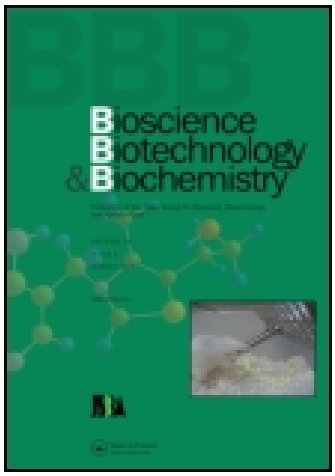


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# Oxidative Stability of Docosahexaenoic Acid-containing Oils in the Form of Phospholipids, Triacylglycerols, and Ethyl Esters

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**The peroxidative stability of docosahexaenoic acid (DHA)-containing oils (DHA at 10.7 mol% of the total fatty acids), in the form of phospholipids (PL), triacylglycerols (TG), and ethyl esters (EE) with the same constituent fatty acids, was investigated in the dark at 25°C in a bulk phase, and compared with that of control palm oil (supplemented with 20% soybean oil). The oxygen absorption of the DHA-containing oil was significantly lower in the form of PL than in the form of TG and EE during a 10-week oxidation, and the oxygen absorption of PL was almost equivalent to that of the control oil. A gas chromatographic analysis showed that 90% of initial DHA was retained in the form of PL after the 10-week oxidation, while TG and EE respectively more rapidly decayed with the loss of 97% and 64% of DHA. Tocopherol in the form of TG and EE had also completely decayed after the oxidation, while 37% of the initial tocopherol remained in the form of PL. The peroxide and carbonyl values of TG and EE showed large increases after the oxidation, but no such increase was observed for PL. These results show that DHA-containing oil in the form of PL was more resistant to the oxidative degradation of DHA than that in the form of TG and EE in a bulk phase.**

**Key words:** oxidative stability; docosahexaenoic acid; oxygen absorption; tocopherol; phospholipid



Docosahexaenoic acid (DHA, 22:6n-3) has received increasing attention in relation to its pathophysiological effects on mammals.[1–6] It is well known that polyunsaturated fatty acids (PUFAs) such as DHA are extremely susceptible to peroxidation, decaying more than 8.5 times faster than α-linolenic acid (18:3n-3),[7] and that the hydroperoxides and secondary aldehydic products formed in oxidized oil are generally toxic.[8–13] The inferior oxidative instability of DHA oil makes its ingestion a matter of concern, even in a diet containing a large amount of exogenous antioxidants.

Although several studies[14–22] on DHA oil autoxidation have been carried out, none have compared the peroxidizability among DHA-containing oils in the form of phospholipids (PL), triacylglycerols (TG), and ethyl esters (EE). We thought it important to know which form would be the most resistant to autoxidation.

In the present study, we investigated the oxidative stability of DHA-containing oils (DHA at 10.7 mol% of total fatty acids) and compared peroxidizability in the form of PL, EE, and TG, which had absolutely the same constituent fatty acids.

## Materials and Methods

*Materials.* DHA-containing oils in the form of PL (Active DHA-10®, 3.1 in the ratio of phosphatidylcholine (PC)/phosphatidylethanolamine (PE)), TG and EE that were used in the present study were provided by Bizen Chemical Co. (Okayama, Japan). These DHA oils were prepared from egg yolk lipids of laying hens that had been fed on a fish oil-supplemented diet.[23] The DHA content of all these oils was 10.7 mol% of the total fatty acids (Table I), and the composition of constituent fatty acids was almost the same among the three oils. As a control, palm oil supplemented with 20% (w/w) soybean oil (from Nippon Oil and Fats Co., Tokyo, Japan) was used.[24] The total tocopherol contents of PL, EE, TG and the control oil were 6.7, 18.9, 35.1, and 6.3 mg/100 g of oil, respectively (Table II). The other reagents and chemicals were commercially available products in extra-pure grade.

