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GAS–LIQUID CHROMATOGRAPHIC IDENTIFICATION OF OZONOLYSIS FRAGMENTS AS A BASIS FOR MICRO-SCALE STRUCTURE DETERMINATIONS

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SUMMARY

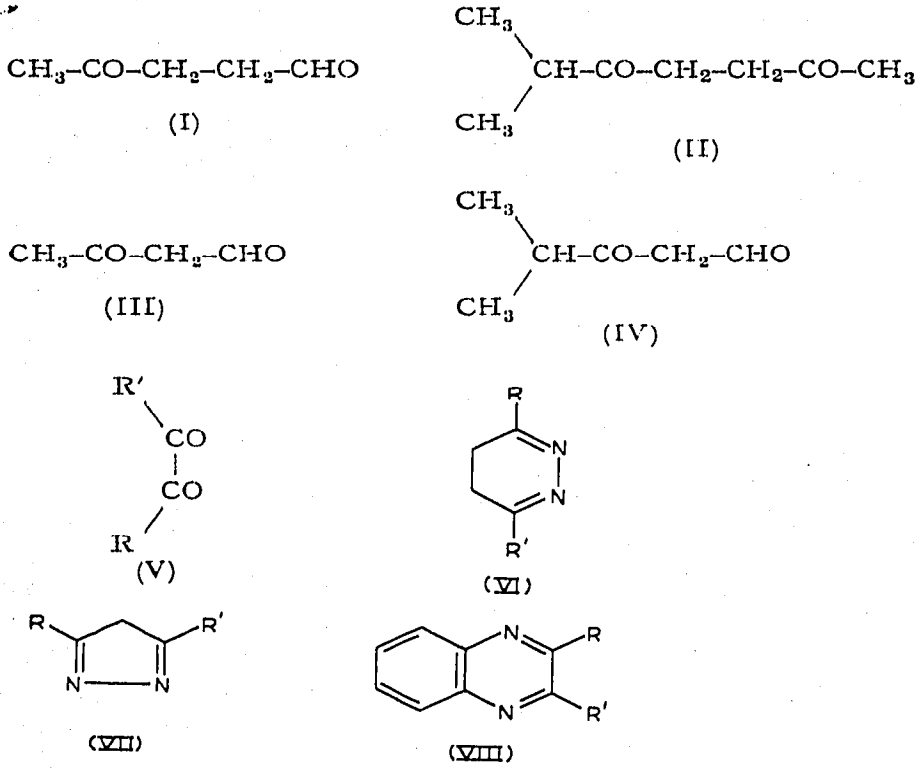
Satisfactory gas–liquid chromatographic procedures have been developed for the identification of twelve molecular fragments that commonly result from micro-scale ozonolysis of terpenes and similar unsaturated compounds. Test runs with model compounds indicate that the method can give useful information concerning unknown structures with as little as 10 μg of material.

INTRODUCTION

Ozonolysis has provided a very valuable approach to problems of structural chemistry but the identification of the resulting molecular fragments has generally involved macro-manipulative methods (determination of physical properties, preparation of derivatives, etc.) and there have been comparatively few reports of a successful scaling down of the technique. However, gas chromatographic analysis has been employed to a limited extent for this purpose and a recent paper by BEROZA AND BIERL[1] describes such a micro-method for locating isolated double bonds in open-chain compounds.

In our work on insect pheromones, we are frequently faced with structural problems concerning polyunsaturated substances, such as terpene hydrocarbons, which are seldom available in more than milligram quantities, and the development of a suitable micro-ozonolysis/gas chromatographic approach has been of prime importance.

The purpose of the present paper is therefore to describe techniques that we have found suitable for the small-scale generation and detection of the salient mono- and di-functional ozonolysis fragments to be expected from terpenes and related substances. By these means, information may be obtained from 10–20 μg of material in most cases, but with the more complex molecules, 2–3 runs, with differing treatments of the ozonide, may be necessary in order to cover the full range of possible fragments. However, in our experience, dialdehydes (except glyoxal) and most tri- and poly-functional derivatives have not proved amenable to gas–liquid chromatography (GLC).



