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# IDENTIFICATION OF HYDROXYALKENALS FORMED FROM OMEGA-3 FATTY ACIDS

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**Summary:** The highly toxic lipid peroxidation product, 4-hydroxynonenal, is formed from the decomposition of hydroperoxides of omega-6 fatty acids. In this study the analogous hydroxyalkenals formed from the decomposition of hydroperoxides of omega-3 fatty acids (eicosapentaenoic acid and docosahexaenoic acid) were isolated and identified using TLC densitometry, HPLC and GC/Mass Spectrometry. The major hydroxyalkenal formed from both fatty acids was a diene analog of 4-hydroxynonenal, 4-hydroxynona(2,6)dienal, while 4-hydroxyhexanal was a minor product. Measurement of specific omega-3 lipid peroxidation products may be important in studies using dietary fish oil.


The potential effect of dietary omega-3 fatty acids on reducing thrombosis, coronary heart disease and certain inflammatory diseases (1-4) has stimulated numerous investigations and promoted the development of the fish oil industry. Although it is generally recognized that the highly unsaturated omega-3 fatty acids oxidize rapidly and that experimental diets must be stringently protected from spoilage (by storage under nitrogen and perhaps an in vitro active antioxidant [5]), relatively little attention has been placed on the consequences of ingestion of omega-3 fatty acids on in vivo lipid peroxidation. In this regard, dietary fish oil has been demonstrated to lower tocopherol levels in plasma, liver and kidney of mice (6). This may play a stress on the normally adequate defense mechanisms against peroxidative injury.

An important product of lipid peroxidation is 4-hydroxynonenal (HNE) which has been reported by Esterbauer and coworkers to exhibit reactivity/toxicity in biological systems (7-11) and to be detected in tissues following exposure to peroxidative stimuli (12,13). The formation of analogous products from omega-3 fatty acids has not been specifically studied, although it has been implied that 4-hydroxyhexenal is an anticipated product (14). In this report we have identified the major hydroxyalkenals formed from the omega-3 fatty acids, eicosapentaenoic acid and docosahexaenoic acid. These results are required to establish Selected Ion Monitoring GC/Mass Spectrometry assays of omega-3 related hydroxyalkenals in biological tissues.

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## Experimental Procedure

Materials. Arachidonic acid, docosahexaenoic acid and eicosapentaenoic acid (each approx. 99%) were purchased from Sigma Chemical Company and shipped on dry ice prior to storage at -20 degrees under nitrogen. O-(2,3,4,5,6-Pentafluorobenzyl)hydroxyl amine hydrochloride was obtained from Aldrich Chemical Company.

Autoxidation and hydroperoxide decomposition. Fatty acids (100 mg for various preparations, n=6) were oxidized to form hydroperoxides by exposure to air as a dried film for 24 hours. The oxidation was monitored by TLC (n-heptane/ethyl acetate/glacial acetic acid, 75:25:1 by volume) and densitometry at 205 and 235 nm (as described below) as well as uv spectrometry of the product in methanol. Hydroperoxides were isolated by reverse phase TLC using chloroform/methanol/water 1:10:0.5 by volume). Hydroperoxides were eluted from the TLC plate for use in decomposition reactions.

Fatty acid hydroperoxides were placed in Erlenmeyer flasks, the methanol was evaporated to dryness and lipid redissolved (1 mg/ml) by vortexing in 0.1 M Tris buffer pH 7.4. Ferric chloride (20$\mu$M) and ascorbic acid (500 $\mu$M) were added and solutions were incubated at 37 degrees C for up to 24 hours. Solutions were acidified to pH 3 and extracted three times with equal portions of chloroform. Pooled chloroform fractions were evaporated under nitrogen (without heating) and redissolved in 5 ml of chloroform. Aliquots (50 $\mu$l) were applied to individual lanes of Whatman LK5D silicic acid TLC plates. Chromatography was run using the straight phase TLC system described above.

TLC densitometry. A Shimadzu CS 9000 dual wavelength TLC scanner was used for densitometric monitoring of absorbance. Absorbance was monitored using the reflectance photo mode and the zig-zag scan mode with single wavelength monitoring.

