Autoxidation of Synthetic Isomers of Triacylglycerol Containing Eicosapentaenoic Acid

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ABSTRACT: Several triacylglycerols (TAG) that contained eicosapentaenoic acid (EPA) were chemically synthesized and stored at 25°C to assess the influence of TAG structure on oxidative stability and formation of oxidation products. Oxidative stability was evaluated by oxygen consumption during storage of the TAG. Autoxidation products of TAG were analyzed by high-performance liquid chromatography (HPLC) and liquid chromatography–mass spectrometry (LC–MS). Results showed that a 2:1 (mole/mole) mixture of trieicosapentaenoylglycerol (EEE) and tripalmitoylglycerol (PPP) was most susceptible to autoxidation. The oxidative stability of TAG that contained EPA and palmitic acid was negatively correlated with the moles of EPA in a single TAG molecule. When TAG with one EPA and two other fatty acids were oxidized, chainlength of constituent fatty acids hardly affected the oxidative stability of EPA-containing TAG molecules, except for stearic acid. HPLC and LC–MS analyses showed that monohydroperoxides were major oxidation products regardless of type of TAG. Bis- and tris-hydroperoxides were formed during autoxidation of EEE and dieicosapentaenoylpalmitoylglycerol. Monohydroperoxy epidioxides were found in all autoxidized TAG. These observations suggested that TAG structure affected the oxidation of TAG with highly unsaturated fatty acids.

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KEY WORDS: Autoxidation, eicosapentaenoic acid, hydroperoxide, oxidative stability, triacylglycerol, triacylglycerol structure.

Eicosapentaenoic acid (EPA) as well as docosahexaenoic acid (DHA), abundant in marine oils, has received much attention because of specific physiological and nutritional effects. However, these highly unsaturated fatty acids (HUFA) are very susceptible to oxidation. This oxidation can produce undesirable flavors and harmful products during storage. Thus, oxidation retardation during storage is required. In general, antioxidants are useful for prevention of oxidation of fats and oils during processing and storage. Unfortunately, natural antioxidants, such as tocopherols, do not inhibit the oxidative deterioration of HUFA-rich fish oil. It has been suggested that the oxidative stability of vegetable oils could be affected by triacylglycerol (TAG) structure (1–7). In previous papers (8,9), we found that the oxidative stability of marine oils was modified by chemical and enzymatic interesterification. These results suggested the possibility that the oxidative stabilities of HUFA depend on TAG structure.

In this paper, several chemically synthesized TAG were used to evaluate the stability of EPA-containing TAG. Oxidation products, mainly hydroperoxides, were identified *via* high-performance liquid chromatography (HPLC) and liquid chromatography–mass spectrometry (LC–MS). We discuss the relation between oxidative stability and structure of HUFA-containing TAG in marine oils.

MATERIALS AND METHODS

Materials. EPA was provided by Nihon Suisan Kaisha Ltd. (Tokyo, Japan). 1-Palmitoylglycerol, 1,2-dipalmitoylglycerol, 1,3-dipalmitoylglycerol, 1,3-dilauroylglycerol, 1,3-dimyristoylglycerol, 1,3-distearoylglycerol, 1,3-dioleoylglycerol, 1,3-dilinoleoylglycerol, glycerol, and tripalmitoylglycerol (PPP) were purchased from Sigma Chemical Co. (St. Louis, MO). α-Tocopherol was purchased from Eisai Co. (Tokyo, Japan).

