Correlation Between ^{1}H NMR and Traditional Methods for Determining Lipid Oxidation of Ethyl Docosahexaenoate

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ABSTRACT: Lipid oxidation includes a complex set of chemical reactions; and no single analytical method is available to give a satisfactory description of lipid oxidation status. High-resolution NMR spectroscopy techniques were tested to establish possible correlations with traditional analytical methods and to study lipid oxidation products. Ethyl esters of all-*cis* 4,7,10,13,16,19-docosahexaenoic acid (DHA) (22:6n-3), with and without added α-tocopherol, were oxidized in the dark at 25°C in an air-circulating oven. Correlations were found between primary oxidation products (PV and conjugated dienes) and the appearance of peaks in the 8.0–10.5 ppm chemical shift region of the ^{1}H NMR spectra. Multivariate data analysis (partial least squares; principal component regression) and the study of specific regions of the spectra obtained made it possible to easily separate samples of esters with and without α-tocopherol based on the oxidation products formed. Based on knowledge about lipid oxidation products formed in marine lipids, selected oxidation products have been studied in the ^{1}H NMR spectra with the aim of finding detection limits and their chemical shift values.

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KEY WORDS: DHA, docosahexaenoate, lipid oxidation, marine lipids, ^{1}H NMR spectroscopy, oxidation of ethylesters.

Lipid oxidation is the most important factor limiting the shelf life of polyunsaturated oils such as vegetable and marine oils. Numerous methods are available to determine lipid oxidation, but none of them gives a completely satisfactory description of the oxidative status. It is therefore necessary to develop methods both to replace the traditional chemical methods and to obtain a better understanding of the oxidation processes of lipids. In addition, it is important to develop methods to avoid the use of unhealthy chemicals. Marine oils are highly susceptible to oxidation based on their high content of PUFA, especially EPA (20:5n-3) and DHA (22:6n-3). During auto-oxidation, hydroperoxides are formed as primary oxidation products; these are unstable and undergo further reaction to give a complex set of secondary products such as aldehydes, ketones, alcohols, and acids (1).

Among the NMR techniques, ^{1}H NMR has been most widely used to study lipid oxidation. Previous work has reported a decrease of the ratios between olefinic (δ 5.1–5.6 ppm) to aliphatic hydrogen atoms (δ 0.6–2.5 ppm) and of aliphatic to diallylmethylene hydrogen atoms (δ 2.6–3.0 ppm) during lipid oxidation. These results were obtained from experiments on vegetable (2–4) and marine lipids (5–9). A correlation has been found between PV and the decrease of these hydrogen ratios (2,5,6,8). All these studies have in common a relatively high oxidation level before any detectable changes in the NMR spectra occur (up to 1000 mequiv/kg). As far as we know, results from lipid oxidation of ethyl docosahexaenoate by using ^{1}H NMR have not been published.

Specific products from lipid oxidation of vegetable oils have previously been detected in the ^{1}H NMR spectra (4,10,11). Hydroperoxides (*H*OO–) at 8.5–8.9 ppm, conjugated dienes (–$(CH_2)_n$–CH(OOH)C*H*=C*H*–C*H*=CH–) at 5.4–6.7 ppm, and specific aldehydes (*H*CO–) at chemical shift values above 9.4 ppm (unsaturated aldehydes at 9.45 and 9.55 ppm, saturated aldehydes at 9.7–9.8 ppm) have been identified.

^{1}H NMR spectroscopy can provide both qualitative and quantitative information about lipid composition. The amount of DHA and n-3 FA has been successfully quantified in fish oils (12). ^{13}C NMR spectroscopy is less sensitive than ^{1}H NMR but has shown potential in the analysis of FA composition, positional distributions of FA in acylglycerols (13,14), and acyl stereospecific analysis of tuna phospholipids (15). ^{13}C NMR has been used to study changes caused by lipid oxidation in canned fatty fish (16).

Compared with most other analytical methods, NMR spectroscopy has the advantage that, in addition to detecting products from lipid oxidation, it also presents signals from other lipids in the system and provides information on how they can be changed during lipid oxidation. The aim of this study was to find detection limits for lipid oxidation products by using ^{1}H NMR, find correlations to classical methods for analyzing lipid oxidation, study changes in lipid composition, and elucidate the effect of the presence of antioxidant in the sample. The sample material in this study is ethyl docosahexaenoate, which is a major component in marine lipids.

