papers and notes on methodology

# Comparison of analytical methods for monitoring autoxidation profiles of authentic lipids

**Ryungsoon Song Kim**[1] **and Frank S. LaBella**

Department of Pharmacology and Therapeutics, University of Manitoba, Faculty of Medicine, 770 Bannatyne Avenue, Winnipeg, Manitoba, Canada R3E OW3

**Abstract** Oxidation profiles of polyunsaturated fatty acids, their esters, and fatty alcohols were compared by several commonly employed analytical procedures. The extent of lipid peroxidation varied with the structure of the specific lipid class. The rate of oxidation was greater for polyenoic lipids than for dienoic counterparts. In general, the maximum diene conjugation and thiobarbituric acid reactivity occurred earlier for acids than for esters or alcohols containing identical numbers of carbon chains and double bonds. For each homologous series there were, initially, increasing levels of conjugated dienes and thiobarbituric acid-reactive products, which then diminished in association with rising levels of carbonyl and fluorescent products. At late stages of oxidation, conjugated diene or thiobarbituric acid reactivity was not indicative of total peroxidation products. Thus, supplementary measurements are required to detect secondary degradation products of polyunsaturated fatty acids.—**Kim, R. S., and F. S. LaBella.** Comparison of analytical methods for monitoring autoxidation profiles of authentic lipids. *J. Lipid Res.* 1987. **28:** 1110–1117.

**Supplementary key words** polyunsaturated fatty acids • fatty esters • fatty alcohols • analytical indices of lipid peroxidation • diene conjugation • thiobarbituric acid assay

Lipid peroxidation (LPO) is a complex process whereby unsaturated fatty acids and lipids are oxidized via free-radical intermediates to a variety of products. The control of LPO is of special significance commercially to the oil and food industries. In biology, the peroxidation of polyunsaturated fatty acids (PUFA), especially arachidonic acid, is of importance pathologically because of membrane damage resulting from LPO.

It is now well known that hydroperoxides are the primary products of the LPO, but secondary degradation products are believed to be formed largely from hydroperoxide decomposition. The complex and dynamic nature of the secondary products make quantitative study of their occurrence in autoxidized lipids very difficult. Thus, even accurately determined analyses of oxidized fats can only reflect the extent of LPO at the particular time of sampling.

Detection methods for products of LPO have been based on loss of lipid substrates, measurement of conjugated dienes, and thiobarbituric acid (TBA)-reactive substances and color reactions specific for peroxide and carbonyl groups. For in vivo studies involving complex biological systems, additional methods, such as the estimation of exhaled alkanes and measurement of an increased chemiluminescence, have been developed (1). Fluorescence analysis of biological extracts was originally described by Fletcher, Dillard, and Tappel (2) and applied to clinical situations (3). This method is based on the detection of fluorescent Schiff bases formed between secondary carbonyls and available amino groups in biological material. Recently, however, Gutteridge, Kerry, and Armstrong (4) reported that autofluorescence can be observed in several autoxidized PUFA in the absence of amino groups.

In the course of studying the relationships between structures of various fatty acids, esters, and fatty alcohols, and their calcium-translocating activities in a two-phase model system (5), it was necessary for us to monitor the extent of peroxidation of these lipids at various stages. The present investigation is an attempt to compare systematically the most popular detection methods of LPO by correlating results from these methods as they reflect various products formed over the entire time course of oxidation.

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Abbreviations: LPO, lipid peroxidation; PUFA, polyunsaturated fatty acids; TLC, thin-layer chromatography; TBA, thiobarbituric acid; TBA-RS, thiobarbituric acid-reactive substances; MDA, malondialdehyde.

[1]To whom correspondence and reprint requests should be addressed.

## MATERIALS AND METHODS

### Materials

Unsaturated fatty acids and their methyl esters and alcohols (over 99% pure) were obtained from Nu Chek Prep., Elysian, MN. 2-Thiobarbituric acid (TBA) was from Eastman Organic Chemicals, Rochester, NY, and 1,1,3,3,-tetramethoxy propane (malonaldehyde bisdimethyl acetal) was from Aldrich Chemical Co., Milwaukee, WI. 2,4-Dinitrophenylhydrazine was obtained from BDH Chemicals, Poole, England, and quinine sulfate was from Nutritional Biochemicals Co., Cleveland, OH. All chemicals used were of reagent grade.

### Autoxidation of lipids

Autoxidation of fatty acids, methyl esters, and alcohols was carried out by applying 5 mg of lipid to the inside wall of a glass tube and allowing the film to undergo autoxidation in air at room temperature for up to 3 weeks. Lipids autoxidized for various lengths of time were kept at −80°C until analysis, after replacing air with $N_2$.

### Analysis of peroxidation products

*Measurement of TBA reactivity.* Duplicate aliquots containing nonoxidized (taken immediately after opening the ampule) or autoxidized lipids (0.1 mg/0.1 ml ethanol), 0.5 ml of distilled water, 2 ml of potassium hydrogen phthalate–HCl buffer (50 mM, pH 3.5), and 1 ml of TBA (1% in 0.05 M NaOH) solution were heated for 15 min at 100°C using a heating block. Each tube contained a boiling glass bead and was topped with a glass ball to prevent loss of reactants by evaporation. The tubes were centrifuged at 5°C and the absorbance was measured at 532 nm using a Bausch and Lomb Spectronic 21 colorimeter. An extinction coefficient of $1.56 \times 10^5 M^{-1}cm^{-1}$ (6) was used for calculating the amount of malondialdehyde (MDA), expressed as the percentage of the original, nonoxidized lipid.

