High-Performance Liquid Chromatography and Spectroscopic Studies on Fish Oil Oxidation Products Extracted from Frozen Atlantic Mackerel

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ABSTRACT: The formation of stable hydroxy derivatives from hydroperoxides produced during the oxidation of linoleic acid methyl ester and fish oil were studied by reverse-phase high-performance liquid chromatography (HPLC), gas chromatography–mass spectrometry (GC–MS) and ^{13}C nuclear magnetic resonance (NMR) spectroscopy. The oxidation products identified were mixtures of four isomeric hydroxy derivatives: 13-hydroxy-9-*cis*,11-*trans*-octadecadienoic,13-hydroxy-9-*trans*,11-*trans*-octadecadienoic, 9-hydroxy-10-*trans*,12-*cis*-octadecadienoic, and 9-hydroxy-10-*trans*,12-*trans*-octadecadienoic acids. The presence of hydroxy compounds was confirmed by ^{13}C NMR, which gave rise to a hydroxy carbon peak at 87 ppm, and by GC–MS, which showed three peaks corresponding to isomeric mixtures of trimethylsilyl ethers of the oxidized linoleic acid methyl ester. The mass spectra scans of the three peaks showed that they represent isomers of molecular weight 382 and are consistent with the molecular formula $C_{22}H_{42}O_3Si$. In oil extracted from stored frozen mackerel, 13-hydroxy-9-*cis*,11-*trans*-octadecadienoic acid was more prominent compared to the model lipid systems. HPLC offered a sensitive means of detection of hydroxy compounds produced both in the initiation and latter stages of oxidation. The effect of antioxidants added to the fish mince prior to storage can also be monitored by HPLC. Thus, the monitoring of lipid oxidation hydroxy derivatives by HPLC is of practical value in the efficient processing and quality control of fish, fish oils, and other fatty foodstuffs in order to enhance the acceptability, nutritional, and safety aspects.

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KEY WORDS: ^{13}C NMR, conjugated hydroxy octadecadienoic acids, freezing, GC–MS, HPLC, hydroperoxides, lipid oxidation products, mackerel (*Scomber scombrus*) fish oil, methyl linoleate, oxidized lipids.

Fish oil is rich in n-3 polyunsaturated fatty acids (PUFA), which have both nutritional and pharmacological applications. This interest has given rise to efforts to improve the chemical and organoleptic characteristics of these oils. Epidemiological studies of pathological cases in humans suggest that high fat intake is associated with atherosclerosis and colon and breast cancer (1–3). Unsaturated fatty acids and cholesterol in fat are easily oxidized during cooking and frozen storage (4). The lipid oxidation chain reaction leading to rancidity yields different kinds of mutagens (4–6), promoters, and carcinogens such as fatty acid hydroxides (7), cholesterol hydroxides (8), endoperoxides, cholesterol and fatty acid epoxides (8–11), enals and other aldehydes (12), and alkoxy and hydroperoxide radicals (12).

Oxidative rancidity is probably more important in fish than in any other food due to the high level of PUFA and the number of potential initiators/promoters present. The process of lipid oxidation is thermodynamically favorable; however, the direct reaction between oxygen and even highly unsaturated lipids is kinetically unfavorable. For the oxidation reaction to proceed, an activating reaction is needed to initiate free radical chain reactions. A number of mechanisms are involved in these activation systems: production of singlet oxygen, generation of partially reduced or free-radical oxygen species such as hydroxyl radical (both enzymatically and nonenzymatically), active oxygen iron complexes, and thermal- or iron-mediated homolytic cleavage of hydroxides (Scheme 1).

Analysis of fatty acid hydroperoxides and their hydroxy derivatives. A characteristic of the formation of hydroperoxides from PUFA is the generation of a conjugated diene as the double bonds rearrange. This rearrangement provides one approach to monitoring the formation of PUFA hydroperoxides, namely the measurement of absorbance at 234 nm against an appropriate blank (13). Other methods for fatty acid hydroperoxide quantitation include iodometric titration (14), xylenol orange reactivity, and glutathione oxidation (15). While these approaches have worked well in many simple systems, they lack sensitivity and specificity for application in complex systems and provide no information about specific hydroperoxides. These deficiencies have led to the development of a variety of chromatographic approaches for determining PUFA hydroperoxides and stable hydroxy compounds in biological systems utilizing both liquid chromatography (LC) and gas chromatography (GC).

