

¹H-NMR Characterization of Epoxides Derived from Polyunsaturated Fatty Acids

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Abstract In recent years, ¹H NMR has been used to study epoxides in lipid oxidation and industrial processes, but the peak assignments reported for monoepoxides and diepoxides have been inconsistent. Lack of clear assignments for chemical shifts of epoxides derived from polyunsaturated fatty acids (PUFA) has also limited the use of ¹H NMR in detecting and quantifying these products during both oxidative degradation and industrial epoxidation. In this study, ¹H NMR was used to characterize the epoxides synthesized from trilinolein, trilinolenin, canola oil, and fish oils by reaction with formic acid and hydrogen peroxide. Assignments for epoxides derived from PUFA in canola oil and fish oil were between 2.90–3.23 ppm and 2.90–3.28 ppm, distinct from other chemical groups in these oils. Chemical shifts of epoxy groups moved downfield with an increasing number of epoxy groups in the fatty acid chain. Hence, peaks for diepoxides appeared at 3.00, 3.09, and 3.14 ppm and for triepoxides at 3.00, 3.16, and 3.21 ppm. Results also suggested that stereoisomers of diepoxides and triepoxides were formed during the epoxidation process under the conditions of this study. These new assignments for di- and tri-epoxide stereoisomers were supported by GC–MS analysis of their methyl esters, H–H COSY experiments,

and a re-evaluation of several previous epoxide-related studies.

Keywords ¹H NMR · Lipid oxidation · Canola oil · Fish oil · Stereoisomer · Diepoxide · Triepoxide

Introduction

Epoxides contain at least one epoxy group, which is a three-member cyclic ether. In industrial processes, epoxides are synthesized from vegetable oils and widely used as intermediates for a number of products, including lubricants [1], plasticizers and chemical feedstocks [2]. Epoxides are also intermediates of lipid oxidation [3], which occurs in lipid-containing foods, such as oils. They are thought to be formed during free radical lipid peroxidation [4, 5].

Several approaches have been reported previously for the determination of epoxides in oil samples, including methods utilizing hydrogen bromide (HBr) [6], 4-(*p*-nitrobenzyl) pyridine (NBP) [7], *N,N*-diethyldithiocarbamate (DTC) [8], and gas chromatography–flame ionization detection (GC-FID) [9]. The HBr method is the most commonly used method to determine epoxides in industrial applications where they are important intermediates. However, secondary lipid oxidation products, such as conjugated dienes and some unsaturated carbonyls, interfere with the HBr method, thereby causing the overestimation of epoxide content in oxidized oils [6]. The NBP, DTC, and GC-FID methods require derivatization and are relatively time-consuming [7–9].

Nuclear magnetic resonance (NMR) has been applied to the study of epoxides in both oxidized and epoxidized oils. For example, it has been used to analyze mono- and

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diepoxides in oxidized sunflower oil [10] and to study epoxidized vegetable oils [2, 11]. Without requiring preparation of derivatives, NMR can be a fast, direct, alternative method for quantification of epoxides, if the chemical shifts of epoxides are known.

An earlier study of monoepoxides had shown the chemical shift of the epoxy protons to be 2.9 ppm, while diepoxides were reported to give signals at 2.9 and 3.1 ppm [12]. However, these findings were not consistent with a number of more recent studies on epoxidized vegetable oils using ^1H NMR [1, 11, 13, 14]. For instance, epoxidized jatropha oil and soybean oil, both of which contain monoepoxides and diepoxides derived from oleic acid and linoleic acid, gave four signals between 2.8 and 3.2 ppm [1, 11, 13, 14]. Based on the previously established assignments for monoepoxides and diepoxides [12], only three of the signals can be explained. Therefore, the currently used assignments may not be appropriate. Furthermore, the applications of ^1H NMR to the quantification of epoxides have been limited to the measurements of monoepoxides and diepoxides [15]. For epoxidized oils which may contain triepoxides, including epoxidized canola oil [2, 16], it is difficult to make assignments because little research has been conducted on ^1H -NMR characterization of triepoxides.

The objectives of this study were to interpret the ^1H -NMR spectra of diepoxides, triepoxides, and epoxidized oils and to evaluate their potential use in quantitative studies of epoxides in oils. Epoxides were chemically synthesized and the reaction progress was followed by observing the changes in the ^1H -NMR spectra. Specifically, we examined epoxides of trilinolein (two double bonds in each fatty acid) and trilinolenin (three double bonds in each fatty acid), and epoxides derived from unsaturated fatty acids in canola oil (0–3 double bonds in each fatty acid) and fish oil (0–6 double bonds in each fatty acid). This information is necessary for both identification and quantification of mixed epoxides in any epoxide-related studies.

Experimental Procedures

Materials

Trilinolein (18:2, *cis*-9, *cis*-12), trilinolenin (18:3, *cis*-9, *cis*-12, *cis*-15), and conjugated methyl linoleate (18:2, *cis*-9, *trans*-11) were purchased from Nu-Chek Prep Inc. (Elysian, USA). Commercial canola oil was obtained from Loblaw's Inc. (Toronto, Canada). Fish oil triacylglycerol (containing tocopherol as antioxidant), stripped fish oil triacylglycerol (without tocopherol), and fish oil ethyl esters (containing tocopherol as antioxidant) were kindly given by Ascenta Health Ltd. (Dartmouth, Canada). Deu-

Table 1 The composition of the major fatty acids in canola oil and fish oil expressed as mass% of total fatty acid mass, obtained from GC analysis

Fatty acid	Canola oil	Fish oil
14:0	/	7.47 ± 0.05
16:0	3.84 ± 0.04	15.51 ± 0.10
18:0	1.77 ± 0.04	2.86 ± 0.03
16:1n-7	/	9.16 ± 0.06
18:1n-7	3.42 ± 0.05	2.93 ± 0.02
18:1n-9	59.96 ± 0.13	8.93 ± 0.04
16:2n-4	/	1.39 ± 0.01
18:2n-6	18.89 ± 0.11	1.02 ± 0.01
18:3n-3	9.63 ± 0.07	0.72 ± 0.01
16:3n-4	/	2.02 ± 0.01
16:4n-1	/	3.02 ± 0.02
18:4n-3	/	2.66 ± 0.02
20:5n-3	/	21.20 ± 0.18
22:5n-3	/	2.34 ± 0.03
22:6n-3	/	8.37 ± 0.08

terated chloroform was purchased from Sigma-Aldrich Co. (St. Louis, USA). All chemicals were of analytical grade.