EXPERIMENTAL PROCEDURES

Materials. Ethyl docosahexaenoate (DHA) (94% purity with 0.5% α-tocopherol added as an antioxidant) was provided as a gift from Pronova A/S (Sandefjord Norway). Ethyl docosahexaenoate (DHA) (22:6n-3) without α-tocopherol (99% pu-

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rity) was purchased from Nu-Chek-Prep (Elysian, MN). Ethylene glycol dimethyl ether (EGDM, 99.5%), used as an internal standard, was purchased from Wako (Tokyo, Japan). Deuterated chloroform ($CDCl_3$, >99.5%) was purchased from Fluka (Buchs, Switzerland). Chemicals for analysis of PV, TBARS, and conjugated diene hydroperoxides were purchased from Merck (Darmstadt, Germany) or Sigma-Aldrich (Milwaukee, WI). Hexanal (98%), *trans-trans*-2,4-heptadienal (90%, tech.), *trans*-2-pentenal (95%), *trans*-2-octenal (94%), *trans*-2-nonenal (97%), and 1-penten-3-ol (99%) were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany).

Sample treatment and oxidation trial. Ethyl esters were oxidized in the dark in an air-circulating oven at 25 ± 0.5°C over a 10-d period and duplicate samples were withdrawn daily. The ethyl esters were placed in dark 10-mL glass bottles (5 mL in each) (i.d 1.8 cm). The samples were flushed with N_2, sealed, and frozen at −80°C until analysis.

Analysis of oxidation. Lipid oxidation was determined by PV (17), conjugated diene hydroperoxides (18), and TBARS (19).

NMR analysis. The ethyl esters were introduced (0.1 mL in 0.6 mL $CDCl_3$) into 5-mm NMR sample tubes (Wilmad, Buena, NJ) using 0.2% EGDM in $CDCl_3$ as an internal standard. The samples were run on a Bruker Avance DRX600 (Karlsruhe, Germany) at ambient temperature (298 K). For each sample a proton NMR spectrum was obtained. Experimental conditions: 600 MHz (^{1}H), center frequency 7.2 ppm, spectral width 16.2 ppm, acquisition time 3.4 s, 64K time domain data points, 50 degree pulse angle, recycle delay 4.4 s. Two hundred fifty-six scans were collected for each sample. The data were processed without zero filling using an exponential window function of 0.2 Hz. In studying the detection limits, the selected oxidation products (hexanal, *t,t*-2,4-heptadienal, *t*-2-pentenal, *t*-2-octenal, *t*-2-nonenal, and 1-penten-3-ol) were diluted from 1.0 to 0.01 mM with $CDCl_3$ containing 0.2% EGDM. ^{1}H NMR spectra for these weight dilution analyses were run under the following conditions: 600 MHz (^{1}H), center frequency 7.2 ppm, spectral width 16.2 ppm, acquisition time 3.4 s, 64K time domain data points, 50 degree pulse angle, recycle delay 4.4 s. Two hundred fifty-six scans were collected for each sample. The data were processed without zero filling using an exponential window function of 0.2 Hz.

Data analysis. XWINNMR (v. 3.1; Bruker) and IGOR Pro (v. 4.07; Wavemetrics, Lake Oswego, OR) were used for integrating and curve-fitting the NMR spectra. Multivariate analyses were performed with a variety of software including a program written in-house as well as SCAN (v 1.0; Minitab; State College, PA); and MATLAB (v 6.5; Mathworks, Natick, MA). All spectra were phased and baseline corrected. Peak picking was then performed, and all peaks greater than about 0.5% of the peak maximum in each spectrum were extracted. Despite the use of an internal reference, it should be noted that there are differential variations in the chemical shifts to different levels within different regions of the spectra (due, for example, to possible pH, temperature, concentration, and intermolecular interaction variations). These variations necessitate the correction of all peak positions to ensure that comparable chemical shifts are associated with their corresponding peak heights in the data matrices used for the analyses. Analyses were performed using different sets of chemical shifts in order to determine the optimal combinations for the various analyses. Since major chemical shift variations can be observed within the 8–10.5 ppm regions for all samples at different degrees of oxidation, this region was of primary interest for many of the calculations attempted. However, we also demonstrated that corresponding (albeit more difficult to discern visually) systematic variations in chemical shifts in the 0–7 ppm region can be readily used for quantitative predictions/modeling of degree of oxidation. In this latter instance, we employed genetic analysis of the entire data sets to objectively and optimally extract the major chemical shifts associated with changes in degree of oxidation.

