Antioxidant Activity of Caffeic Acid and Dihydrocaffeic Acid in Lard and Human Low-Density Lipoprotein†

Jae-Hak Moon and Junji Terao*

Department of Nutrition, School of Medicine, The University of Tokushima, Kuramoto-cho 3, Tokushima 770-0042, Japan

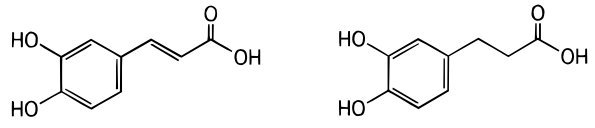
Caffeic acid (CA) has been implied as an important source of natural antioxidants in various agricultural products. We compared the antioxidative activity of CA and dihydrocaffeic acid (HCA) in lard and the low-density lipoprotein (LDL) system to know the role of the 2,3-double bond on the appearance of its antioxidative property. HCA was more effective than CA in enhancing oxidative stability of lard at 60 °C. Inhibition by HCA of copper ion-induced oxidation of human LDL was less effective than that by CA, whereas there was no significant difference between the two compounds in the capacity of 1,1-diphenyl-2-picrylhydrazyl radical scavenging and the activity of lipid peroxyl radical scavenging in solution. It can be concluded that the 2,3-double bond in the structure of CA affects the efficiency of antioxidative activity depending on the environment where the oxidation happens, although it is rarely responsible for its inherent activity.

Keywords: *Antioxidants; caffeic acid; dihydrocaffeic acid; free radical-scavenging activity; low-density lipoprotein*

INTRODUCTION

Lipid peroxidation induces oxidative deterioration of lipid-containing foods. Effective and nontoxic natural antioxidants are therefore required to prevent this event during storage and processing. In addition, the metabolic fate of antioxidants should be known because of not only their safety but also their physiological function after absorption into the body.

Caffeic acid [3-(3,4-dihydroxyphenyl)-2-propenoic acid, CA], which is a hydroxycinnamic acid derivative (Figure 1), is one of the antioxidative compounds in various agricultural products such as coffee beans, potatoes, grains, and vegetables (Friedman, 1997; Gutfinger, 1981; Hudson et al., 1980; Kimura et al., 1985; Ky et al., 1997). CA and some of its related compounds are absorbed into the body after oral administration, and specific metabolites were detected in urine and plasma (Finkle et al., 1962; Goldstein et al., 1984; Herrman, 1956; Horning et al., 1967; Jacobson et al., 1983; Nardini et al., 1995, 1997). In addition, dihydrocaffeic acid [3-(3,4-dihydroxyphenyl)-2-propionic acid, HCA] (Figure 1) is formed by intestinal bacteria as a degradation product of CA (Peppercorn et al., 1971) and eriocitrin (Miyake et al., 1997) which was isolated from lemon fruit (*Citrus limon* B_{URM}. f.) as a flavonoid glycoside (eriodictyol 7-glycoside). It should be also noted that HCA was detected as a catechol metabolite of CA from human plasma (Goldstein et al., 1984). CA may therefore act as an in vivo antioxidant after absorption into the body. However, little is known on the antioxidative activity of HCA. Furthermore, the role of the 2,3-double bond in CA on the antioxidant activity is not known.



Caffeic acid Dihydrocaffeic acid

Figure 1. Structures of caffeic acid (CA) and dihydrocaffeic acid (HCA).

Thus, this study was undertaken to compare the activity of HCA with that of CA in edible oil and human low-density lipoprotein (LDL). We discuss the effectiveness of HCA and CA as antioxidative additives in edible oil and potential antioxidants for preventing oxidative damage in blood circulation.

MATERIALS AND METHODS

Chemicals. CA was obtained from Nacalai Tesque Inc. (Kyoto, Japan) and purified by HPLC (254 nm, 3.0 mL/min) using ODS column (YMC-Pack, 6 × 150 mm, 5 μm; YMC, Kyoto, Japan; water/methanol/acetic acid = 75:25:1, v/v/v). The purified CA showed a single peak in the HPLC chromatogram. HCA was synthesized chemically from CA. Briefly, CA in a solution of methanol reacted with palladium carbon under hydrogen gas bubbling. After filtration of palladium carbon (0.45 μm, GL chromatodisk; GL Science, Tokyo, Japan), HCA was separated from the reaction solution using HPLC (column: YMC-Pack, 6 × 150 mm, 5 μm; YMC, Kyoto, Japan; water/methanol/acetic acid = 72:28:1, v/v/v). The HCA fraction obtained was detected as a single peak in the HPLC chromatogram. HCA fraction was identified by GC−MS analysis after trimethylsilylation using *N*,*O*-bis(trimethylsilyl)acetamide (Tokyo Kasei Co., Ltd., Tokyo, Japan). A QP-5000 mass spectrometer (Shimadzu, Kyoto, Japan) equipped with a fused silica DB-1 capillary column (0.25-mm i.d. × 30 m, 0.25-mm film thickness; G & W Scientific, CA) was used with in electron impact mode (70 eV). The carrier gas was helium at a flow rate of 1.0 mL/min, and the injection port temperature was 250 °C. The column oven temperature was held at 50 °C for