GC Analysis

Both oils were analyzed for their fatty acid profiles via AutoSystem GC-FID (PerkinElmer Inc., Waltham, USA). A modified version of the Global Organization for EPA and DHA Voluntary Monograph for Omega-3 was followed to convert triacylglycerols to fatty acid methyl esters (FAME) [17]. GC conditions were developed by Ref. [18] and FAME were separated on a DB-23 column [(50 %-cyanopropyl)-methylpolysiloxane, 30 m, 0.25 mm ID, 0.25 μm thickness] using helium as the carrier gas. The initial temperature of the oven was held at 153 °C for 2 min and increased to 205 °C at 2.3 °C/min and held for 8.3 min. The FID was set to 270 °C while the injector was operated in split mode at 250 °C [18]. Commercially available reference standards GLC 682 and GLC 455 were obtained from Nu-Chek Prep, Inc. (Elysian, USA) and used to identify the fatty acids. Additionally, fatty acids which are not commercially available were first identified in a menhaden FAME sample by gas chromatography–mass spectrometry (GC–MS); the sample was then used as a reference for retention times in GC-FID. The fatty acid profiles were reported as percent of total mass (Table 1).

Calculation of Number of Double Bonds

The concentrations of double bonds in fatty acid standards and oils were calculated using molecular weight, structure

and, if necessary, the fatty acid proportional data obtained from GC analysis. This information was used to calculate the amount of reagent necessary to prepare fully epoxidized fatty acid glycerol esters and oils. The concentrations of double bonds were estimated to be 6.82, 10.30, and 6.79 mol/kg for trilinolein, trilinolenin, and conjugated methyl linoleate, respectively. For canola and fish oils, the concentrations of double bonds were estimated from the fatty acid proportional data obtained from GC analysis (Table 1) and found to be 4.46 and 7.96 mol/kg, respectively.

Epoxidation

A molar ratio of 1:0.5:1.5 (double bonds:formic acid:hydrogen peroxide) was applied for epoxidation of the standards and oils following the procedures described in a previous work [19]. Moles of 50 and 30 % hydrogen peroxide were calculated by taking into consideration their concentrations in solution. In oxidized oils, epoxy groups are often encountered with adjacent double bonds; thus, the $^1\text{H-NMR}$ characterizations of the epoxides with remaining double bonds nearby are of great interests. To make partially epoxidized trilinolein, the amount of hydrogen peroxide was reduced to only 0.75 mol for each mol of double bonds.

In most cases, the three reagents were simply mixed together and heated at 38 °C for 24 h to allow epoxidation to occur [20, 21]. However, the process using 50 % H_2O_2 with trilinolenin resulted in polymerization. To avoid this, trilinolenin was reacted with 30 % H_2O_2 in two successive incubations at 38 °C. Partially-epoxidized trilinolenin was recovered after incubating for 24 h and analyzed for epoxides by $^1\text{H NMR}$. The recovered product was then mixed with fresh 30 % H_2O_2 /formic acid and reacted for an additional 2 h to generate fully-epoxidized products. Epoxidized oils were extracted by dissolving the reaction mixtures in ethyl acetate, washing in sodium chloride, separating phases by centrifugation, and drying the oil phase under nitrogen according to published procedures [19]. The remaining oil was collected as the epoxidized sample.

The purity of the epoxidized products was verified by thin layer chromatography (TLC) using a plate of silica gel 60 (20 cm \times 20 cm, 250 μm thickness), obtained from EMD Millipore (Darmstadt, Germany), with hexane: ethyl ether (80:20, v/v) and chloroform: methanol (95:5, v/v) as developing solvents and visualization under ultraviolet light (254 nm). The epoxidized products were recovered from the plates with chloroform:methanol (90:10, v/v) and reanalyzed by $^1\text{H NMR}$ for comparison with the epoxidized products without TLC purification.

GC-MS

A Trace GC Ultra gas chromatograph coupled with a PolarisQ mass spectrometer (Thermo Fisher Scientific Inc., Waltham, USA) was used for analysis. The epoxidized trilinolein and trilinolenin were converted into their methyl esters following a base-catalyzed procedures employing sodium methoxide [22] and dissolved in hexane for GC-MS analysis. The analysis was performed in both electron ionization (EI) and chemical ionization (CI) modes. The concentrations of the samples were 0.5 mg/ml for EI and 2 mg/ml for CI. Splitless-injection at 250 °C was used. Separation was performed on a DB-23 capillary column (30 m \times 0.25 mm i.d.) with helium as the carrier gas at 1.2 ml/min. The oven temperature program was initially held at 60 °C for 2 min, then increased to 235 °C at 12 °C/min and held for 40 min. The ionization energy was 70 eV, with multiplier voltage of 1643 V, source temperature at 200 °C, and transfer line at 235 °C. Spectral data were acquired over a mass range of m/z 60–600 in EI mode and m/z 60–400 in CI mode. The emission current was 250 μA for EI and 100 μA for CI. In CI mode, ammonia was used as the reagent gas at a flow rate of 1.3 ml/min.

$^1\text{H-NMR}$ Analysis

All standards and oil samples were prepared for $^1\text{H-NMR}$ analysis by dissolving 0.1 g of sample in 2.0 mL of deuterated chloroform and placing 750 μL of the solution in a 5-mm diameter NMR tube. The acquisition parameters were modified from Ref. [23]: spectral width 10,080 Hz, relaxation delay 3 s, number of scans 64, acquisition time 3.25 s, pulse width 90°. The total acquisition time was 6.9 min. The $^1\text{H-NMR}$ spectra were recorded on a Bruker Avance 500 MHz spectrometer. The signal of non-deuterated chloroform present in the deuterated chloroform was used as a reference for chemical shifts. The assignment of the signals was made taking into consideration previous published assignments [12]. The $^1\text{H-NMR}$ analysis of the epoxidized standards was conducted in duplicate, but the spectra of the epoxidized standards indicated that there was no difference between the two runs of the same standard. Thus, the $^1\text{H-NMR}$ shifts were determined from a single analysis of each oil sample. To verify the $^1\text{H-NMR}$ assignments, H-H correlation spectroscopy (COSY) experiments were conducted with fully-epoxidized trilinolein and trilinolenin using 8 scans, 16 dummy scans, 7.6432 ppm of spectra width, and 1024 \times 128 time domain data points (F2 \times F1).

