Effects of α- and γ-Tocopherols on Formation of Hydroperoxides and Two Decomposition Products from Methyl Linoleate

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ABSTRACT: The antioxidant effects of α- and γ-tocopherols (at 0, 10, 100, 500, and 1000 ppm) were evaluated in a model system based on the autoxidation of methyl linoleate in bulk for 4 d at 40°C. Samples were collected every 24 h and analyzed for the 9 *cis,trans,* 9 *trans,trans,* 13 *cis,trans,* and 13 *trans,trans* isomers of hydroperoxide, hydroxy, and ketodiene oxidation products by high-performance liquid chromatography. Results showed that both α- and γ-tocopherols are effective hydrogen donors as evidenced by their abilities to inhibit the formation of hydroperoxides, hydroxy compounds, and ketodienes and the *cis,trans* to *trans,trans* isomerization of hydroperoxides. Compared with γ-tocopherol, α-tocopherol was a more efficient antioxidant at very low concentrations (10 ppm) but a less efficient antioxidant at the high concentrations (100–1000 ppm). This paradoxical behavior is explained on the basis of differences in ease of hydrogen donation between the two tocopherol homologs. Although α-tocopherol shows some loss of efficiency with increasing concentration, it is not a prooxidant when compared to the control void of antioxidants.

Paper no. J9394 in *JAOCS 77*, 801–806 (August 2000).

KEY WORDS: Antioxidants, autoxidation, hydroperoxides, hydroxy compounds, ketodienes, methyl linoleate, α-tocopherol, γ-tocopherol.

The major antioxidants for vegetable oils are α- and γ-tocopherols (1). They are associated with the polyunsaturated fatty acids mainly to provide protection against autoxidation (2). The classical autoxidation mechanism is a free radical chain reaction which can be described in terms of initiation, propagation, and termination:

Initiation $\quad LH \rightarrow L^{\bullet} + H^{\bullet}$ [1]

Propagation $\quad L^{\bullet} + O_2 \rightarrow LOO^{\bullet}$ [2]

$\quad LOO^{\bullet} + LH \rightarrow LOOH + L^{\bullet}$ [3]

Termination $\quad LOO^{\bullet} + LOO^{\bullet} \rightarrow$ nonradical products [4]

$\quad LOO^{\bullet} + L^{\bullet} \rightarrow$ nonradical products [5]

$\quad L^{\bullet} + L^{\bullet} \rightarrow$ nonradical products [6]

In free radical reactions, tocopherols are known to scavenge propagating peroxyl radicals by at least two mechanisms: (i) hydrogen donation to peroxyl radicals to produce hydroperoxides (Eq. 7) and (ii) formation of adduct compounds between the resulting tocopheroxyl radical and a second peroxyl radical (Eq. 8). Both α- and γ-tocopherols were reported to undergo these reactions with peroxyl radicals (3,4).

$$LOO^{\bullet} + TOH \rightarrow LOOH + TO^{\bullet} \quad [7]$$

$$LOO^{\bullet} + TO^{\bullet} \rightarrow TO\text{-}OOL \rightarrow \text{a variety of products} \quad [8]$$

Although it is generally accepted that α-tocopherol has a higher ability for hydrogen donation than its γ-homolog, the latter was often found a better antioxidant in pure lipid-phase systems especially when comparisons were made using high concentrations (5–10). In fact α-tocopherol was reported to act as a prooxidant rather than an antioxidant when present at high concentrations (11–13). The relatively low potency of α-tocopherol, compared to γ-tocopherol, was attributed to possible participation of α-tocopherol itself or its tocopheroxyl radical in a number of not fully characterized propagative reactions (2,14). These reactions were considered responsible for the reported prooxidant behavior of α-tocopherol when present at high concentrations. In previous works (15,16), no prooxidant effect was found for either α- or γ-tocopherol when present at concentrations between 500–2000 ppm in purified vegetable oil triacylglycerols, and the reasons for the significant variation in relative efficacy seem to be more complex than what might be perceived from reviewing available literature (2). As a matter of fact, α-tocopherol is more efficient than γ-tocopherol at low concentrations and less efficient at high concentrations (15,16). The mechanistic aspects necessary to explain this controversy are not yet clear, and more research is needed to clarify paradoxes in the antioxidant effects of natural tocopherols (2).

