Inhibition of Oxidation in 10% Oil-in-Water Emulsions by β-Carotene with α- and γ-Tocopherols

Marina Heinonen*, Katri Haila, Anna-Maija Lampi, and Vieno Piironen

Food Chemistry Division, Department of Applied Chemistry and Microbiology, University of Helsinki, Finland

ABSTRACT: The effects of low concentrations of β-carotene, α-, and γ-tocopherol were evaluated on autoxidation of 10% oil-in-water emulsions of rapeseed oil triacylglycerols. At concentrations of 0.45, 2, and 20 μg/g, β-carotene was a prooxidant, based on the formation of lipid hydroperoxides, hexanal, or 2-heptenal. In this emulsion,1.5, 3, and 30 μg/g of γ-tocopherol, as well as 1.5 μg/g of α-tocopherol, acted as antioxidants and inhibited both the formation and decomposition of lipid hydroperoxides. Moreover, at a level of 1.5 μg/g, γ-tocopherol was more effective as an antioxidant than α-tocopherol. At levels of 0.5 μg/g, both α- and γ-tocopherol significantly inhibited the formation of hexanal but not the formation of lipid hydroperoxides. Oxidation was effectively retarded by combinations of 2 μg/g β-carotene and 1.5 μg/g γ- or α-tocopherol. The combination of β-carotene and α-tocopherol was significantly better in retarding oxidation than α-tocopherol alone. While γ-tocopherol was an effective antioxidant, a synergistic effect between β-carotene and γ-tocopherol could not be shown. The results indicate that there is a need to protect β-carotene from oxidative destruction by employing antioxidants, such as α- and γ-tocopherol, should β-carotene be used in fat emulsions.
JAOCS 74, 1047–1052 (1997).

KEY WORDS: Antioxidant, β-carotene, emulsion, prooxidant, tocopherols.

Autoxidation of lipids is one of the major factors that diminish food quality. Not only do foods with high amounts of fat oxidize, but low-fat foods are also prone to oxidation. Rancidity problems consequently may be aggravated by decreasing the lipid content from food ingredients. According to Roozen *et al.* (1), lowering the fat content of foods may increase the chances of flavor defects. On the other hand, color is extremely important in food quality. Carotenoids are a family of polyene colorants of which β-carotene, as well as many other carotenoids, such as annatto, capsanthin, capsorubin, lycopene, β-apo-8′-carotenal, ethyl esters of β-apo-8′-carotenic acid and lutein, can be used singly or in combination in various foods as colorants (2). In addition to their use as food colorants, carotenoids can, to some extent, be used to fortify dietary fat as provitamin A (3). However, isolation of the polyene makes carotenoids susceptible to oxidation, and the loss of carotenoid pigment is accelerated by free radicals (4,5).

It is well known that carotenoids, such as β-carotene, are effective singlet oxygen quenchers (6,7). However, the functions of carotenoids as free-radical scavengers in food and other biological systems are not as well defined. The antioxidant efficiency of β-carotene depends on the balance between radical trapping by and autoxidation of β-carotene. Both radical-trapping and autoxidation reactions consume β-carotene. However, according to Kennedy and Liebler (8), autoxidation reactions consume β-carotene without scavenging peroxyl radicals and may thus attenuate β-carotene antioxidant activity. β-Carotene has been demonstrated to possess antioxidant activity in inhibiting autoxidation in homogeneous solutions (9,10), liposomes (8,11), microsomes (12), lipoproteins (13, 14), and corneal endothelial cells (11). On the other hand, a prooxidant effect of β-carotene has been observed during autoxidation of methyl linoleate (15), soybean oil (16), rapeseed oil (RSO) (17), and RSO triacylglycerols (TAG) stripped of tocopherols (18,19). Plausible explanations in clarifying these discrepancies are the amount of oxygen present and the influence of other antioxidants, such as tocopherols. In the present study, the inhibition of lipid oxidation by low concentrations of β-carotene with α- and γ-tocopherols has been investigated during autoxidation of 10% oil-in-water emulsions.

MATERIALS AND METHODS

Reagents. β-Carotene was a gift from Roche Oy (Espoo, Finland), and α-and γ-tocopherols were purchased from E. Merck (Darmstadt, Germany). Organic solvents of high-performance liquid chromatographic (HPLC) grade were purchased from Rathburn Chemicals Limited (United Kingdom). Tween 20 (polyoxyethylene sorbitan monolaurate), decanol, and dinitrophenylhydrazine (DNPH) were obtained from Sigma Chemical Co. (St. Louis, MO).