The following molecular fragmentation derivatives are covered by the present methods: formaldehyde, acetaldehyde, acetone, laevulinaldehyde (I), 6-methylheptan-2,5-dione (II), butan-1-al-3-one (III), 4-methylpentan-1-al-3-one (IV), glyoxal (V, R = R′ = H), methylglyoxal (V, R = H, R′ = CH_3), ethylglyoxal (V, R = H, R′ = C_2H_5), isopropylglyoxal (V, R = H, R′ = $CH(CH_3)_2$) and dimethylglyoxal (V, R = R′ = CH_3). Higher aldehydes (up to C_{10}) are dealt with by BEROZA AND BIERL[1].

EXPERIMENTAL

Gas chromatography

All analyses were carried out on a Carlo Erba Fractovap GB gas chromatograph, fitted with dual flame ionisation detectors and a Hewlett-Packard 3370-A digital integrator. Glass columns (1 or 2 m × 4 mm I.D.) were used and the nitrogen carrier gas flow rate was 30 ml/min. Preparative separations were performed on a Loenco Prep-matic machine with stainless steel columns (2 m × 1 cm I.D.) and thermal conductivity detectors.

The following analytical columns were used: Column A, 2 m of 5 % Carbowax-20M (polyethylene glycol) on Gas-Chrom Z; Column B, 2 m of 5 % OV-225 (cyanopropyl, trifluoropropyl silicone polymer) on Gas-Chrom Z; Column C, 2 m of Porapak P (cross-linked vinylbenzene polymer); Column D, 2 m of Porapak Q (cross-linked vinylbenzene polymer); Column E, 1 m of 1 % Carbowax-20M on Gas-Chrom Z; preparative column, 20 % Carbowax-20M on Gas-Chrom Z.

All columns were conditioned overnight at 220° but operating temperatures varied widely and are indicated in Table I.

TABLE I

SUMMARY OF MICRO-OZONOLYSIS CONDITIONS

Fragment	*Test substance (quantity μg)*	*Solvent*	*Ozonolysis temperature*[a]	*Post-ozonolysis treatment*	*Column and operating temperatures (°C)*
Formaldehyde	carvone (20)	CS_2	D	a	E, 200
	limonene (20)	CS_2	D	a	E, 200
Acetaldehyde	crotonic acid (20)	EtOAc	D	b	C, 110; D, 150
	crotonic acid (20)	EtOAc	D	c	A, 70 for 10 min, then up 4/min
Acetone	farnesol (20)	EtOAc	D	b	C, 110; D, 150
	farnesol (20)	EtOAc	D	c	A, 70 for 10 min, then up 4/min
Laevulinaldehyde (I)	farnesol (10)	CS_2, EtOAc	D	b	A, 60, up 4/min
	squalene (20)	CS_2, EtOAc	D	b	A, 60, up 4/min
	farnesol (10)	EtOAc	D	c	A, 70 for 10 min, then up 4/min
	squalene (20)	EtOAc	D	c	A, 70 for 10 min, then up 4/min
6-Methylheptan-2,5-dione (II)	α-terpinene (10)	CS_2, EtOAc	D	b	A, 60, up 4/min
	α-terpinene (10)	EtOAc	D	c	A, 70 for 10 min, then up 4/min
Butan-1-al-3-one (III)	γ-terpinene (20)	CS_2, EtOAc	D	b	A, 60, up 4/min
	γ-terpinene (20)	EtOAc	D	c	A, 70 for 10 min, then up 4/min
4-Methylpentan-1-al-3-one (IV)	γ-terpinene (20)	CS_2, EtOAc	D	b	A, 60, up 4/min
	γ-terpinene (20)	EtOAc	D	c	A, 70 for 10 min, then up 4/min
Glyoxal (V, R = R′ = H)	*p*-cymene (20)	EtOAc	A	d	A or B, 150
	ethylbenzene (20)	EtOAc	A	d	A or B, 150
	o-xylene (20)	EtOAc	A	d	A or B, 150
	α-terpinene (20)	EtOAc	D	a	A or B, 150
Methylglyoxal (V, R = H, R′ = Me)	α-phellandrene (20)	EtOAc	D	d	A or B, 150
	p-cymene (20)	EtOAc	A	d	A or B, 150
	o-xylene (20)	EtOAc	A	d	A or B, 150
Ethylglyoxal (V, R = H, R′ = Et)	ethylbenzene (20)	EtOAc	A	d	A or B, 150
Dimethylglyoxal (V, R = R′ = Me)	*o*-xylene (20)	EtOAc	A	d	A or B, 150
Isopropylglyoxal (V, R = H, R′ = Isopr)	*p*-cymene (20)	EtOAc	A	d	A or B, 150