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In antioxidant research, the focus is usually on the effects on hydroperoxide formation. However, autoxidation of lipids includes several sequences of radical-mediated reactions, including isomerization and formation of secondary products from hydroperoxides, in which antioxidants have effects. One property which was related to high hydrogen-donating power of antioxidants, especially α-tocopherol, is their effect on the relative distribution of the isomeric species of the hydroperoxides formed during autoxidation (17). Initially formed *cis,trans* peroxyl radicals (kinetic products) rearrange to *trans,trans* isomers (thermodynamic products) during oxidation, and this isomerization is inhibited by hydrogen-donating antioxidants (18). Four isomeric hydroperoxides, *viz.* methyl-13-hydroperoxy-*cis*-9-*trans*-11-octadecadienoate (13*cis*,*trans*), methyl-13-hydroperoxy-*trans*-9-*trans*-11-octadecadienoate (13*trans*,*trans*), methyl-9-hydroperoxy-*cis*-10-*trans*-12-octadecadienoate (9*cis*,*trans*), and methyl-9-hydroperoxy-*trans*-10-*trans*-12-octadecadienoate (9*trans*,*trans*), are known to form upon autoxidation of methyl linoleate. A mechanistic explanation, based on reversible oxygen addition, was provided by Porter *et al.* (19) to explain changes in the position of the hydroperoxyl groups and the stereochemistry of the double bonds in the absence of strong hydrogen donors. The *cis*,*trans*/*trans*,*trans* hydroperoxide ratio was found to be dependent on the temperature, concentration of the fatty acid undergoing oxidation, and the overall hydrogen-donating power of the solvents and solutes (19). Tocopherols, and other strong hydrogen-donors, are believed to increase the *cis*,*trans*/*trans*,*trans* product ratio by trapping initially formed peroxyl radicals before they can undergo rearrangement (17,18). The work of Gottstein and Grosch (9) has shown that, due to its fast hydrogen-donating ability, α-tocopherol is much more efficient in inhibiting the isomerization of methyl linoleate *cis,trans* hydroperoxides than γ-tocopherol. In addition to their effect on hydroperoxide isomer distribution, antioxidants were reported to have effects on the decomposition rate and reaction routes of hydroperoxides. Frankel and Gardner (20) showed that tocopherols inhibit the β-scission of alkoxyl radical formed during hydroperoxide decomposition, and Hopia *et al.* (21) suggested that tocopherols may inhibit hydroperoxide decomposition by scavenging alkoxyl radical, thus increasing formation of hydroxy compounds. The effect of antioxidants on these, and possibly other, reactions is not fully understood, and their importance is yet to be clarified.

To complement previous works on the effects of α- and γ-tocopherols on hydroperoxide formation in purified triacylglycerols (15,16), this paper aims to compare the effects of α- and γ-tocopherols (at 0, 10, 100, 500, and 1000 ppm levels) on the formation of hydroperoxides and two of the secondary decomposition products during the oxidation of bulk methyl linoleate. Beside providing information on whether α-tocopherol will show a prooxidant effect at high concentrations, these data provide additional information on the effects of α- and γ-tocopherols on the relative levels of *cis,trans* and *trans,trans* isomers of methyl linoleate hydroperoxides.

EXPERIMENTAL PROCEDURES

Methyl linoleate (purity >99%; Nu-Chek-Prep. Inc., Elysian, MN) was used as the oxidation substrate without any further purification. Initial hydroperoxide content, analyzed spectrometrically by the thiocyanate method (22), was <5 mmol/kg. α-Tocopherol (purity 98.8%) and γ-tocopherol (98.8%) were purchased from Merck (Darmstadt, Germany). Solvents of high-performance liquid chromatography (HPLC) grade were supplied by Rathburn Chemicals (Walkerburn, United Kingdom). Diethyl ether was purified by silica solid-phase extraction cartridge prior to HPLC analysis to remove peroxides and the stabilizing agent.

