

Fate of Fenitrothion in Forest Trees

V. The Formation of Metabolites in Pinus strobus L. and Their Detection by Gas  
Chromatography and Mass Spectroscopy

by

D.J. Hallett and Pearl Weinberger  
Biology Dept., The University of Ottawa

and

R. Greenhalgh  
Canada Agriculture, Ottawa

and

R. Prasad  
Environment Canada, Ottawa

Chemical Control Research Institute  
Ottawa, Ontario

Information Report CC-X-78

October 1974

# TABLE OF CONTENTS

	Page
ABSTRACT . . . . .	1
RESUME . . . . .	1
INTRODUCTION . . . . .	3
MATERIALS AND METHODS . . . . .	4
(i) Reagents and Solvents . . . . .	4
(ii) Gas Chromatography. . . . .	6
(iii) Thin Layer Chromatography . . . . .	7
(iv) Column Separation . . . . .	7
(v) Biological Application . . . . .	9
(a) Selection and Treatment of Seeds . . . . .	9
(b) Extraction . . . . .	10
(c) Cleanup . . . . .	10
(d) Column Separation . . . . .	10
(e) Growth Studies . . . . .	11
RESULTS . . . . .	11
(i) Characterization of Fenitrothion and Metabolites . . . . .	11
(ii) Gas Chromatograph - Mass Spectroscopy . . . . .	11
(iii) Column Separation . . . . .	25
(iv) Biological Accumulation and Degradation . . . . .	35
DISCUSSION . . . . .	35
SUMMARY . . . . .	38
CONCLUSIONS . . . . .	39
ACKNOWLEDGMENT . . . . .	39
REFERENCES . . . . .	40

# ABSTRACT

Toxic metabolites of fenitrothion: fenitro-oxon, amino-fenitrothion, aminofenitro-oxon, and S-methyl fenitrothion were chemically synthesized. The structure of these compounds was confirmed by gas chromatograph-mass spectroscopy. The compounds were further characterized by gas and thin layer chromatography.

Desmethylfenitrothion, a possible intermediate in the isomeration of fenitrothion to S-methyl fenitrothion was also chemically synthesized. It was derivatized by ethylation, methylation, and butylation. These derivatives were characterized by gas chromatography and the original compound characterized by thin layer chromatography.

Column separation methods were examined for fenitro-oxon, and S-methyl fenitrothion. Activated and deactivated florisil, silica gel, and carbon adsorbents were used.

This methodology was then applied to determine whether fenitrothion or its metabolic breakdown products could be detected in seeds exposed to the parent compound.

White pine seeds, P. strobus were exposed to a  $1.5 \times 10^{-4}$  M solution of fenitrothion, 0,0-dimethyl-0-(4-nitro-3-methyl-phenyl)-phosphorothioate during imbibition at 10°C and 25°C. The seed coat, perisperm and embryo were monitored for residues of the insecticide and metabolites during germination and early seedling growth. Absorption and accumulation of fenitrothion, and accumulation of fenitro-oxon, and S-methyl fenitrothion was demonstrated in the perisperm and embryos of germinated seeds and seedlings. Neither the parent compound nor its metabolites adversely affected the later growth of the pine seedlings.

## RÉSUMÉ

On a fait la synthèse chimique des métabolites toxiques du fénitrothion: fénitro-oxon, aminofénitrothion, aminofénitro-oxon et S-méthyl fénitrothion. On a établi la structure de ces composés au moyen de la chromatographie en phase gazeuse et la spectroscopie de masse et déterminer leurs caractéristiques par chromatographie en phase gazeuse et sur couche mince.

On a aussi synthétisé le fénitrothion déméthylé, un intermédiaire possible de l'isomérisation du fénitrothion donnant le S-méthyl fénitrothion, et obtenu des dérivés de cet intermédiaire par des réactions d'éthylation, méthylation et butylation. Ces dérivés ont ensuite été caractérisés par chromatographie en phase gazeuse, tandis qu'on a fait la même chose au produit de départ, par chromatographie sur couche mince.

