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ORIGINAL PAPER

Docosahexaenoic Acid is More Stable to Oxidation when Located at the *sn*-2 Position of Triacylglycerol Compared to *sn*-1(3)

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Abstract Regio-isomeric effects on the oxidative stability of triacylglycerols (TAG) containing docosahexaenoic acid (DHA) were investigated using two pairs of regio-isomerically pure TAG, namely 1,3-dihexadecanoyl-2-(4,7,10,13,16,19-docosahexaenoyl)glycerol (PDP)/1,2-dihexadecanoyl-3-(4,7,10,13,16,19-docosahexaenoyl)glycerol (PPD) and 1,3-dioctadecenoyl-2-(4,7,10,13,16,19-docosahexaenoyl)glycerol (ODO)/1,2-dioctadecenoyl-3-(4,7,10,13,16,19-docosahexaenoyl)glycerol (OOD) where P, O, and D represent palmitic acid, oleic acid, and DHA respectively. Each pair of regio-isomers was subjected to accelerated auto-oxidation (at 40 or 50 °C inside a dark oven). In each case, the TAG oxidized more slowly when DHA was located at the *sn*-2 position (PDP and ODO) compared to the *sn*-1(3) position (PPD and OOD), as evidenced by slower development of peroxide value, slower depletion of DHA, and slower generation of secondary oxidation products propanal and *trans*, *trans*-2,4-heptadienal. The positional effect on auto-oxidation was more pronounced when DHA occurred in combination with oleic acid than with palmitic acid.

Keywords Auto-oxidation · Docosahexaenoic acid · Omega-3 fatty acid · Oxidative stability · Regio-isomer · Triacylglycerol

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Introduction

Dietary lipids are mainly composed of glycerol esters of fatty acids (FA), i.e. triacylglycerols (TAG), and relatively small amounts of other FA derivatives. Yet, the functional properties of dietary lipids, e.g. oxidative stability and bioactivity, are often discussed in terms of their component FA. This is because, whilst effects of FA structure (e.g. the degree of unsaturation) on oil functionality are well understood, relatively little is known about the effects of positional distribution of FA on the glycerol backbone (regio-isomerism).

Long-chain n-3 polyunsaturated FA (LC n-3 PUFA) such as docosahexaenoic acid (DHA) have been shown to play an important role in human health [1–3], and international nutrition and cardiac associations have begun advocating increased dietary intake of LC n-3 PUFA. The food industry has responded by introducing several DHA-fortified products into the market place. A major obstacle to expanding the product range of DHA-enriched foods is the extremely high susceptibility of DHA to oxidative rancidity, which severely reduces the flavor quality and shelf-life of such products. Whilst it is possible to protect oil from oxidation by technologies such as micro encapsulation, such processing interventions are expensive and the bio-availability of DHA in micro encapsulated products is uncertain. Knowledge of how TAG structure impacts on oxidative stability will assist in producing DHA-fortified foods with minimal use of micro encapsulation.

The literature shows that no consensus has emerged from previous studies on the effect of FA regio-isomerism on oxidative stability. Many of the previous studies were based on comparison of oxidative stability of oil before and after chemical randomization [4–7]. A major deficiency of this approach is, that during chemical randomization, the

natural antioxidants in the oil can be destroyed and various pro-oxidant artifacts introduced, thus preventing direct comparison of the randomized oil with the parent oil. The only way to reach unambiguous conclusions is to compare oxidative stabilities of pure TAG regio-isomers.

Although a relatively small number of studies using synthetic TAG regio-isomers have been conducted [8–11], there have been no studies on the effects of regio-isomerism for DHA-containing TAG. A major impediment to such studies is the difficulty in obtaining pure DHA regio-isomers. We recently reported on practical methods for the syntheses of regio-isomerically pure TAG containing DHA [12]. In this paper we report on the relative oxidative stabilities of two pairs of DHA-containing TAG regio-isomers which were synthesized using this newly published method.

