Inhibition of hydroperoxy-, keto- and hydroxy-FAME by alpha- and delta-tocopherol at Rancimat conditions

Susana Marmesat¹, Arturo Morales¹, M. Victoria Ruiz-Méndez¹, Gloria Márquez-Ruiz² and Joaquín Velasco^{1,*}

*¹Instituto de la Grasa, Consejo Superior de Investigaciones Científicas (CSIC),
Campus Universidad Pablo de Olavide E46, Ctra. de Utrera km 1, E-41013 Sevilla, Spain*

²Instituto de Ciencia y Tecnología de Alimentos y Nutrición, Consejo Superior de Investigaciones Científicas (CSIC), c/ José Antonio Novais, 10, E-28040 Madrid, Spain

*To whom correspondence should be addressed:

Telephone: +34 954 61 15 50

Fax: +34 954 61 67 90

E-mail: jvelasco@ig.csic.es

Abstract

The effects of α - and δ -tocopherol on inhibition of hydroperoxides, keto and hydroxy compounds at Rancimat conditions, i.e. 100 °C and air bubbling, were studied in samples of fatty acid methyl esters (FAME) obtained from high linoleic (HL) and high oleic (HO) sunflower oils. Primary hydroperoxides from methyl linoleate and methyl oleate and secondary keto and hydroxy compounds derived from methyl linoleate hydroperoxides were analyzed by HPLC-UV-ELS. Different tocopherol concentrations, namely, 10, 50, 100, 500 and 1000 mg/kg, were tested. Irrespective of the lipid substrate and the initial concentration of tocopherol, results showed that the content of hydroperoxides accumulated during the induction period was remarkably higher in the samples containing δ -tocopherol. The relative concentrations of oleate hydroperoxides in the HO samples were also higher in the presence of δ -tocopherol. α -Tocopherol was more effective in inhibiting hydroperoxides at low levels, with 100 mg/kg as optimal concentration, while δ -tocopherol displayed optimal protection at 1000 mg/kg. Under the oxidation conditions applied, neither α - nor δ -tocopherol showed a protective effect on hydroperoxide decomposition at any level assayed. Formation of keto- and hydroxydienes was more related to the concentration of their hydroperoxide precursors. Furthermore, both tocopherols gave rise to increased concentrations of ketodienes at 500 and 1000 mg/kg compared to the controls. Such an effect was more pronounced for α -tocopherol and in the HL samples.

Key-words: Tocopherol, hydroperoxides, ketodienes, hydroxydienes, Rancimat.

Introduction

The antioxidant activity of different types of tocopherols has been intensively investigated in a variety of lipid substrates and at different oxidation conditions, as compiled in different reviews [1-3]. The antioxidant properties of tocopherols are mainly due to their ability to donate their phenolic hydrogen to peroxy radicals. According to their chemical structures, the hydrogen-donating power decreases in the order $\alpha > \beta > \gamma > \delta$. It is generally accepted that the relative antioxidant activity of the tocopherols *in vivo* is in the order $\alpha > \beta > \gamma > \delta$; however, there is widespread confusion regarding their relative activities in fats and oils [1]. While the relative order of antioxidant activity found in an organic solvent agreed with their hydrogen-donating power [4], a reversed order, i.e. $\delta > \gamma \approx \beta > \alpha$, was obtained in fats, oils and lipoproteins *in vitro* [1]. Although this is not yet clearly understood, it is known that the activities of tocopherols not only depend on their reactivities towards peroxy radicals, but also on their participation in side reactions that normally reduce and counteract their principal action mode. These side reactions are dramatically affected by tocopherol concentrations, the type of lipid substrate and other factors [1].

In most of the studies addressed to evaluate the antioxidant activities of tocopherols, the peroxide value is normally applied because it provides information on the total hydroperoxides, i.e. the most abundant oxidation products formed at ambient conditions. However, few are the studies in which the inhibitory effects of different types of tocopherols on specific oxidation compounds have been undertaken.

The action of tocopherols goes further beyond their reduction effect on lipid oxidation rate. Tocopherols can also affect the relative abundance of lipid oxidation products because of kinetic reasons. The isomerization of the *cis,trans* forms of methyl linoleate

hydroperoxides to *trans,trans* isomers has been reported to be completely inhibited in the presence of high levels of α -tocopherol [5-7]. However, hydroperoxides isomerized into *trans,trans* isomers at low α -tocopherol concentrations [6, 7]. γ - and δ -Tocopherols also showed the same behavior, although their effects were quite lower than that of α -tocopherol, resulting in a different relative isomeric distribution of hydroperoxides [6, 7].

Tocopherols have also been reported to inhibit hydroperoxide decomposition and affect the composition of derived products. Frankel and Gardner [8] found that the total amount of volatile compounds formed by thermal decomposition from a mixture of methyl linoleate hydroperoxides decreased in the presence of α -tocopherol and that the relative distribution of individual volatiles also was affected. Koskas et al. [6] found that α -, γ - and δ -tocopherols inhibited the decomposition of both 13-*cis,trans* and 13-*trans,trans* linoleic acid hydroperoxides in water and that the activity order of tocopherols was α - < γ - < δ -. Mäkinen et al. [7] also observed that γ - and δ -tocopherols were, in contrast to hydroperoxide isomerization, more efficient inhibitors of the decomposition of methyl linoleate hydroperoxides than α -tocopherol. In addition, Huang et al. [9, 10] observed that α -, γ - and δ -tocopherols inhibited formation of hexanal, a volatile compound formed from hydroperoxides of n-6 polyunsaturated fatty acids, and that, in contrast to hydroperoxide formation, the inhibition of hexanal increased at higher levels of tocopherols. Nevertheless, Mäkinen et al. [7] observed a loss of antioxidant efficiency of tocopherol in inhibiting hydroperoxide decomposition at high antioxidant concentration, being higher for α -, but it was also evident for γ - and δ -tocopherols. These authors suggested that the differences in the relative effects of tocopherols at different concentrations seem to result from a compromise between their

radical scavenging efficiency and their participation in side reactions of peroxidizing nature.