Two experiments were performed, and each oxidation experiment was run in duplicate to confirm the order of activity of antioxidants studied. Methyl linoleate samples (0.5 g) were oxidized in the presence of antioxidants at levels of 10, 100, 500, and 1000 ppm at 40°C in the dark in open vials (15 mL). Antioxidants were added into oil as freshly prepared ethanol solutions, and ethanol was finally removed by purging the oil with nitrogen. Methyl linoleate without antioxidant addition served as the control (n = 4 in each experiment). Since the rate of oxidation varied between the two experiments conducted in this study, we did not combine the results from these experiments but the two experiments were comparable in terms of trends of tocopherol action. Results reported in this paper are mean values from the duplicate determinations in one experiment except for controls which were analyzed in quadruplicate. The repeatability data for controls in the reported experiment, given in Table 1, show that there are larger experimental errors in the determination of peak areas of hydroxy compounds compared to peak areas of hydroperoxides and ketodienes.

Hydroperoxide formation, isomer distribution, and formation of hydroxy and ketodiene compounds were monitored by HPLC as described before (21) with the following modifications: the mobile phase was 12% diethyl ether in heptane with the flow rate of 0.4 mL/min. A Supelcosil column LC-SI 57930 (250 × 2.1 mm, 5 μm particle size; Supelco, Bellefonte, PA), preceded by a Supelcosil precolumn of 20 × 2.1 mm, was used for chromatographic separations. The HPLC apparatus was composed of autosampler (Waters 700 Satellite WISP; Millipore Corporation, Milford, MA), one pump (Waters 501), UV-VIS/diode array detector (Waters 996 PDA), and a computer work station. Data handling was performed using Millenium 2010 software (Waters).

RESULTS AND DISCUSSION

Figure 1 shows plots for the formation of total hydroperoxides, hydroxy compounds, and ketodienes for different concentrations of α- and γ-tocopherols (0, 10, 100, 500, and 1000 ppm) in methyl linoleate. In agreement with previous results obtained with purified vegetable oil triacylglycerols (15,16), α-tocopherol was a more efficient antioxidant than γ-tocopherol at 10 ppm concentrations, but this order of efficiency was