*Analysis by thin-layer chromatography.* The lipids (250 μg of each) were applied on high-performance silica gel thin-layer plates (Whatman LHP-KF) for analysis of the relative amount of oxygenated products. The plates were developed in n-hexane–diethyl ether–acetic acid 100:100:1 (v/v) and detected by spraying with 4% $H_2SO_4$ in ethanol followed by heating. The relative amount of each separated product was estimated as reported before by a densitometer and a computerized digitizer (5). Peroxides were located by appearance of red color when reacted with ferrous thiocyanate (0.2 g of $NH_4SCN$ in 15 ml of acetone plus 10 ml of 4% aqueous ferrous sulfate, freshly prepared) (7) and carbonyl compounds were detected by orange spots on a yellow background when sprayed with 0.4% 2,4-dinitrophenylhydrazine in 2 M HCl (8).

*Measurement of diene conjugation.* For estimation of conjugated dienes, lipids were dissolved in ethanol (0.04 mg/ml) and the spectra of ultraviolet absorption scans between 220 and 300 nm were measured with ethanol as a blank, using the Beckman DU-8 spectrophotometer. The difference between the maximum absorbance at 233 (232–236) nm of the peroxidized lipid and that of a corresponding nonperoxidized lipid was taken as a measure of diene conjugation. A molar extinction coefficient of 27,000 $M^{-1} \cdot cm^{-1}$ (9) was used for calculation of the conjugated dienes and the amount was expressed as the percentage of the original nonperoxidized lipids. Likewise, the absorption at higher wavelengths (260–280 nm) was also determined as a measure of oxodiene (276 nm, $\epsilon m = 22{,}000$) (10) for compounds of $C_{18:2}$ series or of conjugated trienes (268 nm, $\epsilon m = 43{,}400$ and 278 nm, $\epsilon m = 33{,}500$) (11) for compounds of the $C_{18:3}$ and $C_{20:4}$ series.

*Measurement of fluorescence.* The lipids were dissolved in ethanol (1 mg/ml) and the relative fluorescence intensity was measured with a Perkin-Elmer LS-5 Fluorescence Spectrometer at an excitation wavelength of 360 nm and an emission wavelength of 430 nm with reference to quinine sulfate (1 μg/ml in 0.1 M $H_2SO_4$) set at 1000 units.

*Estimation of carbonyl compounds.* The estimation of the carbonyl compounds was measured according to the method proposed by Henick, Benca, and Mitchell (12), slightly modified. In a stoppered test tube, 0.3 ml of trichloroacetic acid solution (4.3% in benzene), 0.5 ml of 2,4-dinitrophenylhydrazine solution (0.05% in benzene, freshly recrystallized), and autoxidized lipids (0.038 mg in 0.5 ml of benzene) were mixed and heated in a water bath at 60°C for 30 min. After cooling to room temperature, 1 ml of KOH solution (4% in absolute ethanol, freshly prepared) was added and the volume was adjusted to 5 ml with absolute ethanol with mixing. After exactly 10 min, the absorbancy at 460 nm was read against a blank prepared in an identical manner but containing benzene (0.5 ml) instead of the sample.

Total carbonyl compounds were estimated only in the ester series according to the method of Henick et al. (12) after determination of mole fractions of both saturated and unsaturated carbonyls by measuring the absorbance at 460 and 430 nm. The amount of total carbonyl compounds was expressed as the percentage of the original lipids.

## RESULTS

Linoleic (18:2), linolenic (18:3) and arachidonic (20:4) acids and the corresponding methyl esters and alcohols were examined by several methods to detect oxidation products. Thin-layer chromatographic profiles of lipids exposed to room air in the dark up to 3 weeks are shown

in **Fig. 1**. The disappearance of the parent fatty acid derivatives and corresponding appearance of oxidized products were noted by densitometric analysis. The rate of oxidation of lipids is known to parallel the degree of unsaturation. The primary oxidation products (hydroperoxides) of linoleic acid were detected after 2 days of oxidation. The highly polar ($R_f = 0$) polymeric products which contained carbonyl groups as detected by 2,4-dinitrophenylhydrazine (8) appeared only after 5 days. In contrast, both linolenic and arachidonic acids yielded primary and secondary oxidation products after 24 hr.

In **Table 1**, several analytical indices of LPO were compared to oxidation products separated chromatographically (see Fig. 1). In **Fig. 2,** the data for linolenic acid are shown as an example to demonstrate more clearly the overall relationships; similar patterns were observed with all other lipids tested. As shown in Fig. 2a, both diene conjugation and TBA reactivity were closely correlated with oxidation products containing mainly monohydroperoxy compounds (HP) as measured by densitometric analysis, with the peak of TBA value lagging slightly behind. The formation of highly polar products (PP) and concomitant loss of unoxidized parent compounds (UNOX) paralleled the increase in fluorescence which reflects the level of polymerized MDA and other secondary products (see Fig. 2b).

