A Rapid Microozonolysis-GLC Procedure for Locating Unsaturation in Olefinic Acids, Including Trienes and Tetraenes[1]

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ABSTRACT

Increased versatility has been achieved in the identification of unknown olefinic fatty acids by ozonolysis. The method has been applied to purified methyl esters containing up to four double bonds. Aldehydic fragments, obtained from esters by the Stein-Nicolaides procedure (2), were determined by GLC on two columns of different polarity. Equivalent chain lengths of each fragment on the two columns provide identification. For monoenoic esters the location of the double bond is clearly indicated by the aldehyde and aldehyde-ester fragments. Dienes are identified by the aldehyde and aldehyde-ester fragments when the original chain length of the ester is known; the dialdehyde fragment provides confirmatory evidence. Trienes and tetraenes are analyzed by interrupting the ozonolysis at various times, thereby producing unsaturated, as well as saturated, aldehydes and aldehyde-esters. Unsaturated fragments locate the central or interior double bonds.

INTRODUCTION

In the GLC analysis of methyl esters prepared from vegetable oils, components are usually identified as to chain length and number of double bonds. Such identification is not specific unless supported by rigorous characterization by chemical or physical means. Ozonolysis has become widely used in determining the location of double bonds in fatty acids. The method reported here can be used without repeated ozonolysis of known materials in order to identify components and is of general applicability to mono- and polyenoic fatty acids, even those containing double bonds more widely separated than the common methylene interruption. The method was used with esters having both *cis* and *trans* unsaturation.

METHODS

A 0.2% solution of purified methyl esters (1 to 10 mg) was prepared in dichloromethane (Matheson Coleman and Bell, superior grade). The solution was cooled in an acetone-dry ice bath and the ozone-oxygen mixture from a Bonner (1) ozone generator was bubbled through at 30 ml/min. The concentration of ozone was such that 5 mg 18:1 was completely ozonized in 1½ min. After this treatment, ozonides were reduced by adding to the solution a few crystals of triphenylphosphine (2), and 15 μliter aliquots of the solution were analyzed in an F&M 402 gas chromatograph equipped with glass columns and flame ionization detectors. One 12 ft x ¼ in. column was packed with 5% LAC-2-R 446 (3) on Chromosorb W-AWDMCS and the other was 4 ft x ¼ in. packed with 5% Apiezon L on Chromosorb W-AWDMCS. Samples were injected onto the two columns simultaneously and the temperature was programmed from 80 to 200 C at 7.5 C/min.

When trienoic or tetraenoic esters were analyzed, the ozonolysis reaction was interrupted three to five times at 15 to 30 sec intervals. After each interval, the entire reaction mixture was reduced with triphenylphosphine and two 15 μliter aliquots were analyzed by GLC as above.

Equivalent chain lengths (ECLs) (3) were calculated with even chain length methyl esters (C_6-C_{22}) as standards using the equation:

$$ECL = S_1 + (S_2 - S_1)\ \frac{t_x - t_{s1}}{t_{s2} - t_{s1}}$$

where: S_1 = chain length of first standard ester; S_2 = chain length of second standard ester; $ts_{(1\ or\ 2)}$ = standard retention time: and tx = retention time of peak. The ECL of each peak was calculated using the two standards closest in retention time to that of the peak. ECLs and relative area percentages for each component were calculated by computer. In the case of monoenoic esters, the computer program provided identification of the fragments and the molar ratio of the parent esters.

In esters with (ω 3) bonds, the ozonides were formed at –23 C in CCl_4 and reduced with triphenylphosphine. The C_3 aldehyde formed was determined by GLC on a 9 ft x ⅛ in. stainless steel column packed with Porapak Q and held at 155 C in an F&M

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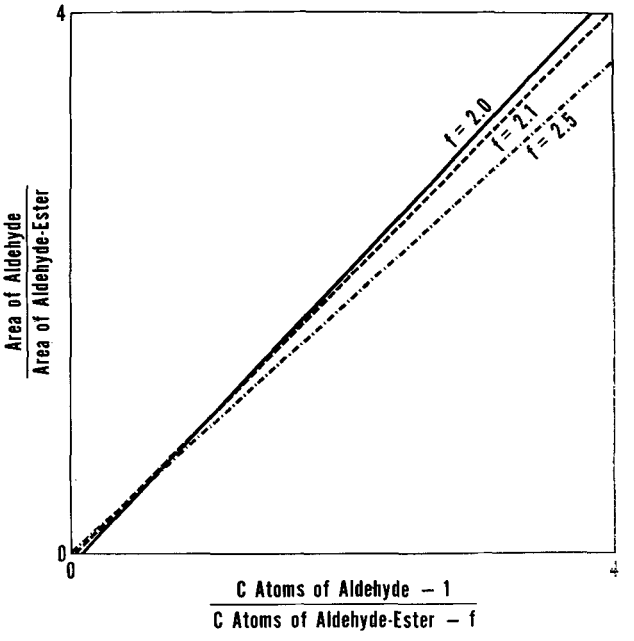

