In Vitro Test for the Effectiveness of Antioxidants as Inhibitors of Thiyl Radical-Induced Reactions with Unsaturated Fatty Acids

Erika Klein and Nikolaus Weber*

Institute for Biochemistry and Technology of Lipids, H.P. Kaufmann-Institute, Federal Centre for Cereal, Potato and Lipid Research, Piusallee 68, D-48147 Münster, Germany

Attacks of thiyl radicals on the double bonds of unsaturated fatty acids lead to stereomutation (cis-, trans-isomerization without double-bond migration) and addition reactions (thioether formation). On the basis of these findings, an in vitro test system has been developed which allows the study of the effectiveness of specific antioxidants in preventing thiyl radical-induced attacks on unsaturated fatty acids. The test involves thermal treatment of a mixture of oleic (*cis*-9-octadecenoic) acid and 1-tetradecanethiol with the antioxidant, followed by measurement of the extent of formation of the products of stereomutation and addition (i.e., elaidic (*trans*-9-octadecenoic) acid and isomeric 9(10)-*S*-tetradecylstearic acids, respectively) by gas chromatography of their methyl esters as a function of antioxidant concentration. Antioxidants such as octyl gallate, ascorbic acid 6-*O*-palmitate, ubiquinone 50 (coenzyme Q_{10}), *rac*-α-tocopherol, and 2,6-di-*tert*-butyl-4-methylphenol (BHT) were tested for their ability to protect the >C═C< double bond of oleic acid against attacks of thiyl radicals generated from 1-tetradecanethiol by heating. The results show that octyl gallate, ascorbic acid 6-*O*-palmitate and, to some extent, ubiquinone 50 (coenzyme Q_{10}) were highly effective in preventing reactions of free thiyl radicals with oleic acid, whereas *rac*-α-tocopherol and BHT were moderately effective.

Keywords: *Antioxidants; in vitro test; unsaturated fatty acids; cis-, trans-isomerization; stereomutation; thiyl radicals*

INTRODUCTION

Free radicals are a highly reactive species of chemical compounds which are induced by the transfer of energy, e.g., in the form of UV irradiation or heating. They are also formed under physiological conditions, particularly by mitochondrial oxidation processes or the hepatic cytochrome P450 redox system. Once formed, free radicals attack other cell constituents which are converted to various chemical derivatives. There is increasing evidence that reactions of free radicals and their derivatives are involved in pathological processes leading to several types of human diseases including atherosclerosis, diabetes, and cancer (*1*). For example, oxidation of low-density lipoproteins (LDL) may play an important role in the initiation and progression of atherosclerosis (*2*−*4*) and physiological thiol compounds such as homocystein may be involved in this mechanism (*5*).

Various chemical properties seem to be involved in the action of thiol compounds, predominantly redox potential and capacity for the formation of reactive thiyl radicals ($RS^{\bullet}$), which attack, e.g., the double bonds of unsaturated fatty acids leading finally to cis, trans-isomerization and addition reactions (*6*−*10*). In feed and food, as well as in organs and tissues, thiyl radicals may arise predominantly from biological thiol compounds such as cystein and glutathion. Typically, in a reaction of oleic (*cis*-9-octadecenoic) acid (**1**) with 1-tetradecanethiol, the tetradecanethiyl radical-induced stereomutation (cis, trans-isomerization without double-bond migration) and addition (thioether formation) lead to formation of elaidic (*trans*-9-octadecenoic) acid (**3**) and isomeric 9(10)-*S*-tetradecylstearic acids (**4**), respectively (Figure 1). The ability of a reagent to affect the reactions induced by free thiyl radicals − among other reactions − can thus modulate either the protective or noxious properties of individual thiol compounds.