Preparation and characterization of TAG fraction and emulsion samples. Commercial RSO was a gift from Van den Bergh Foods (Helsinki, Finland).The TAG fraction was purified from RSO by the chromatographic method described by Lampi *et al.* (20). The RSO TAG fraction was characterized

*To whom correspondence should be addressed at Food Chemistry Division, Department of Applied Chemistry and Microbiology,P.O. Box 27, FIN-00014, University of Helsinki, Finland.
E-mail: Marina.Heinonen@Helsinki.Fi.

by its tocopherol composition as analyzed by HPLC as described by Haila and Heinonen (18) and by its peroxide value (PV) as measured by the ferric thiocyanate method (21,22). The determination limit of tocopherols was 1 μg/g. The amount of γ- and α-tocopherols left in the RSO TAG fraction after purification was 1.2 and 0.1 μg/g, respectively, in the emulsions. β-Carotene (4.5–200 μg/g) in hexane and α- and γ-tocopherols (5–300 μg/g) in ethanol were added to the RSO TAG fraction to produce emulsions with 0.45, 2, and 20 μg/g of β-carotene and 0.5, 1.5, 3, and 30 μg/g of tocopherol. The organic solvents were evaporated under nitrogen.Oil-in-water emulsions (10%) were made with 2.5 g RSO TAG and distilled Milli-Q water with 0.25 g of Tween 20 as the emulsifier. Emulsions were then sonicated (Labsonic U Braun, Allentown, PA) at high speed (200 rpm) for 5 min in an ice bath. The stability of the particle size of the emulsions before and after the oxidation was checked by microscopy (Olympus OMT11, Tokyo, Japan).

Oxidation. The 10% oil-in-water emulsion samples were oxidized uncovered for 4 d in 300-mL flasks at 25°C in the dark in a shaker water bath (Gyrotory Water Bath Shaker Model G76, New Brunswick Scientific Co., Edison, NJ). The effect of β-carotene and α- and γ-tocopherol on autoxidation of the emulsified RSO TAG was followed by measuring PV with the ferric thiocyanate method (21,22) and the decomposition of lipid hydroperoxides into volatile aldehydes. Hexanal, 2,4-heptadienal, and heptenal were determined as their DNPH derivatives by HPLC. For the volatile aldehyde analysis, 5.0-g emulsions were mixed with 230 mL water and with 40 μL decanol. Volatile aldehydes were collected by flushing the samples with nitrogen at 80 mL/min for 16–20 h at 50°C and by chemically trapping them as DNPH derivatives. A Hewlett Packard 1090 liquid chromatograph, equipped with diode array detector, was used to analyze the DNPH derivatives. The column (Spherisorb ODS5 5 μm, 25 × 0.46 cm; Phase Separations, Deeside, United Kingdom) was maintained at 60°C. The mobile phase was a gradient mixture, ranging from 60% acetonitrile in water to 100% acetonitrile, at a flow rate of 1 mL/min. The DNPH derivatives were detected at the wavelength of 360 nm. All analyses were done in duplicate, and the statistical analysis was done by one-way analysis of variance (23).

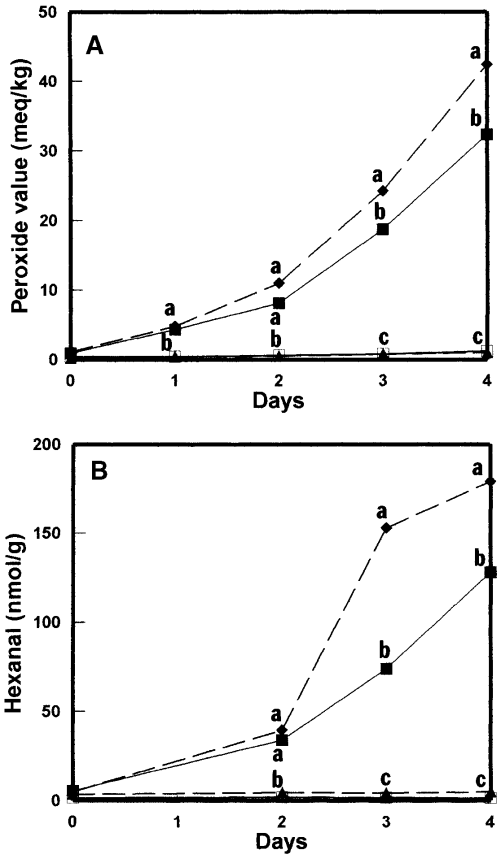

FIG. 1. The effect of 0.45 μg/g β-carotene, 3 μg/g γ-tocopherol, and their combination (0.45 μg/g β-carotene + 3 μg/g γ-tocopherol) on the formation of (A) lipid hydroperoxides and (B) hexanal. Values on the same day marked by the same letter or no letter are not significantly different at $P < 0.05$. ■, Control; ◆, β-carotene; ▲, γ-tocopherol; □, β-carotene + γ-tocopherol.

