constitutes confirmation for these assignments. A few bands appear in the spectrum of 6-aminohexanoic acid which cannot be accounted for from investigations on the long-chain fatty acids nor on the infrared absorption studies of the amine group.

With the exception of a few unassigned bands the spectra confirm the structures of lauraldehyde oxime, adipaldehydic acid oxime and 6-aminohexanoic acid.

The best yields of dodecylamine and of 6-aminohexanoic acid were obtained by the procedure given under Experimental. The yield of the dodecylamine was 48% of the theoretical calculated on the wt of petroselinic acid used, the yield of 6-aminohexanoic acid was 44%.

The yields of the aldehydes from the reduction of the ozonides with zinc-acetic acid was 77–85% of the theoretical. The yields of oximes was in the same range, as calculated from their weights and nitrogen contents, assuming that all the nitrogen present was in the form of oximes.

Various modifications of the procedure for the preparation of the amines were tried: using the procedure given under Experimental but without separating the oximes; using the same procedure except hydroxylamine was added before the reduction of the ozonides with zinc and acetic acid; direct catalytic aminative reduction of the aldehydes in the presence of ammonia and hydrogen; ozonizing methyl petroselinate instead of the acid; and catalytic reduction with hydrogen of the ozonides to aldehydes. None of these procedures gave as high yields as the procedure outlined above. A problem encountered in all procedures was the solubility of the adipaldehydic acid and its oxime in water, and especially in water-alcohol mixtures. For this reason more difficulty was encountered in isolating the adipaldehydic acid and its oxime than would be the case with the corresponding derivatives from oleic acid.

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Determination of Structure of Unsaturated Fatty Acids via Reductive Ozonolysis[1,2]

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Abstract

An improved method of reductive ozonolysis for the determination of structure of unsaturated fatty acids is reported.

The ozonization is carried out at −60 – −70C by adding the sample dissolved in pentane to a .02–.03 M pentane solution of ozone. The reduction is effected by the Lindlar catalyst at 0C in pentane or in other solvents, such as the methyl esters of short-chain fatty acids or in dimethyl phthalate, and the aldehydic fragments are analyzed by gas-liquid chromatography (GLC).

The method is applied to the structural analyses of methyl esters of oleic, linoleic, linolenic, and arachidonic acids. The sensitivity of the method is demonstrated by the analysis of methyl oleate containing a small amount of added methyl linolenate. Mixtures of methyl oleate and petroselinate are analyzed to demonstrate the identification of the simple aldehydes and ester-aldehydes, and to show the potential of the method for quantitative analysis of mixtures of unsaturated esters.

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[2] Presented at the AOCS meeting in Chicago, Ill., 1961.

Introduction

OZONIZATION, followed by oxidative or reductive fission of the ozonides, has been used by many investigators for the determination of structure of unsaturated fatty acids (1,3,6,9,10,11,14,16,18). However, a completely satisfactory procedure has not been developed. In general, the determination of structure by ozonolysis is complicated by side reactions which give spurious results (4,6,8). The products of the ozonization of double bonds are markedly influenced by the conditions of the reaction and may be very complex (2,7). Herein, we describe a procedure which is based on the quantitative and instantaneous formation of true ozonides and their reductive cleavage to aldehydes. The location of the double bonds is based on the analysis of the aldehyde fragments by gas-liquid chromatography (GLC).

Experimental

Methyl Ester Standards. Highly purified methyl oleate, linoleate, linolenate, petroselinate, and arachidonate were obtained from The Hormel Foundation, Austin, Minn. These esters were used without further purification, except for methyl arachidonate, which was further purified by reversed-phase partition chromatography as previously described by the authors (13). No impurities could be detected in these preparations by GLC.

Procedure

Reagents and Solvents. N-pentane (Matheson Co., Inc.) is freed of unsaturated contaminants by the following treatment. Oxygen containing 2–3% ozone generated from a Bonner ozone generator (5) is bubbled through the pentane at −60 – −70C until it takes on a distinct blue color. Then purified nitrogen is bubbled through the solution as it warms up to room temp to remove the dissolved ozone and oxygen. The pentane is then shaken with concentrated sulphuric acid (20:1 v/v) in a separatory funnel, washed with water until neutral, dried, and distilled from P_2O_5.

Heptane and isooctane must be similarly purified when used as solvents for ozonization and/or the reduction. However, when these solvents are only used to dissolve the fragments collected for GLC analysis, they may be used after treatment with P_2O_5 and distillation.