Investigation of the initiation process has proved to be quite difficult, as only a few of the initiating species are usually generated before propagation commences. This is particularly true for fish, as the intermediate hydroperoxides are ex-

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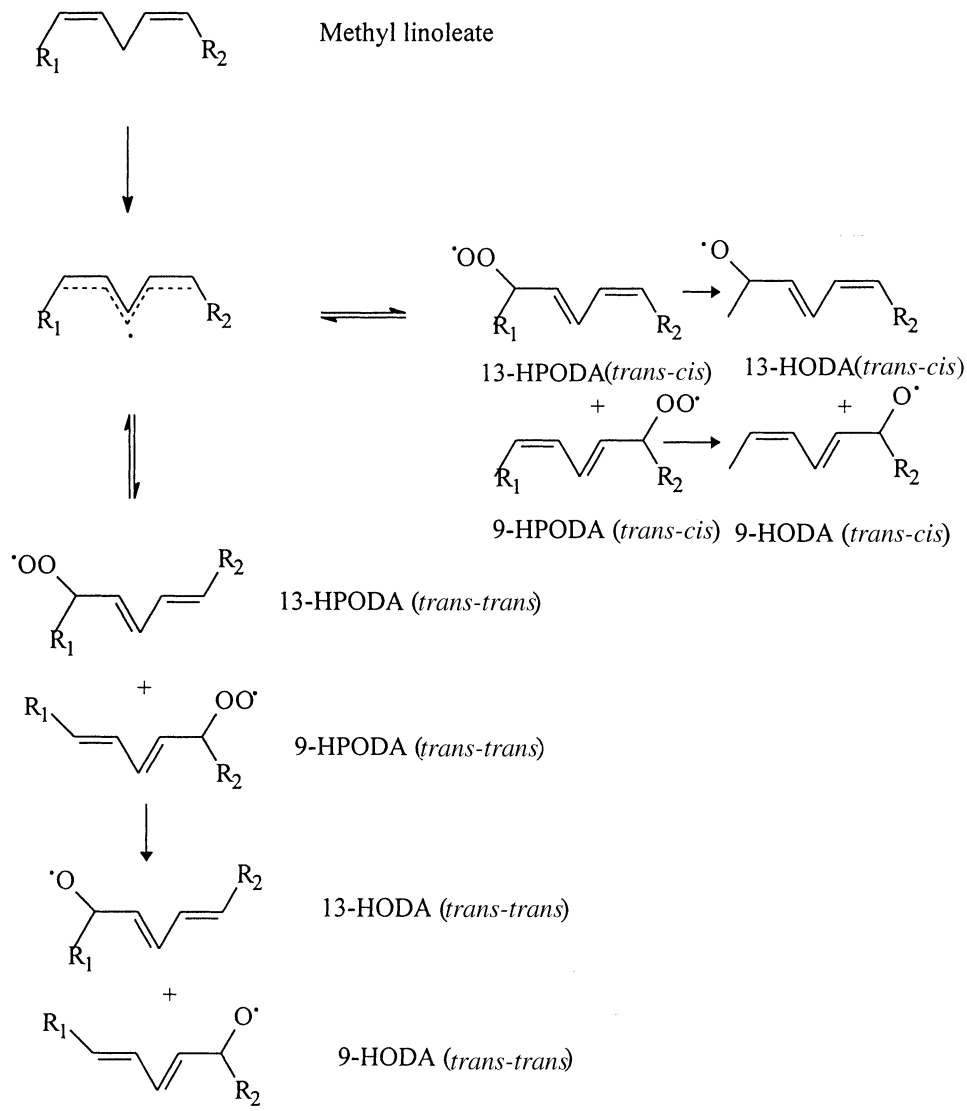

SCHEME 1

tremely unstable due to their highly polyunsaturated nature, and break down almost instantaneously to hydroxy compounds. The use of high-performance liquid chromatography (HPLC) avoids the challenges of preparing the relatively labile hydroperoxides for GC and peroxide value measurements. HPLC methods also allow the direct analysis of esterified forms of the hydroperoxides and hydroxy compounds. Both reversed-phase chromatography with methanol/water-based mobile phase and silica chromatography with chloroform/methanol-based mobile phases have been used successfully (16) to quantitate the hydroperoxides in the different phospholipid classes and cholesterol esters. However, the application of HPLC to the analysis of fish oils obtained from frozen fish has not been reported thus far. The aims of this study were to develop an easy, sensitive, and more specific method to assess lipid oxidation products in frozen fish using HPLC and to confirm the assignment of the hydroxy derivatives from the hydroperoxides by ^{13}C nuclear magnetic resonance (NMR) and GC–mass spectrometry (MS).

