid composition (**Table 1**) similar to that of VOO and a low content of squalene (123 mg/kg), was used to evaluate the action of squalene on the stability of the lipid matrices, because those obtained from VOO contained a significant amount of squalene, which is difficult to eliminate. To investigate the possible synergistic effect of squalene with α-tocopherol and hydroxytyrosol, HOSO (containing 653 mg of α-tocopherol/kg α-tocopherol), PHOSO, and PHOSO spiked with 0.20 mmol of hydroxytyrosol/kg were fortified with squalene. The IT values from Rancimat measurement are shown

Table 1. Fatty Acid Composition of Purified Olive Oil (PVOO) and High-Oleic Sunflower Oil (PHOSO) [Mean Values ± SD ($n = 2$)]

fatty acid	composition (%)	
	PVOO	PHOSO
16:0	9.65 ± 0.22	4.46 ± 0.06
16:1 (n–7 + n–9)	0.84 ± 0.11	0.08 ± 0.01
17:0	0.05 ± 0.01	0.04 ± 0.01
17:1	0.08 ± 0.01	0.05 ± 0.01
18:0	3.70 ± 0.03	4.64 ± 0.08
18:1 (–9 + n–11)	80.11 ± 1.62	70.94 ± 1.53
18:2 (n– 9, 12)	4.23 ± 0.38	17.90 ± 0.43
18:3 (n– 9, 12, 15)	0.48 ± 0.01	0.07 ± 0.01
20:0	0.43 ± 0.01	0.38 ± 0.01
20:1	0.26 ± 0.01	0.24 ± 0.01
22:0	0.12 ± 0.01	1.16 ± 0.02
24:0	0.05 ± 0.01	0.04 ± 0.01

Table 2. Effect of Squalene on the Rancimat Stability of Purified High-Oleic Sunflower Oil (PHOSO), PHOSO Spiked with 0.2 mmol/kg of Hydroxytyrosol (HTy), and High-Oleic Sunflower Oil (HOSO) [Mean Values ± SD ($n = 4$)]

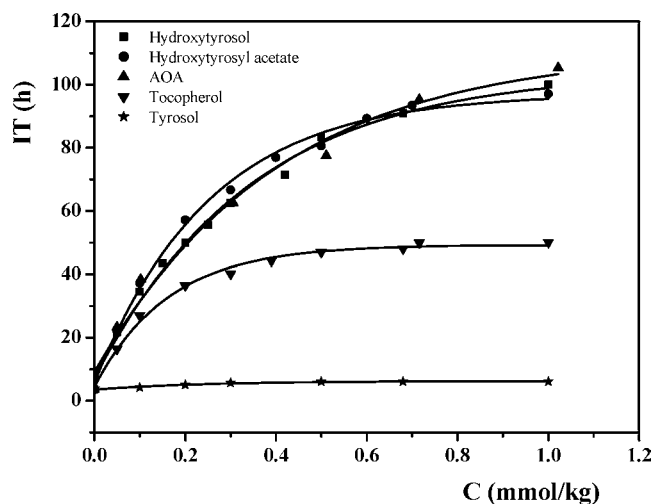| squalene added (mg/kg)	induction time (h)		
	PHOSO	PHOSO + 0.2 mmol/kg of HTy	HOSO
0	2.06 ± 0.09	13.90 ± 0.66	22.0 ± 1.1
2000	2.09 ± 0.08	14.01 ± 0.63	20.5 ± 1.0
4000	2.19 ± 1.00	13.38 ± 0.69	22.3 ± 0.9
6000	2.04 ± 0.07	13.51 ± 0.58	21.5 ± 1.3
8000	2.10 ± 0.08	14.20 ± 0.61	21.9 ± 1.0

Table 3. Effect of Free Oleic Acid on the Rancimat Stability of the Purified Virgin Olive Oil (PVOO) and Aliquots of the Same Matrix Spiked with Various Amounts of Hydroxytyrosol (HTy) [Mean Values ± SD ($n = 4$)]

oleic acid (%)	induction time (h)		
	PVOO	PVOO + 0.03 mmol/kg of HTy	PVOO + 0.30 mmol/kg of HTy
0.0	2.11 ± 0.10	17.0 ± 1.1	61.5 ± 3.6
0.2	2.10 ± 0.10	17.2 ± 1.0	62.0 ± 3.1
0.4	1.79 ± 0.08	17.0 ± 0.7	61.3 ± 3.4
0.6	1.61 ± 0.09	16.7 ± 1.0	59.3 ± 3.4
0.8	1.38 ± 0.11	16.4 ± 0.8	59.9 ± 3.5
1.0	1.29 ± 0.09	16.6 ± 1.0	59.7 ± 3.7