In a previous study carried out in our lab [11], it was found that addition of α -tocopherol in a FAME sample derived from high oleic sunflower oil resulted in changes in the distribution of hydroperoxides from methyl linoleate and methyl oleate. For a given content of total hydroperoxides, determined by the peroxide value, it was found that the relative concentration of methyl linoleate hydroperoxides increased in the presence of α -tocopherol, whereas that of methyl oleate hydroperoxides decreased. Quantitative analysis of methyl linoleate hydroperoxides was performed by an HPLC-UV method developed in our lab [12]. The method allows the quantitative analysis of the main oxidation products of linoleic acid, i.e. hydroperoxy-, keto- and hydroxy-dienes, and it can be directly applied to samples of FAME obtained from vegetable oils. Then the method was adapted to the analysis of vegetable oils by applying an adequate derivatization step to transform the oxidized triacylglycerol molecules into FAME [14]. In addition, the incorporation in series of an Evaporative Light Scattering detector (ELS) proved to be useful for the analysis of methyl oleate hydroperoxides [14, 15].

Compared to a control, increased formation of keto- and hydroxy-dienes, both derived from methyl linoleate hydroperoxides, was also found in the presence of α -tocopherol [11]. It was suggested that such an effect of α -tocopherol could be related to its participation in side reactions, through direct reactions with hydroperoxides to produce secondary compounds.

The results of our previous study led us to go into detail on the inhibitory effects of tocopherol through new kinetic studies. The present work was then undertaken to examine the effect of the type of tocopherol, namely, α - and δ -tocopherol, on the

inhibition of lipid oxidation products. These tocopherols were chosen because they are the two that differ most in their hydrogen-donating abilities [1]. FAME obtained from high oleic (HO) and high linoleic (HL) sunflower oils were used as lipid substrates. Unlike our previous study, in which a UV detector was used only, quantitative analysis of hydroperoxides, keto and hydroxy compounds was performed by HPLC-UV-ELS [14]. Different concentrations of both tocopherols, namely, 10, 50, 100, 500 and 1000 mg/kg, were assayed to study the influence of their hydrogen donating power on the inhibition of compounds. For practical purposes, the oxidation conditions used were those normally applied in the Rancimat test, i.e. 100 °C and air bubbling, which is an accelerated method used to evaluate oxidative stability of oils.

Materials and Methods

Chemicals

(±)- α -Tocopherol (>96%) and (+)- δ -Tocopherol (>90%) were purchased from Sigma-Aldrich® (St. Louis, MO). Diethyl ether stabilized with 1% v/v ethanol (Super purity solvent, HPLC grade) was purchased from Romil, LTD (Cambridge, UK) and n-heptane (99% purity, HPLC grade) from Carlo Erba Reactifs-SDS (Val de Reuil, France). Both HPLC solvents were used as received. All other chemicals and reagents were of analytical grade and obtained from local suppliers.

Samples

Refined HL and HO sunflower oils were acquired in a local supermarket. Their fatty acid compositions were 6.5% C16:0, 0.2% C16:1, 3.2% C18:0, 36.0% C18:1, 52.9% C18:2 and 1.2% others for HL, and 4.5% C16:0, 0.2% C16:1, 2.6% C18:0, 81.3% C18:1, 9.9% C18:2 and 1.5% others for HO sunflower oil. Samples of FAME were obtained from these two oils by transesterification at room temperature with KOH in methanol. Sixty grams of oil were dissolved in 200 mL n-hexane and 20 mL of a solution 2M KOH in methanol was added. Stirring was applied for 30 min and then the medium was neutralized with 50 mL of 1 M H₂SO₄ in methanol. The organic phase was then separated, washed with 80 mL water and filtered through filter paper containing anhydrous Na₂SO₄. The solvent was evaporated in a rotary evaporator at 40 °C. Finally, N₂ was bubbled through the lipid sample to remove the residual solvent. Thin layer chromatography (TLC) was used to confirm that the transesterification reaction was complete. Silica plates and hexane:diethyl ether:acetic acid (80:20:1, v/v/v) as the mobile phase were used. The spots on the plate were revealed with iodine vapor. The FAME were purified by alumina column chromatography according to Yoshida et al.

[16] to obtain FAME devoid of their naturally occurring tocopherols, peroxides and trace metals. The samples obtained showed similar fatty acid compositions to their parent oils.

When indicated, α - and δ -tocopherol were added to the stripped FAME samples. An aliquot of neat α - or δ -tocopherol was weighed into a flat-bottomed round flask and then the FAME sample was added to obtain a tocopherol concentration of 1000 mg/kg sample. Stirring was applied for 15 min at room temperature. Then, five additional samples containing 500, 100, 50 and 10 mg/kg of α - or δ -tocopherol were obtained by dilution using purified FAME.

