Autoxidation of Linoleic Acid and Behavior of its Hydroperoxides With and Without Tocopherols

J.P. KOSKAS, J. CILLARD and **P. CILLARD**, Laboratoire de Botanique et de Biologie Cellulaire 2, Avenue du Professeur Léon Bernard - 35043 - Rennes Cedex - France

ABSTRACT

The autoxidation of linoleic acid dispersed in an aqueous media and the effect of α-, γ- and δ-tocopherols were studied. The quantitative analysis of the hydroperoxide isomers (13-*cis,trans*; 13-*trans,trans*; 9-*trans,cis*; 9-*trans,trans*) by direct high-performance liquid chromatography exhibited a prooxidant activity of α-tocopherol at high concentration (3.8% by weight of linoleic acid). On the other hand, α-tocopherol at lower concentrations (0.38 and 0.038%) and γ- and δ-tocopherols at high concentration (3.8%) were antioxidant. Furthermore, the addition of tocopherols modified the distribution of the geometrical isomers. The formation of the *trans,trans* hydroperoxide isomers was completely inhibited by the highest concentration of the three tocopherols independently of their antioxidant or prooxidant activity and only delayed by the lower concentrations of α-tocopherol. The addition of tocopherols to hydroperoxide isomers reduced the decomposition rate of these isomers in the order α-tocopherol < γ-tocopherol < δ-tocopherol for the *cis,trans* hydroperoxide isomer and α-tocopherol ≪ γ-tocopherol ≃ δ-tocopherol for the *trans,trans* hydroperoxide isomer. With these hydroperoxides, as during linoleic acid autoxidation, α-tocopherol was completely oxidized whatever its initial concentration, while γ-tocopherol underwent partial oxidation and δ-tocopherol was practically unchanged.

INTRODUCTION

Lipid autoxidation is a phenomenon which has important effects in vivo. It is well known that the free radicals formed by lipid peroxidation involve serious damage, particularly to the cell membranes (1). Recently some authors (2,3) have shown that cell division in a tumor clone is correlated with lipid peroxidation. They noted that lipid peroxides are reduced and almost absent in tumors.

Tocopherols are well known to act as antioxidants. In vitro their antioxidant efficacy decreases in the order $\gamma > \delta \gg \alpha$ whereas α-tocopherol is most effective as vitamin E (4). At high concentration, α-tocopherol can cause a prooxidant effect in vitro and consequently increase the autoxidation rate of fatty acids (5,6). We already have shown that the prooxidant effect in an aqueous media depends on two factors: the concentration of α-tocopherol ($> 5 \times 10^{-3}$ mol α-tocopherol/mol linoleic acid) and the solvent: the prooxidant effect occurs more easily in an aqueous system (7,8).

High performance liquid chromatography (HPLC) has allowed more investigations into the hydroperoxides formed by lipid peroxidation. Linoleic acid autoxidation leads to the formation of four hydroperoxide isomers: 13-hydroperoxy-9-*cis*,11-*trans*-octadecadienoic, 13-hydroperoxy-9-*trans*,11-*trans*-octadecadienoic, 9-hydroperoxy-10-*trans*,12-*cis*-octadecadienoic and 9-hydroperoxy-10-*trans*,12-*trans*-octadecadienoic acids. These isomers have been analyzed by HPLC either after reduction to the corresponding hydroxy fatty acids (9,10) or directly (11). The distribution of these isomers is dependent on the temperature and the concentration of linoleic acid (10) and on the presence of an antioxidant (10,12,13).

Using our previous experimental model (7,8), we have investigated the quantitative and qualitative analysis of hydroperoxide isomers formed during the autoxidation of linoleic acid dispersed in an aqueous media with α-, γ- and δ-tocopherols and without tocopherol. Furthermore, we have studied the behavior of hydroperoxides with and without these tocopherols and measured the degradation of tocopherols.