[a] D = dry-ice (−70°); A = ambient (20°).

Solvents

Carbon disulphide was "spectroquality reagent" supplied by Matheson, Coleman and Bell, and ethyl acetate was "nanograde quality" by Mallinckrodt. These solvents were shown by GC to give blanks of acceptable background without the need for further purification.

Test materials and reagents

All test materials listed in Table I were commercial samples, with the exception of α- and γ-terpinenes and α-phellandrene, which were gas chromatographically purified specimens prepared in connection with another investigation[2]. Triphenylphosphine was supplied by Fluka AG (Switzerland) and was powdered finely before use; cyclohexane-1,3-dione (dihydroresorcinol) and *o*-phenylenediamine were not available commercially in sufficiently pure form and needed to be recrystallized to constant m.p. for reliable results.

Ozonolysis

Ozonolyses were conducted in small, cylindrical (5 cm × 1 cm O.D.) bubblers with a tapered inlet tube reaching almost to the bottom. The operating temperature was normally that of a carbon dioxide snow-bath (*ca.* —70°) but aromatic compounds required somewhat higher temperatures and were best run under ambient conditions. Ozonized oxygen from a standard laboratory generator was dried by passage through a tube of anhydrous calcium chloride and then led into the reaction vessel for 2–5 min, according to the size of the sample (10–100 μg). The mixture was then accorded one of four possible treatments, according to the nature of the fragments expected.

Treatment (a) for formaldehyde: the solvent was removed by aeration, an aqueous solution (50 μl) of cyclohexane-1,3-dione (300 μg–1 mg) was then added and the mixture heated on a water-bath for 15 min. Next, the water was removed under reduced pressure and replaced by a little carbon disulphide, when the mixture was ready for GC analysis (Column E).

Treatment (b) for simple aldehydes and ketones (see Table I): a little triphenylphosphine was added to the cold ozonolysis mixture, which was then ready for analysis.

Treatment (c) confirmatory test for fragments identified by treatment (b): the ozonolysis mixture was warmed gently and aerated to remove excess ozone, then treated with an excess of hydrazine hydrate (2 μl), shaken and warmed; 2.5 μl portions of the mixture were then used for analysis.

Treatment (d) for glyoxal and its homologues: the ozonolysis mixture was warmed gently and aerated to remove solvent and excess ozone. The solvent was then made good with fresh material and a little solid *o*-phenylenediamine was added. The mixture was warmed for a few minutes and 2.5 μl portions were then used for analysis. Note: it is essential that excess ozone be completely removed before the addition of the diamine, or ozonolysis derivatives of the latter will appear in the chromatogram.

RESULTS AND DISCUSSION

The method of BEROZA AND BIERL[1] involves ozonolysis of a few micrograms of unsaturated material at —70°, in carbon disulphide or pentyl acetate and reduction

of the ozonide so formed, *in situ*, with triphenylphosphine to give free aldehydes or ketones, which are then detected directly by GC. We have found that this technique also gives satisfactory results with the following bifunctional terpene fragments: laevulinaldehyde (I), 6-methylheptan-2,5-dione (II), butan-1-al-3-one (III) and 4-methylpentan-1-al-3-one (IV). We used ethyl acetate or carbon disulphide as solvent and obtained the best analyses on Carbowax 20M (Column A), temperature-programmed upwards from 60°. Retention times and methylene unit (MU) values[3] are given in Table II.