Various antioxidants such as tocopherols, ascorbic acid, and polyphenols are known to have radical scavenger properties. They are able to trap free radicals and prevent initiation of radical chain reactions. We have developed a test system which allows one to study specifically the effectiveness of antioxidants in reducing thiyl radical-induced reactions with unsaturated fatty acids. This test involves the formation of thiyl radicals by heating 1-tetradecanethiol in the presence of oleic acid and various concentrations of the antioxidant, as well as the quantitative determination by gas chromatography of the methyl esters of the reaction products, i.e., *trans*-9-octadecenoic acid and isomeric 9(10)-*S*-tetradecylstearic acids, respectively. This in vitro test may help to assess the effectiveness of various antioxidants in protecting >C═C< double bonds of chemical and biochemical compounds against thiyl radical-induced reactions.

EXPERIMENTAL PROCEDURES

Chemicals. 1-Tetradecanethiol, oleic acid, octyl gallate, ascorbic acid 6-*O*-palmitate, ubiquinone 50 (coenzyme Q_{10}), and *rac*-α-tocopherol were purchased from Sigma-Aldrich-Fluka (Deisenhofen, Germany). 2,6-Di-*tert*-butyl-4-methylphenol (BHT) was obtained from E. Merck (Darmstadt, Germany). Trimeth-

* To whom correspondence should be addressed. Telephone: +49-251-43510. Telefax: +49-251-519 275. E-mail: ibtfett@uni-muenster.de.

10.1021/jf000725m CCC: $20.00
Published on Web 03/01/2001

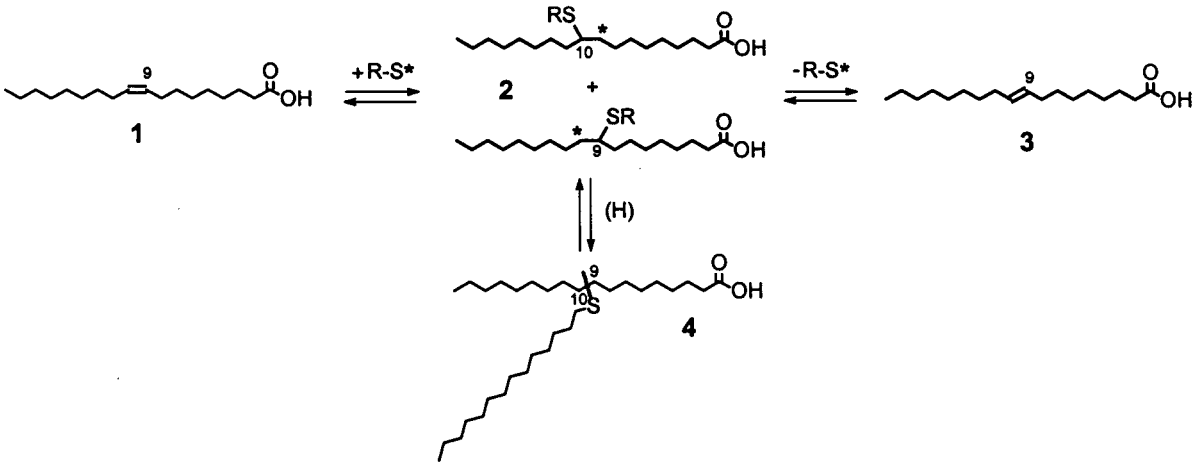

Figure 1. Mechanism of tetradecanethiyl radical-induced reactions of oleic (*cis*-9-octadecenoic) acid (**1**) leading to elaidic (*trans*-9-octadecenoic) acid (**3**) and isomeric 9(10)-*S*-tetradecylstearic acids (**4**) by stereomutation and addition reactions, respectively, with isomeric alkyl radicals (**2**) as intermediates (R, tetradecyl moiety; RS*, tetradecanethiyl radical).

ylsulfonium hydroxide (TMSH) reagent (0.2 M TMSH in methanol) was a product of Macherey-Nagel (Düren, Germany). Isomeric methyl 9(10)-*S*-tetradecylstearates were prepared by UV irradiation of a mixture of oleic acid and 1-tetradecanethiol as described previously (*10*).