Experimental Procedures

TAG Model Compounds

1,3-Dihexadecanoyl-2-(all-*cis*-4,7,10,13,16,19-docosahexaenoyl)glycerol (PDP), and 1,2-dihexadecanoyl-3-(all-*cis*-4,7,10,13,16,19-docosahexaenoyl)glycerol (PPD) were synthesized as previously described (12). 1,3-Dioctadec-*cis*-9-enoyl-2-(all-*cis*-4,7,10,13,16,19-docosahexaenoyl) glycerol (ODO) and 1,2-dioctadec-*cis*-9-enoyl-3-(all-*cis*-4,7,10,13,16,19-docosahexaenoyl)glycerol (OOD) were also synthesized using similar procedures. Regio-isomeric purities of the compounds were established by ^{13}C NMR spectroscopy and lipase-based regiospecific analysis [13].

The compounds were purified immediately prior to use, by chromatography using amino SPE cartridges (Strata SI-1 Silica, 55 μm, 70Å, 20 g/60 mL Giga Tubes (Phenomenex, Lane Cove, NSW, Australia). TAG were eluted with hexane and checked for the presence of hydroperoxides and free fatty acids (FFA) by HPLC. This purification ensured the removal of any traces of FFA and hydroperoxides that could have influenced oxidation rates. The pure TAG were recovered by purging with dry argon to remove most of the eluting solvent followed by high vacuum (<1 micron) treatment to remove residual solvent.

Oxidation of TAG Model Compounds

Samples (50 mg) were placed inside amber-colored glass headspace vials (10 mL), which were then flushed with pure oxygen inside a glove-box, and sealed with PTFE-lined silicone septa. Use of a glove-box was necessary to ensure consistent oxygen content in the vial headspace. The oxygen-filled vials were heated inside a dark oven at 50 °C in the case of PDP/PPD, and at 40 °C in the case of ODO/OOD. Use of headspace vials for oxidation facilitated the quantification of volatile oxidation products by headspace solid phase micro extraction (SPME) analysis. Headspace analyses were performed immediately after the vials were removed from the oven, which were then frozen at −80 °C until required for PV and fatty acid analyses. The oxidation of the PDP/PPD pair was replicated 3 times. Due to limited material, the oxidation of ODO/OOD was performed once only.

Solid phase micro extraction

Solid phase micro extraction was performed with divinylbenzene/carboxen/polydimethylsiloxane fiber (DVB/Carboxen/PDMS, 50/30 μm coating) using a CombiPal SPME autosampler. The fiber was inserted into the sample headspace and the vials incubated for 30 min at the same temperature used for the accelerated oxidation. The fiber was then transferred to the GC injector, and the injector was maintained in the splitless mode for 2 min to desorb the extracted volatile compounds on to the GC column. The split ratio was then changed to 50:1, and the fiber was allowed to remain in the injector for a further 5 min to condition the fiber for the next analysis.

Capillary Gas Chromatography

Gas chromatography analyses were conducted on an Agilent Model 6890 N instrument fitted with a flame ionization detector (FID) and a VOC fused silica capillary column (60 m, 0.32 mm i.d., 1.8 μm film, Agilent, Melbourne). Helium was used as the carrier gas at a constant flow rate of 1.0 mL min^{-1}. The temperature of the injector and the FID detector were held at 220 and 250 °C, respectively. The oven temperature was initially held at −20 °C for 2 min before increasing to 60 °C at the rate of 120 °C min^{-1}, and then to 220 °C at the rate of 7 °C where it was held for a further 10 min. The volatile compounds were identified by retention times. Identities were confirmed by GC–MS analysis (Agilent Model 6890 GC equipped with model 5973 mass spectral detector) using the same analytical column and GC operating conditions. Peaks were quantified using Chemstation software.

Fatty Acid Composition

Fatty acid profiles of the fresh and oxidized TAG model compounds were determined by GC-FID using a BPX-70 fused silica capillary column (25 m, 0.32 i.d., 0.1 μm film).

The temperatures of the GC column, injector, and the FID were maintained at 220, 220, and 240 °C. Fatty acid methyl esters (FAME) were prepared by transesterification with potassium hydroxide/methanol according to the procedure of Bannon et al. [14].