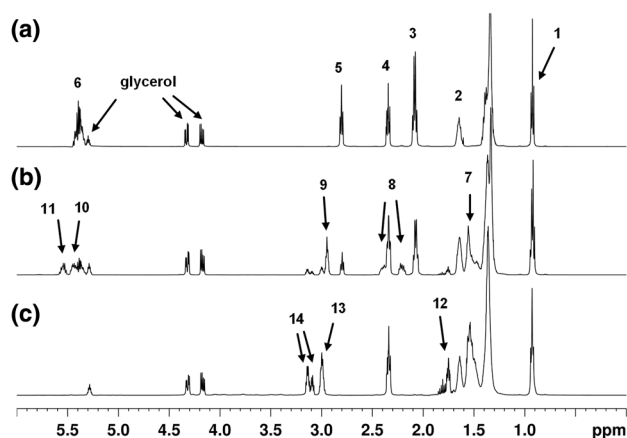


Fig. 1 $^1\text{H-NMR}$ spectra of **a** trilinolein, **b** partially-epoxidized trilinolein and **c** fully-epoxidized trilinolein. The corresponding chemical shift assignments are in Table 2

Identification of an Internal Standard

Vegetable oils primarily consist of triacylglycerols. The fish oil used in this study was also triacylglycerol. The glycerol backbone of these lipids is expected to be stable during oxidation as moisture is not present and hydrolysis of triacylglycerol does not take place [24]; therefore, glycerol protons may be good candidates for internal standards. To test their stability after the epoxidation process, the area ratios of signals of glycerol protons to methyl protons were calculated for both original oils and epoxidized oils. Stripped fish oil triacylglycerol (without tocopherol) and fish oil ethyl esters (containing tocopherol as antioxidant) were epoxidized for comparison.

Results and Discussion

Epoxidation of the Fatty Acids and Oils

The epoxidation process of canola oil was previously monitored by non-aqueous reversed phase liquid chromatography/mass spectrometry (NARP-LC/ESI-MS) in [19], where partially-epoxidized and fully-epoxidized products were reported to form during the reaction. In the current study, formic acid with H_2O_2 successfully epoxidized trilinolein, trilinolenin, canola oil, and fish oils. The progress of epoxidation of trilinolein and trilinolenin was followed by the loss of double bonds and the appearance of epoxide peaks in the $^1\text{H-NMR}$ spectra.

Original trilinolein and trilinolenin did not contain epoxy groups (Figs. 1a, 2a). In the partially-epoxidized trilinolein and trilinolenin (Figs. 1b, 2b, respectively), the signals appearing ~ 3.00 ppm indicated the formation of epoxides, while the remaining double bonds

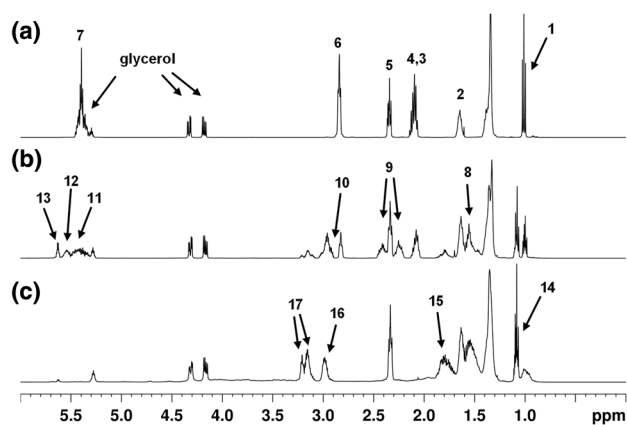













Fig. 2 $^1\text{H-NMR}$ spectra of **a** trilinolenin, **b** partially-epoxidized trilinolenin and **c** fully-epoxidized trilinolenin. The corresponding chemical shift assignments are in Table 3

gave signals ~ 5.50 ppm. In the fully epoxidized trilinolein and trilinolenin, all the double bonds were converted into epoxy groups (Figs. 1c, 2c). This is clear in the $^1\text{H-NMR}$ spectra with the disappearance of the signals due to double bonds (~ 5.50 ppm) and methylene groups adjacent to the double bonds (~ 2.10 ppm), and the appearance of the signals due to epoxy groups (~ 3.00 ppm) and methylene groups adjacent to these epoxy groups (~ 1.50 ppm). In both epoxidation processes, the signals associated with the glycerol backbone remained constant.

In epoxidized trilinolein, three distinct groups of signals (3.00, 3.09, 3.14 ppm) were found for the epoxy groups (Table 2). This result was different from a previous study, which only reported two signals (2.90, 3.10 ppm) for diepoxides derived from methyl linoleate [12]. The difference was likely due to the presence of stereoisomers of diepoxides, as discussed in detail in the next section. The chemical shifts of epoxidized trilinolenin (Fig. 2) were similar to epoxidized trilinolein, with three signals for the epoxy groups (3.00, 3.16, 3.21 ppm) (Table 3). We also noted a clear change in the chemical shift of the signal originally at 1.00 ppm in untreated trilinolenin (Fig. 2). The methyl groups in ω -3 fatty acids show a distinctive signal at 1.00 ppm which differentiates them from the methyl groups (0.88 ppm) in other fatty acids [25]. After epoxidation, the chemical shifts of the methyl groups in trilinolenin moved downfield from 1.00 to 1.08 ppm, suggesting that the epoxy group on the third carbon from the end had a significant influence on the chemical shift of the methyl group. This change was not observed in the epoxidized trilinolein. The signal between 1.25 and 1.45 ppm represented the saturated acyl C4–C7 methylene groups in the fatty acid chain. A slight change in peak shape was observed after epoxidation because of the replacement of double bonds by epoxy

Table 2 ^1H NMR chemical shifts observed from the epoxidation of trilinolein

spectrum ^a	signal	chemical shift ^b (ppm)	protons	functional groups
a)	1	0.88 (t)	$\text{CH}_3\text{-CH}_2\text{-CH}_2\text{-}$	methyl group
	2	1.65 (m)	$\text{-CH}_2\text{-CH}_2\text{-CO-O-}$	methylene group beta to acyl group
	3	2.08 (m)	$\text{-CH}_2\text{-CH}_2\text{-CH=CH-}$	allylic methylene group
	4	2.34 (t)	$\text{-CH}_2\text{-CH}_2\text{-CO-O-}$	methylene group alpha to acyl group
	5	2.80 (t)	$\text{-CH=CH-CH}_2\text{-CH=CH-}$	diallylic methylene group
	6	5.38 (m)	-CH=CH-	olefinic group
b)	7	1.45-1.60(m)	$\text{-CH}_2\text{-CH}_2\text{-CH}$  CH-	methylene group alpha to epoxy group
	8	2.20 (m), 2.40 ^c	-CH  $\text{CH-CH}_2\text{-CH=CH-}$	methylene groups between an epoxy group and an olefinic group
	9	2.95(m)	-CH  $\text{CH-CH}_2\text{-CH=CH-}$	epoxy group beta to olefinic group
	10	5.35-5.50 (m)	-CH  $\text{CH-CH}_2\text{-CH=CH}_2$	olefinic group beta to epoxy group
	11	5.50-5.60 (m)	-CH  $\text{CH-CH}_2\text{-CH=CH-}$	olefinic group beta to epoxy group
c)	12	1.70-1.85 (m)	-CH  $\text{CH-CH}_2\text{-CH}$  CH-	methylene group between two epoxy groups
	13	3.00 (m)	-CH  $\text{CH-CH}_2\text{-CH}$  CH_2	epoxy groups separated by a methylene group
	14	3.09 (m), 3.14 (m)	-CH  $\text{CH-CH}_2\text{-CH}$  CH-	epoxy groups separated by a methylene group

t triplet, *m* multiplet

^a Here, (a), (b), and (c) corresponded to the ^1H NMR spectrum (a), (b), and (c) in Fig. 1

^b This signal overlapped with the signal at 2.34 ppm (t, $\text{-CH}_2\text{-CO-O-}$)

groups; however, their chemical shifts remained in the same range.