Figure 1. Effect of α-tocopherol, hydroxytyrosol, tyrosol, hydroxytyrosyl acetate, and aldehydic form of oleuropein aglycon (AOA) on the Rancimat stability of the purified olive oil matrix (PVOO). For α-tocopherol, hydroxytyrosol, and tyrosol, RSD < 6.0% ($n = 4$). For hydroxytyrosyl acetate and AOA, RSD < 10% ($n = 2$).
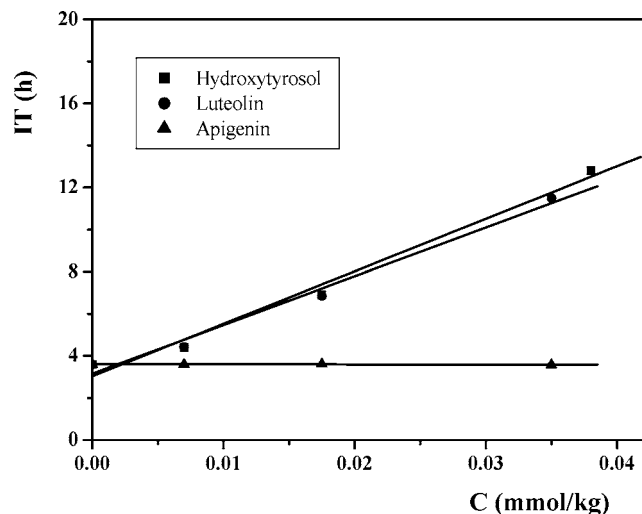
Figure 2. Effect of hydroxytyrosol, luteolin, and apigenin on the Rancimat stability of the purified olive oil matrix (PVOO). RSD < 8% of the mean values ($n = 2$).

in **Table 2**. No statistical differences were found between results within each set of samples, indicating a negligible effect of squalene on the stability of matrices with fatty acid composition similar to that of olive oils. These results are in agreement with those earlier reported for olive oil matrices (*10*), and not in contradiction with the positive effect found in rapeseed oil (*9*), because this oil has a very high unsaturation rate.

Effect of Free Oleic Acid. To test the effect of free fatty acids, matrices of PVOO and PVOO containing 0.03 and 0.30 mmol of hydroxytyrosol/kg were spiked with various amounts of oleic acid. The statistical analysis of the results obtained for the set of PVOO samples (**Table 3**) indicated a slight decrease in IT (0.8 h) for the sample containing 1% of free fatty acid. For the other two sets of samples, the differences were not statistically significant.

Effect of α-Tocopherol, Phenols, and Flavones. To evaluate the antioxidant activity of these compounds, the IT values of the PVOO matrices containing α-tocopherol, hydroxytyrosol, tyrosol, hydroxytyrosyl acetate, AOA, luteolin, and apigenin were determined. The activity of lignans─compounds with two

4-hydroxy-3-methoxyphenyl moieties─was tested using homovanillic acid (4-hydroxy-3-methoxyphenylacetic acid) because it has the same phenolic structure.

Hydroxytyrosol, hydroxytyrosyl acetate, and AOA showed similar antioxidant activities per millimole of substance, whereas the activity of α-tocopherol was significantly lower than that of similar concentrations of these phenols (**Figure 1**). The contribution of tyrosol to oil stability was slight: a small increase of 3 h was observed at concentrations from 0.35 to 1.0 mmol/kg. The addition of homovanillic acid (from 0.05 to 0.25 mmol/kg) did not increase the matrix stability. The flavone luteolin showed an antioxidant activity similar to that of hydroxytyrosol, whereas apigenin did not show any effect (**Figure 2**). These data are in agreement with the fact that antioxidant activity is correlated with the number of phenolic hydroxyls in the molecule (*19*, *29*) and mainly the ortho-disubstitution (*5*, *30*).