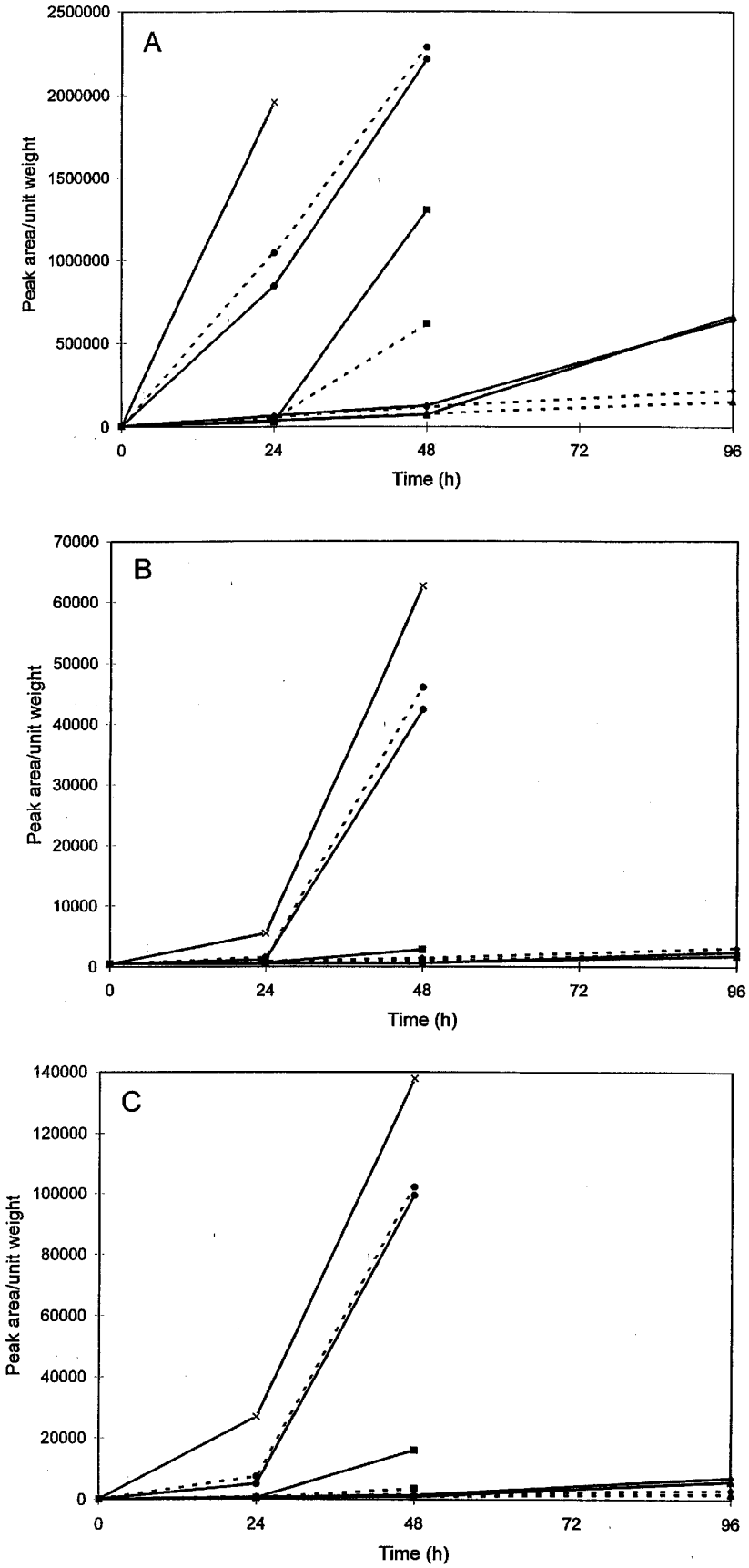

FIG. 1. Effect of α (—)- and γ (----)-tocopherols on the formation of (A) hydroperoxides, (B) hydroxy compounds, and (C) ketodienes from methyl linoleate during 4 d of oxidation at 40°C. Tocopherol concentrations: control (X), 10 ppm (●), 100 ppm (■), 500 ppm (▲), and 1000 ppm (◆).

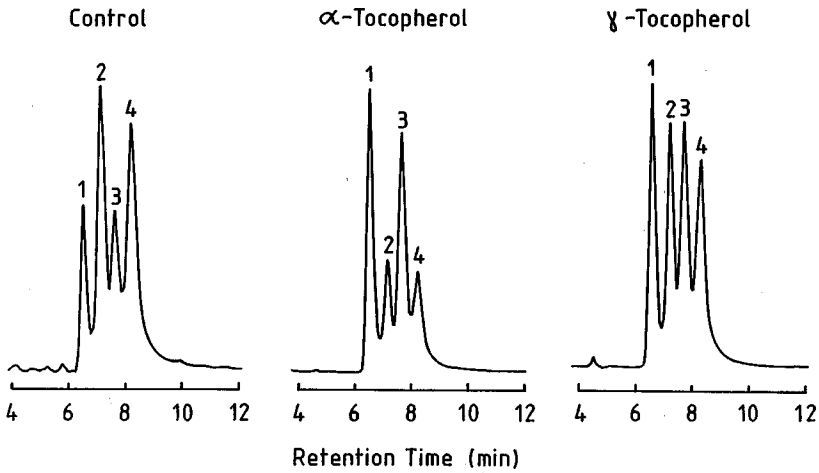

FIG. 2. Parts of high-performance liquid chromatograms showing the effects of α- and γ-tocopherols at 1000 ppm concentrations on the relative levels of hydroperoxide isomers (compared to control) after 48 h of oxidation. Peak identification: (1) methyl-13-hydroperoxy-*cis*-9-*trans*-11-octadecadienoate (13*cis,trans*), (2) methyl-13-hydroperoxy-*trans*-9-*trans*-11-octadecadienoate (13*trans,trans*), (3) methyl-9-hydroperoxy-*cis*-10-*trans*-12-octadecadienoate (9*cis,trans*), and (4) methyl-9-hydroperoxy-*trans*-10-*trans*-12-octadecadienoate (9*trans,trans*). Chromatogram was recorded at 234 nm using diode array detector.

reversed at 100 ppm. Despite this change in relative efficiency, α-tocopherol was still a strong antioxidant and had no prooxidant effect *per se* when present at high concentrations in bulk lipids. Lampi *et al.* (16) proposed that the higher efficiency of α-tocopherol compared to γ-tocopherol may be correlated to a higher stability of the former when both tocopherols are present at low concentrations. These effects were attributed to increased significance of the speed of hydrogen donation when the concentration of tocopherols, with respect to the oxidation rate, is significantly low. When the concentration of tocopherols is high, e.g., α-tocopherol 1000 ppm (Fig. 1A), a loss of efficiency occurs (15,16). Studies on these phenomena are ongoing, and results will be reported shortly.