TABLE II

RETENTION TIMES ON 5% CARBOWAX-20M (COLUMN A)[a]

Ozonolysis fragment	*Retention time (min)*	*MU value*
Butan-1-al-3-one (III)	9.50	12.14
4-Methylpentan-1-al-3-one (IV)	11.29	12.78
Laevulinaldehyde (I)	16.62	14.67
6-Methylheptan-2,5-dione (II)	19.63	15.78

[a] Temperature programmed 60–140° at 4°/min.

TABLE III

RETENTION TIMES ON PORAPAKS P AND Q (COLUMNS C AND D)

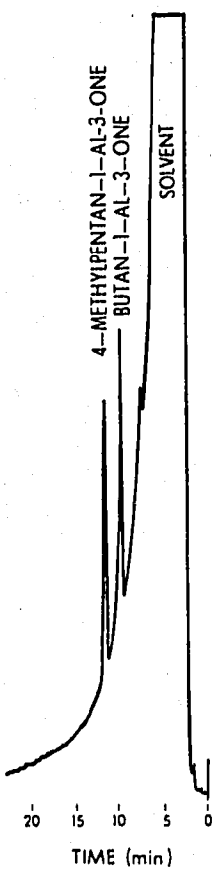
Ozonolysis fragment	*Retention time (min)*	
	Porapak P (110°)	*Porapak Q (150°)*
Acetaldehyde (*ex* ozonolysis)	3.18	3.92
Acetaldehyde (authentic)	3.11	3.82
Acetone (*ex* ozonolysis)	6.80	9.67
Acetone (authentic)	6.75	9.53
Ethyl acetate[a]	17.58	22.50

[a] Small injection; when used as a solvent the retention time was shortened by overloading.

At long retention time (*ca.* 30 min) all trial runs conducted under these conditions (including blanks) showed one large peak that did not interfere with the analysis; otherwise, there were no significant peaks other than those of expected products. Runs with compounds not expected to give rise to any of the four bifunctional derivatives gave blank results. Fig. 1, showing the result from ozonolysis of 20 μg of γ-terpinene to give III and IV, serves as an example.

For the detection of acetaldehyde and acetone residues, we prefer ethyl acetate as the ozonolysis solvent and Porapaks P and Q (Columns C and D) for analysis. Conditions and retention times are given in Table III. Blanks and runs with limonene showed only minor peaks due to impurities remaining in the solvent; these did not interfere seriously with those due to acetaldehyde or acetone. Fig. 2 shows the detection of acetone from ozonolysis of farnesol (20 μg).

Although the above technique gives useful indications of the nature of unsatu-



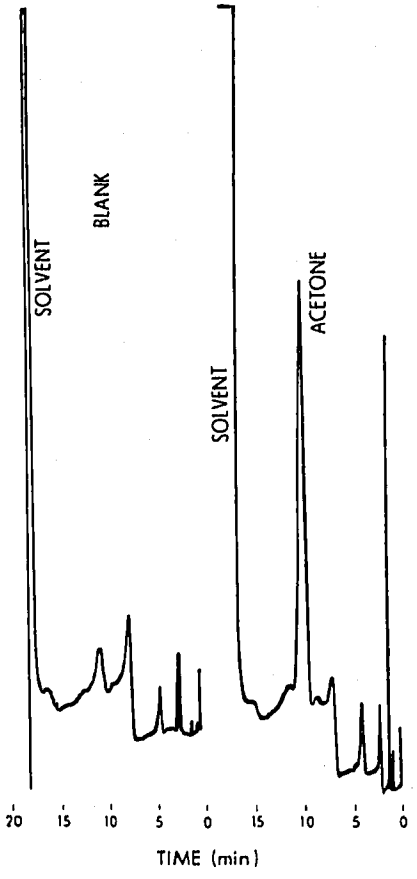


Fig. 1. Gas chromatogram of ozonolysis products of 20 μg γ-terpinene; column A, 60–150°, programmed at 4°/min.

Fig. 2. Gas chromatogram of ozonolysis products of 20 μg farnesol, and ozonolysis blank; column D, 150°.

rated residues in unknown compounds, some confirmatory alternative analysis would be advantageous.

