Analysis of Autoxidized Fats by Gas Chromatography-Mass Spectrometry: III. Methyl Linolenate[1]

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ABSTRACT

The gas chromatography-mass spectrometry (GC-MS) method developed in the preceding papers was extended to the analysis of autoxidation products of methyl linolenate. Four isomeric hydroxy allylic trienes with a conjugated diene system were identified after reduction of the linolenate hydroperoxides. All eight geometric *trans,cis-* and *trans, trans-*conjugated diene isomers of these hydroxy allylic compounds were identified and partially separated by GC of the trimethylsilyl (TMS) ether derivatives. The proportion found of 9- and 16-hydroperoxides was significantly higher (75-81%) than the 12- and 13-hydroperoxides (18-25%). The tendency of the 12- and 13-hydroperoxides to form cyclic peroxides, cyclic peroxide-hydroperoxides, and prostaglandin-like endoperoxides was supported by indirect evidence for the presence of 9,10,12- and 13,15,16-trihydroxyoctadecanoate in hydrogenated derivatives of the highly oxygenated products. The quantitative GC-MS method was used to determine the relative contribution of linolenate, linoleate, and oleate in mixtures to the formation of hydroperoxides.

INTRODUCTION

In the first two papers in this series (1,2), structural investigations were made of autoxidation products of methyl oleate and linoleate by gas chromatography-mass spectrometry (GC-MS). We showed that this analytical tool is powerful for qualitative studies, but quantitative studies require standardization with authentic synthetic compounds. Quantitative GC-MS was also used as a method for determining isomeric hydroperoxide composition of autoxidized mixtures of methyl oleate and linoleate (2). This paper reports an extension of these studies to the autoxidation products of methyl linolenate and to the analysis of its autoxidized mixtures with methyl linoleate and oleate.

EXPERIMENTAL PROCEDURES

Methyl linolenate was prepared from methyl esters of linseed oil and purified by counter-double current distribution (3). It analyzed 99.9% linolenate by GC.

Procedures for autoxidation, analyses, KI and $NaBH_4$ reduction, catalytic hydrogenation, silylation, and GC-MS were the same as before (1). Catalytic hydrogenation of autoxidized linolenate with PtO_2 in 95% EtOH caused no apparent hydrogenolysis of oxidation products because GC analysis showed no increase in stearate. Authentic 9-, 13-, and 16-hydroxyoctadecanoate were derived by $NaBH_4$ reduction of the corresponding keto esters synthesized by literature methods (4). Methyl 12-hydroxyoctadecanoate was derived from ricinoleate by catalytic hydrogenation and purified chromatographically (5). Methyl 9,10,12-trihydroxyoctadecanoate was prepared by hydroxylation of either ricinoleic acid with alkaline $KMnO_4$ to obtain the erythro acids, or methyl ricinoleate with formic acid-H_2O_2 to obtain a mixture of diastereoisomers (6). For these polyhydroxy compounds to achieve com-

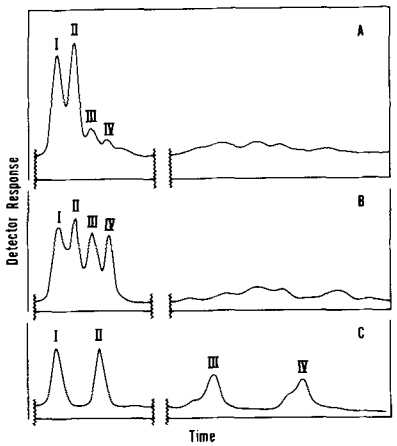


FIG. 1. Gas chromatogram of silyl ethers from hydroxy derivatives of methyl linolenate autoxidized at room temperature to a peroxide value of 1315. A. $NaBH_4$ reduced. B. KI reduced. C. Catalytically hydrogenated.

[1]Presented at the AOCS Meeting, New York, May 1977.