In general, the rate of oxidation was greater in acids than in esters or alcohols as estimated by TBA and diene conjugation analyses. For diene conjugation products (absorbance at 233 nm) of autoxidized $C_{18}$-lipids, the maximum peak appeared earlier for acids than esters or alcohols **(Fig. 3)**. Among homologous compounds with different degrees of unsaturation, the peak values in conjugated dienes appeared earlier in polyenoic lipids than dienoic counterparts (as an example, data for acids are shown in **Fig. 4)**. A similar pattern was observed for the time course of TBA-reactive products (See Fig. 2 and Table 1). The oxodiene compounds in $C_{18:2}$ series were

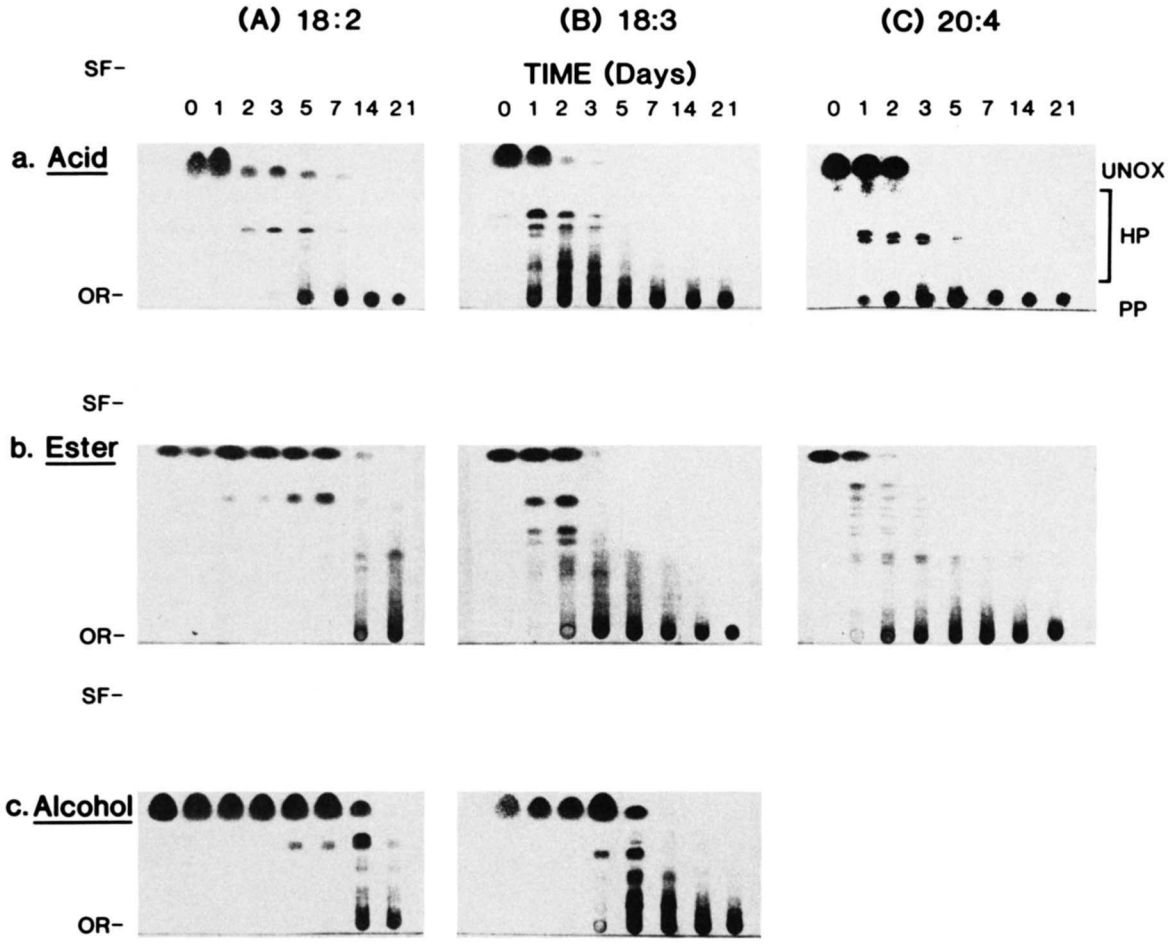


**Fig. 1.** Thin-layer chromatography of autoxidized polyunsaturated fatty acids. The autoxidized lipids were applied on high-performance silica gel TLC plates and developed as described in Materials and Methods. SF, solvent front; OR, origin; UNOX, unoxidized parent fatty acids; HP, oxidation products containing mainly monomeric hydroperoxy compounds; PP, highly polar products ($R_f = 0$); 18:2, linoleic acid; 18:3, linolenic acid; 20:4, arachidonic acid.