Oxidation conditions

Lots of six samples of 8 ± 0.01 g containing 0, 10, 50, 100, 500 and 1000 mg/kg of α - or δ -tocopherol were placed into Rancimat tubes and these were inserted into the heating block of a Rancimat device (Metrohm, Herisau, Switzerland), previously heated at 100 °C. The range of oxidation time studied was chosen from previous Oil Stability Index (OSI) data provided by the Rancimat test at 100°C. The air flow was 20 L h⁻¹ in all experiments. Samplings of 0.5 g were carried out along the induction period and the samples were kept at -35 °C until analyses.

Analysis of oxidation compounds by HPLC

The main oxidation products of methyl linoleate and methyl oleate, namely, hydroperoxides, keto- and hydroxy- compounds, were determined quantitatively by an HPLC-UV-ELS method developed in our lab [12, 14]. The oxidized FAME samples were analyzed in a Waters 600 HPLC chromatograph. The chromatograph was equipped with a Waters 600 pump, a Rheodyne injector valve (20- μ L sample loop), a

silica HPLC column (LiChrospher[®] Si 60, 250 mm x 4 mm i.d., 5 µm particle size) (Merck, Darmstadt, Germany), an HP 1050 Series variable wavelength UV detector (8 mm path length) (Agilent Technologies Inc., Palo Alto, CA), a Waters 2424 Evaporative Light Scattering (ELS) detector, an Agilent 35900E A/D Converter (Agilent Technologies Inc.) and a Waters 600 controller. The separation of analytes was performed in isocratic elution using n-heptane:diethyl ether (82:18, v/v) with a flow rate of 1 mL/min. Ethanol present in diethyl ether as a stabilizer was not removed. Hydroperoxy- and hydroxy-dienes were recorded by the UV detector at 234 nm, while ketodienes were at 268 nm. Hydroperoxides from methyl oleate and methyl linoleate as a whole were detected by the ELS. The ELS conditions applied were 35°C for nebulizer temperature, 40 °C for tube temperature and 25 psi for gas flow. The gas used for nebulization was nitrogen (purity 99.99%). The gain was set at 50. Quantitative data by the UV detector were obtained by applying response factors reported elsewhere [12], while external calibration was applied for quantitative determination of compounds by the ELS, according to a previous report [14].

Analysis of tocopherols

Analysis of α - and δ -tocopherol was carried out by normal-phase HPLC with fluorescence detection following IUPAC standard method 2.411 [17].

Oil stability index (OSI)

The OSI of the FAME samples was determined at 100 °C according to AOCS Official Method Cd 12b-92 [18]. A Rancimat instrument, model 679, from Metrohm (Metrohm, Herisau, Switzerland) was used in this study. The tests were carried out with 2.5 g of oil and with an air flow of 20 L h⁻¹.

176 Statistical analysis

177 Experiments were run in triplicate and results were presented as mean values of three
178 analytical determinations. Comparisons were made by the Student's *t* test using
179 Microsoft Excel 2010 (Microsoft Corporation, Redmond, WA, USA). Linear regression
180 analysis was also performed in Microsoft Excel 2010. Significance was defined at $p <$
181 0.05.

182

Results and discussion

Inhibition of hydroperoxides

Formation of hydroperoxides in the two lipid substrates, HL and HO, followed different reaction kinetics in the presence of α - and δ -tocopherol. In the samples containing α -tocopherol, the formation of hydroperoxides took place at constant rate during the whole induction period (IP), i.e. until the total loss of the antioxidant occurred (**Fig. 1**). As a result, the reaction order for hydroperoxides in both lipid substrates was zero in the presence of α -tocopherol at all the concentrations assayed. On the other hand, hydroperoxides only increased linearly during the first stages of oxidation in the samples containing δ -tocopherol and the reaction rate increased progressively in the presence of substantial contents of the antioxidant. This was a common pattern for all the samples analyzed, i.e. irrespective of the lipid substrate and the initial concentration of δ -tocopherol (**Fig. 1**). Compared to the samples containing α -tocopherol, the content of hydroperoxides accumulated during the IP was remarkably higher in the presence of δ -tocopherol at any initial tocopherol concentration assayed and in both lipid substrates.

Given that hydroperoxides are the major oxidation compounds formed during the IP and that their participation in other reactions is relatively low compared to their formation, a rough estimate of the oxidation rate can be expressed as Eq. 1 [19].

$$\frac{d[\text{LOOH}]}{dt} = k \cdot [\text{LOOH}]^n \quad (1)$$

The kinetic parameters k and n represent a global oxidation rate constant and the reaction order, respectively. By rearrangement and integration of Eq. 1, Eq. 2 is readily obtained, in which $[\text{LOOH}]$ and $[\text{LOOH}]_0$ represent the concentrations of hydroperoxides at time t and 0, respectively.

$$[LOOH]^{1-n} = [LOOH]_0^{1-n} + kt \quad (2)$$

The kinetic parameters k and n were calculated from the experimental data acquired during the whole IP. The results obtained showed that all the samples containing δ -tocopherol presented similar reaction kinetics to the control samples, i.e. in the absence of antioxidant, in terms of the reaction order, which was equal to 0.5 (**Table 1**).