We therefore investigated the GC properties of the hydrazine derivatives of the above-mentioned carbonyl-containing molecular fragments. With acetaldehyde and its homologues, hydrazine hydrate gives the azine directly but with acetone and similar ketones, the hydrazone or the azine, according to which reagent is in excess. With the difunctional fragments, heterocyclic compounds (pyridazinines or pyrazoles) were formed. All of these derivatives have proved amenable to GLC and our trials have shown that they are formed smoothly from the products of micro-ozonolyses. However, under our conditions (treatment c) simple ketones appear only as the hydrazones. The relevant retention times are shown in Table IV.

The identities of the products from micro-ozonolyses, after treatment by our method c, were confirmed either by direct comparison of retention times with authentic samples (derivatives of acetaldehyde, acetone) or by mass spectral analysis. With pyridazinines (VI), mass spectra show very little of the molecular ion, but a very large peak at M-2 is due to ready aromatization of the ring; the pyrazoles (VII) give normal mass spectra.

In practice, this alternative method gave very good results, provided the analysis was performed without undue delay, only expected peaks were seen (limonene gave no major peak) and blanks had a low background level. However, when the hydrazine-containing mixture was allowed to stand, an additional peak (MU 18.47) gradually developed; this was shown to be due to acetohydrazide, by direct comparison with an authentic sample. The formation of this material is doubtlessly due to slow interaction of excess hydrazine with the solvent. Sample chromatograms of micro-ozonolyses worked up by this method are given in Figs. 3–5.

TABLE IV

RETENTION TIMES OF HYDRAZINE DERIVATIVES ON CARBOWAX-20M (COLUMN A)

Derivative of	*Structural type*	*Retention time (min)*[a]		*MU value*
		a	*b*	
Acetaldehyde (*ex* ozonolysis)	azine	10.13		11.86
Acetaldehyde (authentic)	azine	10.11		
Acetone (*ex* ozonolysis)	hydrazone	13.63		12.37
Acetone (authentic)	hydrazone	13.67		
Laevulinaldehyde	pyridazinine	26.49	5.10	15.94
6-Methylheptan-2,5-dione	pyridazinine	29.54	7.60	17.00
Butan-1-al-3-one	pyrazole	32.67	11.93	18.14
4-Methylpentan-1-al-3-one	pyrazole	34.82	16.97	19.01
Ethyl acetate	acetohydrazide		13.62	18.47

[a] a = isothermal at 70° for 10 min, then programmed at 4°/min; b = isothermal at 140°.

The combined data from this treatment and from treatment (b) afford good evidence of the existence, in an unidentified compound, of the fragments concerned.

Glyoxal and its homologues, which result from ozonolysis of conjugated unsaturated systems, readily react, *in situ*, with *o*-phenylenediamine to form quinoxalines (VIII) and these derivatives proved entirely suitable for the purposes at hand. The relevant retention times are given in Table V. Quinoxaline and its methyl homologue were confirmed by direct comparison with authentic samples.

With this technique, it was essential to remove, by aeration of the warm reaction mixture, all traces of excess ozone prior to addition of the *o*-phenylenediamine, otherwise some ozonolysis of this reagent occurred, with the generation of a spurious quinoxaline peak in the chromatogram. Where this precaution was observed, blanks were free from significant peaks in the area of interest. Aromatic substances proved somewhat resistant to ozonolysis under the standard conditions and were best treated at ambient temperature. Figs. 6 and 7 show sample analyses of ozonolysis products from *o*-xylene (20 μg) and a farnesene isomer (20 μg), run under these conditions.

Formaldehyde poses special problems in GC, owing to its volatility and its near-zero response factor in the flame-ionization detector, but it is an important fragment in ozonolysis of terpenoids, wherever terminal double bonds occur. It was therefore necessary to consider derivatives of this simple aldehyde.