TABLE 1. Relative amounts of peroxidized lipids estimated by various analytical methods

| Time | TLC | | | Conjugated Dienes | TBA Assay |
|---|---|---|---|---|---|
| | UNOX | HP | PP | | |
| *days* | | | | | |
| $C_{18:2}$ (Acid) | | | | | |
| 0 | 100.0 | 0.0 | 0.0 | 0.0 | 0.54 ± 0.00 |
| 1 | 99.6 | 0.4 | 0.0 | 26.4 | 1.65 ± 0.15 |
| 2 | 60.9 | 23.6 | 15.5 | 44.6 | 3.84 ± 1.01 |
| 3 | 54.6 | 28.3 | 17.1 | 37.1 | 6.16 ± 1.19 |
| 5 | 16.6 | 30.4 | 53.0 | 25.0 | 5.07 ± 0.75 |
| 7 | 7.5 | 27.6 | 64.9 | 17.9 | 3.70 ± 0.11 |
| 14 | 0.0 | 21.1 | 78.9 | 14.3 | 2.14 ± 0.13 |
| 21 | 0.0 | 11.8 | 88.2 | 12.1 | 1.49 ± 0.05 |
| $C_{18:2}$ (Ester) | | | | | |
| 0 | 100.0 | 0.0 | 0.0 | 0.0 | 0.11 ± 0.00 |
| 1 | 86.6 | 13.4 | 0.0 | 7.0 | 0.51 ± 0.07 |
| 2 | 78.8 | 19.0 | 2.2 | 10.6 | 0.71 ± 0.23 |
| 3 | 73.9 | 22.7 | 3.4 | 12.7 | 1.21 ± 0.03 |
| 5 | 66.5 | 31.8 | 1.7 | 21.8 | 1.29 ± 0.02 |
| 7 | 49.5 | 49.3 | 1.2 | 28.4 | 2.10 ± 0.02 |
| 14 | 2.3 | 34.3 | 63.4 | 13.0 | 1.34 ± 0.07 |
| 21 | 0.0 | 22.9 | 77.1 | 6.9 | 1.00 ± 0.04 |
| $C_{18:2}$ (Alcohol) | | | | | |
| 0 | 100.0 | 0.0 | 0.0 | 0.6 | 0.29 ± 0.00 |
| 1 | 100.0 | 0.0 | 0.0 | 3.2 | 1.00 ± 0.14 |
| 2 | 98.0 | 2.0 | 0.0 | 5.5 | 3.33 ± 0.34 |
| 3 | 84.2 | 15.8 | 0.0 | 10.8 | 3.64 ± 0.53 |
| 5 | 77.0 | 23.0 | 0.0 | 26.5 | 5.54 ± 0.65 |
| 7 | 62.0 | 34.7 | 3.3 | 32.7 | 3.60 ± 0.71 |
| 14 | 8.7 | 12.2 | 79.1 | 10.6 | 2.16 ± 0.03 |
| 21 | 0.0 | 0.0 | 100.0 | 5.4 | 1.69 ± 0.28 |
| $C_{18:3}$ (Acid) | | | | | |
| 0 | 100.0 | 0.0 | 0.0 | 1.7 | 0.19 ± 0.01 |
| 1 | 37.7 | 34.9 | 27.4 | 28.9 | 2.39 ± 0.12 |
| 2 | 14.2 | 28.6 | 57.2 | 17.9 | 3.02 ± 0.46 |
| 3 | 7.4 | 23.8 | 68.8 | 11.4 | 1.87 ± 0.12 |
| 5 | 1.4 | 15.8 | 82.8 | 7.1 | 1.82 ± 0.08 |
| 7 | 0.0 | 6.4 | 93.6 | 5.4 | 1.16 ± 0.03 |
| 14 | 0.0 | 3.3 | 96.7 | 5.0 | 0.69 ± 0.03 |
| 21 | 0.0 | 0.0 | 100.0 | 4.6 | 0.50 ± 0.02 |
| $C_{18:3}$ (Ester) | | | | | |
| 0 | 100.0 | 0.0 | 0.0 | 0.0 | 0.00 ± 0.00 |
| 1 | 71.8 | 26.6 | 1.6 | 30.4 | 0.42 ± 0.12 |
| 2 | 53.3 | 39.7 | 7.0 | 28.1 | 3.12 ± 0.63 |
| 3 | 26.2 | 30.0 | 43.8 | 12.1 | 3.85 ± 1.06 |
| 5 | 2.5 | 5.0 | 92.5 | 6.1 | 3.03 ± 0.63 |
| 7 | 0.0 | 0.0 | 100.0 | 5.1 | 2.27 ± 0.49 |
| 14 | 0.0 | 0.0 | 100.0 | 3.9 | 1.33 ± 0.24 |
| 21 | 0.0 | 0.0 | 100.0 | 2.6 | 0.93 ± 0.11 |
| $C_{18:3}$ (Alcohol) | | | | | |
| 0 | 100.0 | 0.0 | 0.0 | 1.4 | 0.00 ± 0.00 |
| 1 | 94.1 | 3.6 | 2.3 | 7.1 | 0.55 ± 0.14 |
| 2 | 78.7 | 12.8 | 8.5 | 21.1 | 1.87 ± 0.57 |
| 3 | 38.8 | 22.8 | 38.4 | 14.1 | 3.09 ± 0.65 |
| 5 | 7.0 | 25.5 | 67.5 | 5.9 | 1.54 ± 0.27 |
| 7 | 3.8 | 8.6 | 8.6 | 5.9 | 1.27 ± 0.20 |
| 14 | 0.0 | 1.4 | 98.6 | 5.9 | 0.76 ± 0.16 |
| 21 | 0.0 | 0.0 | 100.0 | 3.5 | 0.48 ± 0.10 |

estimated by a lesser secondary absorption maximum at 276 nm; although not always discernible, levels of these compounds indicated that the overall pattern of formation with time was very similar to that of conjugated dienes (233 nm). The maximum formation of oxodiene compounds was much less than that of the primary monohydroperoxides and amounted to 14, 16, and 2.5% of the original lipids for linoleic acid, its methyl ester, and alco-