EXPERIMENTAL PROCEDURES

Atlantic mackerel *(Scomber scombrus)* was supplied by Food Science Laboratories, Torry Research Station, Aberdeen, Scotland. Methyl linoleate, 13-hydroxyoctadecaenoic acid standard, and α-tocopherol were purchased from Sigma-Aldrich (Poole, Dorset, United Kingdom). Sodium (10%) in methanol, *tert*-butyl methyl ether (MTBE), pyridine, and *N*,*O*-bis(trimethylsilyl)acetamide (BSA) with 5% trimethylchlorosilane (TMCS) were all obtained from Fluka Analytika, Buchs, Switzerland. Bonded amino-phase SPE cartridges were purchased from Varian Sample Preparation Products (Walton-on-Thames, Surrey, United Kingdom). Anhydrous sodium sulfate was bought from Fis her

Scientific (Loughborough, United Kingdom). The rest of the reagents used were analytical grade.

Preparation of oxidized methyl linoleate. A model system of oxidized methyl linoleate was prepared by pouring methyl linoleate (3 mL) into quartz cuvettes. The samples were either placed under ultraviolet (UV) radiation for up to 48 h or oxygen was bubbled through for 6 h. Aliquots of 50–100 μL were taken after 1 h and then every 6 h up to 48 h.

Sample treatment. Matched pairs of fish fillets were stored at −20 or −30°C upon arrival in the laboratory. Others were chopped and minced with or without vitamin E [α-tocopherol (200 ppm)] and stored at −10°C. A relatively high temperature of −10°C was used instead of −20 or −30°C in order to accelerate the changes in frozen storage.

Lipid extraction from frozen mackerel. Lipids were extracted from fish fillets by the Bligh and Dyer (17) method. Portions of mackerel (1 g) were chopped and homogenized with a mixture of 20 mL of chloroform and 40 mL of methanol. A further 20 mL of chloroform was added, and the mixture was homogenized for another 30 s. This was followed by the addition of 20 mL of deionized water and further homogenization for 30 s. The final mixture was centrifuged ($3000 \times g$), the bottom layer was collected in a flask, and the organic extract was evaporated using a Buch rotary evaporator. An aliquot (200 μL) of the oil collected was stored for transesterification.

Transesterification. Transesterification was performed according to Schmarr *et al.* (18). Sodium methylate (10%) in methanol (2 mL) diluted with MTBE (4:6, vol/vol) was added to the oil and mixed by vortex for 1 min. The mixture was vortexed and allowed to stand for 1 h. After 1 h, 2 mL of water and 5 mL of chloroform were added. The mixture was shaken for 1 min, and after 5 min of centrifugation at $3000 \times g$ to facilitate phase separation, the upper layer was removed with a Pasteur pipette. An aliquot (2 mL) of 1% (vol/vol) of citric acid in water was added to neutralize excess alkali. The sample was shaken, centrifuged, and the aqueous phase discarded. The chloroform extract was evaporated under a stream of nitrogen in a warm water bath until the volume was reduced to 200 μL. The 200 μL extract was transferred into an SPE cartridge that was conditioned with 5 mL of hexane.

Purification of hydroperoxides by LC fractionation on amino-phase SPE cartridges. To remove traces of moisture and to ensure reproducible retention behavior, a small amount of anhydrous sodium sulfate was placed on top of 500 mg of bonded amino-phase SPE cartridges, which were then conditioned with 5 mL of hexane. The transesterified lipid was redissolved in 250 μL of chloroform, transferred onto the cartridges with a Pasteur pipette, and washed again with 2×2.5 mL of hexane. Polar material containing the components of interest was eluted with 7 mL of acetone and collected in a round-bottom flask. Elution from the SPE cartridge proceeded under gravity only at a flow rate of 0.3–0.5 mL/min.

Analysis of hydroxy compounds by HPLC. The acetone extracts were poured into a 250-mL conical flask and acetone was evaporated under reduced pressure using a rotary evaporator. The residue was reconstituted with 200 μL of eluting buffer. A Hichom Kromasil (Reading, United Kingdom) 100 5C18 column (4.6 mm i.d. × 25 cm) was used for the separation of hydroperoxides using acetic acid/methanol/water (0.1:65:35) solvents.