Partial least squares (PLS) regression (20,21) was employed for additional quantitative analysis of the data. Correlation analysis was performed in Excel (Microsoft, Redmond, WA).

RESULTS AND DISCUSSION

The ^{1}H NMR spectrum has previously been reported to provide specific molecular information about the marine lipids (14). Figure 1 presents the ^{1}H NMR spectrum of the ethyl docosahexaenoate with identification of the main peaks. The signals from the terminal methyl group (–CH_3) of n-3 fatty chains have reported chemical shift values between 0.95 and 0.98 ppm, signals from 22:6n-3 (C=CH–CH_2–CH_2–COO) are found at 2.38 ppm, and other hydrogen atoms from n-3 FA will also appear at other chemical shift values for unsaturated FA, PUFA, and n-3 PUFA (12,14). To the authors' knowledge, no work has been published in which ^{1}H NMR has been used to study specific lipid oxidation products in marine lipids.

By using GC–MS, Kulås *et al.* (22) have reported the formation of different products during lipid oxidation of marine

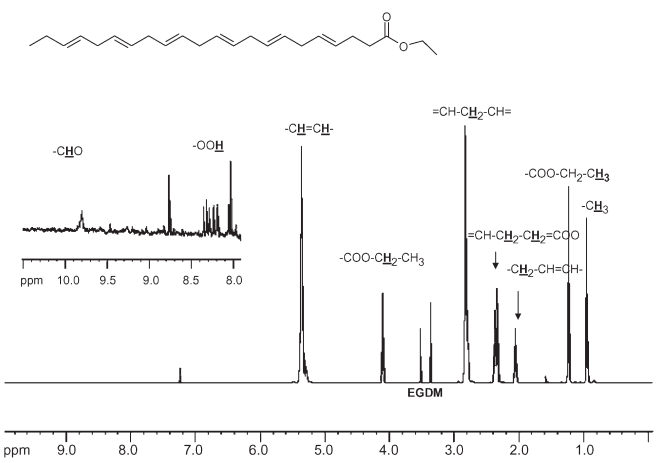
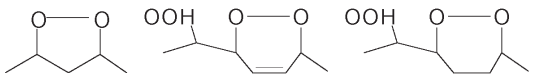


FIG. 1. ^{1}H NMR spectrum of (0–10 ppm) docosahexaenoate with identification of the main peaks (13). The region between 8 and 10.5 ppm has been enlarged (× 30 compared with the whole spectrum) to see the new peaks that are being developed. EGDM, ethylene glycol dimethyl ether.

lipids containing DHA. In our work, standards of selected oxidation products reported by Kulås *et al.* (22) were tested in order to find detection limits and to study chemical shift values under these conditions. The chemical shift values are presented in Table 1, and the detection limits were found to be <0.01 mM, but these limits might be even lower following instrument optimization (e.g., 800 MHz magnet equipped with a cryoprobe).

Table 1 summarizes the chemical shifts selected from a genetic algorithm analysis of the data from the ethyl ester without α-tocopherol added. Forty-five chemical shifts were found to be most significant in relation to the corresponding changes in degree of oxidation in the 0–7.5 ppm region. The results of the analysis constitute an extensive mixture of both obviously major peak intensities and a variety of minor peak intensities, and it reveals that hydrogen signals from n-3 FA, PUFA, and unsaturated FA decrease with increasing degree of oxidation, and the intensity of peaks at 4.3–4.4 and 5.6–6.6 ppm increase. The vinylic hydrogens from the standard oxidation products presented in Table 2 gave signals between 5.1 and 6.9 ppm, depending on whether the source was an alcohol (5.1 and 5.8 ppm) or an alkenal (6.0–6.2 and 6.8–6.9 ppm). According to Claxon *et al.* (10), the conjugated diene olefinic multiplets are also located between 5.4 and 6.7 ppm. Conjugated diene hydroperoxides are primary oxidation products and are expected to increase during auto-oxidation of DHA. The chemical shift signals around 4.3 ppm, which also increased during lipid oxidation, have previously been associated with proton multiplets (–C*H*(OOH)–) (10) and with hydrogens associated with