RESULTS AND DISCUSSION

The effect of β-carotene on autoxidation of 10% oil-in-water emulsions was investigated individually and in combination with α- and γ-tocopherol. For most data points that resulted from concentrations of 0.45, 2, and 20 μg/g in the emulsions, β-carotene had a significant ($P < 0.05$) prooxidant effect on the autoxidation of emulsified TAG when measuring the formation of lipid hydroperoxides (Figs. 1A, 2A, 3, and 4), hexanal (Figs. 1B and 2B), and 2-heptenal (Fig. 2D). The amount of 2,4-heptadienal (Fig. 2C), formed in the presence of β-carotene (2 μg/g), did not significantly differ from that of the control sample. The latter result in the dark is different from the findings by Warner and Frankel (16), who reported that twice as much 2-heptenal was formed in the control sample, compared to soybean oil with higher concentrations (20 ppm) of added β-carotene oxidized under light. In the present study, hexanal was the main volatile aldehyde formed (60–70% of total volatiles), in both the control sample and samples with added β-carotene.

The activity of β-carotene depends on other antioxidants present, the oxidation model used, and the oxygen tension. Data supporting the possibility that β-carotene could be more effective as a chain-breaking antioxidant in a lipid environment at a low oxygen tension than at high oxygen tensions was presented by Burton and Ingold (9) and Palozza and Krinsky (12). At higher oxygen tensions, β-carotene loses its antioxidant activity and shows autocatalytic prooxidant effects, particularly at concentrations above 0.5 mM (9). According to the present data, this prooxidant effect is also true at atmospheric conditions with very low (0.45 and 2 μg/g) and low (20 μg/g) concentrations of β-carotene. These data are in accordance with the authors' previous findings where, in a model of RSO TAG stripped of tocopherols that were au-