The purity of the fully-epoxidized products were verified by TLC. Using hexane:diethyl ether (80:20, v/v) as developing solvent, only one band with $R_f = 0$ was observed for each of the epoxidized products, while R_f values of trilinolein, trilinolenin, canola oil, and fish oil were 0.39, 0.35, 0.42, and 0.39, respectively. The absence of the band due to the substrates for all the epoxidized samples indicated that the triacylglycerols had fully reacted. Use of chloroform: methanol (95:5, v/v) as developing solvent did not allow the separation of substrates (R_f range of 0.62–0.76) and epoxidized products (R_f range of 0.5–0.8). However, with chloroform: methanol (95:5, v/v), trace amounts of polar compounds with $R_f = 0$ were found for epoxidized trilinolenin, while only one broad band (R_f range of 0.5–0.8) was observed for each of epoxidized trilinolein, epoxidized canola oil, and epoxidized fish oil. The products with $R_f = 0$ were more polar than fully-epoxidized triacylglycerols, and were likely due to ring-opened by-products formed during the epoxidation process [2]. Most importantly, the

epoxidized products purified by TLC gave ^1H -NMR signals that were identical to those of the epoxidized products without TLC purification, indicating that the polar band ($R_f = 0$) would not affect the ^1H -NMR assignments of the epoxidized trilinolenin.

GC-MS enabled further confirmation of the structure of the reaction products (Supplementary Figures S-1, S-2). The FAME derived from fully-epoxidized trilinolein and trilinolenin were expected to be methyl 9,10-12,13-diepoxyoctadecanoate (*cis/cis*) and methyl 9,10-12,13-15,16-triepoxyoctadecanoate (*cis/cis/cis*), with the configuration of the epoxy ring retained from the original double bond [26]. GC-MS analysis of the methyl esters of epoxidized trilinolein yielded two peaks with retention times of 22.6 and 24.1 min, and identical EI-MS spectra (% relative intensity): m/z 308 (2), 295 (2), 277 (4), 187 (7), 165 (12), 155 (50), 137 (20), 109 (62), 67 (100). Their EI spectra were consistent with that of methyl 9,10-12,13-diepoxyoctadecanoate reported in a previous study [27]. In positive chemical ionization (PCI) mode, a base peak of m/z 344 [$\text{M} + 18$, addition of NH_4^+] and m/z 327 [$\text{M} + 1$], 309 [$\text{M} - 17$], 295

Table 3 Proton chemical shifts observed from the epoxidation of trilinolenin

spectrum ^a	signal	chemical shift ^b (ppm)	protons	functional groups
a)	1	1.00 (t)	$\underline{\text{C}}\underline{\text{H}}_3\text{-CH}_2\text{-CH=CH-}$	methyl group in ω -3 fatty acid
	2	1.65 (m)	$-\underline{\text{C}}\underline{\text{H}}_2\text{-CH}_2\text{-CO-O-}$	methylene group beta to acyl group
	3	2.08 ^c	$-\text{CH}_2\text{-}\underline{\text{C}}\underline{\text{H}}_2\text{-CH=CH-}$	allylic methylene group
	4	2.11 ^c	$\text{CH}_3\text{-}\underline{\text{C}}\underline{\text{H}}_2\text{-CH=CH-}$	allylic methylene group
	5	2.34 (t)	$-\text{CH}_2\text{-}\underline{\text{C}}\underline{\text{H}}_2\text{-CO-O-}$	methylene group alpha to acyl group
	6	2.84 (t)	$-\text{CH=CH-}\underline{\text{C}}\underline{\text{H}}_2\text{-CH=CH-}\underline{\text{C}}\underline{\text{H}}_2\text{-CH=CH-}$	diallylic methylene group
	7	5.40 (m)	$-\underline{\text{C}}\underline{\text{H}}=\underline{\text{C}}\underline{\text{H}}-$	olefinic group
	8	1.45-1.60 (m)	$-\text{CH}_2\text{-}\underline{\text{C}}\underline{\text{H}}_2\text{-CH} \begin{array}{c} \diagup \text{O} \diagdown \\ \text{CH-} \end{array}$	methylene group alpha to epoxy group
b)	9	2.25 (m), 2.40 ^d	$-\text{CH} \begin{array}{c} \diagup \text{O} \diagdown \\ \text{CH-}\underline{\text{C}}\underline{\text{H}}_2\text{-CH=CH-} \end{array}$	methylene groups between an epoxy group and an olefinic group
	10	2.95 ^c	$-\underline{\text{C}}\underline{\text{H}} \begin{array}{c} \diagup \text{O} \diagdown \\ \underline{\text{C}}\underline{\text{H}}\text{-CH}_2\text{-CH=CH-} \end{array}$	epoxy group beta to olefinic group
	11	5.35-5.50 (m)	$-\text{CH} \begin{array}{c} \diagup \text{O} \diagdown \\ \text{CH-CH}_2\text{-CH=}\underline{\text{C}}\underline{\text{H}}-$	
	12	5.50-5.60 (m)	$-\text{CH} \begin{array}{c} \diagup \text{O} \diagdown \\ \text{CH-CH}_2\text{-}\underline{\text{C}}\underline{\text{H}}=\text{CH-}$	olefinic group beta to epoxy group
	13	5.63 (m)	$-\text{CH} \begin{array}{c} \diagup \text{O} \diagdown \\ \text{CH-CH}_2\text{-}\underline{\text{C}}\underline{\text{H}}=\underline{\text{C}}\underline{\text{H}}\text{-CH}_2\text{-CH} \begin{array}{c} \diagup \text{O} \diagdown \\ \text{CH-} \end{array}$	
	14	1.08 (m)	$\underline{\text{C}}\underline{\text{H}}_3\text{CH}_2\text{-CH} \begin{array}{c} \diagup \text{O} \diagdown \\ \text{CH-} \end{array}$	methyl group in ω -3 fatty acid epoxide
c)	15	1.70-1.85 (m)	$-\text{CH} \begin{array}{c} \diagup \text{O} \diagdown \\ \text{CH-}\underline{\text{C}}\underline{\text{H}}_2\text{-CH} \begin{array}{c} \diagup \text{O} \diagdown \\ \text{CH-} \end{array}$	methylene group between two epoxy groups
	16	3.00 (m)	$-\underline{\text{C}}\underline{\text{H}} \begin{array}{c} \diagup \text{O} \diagdown \\ \text{CH-CH}_2\text{-CH} \begin{array}{c} \diagup \text{O} \diagdown \\ \text{CH-CH}_2\text{-CH} \begin{array}{c} \diagup \text{O} \diagdown \\ \underline{\text{C}}\underline{\text{H}}- \end{array} \end{array}$	epoxy groups separated by a methylene group
	17	3.16 (m), 3.21(m)	$-\text{CH} \begin{array}{c} \diagup \text{O} \diagdown \\ \underline{\text{C}}\underline{\text{H}}\text{-CH}_2\text{-}\underline{\text{C}}\underline{\text{H}} \begin{array}{c} \diagup \text{O} \diagdown \\ \underline{\text{C}}\underline{\text{H}}\text{-CH}_2\text{-}\underline{\text{C}}\underline{\text{H}} \begin{array}{c} \diagup \text{O} \diagdown \\ \text{CH-} \end{array} \end{array}$	