Derivatization of the hydroxy fatty acid to trimethylsilyl ethers (TMS ethers). The acetone fraction was dried under a stream of nitrogen. Then, 100 μL of dry pyridine and 100 μL of BSA + 1% TMCS were added and, after flushing with nitrogen, the vial was sealed. Gently turning the vial and final vortexing ensured good solvation of the residue in the reagent. After a reaction time of 3–4 h at room temperature, the sample was ready for injection into the GC–MS instrument.

GC–MS analysis. GC–MS was carried out on a Fisons MD 800 GC system equipped with an electron impact ion source operated at 70 eV. A Fisons GC 8000 GC system with a DB-1 fused-silica gel capillary column (30 m × 0.32 mm i.d.) was used for sample separation. The injector temperature was kept at 280°C. The temperature program was set at 50–220°C at a rate of 20°C/min and then 5°C/min until 285°C.

Analysis of hydroxy derivatives of hydroperoxides by ^{13}C NMR. Hydroxy derivatives of hydroperoxides from methyl linoleate and fish lipid extract (100 mg) mixed with CD_3Cl (0.5 mL) were analyzed by ^{13}C NMR on a Bruker AC 300E. The experimental conditions were: proton noise decoupling, 4091 data points; spectral width 25 kHz; pulse angle 33°; and pulse repetition time 4.31 s.

RESULTS AND DISCUSSION

Most of the hydroperoxides produced during the oxidation of methyl linoleate were spontaneously reduced to their more stable hydroxy derivatives during the separation of the esterified hydroperoxide fatty acids from the esterified fatty acids. This was confirmed when the standards of hydroperoxyoctadecaenoic acid (HPODA) were passed though the amino-phase SPE cartridges and stable hydroxy derivatives were produced. HPLC provided a simple and very useful method for investigating the initiation, propagation, and termination stages of lipid oxidation in detail. Figure 1 shows the development of hydroxy octadecadienoic acids (HODA) from methyl linoleate oxidized by UV radiation for 1, 6, 24, or 48 h.

After 1 h minor peaks were observed, indicating the commencement of oxidation and the development of the hydroxy compound, HODA (Fig. 1A). A peak started to show at 6 h (Fig. 1B) and became sharper and more prominent after 24 (Fig. 1C) and 48 h (Fig. 1D). Using authentic standards and the results obtained from GC–MS to identify the positions of the hydroxy compounds, it was shown that the peak eluted at 5.5 min corresponded to the 13-HODA (*cis-trans*) and the peak eluted at 9.5 min was assigned to 9-HODA (*trans-cis*).

The mass spectra of the hydroxy compounds formed from methyl linoleate and analyzed by GC–MS are summarized in

Figure 2. The mass spectra scans of peaks 1, 2, and 3, highlighted in Figure 2A, are shown in Figures 2B, C, and D, respectively. All mass spectral scans showed that peaks 1, 2, and 3 represent isomers of molecular weight 382 and are consistent with the molecular formula $C_{22}H_{42}O_3Si$, which is the hydroxy derivative of the hydroperoxide. Ion fragmentation spectra of these peaks indicated that the major high mass fragment was *m/z* 225, which shows the location of the TMS group itself at the C-9 position. The *m/z* 311 peak locates the double bonds in the C-10 and C-12 position. The fragmentation of the compound is illustrated in Figure 2.

Results obtained for methyl linoleate oxidized by UV radiation for 24 h and analyzed by ^{13}C NMR are shown in Figure 3. A peak at 87 ppm corresponding to a carbon atom carrying the hydroxy derivative was observed only for samples oxidized for 24 and 48 h. The peak at 87 ppm was not observed for samples oxidized for 1 or 6 h. In addition, other peaks corresponding to methyl groups (1–10 ppm), CH_2 group (20–35 ppm), olefinic group (130 ppm), and carboxylic group (173 ppm) have been assigned. Although ^{13}C NMR lacks sensitivity for measurements of products generated in the initial stages of oxidation, it is a useful technique for measuring the high concentrations of hydroxy compounds produced during the latter stages of oxidation.

In contrast, the HPLC method was sensitive for analyzing hydroxy compounds in both the initial and latter stages of oxidation. HPLC was also successfully used to detect the hydroxy derivatives not only for simple systems such as methyl linoleate but also for the more complex fish oil extracted from stored frozen Atlantic mackerel.