Syntheses of EPA-containing TAG. The following TAG were chemically synthesized, based on the method of Awl *et al.* (10). Trieicosapentaenoylglycerol (EEE) was synthesized by esterification of EPA with glycerol in the presence of 4-dimethylaminopyridine and *N*,*N*′-dicyclohexylcarbodiimide as catalysts. 1,2(or 2,3)-Dieicosapentaenoyl-3(or 1)-palmitoylglycerol (EEP) and 1,3-dieicosapentaenoyl-2-palmitoylglycerol (EPE) were prepared similarly from 1,2-dipalmitoylglycerol and 1,3-dipalmitoylglycerol. 1,3-Dilauroyl-2-eicosapentaenoylglycerol (LaELa), 1,3-dimyristoyl-2-eicosapentaenoylglycerol (MEM), 1,3-distearoyl-2-eicosapentaenoylglycerol (SES), 1,3-dioleoyl-2-eicosapentaenoylglycerol (OEO), and 1,3-dilinoleoyl-2-eicosapentaenoylglycerol (LiELi) were prepared from the corresponding 1,3-diacylglycerols. Synthesized TAG were purified through a Florisil column (Kanto Chemical Co. Inc., Tokyo, Japan), deactivated with 7% water, with diethyl ether/*n*-hexane (7:93, vol/vol). The purity of all TAG was higher than 95% by HPLC analysis.

Autoxidation. TAG (*ca.* 50 mg = 600 μmol) were supplemented with α-tocopherol (0.1%) in a 10-mL test tube with a W-type silicone rubber cap and stored at 25°C in the dark.

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The headspace gas (20 μL) was collected periodically and then subjected to gas chromatography with a thermal conductivity detector to follow the oxygen uptake during storage of TAG as described in the previous paper (9). Autoxidized EPA-containing TAG were measured for ultraviolet absorbance at 234 nm in an ethanol solution to estimate hydroperoxide concentration prior to HPLC and LC–MS analyses. Experiments were performed in triplicate.

Analysis of autoxidation products. HPLC and LC–MS were used to analyze autoxidation products of synthetic EPA-containing TAG. A reversed-phase C18 column (4.6 × 250 mm; Shiseido Co. Ltd., Tokyo, Japan) was used for HPLC analysis. Methanol/ethanol (1:1, vol/vol) was used for EPP and EEP as a mobile phase, whereas methanol/ethanol (4:1, vol/vol) was used for EEE. Flow rate was 0.7 mL/min except for EEP (0.5 mL/min). TAG and their oxidation products were monitored at 210 nm. The relative ratio of oxidation products was estimated by the area on HPLC chromatograms. LC–MS was performed by the electronspray–ionization mode in a Finnigan Mat (San Jose, CA) TSQ700 MS. The vaporizer was operated at 240°C; the capillary heater was set at 250°C. The electron multiplier was 1000 V. High-purity nitrogen gas was used for the sheath and auxiliary gases, which were set at 70 and 15 psi, respectively. The conditions of LC were the same as those of HPLC.

RESULTS

Oxidation effect of fatty acid composition and glycerol location. TAG that consisted of EPA and palmitic acid were stored at 25°C, and oxygen uptake was monitored (Fig.1). A 2:1 (mole/mole) mixture of EEE and PPP oxidized faster than EEP and EPE. Induction periods were 5 d for the EEE/PPP treatment and 8 d for both the EEP and EPE treatments. TAG oxidative stability was affected by the moles of EPA in a single TAG molecule but not by the specific position of EPA on the glycerol backbone.

When TAG that contained EPA and palmitic acid were stored at 25°C, induction periods were 7, 10, 16, and 17 d for a 1:2 (mole/mole) mixture of EEE and PPP (EEE/PPP, 1:2), a 1:1 (mole/mole) mixture of EEP and PPP (EEP/PPP, 1:1), EPP, and PEP, respectively (Fig. 2). A 1:2 (mole/mole) mixture of EEE and PPP was most rapidly autoxidized among these four TAG samples. The oxidative stability was negatively proportional to the moles of EPA present in each sample. In comparing the oxidative stability of EPP and PEP, EPA at the 2-position appeared to be slightly more stable. These observations indicate that number and position of EPA could affect the oxidative stability of EPA-containing TAG molecules.

Effect of other fatty acids with EPA. Oxidative stability was evaluated for TAG that contained EPA and saturated fatty acids with carbon chainlengths of 12 to 18. As shown in Figure 3, the induction period of LaELa, MEM, and PEP was 16 d, except for SES. In general, saturated fatty acids did not affect the oxidative stability of EPA-containing TAG.