Peroxide Value (PV)

Peroxide value values were determined according to the spectrophotometric method reported by Shantha and Decker [15].

High Performance Liquid Chromatography (HPLC)

Hydroperoxides formed by oxidation of the TAG isomers were analyzed by non-aqueous reversed phase (NARP) HPLC on a Prevail C18 column (250 mm × 4.6 mm i.d., Alltech, Melbourne, Australia) using a modification of the method of Frankel et al. [16]. The instrument used was a HP (Agilent) Series 1050 HPLC system consisting of quaternary HPLC pump, degasser, autosampler and diode array detector (DAD). The DAD was operated at 235 nm. A 35900 ADC interface collected data from a Polymer Labs Model PL-ELS1000 evaporative light scattering detector (ELSD). An isocratic eluent system composed of acetonitrile/dichloromethane (65:35, %v/v) was used at a flow rate of 1.0 ml/min. Each run was terminated by flushing with acetonitrile followed by dichloromethane to remove strongly retained components. Data acquisition and analysis were performed using Chemstation software (LC-3D Rev A.09.03).

Statistical Analysis

The oxidation of PDP/PPD was replicated three times while the ODO/OOD oxidation was performed once only. For each time point in oxidation, headspace and fatty acid analyses were performed once and PV in triplicate. Standard deviations were calculated using Microsoft Office statistical software. Previous studies showed that the coefficients of variation for the fatty acid and headspace analyses were below 1 and 8% respectively.

Results and Discussion

Figure 1a and b respectively show the change in PV (in absorbance units) for the pairs PDP/PPD and ODO/OOD during accelerated oxidation. The PV of PDP remained practically constant for the first 5 h (induction period) before rising sharply and reaching a maximum after approximately 8 h. In contrast, the PV of PPD rose sharply from the outset reaching a maximum after 3 h before beginning to gel after 5 h. This showed that PDP is more resistant to auto-oxidation than PPD. A similar but more pronounced regio-isomeric effect was observed for the oxidation of the ODO/OOD (Fig. 1b).

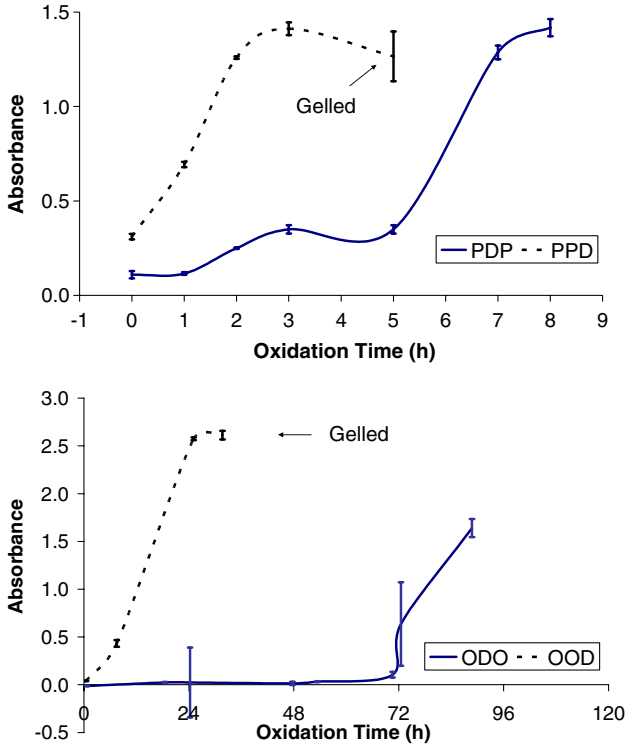

Fig. 1 Development of hydroperoxides (expressed as absorbance at 500 nm) during accelerated auto-oxidation of (**a** *top*) PDP and PPD at 50 °C and (**b** *bottom*) ODO and OOD at 40 °C. The *error bars* represent standard deviation for three replicate measurements

The superior oxidative stability of DHA located at the TAG *sn*-2 position was further confirmed by faster disappearance of DHA in PPD than PDP (Fig. 2a) and OOD than ODO (Fig. 2b). At the start of oxidation the D:P GC peak area ratio was approximately 0.6 for both isomers. This ratio steadily decreased for both isomers as oxidation progressed. However, the D:P ratio decreased at a faster rate for PPD compared with PDP (Fig. 2a). This regio-isomeric effect was more pronounced for the ODO/OOD pair. As the accelerated oxidation progressed, the D:O peak area ratio fell much more sharply for OOD than ODO (Fig. 2b).