FIG. 1. Area vs. calculated response of monoenoic ester ozonolysis products from LAC-2-R 446 column. Variable f = nonresponding carbon atoms.

810 chromatograph equipped with a hydrogen flame detector (4).

Known fatty acid methyl esters of high purity were obtained from commercial suppliers, by preparation from known plant sources, or by synthesis.

RESULTS AND DISCUSSION

The products resulting from the ozonolysis reported by Stein and Nicolaides (2) are essentially all aldehydic in nature. Side products resulting from chain degradation or other anomalies (5) are almost completely absent from the mixture. This method has the further advantages that it involves no transfer of sample to a special reaction vessel, no catalyst preparation (6), no solvent purification, and employs standard injection techniques.

Our chromatographic method, which entails on-column injection into glass columns without preheaters, minimizes polymerization and loss of reactive ozonolysis products. Retention characteristics in the two columns, which have different stationary phases, provide identification of each fragment.

Relative Response of Flame Ionization Detectors to Ozonolysis Products

The aldehyde-ester fragments have been used in the past to quantitate the relative amounts of monoenoic esters (7,8); little has been done quantitatively to relate the aldehyde fragments to the aldehyde-ester fragments and to the parent ester. In the use of the flame ionization detector, quantitation is complicated by the unequal detector response to different organic molecules. The response has been reported to be proportional to the number of carbon atoms in the molecule. Dal Nogare and Juvet (9) describe a correction factor (C-factor) for conversion of area response to weight response. They equate this factor to (molecular weight)/(12 × number of carbon atoms) and suggest that carbon atoms bonded to oxygen be excluded from this calculation. A later report (10), however, indicates that methoxyl carbon atoms do respond to flame ionization detectors and that the absolute response of carboxyl carbon atoms is not clearly defined. In Figure 1 the ratios of the areas of aldehyde fragments to the areas of their corresponding aldehyde-esters from individual monoenoic esters are plotted against the ratios of the number of responding carbon atoms in each fragment. Aldehydic carbon atoms are assumed to have zero response while the number of nonresponding atoms in aldehyde-esters is set equal to the variable (f) so that a one to one relationship between the two ratios can be established. The line with f = 2.1 has unit slope. An additional assumption that the carboxyl carbon

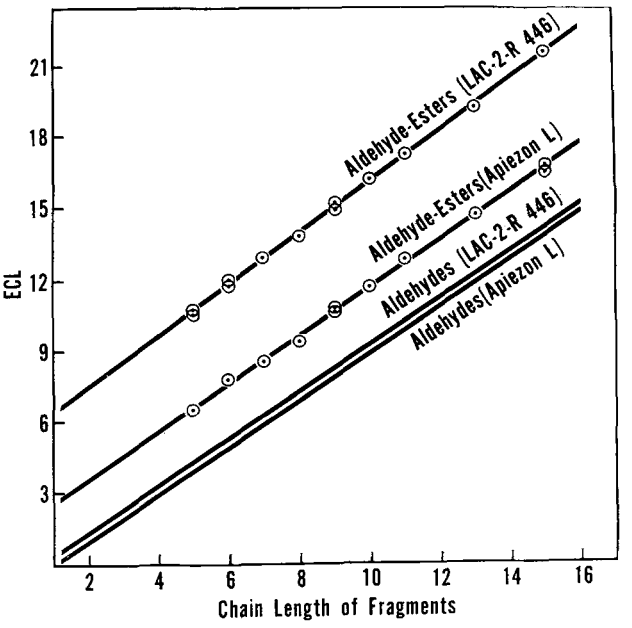

FIG. 2. Equivalent chain lengths (based on saturated methyl esters) of aldehydes and aldehyde-esters. Points on aldehyde lines were omitted for clarity but showed the same relation to the lines as in the aldehyde-ester plots.

is unresponsive and that the methoxyl carbon has nine-tenths the response of a methylene carbon would conform to this result. For practical use, it is adequate to consider that the carbonyl and carboxyl carbon atoms give no response and that the methoxyl carbon atoms respond equally with methylene carbon atoms. Therefore, the response of aldehyde fragments is proportional to number of carbon atoms in the molecule minus one and the aldehyde-ester response is proportional to its number of carbon atoms *minus two*.