The different oxidation kinetics displayed by the samples containing different type of tocopherol along with the kinetic similarities between the samples containing δ -tocopherol and the control samples can be attributed to the different hydrogen donating abilities of the two antioxidants, being much larger for α -tocopherol [1]. Compared to δ -, the larger reactivity of α -tocopherol towards peroxy radicals gave rise to a more effective inhibition of hydroperoxide formation during the whole IP since hydroperoxides increased at constant rate until the antioxidant was completely depleted. The fact that the oxidation rate did not increase until the total loss of α -tocopherol took place is consistent with the fact that such a kinetic pattern was even observed when the lowest antioxidant concentration was assayed (10 mg/kg), emphasizing the enormous hydrogen donating power of α -tocopherol and its effectiveness at low concentrations. On the other hand, δ -tocopherol was not reactive enough and the rate of hydroperoxide formation increased in the presence of still considerably high contents of the antioxidant. In consequence, larger contents of hydroperoxides were accumulated during the IP in the presence of δ -tocopherol compared to α -. Accumulation of oxidation compounds during the IP of lipid model systems and vegetable oils has been reported to decrease when temperature was increased [20, 21] and to increase as the degree of unsaturation of the oil was higher [22]. However, to the best of our knowledge, the results of the present study are the first evidence of that the type of

230 tocopherol can also have a remarkable impact on the total amounts of oxidation
231 products accumulated during the IP.

232 The differences in the hydrogen donating capabilities between α - and δ -tocopherol also
233 gave rise to differences in the relative formation of hydroperoxides from methyl oleate
234 and methyl linoleate in the HO samples. As to the HL samples, the oxidation of methyl
235 oleate was considerably low (not shown), which supports previous results [11]. The
236 concentration of the primary hydroperoxides of methyl oleate can be determined by
237 difference from the results obtained with the two detectors used in series, UV and ELS
238 [14]. In a previous report it was found that substantial contents of methyl oleate
239 hydroperoxides were detected in FAME from HO sunflower oil in the absence of
240 antioxidant [11]. When α -tocopherol was added the oxidation of methyl oleate was
241 remarkably reduced compared to methyl linoleate. In the present study, α -tocopherol
242 considerably inhibited the formation of methyl oleate hydroperoxides in the HO sample
243 at all the concentrations assayed, whereas substantial oxidation of methyl oleate was
244 detected in the presence of δ -tocopherol. In this regard, the relationship between the
245 contents of hydroperoxides detected by ELS and hydroperoxydienes by UV was linear
246 during the entire IP in all the HO samples containing α -tocopherol. No differences were
247 found in such a relationship between the samples containing different initial
248 concentrations of α -tocopherol (**Fig. 2a**). The oleate hydroperoxides did not constitute
249 more than 19 mol% of the analyzed hydroperoxides at the end of the IP in all the
250 samples containing α -tocopherol. In contrast, the formation of hydroperoxides from
251 methyl oleate seemed to be dependent of the initial concentration of δ -tocopherol (**Fig.**
252 **2b**). For a given content of total hydroperoxides formed during the IP, the relative
253 amount of oleate hydroperoxides was lower when the initial concentration of δ -

tocopherol was increased. In addition, unlike the samples containing α -tocopherol, the relative concentration of oleate hydroperoxides formed during the IP increased with the oxidation extent in the presence of δ -tocopherol. In fact, the relationship between total hydroperoxides and hydroperoxydienes was not linear (**Fig. 2b**). The data adjusted well to quadratic polynomial functions (not shown). The oleate hydroperoxides levels at the end of the IP were approximately 50 mol% of the analyzed hydroperoxides in all the samples containing δ -tocopherol.

It has been reported that α -tocopherol was more efficient in inhibiting the isomerization of *cis,trans* methyl linoleate hydroperoxides to the *trans,trans* isomers than δ -tocopherol due to its higher hydrogen donating ability [7]. Nevertheless, no reported data have been found in relation to the relative hydroperoxide formation of methyl oleate and methyl linoleate. The analytical method used in the present study has allowed us to determine how the type of tocopherol can influence the relative formation of hydroperoxides of different unsaturated fatty acids. In the present study, and also in a previous report [11], it was found that α -tocopherol considerably reduced the oxidation of methyl oleate in a HO sample compared to a control, i.e. in the absence of antioxidant. Due to its great reactivity with peroxy radicals, α -tocopherol largely prevented the participation of methyl oleate in the propagation step of the oxidation process. The larger formation of methyl oleate hydroperoxides in the HO samples containing δ -tocopherol could be therefore explained by a slower interaction of the antioxidant with peroxy radicals, thus allowing peroxy radicals to react with methyl oleate in the propagation step to a greater extent.

Given the different kinetics shown by the samples containing each type of tocopherol, the initial rate of inhibited oxidation (W_{inh}) was taken into account to compare the

effects of the two antioxidants [23]. The amounts of hydroperoxides found in the early stages of oxidation for the controls and the samples containing δ -tocopherol were considered for calculation of pseudo zero-order rate constants. As to the samples containing α -tocopherol, all the data obtained during the IP were used in the analysis and, in consequence, the initial rate constants were the same as the global rate constants during the whole IP. As expected, the results showed that the W_{inh} decreased to a great extent in the presence of either tocopherol (**Table 2**). The decrease in W_{inh} was in line with the increase of antioxidant concentration up to 50 and 500 mg/kg for α - and δ -tocopherol, respectively, in the HL sample and up to 50 mg/kg for both tocopherols in the HO sample. Then a significant gradual increase was observed with tocopherol concentration. This significant increase in the oxidation rate was not observed in the rate constants presented in Table 1. When the whole IP was considered in the samples containing δ -tocopherol, the oxidation rate decreased as the initial content of the antioxidant was increased.