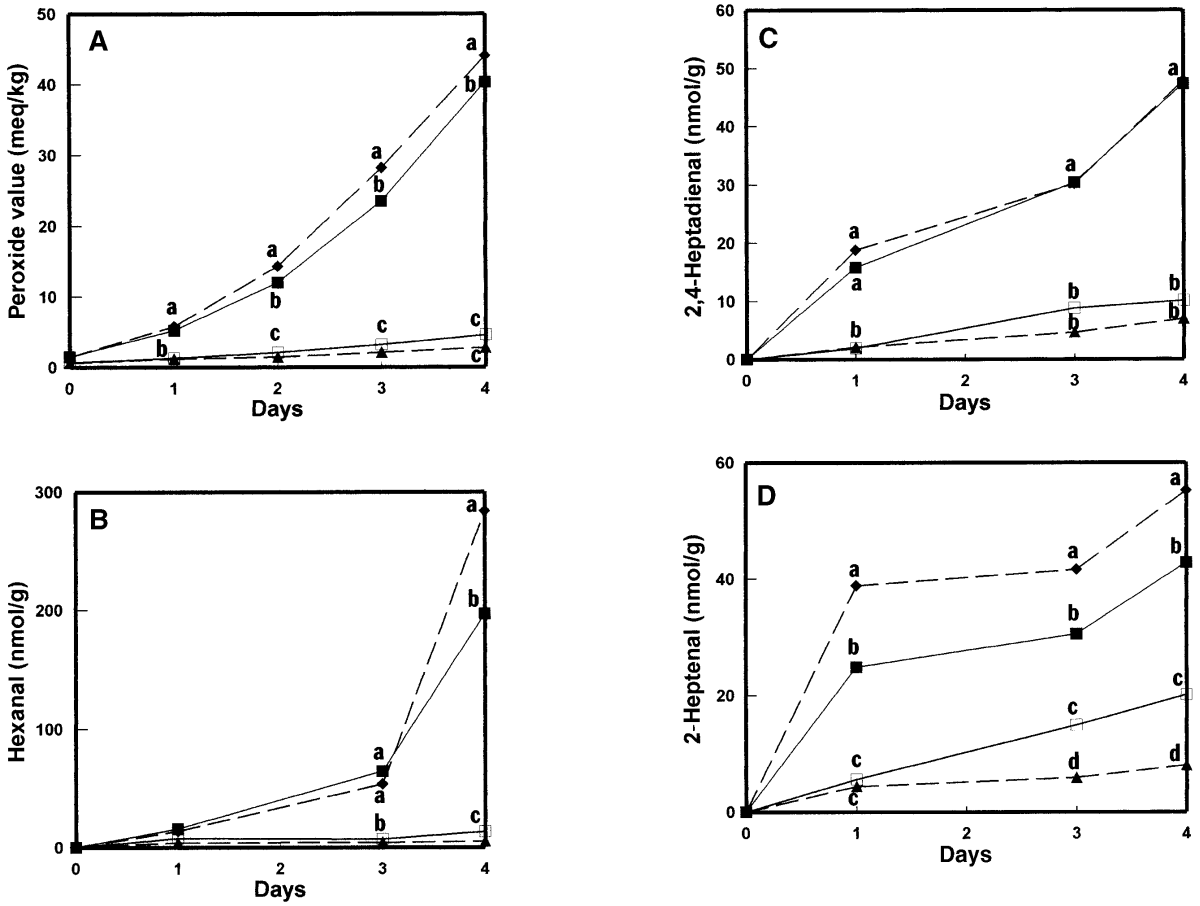

FIG. 2. The effect of 2 μg/g β-carotene, 1.5 μg/g γ-tocopherol, and their combination (2 μg/g β-carotene + 1.5 μg/g γ-tocopherol) on the formation of (A) lipid hydroperoxides, (B) hexanal, (C) 2,4-heptadienal, and (D) 2-heptenal.Values on the same day marked by the same letter or no letter are not significantly different at $P < 0.05$. ■, Control; ◆, β-carotene; ▲, γ-tocopherol; □, β-carotene + γ-tocopherol.

toxidized both in the dark and under light, β-carotene (18), lutein, and lycopene (19) showed prooxidant activity at concentrations of 5–40 μg/g. It has been suggested by Warner and Frankel (16) that free-radical oxidation is promoted by β-carotene in the dark. In reacting with peroxyl radicals, a carbon-centered β-carotene radical is formed that is readily autoxidized, in turn depending on the oxygen partial pressure (9).

In this 10% oil-in-water emulsion, the prooxidant effect of β-carotene was inhibited only in combination with antioxidants, such as α- and γ-tocopherols (Figs. 1, 2, and 3). Apart from food systems, this may also be true in other biological systems, such as in lipoproteins and microsomes, in which antioxidants are naturally present. It is well known that tocopherols protect β-carotene from deterioration (12,17–19, 24). Terao *et al.* (24) reported a protective effect with δ-tocopherol in soybean oil. However, in their study, β-carotene also exhibited antioxidant activity without added δ-tocopherol that was most likely due to the fact that the soybean oil used contained 80 μg/g of α-tocopherol, 620 μg/g of γ-tocopherol, and 220 μg/g of δ-tocopherol. Similarly, Warner and Frankel (16) referred to the effect of natural tocopherols in soybean oil in protecting added β-carotene. In poultry meat that contained 2.4 μg/g β-carotene was a prooxidant, and α-tocopherol counteracted the effects of β-carotene (25).

Both α- and γ-tocopherol were effective in retarding the autoxidation of emulsified triacylglycerols (Figs. 1–5). α-Tocopherol at a concentration of 1.5 μg/g and γ-tocopherol at 1.5, 3, and 30 μg/g inhibited both the formation and decomposition of lipid hydroperoxides. Hexanal was not a predominant volatile aldehyde formed in the presence of tocopherols, but similar amounts of 2,4-heptadienal and 2-heptenal were formed. Significantly ($P < 0.05$) more lipid hydroperoxides, hexanal, 2-heptenal, and 2,4-heptadienal were formed with added α-tocopherol, compared to added γ-tocopherol (Fig. 5). This is in accordance with data on high levels of added tocopherols. For example, Huang *et al.* (26) showed that γ-tocopherol had less antioxidant activity than α-tocopherol at 100 μg/g in bulk oil and in oil-in-water emulsions but had higher antioxidant activity at higher concentrations (250–1000 μg/g) in oil-in-water emulsions.