Analysis of Monoenoic Esters

The products from reductive ozonolysis of monoenoic esters are aldehydes (A) and aldehyde-esters (AE). Ozonolysis of esters of known structure ($16{:}1^{3}$; $16{:}1^{9}$; 18:1 (individual isomers Δ 5 through Δ 12); $20{:}1^{5}$; $20{:}1^{11}$; $22{:}1^{13}$; $24{:}1^{15}$) produced a variety of fragments of known chain length as reference materials. ECLs (based on saturated methyl esters) of these fragments were determined from both Apiezon L and LAC-2-R 446 columns and were then plotted vs. their respective chain lengths. The straight line relationships evident in Figure 2 show that ECLs from the two columns can be used to identify ozonolysis fragments without further comparison with fragments from ozonized reference compounds. Methyl esters were used as standards instead of aldehydes because they are stable and readily available in high purity.

The ECLs of an aldehyde are essentially the same from both Apiezon L and LAC-2-R 446 columns, but those of aldehyde-esters differ markedly. Therefore, each component can be identified from the ECL data. Furthermore, components which overlap in one column are well separated by the other and quantitative relationships can be determined (11). The identification and quantitation of mixtures of monoenes of the same chain length and of simple mixtures of different chain lengths (if the relative proportions of esters of each chain length are known) are easily accomplished. In mixtures of monoenoic esters, peak areas from the ozonolysis fragments are used to calculate the mole percentages of the parent esters from the following relationships:

wt % = area % × C-factor, and C-factor = MW/ (responsive carbon atoms × 12).

Responsive carbon atoms = No. of carbon atoms — [carbonyl + carboxyl carbon(s)].

Therefore wt % $= \dfrac{\text{area \%} \times \text{MW}}{(N-f) \times 12}$, where N = total number of carbon atoms and f =

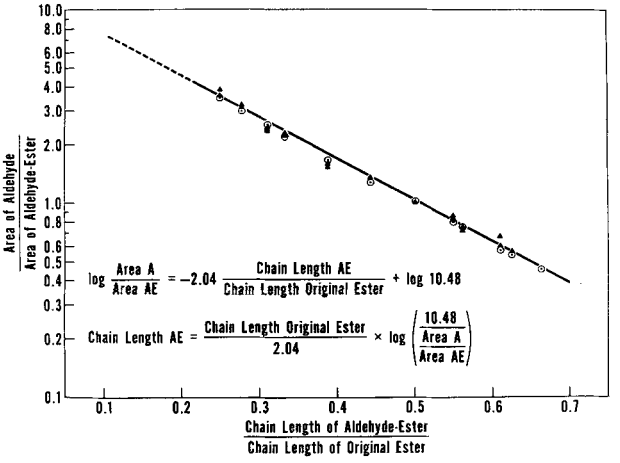

FIG. 3. Graphical determination of double bond position in monoenes from the ratio of peak areas of ozonolysis fragments: Δ from LAC-2-R 446 column, ⊙ calculated (see text).

number of carbonyl + carboxyl carbons.

$$\text{Mole Fraction, } X_a = \frac{\frac{\text{wt }\%_a}{MW_a}}{\sum_{x=1}^{x=n} \frac{\text{wt }\%_x}{MW_x}}$$

$$X_a = \frac{\frac{\text{area }\%_a \times MW_a}{MW_a \times (N-f)_a \times 12}}{\sum_{x=1}^{x=n} \frac{\text{area }\%_x \times MW_x}{MW_x \times (N-f)_x \times 12}}$$

$$X_a = \frac{\frac{\text{area }\%_a}{(N-f)_a}}{\sum_{x=1}^{x=n} \frac{\text{area }\%_x}{(N-f)_x}}$$

The mole fractions of the fragments produced on complete ozonolysis of a monoenoic ester are equal and, in a mixture of pure monoenes, their sum equals mole fraction of the parent ester in the original mixture. The ratios of the mole fractions of the fragments from the parent esters are the same as the ratio of the parent esters, even upon incomplete ozonolysis; the aldehyde fragments serve as well as the aldehyde-ester to establish the ratio.