t triplet, *m* multiplet

^a Here, (a), (b), and (c) corresponded to the ¹H NMR Spectrum (a), (b), and (c) in Fig. 2

^b These two signals overlapped with each other

^c This signal overlapped with the signal at 2.34 ppm (t, $-\text{CH}_2\text{-CO-O-}$)

^d This signal overlapped with signal at 3.00 ppm (m, $-\text{CHOCH-CH}_2\text{-CHOCH-CH}_2\text{-CHOCH-}$)

[M – 31], 291 [M – 35], and 277 [M – 49] were identified for both stereoisomers of methyl 9,10-12,13-diepoxyoctadecanoate, but the spectra were not identical. The first eluted stereoisomer had a more intense *m/z* 291 than *m/z* 295, consistent with a previous study [28]; the later eluting stereoisomer had a more intense *m/z* 295 than *m/z* 291.

The methyl esters of epoxidized trilinolenin had retention times of 38.8, 41.3, 42.2, and 47.1 min, and identical

spectra in EI-MS (% relative intensity): *m/z* 155 (24), 109 (35), 67 (100). The EI spectra of the products were difficult to interpret, although *m/z* 155 was evidence of the presence of a 9,10 epoxy ring, formed from loss of CH₃OH from [HO=CH(CH₂)₇COOCH₃]⁺ [27]. In PCI, the proton adduct of the molecular ion *m/z* 341 [M + 1] was small; however, characteristic peaks of *m/z* 358 [M + 18, addition of NH₄⁺], 323 [M – 17], 305 [M – 35], and 291

[M – 49] were abundant. As few descriptions of MS spectra of triepoxides are available in the literature, comparison with other studies was impossible. However, the base peak m/z 358 [M + 18] and the fragmentation in PCI, and the relatively long retention times suggested that the four stereoisomers of triepoxides were unlikely to be short-chain epoxide monomers or terminal epoxides; methyl 9,10-12,13-15,16-triepoxyoctadecanoate (*cis/cis/cis*) was a much more likely identity of the four stereoisomers.

Impurities in the FAME derived from fully-epoxidized trilinolein and trilinolenin found at 12.6 and 12.7 min by GC–MS had convincing MS library spectral matches with methyl 9-oxo-nonanoate and nonanedioic acid dimethyl ester. These were estimated at <1 % in methyl diepoxyoctadecanoate (*cis/cis*) and <8 % in methyl triepoxyoctadecanoate (*cis/cis/cis*). No other epoxy compounds were observed in GC–MS. Therefore, it was concluded that all the peaks identified for diepoxides and triepoxides in $^1\text{H-NMR}$ were internal epoxides.

The epoxidation method was unsuccessful when applied to conjugated methyl linoleate. Signals found ~3.00 ppm (Supplementary Fig. S-3) could be due to the partial generation of epoxides. The signals between 5.00 and 6.00 ppm indicated that some double bonds were not converted, while signals that occurred between 2.80–4.40 ppm and 8.00–9.50 ppm suggested that other products were formed in addition to epoxides (Supplementary Fig. S-3). It was assumed that, like the other standards, conjugated methyl linoleate would be readily epoxidized by hydrogen peroxide and formic acid; however, the combination of formic acid with both 30 and 50 % hydrogen peroxide was found to be ineffective, with the products of the reactions using both concentrations of reagents giving identical $^1\text{H-NMR}$ spectra. The signals were indistinct, so it was difficult to assign the signals to exact protons.

Epoxidation of the conjugated fatty acids may have been impossible because the structure does not allow the required electron migration. The current epoxidation method utilized the peracid (RCO_3H) formed from the reaction of formic acid and hydrogen peroxide. The intramolecular hydrogen bond of the peracid results in an electrophilic oxygen atom that can be added to the double bond [29]. The addition of oxygen to the double bond is accompanied by the migration of a pair of electrons from the π bond of the original double bond to the new C–O bond, which results in a carbocation adjacent to the double bond in the transition state [30]. Normally, the carbocation will form another carbon–oxygen bond ($-\text{C}-\text{O}-$) with the oxygen, so an epoxy group is formed. However, when the transition state applies to a conjugated diene, the carbocation is conjugated to the adjacent double bond, which generates an allylic carbocation and causes the double bond to shift. This may isomerize with double bond migration,

preventing the formation of the other C–O bond. In addition, the formation of an epoxy group in a conjugated diene will reduce the nucleophilicity of the remaining double bond, making the partially epoxidized structure less likely to undergo further epoxidation [29]. Compared to the $^1\text{H-NMR}$ spectrum of the original conjugated methyl linoleate, hydroperoxides (~8.10 ppm) and unsaturated alcohols or cyclic peroxides (3.40–4.50 ppm) were found in the reaction product [31], which may be due to the shifting of the carbocation during the reaction. Another work [32] found a similar effect when epoxidizing oils using methyltrioxorhenium (MTO), pyridine, methylene chloride, and hydrogen peroxide. Their method was successful in epoxidizing fish oil ethyl esters but ineffective when applied to conjugated fish oil ethyl ester derived from the same fish oil ethyl ester. Since no information about the signals in $^1\text{H-NMR}$ spectra was provided in that study [32], it is impossible to compare the products epoxidized by the MTO method and the method used here.