Methyl caprylate and methyl caprate, used as solvents for catalytic reduction, were obtained from The Hormel Foundation; dimethyl phthalate was obtained from Eastman Organic Chemicals, Rochester, N.Y.

The Lindlar catalyst is a lead poisoned palladium catalyst precipitated on calcium carbonate and is prepared according to the procedure described by Lindlar (12). The palladium (ous) chloride used in its preparation was obtained from Fisher Scientific Co., Fair Lawn, N. J.

The oxygen used in the preparation of ozone should be dried prior to use by passing it through a train consisting of concentrated sulphuric acid and calcium chloride. Finally, the oxygen containing the ozone emerging from the ozone generator should be passed through a U tube packed in dry ice. This U tube serves to trap traces of moisture and precools the gas.

Ozonization. A solution of ozone is prepared by bubbling oxygen containing 2–3% ozone at about 100 ml per min through 10 ml of pentane in a 25 ml round-bottom flask immersed in a dry ice bath at −60 – −70C for 5 min. At the end of this time, the solution has a dark blue color and contains about 0.3 mmole of ozone.

The ozonization is performed by adding the sample (1–50 mg) dissolved in 2–3 ml of purified pentane, cooled as low as possible without causing crystallization of the sample, to the 10 ml of the ozone solution prepared as described above.

The reaction of ozone with double bonds is virtually instantaneous under these conditions. Therefore, the excess dissolved oxygen and ozone are immediately removed after the solutions are mixed by connecting the flask to a water aspirator and evaporating a portion of the solvent. The entire process requires less than one min to complete.

Ten ml of the ozone solution is sufficient to ozonize 50 mg of methyl oleate. This relationship is used as a guide for the ozonization of other esters, the amount of solution or size of the sample, or both, being adjusted, depending on the degree of unsaturation of the sample. After the reaction is complete, the solution should be pale grey in color. If the solution decolorizes completely, indicating an insufficiency of ozone, the flask is connected directly to the ozone generator for several sec to introduce sufficient ozone to complete the reaction. An excess of ozone is indicated by the return of a faint bluish color to the solution. If there is any suspicion that the ozonization is not complete, the sample is analyzed by thin-layer chromatography (TLC) using Silica Gel G as the adsorbent and a solvent system of 2% diethyl ether in petroleum ether, B.P. 30–60C.

Catalytic Reduction of Ozonides. The ozonides of unsaturated fatty acid methyl esters yielding fragments of 5 carbon atoms or longer, may be reduced directly in the pentane solution in which the ozonization is carried out. Prior to fission the vol of the solution is usually reduced to about 2 ml. Generally, and especially the esters yielding short-chain fragments or a mixture of short- and long-chain fragments, the pentane is removed completely by evaporation at 0C under reduced pressure, prior to reduction. Then the ozonides are reduced in about 2 ml of methyl caprylate, other methyl esters of short-chain fatty acids, or dimethyl phthalate which have a relatively long retention time on GLC analysis.

The reduction is carried out as follows. In order to avoid secondary oxidation of the aldehyde fragments, products of the reduction must be protected from oxygen at all times.

About 25 mg. of catalyst and 2 ml. of the solvent are added to the reaction flask (Fig. 1) which is immersed in an ice-water bath at 0C. The solution is saturated with hydrogen while stirring it vigorously by means of a magnetic stirrer. The sample, dissolved in a minimum amount of solvent, is also saturated with hydrogen in a similar manner, usually in a small test tube. Then, with a stream of hydrogen passing over the solution in the reaction flask (Fig. 1), the sample is introduced through the side arm of the flask by means of a syringe. The flask is closed to give a slight positive pressure to hydrogen. The reduction of ozonides of normal straight-chain methyl esters is complete in less than 15 min. Thus, after a reaction period of about 15 min, the side arm containing the stopcock is connected to a series of three U-tubes of the type shown in Figure 1. These are packed in dry ice and filled with purified nitrogen through the T tube A (Fig. 1). The stopcock connecting the reaction flask with the U tubes is opened; simultaneously, nitrogen is passed into the apparatus by the two-way stopcock on top of the reaction flask, over the solution, and through the U-tubes. After about 15 min, the reaction flask is warmed in a water bath to facilitate the distillation of the more volatile fragments. The length of time of this operation and the temp is governed by the volatility of the fragments. This technique increases greatly the sensitivity of the method, permitting the analysis of samples of 1 mg or less.