The number of double bonds in constituent fatty acids with

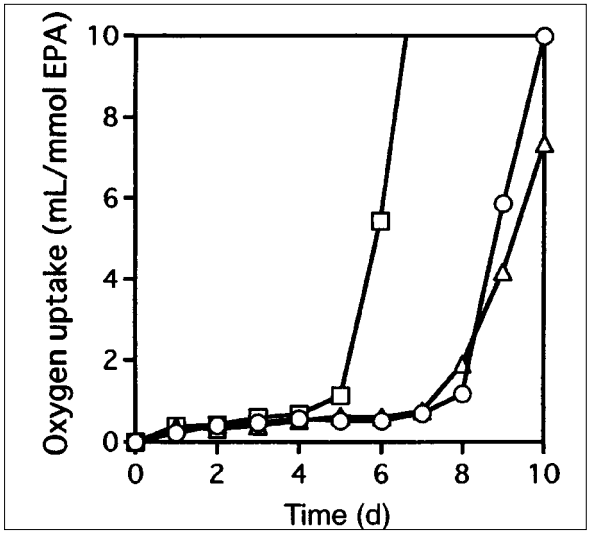

FIG. 1. Oxygen uptake of triacylglycerols containing eicosapentaenoic acid (EPA) and palmitic acid during storage at 25°C: ○, 1,3-dieicosapentaenoyl-2-palmitoylglycerol; △, 1,2(or2,3)-dieicosapentaenoyl-3(or1)-palmitoylglycerol; □, trieicosapentaenoylglycerol/tripalmitoylglycerol (2:1). (E, 20:5; P, 16:0).

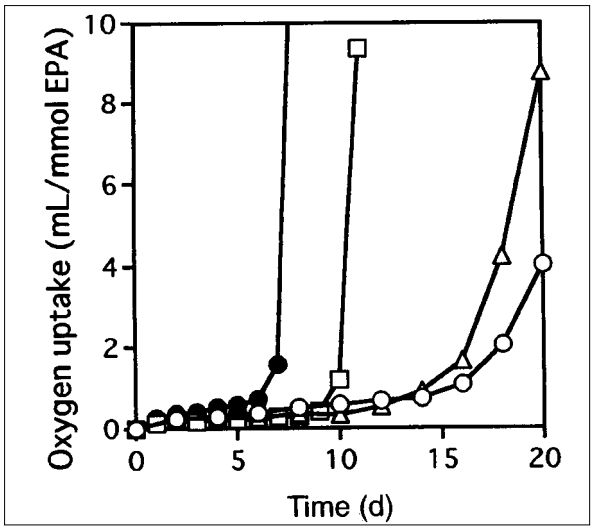

FIG. 2. Oxygen uptake of triacylglycerols of eicosapentaenoic acid and palmitic acid during storage at 25°C: ○, PEP; △, EPP; □, EEP/PPP (1:1); ●, EEE/PPP (1:2). See Figure 1 for abbreviation.

EPA all had some effects on TAG oxidative stability. TAG that consisted of one EPA and two stearic (18:0), oleic (18:1), or linoleic acids (18:2) were incubated at 25°C (Fig. 4). The induction period of LiELi was 12 d and was shorter than OEO (16 d). OEO was the most stable, and the induction period was almost the same as PEP, MEM, and LaELa in Figure 3. The variety of co-constituent fatty acids in TAG with EPA might be related in the oxidative stability as well as number and position of EPA in a TAG.

LC–MS analysis of autoxidation products. Autoxidation products of EPP, EEP, and EEE were compared at the initial stage of