Stereoisomers of Diepoxides and Triepoxides

Unlike the previous assignments of diepoxides [12], three signals were found for diepoxides derived from trilinolein. This difference may be due to the different reagents used for epoxidation. In a previous work, 3-chloroperbenzoic acid (mCPBA) was used as the epoxidation reagent [12] and it may have had a steric effect, leading to a stereospecific reaction. When formic acid was employed for epoxidation, the steric effect may have been inconsequential due to the relatively small size of the formic acid molecule, leading to the formation of both *cis/cis* diepoxide stereoisomers (Fig. 3). Stereoisomers of tetrahydroindene diepoxide have been distinguished using $^1\text{H-NMR}$ [33] and stereoisomers derived from methyl linoleate using peracetic acid have also been discussed since 1966 [34]. Diepoxide stereoisomers have been verified using HPLC–MS [27], GC–FID [35] and GC–MS [26] but remained un-reported by $^1\text{H-NMR}$.

The methyl esters of epoxidized trilinolein gave two peaks in GC–MS with the same EI spectra, and were identified as the two stereoisomers of methyl 9,10-12,13-diepoxyoctadecanoate. The peak area ratio of the two peaks (RT 22.6 and 24.1 min) was 1.6:1, in agreement with the peak area ratio of the signals observed at 3.14 and 3.09 ppm in $^1\text{H-NMR}$ (Fig. 1c). For methyl esters of epoxidized trilinolenin, four potential stereoisomers were observed in GC–MS analysis. The peak area ratio of the four triepoxide stereoisomers was determined to be ~2.9:2:1.9:1 (RT 38.8, 41.3, 42.2, and 47.1 min) in GC–MS, potentially representing four different combinations of the three epoxy groups facing the same and opposite sides. In the $^1\text{H-NMR}$ spectrum of epoxidized trilinolenin (Fig. 2c), the peak area ratio

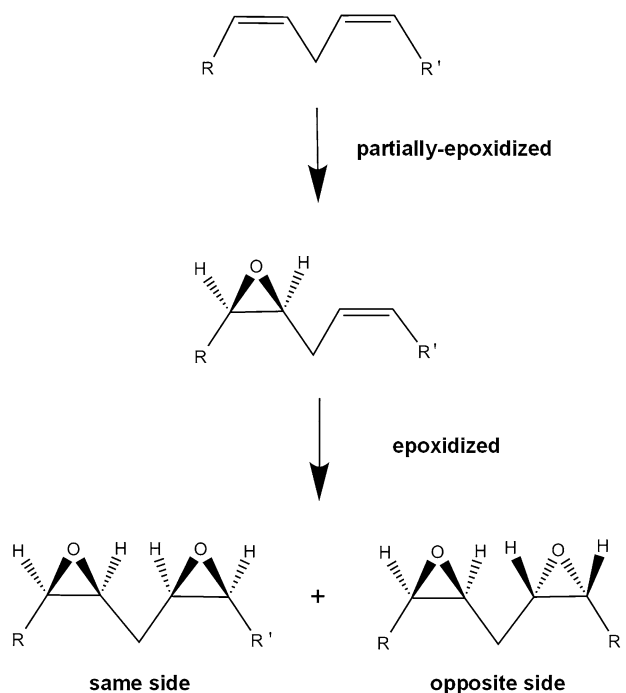


Fig. 3 Stereoisomers of diepoxides derived from methylene-interrupted dienes differed in the relative positions of the two oxygens. The adjacent oxygen atoms can be located either on the same or opposite side

of the two signals at 3.16 and 3.21 ppm was approximately 2:1. Further interpretation is difficult due to a lack of information about these stereoisomers in the literature. However, the methyl esters of epoxidized trilinolein and trilinolenin were analyzed by ^1H NMR. Identical signals were confirmed for diepoxides and triepoxides in their triacylglycerol and methyl ester forms, suggesting little influence of the glycerol backbone.

A mixture of stereoisomers could yield the three signals (3.00, 3.09, and 3.14 ppm) detected here for diepoxides in linoleic acid (Fig. 1). The signal at 3.00 ppm (m, $-\text{CHOCH}-\text{CH}_2-\text{CHOCH}-$) can be assigned to the outer pairs of CH protons while the two signals at 3.09 ppm (m, $-\text{CHOCH}-\text{CH}_2-\text{CHOCH}-$) and 3.14 ppm (m, $-\text{CHOCH}-\text{CH}_2-\text{CHOCH}-$) can be assigned to the inner pairs of CH protons (Fig. 3). The two oxirane oxygens could be located on either the same or opposite sides, causing different shielding and slightly different chemical shifts of the inner protons [36].

Similarly, the three distinct groups of signals found for the triepoxides in linolenic acid at 3.00, 3.16, and 3.21 ppm (m) (Fig. 2) were associated with the three epoxy groups derived from the original double bonds on each fatty acid chain of epoxidized trilinolenin. The signal at 3.00 ppm can be assigned to the outer pair of protons of the triepoxides (m, $-\text{CHOCH}-\text{CH}_2-\text{CHOCH}-\text{CH}_2-\text{CHOCH}-$), while the

signals at 3.16 and 3.21 ppm could be assigned to the inner protons (m, $-\text{CHOCH}-\text{CH}_2-\text{CHOCH}-\text{CH}_2-\text{CHOCH}-$), with the two different resonances due to the relative positions of the two oxirane oxygens separated by a methylene group (Table 3). The relative positions of the two adjacent oxirane oxygens (separated by a methylene group) produce two slightly different electronic environments for the inner protons. The protons with higher electro-negativities could result in higher frequencies of resonances because of less shielding, generating a slightly greater chemical shift.

The ^1H -NMR assignments were further confirmed using the H–H COSY experiments with fully-epoxidized trilinolein and trilinolenin (Supplementary Figs. S-4, S-5). In their COSY spectra, the outer pair of the epoxy protons (3.00 ppm, $-\text{CHOCH}-\text{CH}_2-\text{CHOCH}-$) were coupling with the methylene protons that were only adjacent to one epoxy group (1.45–1.60 ppm, $-\text{CH}_2-\text{CH}_2-\text{CHOCH}-$). The inner pair of the epoxy protons (3.09, 3.14 ppm for epoxidized trilinolein, $-\text{CHOCH}-\text{CH}_2-\text{CHOCH}-$; 3.16, 3.21 ppm for epoxidized trilinolenin, $-\text{CHOCH}-\text{CH}_2-\text{CHOCH}-\text{CH}_2-\text{CHOCH}-$) were coupling with the methylene protons between two epoxy groups (1.70–1.85 ppm, $-\text{CHOCH}-\text{CH}_2-\text{CHOCH}-$).

