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Notes on Methodology

Structure determination of methyl esters of unsaturated fatty acids by gas-liquid chromatography of the aldehydes formed by triphenyl phosphine reduction of the ozonides*

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» Ozonolysis, a useful method for determining positions of unsaturation, has been extensively reviewed and its mechanisms described (1, 2). Although both oxidative and reductive methods have been used for cleaving the ozonolysis products from unsaturated fatty acids, it appears that reduction offers the simpler method when used in conjunction with gas-liquid chromatography. The aldehyde or ketone products from such a reaction sequence do not require a subsequent modification (methyl esterification) before gas chromatography, and the carbonyl compounds are free of over-oxidation products. One widely used method of reduction is that of hydrogenation in the presence of a poisoned catalyst, the usefulness of which has been exemplified with the cleavage of fatty acids (3, 4). Triphenyl phosphine has been shown to be equally useful for the reduction of ozonolysis products (5). In this communication we will show that the use of triphenyl phosphine following ozonolysis of unsaturated fatty esters offers a rapid and simple method of obtaining oxidation products that may be identified by gas-liquid chromatography and are free of by-products.

The unsaturated fatty ester, 1-10 mg dissolved in 1-2 ml of methylene chloride at −65° or methyl caprylate at −35° to −20°, is ozonized by bubbling in a stream of dry oxygen containing approximately 1% ozone. The ozonolysis is performed in 12-ml centrifuge tubes with a simple glass tube drawn to a fine tip as a bubbler, or in a gas bubbling device containing a fritted disc for dispersion of the gas (JM-6615, Scientific Glass Apparatus Co., Bloomfield, N. J.). Ozonization is continued until a blue color develops in the solution at −65° or for about 30 sec after the effluent gas contains ozone (total time 5–10 min). After the excess ozone and oxygen are purged by dry nitrogen, the ozonide is reduced by the addition of a two- to three-fold equivalent excess of triphenyl phosphine either as crystals or dissolved in the solvent being used. The reaction vessel is removed from the cold bath and warmed to room temperature, and a sample of the reaction mixture is injected directly into the gas chromatograph without further treatment.

The choice of solvent will depend upon the ozonolysis products to be examined. Dichloromethane as solvent for the fatty esters permits a gas chromatographic examination of the aldehyde ester fragments, but aldehydes eluted earlier than caproaldehyde are hidden in the solvent front. When the aldehydes are to be examined, methyl caprylate is used, permitting the elution of the short-chain compounds before the chromatogram is obscured by the solvent. Because of the poorer solvent properties and relatively high melting point (−43°) of methyl caprylate, the ozonization in this solvent is performed at −35° to −20° instead of at −65° as with dichloromethane.

The ozone generator used in this study was similar to that of Henne and Perilstein (6). The primary of the high-voltage transformer (Acme Electric Co., Cuba, N.Y., Cat. No. 6015) was attached to a variable transformer. At an oxygen flow of 12 ml/min, the ozone concentration varied from 0.6–1.4 mole % with a primary voltage of 90 and 110 v, respectively.

The gas chromatographic runs were made with a Barber-Coleman Model 10 chromatograph using an argon ionization detector, with radium as the ionizing source.

The methyl esters of some naturally occurring unsaturated fatty acids as well as some synthetic octadecenoic acids[1] were used originally to determine the elution characteristics of the aldehydes and aldehyde esters. Following a selenium isomerization of linoleic acid, Dr. G. A. Dhopeshwarkar of this laboratory isolated a crystalline C_{18} monoene fatty acid mixture that contained all of the positional isomers between positions 6 and 14. After the various aldehydes and aldehyde esters obtained by ozonolysis were identified, the mix-

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[1] Methyl arachidonate was obtained from Hoffmann LaRoche, Inc., Nutley, N. J. Methyl *trans*-6- and *trans*-7-octadecenoate were gifts from Dr. Robert J. Meyer, Morton Salt Co., Chicago, Ill. Methyl elaidate was obtained from California Corp. for Biochemical Research, Los Angeles. Methyl vaccenate and methyl *trans*-12-octadecenoate were obtained from the mixture that comprises the "vaccinic" acid from Nutritional Biochemical Corp., Cleveland, Ohio.

TABLE 1. RELATIVE RETENTION TIMES OF ALDEHYDES AND ALDEHYDE METHYL ESTERS OBTAINED BY OZONOLYSIS OF ISOMERIC METHYL OCTADECENOATES

Chain Length	Retention Time: Aldehydes Relative To Methyl Pelargonate*	Retention Time: Aldehyde Methyl Esters Relative to Methyl Stearate†
C_6	0.17	0.33
C_7	0.26	0.45
C_8	0.43	0.61
C_9	0.67	0.81
C_{10}	1.05	1.09
C_{11}	1.67	1.45
C_{12}	2.60	1.93
C_{13}		2.55
C_{14}		3.38

* Data obtained at 129° and 22.5 ml argon/min using a 10-ft Pyrex column packed with 17% ethylene glycol–succinic acid polymer on 80–100 mesh Johns-Manville Chromosorb deactivated with dimethyldichlorosilane.

† Data obtained at 183° and 28.3 ml argon/min using a 3-ft Pyrex column containing 12% ethylene glycol–succinic acid polymer on 60–100 mesh Johns-Manville Chromosorb deactivated with dimethyldichlorosilane.

ture of monoenes was used as a single source for the relative elution data presented in Table 1.