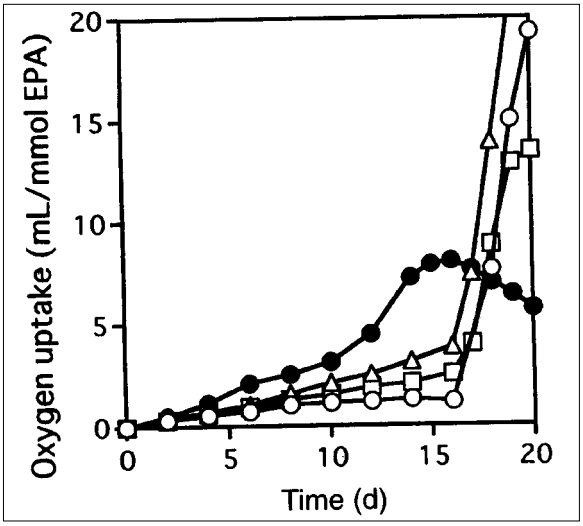

FIG. 3. Oxygen uptake of triacylglycerols of eicosapentaenoic acid and saturated fatty acids during storage at 25°C: ○, LaELa; △, MEM; □, PEP; ●, SES. (La, 12:0; M, 14:0; P, 16:0; S, 18:0; E, 20:5). See Figure 1 for abbreviation.

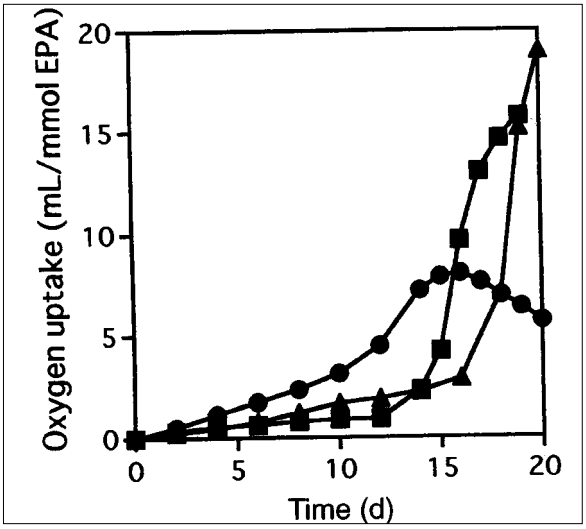

FIG. 4. Oxygen uptake of triacylglycerols of eicosapentaenoic acid and unsaturated fatty acids during storage at 25°C: ●, SES; ▲, OEO; ■, LiELi. (S, 18:0; O, 18:1; Li, 18:2; E, 20:5). See Figure 1 for abbreviation.

oxidation (absorbance of the ethanol solution at 234 nm = 0.16) by HPLC and LC–MS. Figure 5 shows HPLC and LC–MS chromatograms of autoxidized EEE among three EPA-containing TAG molecules. Similar HPLC chromatograms as shown in Figure 5 were obtained for all TAG. Peak 2, next to unoxidized TAG (peak 1), was observed at a retention time (R_t) of about 11 to 12 min for EPP, EEP, and EEE. Peak 2 compounds which gave [M + Na] ions at *m/z* 908, 954, and 1000 for EPP, EEP, and EEE, respectively, were identified as monohydroperoxides of each TAG (Fig. 5B). After reduction with $NaBH_4$, these compounds gave [M + Na] ions corresponding to the monohydroxy TAG at *m/z* 892, 938, and 984 for EPP, EEP, and EEE, respectively.

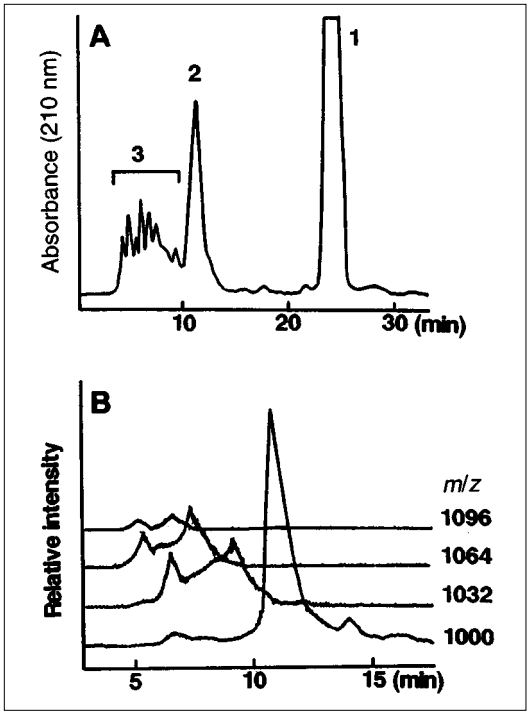

FIG. 5. HPLC (A) and LC–MS (B) chromatograms of autoxidized trieicosapentaenoylglycerol (EEE). Peak 1, unoxidized EEE; peak 2, monohydroperoxides; peak 3, secondary products.