Formaldehyde is frequently characterized as the dimedone derivative (IX, R = CH_3) but this material proved too involatile for our purpose, as did the lower

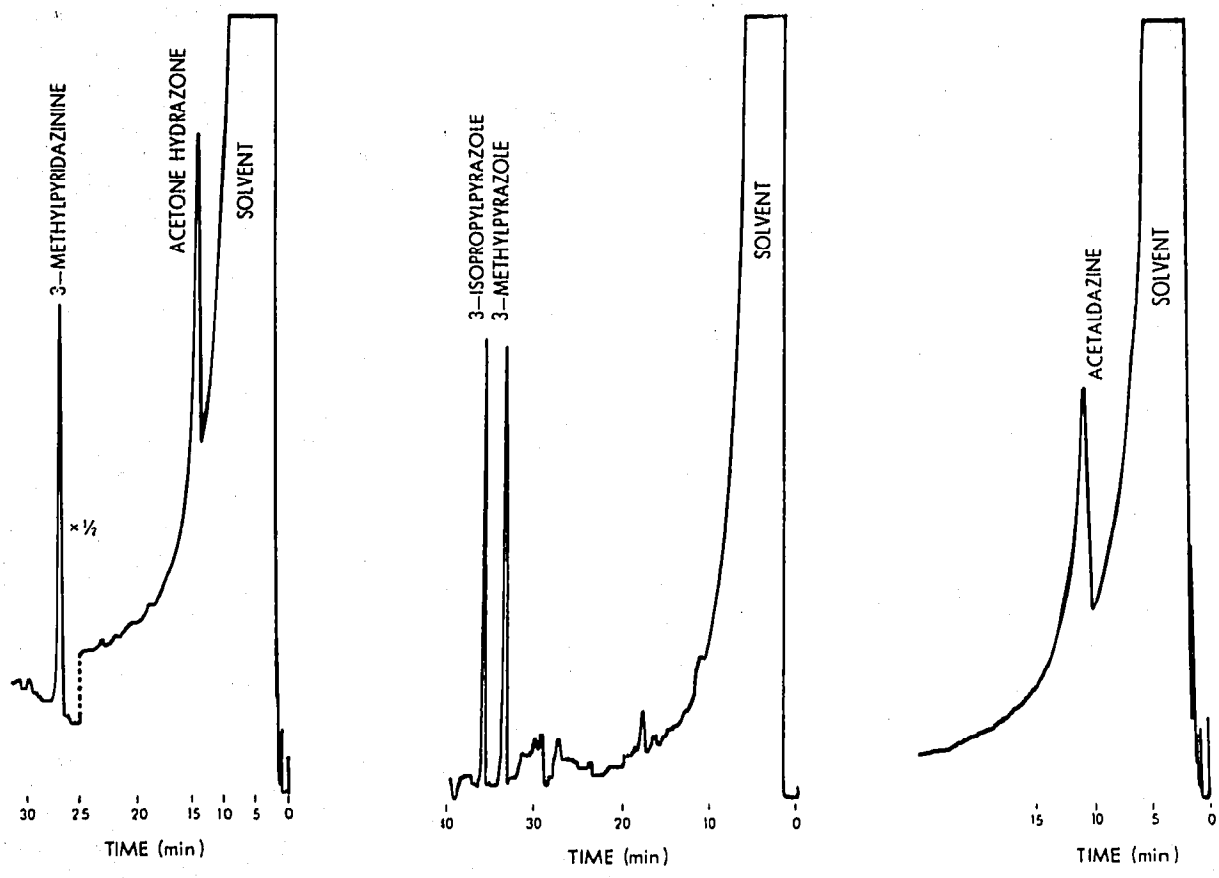


Fig. 3. Gas chromatogram of ozonolysis products of 20 μg farnesol, after treatment with hydrazine hydrate; column A, 70° for 10 min, then programmed at 4°/min.

Fig. 4. Gas chromatogram of ozonolysis products of 20 μg γ-terpinene, after treatment with hydrazine hydrate; column A, 70° for 10 min, then programmed at 4°/min.

Fig. 5. Gas chromatogram of ozonolysis products of 20 μg crotonic acid, after treatment with hydrazine hydrate; column A, 70° for 10 min, then programmed at 4°/min.