In the authors' data, the combination of α- and γ-tocopherol (0.75 + 0.75 μg/g) was less effective than γ-tocopherol (1.5 μg/g) but more effective than α-tocopherol (1.5 μg/g) in

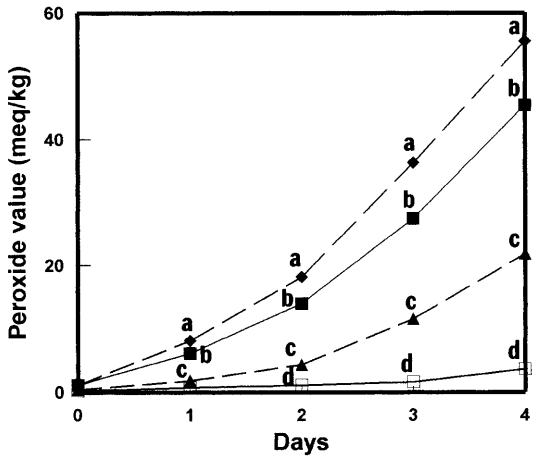

FIG. 3. The effect of 2 μg/g β-carotene, 1.5 μg/g α-tocopherol, and their combination (2 μg/g β-carotene + 1.5 μg/g α-tocopherol) on the formation of lipid hydroperoxides. Values on the same day marked by the same letter or no letter are not significantly different at $P < 0.05$. ■, Control; ◆, β-carotene; ▲, α-tocopherol; □, β-carotene + α-tocopherol.

inhibiting lipid oxidation (Fig. 5). The latter finding was significant ($P < 0.05$) at all data points when measuring lipid hydroperoxides (Fig. 5A) and at days 2 and 3 when measuring 2,4-heptadienal (Fig. 5C). This was not true with high levels of tocopherol mixtures (250–3000 μg/g) that showed no additive or synergistic effects in terms of formation of hydroperoxides in triacylglycerides, although tocopherol mixtures strongly inhibited hexanal formation (27). A level of 0.5 μg/g was too low a concentration of tocopherols to inhibit lipid oxidation of emulsified RSO TAG, measured by formation of hydroperoxides (Fig. 6A). However, formation of hexanal was significantly ($P < 0.05$) inhibited at this low level of α- or γ-tocopherol (Fig. 6B).

The reaction mechanism of interaction between β-carotene and tocopherols is not clear. Palozza and Krinsky (28) reported a hypothesis that addition of both β-carotene and α-tocopherol to a membrane system substantially facilitated the antioxidant character of β-carotene by limiting the production of β-carotene peroxyl radical. Moreover, it was stated that the protection is dose-dependent and occurs only at sufficiently high concentrations of α-tocopherol (12,28,29). According to Handelman *et al.* (29), there is a lack of synergism between β-carotene and α-tocopherol when the levels of α-tocopherol are much lower than those of β-carotene. The present data also showed that low levels of α- or γ-tocopherol (1.5 μg/g), together with low levels of β-carotene (2 μg/g) inhibited formation and decomposition of lipid hydroperoxides at a molar ratio of 1:1 (Figs. 2 and 3). Moreover, while γ-tocopherol was an efficient antioxidant, a synergistic effect of the combination of β-carotene and γ-tocopherol was not shown (Figs. 1 and 2) unlike the combination of β-carotene and α-tocopherol (Fig. 3). In the interaction between α-tocopherol (1.5 μg/g) and β-carotene (2 μg/g), a significant ($P < 0.05$) beneficial effect was seen, compared to the prooxidant effect of β-carotene and the antioxidant effect of α-tocopherol (Fig. 3). However, during autoxidation of RSO TAG (18,19), the beneficial effect was also shown with added γ-tocopherol. These findings add to the evidence that indicates that tocopherols effectively protect β-carotene against free-radical autoxidation as proposed by Frankel (30).

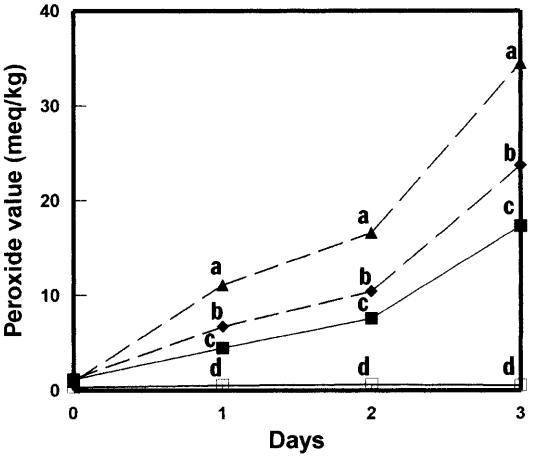

FIG. 4. The effect of 2 and 20 μg/g β-carotene and 30 μg/g γ-tocopherol on the formation of lipid hydroperoxides.Values on the same day marked by the same letter or no letter are not significantly different at $P < 0.05$. ■, Control; ◆, β-carotene; ▲, β-carotene; □, γ-tocopherol.