TABLE I

Mass Spectral Data

Peak	Characteristic fragments m/e (relative abundance)	Identification[a,b] (C-18 OTMS methyl esters)
Figure 1A	$NaBH_4$ reduced-oxidized linolenate	
I	183 (79.7), 365 (2.0) M-15, 380 (1.3) M	12-OH-triene (B)[c]
	223 (23.4)	9-OH-triene (A)
	311 (56.3)	13-OH-triene (C)
II	183 (78.7),[d] 365 (2.0) M-15, 380 (1.7) M	16-OH-triene (D)
	351 (15.9)	
Figure 1B	KI reduced-oxidized linolenate	
I	183 (67.6), 365 (1.8) M-15, 380 (1.5) M	12-OH-triene (B)[c]
	223 (30.1)	9-OH-triene (A)
	311 (55.8)	13-OH-triene (C)
II	183 (95.4), 365 (1.5) M-15, 380 (1.5) M	12-OH-triene (B)[e]
	351 (13.8)	16-OH-triene (D)
III	223 (45.3), 365 (1.7) M-15, 380 (2.5) M	9-OH-triene (A)
	311 (56.7)	13-OH-triene (C)
IV	183 (75.3),[c] 365 (1.6) M-15, 380 (1.5) M	16-OH-triene (D)
	351 (18.4)	
Figure 1C	Hydrogenated-oxidized linolenate	
I	229 (93.9), 259 (100), 371 (4.3) M-15	9-OH-ane
	187 (26.5), 301 (22.7)	12-OH-ane
	173 (12.6), 315 (8.3)	13-OH-ane
II	131 (100), 357 (27.4), 371 (1.7) M-15	16-OH-ane
III	187 (100), 259 (37.2), 503 (1.0) M-59	9,10,12-triOH-ane
IV	131 (36.4), 315 (100), 503 (1.0) M-59	13,15,16-triOH-ane

[a]Based on comparison with reference compounds: methyl 9-, 12-, 13-, 16-hydroxyoctadecanoate, methyl 9,10,12-trihydroxyoctadecanoate, and by analogy with reported fragmentation schemes (9,10).

[b]See structures and fragmentation given in text.

[c]Due to *cis,trans*-12-OH-triene.

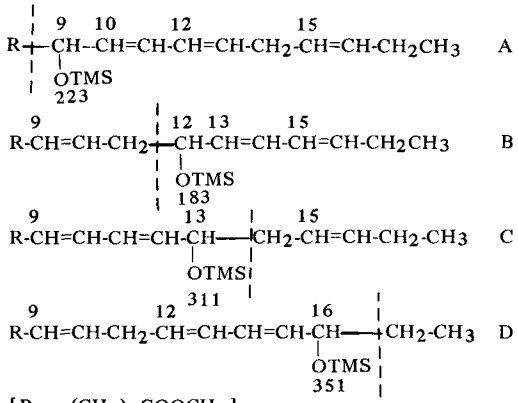
[d]Rearrangement fragment from D.

[e]Due to both *trans,trans*-12-OH-triene and to rearrangement from D.

plete silylation, it was necessary to use pyridine, hexamethyl disilazane, and trimethylchlorosilane (7).

RESULTS

Four isomeric allylic hydroxy trienes with a conjugated *trans,cis/cis,trans* diene system are formed by reducing the corresponding linolenate hydroperoxides (8). The structure of the trimethylsilyl ether (OTMS) derivatives and the expected MS fragmentation are as follows:



When methyl linolenate was autoxidized at room temperature to minimize the formation of *trans,trans*-diene isomers, two prominent peaks I and II were obtained on GC after $NaBH_4$ reduction and silylation (Fig. 1A). MS showed that peak I is due to a mixture of 9-, 12-, and 13-OTMS esters (A, B, and C) by the respective masses 223, 183, and 311; and that peak II is due to the 16-OTMS ester (D) (Table 1). The expected fragment mass for this isomer D, m/e 351, is accompanied by m/e 183, due to the 12-OTMS isomer B. Apparently, on mass spectrometry, there is a rearrangement between D and B, and the fragment of m/e 183 is favored because it has only one oxygenated function or because it tends not to form the high energy ethyl radical. Any corresponding rearrangement expected between the 9- and 13-OTMS isomers (A⇔C) could not be detected because these two isomers are not separated and emerge together within peak I. Identifications of the four hydroxy isomers is confirmed below by analyses of the saturated derivatives.