MATERIALS AND METHODS

Reagents and Chemicals

Linoleic acid was purchased from Koch Light (England), α-, γ- and δ-tocopherols from Hoffmann Laroche (France), Tween 20 and m-cresol from Merck (Germany) and disodium phosphate from Prolabo (France); n-heptane "chromasol" was supplied by S.D.S. (France); methanol, chloroform and glacial acetic acid by Prolabo (France).

Instrumentation

High-performance liquid chromatograph Laboratory Data Control was purchased from Sopares (France) and equipped with a Constametric III pump, a Valco 7000 psi injector and a Spectromonitor III UV as detector set at 234 nm for semi-preparative chromatography and 239 nm for analytical chromatography of hydroperoxides and 280 nm for detection of α-, γ- and δ-tocopherols and α-tocopherolquinone.

Chromatography

Before extraction of hydroperoxides, m-cresol was added to the aqueous sample as internal standard for HPLC analysis. The extraction was then achieved by a mixture of chloroform-methanol (1/1, v/v). After evaporation to dryness under reduced pressure at 30 C, the dry residue was dissolved in diethyl ether. Chromatographs of hydroperoxides were done on a stainless steel column (20 cm × 0.47 cm) of Spherisorb Si 60 (particle size 3-4 μm). The solvent used was composed of n-heptane and acetic acid (100/2.3, v/v), and the flow-rate was 2 mL/min (11).

Two systems were employed: chromatographs of tocopherols and α-tocopherolquinone at high concentrations were done on a reverse phase of Spherisorb O.D.S. C_{18} (particle size 5 μm) column (20 cm × 0.47 cm) and eluted with methanol-water (85/15, v/v) at a flow-rate of 2 mL/min. The aqueous samples with an internal standard were directly injected into HPLC. The internal standards were respectively γ-tocopherol for α-tocopherol, δ-tocopherol and α-tocopherolquinone analysis and δ-tocopherol for γ-tocopherol analysis. For the weak concentrations of α-tocopherol and α-tocopherolquinone, we used adsorption chromatography with a stainless steel column (20 cm × 0.47 cm) of Spherisorb Si60 (particle size 3-4 μm) and a mobile phase composed of n-heptane and 2-propanol (100/1, v/v) at a flow rate of 2 mL/min. This method required extraction of α-tocopherol and α-tocopherolquinone from the aqueous samples. Before the extraction, γ-tocopherol was added as an internal standard. The compounds were extracted by a mixture of n-heptane and ethanol (1/2, v/v). After centrifugation, the supernatant was injected into HPLC.

Linoleic Acid Autoxidation With and Without Tocopherols

Linoleic acid and tocopherols were dispersed in a phosphate buffer solution (14). In all samples, the initial concentration of linoleic acid was 2.5×10^{-3}M, whereas different concentrations of tocopherols were investigated. The initial concentrations of α-tocopherol were respectively 6.25×10^{-5}M (i.e. 3.8% by weight of linoleic acid); 6.25×10^{-6}M

(0.38%), and 6.25×10^{-7}M (0.038%), while γ-tocopherol and δ-tocopherol were used only at an initial concentration of 6.25×10^{-5}M (3.8%).

Hydroperoxides With and Without Tocopherols

Linoleic acid was autoxidized in an aqueous media (8). After 10 days of autoxidation, hydroperoxides were extracted from the media by chloroform-methanol (1/1, v/v). Then they were submitted to semi-preparative chromatography. This chromatography allowed us to get pure 13-*cis,trans* and 13-*trans,trans* hydroperoxide isomers. Since the 9-*trans,cis* and 9-*trans,trans* hydroperoxide isomers were not well separated, we used only the 13-*cis,trans* and 13-*trans,trans* hydroperoxide isomers.

Each hydroperoxide was dispersed with and without tocopherols (α- or γ- or δ-) in a phosphate buffer solution (0.025M, pH 6.9) with 0.5% Tween 20. The reaction mixtures were placed in darkness at room temperature. In the samples, the initial concentrations were respectively 1.28×10^{-4}M for the 13-*cis,trans* and the 13-*trans,trans* hydroperoxide isomers, 4.19×10^{-5}M for α-tocopherol and 6.25×10^{-5}M for γ- and δ-tocopherols.