TABLE V

RETENTION TIMES OF QUINOXALINES

Derivation	*Carbowax-20M* (150°)		*OV-225* (150°)	
	Retention time (min)	*MU value*	*Retention time (min)*	*MU value*
Glyoxal (*ex* ozonolysis)	11.03	19.07	6.17	17.01
Quinoxaline (authentic)	11.00		6.22	
Methylglyoxal (*ex* ozonolysis)	13.51	19.61	8.07	17.74
Methylquinoxaline (authentic)	13.47		8.06	
Ethylglyoxal (*ex* ozonolysis)	16.28	20.11	10.33	18.38
Isopropylglyoxal (*ex* ozonolysis)	16.03	20.06	10.85	18.51
Dimethylglyoxal (*ex* ozonolysis)	19.03	20.51	11.75	18.71
o-Phenylenediamine	*ca.* 35		*ca.* 14	

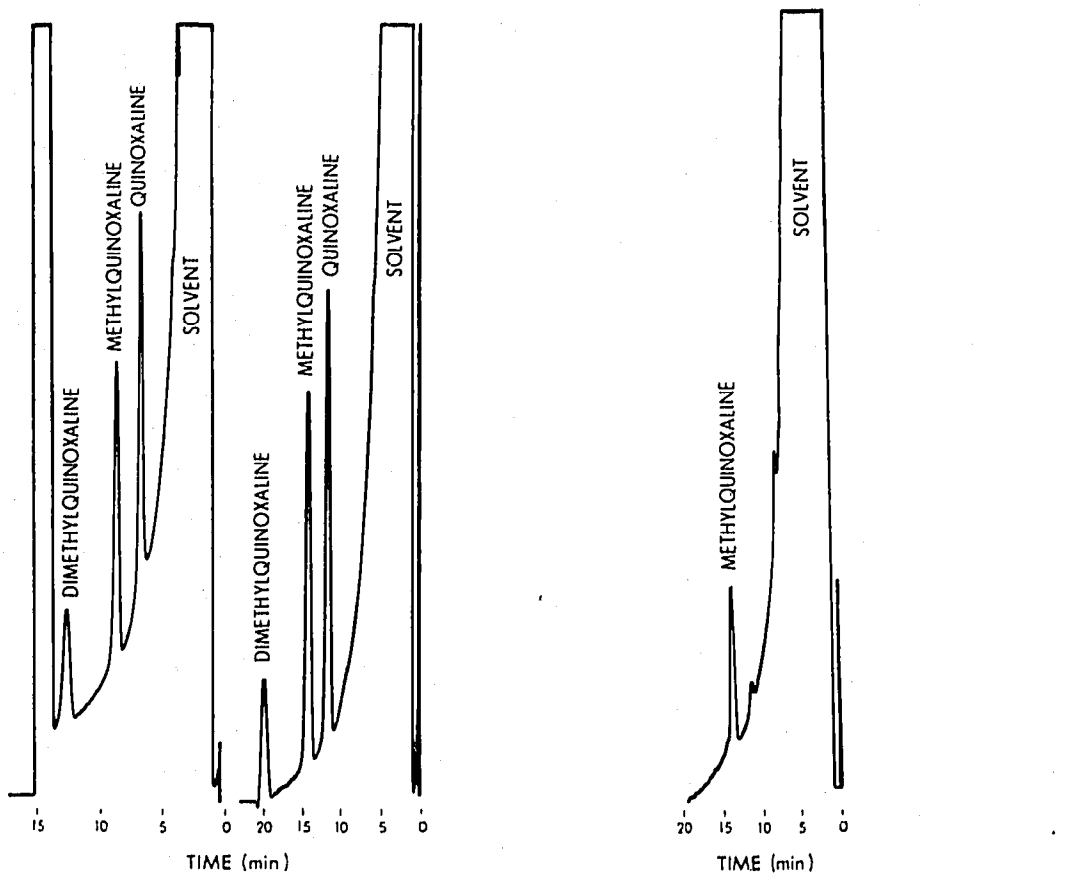
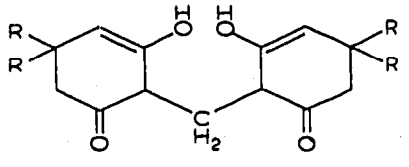


Fig. 6. Gas chromatograms of ozonolysis products of 20 μg *o*-xylene, after treatment with *o*-phenylenediamine. Right, column A, 150°; left, column B, 150°.