*Constituent fatty acid analysis.* Fatty acid methyl esters from the oil samples for each oxidation period were prepared by transmethylation in a 10% methanolic hydrogen chloride solution under reflux for 1 h at 85°C. The methyl esters were extracted with *n*-hexane and were analyzed with a GC-380 gas chromatograph (GL Sciences, Tokyo, Japan) equipped with a flame ionization detector and a CP-SIL 88 capillary column (0.25 mm × 50 m, Chrompack, The Netherlands). The column temperature was 170°C, and the injector and detector temperatures were kept at 250°C. Fatty acid methyl esters were identified by comparing their retention times with those of standard methyl esters.

*Tocopherol determination.* An ethanolic solution of 2,2,5,7,8-pentamethyl-6-hydroxychroman (PMC; Wako Chemical Co., Osaka, Japan) was used as an internal standard for a quantitative analysis of tocopherol in the oil samples. The tocopherol content of each oil sample was determined by a JASCO HPLC system (Tokyo, Japan) in a JASCO Finepak SIL column (5 μm, 250 × 4.6 mm). The mobile phase was a mixture of *n*-hexane–2-propanol (99.5 : 0.5, v/v) delivered at a flow rate of 1.3 ml/min with a JASCO 880-PU pump. The tocopherols were detected by a JASCO 821-FP spectrofluorometer ($Ex = 298$ nm; $Em = 325$ nm) and recorded with a SIC 21J Chromatocorder (System Instruments, Tokyo, Japan). A standard solution (Sigma Chemical Co.) consisting of α-, β-, γ-, δ-tocopherol and PMC was used to identify peak components. Quantification was performed by comparing the peak areas relative to that of the internal standard.

*Headspace gas assay.* The oil samples (each accurately weighed to 50 mg, $n = 10$) were oxidized in a 10 ml W-teflon capped Pyrex tube and then kept at 25°C in the dark for 10 weeks. To evaluate the oxygen uptake into each sample oil during oxidation, the change of oxygen in the headspace gas of the tube was determined at appropriate intervals with a Shimadzu GC-4C gas chromatograph equipped with a thermal conductivity detector and a stainless column (0.3 × 200 cm) packed with

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*Abbreviations*: DHA, docosahexaenoic acid; PUFA, polyunsaturated fatty acid; TG, triacylglycerols; EE, ethyl esters; PL, phospholipids; PV, peroxide value; CV, carbonyl value; TOCO, tocopherol; PI, peroxidizability index.