RESULTS

Effect of Tocopherols on the Formation of Hydroperoxides

The autoxidation of linoleic acid in an aqueous media was accompanied by an increase in the level of hydroperoxides. The maximum concentration of the total hydroperoxides was 180 μg/mL after 12 days of autoxidation (Fig. 1). The addition of tocopherols modified the rate of formation and the level of hydroperoxides, α-Tocopherol at the initial concentration of 3.8% caused an important increase of both level and rate formation of hydroperoxides (Fig. 1). Their maximum concentration was ca. 350 μg/mL after 11 days. With α-tocopherol at the two other concentrations (0.38% and 0.038%), the rate of hydroperoxide formation was reduced, especially with the lowest concentration, which nearly inhibited their formation during the first 8 days (Fig. 1). Nevertheless, the maximum concentrations of hydroperoxides were almost the same as in the sample without tocopherol. They were reached after 18 and 14 days respectively for α-tocopherol at 0.38 and 0.038%.

The addition of γ- and δ-tocopherols at a concentration of 3.8% reduced the rate of formation of hydroperoxides, especially with δ-tocopherol (Fig. 2). Furthermore, these tocopherols exhibited different effects on the maximum concentrations of hydroperoxides. We noted an increase of total hydroperoxides with γ-tocopherol (maximum ca. 250 μg/mL at 31 days) and a decrease with δ-tocopherol (maximum ca. 85 μg/mL at 74 days).

We calculated the percentage of each isomer. In all cases of autoxidation the amounts of 13-isomers (13-*cis,trans* + 13-*trans,trans*) and 9-isomers (9-*trans,cis* + 9-*trans,trans*) were practically the same. On the other hand, the distribution of geometrical isomers was different. It varied over time and with the presence of tocopherols. The autoxidation of linoleic acid without tocopherol showed only *cis,trans* hydroperoxide isomers during the first two days, then the *trans,trans* hydroperoxide isomers appeared and increased over time (Fig. 3). at 11 days, ca. 50% of each geometrical isomer (*cis,trans* and *trans,trans*) were present in the sample, while at 24 days, the percentages were respectively 16 and 84% for the *cis,trans* and the *trans,trans* hydroperoxide isomers.

The addition of tocopherols affected the distribution of the geometrical isomers. With high concentration of α-, γ- and δ-tocopherols (3.8%) only *cis,trans* hydroperoxide isomers were detected. On the other hand, with lower con-

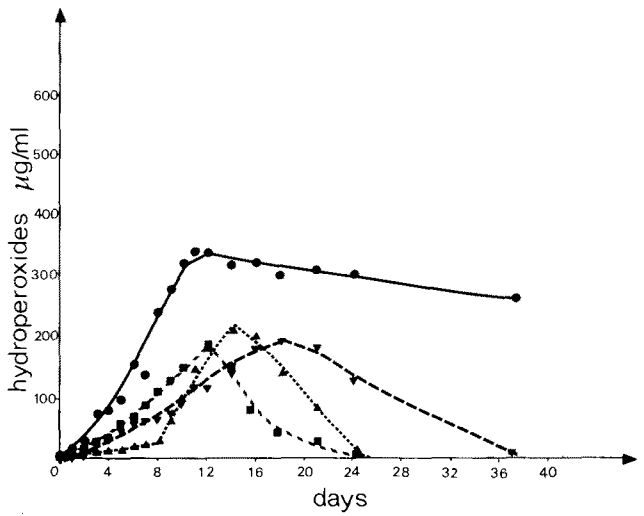

FIG. 1. Total hydroperoxide concentrations (13-*cis,trans* + 13-*trans,trans* + 9-*trans,cis* + 9-*trans,trans*) measured by HPLC during linoleic acid autoxidation in a phosphate buffer solution pH 6.9. ■ - - - linoleic acid without tocopherol; ● —— linoleic acid with α-tocopherol (3.8%); ▼ — — — linoleic acid with α-tocopherol (0.38%); ▲ - - - linoleic acid with α-tocopherol (0.038%).