TABLE 1
Most Significant Chemical Shift Values in Relation to Corresponding Changes in Degree of Oxidation[a]

Chemical shift values[a,b] (ppm)	Assignments	References
6.61 6.58, 6.57, 6.56 6.01, 5.99, 5.98, 5.97	Conjugated diene hydroperoxides (5.4–6.7 ppm) –CH_2–CH(OOH)C*H*=C*H*–C*H*=CH–	3,10
5.63 5.43	Vinylic hydrogens from aldehydes (–C*H*=C*H*–CHO) (6.2–6.8 ppm)	2 Table 2
5.36, 5.33 5.24	Unsaturated fatty acids (–C*H*=C*H*–) (5.34–5.36 ppm)	14
4.15 4.29 4.44, 4.43, 4.42, 4.41	Hydrogen on the peroxy-bearing carbon, –C*H*(OOH)– (4.1–4.2 ppm/4.35 ppm)	3,4 23
	Unsaturated alcohols (–CH(O*H*)–) (4.5–5.0 ppm)	25
	Cyclic peroxide methane hydrogens (epoxides, polymerization) (–C*H*(OO)–) (4.5–4.7 ppm) see structures **1**, **2**, and **3**	23
		
	[**1**] [**2**] [**3**]	
2.96, 2.95, 2.94	PUFA (2.81–2.84 ppm) (=CH–CH_2–CH=)	14
2.49	Aldehydic hydrogens adjacent to carbonyl (-CH_2-CHO)	Table 2
2.41, 2.41	22:6n-3 (DHA) (2.38ppm) (=CH–CH_2–CH_2–COO)	12,14
2.18 2.14 2.04 2.00 1.96, 1.95	Unsaturated FA (–CH_2–CH=CH–) (1.99–2.07 ppm)	14
1.48 1.27, 1.25	Methyl group of selected lipid oxidation products (–CH_3)	Table 2
1.01, 0.99, 0.98, 0.91, 0.90, 0.83	n-3 FA (–CH_3) (0.95–0.98 ppm)	14

[a]These chemical shift values are selected by genetic algorithm analysis of the data from docosahexaenoate without α-tocopherol added in the region between 0 and 7.5 ppm. The corresponding assessment of these peaks is also presented.
[b]Chemical shift values are presented to two decimal places, so fewer than 45 shift values are presented here.
[c]The chemical shift values are collected in groups depending on what hydrogen signals are found in this region. Signals from other products also might be found in these specific regions.

TABLE 2
Chemical Shift Values of Specific Oxidation Products Detected by Using ^{1}H NMR

	M.W.[a]	Purity (%)	Chemical shift values (ppm)
t,t-2,4-Heptadienal	110.16	90[a]	9.5, 9.58 (C*H*O) 6.05, 6.30 (–C*H*=C*H*–CHO) 2.25 (–CH_2–) 1.55 (–CH_3)
1-Penten-3-ol	86.13	99	5.8, 5.15 (–C*H*=CH_2) 4.0 (–CH-(O*H*)–CH=CH_2) 2.1 (–C*H*-(OH)–CH=CH_2) 1.5 (–CH_3)
Hexanal	100.16	98	9.75 (–C*H*O) 2.6, 1.8, 1.6 (–(CH_2)$_n$–) 1.2 (–CH_3)
t-2-Pentenal	84.12	95	9.5 (–C*H*O) 6.9, 6.2 (–C*H*=C*H*–CHO) 2.4 (–(CH_2)$_n$–) 1.1 (–CH_3)
t-2-Octenal	126.2	94[a]	9.5 (–C*H*O) 6.9, 6.1 (–C*H*=C*H*–CHO) 2.4, 1.6 (–(CH_2)$_n$–) 1.16 (–CH_3)
t-2-Nonenal	140.23	97	9.5 (–C*H*O) 6.8, 6.1 (–C*H*=C*H*–CHO) 2.3, 1.3, 1.2 (–(CH_2)$_n$–) 0.8 (–CH_3)

[a]All standards supplied by Sigma-Aldrich (Steinheim, Germany). *t,t*-2,4-Heptadienal and *t*-2-octenal were technical grade.