**Table I.** Changes in the Fatty Acid Composition of DHA Oils during Autoxidation at 25°C

| | Unoxidized | | | | Oxidized for 5 weeks | | | | Oxidized for 10 weeks | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | PL | EE | TG | Control[a] | PL | EE | TG | Control[a] | PL | EE | TG | Control[a] |
| Fatty acid (mol%) | | | | | | | | | | | | |
| 16:0 | 32.5 | 41.6 | 41.8 | 41.7 | 32.7 | 41.2 | 60.2 | 41.4 | 35.3 | 47.4 | 61.8 | 40.6 |
| 16:1 (n-7) | 1.7 | 3.2 | 3.2 | 0.2 | 2.6 | 4.3 | 3.8 | 0.4 | 1.3 | 4.9 | 2.1 | 0.0 |
| 18:0 | 11.1 | 4.8 | 5.1 | 4.3 | 10.7 | 5.3 | 9.8 | 4.5 | 10.8 | 6.0 | 7.4 | 4.2 |
| 18:1 (n-9) | 27.7 | 22.8 | 22.4 | 35.8 | 28.4 | 22.4 | 20.5 | 35.4 | 26.9 | 25.4 | 25.5 | 36.1 |
| 18:2 (n-6) | 11.4 | 11.4 | 10.6 | 17.2 | 11.5 | 11.7 | 1.9 | 17.4 | 11.9 | 9.7 | 1.8 | 18.2 |
| 18:3 (n-3) | 0.2 | 0.6 | 0.6 | 0.8 | 0.3 | 0.6 | 0.8 | 0.9 | 0.3 | 0.5 | 0.3 | 0.9 |
| 20:4 (n-6) | 1.3 | 0.4 | 0.9 | | 1.2 | 0.6 | 0.1 | | 1.2 | 0.6 | 0.3 | |
| 20:5 (n-3) | 1.5 | 3.0 | 3.1 | | 1.1 | 2.6 | 1.9 | | 1.1 | 1.2 | 0.3 | |
| 22:5 (n-6) | 0.3 | 0.9 | 0.9 | | 0.1 | 0.9 | 0.1 | | 0.2 | 0.6 | 0.2 | |
| 22:5 (n-3) | 1.5 | 0.7 | 0.7 | | 1.4 | 0.7 | 0.1 | | 1.4 | 0.4 | 0.1 | |
| 22:6 (n-3) | 10.7 | 10.7 | 10.7 | | 10.0 | 9.4 | 0.9 | | 9.6 | 3.5 | 0.3 | |
| P:M:S[b] | 0.6:0.7:1 | 0.6:0.6:1 | 0.6:0.5:1 | 0.4:0.8:1 | 0.6:0.7:1 | 0.6:0.6:1 | 0.1:0.4:1 | 0.4:0.8:1 | 0.6:0.6:1 | 0.3:0.6:1 | 0.0:0.4:1 | 0.4:0.8:1 |
| n-6/n-3 | 0.9 | 0.8 | 0.8 | 22.3 | 1.0 | 1.0 | 0.6 | 20.2 | 1.1 | 2.0 | 2.4 | 21.5 |
| PI[c] | 123.1 | 127.7 | 129.8 | 19.6 | 113.2 | 117.4 | 23.4 | 20.0 | 111.1 | 54.3 | 10.1 | 20.9 |

Each value represents the mean of three determinations.
The DHA oils were examined in the form of PL (phospholipids), EE (ethyl esters), and TG (triacylglycerols).
[a] Palm oil supplemented with 20% soybean oil.
[b] P:M:S denotes polyunsaturated:monounsaturated:saturated acids.
[c] Peroxidizability index = (% dienoic × 1) + (% trienoic × 2) + (% tetraenoic × 3) + (% pentaenoic × 4) + (% hexaenoic × 5).

**Table II.** Changes in the Tocopherol Contents of DHA Oils during Autoxidation at 25°C

| | Unoxidized | | | | Oxidized for 5 weeks | | | | Oxidized for 10 weeks | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | PL | EE | TG | Control | PL | EE | TG | Control | PL | EE | TG | Control |
| Tocopherol (mg/100 g) | | | | | | | | | | | | |
| α- | 6.3 | 3.8 | 7.8 | 2.9 | 3.4 | 0.0 | 0.0 | 2.0 | 2.5 | 0.0 | 0.0 | 0.8 |
| γ- | 0.2 | 8.9 | 17.0 | 2.2 | 0.0 | 0.0 | 0.0 | 1.8 | 0.0 | 0.0 | 0.0 | 1.2 |
| δ- | 0.2 | 6.2 | 10.3 | 1.2 | 0.1 | 1.4 | 0.1 | 1.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| Total | 6.7 | 18.9 | 35.1 | 6.3 | 3.5 | 1.4 | 0.1 | 4.8 | 2.5 | 0.0 | 0.0 | 2.0 |

Each value is the mean of three determinations.
Sample oils and abbreviations are given in Table I.

5A molecular sieves (GL Science, Tokyo, Japan). Helium was used as the carrier gas (30 ml/min flow rate), the column temperature was kept at 70°C, and the injection and detection temperature were 90°C. The oxygen absorption was expressed as ml/g of oil.

*Measurements of peroxide value (PV) and carbonyl value (CV).* Hydroperoxide formation in the sample oil was evaluated by measuring PV according to the AOCS method[25] and is expressed as meq/kg of oil. The dinitrophenylhydrazine method[26] was employed to measure CV of each oil sample.