Components of complicated peaks (peak 3) on HPLC chromatograms were analyzed by LC–MS. LC–MS analysis indicated that the peak at R_t 9 min of autoxidized EEE might contain monohydroperoxy epidioxides (Fig. 5B). This identification was based on the molecular ion peak ([M + Na], *m/z* 1032), which was shifted to *m/z* 1016, corresponding to trihydroxy EEE after reduction with $NaBH_4$. Similarly, monohydroperoxy epidioxides ([M + Na], *m/z* 940 and 986) were observed at R_t 9 and 12 min for autoxidized EPP and EEP, respectively. On the other hand, peak 3 of autoxidized EEP and EEE might contain bis-hydroperoxides, which give [M + Na] ions of *m/z* 986 and 1016 at R_t 8 and 7 min in addition to monohydroperoxy epdioxides. The [M + Na] ions of bis-hydroperoxides of EEP and EEE were shifted to *m/z* 954 and 984, corresponding to bis-hydroxy EEP and EEE, respectively, after reduction. Moreover, the [M + Na] ion (*m/z* 1064) owing to tris-hydroperoxides was observed at R_t 5.5 min in autoxidized EEE. These results indicate that major autoxidation products of EPA-containing TAG were monohydroperoxides, whereas secondary products depended on varieties of EPA-containing TAG.

The ratio of monohydroperoxides occupied in oxidation products formed during autoxidation was determined for EPP, EEP, and EEE (Fig. 6). Monohydroperoxides accounted for about 70 to 80% of total autoxidation products and did not change after the initial stage of autoxidation (absorbance within the range of 0.04 to 0.16 at 234 nm). Monohydroperoxides were major autoxidation products for all EPA-containing TAG, although the ratio of monohydroperoxides of autox-

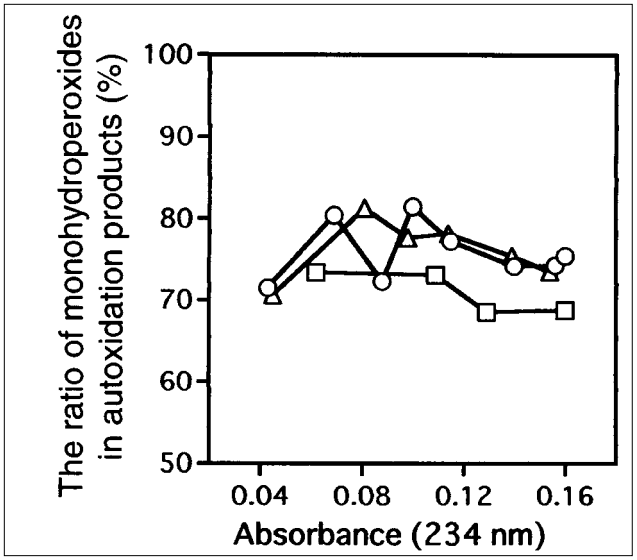

FIG. 6. The concentration (%) of monohydroperoxides in autoxidation products of triacylglycerols that contained eicosapentaenoic acid: ○, EPP; △, EEP/PPP (1:1); □, EEE/PPP (1:2). (E, 20:5; P, 16:0). See Figure 1 for abbreviation.

idized EEE was slightly lower than that of autoxidized EEP and EPP. Moles of EPA esterified to a TAG molecule did not consistently affect the ratio of monohydroperoxides in oxidation products formed during autoxidation of EPA-containing TAG.