† This work was done at the National Food Research Institute, Tsukuba, Japan, where J.-H.M. worked as a Science and Technology Agency fellow.

* Corresponding author (tel, +81-886-31-3111, ext. 2511; fax, +81-886-33-7089; e-mail, terao@nutr.med.tokushima-u.ac.jp).

10.1021/jf9805799 CCC: $15.00
Published on Web 11/12/1998

6 min, before being elevated to 240 °C at 10 °C/min and then kept constant for 5 min. The compound was identified as 3,4-bis(trimethylsilyloxy)hydroxycinnamic acid trimethylsilyl ester by the mass spectrum: *m/z* (rel int) 73 ($SiMe_3^+$, 100%), 179 ($C_9H_7O_4^+$, 89%), 219 [$(SiMe_3^+)_3$, 5%], 267 [$(OSiMe_3^+)_3$, 38%], 383 ($M - CH_3^+$, 8%), 398 (M^+, 50%). *d*-α-Tocopherol (α-Toc) was kindly supplied by Eisai Co. (Tokyo, Japan). 2,2′-Azobis-(2,4-dimethylvaleronitrile) (AMVN), 1,1-diphenyl-2-picrylhydrazyl (DPPH), and cupric sulfate were obtained from Kanto Chemical Co. Inc. (Tokyo, Japan). Methyl linoleate was purchased from Tokyo Kasei Co. Ltd. (Tokyo, Japan). Methyl linoleate was further purified by column chromatography using Florisil (Wako Pure Chemical Co., Osaka, Japan) to remove contaminant hydroperoxides and impurities (Terao et al., 1977). Lard without any additives was a kind gift from Tsukishima Food Industrial Co. Ltd. (Tokyo, Japan). HPLC analysis in this study was carried out under room temperature. All other chemicals and solvents were of analytical grade.

Measurement of Oxidative Stability of Lard. The procedures for the measurement of oxidative stability of lard were followed by the method of Koga et al. (1994). Lard (1.0 g) was measured accurately and spread out in a glass dish (5.0-cm diameter). CA, HCA, or α-Toc (0.4 μmol) dissolved in a solution of methanol/chloroform (2:8, v/v, 3.0 mL) was added to the lard and then evaporated under nitrogen and in vacuo. The mixture was allowed to autoxidize at 60 °C in the dark with circulating air. At regular intervals, the weight gain was recorded.

Measurement of Copper Ion-Induced LDL Oxidation. LDL particles were isolated from heparinized plasma of healthy volunteers by differential density-gradient ultracentrifugation according to the method described previously (Silva et al., 1997). Isolated LDL preparation was used immediately for the experiment or was stored at 4 °C under nitrogen atmosphere until use for a maximum of 1 week. After the solution was incubated for 5 min at room temperature, CA or HCA (each final concentration of 12 μM) dissolved in methanol was added to the reaction solution such that the final concentration of methanol in LDL suspension was 0.5% (v/v). The oxidation reaction was initiated by the addition of cupric sulfate (final concentration of 5 μM) dissolved in PBS buffer (pH 7.4). Cholesteryl ester hydroperoxides (CE-OOH) were measured by HPLC as an index of oxidation. The procedures of CE-OOH determination were the same as those described previously (Silva et al., 1997).

Scavenging of 1,1-Diphenyl-2-picrylhydrazyl (DPPH) Radical. Free radical-scavenging activity of each antioxidant was assayed using a stable free radical, DPPH, according to the method of Blois (1958). The reaction mixture contained 1.0 mL of 0.5 mM DPPH and 0.1 mL of an ethanol solution of the antioxidant at different concentrations (5−350 μM) except for cysteine (dissolved with 100 mM Tris-HCl buffer, pH 7.4). Finally, the total volume of the reaction mixture was adjusted to 2.0 mL with 100 mM Tris-HCl buffer (pH 7.4). After the reaction was carried out at room temperature for 20 min in the dark, the free radical-scavenging activity of each antioxidant was quantified by decolorization at 517 nm.