When methyl linolenate was autoxidized at 60 C, peaks III and IV increased in relative size. These peaks became especially prominent when

KI was used instead of $NaBH_4$ as the reducing agent (Fig. 1B). In our previous paper (2), we observed that the 9- and 13-dienols from autoxidized linoleate are separated by GC according to the conjugated diene configuration, the *trans, trans*-isomers having a longer retention than the *cis,trans*-isomers. Also, it was shown that KI reduction results in an increase in the relative proportion of the *trans,trans*-dienol isomers. Infrared analyses showed that the ratio of the bands at 983 *(trans,trans* plus *cis,trans)* and 945 cm^{-1} *(cis,trans)* was greater in the KI-reduced sample (12.0) compared to the $NaBH_4$-reduced sample (6.5). On the basis of these observations and of the MS data (Table I), the following structural assignments can be made for the gas chromatogram in Figure 1B. Peak I: mixture of *trans,cis*-9-OTMS (m/e 223), -12-OTMS (m/e 183) and -13-OTMS (m/e 311) esters; peak II: mixture of *trans,trans*-12-OTMS (m/e 183) and *trans,cis*-16-OTMS (m/e 351 and m/e 183 by rearrangement); peak III: mixture of *trans, trans*-9-OTMS (m/e 223) and -13-OTMS (m/e 311); and peak IV: *trans,trans*-16-OTMS (m/e 351 and m/e 183 by rearrangement).

The more polar peaks of longer retention than peak IV (Fig. 1A and 1B) are more difficult to identify without proper reference compounds. However, significant mass fragment of m/e 131 and 259 suggests the presence of polyhydroxy esters with OTMS substituents on C-16 and C-9, respectively. Less abundant fragments at m/e 183 and 311 also indicate the presence of polyhydroxy esters with OTMS substitutents on C-12 and C-13, respectively.

After double bond hydrogenation of the autoxidized linolenate, the gas chromatogram showed four well-separated peaks (Fig. 1C). MS identifications (Table I) were confirmed by comparison of chromatographic (GC and TLC) and MS data with those of authentic compounds and by mass chromatography (1,2). The 9-, 12-, and 13-OTMS saturated esters are partially separated in peak I, and the 16-OTMS ester is completely separated in peak II. There was no evidence of any other mono OH ester that might be derived from hydroperoxides on C positions 8, 10, 15, and 17. The tri-OTMS derivatives with end substituents on C-9 (m/e 259) and C-12 (m/e 187) emerge in peak III, and with end substituents on C-13 (m/e 315) and C-16 (m/e 131) emerge in peak IV. These assignments were confirmed by mass chromatography showing a scan of the appropriate masses (Fig. 2). The identity of peak III was further confirmed by peak enhancement resulting when *erythro*-9,10,12-trihydroxyoctadecanoates were added to the sample. The small