TABLE 1. *(Continued)*

| Time | TLC UNOX | TLC HP | TLC PP | Conjugated Dienes | TBA Assay |
|---|---|---|---|---|---|
| *days* | | | | | |
| $C_{20:4}$ (Acid) | | | | | |
| 0 | 100.0 | 0.0 | 0.0 | 1.9 | 0.32 ± 0.07 |
| 1 | 66.1 | 15.8 | 18.1 | 25.0 | 4.00 ± 0.32 |
| 2 | 47.1 | 4.8 | 48.1 | 14.3 | 3.23 ± 0.04 |
| 3 | 2.0 | 4.0 | 94.0 | 13.6 | 3.00 ± 0.06 |
| 5 | 0.0 | 0.9 | 99.1 | 13.0 | 2.39 ± 0.01 |
| 7 | 0.0 | 0.0 | 100.0 | 12.5 | 1.87 ± 0.01 |
| 14 | 0.0 | 0.0 | 100.0 | 11.4 | 1.27 ± 0.00 |
| 21 | 0.0 | 0.0 | 100.0 | 11.4 | 0.93 ± 0.02 |
| $C_{20:4}$ (Ester) | | | | | |
| 0 | 100.0 | 0.0 | 0.0 | 0.0 | 0.43 ± 0.01 |
| 1 | 66.7 | 30.0 | 3.3 | 28.7 | 3.24 ± 0.70 |
| 2 | 18.3 | 34.4 | 47.3 | 39.2 | 4.02 ± 0.56 |
| 3 | 4.5 | 30.1 | 65.4 | 15.0 | 1.80 ± 0.11 |
| 5 | 0.0 | 0.0 | 100.0 | 8.9 | 4.35 ± 0.44 |
| 7 | 0.0 | 0.0 | 100.0 | 8.4 | 3.12 ± 0.11 |
| 14 | 0.0 | 0.0 | 100.0 | 8.4 | 2.00 ± 0.09 |
| 21 | 0.0 | 0.0 | 100.0 | 8.4 | 1.42 ± 0.06 |

Densitometric values from TLC plates (UNOX, HP, PP) are expressed as relative percentages. Conjugated dienes and TBA-reactive substances (see Methods) are expressed as the percentage of original unoxidized lipid. Single UV or densitometry determinations were made at various time periods. TBA values are means ( ± SEM) from three separate experiments in which duplicate measurements were made.

hol, respectively. Similarly, the conjugated triene (dihydroperoxides) content as measured by lesser defined absorption at 268 and 278 nm, appeared to coincide with that of conjugated dienes. The maximum levels of conjugated trienes were much lower than those of the primary monohydroperoxides, amounting to 6, 11, and 3.5% for acid, ester, and alcohol of $C_{18:3}$ series and 3 and 7% for $C_{20:4}$ acid and ester, respectively.

The relative fluorescence intensity of PUFA increased with degree of unsaturation (**Fig. 5**), with the lowest value for linoleic acid as seen in TBA reactivity. The production of carbonyls, as an estimate of a class of secondary oxidation products, also increased with degree of unsaturation. The methyl esters of arachidonic and linolenic acids formed carbonyls more rapidly and to a greater extent than did linoleic acid ester **(Fig. 6).**

## DISCUSSION

The formation of hydroperoxides of autoxidized lipids estimated by densitometric analysis, although less accurate, compared well with the level of conjugated dienes determined spectrophotometrically. However, the relative proportions of the unchanged lipids, hydroperoxides and polymerized polar substances do not reflect the true levels present at any time because of limited detectability of this method and partly due to the loss of volatile carbonyl compounds with advanced autoxidation. Thus, after 3 weeks of oxidation, the total products remaining corresponded to approximately two-thirds of the starting material. The rate of autoxidation depended on the type and degree of unsaturation of parent lipids, probably due to the difference in relative rates of propagation and termination of the respective peroxy radicals. Comparison of authentic PUFA, esters, and alcohols containing identical numbers of carbon chains and double bonds demonstrated considerable differences in overall oxidation profiles. Esters and alcohols appear to have a slower rate of formation than corresponding acids of both primary and secondary oxidation products. The lipid composition of a given type of biomembrane, therefore, will influence its susceptibility to peroxidation.

