A Rapid Method for Determining the Oxidation of n-3 Fatty Acids

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The stability of unsaturated fatty acids to oxidation was monitored by following gas chromatographic (GC) analyses of headspace volatiles in comparison to changes in polyunsaturated fatty acids (PUFA) and increases in malonaldehyde *via* the 2-thiobarbituric (TBA) assay. Pure standards of linoleic acid (Lo) and n-3 fatty acids [eicosapentaenoic (EPA) and docosahexaenoic acid (DHA)] were added to headspace vials, equilibrated in air for 10 min, followed by heating at 80°C in teflon-capped vials for different time intervals. Headspace analysis showed increases in acetaldehyde, propenal, and propanal, corresponding to the oxidation of n-3 fatty acids, whereas hexanal production corresponded to losses of linoleic acid. The analysis of propanal by GC-headspace after only five minutes of heating appeared to be the most effective method of monitoring the oxidation of n-3 fatty acids, as indicated by correlations between TBA values and loss of PUFA. The oxidation of Lo, EPA and DHA appeared to be a function of the number of double bonds. Correlations between PUFA depletion, TBA values and volatile formation indicate that under the prescribed conditions of this experiment, GC-headspace analysis of propanal and pentane/hexanal is an excellent method for following the oxidation of selected n-3 fatty acids and linoleic acid.

KEY WORDS: Omega-3 fatty acid, oxidation, 2-thiobarbituric acid, volatile gas chromatographic headspace analysis.

Lipid oxidation has long been recognized as the major cause for the deterioration of unsaturated lipids. In the presence of oxygen, hydroperoxides are formed. These primary products are rapidly decomposed to form a variety of secondary products, including aldehydes, ketones, alcohols, hydrocarbons and other products. Many of the products resulting from oxidation are low-molecular weight volatile compounds, which produce off-flavors and odors in foods (1,2). Many of these compounds have been associated with increased risk of cancer and other degenerative diseases (3,4). Therefore, more effective methods to detect and quantitate these oxidation products in foods and biological systems are important to the food industry as well as to researchers studying oxidation and its consequences.

Oxidation can be measured by a number of methods that include oxygen consumption of polyunsaturated fatty acids (PUFA), lipid hydroperoxide formation, thiobarbituric acid (TBA) assay of malonaldehyde (MDA) and other TBA-reactive substances (TBARS), gas chromatography/mass spectrometry (GC/MS) of volatile compounds, and sensory evaluation (5). The TBA reaction is one of the most widely used methods for determining the extent of lipid oxidation in foods and in biological systems. However, due to the limitations of the TBA assay, it is often recommended that more than one method of measuring lipid oxidation be used (6). Gas chromatographic measurement of headspace volatiles resulting from lipid oxidation has often been used as an objective tool to compare chemical measurements of oxidation to sensory panel evaluation of off-aromas and off-flavors. Due to the abundance and simpler structure of linoleic acid (Lo) in our food supply, its oxidation pattern has been studied extensively. However, the oxidation pattern of n-3 PUFA is not as well defined, with limited information available on oxidation products that might be used to follow the deterioration pattern of food products containing large amounts of n-3 fatty acids. Since several health reports have recommended increased dietary consumption of fish and other foods rich in n-3 fatty acids, it is important to determine the oxidation pattern and the breakdown products that could result from the oxidation of such foods. The major objectives of this study were i) to establish a rapid and economical GC-headspace method to determine major volatile decomposition products of n-3 PUFA, whose major fatty acids are eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), and ii) to compare the effectiveness of the GC-headspace technique to one or more of the classical techniques, such as the TBA assay and change in PUFA content.

EXPERIMENTAL METHODS

Sample preparation. Commercial preparations of Lo (18:2n-6) (Sigma Chemical Co., St. Louis, MO), EPA (20:5n-3) and DHA (22:6n-3) (Nuchek Prep, Elysian, MN) were used throughout the study. All samples were dissolved in iso-octane for gravimetric transfer into 5-mL Hewlett Packard (HP) (Avondale, PA) headspace vials. The iso-octane was evaporated under a stream of nitrogen, followed by equilibration of the vials in air for 10 min. After equilibration vials were sealed with teflon-lined silica septa before heating.