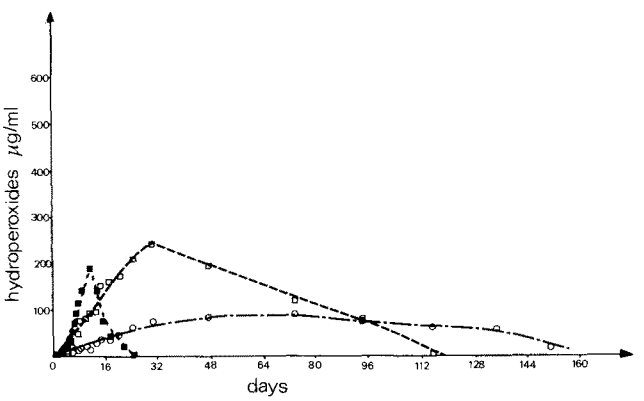

FIG. 2. Total hydroperoxide concentrations (13-*cis,trans* + 13-*trans,trans* + 9-*trans,cis* + 9-*trans,trans*) measured by HPLC during linoleic acid autoxidation in a phosphate buffer solution pH 6.9. ■ - - - linoleic acid without tocopherol; □ — — — linoleic acid with γ-tocopherol (3.8%). ○ —— - linoleic acid with δ-tocopherol (3.8%).

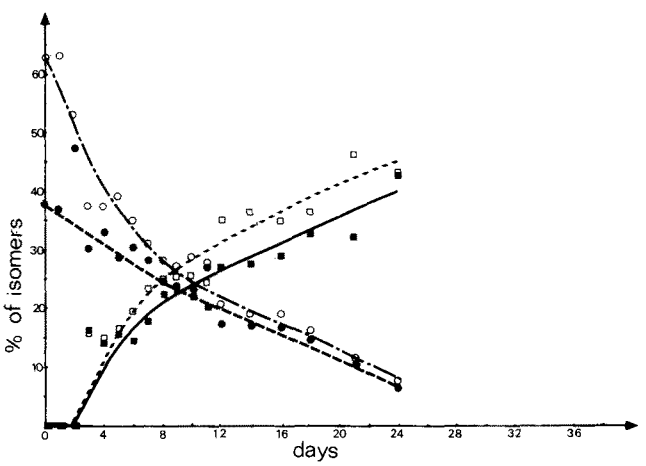

FIG. 3. Distribution of the hydroperoxide isomers detected during the autoxidation of linoleic acid without tocopherol in a phosphate buffer solution pH 6.9. ○ - —— - 13-*cis,trans*, ■ —— 13-*trans,trans*, ● — — — 9-*trans,cis*, □ - - - 9-*trans,trans*.

centrations of α-tocopherol (0.38% and 0.038%) the distribution of the geometrical isomers was slightly modified with regard to the autoxidation of linoleic acid without tocopherol. The formation of the *trans,trans* hydroperoxide isomers was delayed only 1-2 days.

The quantitative analysis of hydroperoxide isomers by HPLC showed that the concentrations of the 13-*cis,trans* and the 13-*trans,trans* hydroperoxides of linoleic acid decreased rapidly in the aqueous media. No hydroperoxide was detected after 10 days (Fig. 4). Nevertheless, the degradation rate of the 13-*cis,trans* hydroperoxide isomers was slightly superior to the one of the 13-*trans,trans* hydroperoxide isomer.