Radical Scavenger Test. Solutions in isohexane of oleic acid (20 μmol) and 1-tetradecanethiol (60 μmol) were mixed in brown, screw-capped Teflon-lined 1 mL autosampler vials with various proportions (0.1, 0.5, 1, 5, 10, 50, and 100 μmol) of one of the above antioxidants which were dissolved in dichloromethane or dichloromethane/methanol (9:1, v/v). Samples without antioxidant were used as blanks. The solvents were removed in a stream of nitrogen at room temperature and then in vacuo at 45 °C for 10 min. Finally, the vials were heated in a heating block at 100 °C for 10 min in a nitrogen atmosphere. After cooling, the reaction mixtures were dissolved in 0.6 mL of methyl *tert*-butyl ether/methanol (9:1, by vol.), and a 20 μL aliquot was used for GC derivatization.

Derivatization for GC. Under standard conditions, 40 μL of TMSH reagent was added to a 20-μL aliquot of reaction mixture containing about 1 μmol 1-tetradecanethiol and 0.3 μmol oleic acid, as well as varying concentrations of antioxidants (the proportion of derivatization reagent was doubled for samples containing more than 10 μmol antioxidant). The derivatization mixtures, 1−2 μL, were injected into the gas chromatograph (*11*, *12*).

Gas Chromatography. A Hewlett-Packard (Böblingen, Germany) HP-5890 Series II gas chromatograph equipped with a flame ionization detector was used. Separations were carried out on a 0.1 μm Quadrex 400-5HT (Quadrex Corp., New Haven, CT) fused silica capillary column, 25 m × 0.25 mm i.d., using hydrogen as the carrier gas (column pressure 50 kPa) initially at 120 °C for 2 min, followed by linear programming from 120 °C to 180 °C at 5 °C × min^{-1} and from 180 °C to 380 °C at 20 °C × min^{-1}, the final temperature of 380 °C was held for 6 min. The split ratio was 1:10. Peaks in gas chromatograms were assigned by comparison of their retention times with those of known standards. Peak areas and percentages were calculated using Hewlett-Packard 3365 Series GC ChemStation software. Stereomutation of oleic acid, as well as the formation of thiol adducts, was checked by GC after derivatization with TMSH of the reaction mixture to methyl *cis*-9- and *trans*-9-octadecenoates, isomeric methyl 9(10)-*S*-tetradecylstearates, and methyl tetradecanesulfide (*11*, *12*) using the GC conditions described above.

Data Analysis. Data of radical scavenger tests are expressed as means ± SEM of three independent experiments ($n = 3$) per antioxidant concentration, whereas data of blanks (without antioxidant) are given as mean ± SEM ($n = 16$). The difference between the proportion of elaidic acid in the blanks and that of maximum inhibition (Δ_{max}) was calculated, whereas the concentration of antioxidant which reduced stereomutation by 50% ($C_{\Delta 50}$) was determined graphically (cf. Figure 2) (*13*). Similarly, data of radical inhibition tests were analyzed from the formation of isomeric 9(10)-*S*-tetradecylstearic acids, i.e., the products of addition reaction.

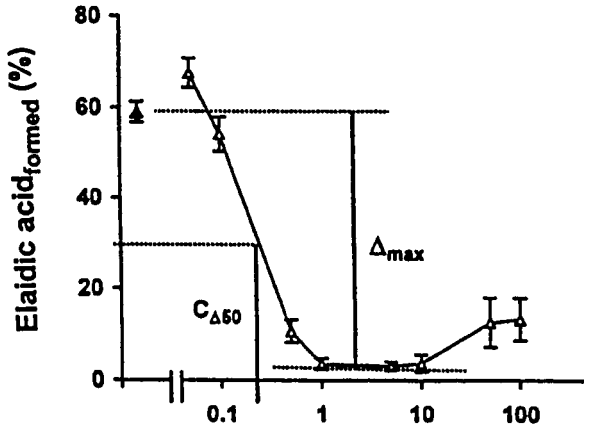

Figure 2. Graphic determination of the effectiveness of octyl gallate as scavenger of thiyl radicals (▲, blank, without antioxidant, $n = 16$; △, other determinations in the presence of various concentrations of octyl gallate, $n = 3$; Δ_{max}, difference between the proportion of elaidic acid in the blanks and that of maximum inhibition; $C_{\Delta 50}$, concentration of octyl gallate which reduced stereomutation by 50%).