The volatile products from auto-oxidation of PDP and PPD were basically the same and included 2-propenal, propanal, 2-butenal, 1-penten-3-ol, 1-penten-3-one, 2-ethyl furan, *trans*-2-pentenal, 2-(1-pentyl)furan, *cis*, *trans*-2,4-heptadienal, *trans*, *trans*-2,4-heptadienal, 3,5-octadiene-2-one, and *trans*, *trans*-2,4-nonadienal. The volatile oxidation

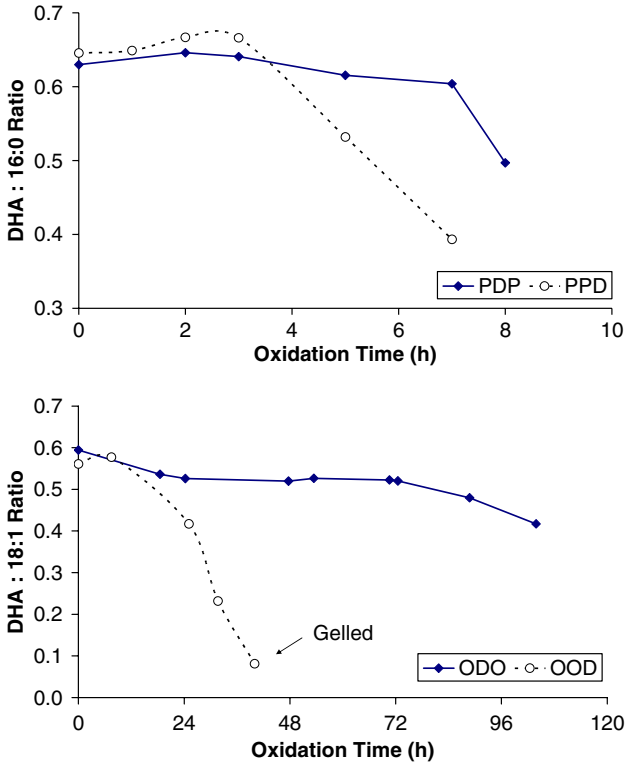

Fig. 2 Depletion of DHA during accelerated auto-oxidation of (**a**) PDP and PPD at 50 °C and (**b**) ODO and OOD at 40 °C. Values shown are GC/FID peak areas for DHA methyl ester relative to (**a** *top*) methyl palmitate in the case of PDP/PPD, and (**b** *bottom*) methyl oleate in the case of ODO/OOD

products from ODO and OOD contained nonanal, 2-decenal and 2-undecenal, which are derived from oleic acid, in addition to those found for PDP/PPD. There were also several other compounds common to both pairs of isomers but they were not fully characterized.

Propanal has widely been used as an oxidation marker for oils containing n-3 PUFA, especially fish oils where EPA and DHA are abundant [17–20]. Other oxidation markers include 1-penten-3-one [21, 22] and *trans*, *trans*-2,4-heptadienal [21–23]. Figure 3a shows the development of propanal during auto-oxidation of PPD and PDP. PPD attained the maximum for propanal in approximately 10 h whereas PDP required approximately 14 h. The corresponding times for *trans*, *trans*-2,4-heptadienal were 7, 10 h respectively (Fig. 3b). Figure 4a and b, respectively, show the development of propanal and *trans*, *trans*-2,4-heptadienal during auto-oxidation OOD and ODO. For OOD, the concentration of propanal and *trans*, *trans*-2,4-heptadienal attained maxima in approximately 40 and 34 h, respectively, whereas for ODO, the maxima were not reached even after 100 h, providing further evidence that DHA is more resistant to auto-oxidation when located at the *sn*-2 position of the TAG.