*Statistical analyses.* Each value is expressed as the mean value and standard deviation (SD). All data were subjected to an analysis of variance (ANOVA) and Student's *t*-test.

## Results and Discussion

The headspace oxygen uptake of each DHA oil and of the control oil during autoxidation at 25°C for 10 weeks is given in Fig. 1. The induction period for the oxidation of TG (25 days) was much shorter than that of EE (57 days), but neither PL nor the control palm oil showed any clear induction period. During the 10-week oxidation, the oxygen uptake was most evident in the form of TG and then by EE. Oxygen uptake in the form of PL was almost equivalent to that of the control oil and was low.

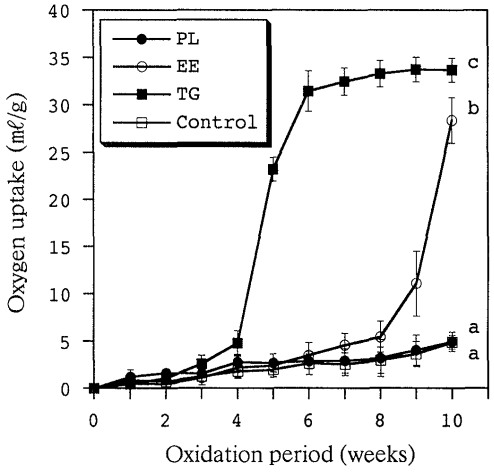


**Fig. 1.** Changes in the Oxygen Absorption of DHA-containing Oils during Autoxidation at 25°C.

Each value is the mean ± SD of 10 vials for each oil.
[a-c] Means not followed by a common superscript letter are significantly different at $p < 0.01$. DHA oil (50 mg/test tube) in the form of phospholipids (PL), ethyl esters (EE), and triacylglycerols (TG) was autoxidized, and its peroxidizability was compared with that of control oil (palm oil supplemented with 20% soybean oil).

Changes in the fatty acid composition of the oil samples during autoxidation are shown in Table I. Before oxidation, the major PUFAs in PL, EE, and TG were DHA (10.7 mol%) and linoleic acid (10.6–11.4 mol). The control oil contained linoleic acid (17.2 mol%) as the major polyunsaturate and no DHA. The DHA content in the form of TG showed the largest decrease (from 10.7 to 0.9 mol%) during a 5-week oxidation, while that of EE and PL showed a very slight decrease (from 10.7 to 9.4 and 10.0 mol%, respectively). The DHA contents of PL, EE, and TG after the 10-week oxidation were 9.6 (corresponding to 89.7% of initial DHA), 3.5 (32.7%), and 0.3 mol% (2.8%), respectively. Changes in P:M:S (polyunsaturates:monounsaturates:saturates), the n-6/n-3 ratio and peroxidizability index (PI) were evident in all forms, but less extensive in the PL and control oil. It was also noticed that 18:2n-6 was rather more stable in all three DHA oils against autoxidation for 10 weeks (Table I).

The change in tocopherol content of each oil sample during oxidation is shown in Table II. The sample oils before oxidation contained α-, γ-, and δ-tocopherols, and the total tocopherol concentration of PL was almost equivalent to that of the control oil. The total tocopherol contents of EE and TG were three- and five-times higher, respectively, than those of the PL oil. The tocopherols present in the form of EE and TG had compeltely decayed after the 10-week oxidation, but in PL and the control oil, tocopherol was respectively retained as 37.3% and 34.9% of the initial amount. For DHA-containing TG, γ-tocopherol was seen to decay most rapidly during the oxidation.

As shown in Fig. 2, PV and CV of the DHA oils in the form of TG and EE increased more markedly than in the form of PL and control oil during the oxidation, even though the initial tocopherol content of TG and EE was much higher than that of PL and the control oil (Table II).