A correlation was found when the logarithms of the ratios of the peak areas of the aldehydes to the corresponding aldehyde-esters were plotted vs. the ratios of the position of the double bond to the original chain length (Fig. 3). The calculated points, $\log[(N_A - 1)/(N_{AE} - 2)]$, vs. position of the double bond/chain length of original ester, are essentially the same as those found from the peak area ratios from the LAC-2-R 446 column. The relationship between the areas from fragments and the double bond position allows calculation of the position of the double bond from the peak areas of the aldehyde and aldehyde-ester if the parent chain length is known. Location of the double bond is a check on the identification of the aldehyde-ester made from retention data.

The ozonolysis procedure used permitted detection of some components not usually found from $KMnO_4/KIO_4$ oxidation (12). For example, analysis of $KMnO_4/KIO_4$ cleavage of 16:1[3] showed only one product, tridecanoic acid (13,14). Using the above ozonolysis procedure both 13A and 3AE were observed.

Analysis of Dienoic Esters

The products formed from the ozonolysis of dienoic methyl esters are aldehydes, aldehyde-esters and dialdehydes. To establish the positions of the olefinic bonds in pure single component esters, only correct identification of the aldehyde and aldehyde-ester fragments are necessary, although dialdehydes supply confirmatory evidence.

An example of a dienoic methyl ester from which the dialdehyde fragment is a major component is the 22:2[5,13] from *Limnanthes douglassii* (15, 16). The eight carbon dialdehyde (AA) has ECLs of 13.9 (LAC-2-R 446) and 8.8 (Apiezon L). A plot of ECLs of several

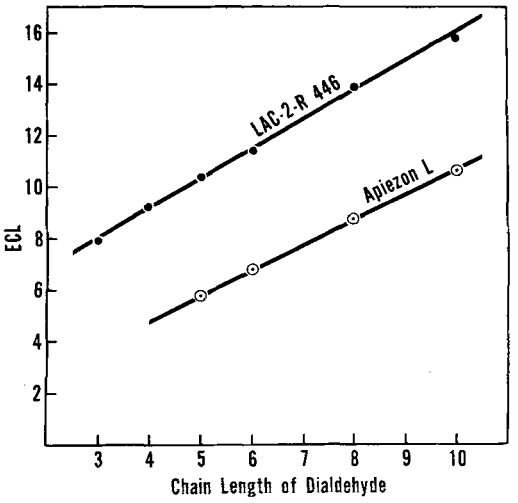

FIG. 4. Equivalent chain length (based on saturated methyl esters) of dialdehydes.

dialdehydes vs. their chain length is shown in Figure 4. The sources of these dialdehydes are listed in Table I. The detector response to dialdehydes is analogous to that of other aldehydic components in that neither carbonyl carbon atom responds to flame ionization. Therefore, 3AA has only one responding carbon atom and is not easily detected.

Another example of the superiority of the ozonolysis procedure for double bond location is shown by its application to the allenic diene, 5,6-octadecadienoic acid from *Leonotis nepetaefolia* (17). When the methyl ester of this acid was oxidized by $KMnO_4/KIO_4$ (12) only lauric acid was observed (17). Addition of acetic acid before oxidation was necessary before glutaric acid was detected (17). Ozonolysis revealed two products, 5AE and 12A, which clearly define the positions of the double bonds.

Analysis of Trienoic and Tetraenoic Esters

Complete ozonolysis of trienoic and tetraenoic esters and identification of the resulting aldehyde and aldehyde-ester fragments to reveal the structure of the esters is well documented (6,18). In both reports, the unsaturation in the esters is either all conjugated or all methylene-interrupted. Complete ozonolysis, therefore, resulted in aldehydes and aldehyde esters which served as identifying fragments.

TABLE I
Source of Known Dialdehydes

Dialdehyde	Source	Reference
3AA	$20:4^{5,11,14,17}$	25
4AA	$18:3^{5,9,12}$	21
5AA	Aldrich Chem. Co.	—
6AA	$20:3^{5,11,14}$	25
8AA	$22:2^{5,13}$	17
10AA	$18:3^{5,8,16}$	22

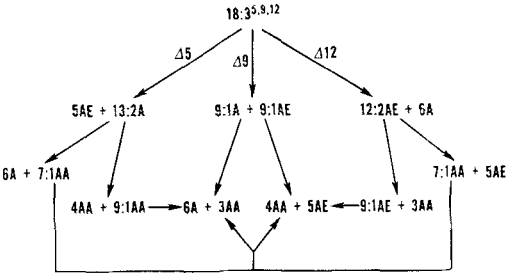

FIG. 5. Reaction scheme for ozonolysis of $18:3^{5,9,12}$.