The fragments in the traps usually are combined in about 0.5 ml of heptane or a higher boiling hydro-

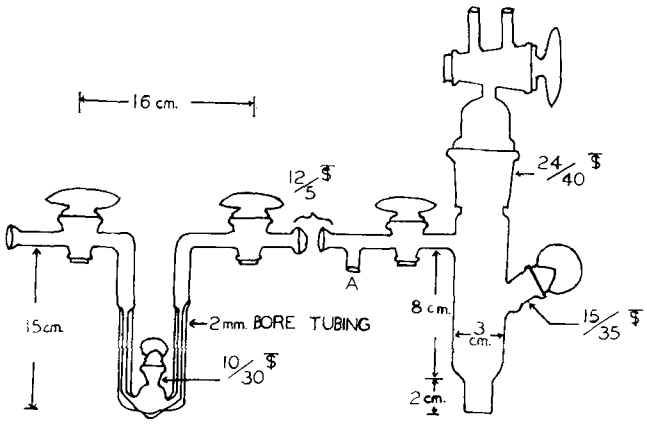

FIG. 1. U-tube and reaction flask.

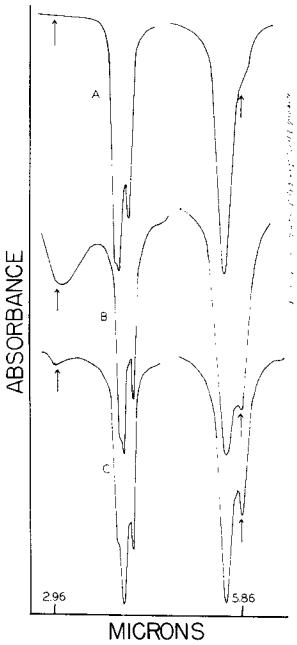

FIG. 2. Infrared spectra of the products of the ozonization of methyl oleate: (A) in pentane and (B) in ethyl acetate as described in text; (C) by bubbling oxygen containing ozone through a pentane solution of the sample.

carbon, depending on the nature of the fragments, and analyzed by GLC. The solution in the reaction flask is also analyzed by GLC in order to provide a complete analysis of the fragments.

GLC analyses were carried out with an F and M Model 609 Hydrogen Flame Apparatus (F and M Scientific Corp., Willmington, Del.), using a 12 ft x ¼ in. column packed with Chromosorb W containing 30% silicone or a 6 ft x ¼ in. column containing 15% ethylene glycol succinate polyester on Chromosorb W. The silicone phase consisted of the residue from a molecular distillation of Dow Corning Silicone Fluid 200 (viscosity 10 cs) (15). The analyses were performed using temperature programming.

Results

The infrared spectra of the products of methyl oleate ozonized by the procedure described above, (a) in pentane, (b) in ethyl acetate, (c) by bubbling oxygen containing 2–3% ozone through a pentane solution of the sample as commonly practiced, are shown in Figure 2, A, B, and C, respectively. The ozonide band (not shown) occurs at 9.05 μ.

These results showed that secondary reaction products exhibiting absorption bands in the hydroxyl and carbonyl region of the spectrum were formed, except when the reaction was carried out in pentane as directed above.

Thin-layer chromatographic analysis (Fig. 3) also showed that little true ozonide was formed when the ozonization was carried out in polar solvents.

Figure 4 shows the pertinent regions of the infrared spectra of the products of the reduction of methyl oleate ozonide by palladium on charcoal and the Lindlar catalyst. These analyses showed that appreciable amounts of alcohols and acids were formed, and the yields of aldehydes were low when the reduction was carried out with palladium on charcoal. In contrast, reduction with the Lindlar catalyst was accompanied by little side reaction.

The GLC analyses of the fragments obtained on the ozonolysis of about 10 mg. samples of linoleate, linolenate, and arachidonate are shown in Figures 5, 6, and 7, respectively. Two analyses were made on each sample: one on the distilled fragments, and one on the residual solution. In these analyses the reaction flask was warmed at a temperature of 45C for about 15 min.

Identification of malonaldehyde (Figs. 5,6,7) was made on the basis of the isolation of this compound from the products of the reductive ozonolysis of methyl linoleate. The isolation was accomplished by extracting a pentane solution of the reaction mixture with water. The slight amount of pentane and hexanol that was extracted into the water was removed by evaporation of a small amount of the water at room temperature by means of a water aspirator. Most of the malonaldehyde remained in the water under these conditions because of its high solubility in this solvent. The presence of malonaldehyde in the water was checked by analysis of an aliquot of the aqueous solution by GLC. The removal of traces of pentane and hexanol by evaporation was also followed by GLC. When the aqueous solution showed only one peak on GLC analysis corresponding to that designated as malonaldehyde in Figures 5–7, a small amount of thiobarbituric acid reagent was mixed with it. The spectral properties of the derivative that