Measurement of Peroxyl Radical-Scavenging Activity in Solution. The peroxyl radical-scavenging activities of CA and HCA were determined by measuring the inhibition of an AMVN-induced peroxidation of methyl linoleate in a solution (Terao et al., 1993). The reaction mixture contained 100 mM methyl linoleate, 80 μM antioxidant, and 10 mM AMVN in a solution (1.0 mL) of *n*-hexane/2-propanol/ethanol (8:3:1, v/v/v) and was incubated in the dark at 37 °C. At specific intervals, an aliquot of the reaction mixture (10.0 μL) was withdrawn and injected onto the HPLC column. The HPLC conditions employed and the calculation of kinetic parameters of peroxyl radical-scavenging activity for antioxidants have been described previously (Burton et al., 1981; Nagao et al., 1990; Terao, 1989; Yamamoto et al., 1982).

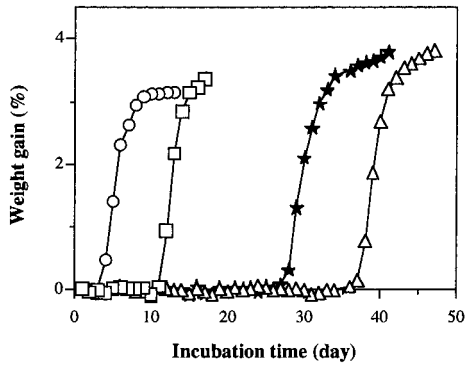


Figure 2. Effect of CA, HCA, and α-Toc on autoxidation of lard at 60 °C. Antioxidants (0.4 μmol) were mixed with lard (1.0 g): (○) no addition; (★) caffeic acid; (△) dihydrocaffeic acid; (□) α-tocopherol. The data are representative of two experiments.

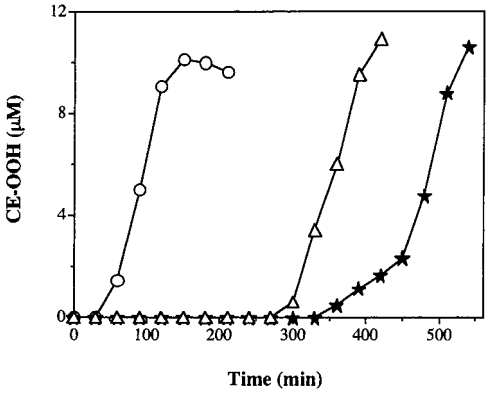


Figure 3. Effect of CA and HCA on copper ion-induced oxidation of human LDL. LDL (0.4 mg of protein/mL) was incubated at 37 °C in the absence or presence of CA or HCA. At the indicated times, aliquots were withdrawn and the levels of cholesteryl ester hydroperoxides (CE-OOH) were determined as described in Materials and Methods. Final concentrations of cupric sulfate and CA/HCA were 5 and 12 μM, respectively, and initial concentration of α-tocopherol was 12.5 nmol/mg of protein: (○) no addition; (★) caffeic acid; (△) dihydrocaffeic acid. The data are representative of two experiments.

RESULTS

Effect of CA, HCA, and α-Toc on the Oxidative Stability of Lard. Lard with or without additives showed a rapid weight gain after an induction period when stored at 60 °C (Figure 2). The end of the induction period was easily recognized by a sharp weight gain. The induction periods of lard with and without antioxidants were 5, 11, 28, and 38 days for control (without additive), α-Toc, CA, and HCA, respectively. Therefore, CA and HCA were more effective than α-Toc on the improvement of oxidative stability. Interestingly, HCA showed a longer induction period than CA.

Effect of CA and HCA on the Copper Ion-Induced LDL Oxidation. LDL isolated from human plasma was oxidized with copper ion at 37 °C under air. Antioxidant activity was determined by the inhibition of CE-OOH formation with or without CA and HCA (Figure 3). Control (no external addition of antioxidant) showed some induction period at the initial stage of the oxidation reaction. It could be derived from the internal antioxidants present in LDL such as α-Toc (12.5 nmol/mg of protein LDL). Although the addition of HCA gave a significant increase in the induction period, it was shorter than that obtained by CA.