The chromatograms gave no indication that by-products are formed by alternative oxidation pathways. In confirmation of this observation, methyl elaidate, completely free of impurities detectable by gas chromatography, gave only the expected products after ozonolysis. Impurities, which would indicate over-oxidation, were not found by using conditions that would have revealed less than 1% of contaminants.

The completeness of the conversion of the unsaturated fatty acid esters to the carbonyl compounds was estimated by including in the ozonolysis solutions a known amount of methyl stearate, methyl pelargonate, or methyl caproate as internal standards. A comparison of the peak areas expected and found is presented in Table 2. Because the detector was not calibrated with respect to the relative response of saturated esters as compared to aldehydes or aldehyde esters, these results are only semi-quantitative. But they do show that the yield of cleavage products was very near the expected values except when the ozonolysis was performed at 0° where there was a definite decrease in yield. Gas chromatograms of reaction solution stored at −20° for 5 days were not materially different.

Malondialdehyde, the product formed by reductive ozonolysis of polyunsaturated fatty acids containing double-bonded carbons separated by a methylene group, had a retention time of 0.34 relative to caproaldehyde.

TABLE 2. RECOVERIES OF PRODUCTS OF OZONOLYSIS OF METHYL ESTERS OF UNSATURATED FATTY ACIDS

Substrate*	Compounds Compared	Theoretical Mass Ratio	Chromatographic Peak Area Ratio (uncorrected)†
Methyl oleate containing methyl stearate and pelargonate at 0°	C_9 aldehyde ester / methyl stearate	0.89	0.32
	pelargonaldehyde / methyl pelargonate	0.63	0.38
Methyl oleate containing methyl stearate and pelargonate at −65°	C_9 aldehyde ester / methyl stearate	0.81	0.74
		0.94	1.20, 1.12‡
	pelargonaldehyde / methyl pelargonate	0.63	0.62, 0.60§
		0.60	0.67
Methyl linoleate containing methyl stearate and caproate at −20°	C_9 aldehyde ester / methyl stearate	0.84	0.93
	caproaldehyde / methyl caproate	0.40	0.40
Methyl linolenate containing methyl stearate at −35°	C_9 aldehyde ester / methyl stearate	0.85	0.94

* Methyl oleate was obtained from the Hormel Foundation, Austin, Minnesota. Methyl linoleate and linolenate were obtained from Mann Research Laboratories, New York City.

† The aldehyde data were obtained at 136° and 23–40 ml argon/min using a 10-ft Pyrex column packed with 17% ethylene glycol–succinic acid polymer on 80–100 mesh Johns-Manville Chromosorb deactivated with dimethyldichlorosilane. The aldehyde ester data were obtained at 201° and 78.5 ml argon/min using a 6-ft column packed with 13% ethylene glycol–succinic acid polymer on 60–100 mesh fire brick deactivated with dimethyldichlorosine.

‡ Duplicate runs.

§ Stored at −20° for 5 days.

Since both malondialdehyde and caproaldehyde are formed in the reductive ozonolysis of linoleic and arachidonic esters, those aldehydes may be used for identification purposes. However, the response of the argon detector to malondialdehyde is very low compared to that of caproaldehyde. Arachidonic acid theoretically yields approximately twice the mass of malondialdehyde as of caproaldehyde, but the chromatogram had seven times as large an area for caproaldehyde as for malondialdehyde. Propionaldehyde and malondialdehyde, from the ozonolysis of methyl linolenate, were not resolved on the columns used. Propionaldehyde was eluted with a retention time of 0.36 relative to caproaldehyde.

Triphenyl phosphine has a retention time relative to methyl stearate of 9.7 and thus does not normally interfere with chromatography of the ozonolysis products, but allowance should be made for its elution during subsequent analyses. The triphenyl phosphine oxide

reaction product is eluted after the phosphine on nonpolar columns, but its elution has not been observed from the polyester columns used in this study.

Although the graphs of the log of the retention time *versus* the carbon chain length for the aldehydic products always formed a straight line nearly parallel to that for the methyl esters of saturated fatty acids, the retention of the aldehydes relative to the methyl esters of fatty acids differed with individual columns. On a similarly prepared column, for example, the retention times for the aldehyde esters relative to the methyl esters of fatty acids were 85% of those reported in Table 1. This was probably due to the use of a different batch of ethylene glycol–succinic acid polymer in the column packing and a different "in use" age for the column. The simple expedient of chromatographing known standards will prevent a difference of this nature from interfering with identifications.

In investigating fatty acid structures, one is often concerned with the possible presence of a small percentage of isomeric compounds. Many degradative methods give a mixture of products that precludes identification of such minor components. The method described here, however, gives products in yields that are nearly stoichiometric and free from by-products. The method has the further advantages of being rapid and requiring a minimum of manipulation and sample.

The only potential exposure to triphenyl phosphine (a toxic compound) that is not easily controlled by the use of a hood is contact with the effluent from the gas chromatographic column. To avoid this, the effluent should be trapped.

It has been reported (7) that phosphoric acid causes corrosion of flame ionization detectors. It is possible that triphenyl phosphine yields phosphoric acid in this type of cell and could result in damage.

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