The TBA method detects a variety of peroxides and secondary oxidation products which decompose under specific conditions of acid-heating to release MDA. The peak production of TBA-reactive substance from autoxidized lipids paralleled that of hydroperoxides, and the level gradually decreased to control (nonoxidized) values. Thus, hydroperoxides, which are stable in pure form, appear to be the major precursors of MDA found in autoxidized PUFA as has been recognized for a long time. However, only a small proportion of the original lipid loss could be accounted for as MDA estimated by TBA assay, even for the compounds with three or more double bonds; the latter represent the major contributors of TBA-reactivity via endoperoxide intermediates. The levels of maximum MDA for each lipid ranged from 7 to 18% of

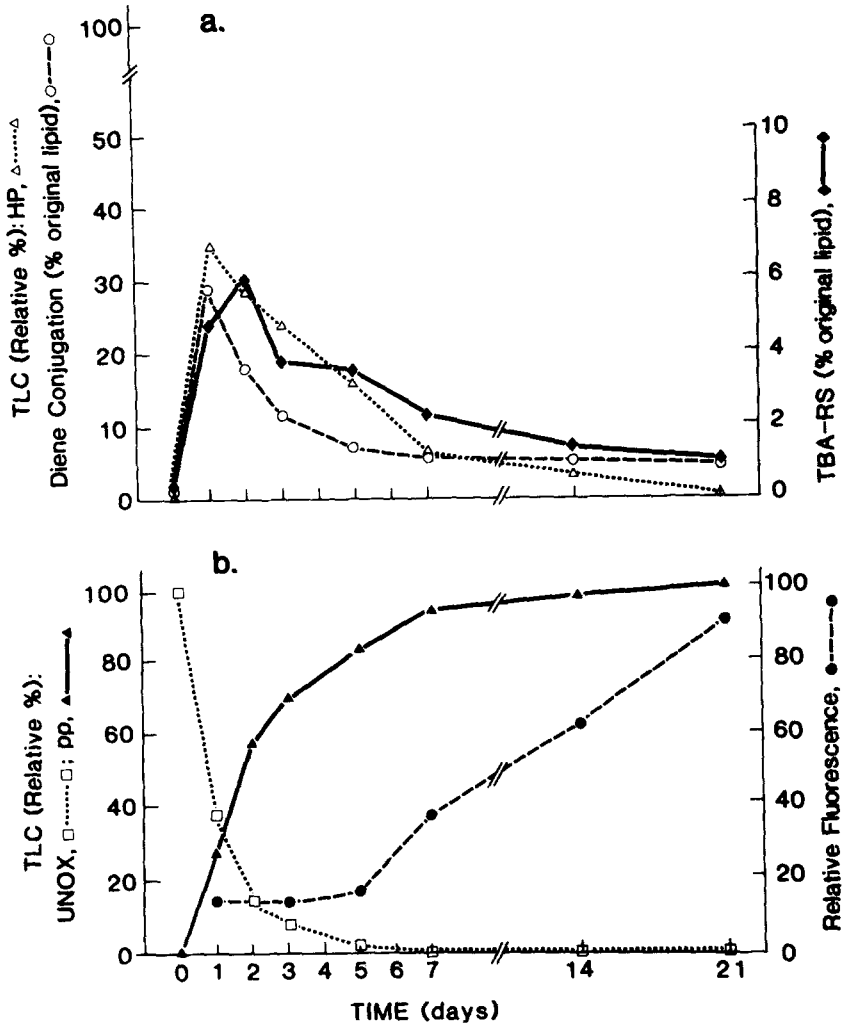


**Fig. 2.** Comparison of various LPO indices of autoxidized linolenic acid. a, See legend to Table 1. b, The relative fluorescence (1 mg lipid/ml ethanol) was measured at an excitation wavelength of 360 nm and an emission wavelength of 430 nm with reference to quinine sulfate (1 μg/ml 0.1 M $H_2SO_4$) which was set at 1000.

corresponding hydroperoxides, based on the estimation of conjugated dienes. Similarly, only 5% of hydroperoxides, estimated by iodometric titration, was detected as MDA in the TBA assay (13). Thus, TBA assay does not give quantitative information but serves as a relative index of lipid peroxidation. The disappearance of the TBA-MDA complex is accompanied by the formation of highly polar products, probably resulting from polymerization of the secondary oxidation products. When the TBA assay was carried out at pH 3.5, pure linoleate hydroperoxides were reactive as reported by Ohkawa, Ohishi, and Yagi (14), and Asakawa and Matsushita (13).

Many peroxidized samples contain complex mixtures of aldehydes other than MDA. The colorimetric determination of carbonyl compounds as an indicator of rancidity (15) has been utilized mainly in the food industry. The procedure is based on the reaction of 2,4-dinitrophenylhydrazine reagent with carbonyls to form 2,4-dinitrophenylhydrazones. This method measures, mainly, the

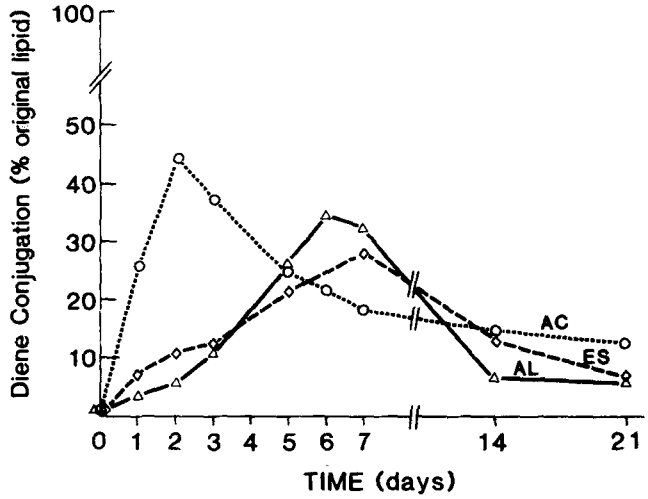


**Fig. 3.** Comparison of levels of conjugated dienes in autoxidized $C_{18}$ acid, ester, and alcohol. At various stages of oxidation, PUFA was dissolved in ethanol (40 μg/ml) and the absorbance at 233 nm was monitored. Ac, acid; ES, ester; AL, alcohol.