These new observations of stereoisomers are supported by re-examination of previous ^1H -NMR analyses of epoxidized soybean oil [1, 11, 13]. In those studies, four clear signals were found for epoxides. Soybean oil typically contains 50–60 % linoleic acid and 20 % oleic acid, with only 7 % linolenic acid. The amount of epoxidized linolenic acid may have been so low that the signals for the triepoxides were not clear. Based on the assignments made in this study, the four intense signals could be assigned to the monoepoxides (one signal, 2.90 ppm) and the diepoxides (three signals, 2.95–3.15 ppm). The shapes of the three signals associated with diepoxides in our study were similar to those signals assigned for diepoxides in the previous studies [1, 11, 13].

^1H -NMR Interpretation of Epoxidized Oils

In this study, the signals associated with epoxides appeared between 2.90 and 3.23 ppm for epoxidized canola oil (Fig. 4), and between 2.90 and 3.28 ppm for epoxidized fish oil (Fig. 5), while the shifts due to protons associated with the glycerol backbone remained at 4.18 ppm, 4.33 ppm (dd, $-\text{CH}_2-\text{O}-\text{CO}-\text{R}$) and 5.30 ppm (m, $>\text{CH}-\text{O}-\text{CO}-\text{R}$) for both oils. Unlike the lipid standards (trilinolein and trilinolenin), two signals (0.88, 1.00 ppm) were observed for methyl groups in fresh canola oil and fresh fish oil. The signal associated with the methyl groups in ω -3 fatty acids moved from 1.00 to 1.08 ppm after epoxidation, but the signal associated with methyl groups in other fatty acids remained the same at 0.88 ppm.

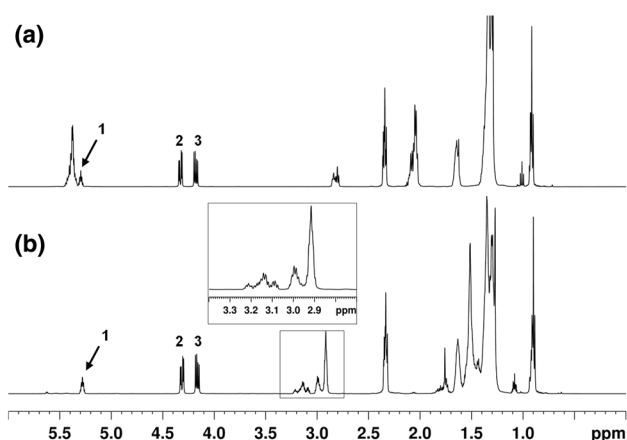


Fig. 4 $^1\text{H-NMR}$ spectrum of **a** canola oil (*top*) and **b** epoxidized canola oil (*bottom*). The signals between 2.90 and 3.23 ppm are due to epoxides. Signals 1, 2, and 3 are associated with the esterified glycerols in oil

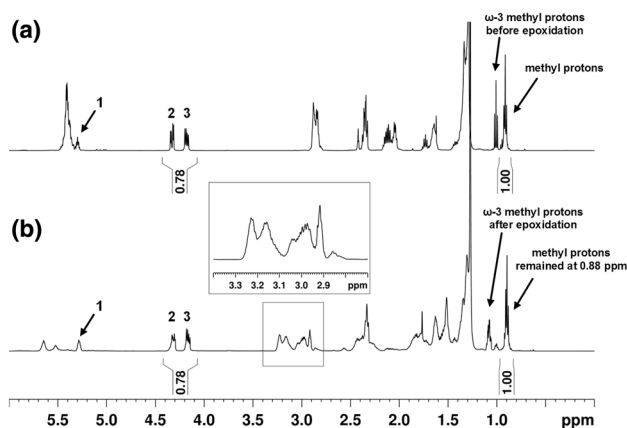


Fig. 5 $^1\text{H-NMR}$ spectrum of **a** fish oil (*top*) and **b** epoxidized fish oil (*bottom*) with integration. The signals between 2.90 and 3.28 ppm are due to epoxides. Signals 1, 2, and 3 are associated with the esterified glycerols in oil. Signals 2 and 3 are assigned to the *sn*-1 and *sn*-3 protons of esterified glycerol which have been integrated before (**a**) and after (**b**) epoxidation by taking the peak of methyl protons at 0.88 ppm, which does not include methyl protons in ω -3 fatty acids, as an internal reference

By examining the spectrum of the epoxidized canola oil, it was clear that almost all the signals due to the double bonds disappeared (Fig. 4). Only a small peak was discovered at 5.60 ppm which might be due to the very few unconverted double bonds remaining. Assuming that all the double bonds in the canola oil had been converted into epoxy groups, it was expected that the ratio of the signals due to the monoepoxides, diepoxides, and triepoxides in the epoxidized canola oil (Fig. 4) would be similar to the ratio of the content of oleic acid, linoleic acid, and linolenic acid in the canola oil (Table 1). The spectrum supports this (Fig. 4), showing the signal associated with monoepoxides

(2.92 ppm) as the most intense among the signals between 2.90 and 3.23 ppm, because the canola oil used in this study contained 59.96 % oleic acid (Table 1). The three signals associated with diepoxides (3.00, 3.09, 3.14 ppm) were less intense, as the canola oil contained 18.89 % linoleic acid. The signals associated with triepoxides (3.00, 3.16, 3.21 ppm) were the least intense because the canola oil contained only 9.63 % linolenic acid and the signals of triepoxides coincided with those of diepoxides. The $^1\text{H-NMR}$ spectrum of the epoxidized canola oil obtained in this experiment was consistent with a previous study [2]. Therefore, epoxidized canola oil can be successfully interpreted by the assignments made in this work.

The signals assigned to the mixed PUFA epoxides in fish oil appeared between 2.90 and 3.28 ppm, which differed from the signals of other chemical groups in fish oil triacylglycerols (Fig. 5). Based on the fatty acid composition of fish oil (Table 1) and the assignments of the signals given in Tables 2 and 3, the sharp peak at 2.92 ppm in epoxidized fish oil was assigned to monoepoxides derived from mono-unsaturated fatty acids, while the signals between 2.95 and 3.28 ppm were likely due to the epoxides derived from PUFA. These are the first presented $^1\text{H-NMR}$ spectra of mixed epoxides derived from PUFA in fish oil triacylglycerols. For fish oil ethyl esters, the signals associated with the methylene protons of the ethyl group were ~ 4.20 ppm (m, $\text{CH}_3\text{-CH}_2\text{-O-CO-R}$), while the chemical shift of the methyl protons of the ethyl group was 1.30 ppm (t, $\text{CH}_3\text{-CH}_2\text{-O-CO-R}$) [37, 38]. Hence, the signals appearing between 2.90 and 3.28 ppm could be used for identification of epoxides in both fish oil triacylglycerol and ethyl esters.