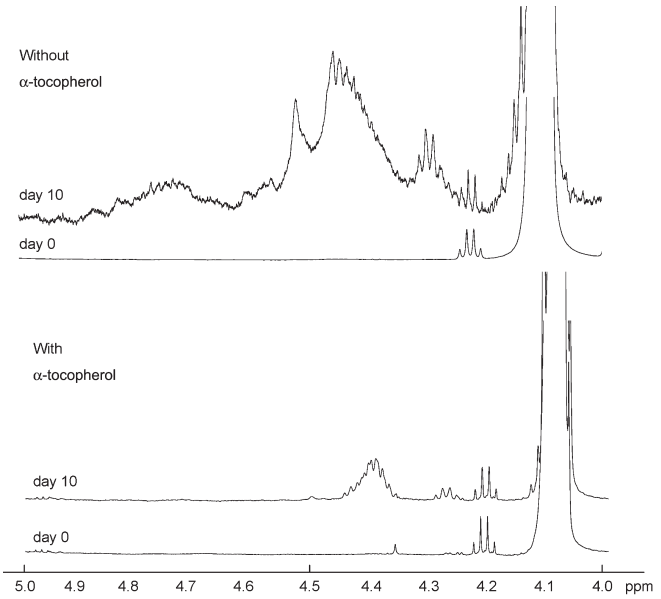


FIG. 2. An expansion of the region between 4 and 5 ppm in the ^{1}H NMR spectrum of (A) docosahexaenoate without α-tocopherol and (B) docosahexaenoate with α-tocopherol. Two spectra from samples stored at 25°C are presented for each series, at day 0 (bottom spectrum) and day 10 (top).

cyclic peroxide methane (23). An enlargement of this part of the spectrum (4–5 ppm) is presented in Figure 2, and formation of new peaks is clearly observed during lipid oxidation.

The aldehydes (–C*H*O) (Table 2) also gave signals in the downfield region of the spectrum (9.5–9.57 ppm). Figure 3 demonstrates the large differences between the unoxidized and most severely oxidized samples in each series studied (with/without antioxidant) in the 8–10 ppm region. The spectra are enlarged to make it possible to detect these relatively small peaks. The figure illustrates the significant development of peaks in this area. The peaks from aldehydic hydrogens are only found at detectable levels in the series with no α-tocopherol added. In both series, the peak intensities are increasing within the region between 8 and 9 ppm, which represents signals from a broad range of hydroperoxides (–OO*H*) (4,10). DHA (*all-cis* 4,7,10,13,16,19-DHA) generally produces ten (4-, 7-, 8-, 10-, 11-, 13-, 14-, 16-, 17-, and 20-) hydroperoxides (1). However, different geometrical isomers of these hydroperoxides are expected to be formed, and their identities have previously been shown to be varying based on temperature and degree of lipid oxidation (1), leading to even more hydroperoxides. In this trial, eight individual signals are detected in the series with α-tocopherol, while additionally some smaller peaks are detected in the series without α-tocopherol added. The chemical shift values of the hydroperoxide hydrogens vary between the series, perhaps as a result of differences in the chemical environment. However, differences in *cis-trans* vs. *trans-trans* hydroperoxides will also affect the chemical shift values of the signals from hydroperoxides formed (23), and different shift values for the hydrogen on the hydroperoxy-bearing-group carbon are reported (4) for different geometrical isomers. The changes in peak intensities between 4.35 and 4.5 ppm indicates a cyclization (formation of epoxides) (23), which is supported by the observation of increased viscosity during oxidation. Any cyclization will affect the chemical shift values of the neighboring hydrogens included the hydroperoxide hydrogen (–OO*H*). A movement of the chemical shift values for the same peaks was also observed during the oxidation time. The different peak development

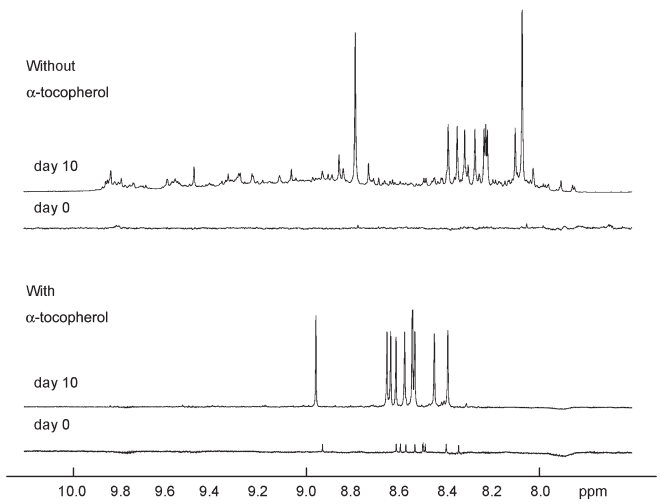


FIG. 3. An expansion of the downfield region (8–10.5 ppm) of the ^{1}H NMR spectrum of (A) docosahexaenoate without α-tocopherol and (B) docosahexaenoate with α-tocopherol. Two spectra from samples stored at 25°C, are presented for each series, at day 0 (bottom spectrum) and day 10 (top).