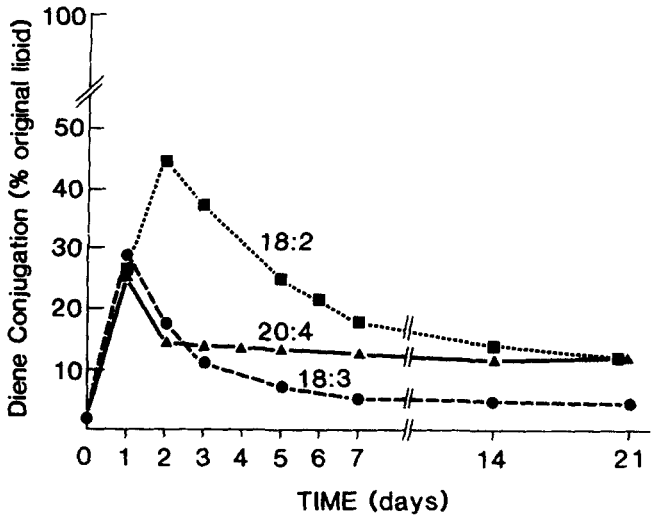


**Fig. 4.** Comparison of levels of conjugated dienes in autoxidized polyunsaturated fatty acids. See legends to Fig. 1 and 3.

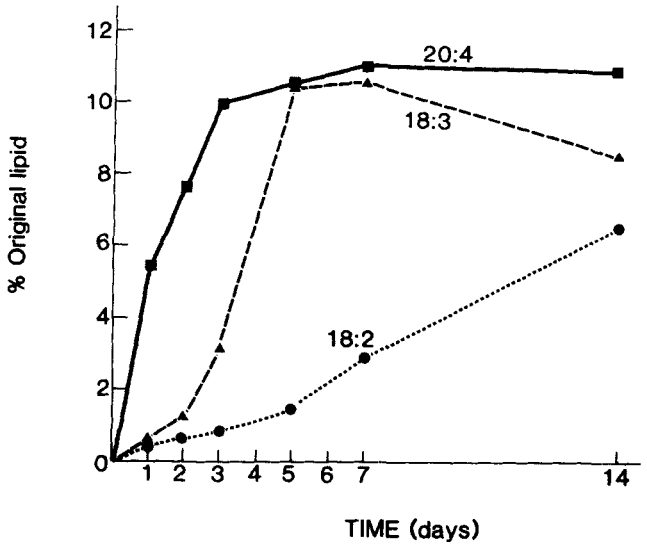


**Fig. 6.** Comparison of levels of carbonyl compounds in autoxidized PUFA methyl esters. The carbonyl compounds in the autoxidized lipids were reacted with 2,4-dinitrophenylhydrazine reagent to form dinitrophenylhydrazones, and the absorbancy of the developed color, after addition of KOH, was measured at 460 nm. Shown are the means of two separate samples whose individual values did not differ by more than 5%.

nonvolatile carbonylic substances of high molecular weight, for free MDA does not form dinitrophenylhydrazone derivatives (16). The most reliable and widely used of the analytical methods is that of Henick et al. (12) which differentiates between saturated and unsaturated carbonyl compounds by measuring absorbance at two wavelengths.

Fluorescence is a sensitive measure of lipid autoxidation in which secondary carbonyls, such as MDA, form complexes with amino groups to form Schiff bases (17). However, autofluorescence occurs during the autoxidation of PUFA in the absence of amino groups (4, 18). Both ultraviolet (excitation 340 nm; emission 390 nm) and visibly (excitation 360 nm; emission 430 nm) fluorescing materials have been recovered from oxidized PUFA and ascribed to the presence of endoperoxides and carbonyl polymers (4).