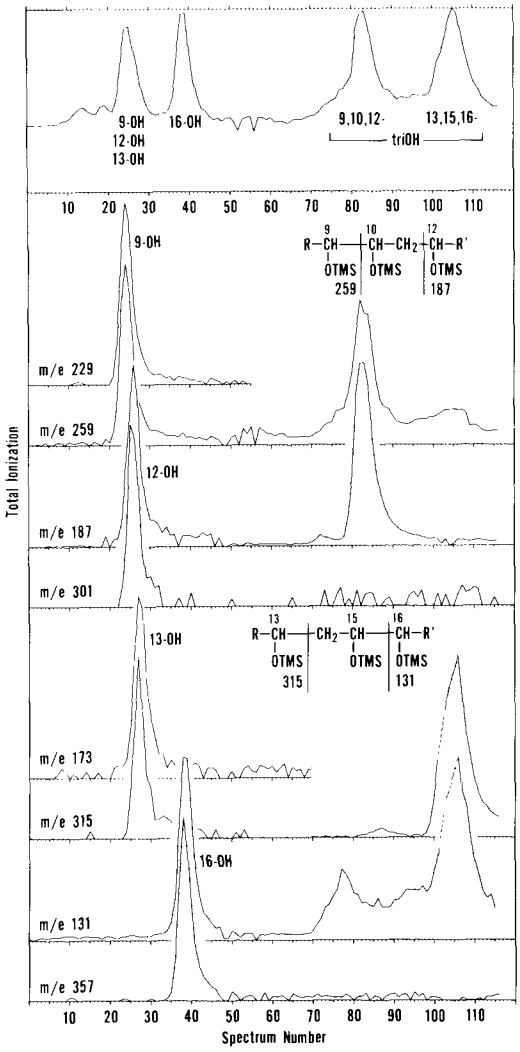


FIG. 2. Mass chromatography of hydrogenated-autoxidized methyl linolenate (peroxide value 1315).

shoulder of lower retention was also enhanced when the mixture of diastereoisomeric 9,10,12-trihydroxy esters was added to the sample. The MS of fractions from peak III obtained with those mixtures were identical to those of the original hydrogenated oxidized sample. These results are consistent with the presence of 9,10,12-triOTMS esters (peak III) and 13,15,16-triOTMS esters (peak IV).

Quantitative GC-MS analysis was carried out by the same computer summation method standardized in the preceding papers for autoxidized oleate and linoleate (1,2). The results obtained with artificial mixtures of synthetic 9-, 12-, 13-, and 16-hydroxyoctadecanoate were

TABLE II

Analysis of Synthetic Hydroxyoctadecanoates by GC-MS and by GC

Known mixtures	GC-MS (rel. %)[a]				GC (rel. %)[b]	
	9-OH	12-OH	13-OH	16-OH	9- + 12- + 13-OH	16-OH
Mixture 1	24.8	24.8	26.3	24.1	75.9	24.1
Found	23.0	25.1	26.9	25.0	76.2	23.8
Mixture 2	29.2	10.6	10.7	49.5	50.5	49.5
Found	27.6	11.6	13.0	47.8	50.5	49.5
Mixture 3	15.9	35.8	31.7	16.6	83.4	16.6
Found	14.7	36.5	32.6	16.2	83.4	16.6
Mixture 4	31.4	21.8	18.4	28.4	71.6	28.4
Found	28.6	24.0	18.9	28.5	71.5	28.5
Mixture 5	20.3	28.7	28.8	22.2	77.8	22.2
Found	18.4	29.6	29.8	22.2	78.7	21.3
Mean rel. % for 5 mixtures	24.3	24.3	23.2	28.2	71.8	28.2
Found	22.4	25.3	24.3	28.0	72.1	27.9
Standard deviation		0.63			0.28	

[a]Based on computer summation of masses 229+259 for 9-OH isomer, masses 187+301 for 12-OH isomer, masses 173+315 for 13-OH isomer, and masses 131-357 for 16-OH isomer (OTMS derivative).

[b]Based on separation shown in Figure 1C: Peak I = 9- + 12- + 13-OH isomer, Peak II = 16-OH isomer.