In both lipid substrates, the initial oxidation rate was lower in the samples containing α -tocopherol when the concentration was equal to or lower than 100 mg/kg (**Table 2**). No significant differences between the two tocopherols were found at 500 mg/kg and δ -tocopherol was more effective in reducing the oxidation rate at 1000 mg/kg in the two lipid substrates.

For a better characterization of the inhibitory effect of the two antioxidants on hydroperoxides, the IP, which was determined as the time at which oxidation was accelerated or when the total loss of tocopherol took place, was also evaluated. While the W_{inh} reflects the antioxidant strength, the IP estimates the effectiveness of an antioxidant in terms of its ability to terminate the chain radical process by reacting with

the peroxy radicals, which are responsible for the duration of the IP. The results obtained showed differences in the effectiveness of the two tocopherols between the two lipid substrates (**Fig. 3a-b**). No significant differences were found between the two tocopherols at 10 and 500 mg/kg in the HL sample. However, α - was more effective than δ -tocopherol at 50 and 100 mg/kg and the opposite was found at 1000 mg/kg. As to the HO samples, no significant differences were found between the two antioxidants at low concentrations, i.e. 10 and 50 mg/kg, and δ - was significantly more effective than α -tocopherol from 100 mg/kg. These results were consistent with the oxidative stability index (OSI) values provided by the Rancimat test (**Fig. 3a'-b'**).

The stabilization factor (F), defined as the ratio between the IPs in the presence and absence of antioxidant, increased linearly with the initial concentration of both tocopherols up to 100 mg/kg in the two lipid substrates. Afterwards, deviation from linearity took place (**Fig. 4**). It is known that the absence of linearity is due to the participation of the antioxidant in side reactions, generating free radicals during the propagation and the consequent loss of efficacy [23]. Therefore, both tocopherols showed loss of effectiveness from concentrations larger than 100 mg/kg, which is consistent with the loss of efficacy in reduction the initial oxidation rate shown by the two tocopherols. Yanishlieva et al. [24] suggested that both α - and γ -tocopherol lose their efficacy at high concentrations owing to their direct reaction with hydroperoxides, giving alkoxyl radicals that may amplify the rate of lipid oxidation by participating in propagation reactions. In addition, it was also suggested that tocopheroxyl radicals can also participate in a number of peroxidizing reactions propagating the oxidation chain. These authors found that α -tocopherol participated more readily in side reactions than γ -tocopherol, which was attributed to its higher hydrogen-donating power.

In order to determine the most optimal concentrations of the two tocopherols, the kinetic parameter A proposed by Yanishlieva and Marinova [25] to evaluate antioxidant activity was examined (**Table 3**). This parameter is calculated by the ratio between the parameter F and the oxidation rate ratio (ORR), defined as the ratio between the initial oxidation rates in the presence and absence of antioxidant. Results showed that α -tocopherol was most effective at 100 mg/kg, while δ -tocopherol displayed optimal protection at 1000 mg/kg in both lipid substrates. If data in Table 1 had been considered for calculation of A instead of the initial oxidation rates, the optimal concentration for δ -tocopherol would have also been the highest concentration assayed, i.e. 1000 mg/kg. Although α -tocopherol was obviously more effective in prolonging the IP at 1000 mg/kg than at 100 mg/kg, the opposite was found for the reduction of the initial oxidation rate. Unlike the Rancimat test, which provides the time at which the antioxidants are exhausted, the parameter A also takes into account the ability of an antioxidant to change the initial oxidation rate. From a practical point of view, this is essential because the appearance of rancidity occurs at relatively low oxidation extents, which are normally reached far earlier than when the antioxidants are completely consumed.

Inhibition of keto- and hydroxy-dienes

Supporting previous studies [14], secondary oxidation products of methyl oleate hydroperoxides were not detected by the ELS in any of the samples analyzed. In those samples in which methyl oleate hydroperoxides were formed significantly, i.e. the control HO sample and the HO samples containing δ -tocopherol, the absence of such compounds could be attributed to the low sensitivity of the ELS compared to the UV detector.

Keto- and hydroxy-dienes showed similar patterns to their precursors, i.e. hydroperoxydienes, in the samples containing tocopherol. Slight formation of ketodienes was observed and, as expected, a sharp increase took place when tocopherol was completely depleted (not shown). On the other hand, hydroxydienes were not detected in the presence of antioxidant in the samples containing 10, 50 and 100 mg/kg of either α - or δ -tocopherol, whereas in the samples containing 500 and 1000 mg/kg formation of the hydroxy compounds was observed when the content of remaining tocopherol was low (not shown). Similarly, a sharp increase of hydroxydienes occurred when tocopherol was exhausted.

As more hydroperoxides were formed during the IP in the samples containing δ -tocopherol, more decomposition products were also found in these samples. However, neither α - nor δ -tocopherol at levels lower than 500 mg/kg showed a protective effect on the hydroperoxide decomposition. Thus, for a given content of hydroperoxydienes, similar concentrations of ketodienes were found between the samples containing tocopherol and the control samples (**Fig. 5a-a'**).