The addition of tocopherols (α- or γ- or δ-tocopherols) increased the stability of both hydroperoxide isomers. The efficacy of tocopherols to protect hydroperoxides increased in the order α- < γ- < δ-tocopherol for the 13-*cis,trans* and α- ≪ γ- ≃ δ-tocopherol for the 13-*trans,trans*. Furthermore, with α- and γ-tocopherols, the 13-*trans,trans* hydroperoxide isomer degraded more slowly than the 13-*cis,trans* hydroperoxide isomer while with δ-tocopherol only a slight difference was observed for. the degradation rate of both hydroperoxide isomers (Fig. 4).

Oxidation of Tocopherols

The HPLC analysis of α-tocopherol showed that in the presence of linoleic acid, α-tocopherol was completely oxidized within 14 days at the same rate whatever its initial concentration (Fig. 5). On the other hand, α-tocopherol dispersed under the same conditions but without linoleic acid was almost stable. The oxidation of α-tocopherol with linoleic acid gave rise to the formation of α-tocopherolquinone, identified as one of the oxidation products of α-tocopherol. In all cases, the concentration of α-tocopherolquinone increased until the complete oxidation of α-tocopherol and then decreased. When α-tocopherol at the initial concentrations of 3.8%, 0.38% and 0.038% was completely degraded, the formation of α-tocopherolquinone corresponded to respectively 22%, 45% and 40% of the total oxidation products of α-tocopherol. Without linoleic acid, α-tocopherol exhibited good stability in the aqueous media whatever its initial concentration, since less than 10% of α-tocopherol was oxidized after 37 days. The quantitative analysis of γ- and δ-tocopherols showed that their degradation was slower than that of α-tocopherol (Fig. 5). Thus, at 60 days we noted that 32% and 92% of γ- and δ-tocopherols were still present in the samples respectively.

When tocopherols were dispersed with hydroperoxides in an aqueous media, the concentrations of γ- and δ-tocopherols remained practically constant, whereas the concentration of α-tocopherol decreased rapidly. The degradation rate of α-tocopherol was the same with the 13-*cis,trans* and the 13-*trans,trans* hydroperoxides (Fig. 6). α-Tocopherol was not detected in the samples after 25 days. Simultaneously, the rate of formation of α-tocopherolquinone was evaluated. When α-tocopherol was dispersed with hydroperoxides, the concentration of α-tocopherolquinone increased rapidly until the complete oxidation of α-tocopherol at 25 days. At this time, α-tocopherolquinone represented 20% of the total oxidation products of α-tocopherol. The rate of formation of α-tocopherolquinone was the same with the 13-*cis,trans* and the 13-*trans,trans* hydroperoxides.

DISCUSSION

An increase in the concentration of α-tocopherol (from 0.038% to 3.8%) caused a conversion of its antioxidant activity to a prooxidant activity. This phenomenon resulted in an increase of hydroperoxide formation. This observation agreed well with the increase of the conjugated dienes noted previously under the same conditions (7.8). On the other hand, the γ- and δ-tocopherols at high concentration (3.8%) exhibited an antioxidant activity. α-,

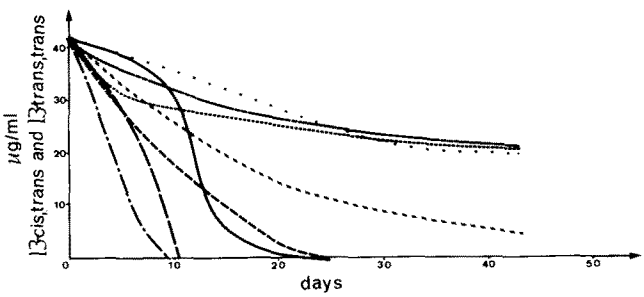

FIG. 4. HPLC measurements of the 13-*cis,trans* and the 13-*trans,trans* hydroperoxides of linoleic acid dispersed with and without tocopherols in a phosphate buffer solution pH 6.9. 13-*cis,trans:* alone ——·, with α-tocopherol — — —, with γ-tocopherol — · —, with δ-tocopherol ----. 9-*trans,trans:* alone ——, with α-tocopherol ——, with γ-tocopherol - - -, with δ-tocopherl ——··——.