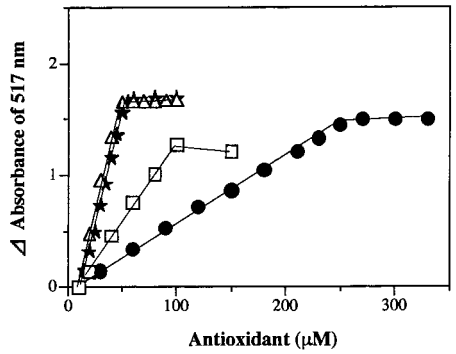


Figure 4. DPPH radical-scavenging effect of antioxidants. The reaction mixture contained 0.5 mM DPPH (1.0 mL) and an ethanol solution (0.1 mL) of the antioxidant at different concentrations (5−350 μM) except for cysteine (dissolved with 100 mM Tris-HCl buffer, pH 7.4). Absorbance of each reaction mixture was monitored at 517 nm as described in Materials and Methods after the total volume of the reaction mixture was finally adjusted to 2.0 mL with 100 mM Tris-HCl buffer (pH 7.4): (★) caffeic acid; (△) dihydrocaffeic acid; (□) α-tocopherol; (●) cysteine. The data are representative of two experiments.

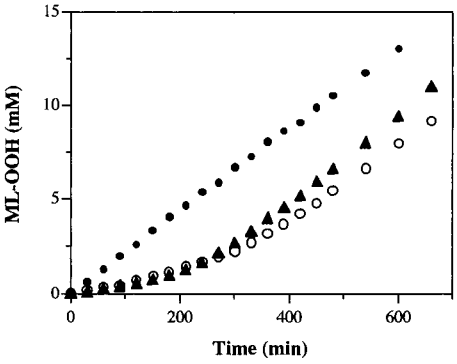


Figure 5. Effects of CA and HCA on AMVN-initiated oxidation of methyl linoleate in solution. The reaction system consisted of methyl linoleate (100 mM), AMVN (10 mM), and antioxidants (80 μM) in a mixture of *n*-hexane/2-propanol/ethanol (8:3:1, v/v/v): (○) caffeic acid; (▲) hydrocaffeic acid; (●) no addition. The data are representative of two experiments.

Scavenging Effect of CA and HCA on DPPH Radical. Figure 4 shows the relationship between the concentration of DPPH trapped (indicated by the decrease of 517 nm) and the concentration of antioxidants in the DPPH radical-scavenging assay. The number of DPPH radicals trapped by antioxidants was calculated from the assumption that one molecule of cysteine scavenges one molecule of DPPH radical. The numbers of CA, HCA, and α-Toc were calculated to be 5.0, 5.0, and 2.5, respectively. Thus, both CA and HCA can scavenge five DPPH radicals regardless of the structual difference in the 2,3-double bond. In addition, the DPPH radicals to be scavenged by CA and HCA were 2 times more than that by α-Toc.

Peroxyl Radical-Scavenging Activity of CA, HCA, and α-Toc in Solution. We determined the peroxyl radical-scavenging activities of CA, HCA, and α-Toc by measuring the inhibition of free radical oxidation of methyl linoleate in solution (Figure 5). The induction period was obtained from the accumulation rate of ML-OOH when CA, HCA, or α-Toc was added to the reaction mixture. The values of chain propagation reaction rate for CA (3.36×10^{-7} Ms^{-1}) was quite similar to that of HCA (3.82×10^{-7} Ms^{-1}), although the rate of oxidation reaction during induction periods (R_{inh}, 0.85×10^{-7} Ms^{-1}) and the term of induction period (t_{inh}, 14.3×10^3 s) for HCA were not significantly different from those of CA (R_{inh}, 1.13×10^{-7} Ms^{-1}; t_{inh}, 18.0×10^3 s). Consequently, it is concluded that CA and HCA scavenge lipid peroxyl radical by a similar mechanism, and their activities in solution are within the same magnitude.

DISCUSSION

Considerable studies have demonstrated that the *o*-dihydroxyl structure is essential for free radical-scavenging and metal-chelating effects in hydroxycinnamic acid derivatives (Chen et al., 1997; Laranjinha et al., 1994; Nardini et al., 1995; Terao et al., 1993). However, the role of the conjugated double bond at the 2,3-position on its antioxidant activity has not been verified as far as we know. Our attention was focused on the contribution of the 2,3-double bond on its antioxidant activity. We therefore compared the antioxidant activity of CA with that of its derivative containing no 2,3-double bond, HCA, in lard, and we also examined their antioxidant capacities on copper ion-induced oxidative modification of human LDL.