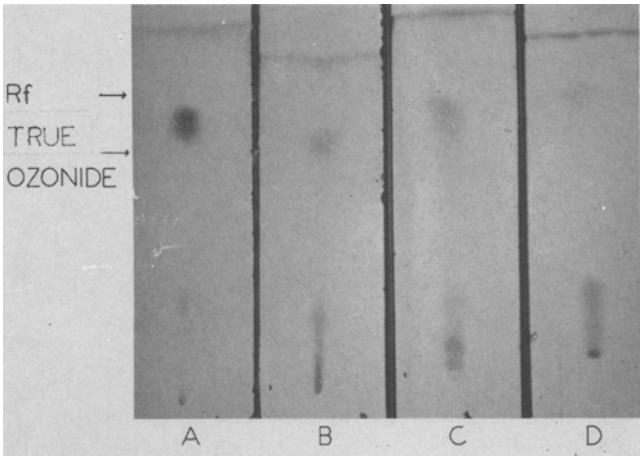

FIG. 3. TLC of the product of the ozonization of methyl oleate—1 min at −60C, in: A, pentane; B, methylene chloride; C, ethyl acetate; D, methyl alcohol. Adsorbent Silica-Gel G solvent system 20% diethyl ether in petroleum ether, B.P. 30–60C.

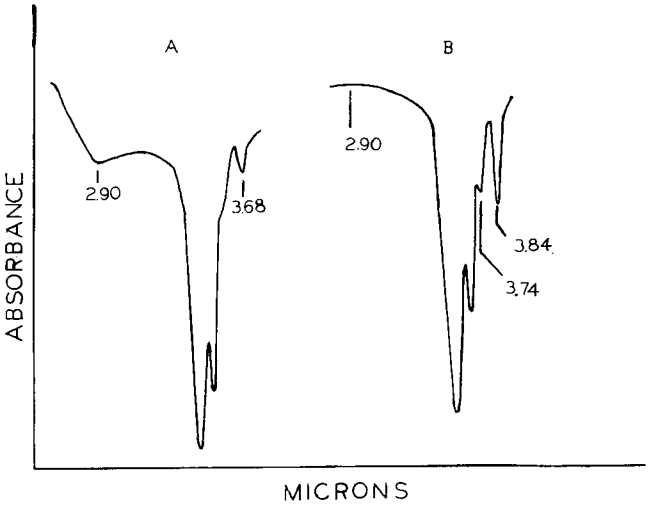

FIG. 4. Infrared spectra of the products of the reduction of methyl oleate ozonide: A, using palladium on charcoal; B, using the Lindlar catalyst.