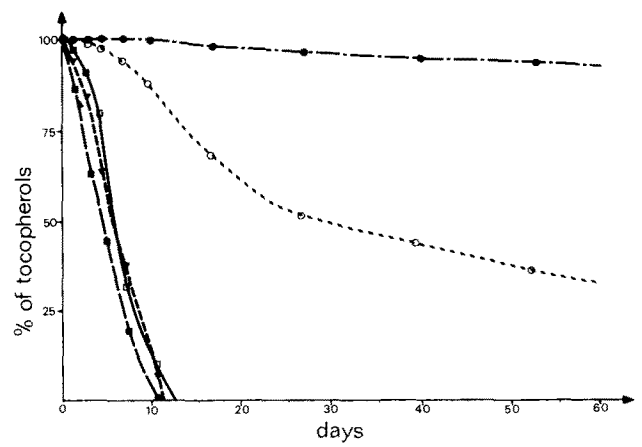

FIG. 5. Change of the percentage of α-, γ- and δ-tocopherols during the autoxidation of linoleic acid in a phosphate buffer solution pH 6.9. —— α-tocopherol (3.8%); — — — α-tocopherol (0.38%); —— α-tocopherol (0.038%); — · — γ-tocopherol (3.8%); ——·—— δ-tocopherol (3.8%).

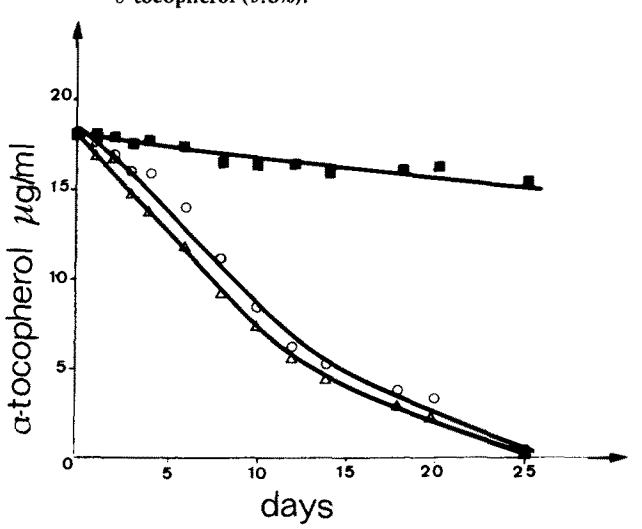

FIG. 6. HPLC measurements of α-tocopherol dispersed with and without the 13-*cis,trans* and 13-*trans,trans* hydroperoxides of linoleic acid in a phosphate buffer solution pH 6.9. —— α-Tocopherol: ■ alone; △ with 13-*cis,trans;* ○ with 13-*trans,trans.*

γ- and δ-Tocopherols at high concentration (3.8%) inhibited the formation of *trans,trans* hydroperoxide isomers, while α-tocopherol at lower concentrations (0.38 and 0.038%) only delayed the formation of these hydroperoxide isomers. This result showed that the inhibition of the formation of these last hydroperoxide isomers was not related to the antioxidant or prooxidant activity of tocopherols but was dependent on their initial concentration in the media.

Porter et al. (10) proposed a mechanism to explain the effect of phenols on the *cis,trans/trans,trans* hydroperoxide product ratios. The same mechanism was applied by Peers et al. (12) to explain the effect of α-tocopherol at high concentration on the inhibition of the formation of the *trans,trans* hydroperoxide isomers. These workers related the decrease in the proportion of *trans,trans* hydroperoxide isomers to the strong H-donating ability of α-tocopherol to a peroxyl radical.

The rate constants k for abstraction by peroxyl radicals of the hydrogens of some phenols and especially of tocopherols have been calculated by Burton and Ingold (15). These workers found different values of k for α-, γ- and δ-tocopherols, i.e., respectively 23.5, 15.9 and 6.5 × 10^{-5}M/S. In spite of this difference, all these tocopherol isomers inhibited at high concentration (3.8%) the formation of the *trans,trans* hydroperoxide isomers.