Figure 3 summarizes the overall changes of DHA, tocopherols (TOCOs), PV and CV of the DHA oil samples during oxidation. A rapid accumulation of peroxides and carbonyls was evident, especially for TG and EE, and was accompanied by extensive breakdown of DHA and the

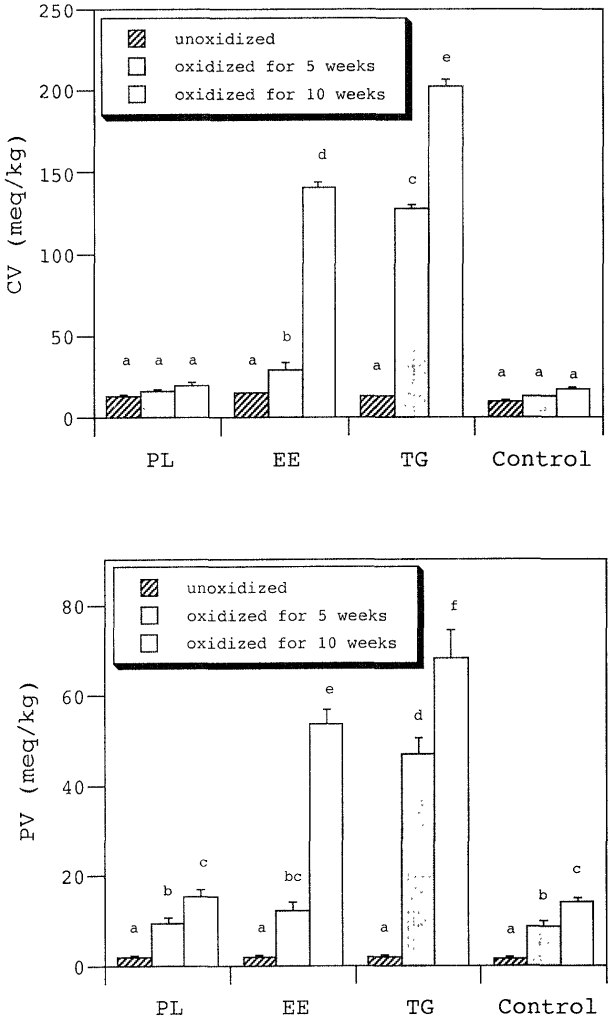


**Fig. 2.** Changes in Peroxide Value (PV) and Carbonyl Value (CV) of DHA-enriched Oils during Autoxidation at 25°C.

Each value is the mean ± SD of 5 samples for each oil.
[a–f] Bars with the same superscript letter are not significantly different at $p<0.01$. DHA-containing oils in the form of phospholipids (PL), ethyl esters (EE), and triacylglycerols (TG) were autoxidized, and the peroxidizability was compared with that of control oil (palm oil supplemented with 20% soybean oil).