TABLE III

GC-MS Analysis[a] of Isomeric Hydroxyoctadecanoate from Autoxidized Methyl Linolenate

Peroxide value	Temp., C	Relative percent			
		9-OH	12-OH	13-OH	16-OH
134	25	28.1	11.4	11.4	49.1
495	25	31.7	10.7	10.9	46.7
1315	25	31.9	8.2	10.0	49.9
337	40	30.4	11.9	13.0	44.8
710	40	33.5	10.6	11.9	44.1
1183	40	35.1	10.0	12.2	42.7
212	60	26.6	10.3	11.1	52.0
639	60	33.4	10.9	12.5	43.4
1130	60	28.2	13.1	11.7	47.0
466	80	34.8	13.2	10.5	41.4
1839	80	33.5	8.9	12.5	45.1

[a]See footnote a in Table II.

quantitatively reliable as shown by a calculated standard deviation of 0.63 between known compositions and experimental values (Table II). A further check was made by quantitative GC analysis of the 16-hydroxy isomer which was separated from the other three hydroxy isomers (Fig. 1C). The standard deviation from the known mixtures calculated to be 0.28.

Samples of methyl linolenate oxidized to different peroxide values and at different temperatures show that the proportion of 9- and 16-hydroxy esters was significantly higher (75-82%) than the 12- and 13-hydroxy esters (18-25%) (Table III). In a previous paper (8), we estimated the hydroperoxides from linolenate autoxidized at 37 C by analyzing cleavage products of monoenes derived by dehydration from hydroxyoctadecanoate derivatives. The distribution obtained (30.2% 9-OH, 10.7% 12-OH, 9.8% 13-OH, 48.1% 16-OH) is in good agreement with out present GC-MS analyses in Table III.

The quantitative GC-MS method was also applied to the analysis of autoxidized mixtures of linolenate with linoleate and with oleate. Two different binary mixtures and one ternary

TABLE IV

GC-MS Analysis of Autoxidized Mixtures of Oleate (01):Linoleate (Lo):Linolenate (Ln)

Mixtures 01:Lo:Ln	PV	Temp. C	Relative percent							Hydroperoxides, %[a]		
			8-OH	9-OH	10-OH	11-OH	12-OH	13-OH	16-OH	01	Lo	Ln
0:1:1	200	60	---	36.3	---	---	3.9	43.2	16.6	---	64	36
	491	60	---	42.1	---	---	3.6	36.7	17.6	---	62	38
	1197	60	---	44.1	---	---	3.4	38.1	14.4	---	68	32
0:1:1	401	80	---	38.3	---	---	4;1	34.5	23.1	---	52	48
	1238	80	---	41.0	---	---	5.2	38.8	15.0	---	64	36
0:9:1	340	80	---	35.2	---	---	0.1	36.9	27.9	---	68	32
	1708	80	---	47.9	---	---	1.6	46.6	3.9	---	90	10
1:0:1	120	60	5.2	29.3	5.3	4.8	6.8	12.1	36.5	24	---	76
	574	60	6.0	27.3	5.7	4.5	6.8	8.4	41.3	15	---	85
	763	60	6.7	29.5	5.6	5.8	8.0	9.4	35.0	24	---	76
1:0:1	552	80	5.3	21.2	2.9	3.1	5.6	13.5	48.4	4	---	96
	1181	80	9.7	21.3	8.6	8.2	11.1	8.4	32.7	22	---	78
9:0:1	530	80	24.5	24.0	20.7	24.6	1.2	1.3	3.7	91	---	9
	1353	80	25.8	20.9	20.9	24.2	0.9	0.9	6.2	89	---	11
1:1:1	114	60	2.6	35.7	2.0	2.3	5.0	28.1	24.4	9	39	52
	563	60	3.1	41.0	2.1	2.3	3.6	32.5	15.4	9	57	34
	1071	60	3.3	41.4	2.2	2.3	3.1	33.5	14.2	9	60	31

[a]Assuming that 12-OH + 16-OH = 56.5% in autoxidized linolenate (Table III) and that 10-OH + 11-OH = 50% in autoxidized oleate (1). Ln-hydroperoxide = (12-OH + 16-OH) ÷ 0.565. 01-hydroperoxides = (10-OH + 11-OH) X2. Lo-hydroperoxides = 100 - (Ln-OOH + 01-OOH).