It is unlikely that the 2,3-double bond of CA is the radical-targeting site because no difference was observed between their DPPH radical-scavenging capacities (Figure 4). Furthermore, the inhibitory effects of CA and HCA on radical chain oxidation of methyl linoleate in solution were indistinguishable (Figure 5); that is, the olefinic double bond in CA hardly affects its inherent chain propagating peroxyl radical-scavenging ability in solution. Nevertheless, antioxidant activity in the autoxidation of lard increased in the order of α-Toc < CA < HCA (Figure 2). This result suggests that the olefinic double bond in CA is an important factor for enhancing antioxidant activity in bulk oil autoxidation.

Nardini et al. (1997) demonstrated that CA accumulated at ca. 1.0 μg/mL concentration in nonfasting rats (plasma) fed a 0.8% CA-containing diet. In addition, HCA was found in human plasma as a metabolite of CA after dietary supplementation and was suggested to be formed by the intestinal bacteria (Goldstein et al., 1984). Therefore, both CA and HCA seem to accumulate in human plasma by the intake of CA-rich food. Our copper ion-induced LDL oxidation study (Figure 4) demonstrated that HCA has considerable antioxidant capacity on oxidative modification of LDL. Several works (Abu-Amsha et al., 1996; Meyer et al., 1998; Nardini et al., 1995) have shown that among hydroxycinnamic acids, CA exerts an effective inhibition of copper ion-induced LDL in vitro. In addition, this study first demonstrated that HCA is also a possible candidate for the antioxidant in blood circulation. *o*-Dihydroxyl structure of HCA seems to be responsible for the radical-scavenging activity as well as the copper ion-chelating activity, similarly to CA (Nardini et al., 1995). It is very likely that CA acts as an in vivo antioxidant after absorption as well as a food antioxidant for the prevention of degradation of food quality. In addition, HCA, a possible metabolite of CA containing the *o*-dihydroxyl structure, may also play a role in the antioxidant capacity of dietary CA.

In conclusion, both CA and HCA can be effective antioxidants for lard and the human plasma LDL oxidation system. The conjugated olefinic double bond

of CA in the side chain of the catechol group affects the efficiency of antioxidant activity of CA depending on the environment where the oxidation happens.

LITERATURE CITED

Abu-Amsha, R.; Croft, K. D.; Puddey, I. B.; Proudfood, J. M.; Beilin, L. J. Phenolic content of various beverages determines the extent of inhibition of human serum and low-density lipoprotein oxidation in vitro: identification and mechanism of action of some cinnamic acid derivatives from red wine. *Clin. Sci.* **1996**, *91*, 449−458.

Blois, M. S. Antioxidant determinations by the use of a stable free radical. *Nature* **1958**, *181*, 1199−1200.

Burton, G. W.; Ingold, K. U. Autoxidation of biological molecules. 1. The antioxidant activity of vitamin E and related chain-breaking phenolic antioxidants in vitro. *J. Am. Chem. Soc.* **1981**, *103*, 6472−6477.

Chen, J. H.; Ho, C. T. Antioxidant activities of caffeic acid and its related hydroxycinnamic acid compounds. *J. Agric. Food Chem.* **1997**, *45*, 2374−2378.

Finkle, B. J.; Lewis, J. C.; Corce, J. W.; Lundin, R. E. Enzyme reactions with phenolic compounds: formation of hydroxystyrenes through decarboxylation of 4-hydroxycinnamic acids by *Aerobacter*. *J. Biol. Chem.* **1962**, *237*, 2926−2931.

Friedman, M. Chemistry, biochemistry, and dietary role of potato polyphenols. A review. *J. Agric. Food Chem.* **1997**, *45*, 1523−1540.

Goldstein, D. S.; Stull, R.; Markey, S. P.; Marks, E. S.; Keiser, H. R. Dihydrocaffeic acid: a common contaminant in the liquid chromatographic-electrochemical measurement of plasma catecholamines in man. *J. Chromatogr.* **1984**, *311*, 148−153.

Gutfinger, T. Polyphenols in olive oils. *J. Am. Oil Chem. Soc.* **1981**, *58*, 966−968.

Herrman, V. K. Uber Kaffeesaure and chlorogensaure. *Pharmazie* **1956**, *11*, 433−449.

Horning, M. G.; Boucher, E. A.; Moss, A. M. The study of urinary acids and related compounds by gas-phase analytical methods. *J. Gas Chromatogr.* **1967**, *5*, 297−302.