An interesting fact is the shape of the curves representing IT versus antioxidant concentration (**Figures 1** and **2**). At low concentrations, a linear relationship can be assumed, whereas over a wide concentration range, the experimental results fulfill

Table 4. Induction Times (IT) of the Purified Olive Oil Matrix (PVOO) Spiked with Different Amounts of Hydroxytyrosol (HTy) and Mixtures of Hydroxytyrosol with Hydroxytyrosyl Acetate (HTyAc) and Tyrosol (Ty) [Mean Values ± SD (*n* = 4)]

[HTy] (mmol/kg)	IT (h)	[HTy + HTyAc] (mmol/kg)	IT (h)	[HTy + Ty] (mmol/kg)	IT (h)
0.1	34.5 ± 1.5			0.1 + 0.1	36.3 ± 1.3
0.2	50.0 ± 2.5	0.1 + 0.1	52.1 ± 2.6	0.2 + 0.2	53.2 ± 2.7
0.3	62.5 ± 3.4			0.3 + 0.3	66.0 ± 3.2
0.4	74.1 ± 3.9	0.3 + 0.1	72.4 ± 4.0	0.4 + 0.4	76.7 ± 4.2
0.6	90.6 ± 5.6	0.5 + 0.1	88.7 ± 5.5	0.5 + 0.5	92.1 ± 5.3
0.8	100.0 ± 6.5	0.7 + 0.1	100.1 ± 6.4		
1.1	104.8 ± 7.0	1.0 + 0.1	108.8 ± 7.1		
0.5	83.3 ± 4.9	0.25 + 0.25	82.1 ± 4.8		
1.0	100.0 ± 6.7	0.50 + 0.50	103.6 ± 6.9		

a sigmoid function—that is, the induction time remains practically constant in the high concentration range. These results are in disagreement with a linear relationship reported (*30*) for the Rancimat stability at 120 °C, probably due to a difference in the oxidation mechanism at 120 °C with respect to that at 100 °C.

Effect of Mixtures of Antioxidants. To evaluate the effect of mixtures of phenols having antioxidant effect, the IT values of PVOO matrices spiked with the mixtures HTy/HtyAc and HTy/Ty were determined. In **Table 4**, it can be seen that the effects of variable ratios of hydroxytyrosol and hydroxytyrosyl acetate were similar to those of a single one at the same millimolar concentration. The mixtures of hydroxytyrosol and tyrosol at the same millimolar amounts also showed additive effects, although the results were not statistically significant at concentrations of hydroxytyrosol >0.30 mmol/kg. These results indicate that the antioxidant effect of the total phenolic fraction of an olive oil is similar to the effect of hydroxytyrosol at a millimolar concentration equal to the sum in millimoles of the *o*-diphenolic compounds present in the fraction, lightly increased by the action of tyrosol.

The effect of mixtures of hydroxytyrosol and α-tocopherol was tested using matrices with various levels of α-tocopherol (0.05, 0.1, 0.2, 0.39, and 0.71 mmol/kg). From each matrix containing a particular concentration of α-tocopherol, a set of samples with various concentrations of hydroxytyrosol was prepared. The plots of IT values versus hydroxytyrosol concentration for each sample are depicted in **Figure 3**. For concentrations of hydroxytyrosol <0.2 mmol/kg, the presence of α-tocopherol originated a stability increase smaller than the sum of the effects of the separate antioxidants, in agreement with data reported earlier (*5*). For concentrations of hydroxytyrosol close to 0.2 mmol/kg, the presence of α-tocopherol did not produce significant effects on stability at any concentration—that is, the matrix behaves as if it contains only hydroxytyrosol. For concentrations of hydroxytyrosol >0.2 mmol/kg, the presence of α-tocopherol at any concentration originated a decrease in the stability in comparison with that of the matrix containing only hydroxytyrosol. For each hydroxytyrosol concentration, the stability diminished with increasing α-tocopherol concentration. These results indicate that for olive oils, which naturally contain *o*-diphenols and α-tocopherol, the Rancimat stability will depend on the concentrations of both kinds of compounds and on the ratio between them.