ACKNOWLEDGMENTS

The authors thank S. Lievonen and L. Varo for technical assistance, Roche Oy for kindly providing the carotenoid standards, and The Academy of Finland and Juho Vainio Foundation for financial support.

REFERENCES

1. Roozen, J.P., E.N. Frankel, and J.E. Kinsella, Enzymic and Autoxidation of Lipids in Low Fat Foods: Model of Linoleic Acid in Emulsified Hexadecane, *Food Chem. 50*:33–38 (1994).
2. European Parliament and Council Directive 94/36/EC, Colours for Use in Foodstuffs, *Off. J. Eur. Comm. 237*:13–29 (1994).
3. Johnson, L.E., Food Technology of the Antioxidant Nutrients, *CRC Critical Rev. Food Sci. Nutr. 35*:149–159 (1995).
4. Carnevale, J., E.R. Cole, and G. Crank, Fluorescent Light Catalyzed Autoxidation of β-Carotene, *J. Agric. Food Chem. 2*:462–463 (1979).
5. Christophersen, A.G., J. Huang, K. Joergensen, and L.H. Skibstedt, Photobleaching of Astaxanthin and Canthaxanthin. Quantum-Yields Dependence of Solvent, Temperature, and Wavelength of Irradiation in Relation to Packaging and Storage of Carotenoid Pigmented Salmonoids, *Z. Lebensm. Unters. Forsch. 192*:433–439 (1991).
6. Di Mascio, P., S. Kaiser, and H. Sies, Lycopene as the Most Efficient Biological Carotenoid Singlet Oxygen Quencher, *Arch. Biochem. Biophys. 274*:532–538 (1989).
7. Conn, P.F., W. Schalch, and T.G. Truscott, The Singlet Oxygen and Carotenoid Interaction, *J. Photochem. Photobiol. B: Biol. 11*:41–47 (1991).
8. Kennedy, T.A., and D.C. Liebler, Peroxyl Radical Scavenging by β-Carotene in Lipid Bilayers, *J. Biol. Chem. 267*: 4658–4663 (1992).
9. Burton, G.W., and K.U. Ingold, β-Carotene: An Unusual Type of Lipid Antioxidant, *Science 244*:569–573 (1984).