RESULTS AND DISCUSSION

Free radicals, including thiyl radicals, are involved in the pathology of many types of human diseases (*1*, *5*) leading to the development of various radical scavengers for the treatment of such disorders. Thiyl radicals are formed from thiol compounds by physical, chemical, and biochemical energy transfer. They reversibly attack the double bonds of unsaturated fatty acids leading to mixtures of cis- and trans-fatty acids as well as addition products (thioethers) as is shown in Figure 1 (*6*, *8*−*10*, *14*). Both reactions, i.e., cis/trans-isomerization and addition, are radical chain reactions caused by thiyl radicals. Key intermediates are the isomeric alkyl radicals **2** (cf. Figure 1) which are formed by the addition of an alkanethiyl radical (RS$^{\bullet}$) to the >C=C< double bond. Alkyl radicals **2** react either by β-elimination of an alkanethiyl radical leading to a mixture of the geometrical isomers **1** (*cis*-9-octadecenoic acid) and **3** (*trans*-9-octadecenoic acid), or by addition of hydrogen

Table 1. Inhibition of Thiyl Radical-Induced Stereomutation of Oleic Acid to Elaidic Acid by Various Concentrations of the Different Antioxidants

	elaidic acid (*trans*-9-octadecenoic acid) formed (%)[a] in the presence of various concentrations of antioxidants (μmol/test)							
antioxidant	0.05	0.1	0.5	1	5	10	50	100
octyl gallate	67.6 ± 3.3	54.1 ± 3.8	10.7 ± 2.5	3.6 ± 1.2	3.1 ± 0.9	3.7 ± 1.9	12.7 ± 5.3	13.3 ± 4.5
ascorbic acid 6-*O*-palmitate	75.4 ± 0.3	68.7 ± 2.7	14.4 ± 4.5	10.5 ± 2.7	4.5 ± 1.3	5.0 ± 0.7	4.0 ± 0.7	4.8 ± 1.7
ubiquinone 50	50.2 ± 2.2	45.9 ± 2.0	29.1 ± 0.6	24.3 ± 1.4	8.8 ± 0.7	4.8 ± 0.1	4.5 ± 2.0	-
rac-α-tocopherol	55.8 ± 2.3	54.4 ± 0.8	65.9 ± 2.9	64.9 ± 3.4	50.5 ± 5.8	36.8 ± 7.9	16.8 ± 5.2	7.3 ± 0.6
2,6-di-*tert*-butyl-4-methylphenol (BHT)	68.2 ± 1.9	72.7 ± 2.4	67.4 ± 2.1	56.7 ± 5.6	29.3 ± 3.1	19.7 ± 0.8	26.7 ± 2.6	27.5 ± 0.4

[a] Mean ± SEM ($n = 3$); blank (without antioxidant), 59.0 ± 2.3 ($n = 16$).

Table 2. Inhibition of Thiyl Radical-Induced Formation of Addition Products, i.e., Isomeric 9(10)-*S*-Tetradecylstearic Acids, from Oleic Acid and 1-Tetradecanethiol by Various Concentrations of the Different Antioxidants

	isomeric 9(10)-*S*-tetradecylstearic acids formed (%)[a] in the presence of various concentrations of antioxidants (μmol/test)							
antioxidant	0.05	0.1	0.5	1	5	10	50	100
octyl gallate	4.1 ± 0.6	2.0 ± 0.5	0.2 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	0.5 ± 0.2
ascorbic acid 6-*O*-palmitate	9.5 ± 0.6	6.0 ± 1.1	0.5 ± 0.2	0.4 ± 0.1	0.1 ± 0.1	0.2 ± 0.1	0.1 ± 0.1	0.3 ± 0.1
ubiquinone 50	4.8 ± 0.4	4.3 ± 0.5	1.6 ± 0.1	1.0 ± 0.1	0.2 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	-
rac-α-tocopherol	3.9 ± 0.1	3.7 ± 0.1	6.1 ± 0.7	5.9 ± 0.3	4.4 ± 0.9	2.4 ± 1.0	0.5 ± 0.20	0.1 ± 0.1
2,6-di-*tert*-butyl-4-methylphenol (BHT)	10.2 ± 0.3	8.8 ± 1.2	5.1 ± 0.3	3.6 ± 0.7	1.6 ± 0.5	0.7 ± 0.1	0.7 ± 0.1	0.6 ± 0.1

[a] Mean ± SEM ($n = 3$); blank (without antioxidant), 5.6 ± 0.4 ($n = 16$).