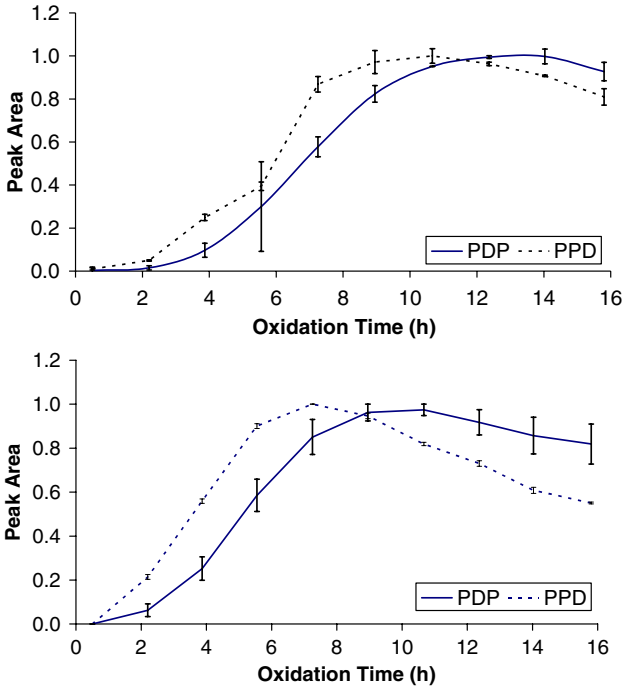

Fig. 3 Development of volatile oxidation products **a** propanal and **b** *trans*, *trans*-2,4-heptadienal during the auto-oxidation of PDP and PPD at 50 °C. Normalized GC/FID peak areas are shown. The *error bars* represent standard deviation for three replicate oxidations

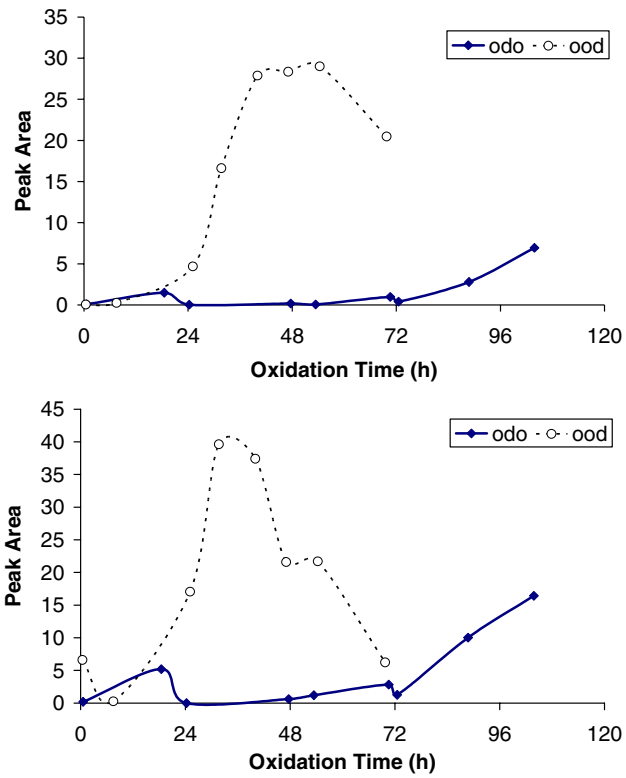

Fig. 4 Development of volatile oxidation products, propanal (**a** *top*) and *trans*, *trans*-2, 4-heptadienal (**b** *bottom*), during the auto-oxidation of ODO and OOD at 40 °C. Normalized GC/FID peak areas are shown