To investigate the behavior of each antioxidant during the oxidation process, aliquots of a matrix containing hydroxytyrosol (0.7 mmol/kg) and α-tocopherol (0.36 mmol/kg) were analyzed at various times during their oxidation in the Rancimat apparatus (**Figure 4**). At the beginning of the process, the hydroxytyrosol
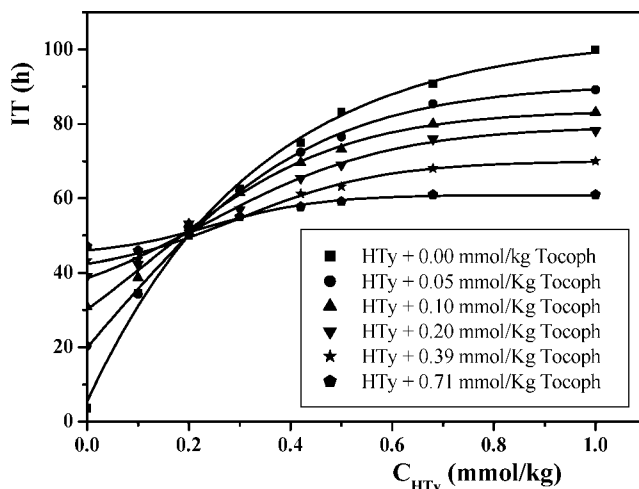

Figure 3. Rancimat stability of sets of the purified olive oil matrix (PVOO), each one spiked with constant amounts of α-tocopherol (0.05, 0.10, 0.20, 0.39, and 0.71 mmol/kg) and variable amounts of hydroxytyrosol. RSD < 10% (*n* = 2) except for hydroxytyrosol + 0.00 α-tocopherol, for which RSD < 6.0% (*n* = 4).
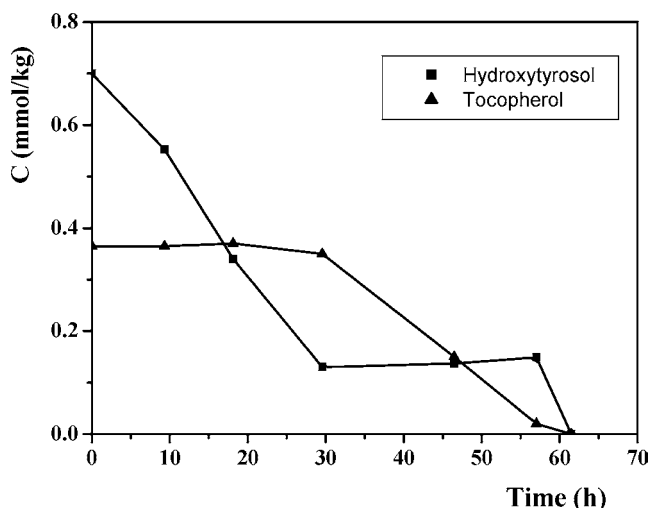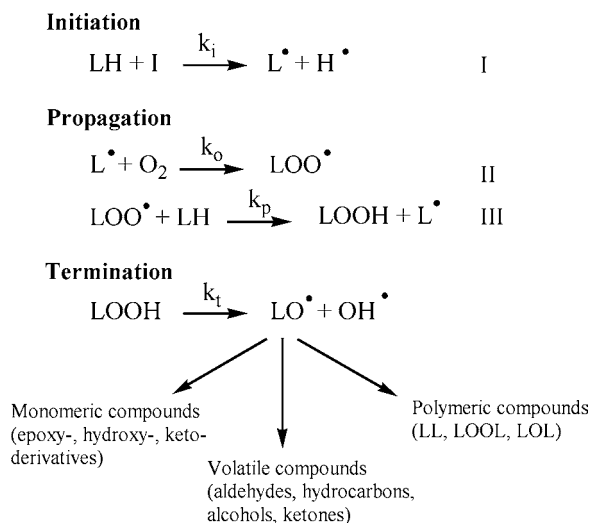

Figure 4. Evolution of antioxidant concentrations in the purified olive oil matrix (PVOO) spiked with a mixture of antioxidants (0.70 mmol/kg hydroxytyrosol and 0.36 mmol/kg α-tocopherol) during the time of oxidation in a Rancimat apparatus at 100 °C. RSD < 10% of the mean values (*n* = 2).

concentration decreased while the α-tocopherol concentration remained constant. When hydroxytyrosol concentration got down to ∼0.13 mmol/kg, it then remained constant, while α-tocopherol decreased. When the IT was reached, both antioxidants had disappeared. These results suggest that *o*-diphenols prevent the oxidation of α-tocopherol, and only when their concentration is low does α-tocopherol have an antioxidant function.