Table 3. Comparison of the Effectiveness of Various Antioxidants as Radical Scavengers in Thiyl Radical-Induced Reactions Using the Inhibition of both the Stereomutation of Oleic Acid to Elaidic Acid and the Addition of 1-Tetradecanethiol to Oleic or Elaidic Acids

	stereomutation			addition		
antioxidant	Δ_{max} (%)	$C_{\Delta 50}$ (μmol)	effectiveness[a] (μmol^{-1})	Δ_{max} (%)	$C_{\Delta 50}$ (μmol)	effectiveness[a] (μmol^{-1})
octyl gallate	56	0.23	243	5.5	0.08	67.5
ascorbyl 6-*O*-palmitate	56	0.3	187	5.5	0.25	22.0
ubiquinone 50	54	0.4	135	5.5	0.24	22.9
rac-α-tocopherol	52	15.1	3.4	5.5	8.3	0.7
2,6-di-*tert*-butyl-4-methyl-phenol (BHT)	40	3.8	10.5	5.0	0.46	10.9

[a] Defined as product $\Delta_{max} \times (C_{\Delta 50})^{-1}$, with Δ_{max} given as maximum inhibition of stereomutation (%) or as maximum inhibition of addition reaction (%) and $(C_{\Delta 50})^{-1}$ as the reciprocal concentration of antioxidant which leads to a 50% inhibition of stereomutation and addition reactions, respectively (*13*).

leading to the isomeric thioether derivatives (**4**) of 9-octadecenoic acid.

We present data of an in vitro test which uses the inhibition of thiyl radical-induced reactions on >C=C< double bonds leading to stereomutation and addition reactions as a measure of the radical-inhibiting activity of synthetic and natural antioxidants. This test may help to assess the efficiency of antioxidants in protecting unsaturated fatty acids such as oleic and linoleic acids against the attacks of thiyl radicals (cf. Figure 1).

The effectiveness of octylgallate and other antioxidants as radical scavengers in thiyl radical-induced reactions was tested for stereomutation reaction of oleic (*cis*-9-octadecenoic) acid (**1**) to elaidic (*trans*-9-octadecenoic) acid (**3**) in the above system by three independent experiments per antioxidant concentration (Figure 2). The results of gas chromatographic analyses, i.e., the proportions of elaidic acid formed, are given as mean ± SEM ($n = 3$). Test mixtures without antioxidant were used as blanks. The maximum difference between the proportion of elaidic acid formed in the blanks and that formed in the presence of various antioxidant concentrations is shown as maximum inhibition (Δ_{max}), whereas the concentration of antioxidant which reduced stereomutation by 50% ($C_{\Delta 50}$) was determined graphically as demonstrated in Figure 2.

The inhibition of stereomutation reaction of oleic (*cis*-9-octadecenoic) acid (**1**) to elaidic (*trans*-9-octadecenoic) acid (**3**) by various concentrations of different antioxidants is shown in Table 1. It is obvious from these results that, under the conditions described, 1 to 5 μmol of octyl gallate reduced cis-, trans-isomerization of oleic acid by >90%. Similar results were obtained for ascorbic acid 6-*O*-palmitate and, to some extent, for ubiquinone 50 as the antioxidants (Table 1). In contrast, the data obtained with BHT and *rac*-α-tocopherol revealed that both antioxidants were less effective in reducing stereomutation of oleic acid as compared to octyl gallate and ascorbic acid 6-*O*-palmitate. The slight increase of stereomutation reaction observed with 50 and 100 μmol octyl gallate/test may be due to prooxidant effects which are known to occur in the presence of high concentrations of antioxidants (*15*).