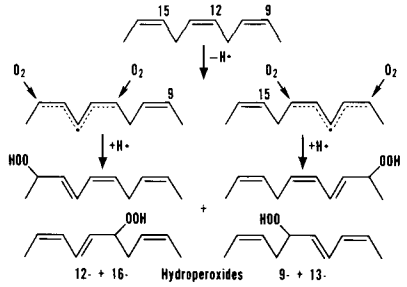


FIG. 3. Mechanism of linolenate autoxidation.

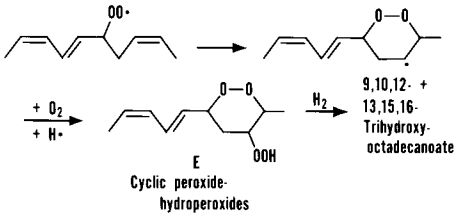


FIG. 4. 1,4-Cyclization of 12- and 13-hydroperoxides of linolenate.

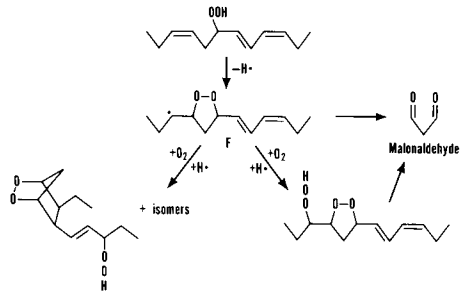


FIG. 5. Prostaglandin-like endoperoxides and malonaldehyde formation by 1,3-cyclization of 12- and 13-hydroperoxides of linolenate (14).

mixture were autoxidized at different levels, and the hydroxyoctadecanoate TMS derivatives were analyzed by GC-MS. With these mixtures, the 9-hydroxy ester comes from the hydroperoxides of all three fatty esters and the 13-hydroxy ester from both linoleate and linolenate hydroperoxides. The 8-, 10-, and 11-hydroxy esters come from oleate hydroperoxides whereas the 12- and 16-hydroxy esters come only from linolenate hydroperoxides. To estimate the different hydroperoxides in these autoxidized mixtures, one can use the values for the 12- and 16-hydroxy esters which are unique for the linolenate hydroperoxides, and the values for the 10- and 11-hydroxy esters which are unique for the oleate hydroperoxides. The different hydroperoxides were thus estimated in autoxidized mixtures by assuming that the sum of the 12- and 16-hydroxy esters are formed in the same proportion (averaging 56.5%) as in pure linolenate, and that the sum of the 10- and 11-hydroxy esters form 50% of the oleate hydroperoxides.

With equal binary mixtures of esters autoxidized at levels below 10% (PV less than 610), the proportion of linolenate hydroperoxides varied from 36 to 48% compared to 52 to 64% for linoleate hydroperoxides, and was much greater than that of oleate hydroperoxides (Table IV). At higher peroxide values, the proportion of linoleate hydroperoxides was even larger than that of linolenate hydroperoxides which in turn was larger than that of oleate hydroperoxides.

With the linoleate-linolenate mixture containing 10% linolenate autoxidized to a peroxide value of 340, 32% of the hydroperoxides originated from linolenate. At the more advanced levels of oxidation, as expected, the dominant hydroperoxide originates from the dominant fatty ester in the mixture.

With the oleate-linoleate-linolenate mixtures autoxidized to a PV of 114, 52% of the hydroperoxides were derived from linolenate and 39% from linoleate. This ratio is reversed at the higher peroxide levels. In all samples, the contribution from oleate hydroperoxides remained constant at 9%.