In addition to the volatile secondary oxidation products, we also measured the primary oxidation products of ODO and OOD by HPLC. Figure 5a and b, respectively, show the development of polar products during oxidation of ODO and OOD at 40 °C. There was no notable change in the HPLC chromatogram of ODO during the first 24 h. In contrast, the chromatogram of OOD changed dramatically during this time with the appearance of a cluster of peaks in the region where monohydroperoxides were expected to appear. For ODO, no appreciable production of monohydroperoxides was observed until after four days of oxidation. These results concur with the other oxidative stability measurements discussed above, showing that DHA is more stable to oxidation when located at the *sn*-2 position of the TAG. Although we did not attempt to characterize the individual components of the detected monohydroperoxides, we observed a close similarity between our results and those recently reported for products of oxidized DHA by Lyberg and Adlercreutz [24] who used similar HPLC conditions to ours. They identified the first peak in the cluster, which provided the largest contribution to the total amount of DHA monohydroperoxides as the 20-monohydroperoxy-DHA and reported that, although all isomers decreased after reaching a maximum, the 20-monohydroperoxy isomer continued to increase relative to the others. We also observed that the sharp peak in our chromatogram (MHP) eluting at approximately 4 min grew more rapidly than any other component within the monohydroperoxide cluster (Fig. 5b) suggesting that this peak was due to 20-monohydroperoxy-DHA.

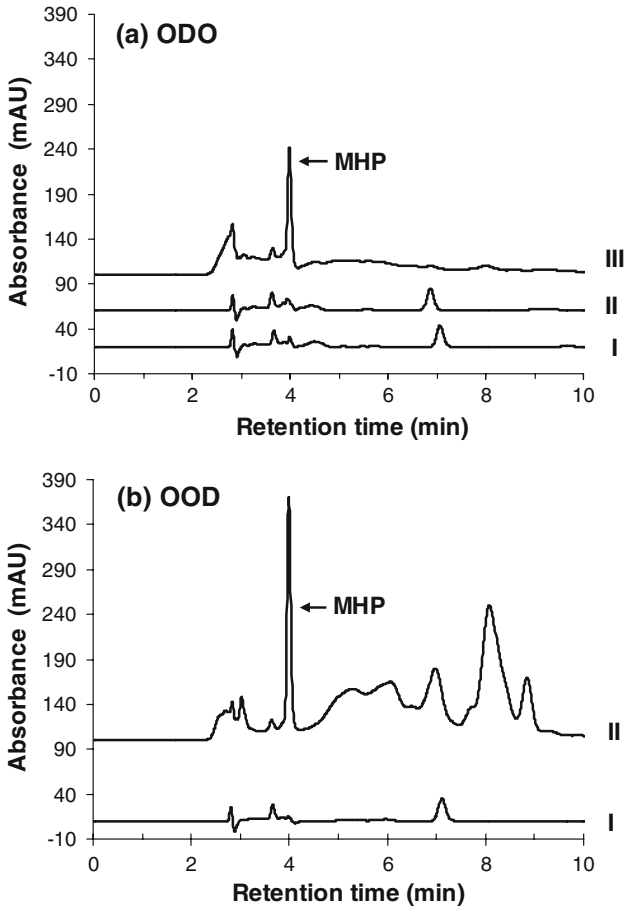

Fig. 5 Development of primary oxidation products during the autoxidation of (**a**) ODO and (**b**) OOD at 40 °C as analyzed by reversed phase HPLC using UV detection at 235 nm. The chromatograms (*i*), (*ii*), and (*iii*), respectively, represent oxidation products after 0.5, 24, and 103 h. The 103 h chromatogram for OOD is not shown as the OOD gelled after 24 h. MHP denotes the dominant component of the DHA monohydroperoxides

Several, albeit sporadic, attempts have been made to elucidate the effects of regio-isomerism on the oxidative stability of polyunsaturated TAG. Essentially, three differently strategies have been used: (1) comparison of oxidative stability of oil before and after chemical randomization, (2) comparison of oxidative stability of synthetic TAG regio-isomers, and (3) determination of the relative abundance of regio-isomeric TAG hydroperoxides. A major deficiency of the randomization approach is the destruction of natural antioxidants and / or generation of pro-oxidants during the chemical randomization process. Oxidative stability studies based on chemical randomization have often produced contradictory results.