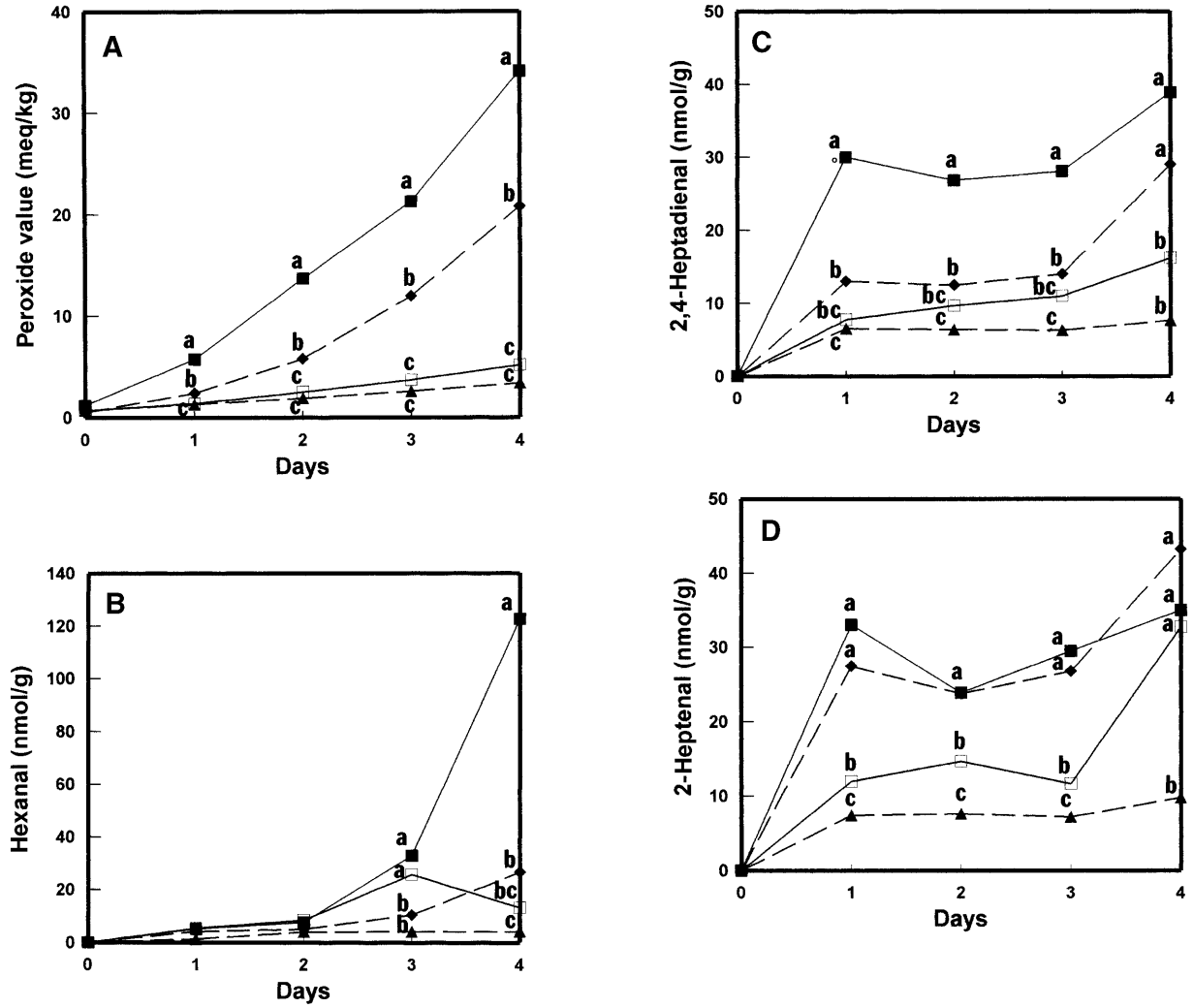

FIG. 5. The effect of 1.5 μg/g α-tocopherol, 1.5 μg/g γ-tocopherol, and their combination (0.75 μg/g α-tocopherol + 0.75 μg/g γ-tocopherol) on the formation of (A) lipid hydroperoxides, (B) hexanal, (C) 2,4-heptadienal, and (D) 2-heptenal. Values on the same day marked by the same letter or no letter are not significantly different at $P < 0.05$. ■, Control; ◆, α-tocopherol; ▲, γ-tocopherol; □, α-tocopherol + γ-tocopherol

10. Terao, J., Antioxidant Activity of β-Carotene-Related Carotenoids in Solution, *Lipids 24*:659–661 (1989).
11. Krinsky, N.I., and S.M. Deneke, Interaction of Oxygen and Oxy-Radicals with Carotenoids, *J. Natl. Cancer Inst. 69*: 205–209 (1982).
12. Palozza, P., and N. Krinsky, The Inhibition of Radical-Initiated Peroxidation of Microsomal Lipids by Both α-Tocopherol and β-Carotene, *Free Rad. Biol. Med. 11*:407–414 (1991).
13. Esterbauer, H., H. Puhl, M. Dieber-Rotheneder, G. Waeg, and H. Rabl, Effect of Antioxidants on Oxidative Modification of LDL, *Ann. Med. 23*:573–581 (1991).
14. Jialal, I., E.P. Norkus, L. Cristol, and S.M. Grundy, β-Carotene Inhibits the Oxidative Modification of Low-Density Lipoprotein, *Biochim. Biophys. Acta 1086*:134–138 (1991).
15. Kigoshi, M., and E. Niki, Inhibition of Oxidation of Lipids by Carotenoids, in *Oxygen Radicals*, edited by K. Yagi, M. Kondo, E. Niki, and T. Yoshikawa, Elsevier Science Publishers, London, 1992, pp. 635–656.
16. Warner, K., and E.N. Frankel, Effects of β-Carotene Light Stability of Soybean Oil, *J. Am. Oil. Chem. Soc. 64*:213–218 (1987).
17. Suzuki, T., R. Usuki, and T. Kaneda, The Role of Carotenoids in the Oxidative Deterioration of Edible Oils, *J. Jpn. Oil Chem. Soc. 38*:486–491 (1989).
18. Haila, K., and M. Heinonen, Action of β-Carotene on Purified Rapeseed Oil During Light Storage, *Lebensm. Wiss. Technol. 27*:573–577 (1994).
19. Haila, K., S. Lievonen, and M. Heinonen, Effects of Lutein, Lycopene, Annatto, and γ-Tocopherol on Autoxidation of Triacylglycerides, *J. Agric. Food Chem. 44*:2096–2100 (1996).
20. Lampi, A.-M., A. Hopia, P. Ekholm, and V. Piironen, Method for the Preparation of Triacylglycerol Fractions from Rapeseed and Other Oils for Autoxidation Studies, *Lebensm. Wiss. Technol. 25*:386–388 (1992).
21. FIL-IDF 74, Anhydrous Milk Fat. Determination of the Peroxide Value, *International Dairy Federation* (1974).
22. Ueda, S., T. Hayashi, and M. Namiki, Effect of Ascorbic Acid on Lipid Autoxidation in a Model System, *Agric. Biol. Chem. 50*:1–7 (1986).
23. Wagner, S.F., Analysis of Variance, in *Introduction to Statistics,* Harper Perennial, New York, 1992, pp. 239–273.
24. Terao, J., R. Yamauchi, H. Murakami, and S. Matsushita, Inhibitory Effects of Tocopherols and β-Carotene on Singlet Oxy-