One major peak, assigned as 13-HODA (*cis-trans*) was observed in chromatograms of oil obtained from fish stored at both −20 and −30°C (Fig. 4). However, for mackerel stored at −20°C the peak was bigger and two extra minor peaks were evident; these were absent from the spectra obtained from control fish fillets stored at −30°C. In contrast, fish stored at −10°C gave a higher amount of 9-HODA (Fig. 5). Thus the rate of oxidation, temperature, and oxidizing conditions appear to influence the nature of the products. When α-tocopherol was added to the minced fish prior to storage at −10°C (Fig. 5), fewer, smaller peaks were observed in the chromatogram of fish stored with α-tocopherol, whereas a mixture of peaks was evident in the chromatograms of the control fish. Similarly, in contrast to results obtained for methyl linoleate oxidized under UV radiation indicating 9-HODA as the major

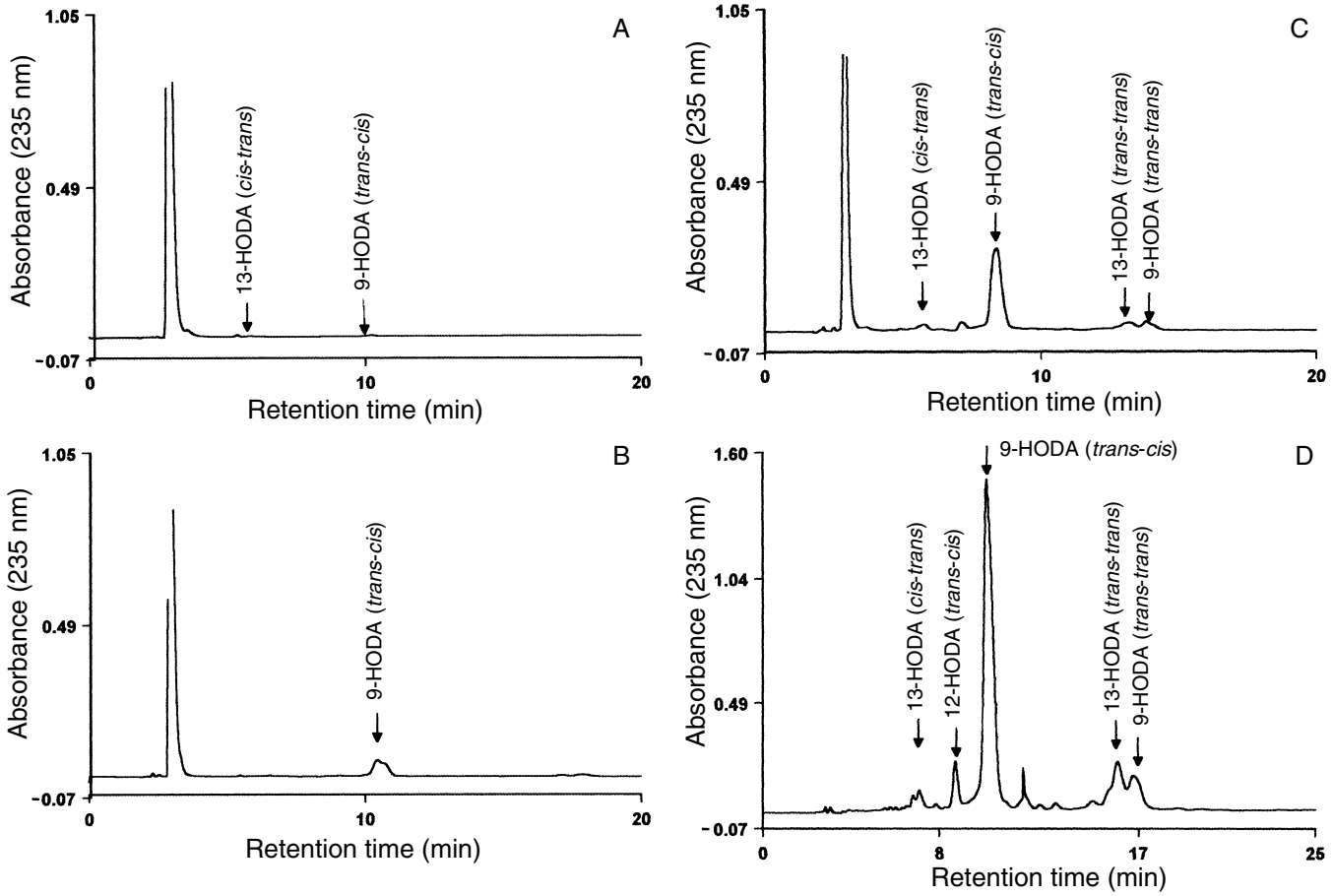

FIG. 1. High-performance liquid chromatogram (HPLC) of methyl linoleate oxidized under ultraviolet radiation for (A) 1 h, (B) 6 h, (C) 24 h, and (D) 48 h. 13-HODA, 13-hydroxyoctadecadienoic acid; 9-HODA, 9-hydroxyoctadecadienoic acid.