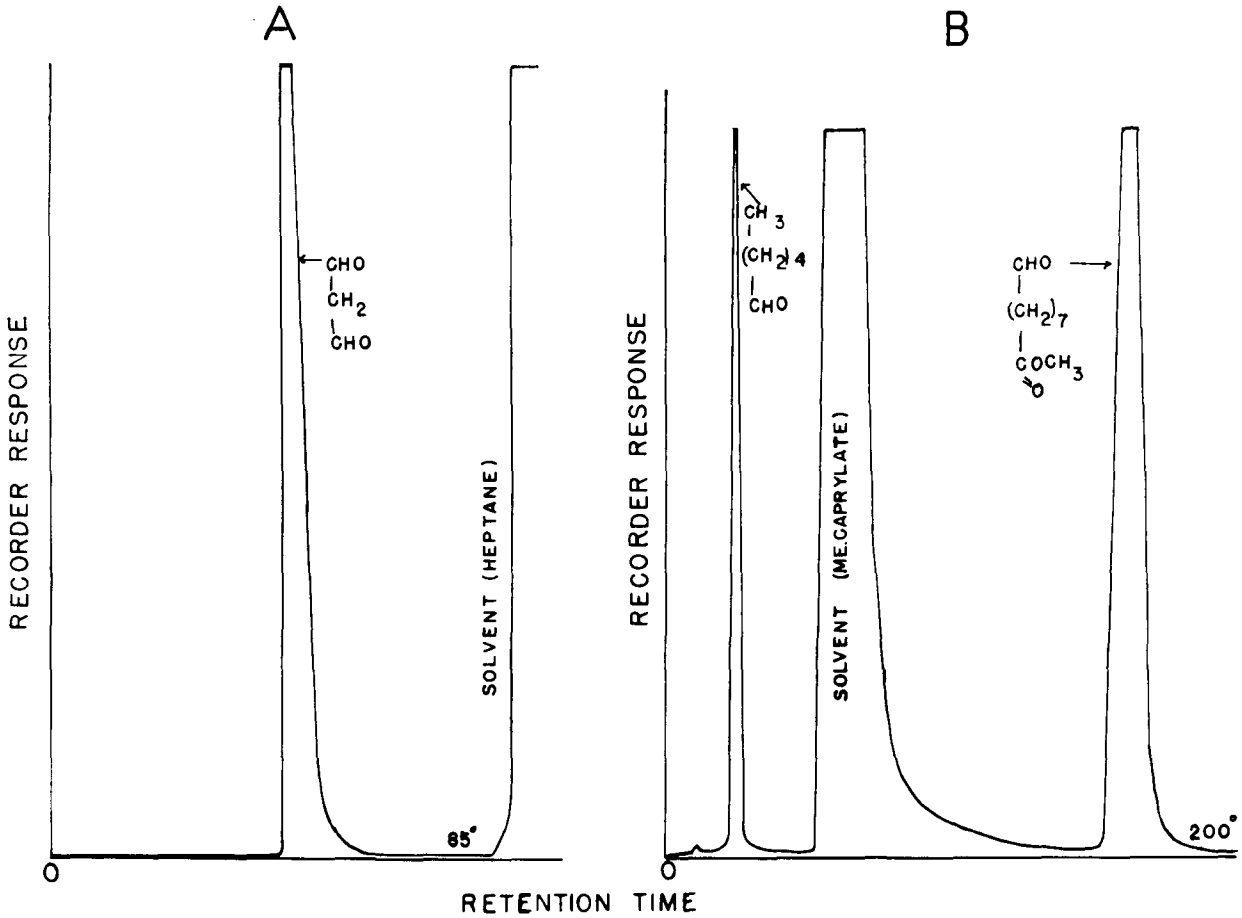

FIG. 5. GLC of the fragments of the reductive ozonolysis of methyl linoleate: A, analysis of distilled fragments; B, analysis of residual solution (methyl caprylate).