Kinetic Approach. The autoxidation in fatty materials is known to be a free radical chain process that, in homogeneous solution at sufficient oxygen pressures, proceeds by the mechanism shown in **Scheme 1** (*31*): LH an unsaturated alkyl chain and L• is the carbon-centered radical formed by abstraction of an allylic hydrogen atom by an initiator (I). The carbon-centered radical (L•) rapidly reacts with molecular oxygen to give the chain-carrying peroxyl radical (LOO•); this reacts with more alkyl chains to produce hydroperoxides (LOOH), which lead to numerous non-radical compounds. Antioxidant (AH) behaves as a chain oxidation breaker, competing with the substrate (LH)

Scheme 1. Simplified Scheme for Autoxidation

Initiation

$$LH + I \xrightarrow{k_i} L^\bullet + H^\bullet \qquad I$$

Propagation

$$L^\bullet + O_2 \xrightarrow{k_o} LOO^\bullet \qquad II$$

$$LOO^\bullet + LH \xrightarrow{k_p} LOOH + L^\bullet \qquad III$$

Termination

$$LOOH \xrightarrow{k_t} LO^\bullet + OH^\bullet$$

Monomeric compounds (epoxy-, hydroxy-, keto- derivatives)

Volatile compounds (aldehydes, hydrocarbons, alcohols, ketones)

Polymeric compounds (LL, LOOL, LOL)

by peroxyl radicals formed during the propagation step, yielding a stabile radical ($A^\bullet$) through reaction IV:

$$LOO^\bullet + AH \xrightarrow{k_p'} LOOH + A^\bullet \qquad (IV)$$

It has been suggested (*32*) that this reaction proceeds via an association between the antioxidant and the radical peroxide to yield an intermediate complex which slowly decomposes according to eq V:

$$LOO^\bullet + AH \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} [LOO^\bullet \text{---} AH] \xrightarrow{k_2} LOOH + A^\bullet \quad (V)$$
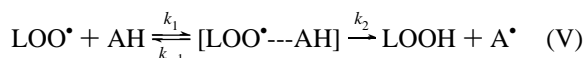
Assuming this hypothesis and that the IT is directly related to the reaction rate of the antioxidant with the peroxyl radical, the relationship between IT and concentration of the intermediate complex can be formulated by eq VI:

$$IT = Kk_2[LOO^\bullet \text{---} AH] \qquad (VI)$$

Assuming that the decomposition of the intermediate complex occurs more slowly than the dissociation, the dissociation constant (K_{dis}) is expressed by eq VII:

$$K_{dis} = k_{-1}/k_1 = [LOO^\bullet][AH]_0/[LOO^\bullet \text{---} AH] \quad (VII)$$

Taking into account that [AH] is very much higher than [LOO$^\bullet$], the antioxidant concentration [AH] will be equal to the initial concentration [AH]$_0$, and the maximum concentration of peroxide radical [LOO$^\bullet$]$_0$ will be equal to [LOO$^\bullet$- - -AH] + [LOO$^\bullet$]. Mathematical calculations yield eq VIII, relating the IT with the initial concentration of antioxidant:

$$IT = \frac{IT_{max}}{1 + K_{dis}/[AH]_0} \qquad (VIII)$$

To calculate K_{dis} and IT_{max}, eq VIII can be transformed to the linear eq IX

$$\frac{1}{IT} = \frac{1}{IT_{max}} + \frac{K_{dis}}{IT_{max}}\frac{1}{[AH]_0} \qquad (IX)$$

where IT_{max} is the maximum induction time, K_{dis} the dissociation constant of the intermediate complex ($K_{dis} = k_{-1}/k_1$), and [AH]$_0$ the initial antioxidant concentration.