Our investigation of the behavior of hydroperoxides dispersed with and without tocopherols in an aqueous media showed that δ-tocopherol was the most effective in protecting hydroperoxides from decomposition. γ-Tocopherol showed the same efficiency as δ-tocopherol at preventing the decomposition of the 13-*trans,trans* hydroperoxide isomer, but it was obviously less effective for the 13-*cis,trans* hydroperoxide isomer. α-Tocopherol was less active in preventing hydroperoxide decomposition. The interaction between α-tocopherol and the hydroperoxides of methyl linoleate was investigated by Gruger and Tappel (16) and Igarashi et al. (17), respectively, in ethanol and in n-hexane. These workers found that α-tocopherol did not react directly with hydroperoxides. Nevertheless, they showed that α-tocopherol reacted with peroxy radicals released from the dissociation of hydroperoxide by ferric ion to produce α-tocopherolquinone. In our experimental model using an aqueous media, we noted simultaneously the total degradation of the hydroperoxides of linoleic acid and the complete oxidation of α-tocopherol within 25 days. Nevertheless, the γ- and δ-tocopherols were quite stable with the hydroperoxides although a degradation of these last compounds was observed. Total oxidation of α-tocopherol at different concentrations also was observed during linoleic acid autoxidation. On the other hand, we noted an incomplete oxidation of γ-tocopherol and practically no oxidation of δ-tocopherol. These results agreed with the observations of Ideda and Fukuzumi (18), who noted that the oxidation of tocopherols with methyl linoleate increased in the order δ- < γ- < α-tocopherols.

ACKNOWLEDGMENTS

The Langlois Foundation (Rennes, France) and the Foundation for Medical Research in France (Paris) provided financial assistance. S. Le Tolguenec provided technical assistance.

REFERENCES

1. Mead, J.F., Free Radicals in Biology, Vol. 1, Edited by W. Pryor, Acad. Press, 59 (1976).
2. Liepkalns, V.A.; C. Icar-Liepkalns, and D.G. Cornwell, Cancer Letters, 15, 173 (1982).
3. Apffel, C.A., Prog. Exp. Tumor Res., 22:317 (1978).
4. Parkhurst, R.M., W.A. Skinner, and P.A. Sturm, JAOCS 45: 641 (1968).
5. Labuza, T.P., CRC Crit. Rev. Food Technol., 2:355 (1971).
6. Naudet, M., Labo-Pharma. Prob. Tech., 215:61 (Nov. 1972).
7. Cillard, J.; P. Cillard, and M. Cormier, JAOCS 57:252 (1980).
8. Cillard, J.; P. Cillard, and M. Cormier, Ibid. 57:255 (1980).
9. Chan, H.W.S., and G. Levett, Lipids 12:99 (1977).
10. Porter, N.A.; B.A. Weber, H. Weenen and J.A. Khan, J. Am. Chem. Soc., 102:5597 (1980).
11. Koskas, J.P.; J. Cillard, and P. Cillard, J. Chromatogr., 258:280 (1983).
12. Peers, K.E.; D.T. Coxon, and H.W.S. Chan, J. Sci. Food Agric., 32:898 (1981).
13. Weenen, H. and N.A. Porter, J. Am. Chem. Soc., 104:5216 (1982).
14. Cillard, J., and P. Cillard, JAOCS 57:39 (1980).
15. Burton, G.W., and K.U. Ingold, J. Am. Chem. Soc. 103:6472 (1981).
16. Gruger, E.H. Jr., and A.L. Tappel, Lipids 5:326 (1970).
17. Igarashi, O.; H. Matsukawa, and C. Inagaki, J. Nutr. Sci. Vitaminol., 22:267 (1976).
18. Ikeda, N., and K. Fukuzumi, JAOCS 54:360 (1977).

[Received March 5, 1984]