PUFA in natural oils are preferentially distributed at the TAG *sn*-2 position and chemical randomization has the effect of increasing the PUFA content at the *sn*-1 and *sn*-3 positions. On this basis, Wada and Koizumi [6] who found randomized soybean oil which oxidized more rapidly than the parent oil concluded that PUFA is more resistant to oxidation when located at *sn*-2. However, Park et al. [5] who also observed that chemically randomized soybean oil oxidized more rapidly than the parent oil, found that when the tocopherols level in the randomized oil was restored to the original level there was no difference in the oxidation rates, suggesting that position of the PUFA did not influence oxidative stability. On the other hand, Neff and List [25] reported that chemical randomization of high stearic soybean oil improved oxidative stability suggesting that PUFA is more stable when located at the *sn*-1 and *sn*-3 positions. Furthermore, it has been reported that oxidative stability of fish oil increases after chemical randomization [26]. This was attributed to a reduction in the amount of highly labile TAG species such as tri-eicosapentaenoyl glycerol (EEE) and tri-docosahexaenoyl glycerol (DDD) rather than any regio-isomeric effects.

Thus it is imperative to use pure TAG regio-isomers to determine the effect of regio-isomerism on oxidative stability. Endo et al. [11] working with synthetic regio-isomers reported that PEP (where P is palmitic acid and E is eicosapentaenoic acid) was slightly more resistant to oxidation than PPE. The results of the present study, which to our

knowledge is the first time positional effects on oxidative stability of DHA have been examined using model DHA-containing TAG concur with the findings of Endo et al. [11]. With model TAG, we have unambiguously demonstrated not only that DHA is more stable to oxidation when located at the *sn*-2 position, but also that this positional effect is more pronounced when DHA occurs in the TAG in combination with other unsaturated FA.

To explain the superior oxidative stability of PUFA located at the *sn*-2 position, Raghuveer and Hammond [27] proposed a theory based on the hexagonal packing of TAG acyl chains in the molten state. They suggested that the TAG structure affected the rate of oxidation by altering the effective concentration of the substrate, thus affecting the propagation rate. They also suggested that, because of the hexagonal packing of the acyl chains in liquid fat, acyl groups attached to the *sn*-1 and *sn*-3 positions of the same TAG molecule could readily interact with each other, and if one became oxidized, the other was likely to be oxidized too [4]. However, the same group analyzing the hydroperoxide formed by auto-oxidation of corn oil found no evidence that any particular TAG is singled out for oxidation, and suggested rather that TAG structure influences oxidative stability by altering the rate of decomposition of hydroperoxides. Miyashita et al. [9] concluded that LLLn (where L is linoleic acid and Ln is linolenic acid) is more resistant to oxidation than LLnL on the basis of the amounts of hydroperoxides formed from each isomer, and they attributed this difference to higher interactions between Ln and L residues (in LLnL) than between the two L residues (in LLLn). This explanation concurs with our observation that ODO is more stable to oxidation than OOD.