Capillary GC-headspace analysis. Sample vials were heated at 80°C in heating blocks for periods of 0, 5, 35, 65 and 95 min. With a heated, gas-tight syringe, 1 mL of headspace volatile was injected directly into a Model 5890A Hewlett Packard gas chromatograph. The GC was equipped with a flame ionization detector, HP 3393A integrator, and an IBM PC-2 computer for data handling and storage. A 30 m × 0.32 mm i.d. fused-silica capillary column (DB-5, J&W Scientific Co., Folsom, CA) with a 1-μm film thickness was used for separation. The column inlet pressure was 11 PSI (76 KPa). Helium was used as the carrier gas with a flow rate of 0.5 mL/min, a split ratio of 7:1, with injector and detector set at 250°C and 270°C, respectively. The GC was temperature programmed from 50°C (1-min hold) to 110°C at 4°C/min and from 110°C to 220°C at 20°C/min with a 1-min final hold. Volatiles were identified by comparison of retention times to reference standards (Polyscience Corp., Niles, IL; Aldrich Chem. Co. Inc., Milwaukee, WI).

GC/MS analysis. Confirmation of volatile identities was accomplished on a Hewlett-Packard Model 5985 GC/MS system containing a DB-5 capillary column. The ionization energy was 70 eV and the scan range was from 38–260 *m/z*. One hundred mg of Lo or DHA was heated in 5-mL headspace vials for 100 min in a heating block set at 80°C. With 5-mL gas-tight heated (80°C) syringes, 100 μL of headspace volatiles was injected onto a DB-5 silica

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capillary column (30 m × 1.0 mm i.d., J&W Scientific Co.). The GC conditions were the same as those described for capillary-GC analysis of headspace volatiles. Based on relative retention times generated from the headspace-GC technique, GC/MS system, and the ionization patterns recorded from the GC/MS system, most of the volatile compounds could be identified.

Fatty acid analysis. Loss of fatty acids due to heating was determined on fatty acid methyl esters (FAME) of Lo, EPA and DHA, which were prepared as described by Morrison and Smith (7). Prior to methylation, nonadecanoic acid (C19:0) was added as an internal standard to each sample. Prepared FAME were injected onto a Model 5890A Hewlett Packard GC containing a DB-225 silica capillary column (30 m × 0.25 mm i.d., J&W Scientific Co. The GC was temperature programmed from 180°C to 230°C at a rate of 2°C/min. Fatty acid loss was estimated by calculating the difference between the amount of initial fatty acids present and the amount of fatty acids remaining after various periods of heating at 80°C.

TBA analysis. The TBA assay also was used as an index of fatty acid oxidation loss due to heating. The TBA number (μmoles/g) was determined according to the procedure of Ke and Woyewoda (8). The TBA assay was performed at intervals of 5, 15, 35, 65, 95 and 155 min on fatty acid samples that had been heated at 80°C in air.

Statistical analysis. Pearson correlation coefficients were used to compare chemical measurements to each other (9). An analyses of variance was used to determine the effects of heating on fatty acid loss and on TBA values of Lo, EPA and DHA, with significant means submitted to Duncan multiple range test. All analyses were performed in duplicate, and the experiment was replicated two times.

RESULTS AND DISCUSSION

Major volatiles. GC analysis of the headspace volatiles produced from the oxidation of EPA and DHA indicated a qualitatively similar pattern (Fig. 1). Propenal, propanal and acetaldehyde were the major volatiles produced from the heating of EPA and DHA. They comprised approximately 65% of the total volatiles. Table 1 summarizes the peak areas of GC-headspace volatiles produced from the oxidation of EPA and DHA after 95 min of heating at 80°C. After 5 min of heating, propenal and acetaldehyde