Similarly, the inhibition of thiyl radical-induced addition reactions was determined for various concentrations of antioxidants (Table 2). These results show that isomeric 9(10)-*S*-tetradecylstearic acids (**4**), derived from the addition of 1-tetradecanethiol to oleic and elaidic acids, are formed in relatively small amounts (around 6%) as compared to elaidic acid (around 60%) derived from stereomutation reaction of oleic acid (Table 1). However, the trend of the inhibition of the addition

reaction by the different antioxidants (Table 2) was similar to those found for the stereomutation reaction (Table 1).

Table 3 shows the comparison of the effectiveness of various antioxidants as inhibitors for thiyl radical-induced stereomutation or addition reactions. It is obvious from these results that both methods reveal a similar order of radical scavenging activities showing highest effectiveness for octyl gallate and ascorbic acid 6-*O*-palmitate, followed by ubiquinone 50, and rather low effectiveness for BHT and *rac*-α-tocopherol.

It is obvious from the data given in Tables 1 and 2, as well as from the mechanism of both reactions, i.e., stereomutation and addition (cf. Figure 1), that the formation of alkyl radical intermediates (**2**) is most probably inhibited by the reaction with the various antioxidants. In contrast, the interruption of the radical chain reaction by dimerization of two alkanethiyl radicals (RS$^{\bullet}$) to dialkyl disulfides (RS−SR) seems to be not inhibited by the antioxidants used. This is evident from our results that the formation of ditetradecyl disulfide by dimerization of two thiyl radicals was generally low (<1%) under the conditions described and it was little influenced by moderate concentrations (≤ 10 μmol/test) of antioxidants. However, high antioxidant concentrations (with the exception of BHT) obviously increased ditetradecyl disulfide formation (data not shown).

Results similar to those observed for oleic acid were obtained for the stereomutation reaction of linoleic acid in the presence of thiyl radicals. However, the pattern of geometrical isomers formed was much more complicated (*8*−*10*), and high molecular products of an addition reaction containing more than one *S*-tetradecyl moiety may not be detected using the GC method described (data not shown).

In contrast to the attacks of thiyl radicals, free radical autoxidation, and singlet oxidation, of oleic acid leads predominantly to hydroperoxy derivatives with positional and geometrical isomerization of the double bond (*16*). Typical primary and secondary reaction products such as hydroperoxy, epoxy, and hydroxy derivatives of oleic acid, however, have not been detected in blanks in the absence of both 1-tetradecanethiol and antioxidants (data not shown).

Thiol compounds which are ubiquitous components of plant and animal food, or which have been added, e.g., cysteine, to food and feed may lead to the formation of undesirable trans-fatty acids from the corresponding cis-fatty acids, particularly if and when heat treatment is required in food processing. The unexplained occurrence of small proportions of trans-fatty acids in native vegetable oils may be due to thiyl radical-induced stereomutation reactions. Moreover, our results demonstrate that the use of thiol compounds as antioxidants (as described recently by Papadopoulou and Roussis (*17*)) is not recommended for fats and oils. The method described here gives one the opportunity to check the ability of specific individual antioxidants to inhibit the formation of trans-fatty acids and thioether derivatives of unsaturated fatty acids which may arise from thiyl radical-induced stereomutation and addition reactions in food and feed.

To summarize, an in vitro test system has been developed which allows the study of the effectiveness of specific antioxidants in preventing thiyl radical-induced attacks on unsaturated fatty acids leading to stereomutation and addition reactions. Our results show that octyl gallate and ascorbic acid 6-*O*-palmitate and, to some extent, ubiquinone 50 (coenzyme Q_{10}) were highly effective in preventing stereomutation and addition reactions of free tetradecanethiyl radicals with oleic acid, whereas *rac*-α-tocopherol and BHT were moderately effective.

LITERATURE CITED

(1) Halliwell, B.; Gutteridge, J. M. C., Eds. *Free Radicals in Biology and Medicine*, 3rd ed.; Oxford University Press: Oxford, UK, 1999.