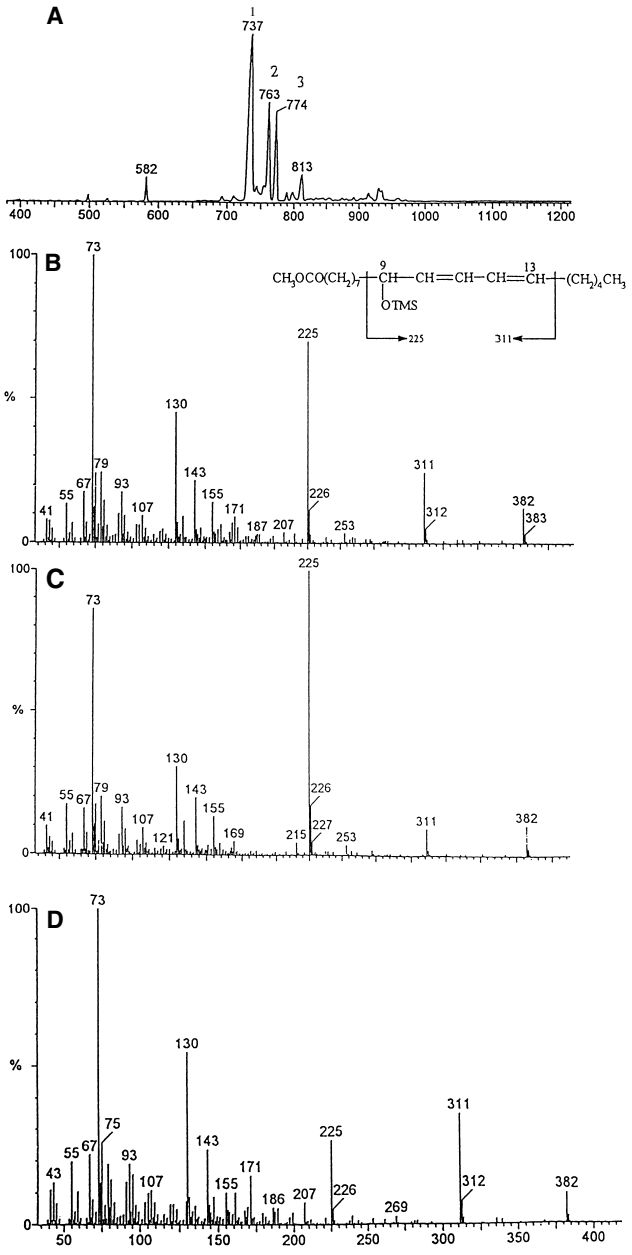

FIG. 2. (A) Gas chromatogram of methyl linoleate oxidized for 24 h. (B) Mass spectrum of Peak 1, (C) spectrum of Peak 2, and (D) spectrum of Peak 3, all indicated in the gas chromatogram.

product, 13-hydroxylinoleate (*cis-trans*) configuration was found for methyl linoleate oxidized with oxygen (Fig. 6). The production of different hydroxides probably arises from the different methods of oxidation employed, either oxygen or UV radiation.

We have found from the results obtained from the trial study of fish stored at different temperatures that the immediate oxidation product detectable at 235 nm is linoleate hydroperoxide. Although other PUFA, such as docosahexaenoic acid (DHA), may be involved in the oxidation and are probably responsible for the minor peaks observed in the HPLC

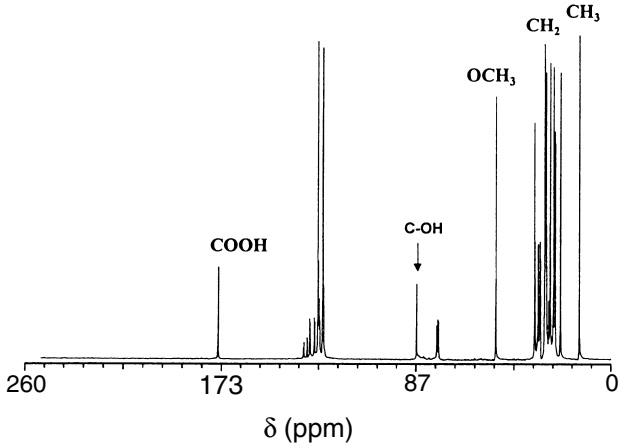

FIG. 3. ^{13}C nuclear magnetic resonance spectrum of methyl linoleate oxidized for 24 h.