HPLC. Preparative HPLC of hydroxyalkenals was done using a Beckman System Gold semipreparative HPLC system equipped with a Model 166 uv detector, Model 126 dual pumps and a Foxy fraction collector with a Model 2150 peak separator. Initial separation of hydroxyalkenals was conducted with a 5$\mu$ Ultrasphere ODS column (10 mm x 25.0 cm) which was preceded by a small Ultrashere ODS guard column (5$\mu$, 4.6 x 4.5 cm). Elution was isocratic using 65% methanol in water and 2.0 ml/min. The recovered peak of the major hydroxyalkenals of each major fatty acid contained minor contaminants of earlier peaks which were removed by a second step of preparative HPLC using an analytical ODS column (4.6mm x 25cm) using 65% methanol and 1.0 ml/min.

Reverse phase HPLC of hydrazones was accomplished using an analytical Ultrasphere ODS column and a gradient system as described by Buffinton et al.(15).

GC/Mass Spectrometry Purified hydroxyalkenals were converted to pentafluorobenzyl oximes/ TMS ethers by the method of Van Kuijk et al. (14). Negative ion chemical ionization mass spectrometry analysis was obtained using a Ribermag R10-10C GC/MS system. A 10 meter SPB1 GC column was used with an initial temperature of 100 degrees C and, following a 1 minute hold, increasing the temperature 20 degrees/min to a final temperature of 250 degrees C.

## Results and Discussion

Exposure of hydroperoxides of eicosapentaenoic acid or of docosahexaenoic acid to iron (20 $\mu$M) and ascorbic acid (500 $\mu$M) for periods up to 24 hours resulted in a decomposition of the hydroperoxide (monitored using TLC densitometry at 235nm) and the formation of a variety of secondary peroxidation products. These products, which became prominent by 18-24

hours, were monitored by TLC densitometry at 220 nm (the approximate wavelength maximum of 4-hydroxynonenal) and compared with those formed from arachidonic acid (Figure 1A,B) . The two omega-3 fatty acids exhibited similar profiles which included 2 prominent peaks as well as considerable material of lower mobility. A single prominent peak was obtained from the arachidonic acid-derived sample (Figure 1A). This corresponded in mobility to standard 4-hydroxynonenal (which was previously purified from a similar preparation and identified by GC/Mass Spectrometry as a pentafluorobenzyl oxime/TMS ether). Ultraviolet spectra of the major 220 nm TLC bands of the fatty acid products were taken by the densitometer itself, using the difference function to subtract background due to the TLC plate. The major component of the arachidonic acid products exhibited a uv spectrum characteristic of 4-hydroxynonenal with an Absorbance maxima at 220-222 nm and a minor chromophore at 270 nm (Figure 2). Identical spectra were obtained by applying standard 4-hydroxynonenal to a separate lane of the TLC plate and focusing on the resulting band. One of the TLC fractions from the docosahexaenoic acid products (the component of highest mobility) exhibited a similar uv spectrum (Figure 2B) while the other fraction exhibited a different spectrum with a prominent maximum at 280 nm (Figure 2C). Similar TLC profiles (two prominent peaks) and spectra resulted from the corresponding fractions from eicosapentaenoic acid.

Reverse phase HPLC was used to separate the components of the major 220 nm absorbing TLC fractions, monitoring at 220 nm. The major TLC

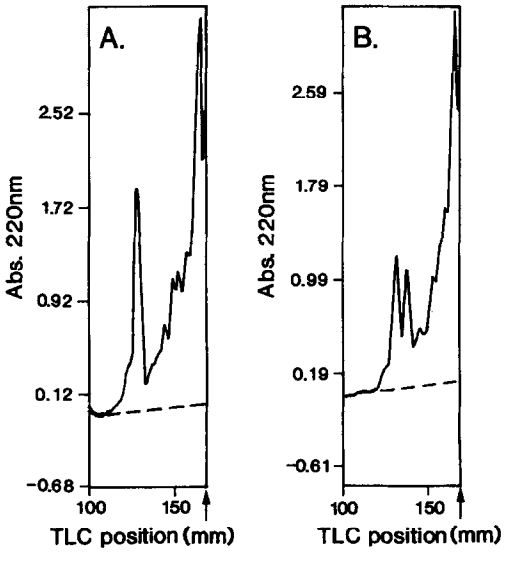


Figure 1. TLC densitometric scans (220 nm) of decomposition products of hydroperoxides formed from arachidonic acid (panel A) or of docosahexaenoiec acid (panel B). Generation of decomposition products and chromatography are described in the text. TLC position refers to the distance from the top of the plate with the origin (top of the loading zone) designated by the arrow (at position 170).