DISCUSSION

In previous papers (8,11), we formulated a mechanism for linolenate autoxidation based on that of methyl linoleate. Hydrogen abstraction on C-11 and C-14 of linolenate produces two pentadiene radicals, each having two equivalent sites for O_2 attack: C-9 and C-13 on one hand, and C-12 and C-16 on the other hand (Fig. 3). As with linoleate (11), the products expected by this scheme would be equal amounts of 9-, 13-, 12-, and 16-hydroperoxides with *cis,trans/trans,cis*-conjugated diene systems. However, our present GC-MS results show a significantly larger concentration of the 9- and 16-hydroperoxides than the 12- and 13-hydroperoxides. This distribution is in remarkably good agreement with our earlier results based on an entirely different chemical characterization scheme (8). We suggested at that time that either the pentadiene radicals prefer to react with O_2 at the end C-9 and C-16 positions, or that the 12- and 13-hydroperoxide isomers are more easily decomposed. Steric factors might indeed be invoked for greater attack of O_2 on C-9 and C-16 than on C-12 and C-13 on one hand, and greater attack on C-16 than on C-9 on the other hand.

Following our earlier work, Haverkamp Begemann et al. (12) identified isomeric cyclic

peroxide-hydroperoxides among the more polar products of linolenate autoxidation. The peroxides with a six-membered ring between C-9 and C-12 on one hand, and between C-13 and C-16 on the other hand, are derived from the corresponding 12- and 13-hydroperoxides of linolenate. Indeed, Gunstone (13) suggested that the reducted yield of 12- and 13-hydroperoxides we reported (8) may be due to their unique 1,5-diene structure leading to the formation of the six-membered cyclic peroxide-hydroperoxides (Fig. 4).

The reduced yields of 12- and 13-hydroperoxides from linolenate can also be explained by their tendency to cyclize into prostaglandin-like endoperoxides. Pryor et al. (14) reported evidence for endoperoxides from autoxidized linolenate with structures related to those of the endoperoxides formed from arachidonic acid in the biosynthetic pathway established for prostaglandin (15). They formulated the internal 12- and 13-hydroperoxides as the source of endoperoxides which on decomposition give malonaldehyde and a positive TBA test (Fig. 5). The chemistry of linolenate autoxidation becomes particularly important today if the suggestion of Pryor et al. (14) is valid that some of the symptoms of lipid peroxidation in vivo could originate from nonenzymatically produced prostaglandins or their stereoisomers.

The higher oxygenated products indicated in this paper by GC-MS in reduced or hydrogenated oxidized linolenate include trihydroxy esters. The 9,10,12- and 13,15,16-trihydroxy esters determined in this paper and identified by Haverkamp Begemann et al. (12) can be produced by hydrogenation from either the six-membered cyclic peroxide-hydroperoxide of type E or from the endoperoxyhydroperoxides of type F formulated by Pryor et al. (14).

In the previous paper of this series, we analyzed the hydroperoxides from different autoxidized mixtures of oleate and linoleate (2). Linoleate hydroperoxides dominated the products from different mixtures at low levels of oxidation. In the present study, with equal mixtures of esters autoxidized at levels below 10%, the proportion of linolenate hydroperoxides actually detected was less than that of linoleate but much greater than that of oleate hydroperoxides. These results reflect not only the greater ease of hydrogen abstraction from linolenate and linoleate compared with that of oleate, but also suggest the loss of linolenate hydroperoxides by cyclization and secondary oxidation. When the mixtures contained only 10% linolenate, the proportion of linolenate hydroperoxides was considerably greater. In mixtures containing the same initial concentration of linoleate and linolenate, the higher level found of linoleate hydroperoxide can be explained by the difference in relative rates of propagation and termination of the respective peroxy radicals. Although predominant autoxidation of linolenate is likely, linolenate hydroperoxides would be expected to decompose more readily and not accumulate to as high a level as the other hydroperoxides (11).

The GC-MS approach is thus useful in determining the origin of hydroperoxides formed in autoxidized mixtures of unsaturated fatty esters. The next paper in this series will deal with a study of oxidation products from mixtures of unsaturated fatty esters in vegetable oils to determine their relative role as precursors of off-flavors.

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