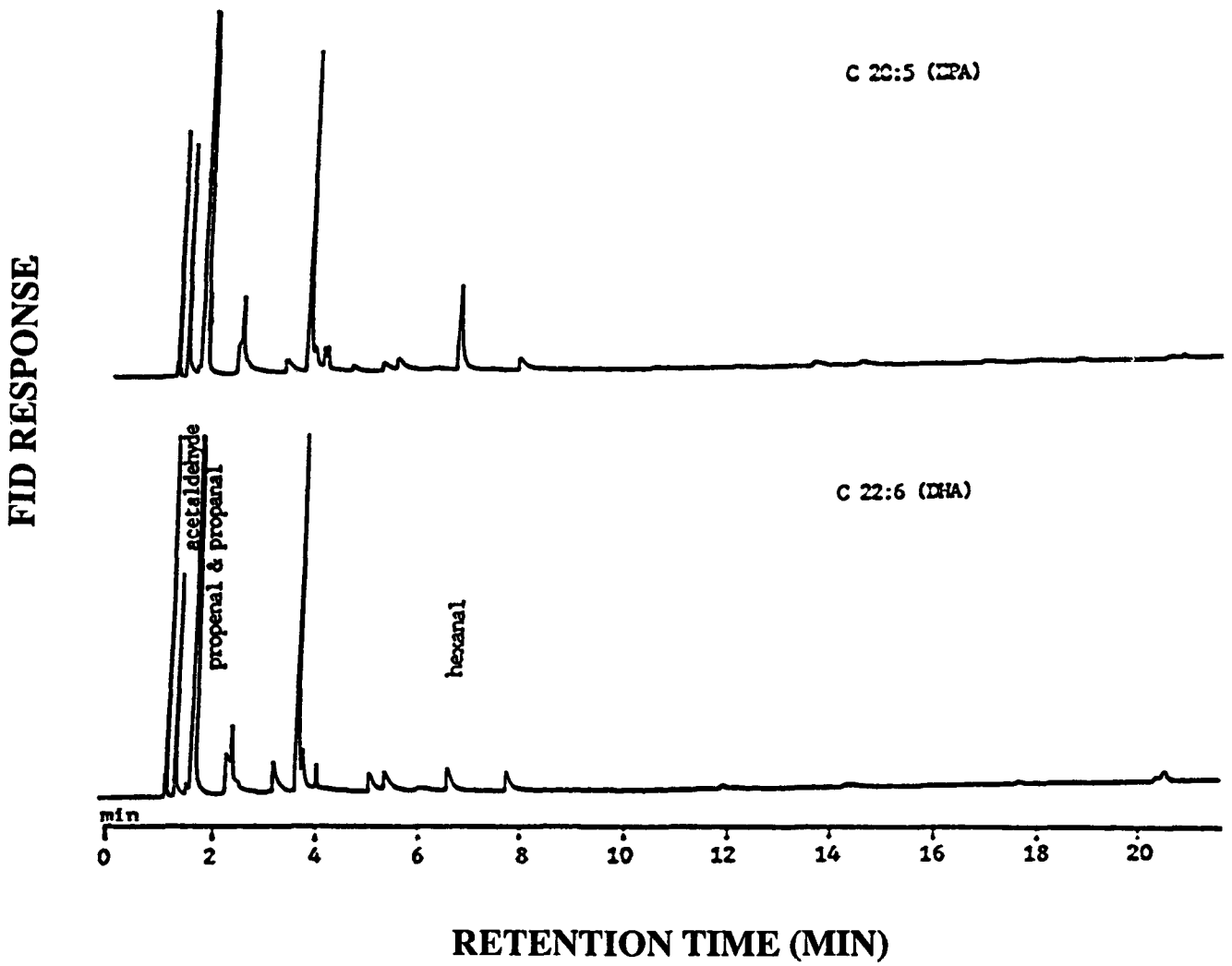

FIG. 1. Gas chromatogram of headspace volatiles from thermally oxidized eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) following heating for 95 min at 80°C in air.

TABLE 1

The Peak Areas of GC-Headspace Volatiles Produced from Heating Docosahexaenoic Acid (DHA) and Eicosapataenoic Acid (EPA) at 80°C