When the higher concentrations of both tocopherols were assayed, i.e. 500 and 1000 mg/kg, it was observed that formation of ketodienes was favored with respect to their precursors, i.e. hydroperoxydienes. In this regard, for a given concentration of hydroperoxydienes, significantly larger contents of ketodienes were found in these samples compared to the controls. This effect was more pronounced in the samples containing α -tocopherol and in the more unsaturated sample, i.e. the HL sample (**Fig. 5**). As to hydroxydienes, such an effect was not observed. In this regard, their contents were similar to those found in the controls for a given content of hydroperoxydienes (not shown).

374 From the results obtained it can be said that under the oxidation conditions applied,
375 neither α - nor δ -tocopherol showed a protective effect on hydroperoxide decomposition
376 at any level assayed. Formation of keto- and hydroxy-dienes was more related to the
377 concentration of their hydroperoxide precursors. However, at high tocopherol
378 concentrations, i.e. 500 and 1000 mg/kg, both tocopherols gave rise to increased
379 concentrations of ketodienes compared to the control samples, whereas such an effect
380 was not observed for hydroxydienes. These results are in agreement with previous
381 studies [11] that showed increased formation of keto- and also, in a lower extent,
382 hydroxy-dienes in the presence of 500 mg/kg α -tocopherol compared to a control. It
383 was suggested that due to its high hydrogen-donating capability, α -tocopherol could
384 react directly with hydroperoxides, yielding alkoxyl radicals, as commented above, and
385 so acting as a decomposer of hydroperoxides [24, 26]. The larger effect found for α -
386 compared to δ -tocopherol in this study can be attributed to its higher hydrogen-donating
387 power. These results are in agreement with those reported by Mäkinen et al. [7], who
388 observed a major decomposing effect of α - than δ -tocopherol on pure linoleate
389 hydroperoxides. Similarly, the fact that the increased formation of ketodienes compared
390 to a control sample was only observed at high concentrations of tocopherols can also be
391 attributed to the increase of the hydrogen-donating activity with the antioxidant
392 concentration [7]. In addition, the fact that such an effect was more pronounced in the
393 HL than in the HO sample could be related to the much higher relative concentration of
394 the more reactive methyl linoleate hydroperoxides found in the HL sample. In this
395 respect, Yanishlieva et al. [24] found rate constants for the participation of α - and γ -
396 tocopherol in one side reaction that were higher in purified soybean oil than in
397 sunflower oil. The authors suggested that these results may have been due to the higher
398 reactivity of the linolenate hydroperoxides that are formed in soybean oil.

The fact that a protective role of tocopherols on the decomposition of hydroperoxides was not observed in the present study is in contrast with the studies of Mäkinen et al. [7, 27]. This could be related to differences in the oxidation conditions applied. Specifically, the oxidation temperature used in the present study (100 °C) was quite different from that applied by Mäkinen and coworkers (40 °C). The isomerization of the *cis,trans* methyl linoleate hydroperoxides to the *trans,trans* isomers has been reported to precede the hydroperoxide decomposition and hence the protective effect of tocopherol seems to be due to its capability to inhibit such an isomerization reaction [27]. In this regard, the larger formation of *trans,trans* isomers of hydroperoxides with temperature [28] could explain the lack of tocopherol activity in protecting hydroperoxides from decomposition found in the present study.

Conclusions

The results of this study have shown that different relative concentrations of oxidation products and different global amounts of compounds formed during the IP may result from each type of tocopherol. The combination of the IPs with the initial oxidation rate data to evaluate the efficacy of antioxidants under conditions similar to the Rancimat test constitutes a more complete approach than the Rancimat test, i.e. the OSI values. Unlike the Rancimat test, which evaluates the time at which antioxidants are completely consumed, such an approach also takes into account the initial stages of lipid oxidation. Rancidity of oils normally appears at very low oxidation extents, which are reached in the presence of elevated contents of antioxidants. Obviously, the Rancimat test is a convenient method that provides useful information on the oxidative stability of oils. The stability order of a series of oils provided by the Rancimat should be similar to the one that would be obtained at room temperature, provided that oxygen is not limiting.

Nevertheless, the different influence of the type and concentration of tocopherol on reducing the initial oxidation rate and prolonging the IP may result in changes in the expected order of stability at room temperature predicted by Rancimat.

Acknowledgments

This work was funded by the Spanish Ministry of Economy and Competitiveness through project AGL2013-45110-R and CSIC through a PIE project with reference 201270E134. The authors also thank the Spanish Ministry of Economy and Competitiveness for supporting Susana Marmesat with a “Juan de la Cierva” contract with reference JCI-2011-08874.