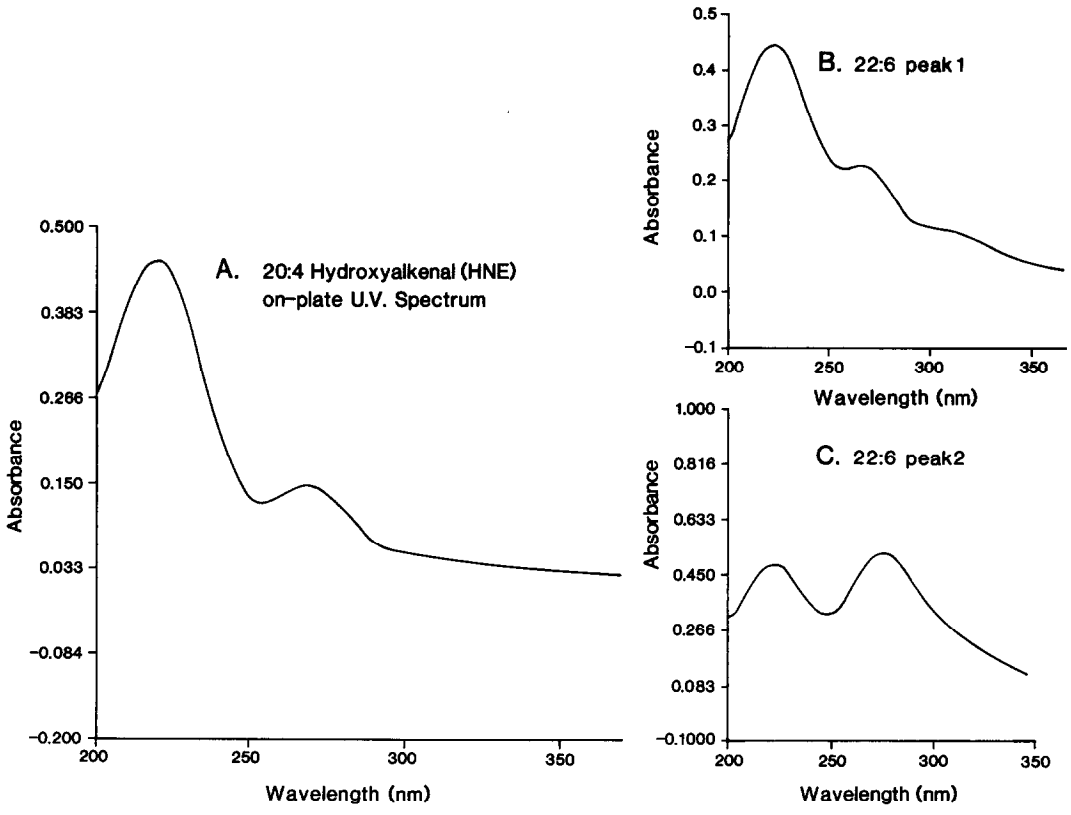


Figure 2. TLC densitometric uv spectra of the major 220 nm densitometric peaks from arachidonic acid and docosahexaenoic acid. Decomposition products were isolated and separated by TLC as described in Fig 1. The TLC densitometer was focused on particular peaks defined by the scans shown in Fig 1 and then used to record uv spectra. The uv spectrum of the arachidonic acid-derived product at TLC position 128 (panel A, Fig 1) is shown in panel A and the spectra of the docosahexaenoic acid products at TLC positions 131 and 138 (panel B of Fig 1) are shown in panels B and C, respectively.

fraction from arachidonic acid gave a single peak (time=15.1 min) which corresponded to standard 4-hydroxynonenal. TLC fraction 1 (higher mobility) from docosahexaenoic acid gave a single prominent peak on HPLC which eluted earlier (time=12 min) than 4-hydroxynonenal. The corresponding TLC fraction from eicosapentaenoic acid gave the same HPLC peak. TLC band 2 from docosahexaenoic acid or eicosapentaenoic acid gave on HPLC (220 nm) a component at 7.9 min. This compound was subsequently shown to exhibit a uv spectrum similar to the hydroxyalkenals (Absorbance maximum at 222 nm). Numerous other compounds of Absorbance maximum of approximately 280 were also present in TLC band 2. The same HPLC profile was found in the corresponding TLC band from eicosapentaenoic acid (data not shown).