When comparing the epoxidized fish oil to the epoxidized canola oil, it was noted that the signals associated with the mixed epoxides moved slightly downfield in fish oil. This might be because more epoxy groups in a fatty acid chain were present in epoxidized fish oil than in epoxidized canola oil. The involvement of more epoxy groups might lead to less shielding of the protons associated with the epoxy groups, which may contribute to a slightly higher frequency of proton resonance. This information may be useful for identification of the compounds containing more than three epoxy groups.

In this study, epoxide generation in trilinolein and trilinolenin was a model system for synthesis of epoxidized oils. Thus, the $^1\text{H-NMR}$ assignments made in this study may be useful for identification of epoxy products and can be used to follow industrial synthesis or test the degree of epoxidation in an unknown oil. It should be noted that during the oxidation of edible oils, the generation of epoxides and their specific structures depend on both the oil composition and the oxidation conditions [39]. Not all of the epoxides observed in this work may necessarily appear in oxidized edible oils. Further, epoxides are present along

with other oxidation products in oxidized lipids. In our study, the chemical shifts of epoxides derived from these oils did not coincide with other chemical groups, a necessary requirement if ^1H NMR is to be used to quantify epoxides in canola oil and fish oil. Their signals were also distinct from those of primary lipid oxidation products, such as conjugated dienes (5.40–6.58 ppm) and hydroperoxides (8.30–8.90 ppm), and other secondary/further lipid oxidation products which gave signals at 3.43–3.62 ppm (alcohols), 6.08–6.82 ppm (double bond conjugated with a keto group), and 9.49–9.79 ppm (aldehydes) [15]. Therefore, a significant advantage of ^1H NMR is the ability to simultaneously detect epoxides and other oxidation products in oxidized oils.

Identification of an Internal Standard

One of the objectives of this study was to investigate the potential of applying ^1H NMR to quantitative studies of epoxides produced during lipid oxidation. It was thus important to identify an internal standard for quantification. In a previous study, the signal at 0.88 ppm due to methyl groups was taken as the internal standard for quantification in oil samples [12]. However, in oxidized oils, carbonyls and alkanes of shorter chain length are produced by the secondary scissions of the intermediate products of lipid oxidation, especially at high temperature [39]. That is, the breakdown of hydroperoxides could result in the generation of more methyl groups during lipid oxidation. Thus, the signal at 0.88 ppm does not remain constant as oxidation proceeds and it may not be a good internal standard for quantitative studies with oxidized oils. As an alternative, the signals associated with the esterified glycerol backbone of triacylglycerols were considered. In the ^1H -NMR spectrum of fish oil, they are clear both before and after epoxidation (Fig. 5) as Signals 1, 2, and 3. Specifically, the signals associated with the protons of *sn*-1 and *sn*-3 esterified glycerols (4.18 and 4.33 ppm) are good candidates as internal standards for triacylglycerol oils as they remain constant as long as no hydrolysis occurs.

It is important to note that a change in the shape of the glycerol signals occurred in the ^1H -NMR spectra of epoxidized fish oil compared to the original fish oil. This change was not observed for the epoxidized canola oil. Such a modification might indicate a change in structure, making those signals at 4.18 and 4.33 ppm (dd, $-\text{CH}_2-\text{O}-\text{CO}-\text{R}$) associated with glycerol inappropriate as internal standards. However, as the *sn*-1, 3 glycerol protons were well separated from those signals associated with epoxides and double bonds, the distortion of the peak shape could not be due to their overlap with signals of epoxy protons and double bonds. To investigate the stability of the glycerol protons, Signal 2 and Signal 3 (Fig. 5) were integrated

before and after epoxidation by taking the signal of methyl protons (not including methyl groups in ω -3 fatty acids) at 0.88 ppm as an internal reference. Because epoxidation, rather than oxidation, had taken place, these methyl protons should remain stable, allowing us to use their signal as an internal reference to verify the stability of the esterified glycerol protons. The ratio of the total area of Signal 2 and Signal 3 to the area of methyl groups was found to be ~ 0.78 for both fresh fish oil and epoxidized fish oil. Thus, it can be inferred that the area of signals due to glycerol remains relatively constant during epoxidation, allowing the use of the glycerol signals as an internal reference for quantification of epoxides despite the change in shape of the signal.

The proposed method of epoxidation was also conducted on stripped fish oil (without tocopherol) as well as fish oil ethyl esters (containing tocopherol), but the same change was observed in the shape of the signals associated with esterified glycerol and ethyl groups after epoxidation. The change in the shape was not observed for fish oils with lower concentrations of epoxides (<30 % of double bonds converted into epoxy groups). It is likely that the change in the shape occurs when the concentration of epoxides reaches some threshold level. In fish oils, the double bonds can be located either close or distant to the ester groups. When the original double bonds were converted into epoxy groups, the hybridization of the carbon atoms changed from sp^2 to sp^3 , which would allow rotation about the single bond. The presence of epoxides derived from $\Delta 4$ unsaturated fatty acids coupled with such rotation might allow the epoxy groups to be closer to the protons of the glycerol backbone than those in canola oil. This would have an influence on the protons in the esterified glycerol or ethyl groups and offers an explanation for the change in shape of the signals after epoxidation of fish oils.

Conclusion

In this study, epoxidation of trilinolein, trilinolenin, canola oil, and fish oils by formic acid and hydrogen peroxide was followed by ^1H NMR to determine epoxide peak patterns and chemical shifts. Consistent with previous studies, monoepoxides gave a signal at 2.90 ppm. New ^1H -NMR assignments were made for diepoxides (3.00, 3.09, 3.14 ppm) and triepoxides (3.00, 3.16, 3.21 ppm). These assignments for epoxides have been successfully applied to evaluate the ^1H -NMR spectra of epoxidized oils in several previous studies, as well as in this work. The signals associated with the mixed epoxides in epoxidized canola oil and fish oils were consistent with the standards and were located between 2.90–3.23 ppm and 2.90–3.28 ppm, respectively. The chemical shifts of epoxides were distinct from hydroperoxides, alcohols, acids, and aldehydes, so it is possible to distinguish

multiple lipid oxidation products in a single analysis using ^1H NMR. This information will facilitate both identification and quantification of epoxides in oil samples. The observation that the chemical shifts of epoxy groups move downfield when more epoxy groups are present in a hydrocarbon chain will also be useful in determining the structure of oxirane-containing products. The current study was conducted with epoxide concentrations at 50 mM. Signals associated with the protons of *sn*-1 and *sn*-3 esterified glycerols (4.18 and 4.33 ppm) were proposed as internal standards for quantification of epoxides in triacylglycerol oils. Further studies are needed to validate this approach and determine the lower detection limits of epoxides by ^1H NMR.

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