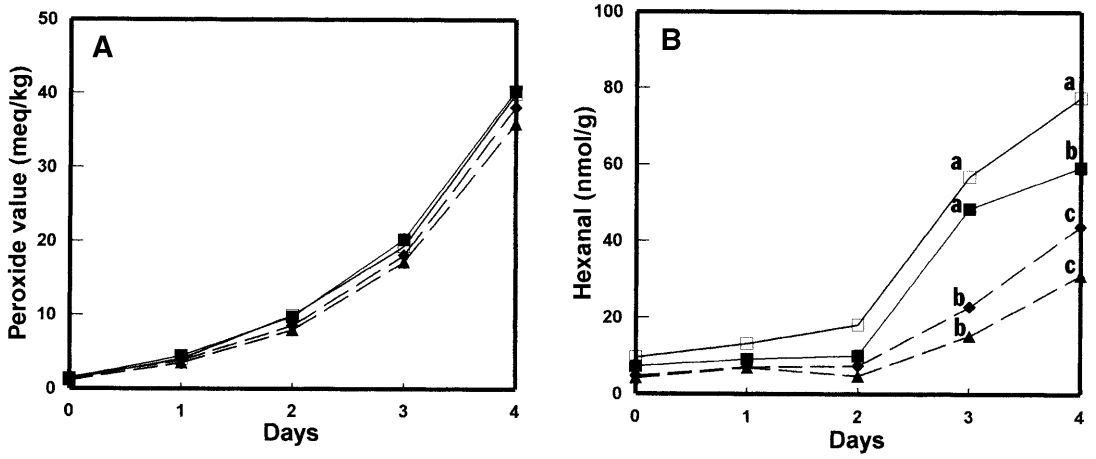

FIG. 6. The effect of 0.5 μg/g α-tocopherol, 0.5 μg/g γ-tocopherol, and their combination (0.25 μg/g α-tocopherol + 0.25 μg/g γ-tocopherol) on the formation of (A) lipid hydroperoxides and (B) hexanal. Values on the same day marked by the same letter or no letter are not significantly different at $P < 0.05$. ■, Control; ◆, α-tocopherol; ▲, γ-tocopherol; □, α-tocopherol + γ-tocopherol.

gen-Initiated Photooxidation of Methyl Linoleate and Soybean Oil, *J. Food Proc. Preserv. 4*:79–93 (1980).
25. King, A.J., T.G. Uijttenboogaart, and A.W. de Vries, α-Tocopherol, β-Carotene and Ascorbic Acid as Antioxidants in Stored Poultry Muscle, *J. Food Sci. 60*:1009–1012 (1995).
26. Huang, S.-W., E.N. Frankel, and J.B.German, Antioxidant Activity of α- and γ-Tocopherols in Bulk Oils and in Oil-in-Water Emulsions, *J. Agric. Food. Chem. 42*:2108–2114 (1994).
27. Huang, S.-W., E.N. Frankel, and J. B. German, Effects of Individual Tocopherols and Tocopherol Mixtures on the Oxidative Stability of Corn Oil Triacylglycerides, *J. Agric. Food. Chem.* 43:2345–2350 (1995).
28. Palozza, P., and N. Krinsky, β-Carotene and α-Tocopherol Are Synergistic Antioxidants, *Arch. Biochem. Biophys. 297*: 184–187 (1992).
29. Handelman, G.J., F.J.G.M. van Kuik, A. Chatterjee, and N.I. Krinsky, Characterization of Products Formed during the Autoxidation of β-Carotene, *Free Rad. Biol. Med. 10*:427–437 (1991).
30. Frankel, E.N., Antioxidant and Nutritional Effects of Tocopherols, Ascorbic Acid and β-Carotene in Relation to Processing of Edible Oils, *Bibl. Nutr. Dieta 43*:297–312 (1989).

[Received March 25, 1996; accepted April 30, 1997]