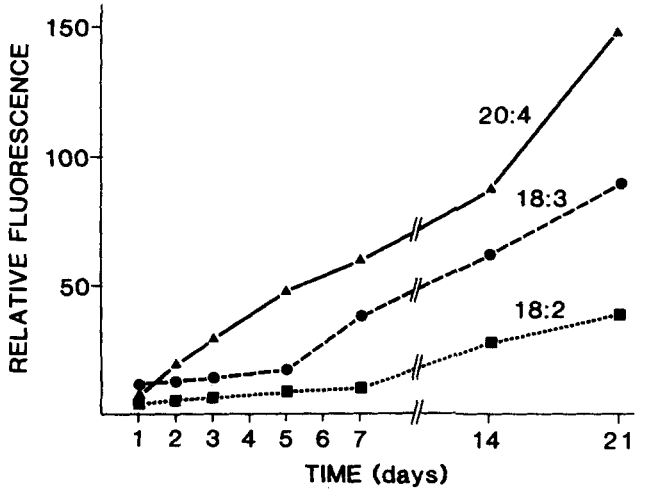


**Fig. 5.** Comparison of relative fluorescence of autoxidized PUFA. See legends to Figs. 2.

We found that fluorescence intensity and total carbonyls in autoxidized lipids increase in parallel and are accompanied by the formation of highly polar substances, the loss of parent PUFA, and a decrease in diene conjugation and TBA reactivity. Since diene conjugation and most of the TBA reactivity appears to derive from lipid peroxides, these two parameters of LPO should vary inversely with the levels of secondary carbonyls and autofluorescence as was demonstrated in this study. It has been reported that, during the process of polymerization, MDA loses substantial amounts of TBA-reactivity (19). In addition, many carbonyls are oxidized during the heating steps and lose TBA-reactivity. This phenomenon is reflected in the present study where low levels of TBA reactivity are associated with high levels of fluorescence and carbonyls. There was a slight lag between the time courses for generation of carbonyls and of fluorescence from linolenic and arachidonic acids (fluorescence) and their methyl esters (carbonyls), whose overall oxidation profiles are similar (Fig. 1). This is most likely due to the slower rate of polymerization of carbonyls to fluorescent products. Considerably lower values for carbonyl, fluorescence, and TBA-reactivity were obtained from autoxidized linoleate.

Of the many methods proposed for the detection of lipid peroxides in biological and food systems, determination of conjugated dienes or TBA-reactivity has been most widely employed. Since levels of both conjugated dienes

and TBA-reactivity decreased with progress of autoxidation, estimation of LPO in pure lipids by either method alone would not appear to be a reliable index of peroxidation. For example, determination of diene conjugation or TBA-reactivity failed to detect any oxidation products of PUFA with three or more double bonds, when measured later than 1 week after the initiation of autoxidation. Therefore, as with many biological processes, parallel and sequential determinations by a variety of methods will ensure overall evaluation of both primary and secondary products of LPO.

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## REFERENCES

1. Slater, T. F. 1984. Overview of methods used for detecting lipid peroxidation. *Methods Enzymol.* **105:** 283–293.
2. Fletcher, B. L., C. J. Dillard, and A. C. Tappel. 1973. Measurement of fluorescent lipid peroxidation products in biological systems and tissues. *Anal. Biochem.* **52:** 1–9.
3. Wickens, D., M. H. Wilkins, J. Lunec, G. Ball, and T. L. Dormandy. 1981. Free-radical oxidation (peroxidation) products in plasma in normal and abnormal pregnancy. *Ann. Clin. Biochem.* **18:** 158–162.
4. Gutteridge, J. M. C., P. J. Kerry, and D. Armstrong. 1982. Autoxidized and lipoxidase-treated polyunsaturated fatty acids autofluorescence associated with the decomposition of lipid peroxides. *Biochim. Biophys. Acta.* **711:** 460–465.
5. Kim, R. S., and F. S. LaBella. 1985. Calcium translocation by fatty acid derivatives in a two-phase partition model. Structure-activity relationships. *Biochim. Biophys. Acta.* **833:** 386–395.
6. Willis, E. D. 1969. Lipid peroxide formation in microsomes. General considerations. *Biochem. J.* **113:** 315–324.
7. Stahl, E. 1956. Dunnschift-chromatographie (Methode, Einfluss-faktoren und einige Anwendungsbeispiele). *Pharmazie (Beih.)* **11:** 633.
8. Reitsema, R. H. 1954. Characterization of essential oils by chromatography. *Anal. Chem.* **26:** 960–963.
9. Hamberg, M., and B. Samuelsson. 1967. Oxygenation of unsaturated fatty acids by the vesicular gland of sheep. *J. Biol. Chem.* **242:** 5344–5354.
10. Vioque, E., and R. T. Holman. 1962. Characterization of the ketones formed in the oxidation of linoleate by lipoxidase. *Arch. Biochem. Biophys.* **99:** 522.
11. Coxon, D. T., K. E. Peers, and N. M. Rigby. 1984. Selective formation of dihydroperoxides in the alpha-tocopherol inhibited autoxidation of methyl linoleate. *J. Chem. Soc. Chem. Commun.* 67–68.
12. Henick, A. S., M. F. Benca, and J. H. Mitchell, Jr. 1954. Estimating carbonyl compounds in rancid fats and foods. *J. Am. Oil Chem. Soc.* **31:** 88–91.
13. Asakawa, T., and S. Matsushita. 1980. Coloring conditions of thiobarbituric acid test for detecting lipid hydroperoxides. *Lipids.* **15:** 137–140.
14. Ohkawa, H., N. Ohishi, and K. Yagi. 1978. Reaction of linoleic acid hydroperoxide with thiobarbituric acid. *J. Lipid Res.* **19:** 1053–1057.
15. Melton, S. L. 1983. Methodology for following lipid oxidation in muscle foods. *Food Technol.* **37:** 105–111.
16. Kwon, T. W., and B. M. Watts. 1964. Malonaldehyde in aqueous solution and its role as a measure of lipid oxidation in foods. *J. Food Sci.* **29:** 294–302.
17. Chio, K. S., and A. L. Tappel. 1969. Synthesis and characterization of the fluorescent products derived from malonaldehyde and amino acids. *Biochemistry.* **8:** 2821–2827.
18. Gutteridge, J. M. C., J. Lunec, and A. D. Heys. 1978. The detection of native fluorescence in peroxidized fatty-acids. *Anal. Lett.* **7:** 537–544.
19. Gutteridge, J. M. C. 1975. The use of standards for malondialdehyde. *Anal. Biochem.* **69:** 518–526.