Table 5. Values of Maximum Induction Time (IT_{max}), Dissociation Constant (K_{dis}), and Activation onstant (K_{act}) Calculated for the Aldehydic Form of Oleuropein Aglycon (AOA), Hydroxytyrosyl Acetate (HTyAc), Hydroxytyrosol (HTy), Mixtures of HTy and HTyAc, and α-Tocopherol from Equation IX [Mean Values ± SD (*n* = 2)]

antioxidant	IT_{max} (h)	K_{dis} (kg/mmol)	K_{act} (mmol/kg)
AOA	111 ± 7	0.19 ± 0.02	5.3 ± 0.6
HTyAc	116 ± 6	0.20 ± 0.01	5.0 ± 0.4
HTy	106 ± 6	0.20 ± 0.02	5.0 ± 0.5
HTy + HTyAc	118 ± 7	0.20 ± 0.02	5.0 ± 0.5
α-tocopherol	68 ± 4	0.28 ± 0.02	3.6 ± 0.3

Table 6. Induction Times at 100 °C of Virgin Olive Oil (VOO) and Olive Oil with Low Phenolic Content (LPOO) Spiked with Hydroxytyrosol (HTy) and α-Tocopherol (α-Toc), in Comparison with the Theoretical Values Calculated from the Results Obtained with Lipid Matrices Spiked with HTy, Ty, and α-Toc [Mean Values ± SD (*n* = 4)]

oil	o-diphenols	α-Toc	induction time (h)	
(mmol/kg)	(mmol/kg)	(mmol/kg)	theor[a]	exptl
VOO	0.55 ± 0.02	0.38 ± 0.02	65 + 3 = 68	72.3 ± 4.8
VOO + 0.25 α-Toc	0.55 ± 0.02	0.63 ± 0.03	61 + 3 = 64	70.8 ± 4.2
VOO + 0.30 HTy	0.85 ± 0.03	0.38 ± 0.02	70 + 3 = 73	80.3 ± 5.0
LPOO	0.04 ± 0.01	0.37 ± 0.03	42	39.2 ± 2.1
LPOO + 0.25 α-Toc	0.04 ± 0.01	0.62 ± 0.03	45	43.1 ± 2.4
LPOO + 0.30 HTy	0.34 ± 0.01	0.37 ± 0.03	57	57.3 ± 3.3
LPOO + 0.30 HTy + 0.25 α-Toc	0.34 ± 0.01	0.62 ± 0.03	56	53.3 ± 3.1

[a] Effect of o-diphenols and α-Toc calculated from **Figure 3**. Effect of monophenols = 3 h.

Applying eq IX to the IT values obtained from matrices containing various concentrations of a single antioxidant, linear functions with good correlations ($r > 0.997; p < 0.0001$) were obtained. These results indicate that the kinetic model is appropriate. **Table 5** shows the values of IT_{max}, K_{dis}, and K_{act} (activity constant = $1/K_{dis}$). The values of IT_{max} and K_{act} were similar for all o-diphenolic compounds and higher than for α-tocopherol.

The IT values obtained from spiked matrices were compared with those from the virgin olive oil (VOO), olive oil with low phenolic content (LPOO) obtained from the VOO by extraction with methanol/water, and both spiked with α-tocopherol and hydroxytyrosol. The experimental IT values (**Table 6**) indicated that the addition of hydroxytyrosol (0.30 mmol/kg) significantly increased the stability of VOO and LPOO, whereas the addition of α-tocopherol (0.25 mmol/kg) slightly decreased the stability of VOO and increased that of LPOO. This behavior is in accordance with the results obtained from the lipidic matrix (PVOO). For virgin olive oil (VOO), the theoretical values of IT values were slightly lower than experimental ones, indicating that concentrations of o-diphenols, monophenols, and α-tocopherol explain >90% of the stability. For methanol-extracted olive oil (LPOO), the theoretical IT values were similar to experimental ones. The small increase of IT found in VOO can be due to the presence of other minor antioxidant compounds soluble in methanol/water.

In summary, the o-diphenols present in virgin olive oils are the most active antioxidants, whereas α-tocopherol increases or decreases the Rancimat stability depending on the concentration of o-diphenols. These results explain the small contribution of α-tocopherol to the stability of virgin olive oils reported by various authors (*5*−*7*), because these oils usually contain high or intermediate concentrations of o-diphenolic compounds. Other

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minor components contribute in a low proportion to the Rancimat stability of virgin olive oils.

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