Although lipid hydroperoxides are rather stable under favorable conditions of low temperatures, antioxidant protection, dilute solutions, and absence of metal catalysts, they dis-

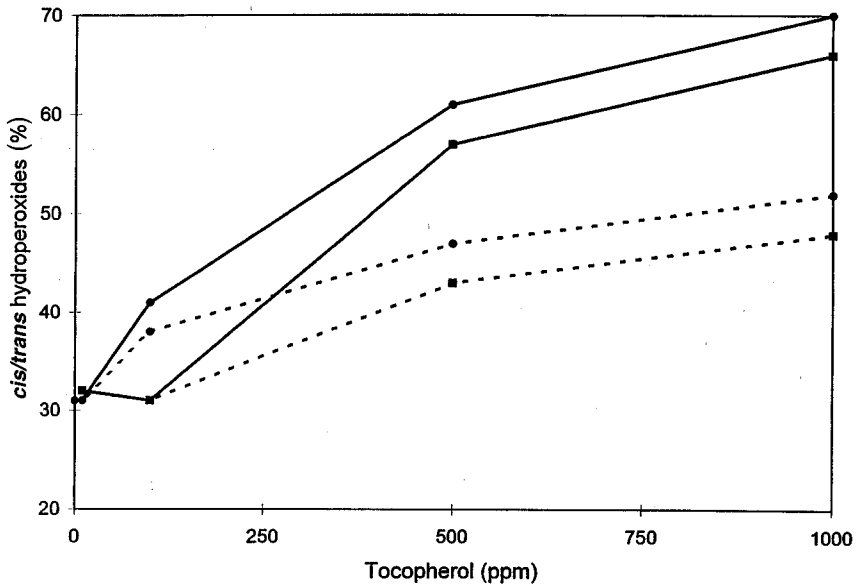

FIG. 3. Effect of α (—)- and γ (-----)-tocopherol concentrations on the percentages of total *cis,trans* hydroperoxides after 24 (●) and 48 (■) h of oxidation.

TABLE 1
Repeatability Data for the Analysis of Total Hydroperoxides, Hydroxy Compounds, and Ketodienes in Controls (n = 4)[a]

Heating time at 40°C (h)	Peak area/μg oxidized methyl linoleate		
	Hydroperoxides	Hydroxy compounds	Ketodienes
0	5 594 ± 74.3 (1)	490 ± 27.9 (6)	240 ± 10.9 (5)
24	1 901 051 ± 66 186.3 (3)	6 280 ± 948.2 (15)	27 286 ± 637.6 (2)
48	2 096 989 ± 114 020.1 (5)	68 963 ± 8 725.2 (13)	136 415 ± 2 858.3 (1)

[a]Results are expressed as means ± standard deviation (coefficient of variation, %).

sociate under unfavorable conditions to give a wide number of volatile, nonvolatile, and polymeric oxidation products (23). Of the secondary oxidation products formed from lipid hydroperoxides, it was possible to co-analyze the 18-carbon hydroxy and ketodiene oxidation products with hydroperoxides using normal-phase HPLC and diode-array detection (21). Results presented in Figure 1 (B and C) show that hydroxy- and ketodiene-compounds are formed in controls and tocopherol-containing methyl linoleate and that their formation propagates after induction periods. Both tocopherols did inhibit formation of hydroxy- and ketodiene-oxidation products (Fig. 1B and 1C) in a similar way to their inhibition of hydroperoxide formation (Fig. 1A). Again, α-tocopherol was more efficient than γ-tocopherol in inhibiting formation of both hydroxy- and ketodiene-derivatives at 10 ppm, but this order of efficiency is reversed at 100 ppm and higher concentrations.