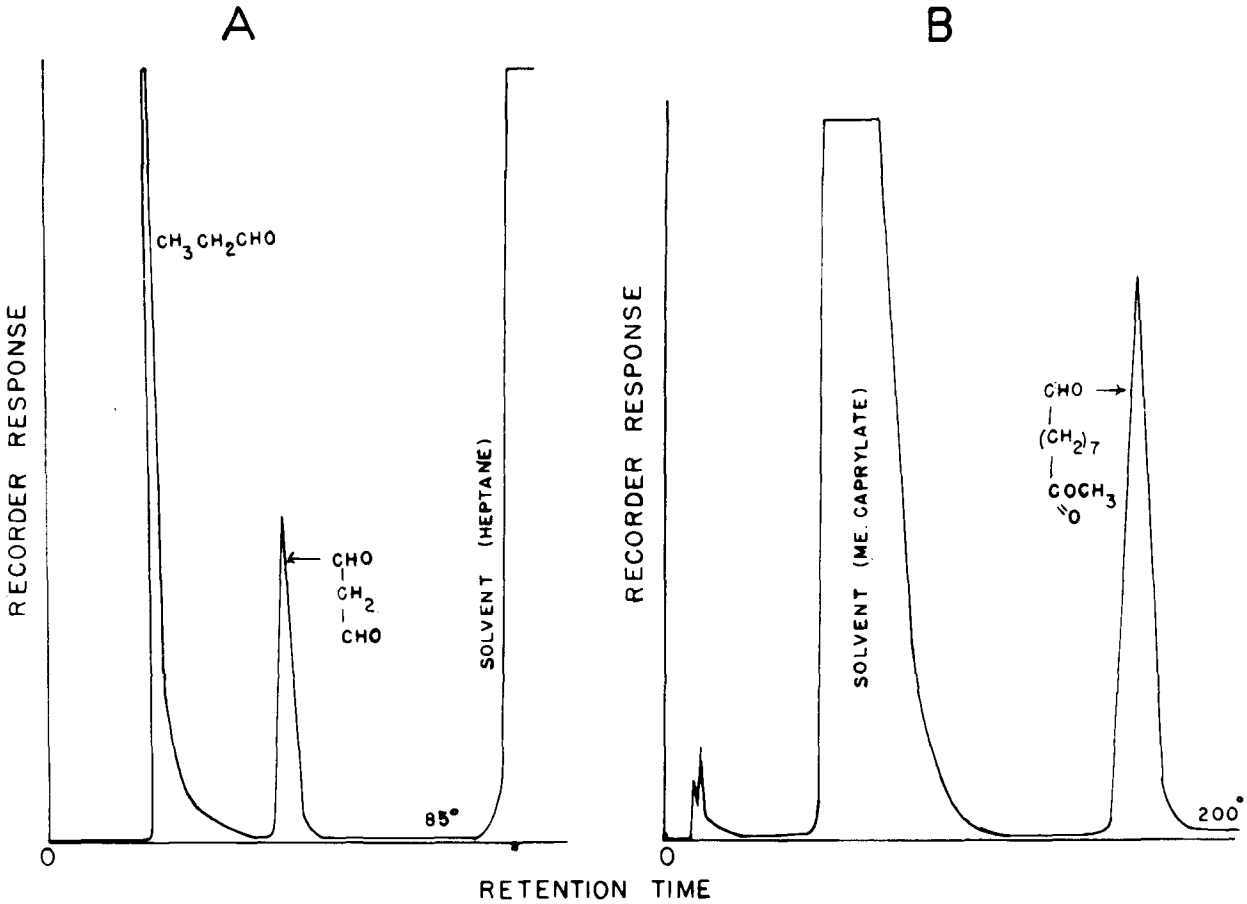

FIG. 6. GLC of the fragments of the reductive ozonolysis of methyl linolenate: A, analysis of the distilled fragments; B, analysis of residual solution (methyl caprylate).