Identified fraction	Retention time (min)	DHA Heating times (min)					EPA Heating times (min)				
		5	15	35	65	95	5	15	35	65	95
*[a]	1.21	nd[c]	35	131	137	140	nd	27	39	76	183
*	1.26	nd	1674	5701	9477	9264	nd	522	1479	2860	4465
Acetaldehyde	1.41	80	1496	3571	6381	6097	nd	821	1560	2562	5220
*	1.62	nd	nd	188	558	586	nd	196	209	387	410
Propenal	1.70	nd	43956	101470	133016	115529	nd	11772	26656	39925	58050
Propanal	1.73	3751	16178	38014	56110	50899	1331	7253	13947	20987	36931
2-Buten-1-ol	2.33	nd	209	3642	7564	6663	nd	nd	nd	nd	1399
Butanal[b]	2.42	nd	2312	5118	7231	6448	nd	nd	1878	3357	4521
1-Penten-3-ol	3.20	nd	737	3784	5930	5097	nd	nd	468	1626	2589
MW 96	3.64	nd	9931	23873	31099	27754	nd	4238	8578	11971	16914
*	3.76	nd	1218	4619	6235	5479	nd	nd	nd	1375	2713
2-Pentenal[b]	4.05	nd	428	1190	2250	2098	nd	nd	774	1373	1995
*	5.06	nd	464	1968	3512	2958	nd	nd	nd	1363	1771
*	5.34	nd	nd	2971	5307	4879	nd	nd	nd	1849	3027
*	5.91	nd	nd	nd	873	1290	nd	nd	nd	nd	nd
Hexanal[b]	6.52	nd	373	2756	4578	4311	nd	600	2548	4459	7130
MW 110	7.64	nd	2397	4669	5002	3568	nd	nd	1103	1651	1685
*	11.77	nd	nd	nd	299	283	nd	nd	nd	nd	nd
Octanal[b]	14.14	nd	nd	850	1163	747	nd	nd	nd	nd	nd
Total area		3831	81407	204513	286719	254088	1331	25429	59239	95821	149003

[a]Peaks unidentified.

[b]Peaks tentatively identified by GC, but not confirmed by GC/MS analyses.

[c]nd, Nondetectable.

were identified as the major volatiles generated during the thermal oxidation of EPA and DHA. The increase in area counts of acetaldehyde for EPA appears to be linear up to 65 min of heating, whereas propenal increased in a linear pattern up to 35 min of heating (Fig. 2). The oxidation of DHA (measured by the production of propanal and acetaldehyde) followed a similar pattern to the oxidation of EPA, except that the rate of oxidation of DHA was faster (Fig. 3). Comparison of the peak areas of propanal and acetaldehyde indicate that, while both could be used as indicators of n-3 fatty acid oxidation, the peak area of acetaldehyde is much smaller and, thus, might not be as sensitive to minor oxidative changes for samples containing trace quantities of PUFA.

Propenal was the major volatile produced from the heating of EPA and DHA. It accounted for 50% of total volatile compounds produced. When propenal production is used as an indicator of oxidation, the oxidation of DHA peaked at approximately 65 min of heating, whereas EPA oxidation was much slower and continued at a near-linear rate throughout 65 min of heating (Fig. 4). In contrast to propanal, which was produced after only 5 min of heating, propenal was not detected in EPA or DHA after 5 min of heating, but was detected at the 15-min measurement interval. The other minor volatiles included 1-penten-3-ol, 2-pentenal, hexanal and octanal. These volatiles also may be used to follow the oxidation of n-3 PUFA. Though these volatiles had similar patterns, they were not detected until after 5 min of heating the fatty acids (Table 1). These results are consistent with those obtained by Frankel and Tappel (10), who reported that propanal production could be used as an indicator of n-3 PUFA peroxidation in biological systems.

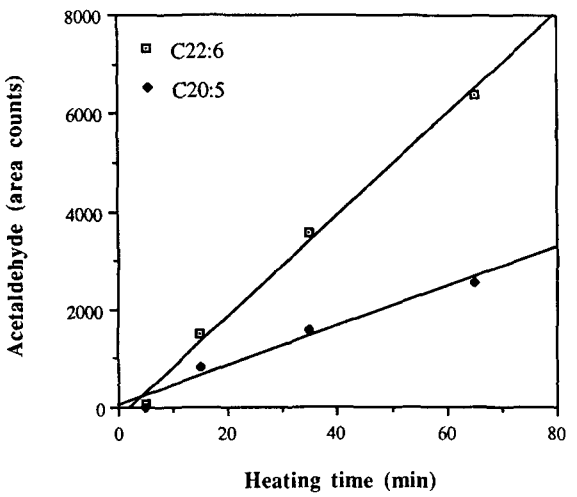

FIG. 2. Slopes and r values of acetaldehyde produced from eicosapentaenoic acid (C20:5) and docosahexaenoic acid (C22:6) heated for 5, 15, 35 and 65 min at 80°C in air.