between the ethyl ester with and without α-tocopherol (Figs. 2 and 3) enables us to separate the two groups based on the lipid oxidation product formed and thereby gain information about the reaction product. α-Tocopherol also seems to play a role in preventing cyclization (epoxidation) of FA (Fig. 2) since the signals at 4.35–4.5 ppm are smaller in the samples with α-tocopherol after oxidation compared to the samples without α-tocopherol.

Table 3 presents the results from classical analysis of lipid oxidation for both series. During the 10 d of oxidation at 25°C, PV reached values far above what are acceptable limits for pharmaceutical n-3 products, which is below 10 mequiv/kg (24). Regression analysis of each of the classical oxidation methods and the increase of peak intensity at specific areas of the ^{1}H NMR spectra gave generally good correlation. Correlations were found between PV and the increase of peak intensity at 8–10.5 ppm (R^2 = 0.95/with α-toc, R^2 = 0.97/without α-toc, where toc = tocopherol), 5.4–6.6 ppm ($R^2 > 0.97$), and 4.2–4.9 ppm (R^2 = 0.99), respectively. Additionally, the conjugated dienes and the same chemical shift areas showed a good correlation (R^2 between 0.95 and 0.99). Because of increasing viscosity during lipid oxidation of ethylester without α-tocopherol, the TBARS value could only be detected at day 0. A correlation was found between TBARS and increase of peak intensity between 8 and 10.5 ppm (R^2 = 0.97) for the ethyl ester with added α-tocopherol. PLS analyses were performed on the data from classical analysis and the data from ^{1}H spectrum. These results are presented in Figure 4.

^{1}H NMR is a good method to study changes in the lipid compositions under these conditions. It is impossible to identify a small, selected interval corresponding to the lipid oxidation products, and it is difficult to identify the lipid oxidation products by comparing chemical shift values gained in other work. The chemical shift values are highly dependent on the environmental conditions such as temperature and pH. Even in samples analyzed under the same conditions, changes in the

TABLE 3
Data from Classical Analysis of Lipid Oxidation (PV, conjugated dienes, TBARS) During Dark Storage at 25°C of Docosahexaenoate with α-Tocopherol (withα) and Without α-Tocopherol (withoutα)

Days of oxidation	PV (mequiv)		Conjugated dienes (%)		TBARS (μg/g)[a]	
	Withoutα	Withα	Withoutα	Withα	Withoutα	Withα
0	4.05 ± 0.45	33.75 ± 1.52	0.21 ± 0.01	0.62	0.16	2.59 ± 1.14
1	88.04 ± 1.75	43.32 ± 1.72	0.60 ± 0.02	0.90	—	3.30 ± 0.40
2	228.7 ± 14.0	56.45 ± 4.65	1.27 ± 0.05	0.93	—	3.21 ± 0.08
3	434.4 ± 16.9	89.48 ± 13.38	2.61 ± 0.09	0.97	—	3.54 ± 0.04
4	381.0 ± 18.1	107.74 ± 9.35	3.98 ± 0.08	1.01	—	4.03 ± 0.06
5	N.A.	120.79 ± 18.5	N.A.	1.14	—	4.15 ± 0.23
6	N.A.	206.45 ± 12.3	N.A.	1.12	—	5.19 ± 0.94
7	N.A.	N.A.	N.A.	N.A.	—	n.a
8	1352 ± 36.6	281.45 ± 20.3	8.62 ± 0.24	1.39	—	5.32 ± 1.38
9	n.a	458.92 ± 34.1	n.a	1.81	—	5.01 ± 0.65
10	1714.6 ± 58.3	893.92 ± 30.3	8.77 ± 0.23	3.62	—	11.42 ± 1.24

[a]Except for the control sample (day 0) it was not possible to carry out TBARS analysis for docosahexaenoate in the absence of added oxidant, owing to small sample volume, high viscosity, and problems on predicting the oxidation level. N.A., not analyzed.