Following purification (greater than 98% at 220 nm) on HPLC, the major hydroxyalkenals from the omega-3 fatty acids were derivatized to form pentafluorobenzyl oximes/TMS ethers and analyzed by Negative Ion Chemical Ionization GC/Mass Spectrometry. Standard 4-hydroxynonenal and the

hydroxyalkenal isolated from arachidonic acid decomposition were analyzed in parallel for comparison. All of the hydroxyalkenals exhibited total ion currents with dual peaks which represent syn and anti isomers of the oxime group (14). The 4-hydroxynonenal standard and the arachidonic acid decomposition product gave essentially identical negative ion mass spectra to that reported for the compound (14) including ions specific to 4-hydroxynonenal (152, 283, 303, 373, 403) and pentafluorobenzyl-associated ions (167, 196). The mass spectra of the major docosahexaenoic acid hydroxyalkenal decomposition product (from TLC fraction 1) for either syn or anti isomers (shown in Fig. 3 for syn isomer), revealed a series of ions (150, 281, 301, 371, 401) that were 2 atomic units less than the specific ions for 4-hydroxynonenal as well as the same pentafluorobenzyl related ions. It is apparent that the major omega-3 related hydroxyalkenal is a diene analog of 4-hydroxynonenal. Consideration of the possible sites of an iron-induced scission reaction on an omega-3 fatty acid suggests formation of 4-hydroxynona(2,6)dienal by reaction at the same relative position as with the formation of 4-hydroxynonenal from arachidonic acid. The mass spectrum of the minor hydroxyalkenal decomposition product of docosahexaenoic acid (from TLC fraction 2) included all of the specific (110, 241, 261, 291, 331) and pentafluorobenzyl-related ions described previously for 4-hydroxyhexenal (14). The mass spectra of the hydroxyalkenals isolated from eicosapentaenoic acid were the same as their corresponding fractions from docosahexaenoic acid.

Dinitrophenylhydrazine derivatives of hydroxyalkenals separate readily by TLC (16) and offer an alternative means of analyzing these products which avoids problems with volatility. In an effort to quantitate the relative formation of the 4-hydroxynonadienal and 4-hydroxyhexenal from docosahexaenoic acid, dinitrophenyl hydrazones of the decomposition products formed from the hydroperoxides of docosahexaenoic acid were derivatized with dinitrophenylhydrazine directly from the iron-ascorbic acid reaction mixtures

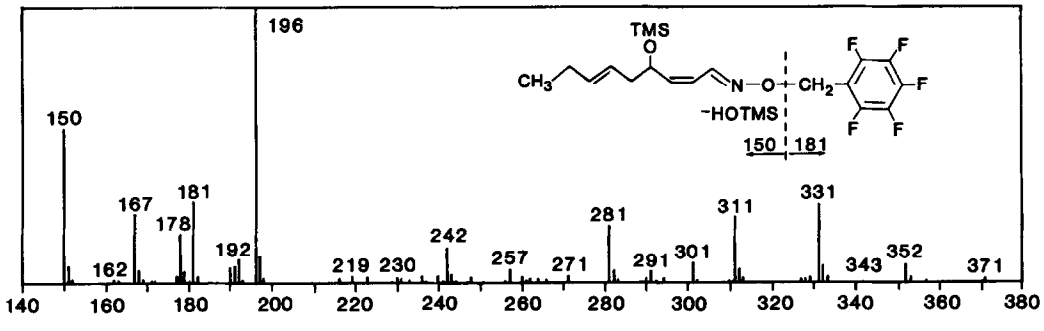


Figure 3. Negative ion chemical ionization mass spectrum of PFB-oxime/TMS ethers of the major hydroxyalkenal formed from docosahexaenoic acid. A fragment at 401 of abundance comparable to the 167 fragment was also observed.

(and thus avoiding potential losses during solvent evaporation). For comparison, standard hydrazones of 4-hydroxynonadienal and 4-hydroxyhexenal were formed from the purified products. Reverse phase HPLC (360nm) was run on the hydroxyalkenal-hydrazone TLC fraction (isolated according to [16]) of the docosahexaenoic acid decomposition products. 4-Hydroxynona(2,6)dienal was the major product (68 ± 5.5 percentage of total hydrazones, n=4) while 4-hydroxyhexenal was less prominent (17 ± 6.3 percent).

In view of the susceptibility of the highly unsaturated omega-3 fatty acids to peroxidation, it may be important to measure the specific products of their peroxidation in biological tissues exposed to oxidative stress. Measurement of omega-3-derived hydroxyalkenals by the Selected Ion Monitoring GC/Mass Spectrometry method (14) should complement assays of lipid hydroperoxide precursors (such as the chemiluminescent HPLC methods (17,18). The significance of omega-3 fatty acids in promoting lipid peroxidation in vivo may then be evaluated.

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