The patterns of volatiles produced from the oxidation of Lo and n-3 PUFA are different (Fig. 5). Hexanal and pentane were the major volatile compounds produced from the oxidation of Lo, whereas only minor amounts of hexanal and no pentane are produced from DHA oxidation. Hesanal and pentane accounted for 80% of the total volatiles produced (Table 2). Neither pentane nor hexanal was detected until after 35 min of heating. Thus, the use of hexanal as an indicator of n-3 fatty acid oxidation does

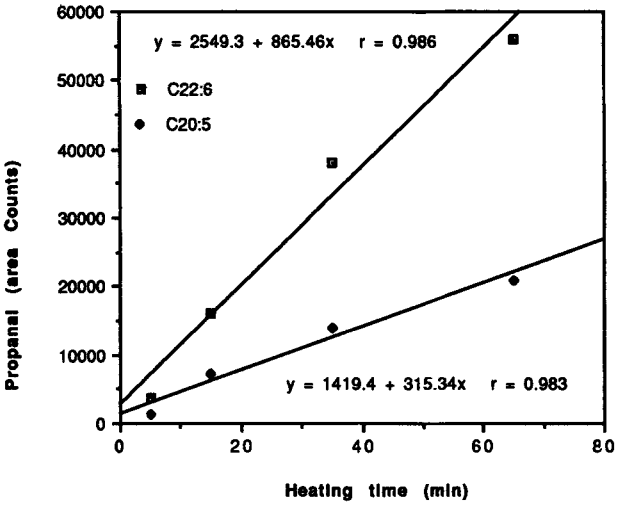

FIG. 3. Slopes and r values of propanal produced from eicosapentaenoic acid (C20:5) and docosahexaenoic acid (C22:6) heated for 5, 15, 35 and 65 min at 80°C in air.

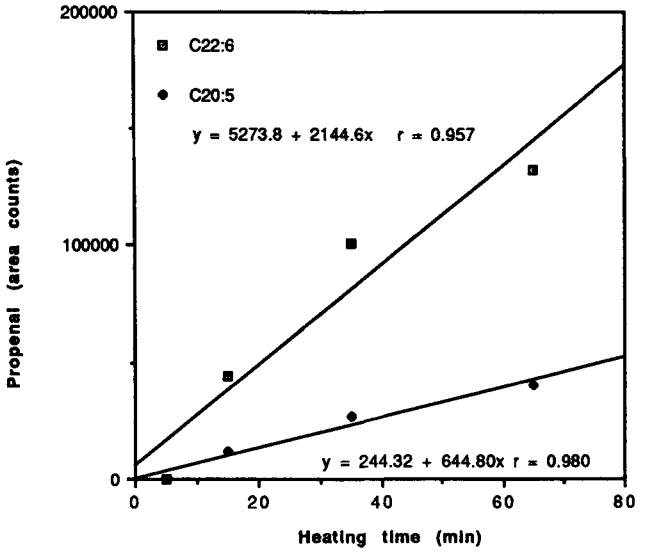

FIG. 4. Slopes and r values of propenal produced from eicosapentaenoic acid (C20:5) and docosahexaenoic acid (C22:6) heated for 5, 15, 35 and 65 min at 80°C in air.