Hudson, B. J. F.; Mahgoub, S. E. O. Naturally occurring antioxidants in leaf lipids. *J. Sci. Food Agric.* **1980**, *31*, 646−650.

Jacobson, E. A.; Newmak, H.; Baptista, J.; Bruce, W. R. A preliminary investigation of the metabolism of dietary phenolics in human. *Nutr. Rep. Int.* **1983**, *28*, 1409−1417.

Kimura, Y.; Okuda, H.; Okuda, T.; Hatano, T.; Agata, I.; Arichi, S. Studies on the activities of tannins and related compounds from medicinal plants and drugs. VII. Effects of extracts of leaves of *Artemisia* species, and caffeic acid and chlorogenic acid on lipid metabolic injury in rats fed peroxidized oil. *Chem. Pharm. Bull.* **1985**, *33*, 2028−2034.

Koga, T.; Terao, J. Antioxidant activity of a novel phosphatidyl derivative of vitamin E in lard and its model system. *J. Agric. Food Chem.* **1994**, *42*, 1291−1294.

Ky, C.-L.; Noirot, M.; Hamon, S. Comparison of five purification methods for chlorogenic acids in green coffee beans (*Coffea* sp.). *J. Agric. Food Chem.* **1997**, *45*, 786−790.

Laranjinha, J. A. N.; Almeida, L. M.; Madeira, V. M. C. Reactivity of dietary phenolic acids with peroxyl radicals: Antioxidant activity upon low-density lipoprotein peroxidation. *Biol. Pharmacol.* **1994**, *48*, 487−494.

Meyer, A. S.; Donovan, J. L.; Pearson, D. A.; Waterhouse, A. L.; Frankel, E. N. Fruit hydoxycinnamic acids inhibit human low-density lipoprotein oxidation in vitro. *J. Agric. Food Chem.* **1998**, *46*, 1783−1787.

Miyake, Y.; Yamamoto, K.; Osawa, T. Metabolism of antioxidant in lemon fruit (*Citrus limon* B_{URM}. f.) by human intestinal bacteria. *J. Agric. Food Chem.* **1997**, *45*, 3738−3742.

Nagao, A.; Terao, J. Antioxidant activity of 6-phosphatidyl-*l*-ascorbic acid. *Biochem. Biophys. Res. Commun.* **1990**, *172*, 385−389.

Nardini, M.; D'Aquino, M.; Tomassi, G.; Gentili, V.; Felice, M. D.; Scaccini, C. Inhibition of human low-density lipoprotein oxidation by caffeic acid and other hydroxycinnamic acid derivatives. *Free Radicals Biol. Med.* **1995**, *19*, 541−552.

Nardini, M.; Natella, F.; Gentili, V.; Felice, M. D.; Scaccini, C. Effect of caffeic acid dietary supplementation on the antioxidant defense system in rat: An *in vivo* study. *Arch. Biochem. Biophys.* **1997**, *342*, 157−160.

Peppercorn, M. A.; Goldman, P. Caffeic acid metabolism by bacteria of the human gastrointestinal tract. *J. Bacteriol.* **1971**, *108*, 996−1000.

Silva, E. L.; Tsushida, T.; Terao, J. Inhibition of mammalian 15-lipoxygenase-dependent lipid peroxidation in low-density lipoprotein by quercetin and quercetin monoglucosides. *Arch. Biochem. Biophys.* **1997**, *349*, 313−320.

Terao, J. Antioxidant activity of β-carotene-related carotenoids in solution. *Lipids* **1989**, *24*, 659−661.

Terao, J.; Matsushita, S. Products formed by photosensitized oxidation of unsaturated fatty acid esters. *J. Am. Oil Chem. Soc.* **1977**, *54*, 234−238.

Terao, J.; Matsushita, S. The peroxidizing effect of α-tocopherol on autoxidation of methyl linoleate in bulk phase. *Lipids* **1986**, *21*, 255−260.

Terao, J.; Karasawa, H.; Arai, H.; Nagao, A.; Suzuki, T.; Takama, K. Peroxyl radical scavenging activity of caffeic acid and its related phenolic compounds in solution. *Biosci. Biotechnol. Biochem.* **1993**, *57*, 1204−1205.

Yamamoto, Y.; Niki, E.; Kamiya, Y. Oxidation of lipids. V. Oxidation of methyl linoleate in aqueous solution. *Bull. Chem. Soc.* **1982**, *55*, 1548−1550.

Received for review June 1, 1998. Revised manuscript received September 29, 1998. Accepted October 2, 1998.

JF9805799