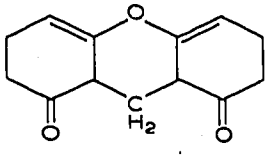
Fig. 7. Gas chromatogram of ozonolysis products of an insect-derived farnesene isomer (*ca.* 20 μg), after treatment with *o*-phenylenediamine; column A, 150°.

homologue from reaction with cyclohexane-1,3-dione (IX, R = H). However, the latter, when heated to *ca.* 90° in aqueous solution, was smoothly converted into the corresponding pyran derivative (X) which was amenable to GC.

On a short column of Carbowax (Column E), this substance had a retention time of 10.2 min at 200°; mass spectral analysis confirmed the constitution (X).



(IX)



(X)

On the micro-scale, ozonolysis was best performed in carbon disulphide solution, to keep background peaks low and it was essential to use a freshly prepared solution of the cyclohexane-1,3-dione each day. Blank runs showed a number of small early peaks, outside the area of interest.

Trial runs on samples as low as 20 μg of carvone and limonene showed the expected peak at 10.2 min but with non-vinylic terpenes and *p*-cymene, no such peak was obtained, even on a 100 μg scale. Thus the method provides a useful means for detecting formaldehyde residues in micro-scale ozonolysis (Fig. 8).

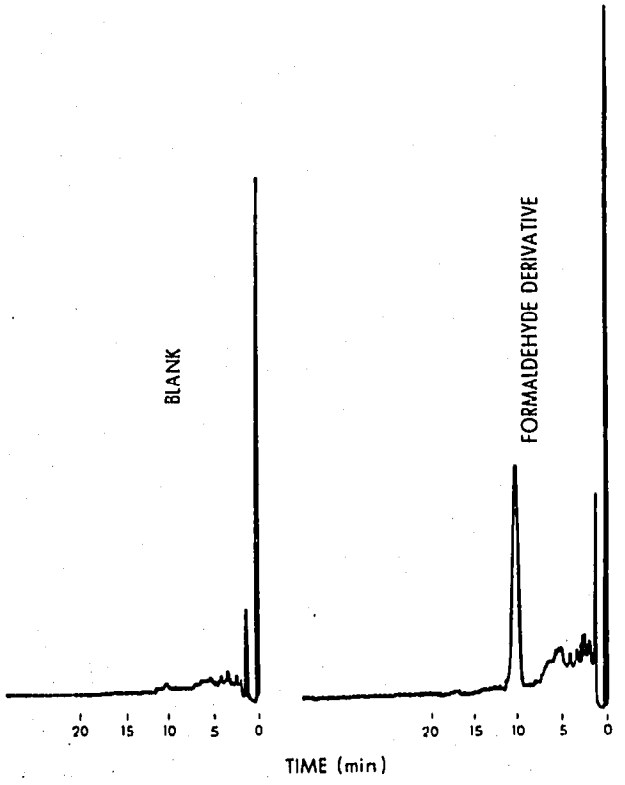


Fig. 8. Gas chromatogram of ozonolysis products of 20 μg limonene, after treatment with cyclohexane-1,3-dione, and ozonolysis blank; column E, 200°.

CONCLUSIONS

The methods here described afford a useful approach to structural problems of unsaturated compounds where only fractions of a milligram of material are available for study. Most of the common mono- and di-functional fragments to be expected may now be detected in the gas chromatogram, but di-aldehydes containing one or more active methylene groups are an exception. These latter substances are so reactive that they do not survive for an appreciable interval after liberation from the ozonide.

Tests on two terpenes of unknown structure, namely an insect-derived farnesene isomer (Fig. 7) and the *Nasutitermes* scent-trail pheromone (nasutene) have demonstrated the value of the new approach.

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