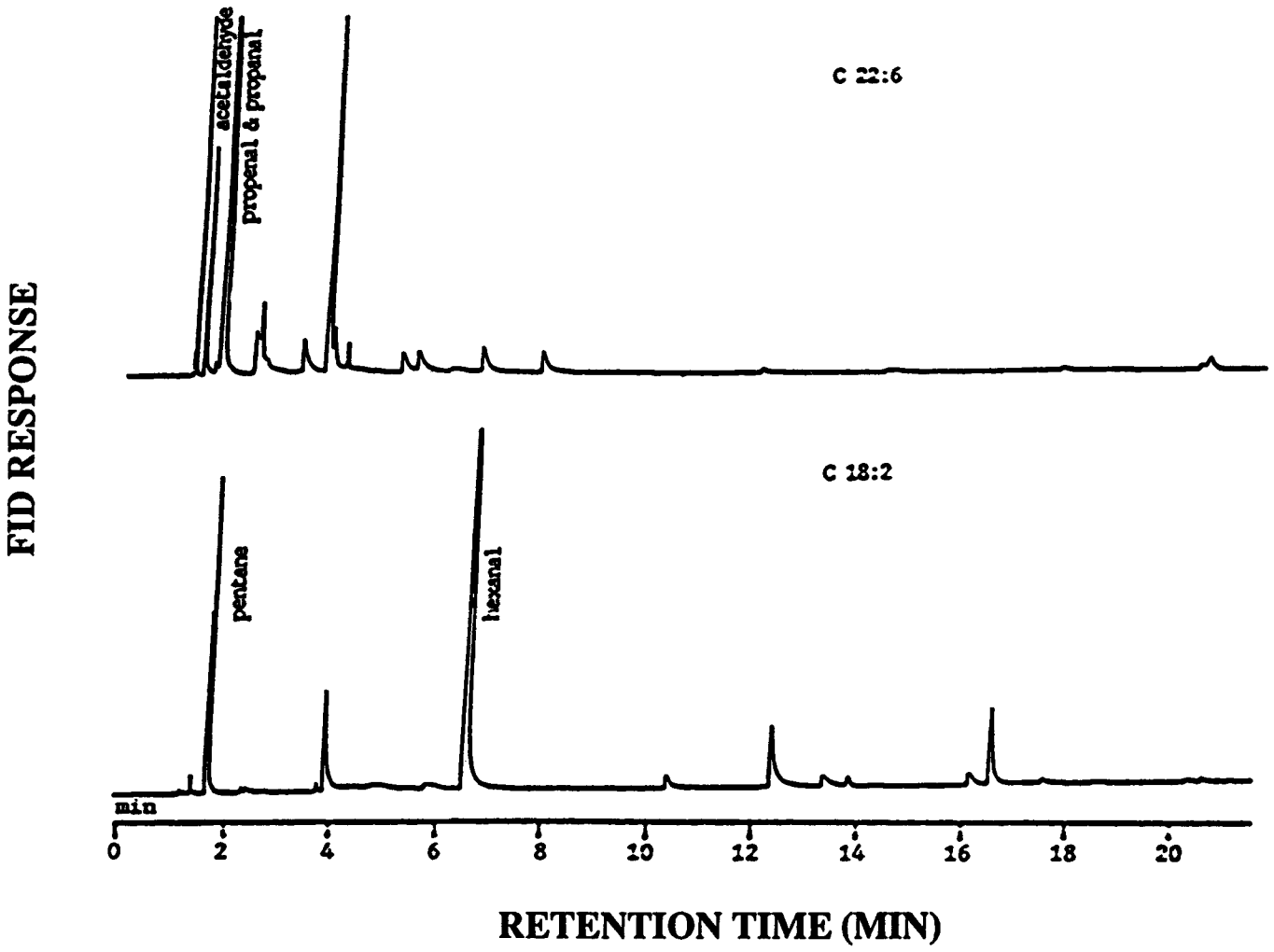

FIG. 5. Gas chromatogram of headspace volatiles from thermally oxidized docosahexaenoic acid (DHA) and linoleic acid (C18:2) after heating for 95 min at 80°C in air.

TABLE 2

The Peak Areas of GC-Headspace Volatiles Produced from Heating Linoleic Acid (Lo) at 80°C

Identified fraction	Retention time (min)	C18:2 Heating time (min) 5	15	35	65	95
*[a]	1.24	nd[c]	nd	41	nd	43
*	1.44	nd	nd	nd	47	518
*	1.72	nd	nd	nd	335	3166
Pentane	1.76	nd	nd	139	2324	7160
Butanal[b]	2.42	nd	nd	nd	nd	57
*	2.48	nd	nd	1	1	122
Pentanal[b]	3.96	nd	nd	1	1	126
2-Pentenal[b]	4.06	nd	nd	1	1233	5369
*	5.84	nd	nd	1	nd	436
Hexanal	6.52	nd	nd	3186	28277	90517
Heptanal[b]	10.36	nd	nd	nd	nd	944
2-Heptanal[b]	12.32	nd	nd	1	2594	5591
2-Pentyl-1-furan	13.31	nd	nd	1	nd	1614
2(1-Pentenyl)-furan	13.78	nd	nd	1	1	634
*	16.04	nd	nd	1	1	1428
2-Octenal[b]	16.41	nd	nd	1	1750	5800
Total ara				3365	36558	123525

[a]Peaks unidentified.