References

1. von Schacky C, Harris WS (2007) Cardiovascular benefits of omega-3 fatty acids. Cardiovasc Res 73:310–315
2. Ruxton CHS, Reed SC, Simpson MJA, Millington KJ (2004) The health benefits of omega-3 polyunsaturated fatty acids: a review of the evidence. J Hum Nutr Diet 17:449–459
3. Nettleton JA, Katz R (2005) N-3 Long-chain polyunsaturated fatty acids in type 2 diabetes: a review. J Am Diet Assoc 105:428–440
4. Lau FY, Hammond EG, Ross PF (1982) Effect of randomization on the oxidation of corn oil. J Am Oil Chem Soc 59:407–411
5. Park DK, Terao J, Matsushita S (1983) Influence of interesterification on the autoxidative stability of vegetable oils. Agric Biol Chem 47:121–123
6. Wada S, Koizumi C (1986) Influence of random interesterification on the oxidation rate of soybean oil triglyceride. Yukagaku 35:549–553
7. Ledochowska E, Wilczynska E (1998) Comparison of the oxidative stability of chemically and enzymatically interesterified fats. Fett Lipid 100:343–348
8. Park DK, Terao J, Matsushita S (1983) Influence of the positions of unsaturated acyl groups in glycerides on autoxidation. Agric Biol Chem 47:2251–2255
9. Miyashita K, Frankel EN, Neff WE, Awl RA (1990) Autoxidation of polyunsaturated triacylglycerols III. Synthetic triacylglycerols containing linoleate and linolenate. Lipids 25:48–53
10. Frankel EN, Selke E, Neff WE, Miyashita K (1992) Autoxidation of polyunsaturated triacylglycerols IV. Volatile decomposition products from triacylglycerols containing linoleate and linolenate. Lipids 27:442–446
11. Endo Y, Hoshizaki S, Fujimoto K (1997) Autoxidation of synthetic isomers of triacylglycerol containing eicosapentaenoic acid. J Am Oil Chem Soc 74:543–548
12. Fraser BH, Perlmutter P, Wijesundera C (2007) Practical synthesis of triacyleglycerol regioisomers containing long-chain polyunsaturated fatty acids. J Am Oil Chem Soc 84:11–21
13. Shen Z, Wijesundera C (2006) Evaluation of ethanolysis with immobilized *Candida antarctica* lipase for regiospecific analysis of triacylglycerols containing highly unsaturated fatty acids. J Am Oil Chem Soc 83:923–927
14. Bannon CD, Craske JD, Hilliker AE (1985) Analysis of fatty acid methyl esters with high accuracy and reliability IV. Fats with fatty acids containing four or more carbon atoms. J Am Oil Chem Soc 62:1501–1507
15. Shantha NC, Decker EA (1994) Rapid, sensitive, iron-based spectrophotometric methods for determination of peroxide values of food lipids. J AOAC Int 77:421–424
16. Frankel EN, Neff WE, Miyashita K (1990) Autoxidation of polyunsaturated triacylglycerols II Trilinolenoylglycerol. Lipids 25:40–47
17. Boyd LC, King MF, Sheldon B (1992) A rapid method for determining the oxidation of n-3 fatty acids. J Am Oil Chem Soc 69:325–330
18. Frankel EN (1993) Formation of headspace volatiles by thermal decomposition of oxidized fish oils vs. oxidized vegetable oils. J Am Oil Chem Soc 70:767–772
19. Min H, McClements DJ, Decker EA (2003) Impact of whey protein emulsifiers on the oxidative stability of salmon oil-in-water emulsions. J Agric Food Chem 51:1435–1439
20. Augustin MA, Sanguansri L, Bode O (2006) Maillard reaction products as encapsulants for fish oil powders. J Food Sci 71:E25–E32
21. Venkateshwarlu G, Let MB, Meyer AS, Jacobsen C (2004) Chemical and olfactometric characterization of volatile flavor compounds in a fish oil enriched milk emulsion. J Agric Food Chem 52:311–317
22. Aidos I, Jacobsen C, Jensen B, Luten Van Der Padt A, Boom RM (2002) Volatile oxidation products formed in crude herring oil under accelerated oxidative conditions. Eur J Lipid Technol 104:808–818
23. Lee H, Kizito SA, Weese SJ, Craig-Schmidt MC, Lee Y, Wei CI, An H (2003) Analysis of headspace volatile and oxidized volatile compounds in DHA-enriched fish oil on accelerated oxidative storage. J Food Sci 68:2169–2177
24. Lyberg AM, Adlercreutz P (2006) Monitoring monohydroperoxides in docosahexaenoic acid using high-performance liquid chromatography. Lipids 41:67–76
25. Neff WE, List GR (1999) Oxidative stability of natural and randomized high-palmitic- and high-stearic-acid oils from genetically modified soybean varieties. J Am Oil Chem Soc 76:825–831
26. Kimoto H, Endo Y, Fujimoto K (1994) Influence of interesterification on the oxidative stability of marine oil triacylglycerols. J Am Oil Chem Soc 71:469–473
27. Raghuveer KG, Hammond EG (1967) The influence of triglyceride structure on the rate of autoxidation. J Am Oil Chem Soc 44:239–243