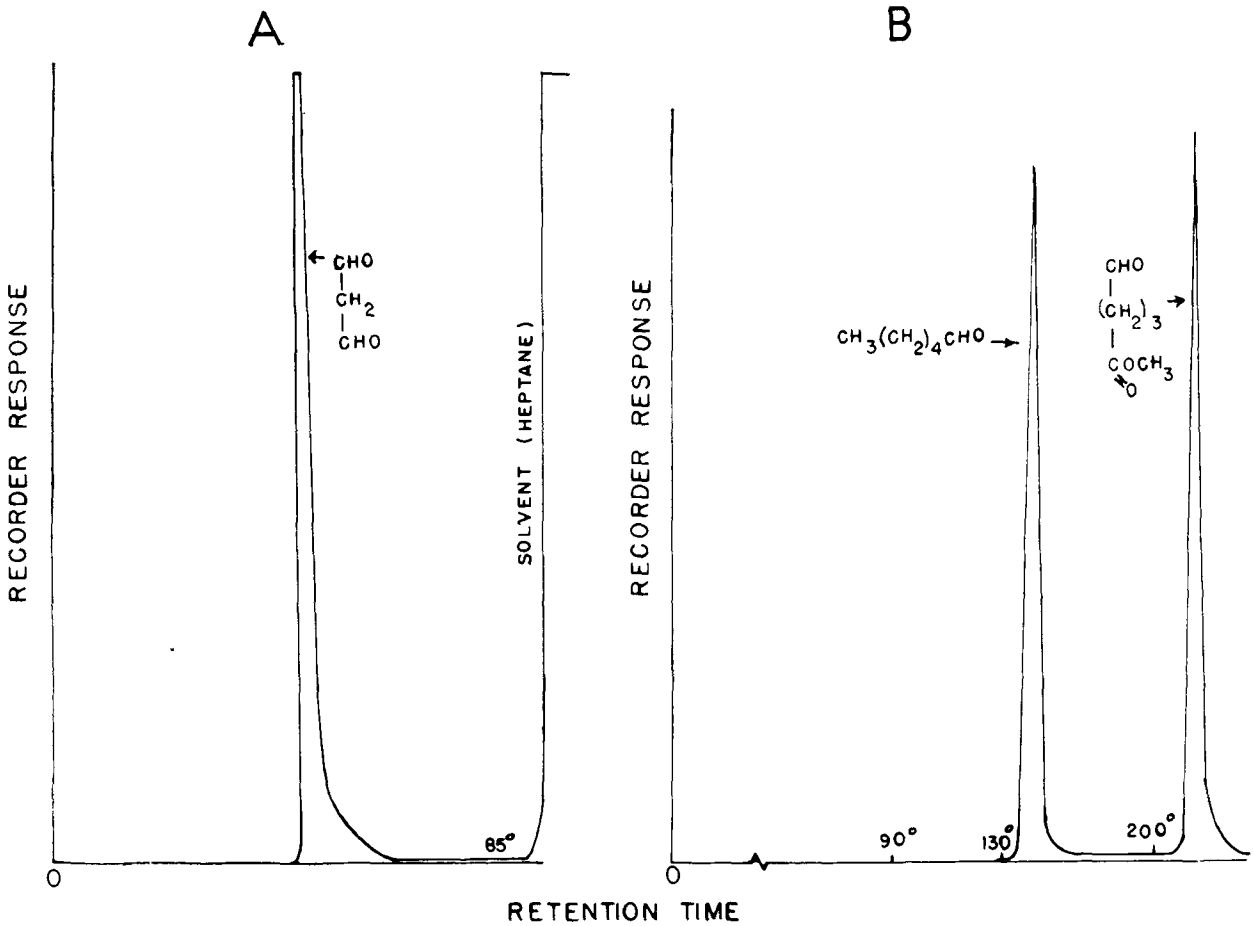

FIG. 7. GLC of the fragments of the reductive ozonolysis of methyl arachidonate: A, analysis of the distilled fragments; B, analysis of the residual solution (dimethyl phthalate).

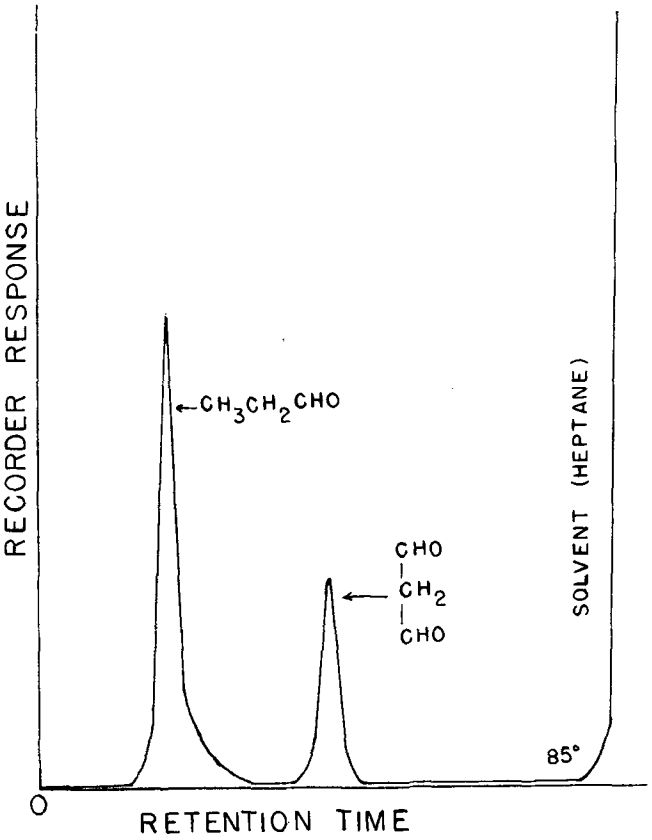

FIG. 8. GLC of the distilled fragments of the reductive ozonolysis of methyl oleate containing 2% methyl linolenate.

formed were identical to the malonaldehyde-TBA derivative reported by Sinnhuber et al. (17).

The sensitivity of the method is demonstrated in Figure 8 by the results on a 25 mg sample of methyl oleate containing 2% added methyl linolenate. The peaks for propional and malonaldehyde (Fig. 8) which originate from the methyl linolenate, are easily detected.

Figure 9 shows the GLC analyses of the products of the reductive ozonolysis of a mixture of methyl oleate and methyl petroselinate on polar and nonpolar phases. These analyses were performed to demonstrate that simple aldehydes and half-ester-half-aldehyde fragments may be distinguished from each other in a mixture by this technique.

Quantitative analysis of several mixtures of methyl oleate and methyl petroselinate are presented in Table I.

TABLE I

Analyses of Mixtures of Methyl Oleate and Petroselinate by Reductive Ozonolysis

Mixture	Composition	
	Known	Found (*GLC analysis of fragments)
	(% wt.)	*(% area of total area)*
A	48.8 oleate 51.2 petroselinate	49 51
B	74.2 oleate 25.8 petroselinate	73 27
C	25.5 oleate 74.5 petroselinate	27 73

* The areas of the peaks for nonanal and methyl nonanoate-9-al, were used to calculate the percentage of methyl oleate. The areas of the peaks for dodecanal and methyl hexanoate-6-al were used to calculate the percentage of methyl petroselinate.