(2) Regnström, J.; Nilsson, J.; Tornvall, P.; Landou, C.; Hamsten, A. Susceptibility to low-density lipoprotein oxidation and coronary atherosclerosis in man. *Lancet* **1992**, *339*, 1183−1186.

(3) Ross, R. The pathogenesis of atherosclerosis: A perspective for the 1990s. *Nature* **1993**, *362*, 801−809.

(4) Steinberg, D.; Parthasarathy, S.; Carew, T. E.; Khoo, J. C.; Witztum, J. L. Beyond cholesterol. Modifications of low-density lipoprotein that increase its atherogenicity. *New Engl. J. Med.* **1989**, *320*, 915−924.

(5) Stamler, J. S.; Slivka, A. Biological chemistry of thiols in the vasculature and in vascular-related disease. *Nutr. Rev.* **1996**, *54*, 1−30.

(6) Griesbaum, K. Probleme und Möglichkeiten der radikalischen Addition von Thiolen an ungesättigte Verbindungen. *Angew. Chem.* **1970**, *82*, 276−290.

(7) Kircher, H. W. The elaidinization of methyl oleate with mercaptans. *J. Am. Oil Chem. Soc.* **1964**, *41*, 351−354.

(8) Schöneich, C.; Dillinger, U.; von Bruchhausen, F.; Asmus, K.-D. Oxidation of polyunsaturated fatty acids and lipids through thiyl and sulfonyl radicals: Reaction kinetics and influence of oxygen and structure of thiyl radicals. *Arch. Biochem. Biophys.* **1992**, *292*, 456−467.

(9) Schwinn, J.; Sprinz, H.; Drössler, K.; Leistner, S.; Brede, O. Thiyl radical-induced cis/trans-isomerization of methyl linoleate in methanol and of linoleic acid residues in liposomes. *Int. J. Radiat. Biol.* **1998**, *74*, 359−365.

(10) Weber, N.; Klein, E.; Vosmann, K.; Mukherjee, K. D. Antioxidants eliminate stereomutation and thioether formation during lipase-catalyzed thioesterification and transthioesterification for the preparation of uniform cis- and trans-unsaturated thioesters. *Chem. Phys. Lipids* **2000**, *105*, 215−223.

(11) Schulte, E. Gas Chromatography of acylglycerols and fatty Acids with capillary columns. In *Analysis of Lipids*, Mukherjee, K. D., Weber, N., Eds.; CRC Press: Boca Raton, FL, 1993; pp 139−148.

(12) Vosmann, K.; Klein, E.; Weber, N. *S*-Methyl derivatives from thiol compounds by the pyrolytic reaction with trimethylsulfonium hydroxide. *Lipids* **1998**, *33*, 1037−1041.

(13) Sanchez-Moreno, C.; Larrauri, J. A.; Saura-Calixto, F. A procedure to measure the antiradical efficiency of polyphenols. *J. Sci. Food Agric.* **1998**, *76*, 270−276.

(14) Chatgilialoglu, C.; Ferreri, C.; Ballesteri, M.; Mulazzani, Q. G.; Landi, L. cis-trans Isomerization of monounsaturated fatty acid residues in phospholipids by thiyl radicals. *J. Am. Chem. Soc.* **2000**, *122*, 4593−4601.

(15) Satue, M. T.; Huang, S.-W.; Frankel, E. N. Effect of natural antioxidants in virgin olive oil on oxidative stability of refined, bleached, and deodorized olive oil. *J. Am. Oil Chem. Soc.* **1995**, *72*, 1131−1137.

(16) Frankel, E. N. Chemistry of free radical and singlet oxidation of lipids. *Prog. Lipid Res.* **1985**, *23*, 197−221.

(17) Papadopoulou, D.; Roussis, I. G. Inhibition of corn oil oxidation by thiols. *Ital. J. Food Sci.* **2000**, *12*, 239−242.

Received for review June 19, 2000. Revised manuscript received December 28, 2000. Accepted January 4, 2001.

JF000725M