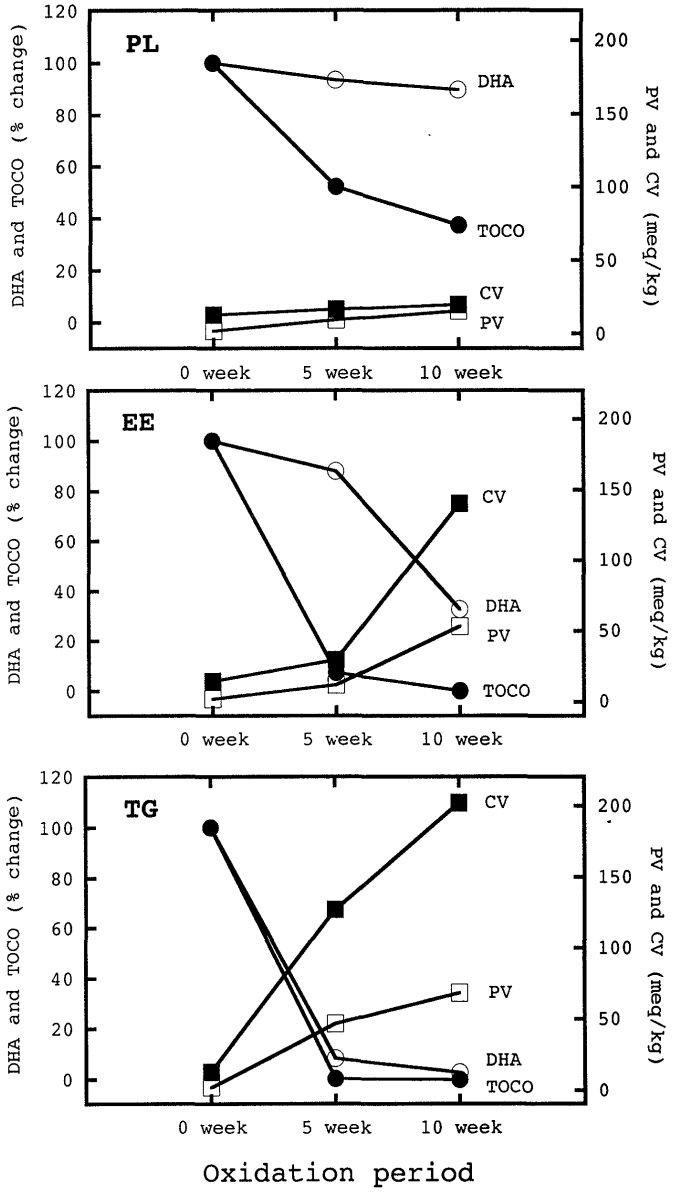


**Fig. 3.** Overall Relationship of the Changes in DHA, Total Tocopherol (TOCO), Peroxide Value (PV), and Carbonyl Value (CV) of DHA-containing Oils in the Form of PL (Upper), EE (Middle), and TG (Bottom) during Autoxidation at 25°C.

tocopherols. This may suggest that lipid radicals derived from DHA can exhaust tocopherols as a proton donor. In the form of PL, these changes were rather small in comparison with those of TG and EE. Thus, among the three DHA oils, the form of PL appears to have been the most advantageous and stable against peroxidation in the bulk phase when comparing the peroxidizability of oils with the same constituent fatty acids. Since the PC/PE ratio of PL presently used was 3.1, any difference in phospholipid composition would also significantly affect the oxidative stability of the PL oil. The DHA contents of PC and PE in the PL oil were 8.1% and 19.6%, respectively. The high stability of PL oil against autoxidation could be explained by the synergistic effects of tocopherols in preventing peroxidation.[27-29] The antioxidative synergy between tocopherols and PL seems to be closely associated with the suppressive effect of PL on the decomposition of tocopherols[27] that was observed in the present study. The chelating action as an antioxidative function of PL may also have been involved. Miyazawa *et al.*[30] have reported that the antioxidative effects of PL accompany reductive conversion of lipid hydroperoxide to the corresponding hydroxyl compound by a polar head group such as unesterified choline and ehtanolamine.

The ingestion of such highly unsaturated oils as fish and DHA oils causes tissue membrane phospholipid peroxidation and exhausts α-tocopherol in mammals.[31] Takeuchi *et al.*[32] have reported that marine oil containing phospholipids showed resistance to autoxidation. They showed that hydroperoxide formed in a phospholipid-containing salmon egg oil was much slower than that of highly purified salmon oil containing no phospholipids. Although the ingestion of DHA-containing PL oil provides several pathophysiological effects in mammals, membrane lipid peroxidation should be attenuated as a result of the incorporation of DHA into the membrane lipids, together with the decay of α-tocopherol.

In conclusion, DHA-containing oil in the form of PL was most advantageous and effective in preventing the oxidative deterioration of DHA oils in a bulk phase. Although, based on the present results, lower peroxidizability in the tissue lipids of mammals would be expected when DHA is ingested in the form of PL oil, no such beneficial effect was observed in our preliminary experiments with rats. Therefore, the prevention of lipid peroxidation and of α-tocopherol exhaustion in the digestive tract, blood, and tissue organelles remains of concern when DHA oil, fish oil, and DHA capsules are ingested.

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