[b]Peaks tentatively identified by GC, but not confirmed by GC/MS.

[c]nd, Nondetectable.

TABLE 3

Correlation Coefficients Among TBA Value, Fatty Acid Loss, and Major Volatiles Produced from Heating of n-3 PUFA (C20:5 + C22:6) and C18:2 (n-6)

Major volatiles	n-3 PUFA (20:5 + C22:6) TBA[a]	Fatty acid loss	n-6 Fatty acid (C18:2) TBA	Fatty acid loss
Acetaldehyde	0.63**	0.71**		
Propenal	0.73**	0.75**		
Propanal	0.77**	0.76**		
Hexanal		0.63**	0.79**	0.94**
Pentane			0.82**	0.93**

[a]TBA values obtained over a 15 min period of heating.
**$p < 0.01$.

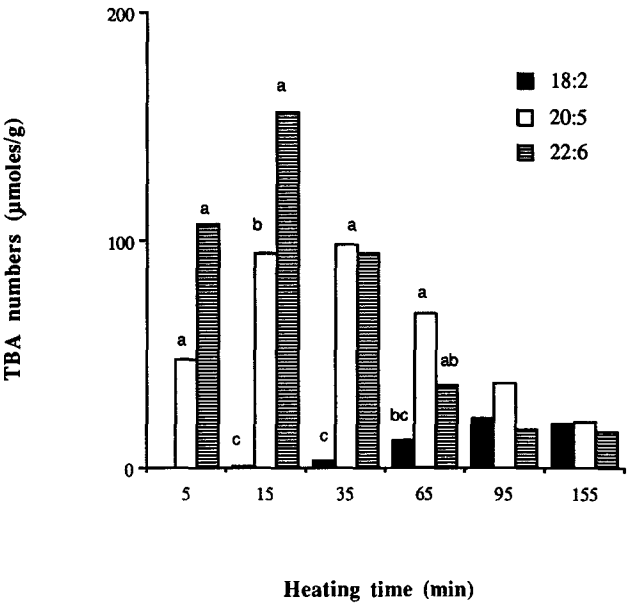

FIG. 6. Thiobarbituric acid (TBA) values of linoleic acid (Lo), eicosapentaenoic acid (C20:5), and docosahexaenoic acid (C22:6) upon heating for 5, 15, 35, 65, 95 and 155 min at 80°C in air. Columns with a, b, c denote significant differences between treatment means at $p < 0.05$.

not appear to be as sensitive as propanal, propenal or acetaldehyde production and, therefore is not a good indicator for n-3 fatty acid oxidation (Tables 1 and 2). The volatile composition pattern produced in the thermal oxidation of Lo is similar to that formed in previous studies (10–12). Both pentane and hexanal are believed to be derived from the decomposition of Lo *via* the 13-hydroperoxide pathway (11).

TBA value and fatty acid loss. The TBA values of Lo, EPA and DHA after heating at 80°C for 5, 15, 35, 65, 95 and 155 min are shown in Figure 6. Both EPA and DHA were oxidized rapidly after 5 min of heating and reached a maximum after 15 min of heating. The concentration of TBARS from DHA was 1.6 times higher than that of EPA. Linoleic acid was much more stable and did not produce significant amounts of TBARS until after 35 min of heating. The TBA value is a linear function of the PUFA unsaturation upon 15 min of heating (Fig. 7).