If an ester has isolated olefinic bonds, complete ozonolysis would not unequivocally locate the positions of unsaturation. For example, $20:4^{5,11,14,17}$ ester, upon complete ozonolysis, yields 5AE, 3A, 6AA, and 3AA as the only products. These products could also result from complete ozonolysis of two other esters: $20:4^{5,8,11,17}$ and $20:4^{5,8,14,17}$. Schlenk solved this problem in the characterization of the $20:4^{5,11,14,17}$ ester from *Ginkgo* lipids (19) by ozonolysis of the ester before and after alkali-isomerization, thereby locating the bonds that were methylene-interrupted. By interrupting the ozonization at various stages before completion, unsaturated fragments (20) are produced as well as the products from complete reaction. The unsaturated intermediates serve to locate the interior olefinic bonds. For example, the "interrupted ozonolysis" method, when applied to the $20:4^{5,11,14,17}$ ester, produced two structure-determining unsaturated fragments, 11:1AE and 6:1A. These fragments together with the end products (5AE, 3A, 6AA, and 3AA) determine the locations of all double bonds.

A simpler ester, $18:3^{5,9,12}$ (21), serves to illustrate the many possible unsaturated fragments that can form during interrupted ozonolysis (Fig. 5). The fragments necessary to locate the double bonds correctly are: 5AE, 6A, 9:1AE, or 9:1A (Fig. 6 and 7). The other fragments supply confirmatory evidence. The 3AA from an ester such as the $20:4^{5,11,14,17}$ or $18:3^{9,12,15}$ is quite evident as two of these fragments are produced per ester molecule.

Identification of unsaturated fragments was made on the basis of their ECLs on both the LAC-2-R 446 and the Apiezon L columns. The ECLs of fatty acid methyl esters are

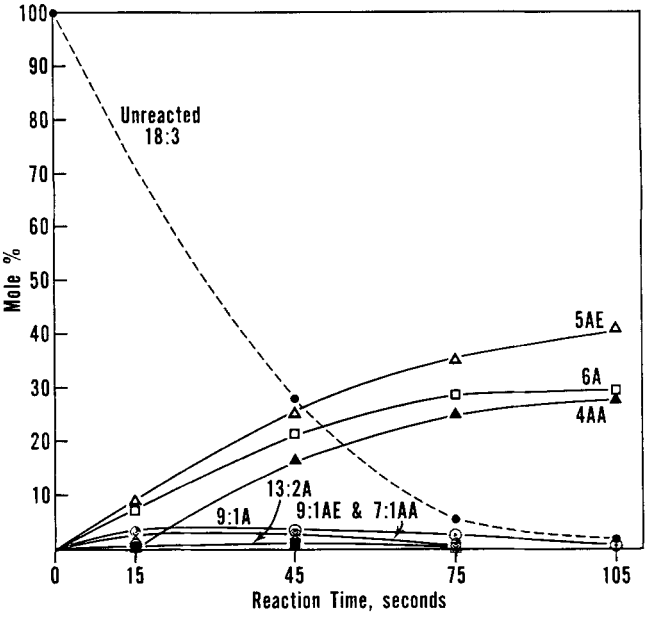

FIG. 6. Concentration of ozonolysis fragments from $18:3^{5,9,12}$ vs. reaction time.

greater by 0.4 units per double bond on the polar LAC-2-R 446 column than their saturated analogues and unsaturated esters usually have ECLs approximately 0.3 units less than their saturated analogues on the Apiezon L column (3). Similarly, 9AE has an ECL of 15.0 on LAC-2-R 446 while the ECL of the 9:1AE is 15.4; the ECLs on Apiezon L are: 9AE = 10.8 and 9:1AE = 10.5. This relationship holds for all chain lengths. Table II shows the ECLs of the components formed from ozonolysis of $18:3^{5,9,12}$ ester.