References

- 1 Kamal-Eldin A, Appelqvist L Å (1996) The chemistry and antioxidant properties of tocopherols and tocotrienols. *Lipids* 31(7):671-701
- 2 Kamal-Eldin A (2006) Effect of fatty acids and tocopherols on the oxidative stability of vegetable oils. *Eur J Lipid Sci Technol* 108(12):1051-1061
- 3 Seppanen CM, Song Q, Csallany AS (2010) The antioxidant functions of tocopherol and tocotrienol homologues in oils, fats, and food systems. *J Am Oil Chem Soc* 87(5):469-481
- 4 Burton GW, Ingold, KU (1981) Autoxidation of biological molecules. 1. Antioxidant activity of vitamin E and related chain-breaking phenolic antioxidants in vitro. *J Am Chem Soc* 103(21):6472-6477
- 5 Peers KE, Coxon DT, Chan H W-S (1981) Autoxidation of methyl linolenate and methyl linoleate: The effect of α -tocopherol. *J Sci Food Agric* 32:898-904
- 6 Koskas JP, Cillard J, Cillard P (1984) Autoxidation of linoleic acid and behavior of its hydroperoxides with and without tocopherols. *J Am Oil Chem Soc* 61:1466-1469
- 7 Mäkinen M, Kamal-Eldin A, Lampi A-M, Hopia A (2001) α -, γ , and δ -Tocopherols as inhibitors of isomerization and decomposition of *cis,trans* methyl linoleate hydroperoxides. *Eur J Lipid Sci Technol* 103:286-291
- 8 Frankel EN, Gardner HW (1989) Effect of α -tocopherol on the volatile thermal decomposition products of methyl linoleate hydroperoxides. *Lipids* 24(7):603-608
- 9 Huang SW, Frankel EN, German JB (1994) Antioxidant activity of α - and γ -tocopherols in bulk oils and in oil-in-water emulsions. *J Agric Food Chem* 42(10):2108-2114

- 456 10 Huang SW, Frankel EN, German JB (1995) Effects of individual tocopherols and
457 tocopherol mixtures on the oxidative stability of corn oil triglycerides. *J Agric Food*
458 *Chem* 43(9):2345-2350
- 459 11 Morales A, Dobarganes C, Márquez-Ruiz G, Velasco J (2012) Formation of
460 hydroperoxy-, keto- and hydroxy-dienes in FAME from oils: influence of
461 temperature and addition of α -tocopherol. *J Am Oil Chem Soc* 89:675-684
- 462 12 Morales A, Dobarganes C, Márquez-Ruiz G, Velasco J (2010) Quantitation of
463 hydroperoxy-, keto- and hydroxy-dienes during oxidation of FAMEs from high-
464 linoleic and high-oleic sunflower oils. *J Am Oil Chem Soc* 87:1271-1279
- 465 13 Morales A, Marmesat S, Dobarganes C, Márquez-Ruiz G, Velasco J (2012)
466 Quantitative analysis of hydroperoxy-, keto- and hydroxy-dienes in refined
467 vegetable oils. *J Chromatogr A* 1229:190-197
- 468 14 Morales A, Marmesat S, Dobarganes C, Márquez-Ruiz G, Velasco J (2012)
469 Evaporative light scattering detector in normal-phase high-performance liquid
470 chromatography determination of FAME oxidation products. *J Chromatogr A*
471 1254:62-70
- 472 15 Morales A, Marmesat S, Ruiz-Méndez MV, Márquez-Ruiz G, Velasco J (2014)
473 Formation of oxidation products in vegetable oils analysed as FAME derivatives by
474 HPLC-UV-ELSD. *Food Res Int* 62:1080-1086
- 475 16 Yoshida H, Kondo I, Kajimoto G (1992) Participation of free fatty acids in the
476 oxidation of purified soybean oil during microwave heating. *J Am Oil Chem Soc*
477 11:1136-1140
- 478 17 IUPAC (1992) Standard Methods for the Analysis of Oils, Fats and Derivatives, 7th
479 edn. International Union of Pure and Applied Chemistry, Blackwell Scientific,
480 Oxford, UK

- 481 18 AOCS (1998) Official Methods and Recommended Practices of the American Oil
482 Chemists' Society, 5th edn., edited by D Firestone, AOCS Press, Champaign,
483 Illinois.
- 484 19 Márquez-Ruiz G, Martín-Polvillo M, Dobarganes C (2003) Effect of temperature
485 and addition of α -tocopherol on the oxidation of trilinolein model systems. *Lipids*
486 38(3):233-240
- 487 20 Márquez-Ruiz G, Martín-Polvillo M, Dobarganes C (1996) Quantitation of oxidized
488 triglyceride monomers and dimers as an useful measurement for early and advanced
489 stages of oxidation. *Grasas y Aceites*, 47(1-2):48-53
- 490 21 Márquez-Ruiz G, Martín-Polvillo M, Velasco J, Dobarganes C (2008) Formation of
491 oxidation compounds in sunflower and olive oils under oxidative stability index
492 conditions. *Eur J Lipid Sci Technol* 110(5):465-471
- 493 22 Martin-Polvillo M, Marquez-Ruiz G, Dobarganes MC (2004) Oxidative stability of
494 sunflower oils differing in unsaturation degree during long-term storage at room
495 temperature. *J Am Oil Chem Soc* 81(6):577-583
- 496 23 Yanishlieva NV, Marinova EM (2003) Kinetic evaluation of the antioxidant activity
497 in lipid oxidation. In: Kamal-Eldin A (ed) *Lipid oxidation pathways*. AOCS Press,
498 Champaign, pp 85-110
- 499 24 Yanishlieva NV, Kamal-Eldin A, Marinova EM, Toneva AG (2002) Kinetics of
500 antioxidant action α - and γ -tocopherols in sunflower and soybean triacylglycerols.
501 *Eur J Lipid Sci Technol* 104:262-270
- 502 25 Yanishlieva NV, Marinova EM (1992) Inhibited oxidation of lipids. I: Complex
503 estimation of the antioxidative properties of natural and synthetic antioxidants. *Fat*
504 *Sci Technol* 94: 374-379