The percentage of fatty acid loss upon heating is shown in Figure 8. Comparison of the losses for the three fatty acids (Lo, EPA and DHA) revealed that the degree of unsaturation affected the rate of PUFA decline, with EPA and DHA loosing 60% and 95%, respectively, after 65 min. In contrast, linoleic acid showed a loss of only 22% after

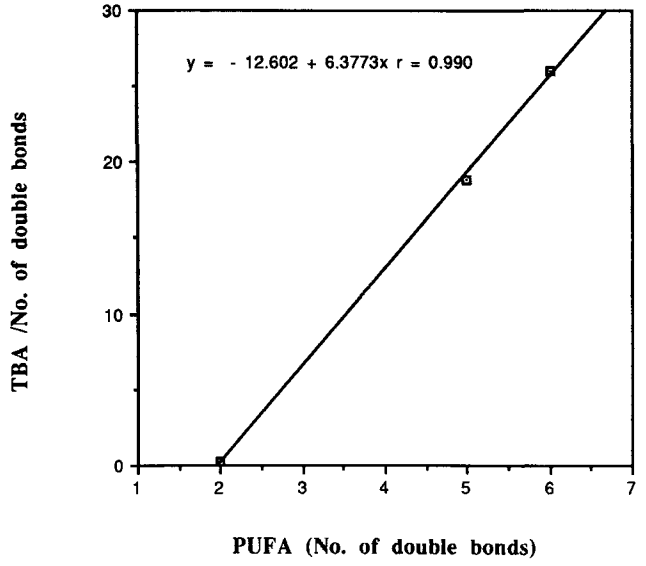

FIG. 7. Thiobarbituric (TBA) values/number of double bonds as a function of polyunsaturated fatty acid double bond content after 15 min of heating at 80°C in air.

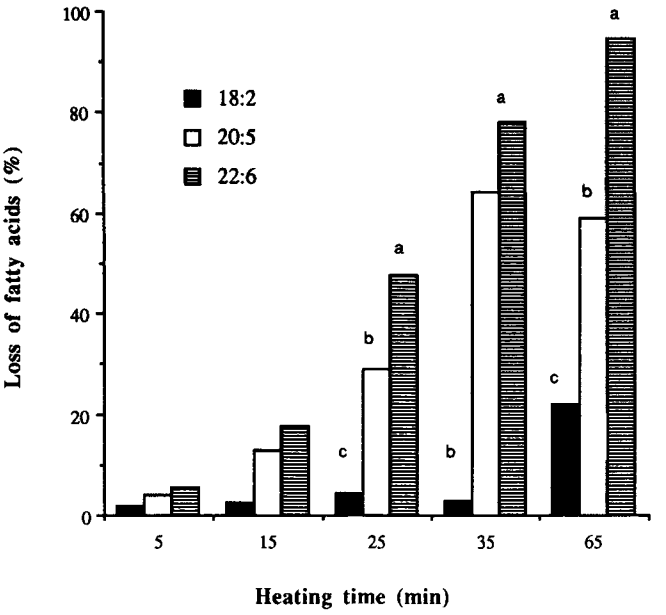

FIG. 8. Percentage of fatty acids loss following heating for 5, 15, 25, 35 and 65 min at 80°C in air. Columns with a, b, c denote significant differences between treatment means at $p < 0.05$.

65 min of heating at 80°C. Our results are in agreement with those of Cho *et al.* (13), who found that the relative oxidation rate of Lo to EPA and to DHA was on the order of 1 to 5.2 to 8.5, when using oxygen uptake as the basis for determining the rate of oxidation. The results of Bruna *et al.* (14) differed from our results as well as those of Cho *et al.* (13), in that they demonstrated that in an aqueous solution, the rate of oxidation of EPA and DHA was less than that of Lo.

Relationship of major volatiles to TBA value and fatty acid loss. The correlation between fatty acid losses, TBA values and major volatiles produced after heating of pure fatty acids is shown in Table 3. The decrease of n-3 PUFA was significantly correlated with acetaldehyde, propenal, propanal and hexanal production ($p < 0.01$). The TBA value also correlated with the release of propenal, propanal and acetaldehyde. Pentane and hexanal production were highly correlated with TBA values and loss of Lo. In summary, direct analysis of propanal may provide a quick and economical method of monitoring the oxidation of foods and biological materials containing n-3 fatty acids. Research on the usefulness of the technique in measuring the oxidation of lipid materials (*i.e.*, oils, foods, etc.) containing significant quantities of n-3 fatty acids is continuing.

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