DISCUSSION

The oxidative stability of TAG molecules with different numbers and glycerol positions of EPA was evaluated to understand the relation between oxidative stability and structure of EPA-containing TAG. Data showed that TAG structure relative to fatty acid composition and location could be an important factor in determining the oxidative stability of TAG that contain EPA. The oxidative stability of EPA-containing TAG depended primarily on the moles of EPA in a TAG molecule. The EEE TAG species were most unstable, while those containing one mole of EPA were most resistant to autoxidation. This result means that fish oil with high concentrations of EPA could be very susceptible to oxidation. In that regard, fish oil after acidolysis with oleic acid was more stable than the corresponding oil mixed with oleic acid (8). Frankel *et al.* (11) assessed the oxidative stability of synthetic TAG that consisted of linoleic acid (L) and linolenic acid (Ln) and found that more volatile decomposition compounds were formed in autoxidation of a mixture of LLL and LnLnLn than LLLn and LLnL.

The distribution of fatty acids on glycerol also affects oxidative stability. Raghuveer and Hammond (1) and Wada and Koizumi (3) reported that TAG with unsaturated fatty acids located at the 2-position of soybean oil were more stable than those at the 1- or 3-position. On the other hand, Miyashita *et al.* (12) found that LLnL was somewhat more unstable than LnLL. In our previous paper (9), we reported that TAG with two or three HUFA were very unstable while whale oil with HUFA preferentially distributed at the 1- or 3-position was stable. In the present studies, we could not find any difference in the stability of EEP and EPE. These results may be due to the high concentration of EPA in the present studies. As already shown, TAG with high levels of EPA were so susceptible to oxidation that it would be necessary to store them at a lower temperature or mix them with more stable TAG to evaluate the effect of the TAG structure. For TAG with one mole of EPA, we found that PEP was slightly more stable than EPP. That was in agreement with previous research (1,3).

As to the properties of coexisting fatty acids, we could not find any marked effect of saturated fatty acids on the oxidative stability of EPA-containing TAG except for stearic acid. Wada and Koizumi (3) reported that saturated fatty acids did not affect the oxidative rate of unsaturated fatty acids coexisting in TAG, while Park *et al.* (13–15) found that the oxidative stability of unsaturated TAG was enhanced with short-chain saturated fatty acids. Neff *et al.* (5,7) also reported that oxidative stability of soybean oil was affected by TAG composition and structure. A different type of oxygen absorption curve of SES observed in the present studies may be due to the physical state of the TAG. In this experiment, TAG were stored at 25°C, at which SES is solid, while other TAG were liquid. Among unsaturated fatty acids, linoleic acid enhanced the oxidation of EPA-containing TAG, which readily produced free radicals during autoxidation.

The conjectured structure of hydroperoxides found in autoxidized EPA-containing TAG is shown in Figure 7. HPLC and LC–MS analyses showed that major autoxidation products of EPA-containing TAG were monohydroperoxides (Fig. 7A,C,F), regardless of TAG structure, while secondary products were affected by varieties of EPA-containing TAG. The presence of monohydroperoxy epidioxides (Fig. 7B,D,G) was recognized in all TAG, while bis-hydroperoxides (Fig. 7E,H) were found in the autoxidized EEP and EEE. Autoxidized EEE also might contain tris-hydroperoxides (Fig. 7I). Yamauchi *et al.* (16,17) found monohydroperoxides as the predominant components and monohydroperoxy epdioxides as minor components in oxidation products of methyl eicosapentaenoate. Frankel (18) reported that the most abundant hydroperoxides formed during autoxidation of trilinoleoylglycerol and trilinolenoylglycerol were monohydroperoxides. Smaller amounts of other hydroperoxides, such as monohydroperoxy epidioxide and bis- and tris-hydroperoxides, also were formed. Our results were similar. However, the analysis of autoxidation products suggested that the TAG structure could modulate the autoxidation mechanism of EPA in TAG. The formation of monohydroperoxy epidioxides showed that the free-radical reaction that led to cyclization might take place in intra-fatty acid during autoxidation of all EPA-containing TAG. On the other hand, the presence of bis- and tris-hydroperoxides showed that the free-radical reaction might take place in inter-fatty acids during autoxidation of EEP and EEE. Instability of EEE might be due to its susceptibility to