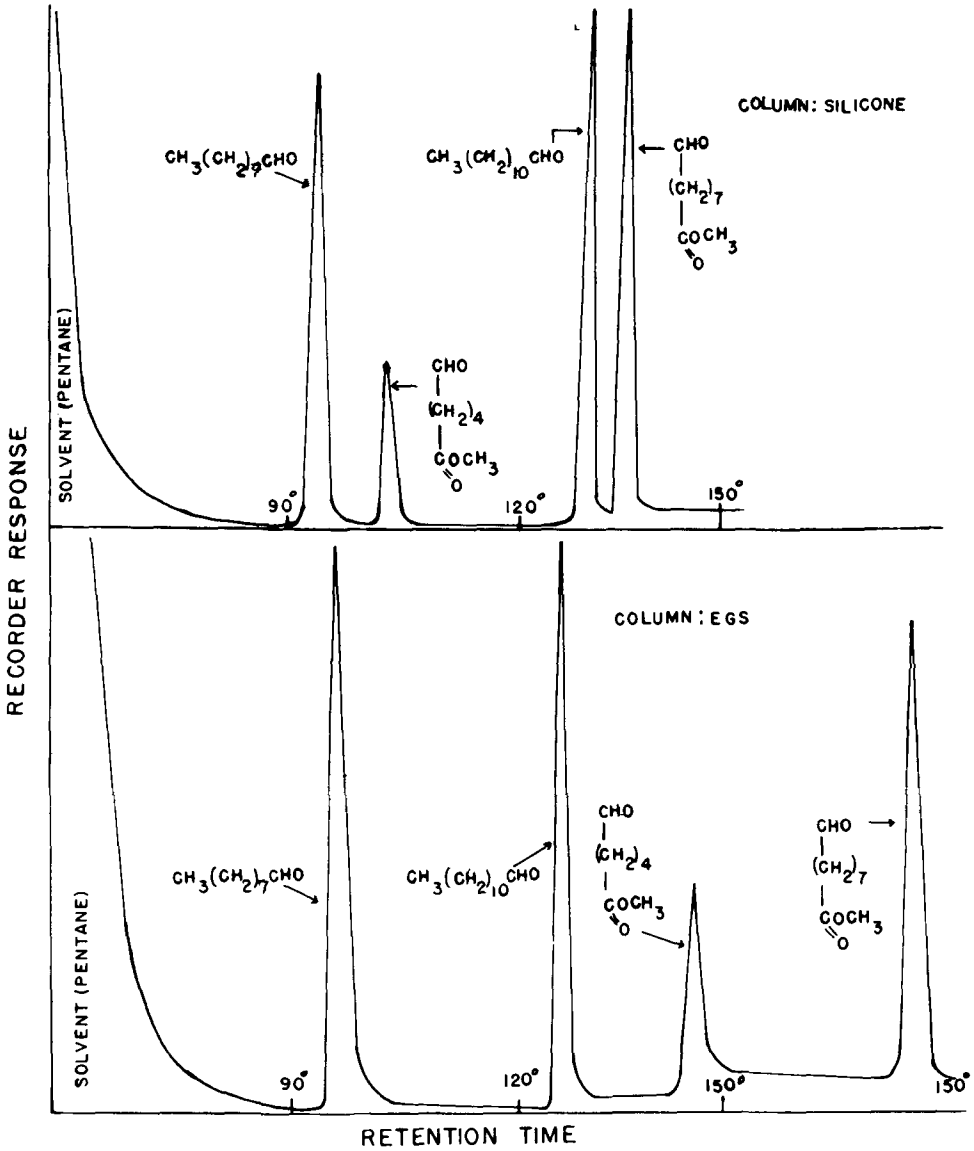

FIG. 9. GLC of the fragments of the reductive ozonolysis of an equal molar mixture of methyl oleate and methyl petroselinate on nonpolar (silicone) and polar (ethylene glycol succinate polyester) phases.

Discussion

Perhaps the most significant feature of the method of ozonolysis reported here is that it permits, essentially, a complete analysis of the fragments corresponding to the position of all of the double bonds because both the ozonization and reduction are uncomplicated by side reactions.

TLC played an important role in working out the conditions of the method, because by means of it, the extent and nature of both the ozonization and reduction could be analyzed.

Simple aldehydes may be identified by comparison with reference compounds which are available from commercial sources. Although half-ester-half-aldehyde standards are not readily available, calibration curves of these compounds, as well as simple aldehydes, can be prepared from the analyses of selected methyl esters.

Since the ozonization and subsequent reduction are essentially free of side reactions and no fractionation procedures need be used which result in loss of fragments, the method may be used for quantitative analysis of mixtures of fatty acid esters. Its greatest value may be for the analysis of mixtures of methyl esters such as positional isomers which cannot be separated quantitatively by GLC. The results of the analyses of mixtures of methyl oleate and petroselinate demonstrate this potential of the method.

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