chromatograms, the discussion has concentrated on linoleate, which gave the largest peak and was verified using standards and GC–MS. The fatty acid profile of Atlantic mackerel is illustrated in Table 1. Although linoleic acid is not present in

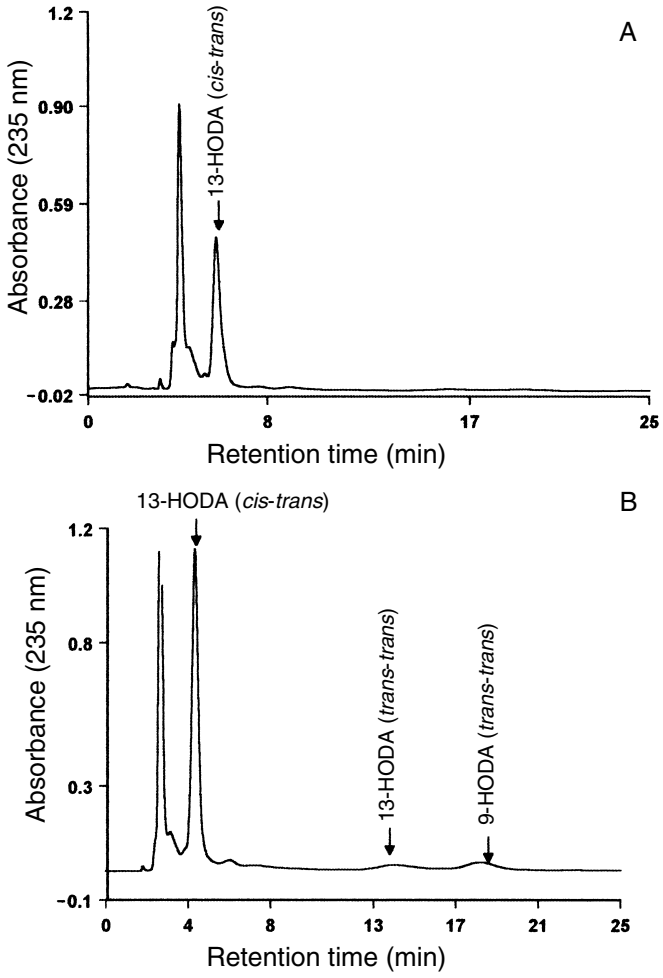

FIG. 4. HPLC of fish oil extracted from Atlantic mackerel stored for 6 mon at (A) –30°C and (B) –20°C. See Fig. 1 for abbreviations.