- 505 26 Kamal-Eldin A, Mäkinen M, Lampi A-M, Hopia A (2002) A multivariate study of
506 α -tocopherol and hydroperoxide interaction during the oxidation of methyl
507 linoleate. Eur Food Res Technol 214:52-57
- 508 27 Mäkinen M, Hopia A (2000) Effects of α -tocopherol and ascorbyl palmitate on the
509 isomerization and decomposition of methyl linoleate hydroperoxides. Lipids
510 35:1215-1223
- 511 28 Frankel EN (2005) Hydroperoxide formation. In: Frankel EN (ed) Lipid oxidation.
512 The Oily Press, Bridgwater (UK), pp 25-50
513

Figure legends

Fig. 1. Oxidative patterns found for FAME containing α - or δ -tocopherol at 100 °C. (**a-a'**) HL samples containing 100 and 1000 mg/kg tocopherol. (**b-b'**) HO samples containing 100 and 1000 mg/kg tocopherol. (Circles) Samples containing α -tocopherol. (Squares) Samples containing δ -tocopherol. Solid symbols correspond to hydroperoxides and hollow symbols to tocopherol.

Fig. 2. Relationships between hydroperoxides detected by ELS and hydroperoxydienes by UV in the HO samples. (**a**) Samples containing α -tocopherol. (**b**) Samples containing δ -tocopherol. The linear equation found for the data in **A** was $y=1.21x$ with a correlation coefficient of 0.9984.

Fig. 3. Induction periods and the oxidative stability index (OSI) values determined in Rancimat. (**a-a'**) HL samples. (**b-b'**) HO samples. The induction periods for the HL and HO control samples were 0.7 and 1.7 h, respectively. The OSI values for the HL and HO control samples were 2.23 and 5.12 h, respectively. Error bars express standard deviation and asterisks denote significant differences ($p < 0.05$).

Fig. 4. Influence of tocopherol concentration on the stabilization factor (F). (**a**) HL samples. (**b**) HO samples. (Circles) Samples containing α -tocopherol. (Squares) Samples containing δ -tocopherol. Data obtained from the OSI values.

Fig. 5. Relationships between keto- and hydroperoxy-dienes in the HL (**a-c**) and HO (**a'-c'**) samples at different concentrations of tocopherols: 100 mg/kg (**a-a'**), 500 mg/kg (**b-b'**) and 1000 mg/kg (**c-c'**). (Triangles) Control samples, (squares) samples containing α -tocopherol, (circles) samples containing δ -tocopherol.

Table 1 Global rate constants (k (mmol/kg s)^{0.5} x 10⁴) and linear correlation coefficients (r) for hydroperoxide formation during the induction period of samples containing δ -tocopherol considering a reaction order of 0.5.

Toc (mg/kg)	HL		HO	
	k	r	k	r
0	13.8 ± 0.4a	0.9986	5.0 ± 0.3a	0.9957
10	5.2 ± 0.4b	0.9974	1.10 ± 0.09b	0.9899
50	2.6 ± 0.2c	0.9913	0.54 ± 0.02c	0.9968
100	1.9 ± 0.1d	0.9949	0.54 ± 0.02c	0.9978
500	1.31 ± 0.09e	0.9917	0.32 ± 0.01d	0.9959
1000	1.13 ± 0.05f	0.9941	0.33 ± 0.01d	0.9942

Different letters for different treatments in each lipid sample denote significant differences ($p < 0.05$).

Table 2 Initial oxidation rates (W (mmol/kg s) x 10⁴) for hydroperoxide formation.

Toc (mg/kg)	HL- α	HL- δ	HO- α	HO- δ
0	25.6 $\pm$ 3.1d	25.6 $\pm$ 3.1e	3.5 $\pm$ 0.9f	3.5 $\pm$ 0.9e
10	4.0 $\pm$ 0.4bA	7.9 $\pm$ 0.9dB	0.40 $\pm$ 0.02bA	1.17 $\pm$ 0.13cB
50	2.0 $\pm$ 0.1aA	5.8 $\pm$ 0.2cB	0.36 $\pm$ 0.02aA	0.61 $\pm$ 0.02aB
100	2.2 $\pm$ 0.1aA	5.0 $\pm$ 0.5bB	0.43 $\pm$ 0.01cA	0.79 $\pm$ 0.13bB
500	4.1 $\pm$ 0.2bA	3.7 $\pm$ 0.3aA	1.11 $\pm$ 0.03dA	1.07 $\pm$ 0.13cA
1000	6.5 $\pm$ 0.2cB	4.6 $\pm$ 0.5bA	1.56 $\pm$ 0.06eB	1.12 $\pm$ 0.16cA

Different lower case letters for different tocopherol concentrations in each sample denote significant differences ($p < 0.05$). Different capital letters for different antioxidants in each lipid substrate denote significant differences ($p < 0.05$).

Table 3 Antioxidant activity (F/ORR) of α - and δ -tocopherol on the inhibition of hydroperoxides.

Toc (mg/kg)	HL- α	HL- δ	HO- α	HO- δ
10	18	8	26	9
50	93	21	78	54
100	115	36	97	63
500	114	111	61	102
1000	83	157	49	133

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5