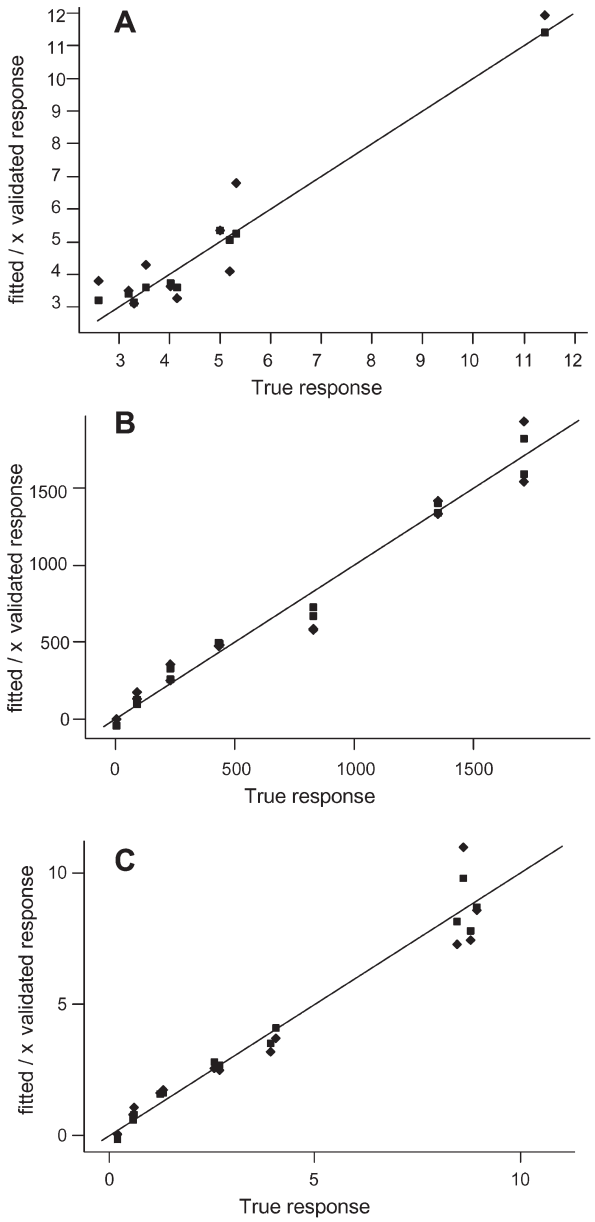


FIG. 4. Partial least squares predictions for the series of docosahexaenoate (A) with α-tocopherol for the TBARS variable, (B) without α-tocopherol for the PV variable, and (C) without α-tocopherol for the conjugated diene variable. NMR predictions used in A, B, and C are selected by using the chemical shifts identified by genetic algorithm analysis. The predicted values are shown by squares (■) and the cross-validated values are shown by diamonds (◆).

chemical shift values from the same hydrogens are detected. During oxidation, chemical shift values varied and it was necessary to do line fitting.

In this work the integration of the specific regions in order to calculate the ratio between olefinic and aliphatic hydrogen atoms and between aliphatic and diallylmethylene hydrogen atoms did not show any significant changes during the 10 d of oxidation. This is in contradiction to what has been reported in other research (5–9).

The new resonances developed in the downfield region of the spectra (9–10.5 ppm) are easy to relate to the lipid oxidation products since there should be no other components in this shift area for lipid extract. When comparing the spectra from vegetable oils oxidized at elevated temperatures (4,10) with the ethyl esters of DHA, we see that much of the same lipid oxidation products are formed in the DHA even if it is oxidized at room temperature (25°C) for a few days.

It is necessary to use special integration of certain areas of the spectra (XWINNMR and IGOR Pro) or multivariate data analysis of the spectra to be able to quantify the changes during the lipid oxidation period. The hydrogen signals from the FA are so much higher than signals that are due to lipid oxidation products that specific parts of the spectra need to be enlarged (10×) even to see the signals. Our results also demonstrate the self-consistency of the relationships among the samples and the corresponding degrees of oxidation. Hierarchical clustering analysis (peaks >0.2% of the peak maxima) separates the samples (without α-tocopherol) into major clusters based on their groups day 0, day 1–4 and day 8–10.

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