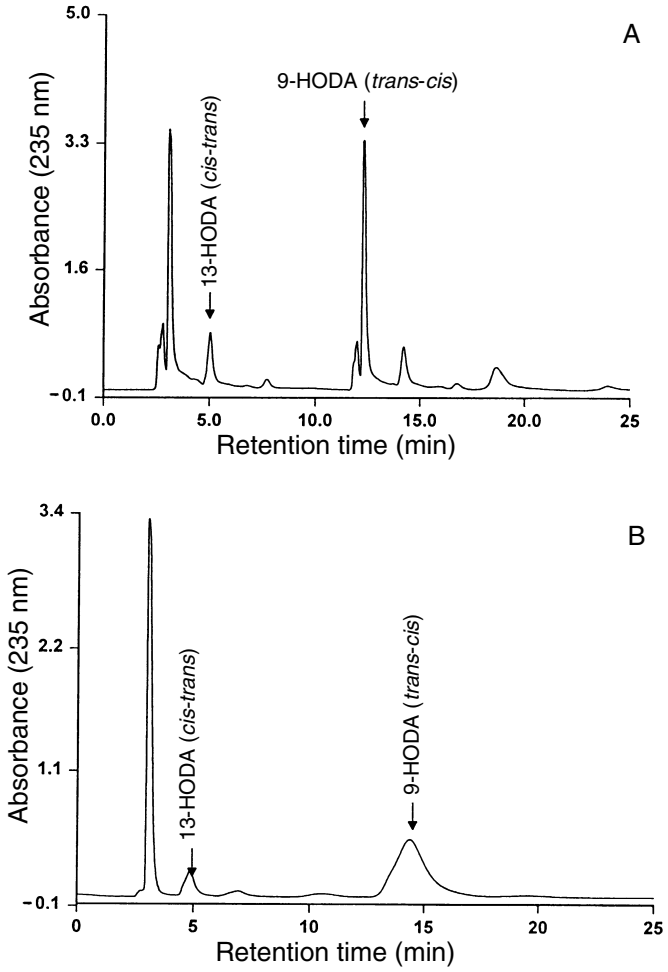

FIG. 5. HPLC of fish oil extracted from Atlantic mackerel stored at –10°C, (A) with α-tocopherol and (B) without α-tocopherol. See Figure 1 for abbreviations.

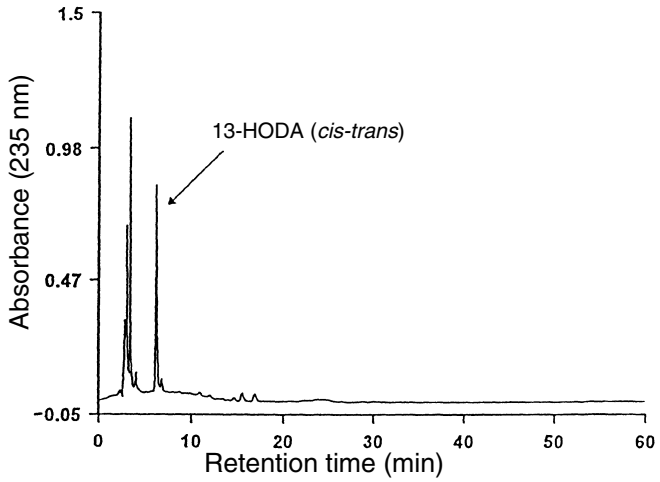

FIG. 6. HPLC chromatogram of methyl linoleate oxidized with oxygen for 4 h. See Figure 1 for abbreviations.

large amounts, Zhang and Chem (25) have demonstrated that conjugated linoleic acids, in the form of both free fatty acids and triglycerols, are extremely unstable, to the same extent as DHA, but they oxidize considerably faster than linolenic and arachidonic acids. This may present one explanation for the detection of linoleate hydroperoxides during the oxidation of Atlantic mackerel lipids. However, it is envisaged that further work will be undertaken on the oxidation of the ω-3 fatty acids DHA and eicosapentaenoic acid to enable the identification of the other minor hydroxide peaks observed in the HPLC chromatogram.

It was interesting to note that after moderate oxidation of low density lipoproteins from blood plasma, the main oxidation product was cholesterol linoleate hydroperoxide (26–28). Recent studies also showed the stability of 9-HODA and 13-HODA compared to 9-HPODA and 13-HPODA (29–31). In these studies, it was observed that there was a strong increase in hydroxy fatty acids derived from linoleic acid in many pathological cases (29–35). Thus, oxidation of linoleic acid seems to be a common process in mammals after tissue injury, causing cell death. This might also be the case in fish lipid oxidation, as observed in this study.

TABLE 1
Fatty Acid Profile of Atlantic Mackerel Lipids Extracted from White Muscle of Nine Different Fillets[a]

Peak	Assignment	Total[b] (%, ±SD)
Myristic acid	14:0	6.72 (0.53)
Palmitic acid	16:0	14.73 (1.84)
Palmitoleic acid	16:1	5.74 (0.45)
Stearic acid	18:0	3.75 (0.37)
Oleic acid	18:1n-9/n-7	17.76 (0.57)
Linoleic acid	18:2n-6	1.77 (0.20)
Linolenic acid	18:3n-6/n-3	1.78 (0.10)
Eicosenoic acid	20:1n-9	8.79 (1.82)
Octadecatetraenoic acid	18:4n-4	3.80 (0.21)
Behenic acid	22:0	0.81 (0.03)
Erucic acid/cetoleic	22:1n-9/n-11	14.82 (0.46)
EPA	20:5n-3	5.83 (0.14)
DHA	22:6n-3	14.84 (1.10)
Total of saturates		24.81
Total of monoenes		46.18
Total of polyenes		25.39

[a]Values are mean values of three samples and each sample was pooled from three fillets
[b]Wet weight basis. EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

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