TABLE 2
Effect of Tocopherol Concentration on Ketodiene/Hydroxy Compound Ratio (at 24 h of oxidation at 40¡C)[a]

Tocopherol concentration	Ketodienes/hydroxy compounds	
	α-T	γ-T
Control	4.5	4.5
10	4.3	4.7
100	0.8	0.7
500	0.8	0.8
1000	MV	0.9

[a]Means of duplicate determinations except for controls where n = 4. Abbreviations: α-T, α-tocopherol; γ-T, γ-tocopherol; MV = missing value.

As mentioned in the introduction, antioxidants not only inhibit formation of total hydroperoxides but also affect the relative levels of isomeric hydroperoxides. Figure 2 presents parts of the HPLC chromatograms, showing the hydroperoxide isomers formed at 48 h in control samples and in samples containing 1000 ppm levels of α- or γ-tocopherols. The percentages of *cis,trans* isomers of hydroperoxides in samples containing different levels of tocopherols after 24 and 48 h of heating are presented in Figure 3. As with the findings of Gottstein and Grosch (9), both α- and γ-tocopherols increased the relative abundance of the *cis,trans* hydroperoxides and, in accordance with a higher hydrogen-donating power, the effect was much greater for α-tocopherol than for γ-tocopherol. Figure 3 also shows that the percentages of *cis,trans* hydroperoxides decreased with increasing oxidation time (level).

Besides the experimental repeatability data, Table 1 also shows that more hydroxy than ketodiene compounds were present in the controls in the beginning of the oxidation, but higher amounts of ketodienes were present after 24 and 48 h of oxidation. The exact mechanism responsible for formation of hydroxy- and ketodiene-products during the autoxidation of methyl linoleate is not fully understood, and the kinetic and thermodynamic factors governing their formation have not been reported before and warrant future research attention. These derivatives can either be formed from peroxyl or alkoxyl radicals of methyl linoleate. Both products can be formed from alkoxyl radicals as follows:

$$R_1R_2CHO^{\bullet} + RH \rightarrow R_1R_2CHOH + R^{\bullet} \quad [9]$$

$$R_1R_2CHO^{\bullet} + ROO^{\bullet} \rightarrow R_1R_2C{=}O + ROOH \quad [10]$$

Another mechanism which can be responsible for the formation of both compounds is the Russell mechanism of disproportionation of hydroperoxyl radicals, which dictates that equal amounts of hydroxy- and ketodiene-compounds are produced (24):

$$2\,R_1R_2CHOO^{\bullet} \rightarrow R_1R_2\text{-}CH\text{-}OOOO\text{-}HCR_1R_2 \rightarrow R_1R_2CHOH + R_1R_2C{=}O + {}^1O_2 \quad [11]$$

Other mechanisms that might be responsible for the formation of only ketodienes (12,13) in controls and in samples containing low levels of tocopherols may include hydroxyl radical elimination after a 1,3- or a 1,5-hydrogen shift (25):

$$R_1R_2CHOO^{\bullet} \rightarrow R_1R_2\text{-}C^{\bullet}\text{-}OOH \rightarrow R_1R_2C{=}O + {}^{\bullet}OH \quad [12]$$

$$R_1R_2CHOO^{\bullet} + O_2 \rightarrow R_1R_2\text{-}CH\text{-}OOOO^{\bullet} \rightarrow R_1R_2\text{-}C^{\bullet}\text{-}OOOOH \rightarrow R_1R_2C{=}O + {}^{\bullet}OH + {}^1O_2 \quad [13]$$

Table 2 presents data on the relative levels of ketodiene/hydroxy as affected by α- and γ-tocopherols after 24 h of autoxidation. The ketodiene/hydroxy ratio decreased in samples containing tocopherols as compared with controls, indicating that relatively more ketodienes are formed at higher oxidation levels. To further investigate the effects of tocopherols on these secondary oxidation products, we are currently performing more detailed experiments on hydroperoxide decomposition.

In summary, results presented in this work support the previous claims (15,16) that α-tocopherol is not a prooxidant with

respect to hydroperoxide formation in pure lipid models and extend them to show that both α- and γ-tocopherols are effective hydrogen donors as evidenced by their abilities to increase the *cis,trans/trans,trans* ratio of hydroperoxides as well as to inhibit formation of hydroxy- and ketodiene-derivatives.

ACKNOWLEDGMENT

This work was financially supported by the Academy of Finland.

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[Received September 16, 1999; accepted June 7, 2000]