Confirmation of the unsaturated nature of the components formed early in the ozonization is provided by their decrease and disappearance as ozonolysis continues. Figure 6 illustrates this phenomenon. As expected, the molar amounts of the 5AE, 4AA, and 6A increase throughout the experiment, but the 9:1A, 9:1AE, 7:1AA, and 13:2A increase for a time and then de-

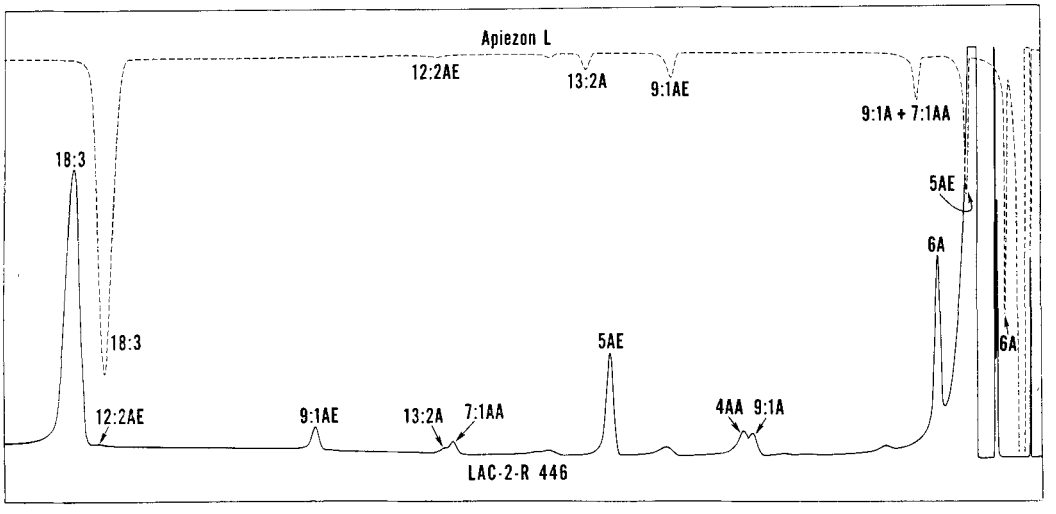

FIG. 7. GLC of ozonolysis products of $18:3^{5,9,12}$ after 45 sec reaction time from LAC-2-R 446 and Apiezon L columns.

TABLE II

Ozonolysis Products of $18:3^{5,9,12}$

Reaction time	Component	ECL LAC-2-R 446	ECL Apiezon L	Area % (LAC-2-R 446)	Ionizable carbons	Mole %
sec						
45	6A	5.2	5.2	12.0	5	20.3
	5AE	10.9	6.8	11.8	4	25.0
	9:1A	8.7	7.8	3.1	8	3.3
	9:1AE	15.4	10.5	2.7	8	2.9
	13:2A	13.3	11.8	1.1	12	0.8
	12:2AE	18.3	13.3	tr	12	tr
	7:1AA	13.2	7.6	1.8	5	3.0
	4AA	9.1	4.0	4.0	2	16.9
	18:3	19.4	17.7	59.0	18	27.8
105	6A	5.2	5.2	37.3	5	29.6
	5AE	10.9	6.8	41.3	4	41.0
	9:1A	8.7	7.8	0.2	8	0.1
	4AA	9.1	4.0	14.0	2	27.7
	18:3	19.4	17.7	7.1	18	1.6

crease. The 9:1A is the unsaturated fragment produced in the largest amount and is the only one remaining after 105 sec (Table II).

The allenic triene, $18:3^{5,6,16}$ from *Lamium purpureum* (22), was investigated by the interrupted ozonolysis method. After 30 sec ozonization, four major components were detected: 5AE, 10AA, 16:2AE (ECL: LAC-2-R 446 = 23.8, Apiezon L = 17.4), and unreacted 18:3. The 16:2AE was 28% of the total peak area. The trienoic ester disappeared completely before a significant decrease (to 19% at 2 min) in the 16:2AE peak area was observed. Three minutes were required to completely ozonize the 16:2AE. Since the double bonds of this component are allenic, it appears that such bonds react more slowly than isolated bonds.

Three other esters with isolated double bonds were analyzed. These and their identifying products are as follows: $18:3^{3,9,12}$ (23), 3AE, 6A, 6AA, 9:1A and 9:1AE; $20:3^{5,11,14}$ (24,-25), 5AE, 6AA, 6A, 9:1A and 11:1AE; and $20:4^{5,11,14,17}$ (19,24,25), 5AE, 3A, 6AA, 6:1A and 11:1AE.

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