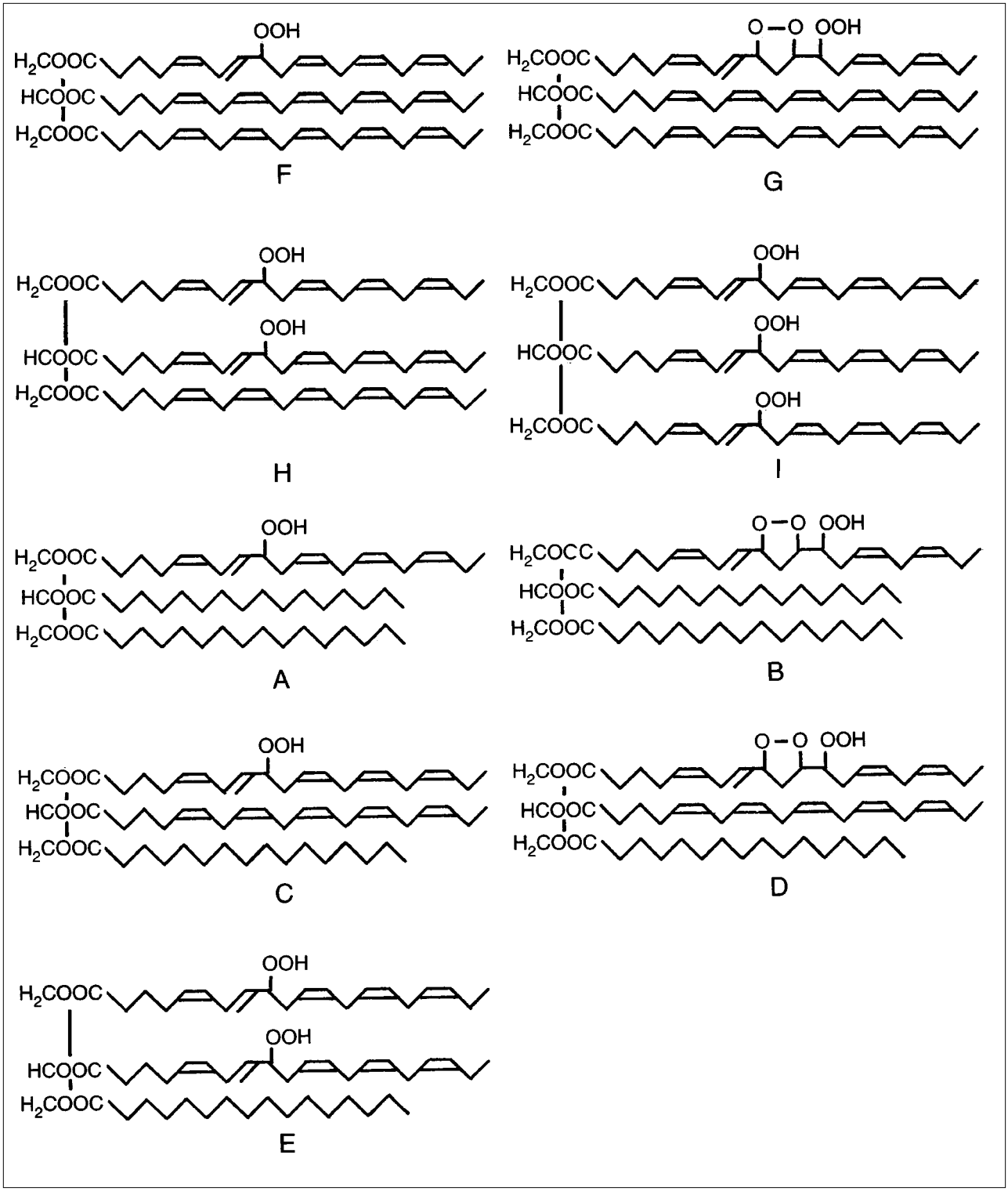

FIG. 7. Conjectured structure of hydroperoxides formed during autoxidation of triacylglycerols of eicosapentaenoic acid.

free-radical reactions in both intra- and inter-fatty acids. Hence, analyzing positional isomers of hydroperoxides and their composition would be necessary to know the detailed autoxidation mechanism of EPA-containing TAG.

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