Des méthodes de partage sur colonne pour le fénitro-oxon et le S-méthyl fénitrothion ont été étudiées au moyen du florisil, du gel de silice et du charbon activés et désactivés.

On a ensuite appliqué cette méthode pour déterminer si le fénitrothion ou les produits de sa décomposition par le métabolisme pouvaient être détectés dans des semences exposés au fénitrothion.

On a mis à tremper des semences de pin blanc, *P. strobus*, dans une solution de fénitrothion ou thionophosphate de 0,0-diméthyle 0-(méthyl-3 nitro-4 phényle)  $1.5 \times 10^{-4}$  M à 10° C et 25° C. On a examiné le tégument, le péricarpe et l'embryon au cours de la germination et le début de croissance des jeunes pousses pour découvrir des traces d'insecticide et de métabolites. Les résultats ont démontré qu'il

y avait eu absorption et accumulation de fénitrothion, et accumulation de fénitro-oxon et de S-méthyl fénitrothion dans le péricarpe et l'embryon des semences et les jeunes pousses ayant germé. Ni l'insecticide initial ni ses métabolites n'ont eu d'effet contraire sur la croissance ultérieure des jeunes pousses de pin.

## INTRODUCTION

Fenitrothion, 0,0-dimethyl-0-(4-nitro-3-methyl-phenyl)-phosphorothioate, is a widely used insecticide in Canada. In 1969 it replaced DDT as the insecticide of choice for the control of spruce budworm in Canadian forests. The recommended rate of application of fenitrothion is 2 to 4 ounces per acre. This dosage is adequate to prevent defoliation by lepidopterous insects (Fettes, 1968).

Miyamoto (1963, 1965) showed that fenitrothion penetrated into the tissues of rice plants. Residual amounts of the insecticide, its oxygen analogue and the decomposition products; desmethyl fenitrothion, dimethyl phosphorothioic acid and phosphorothioic acid were found in rice grains of plants sprayed at the heading stage of growth. Fenitro-oxon was found in trace amounts of from 1 to 10 days after application. Similarly some workers determined the persistence of  $^{32}\text{P}$  labelled fenitrothion in the cocoa tree (Sundaram and Sundaram 1969), and found that it was absorbed rapidly when applied to leaves. Fenitrothion was completely metabolized within the first 5 days after application and fenitro-oxon, which was first detected in the plant 5 days after application, was completely decomposed by the eleventh day.

In Canadian forests, the persistence of fenitrothion was studied on balsam fir and mixed spruce foliage (Yule and Duffy, 1972). After spraying, 50 percent of the fenitrothion deposits were lost from the foliage in 4 days and 70 to 85 percent within 2 weeks.

Hollingworth, et. al. (1973) have shown that the major pathway of biological breakdown of fenitrothion in mammals is via demethylation

through a glutathion S-alkyl transferase enzyme to form desmethylfenitrothion. This metabolite is non-toxic and is found to be excreted in the urine.

The work presented here is an in-depth study of the identification and quantitative evaluation of fenitrothion and its metabolic products during the germination and early seedling growth of white pine, Pinus strobus seeds. The chemical synthesis and positive identification of S-methyl fenitrothion, aminofenitrothion, aminofenitro-oxon and desmethylfenitrothion is also described. The structures of these compounds were confirmed by gas chromatograph-mass spectroscopy. The molecules were characterized by gas and thin layer chromatography. Desmethylfenitrothion was derivatized for quantitation by gas chromatography. Column cleanup and separation methods for fenitrothion, fenitro-oxon, and S-methyl fenitrothion are also quantitatively evaluated. These methods are sensitive and suitable for the identification of toxic metabolites when environmental concern requires monitoring for them.

#### MATERIALS AND METHODS

##### (1) Reagents and Solvents:

Silica gel, 60 to 100 mesh was obtained from Grace Co. Ltd. (grade 950) and Florisil, 60 to 100 mesh was obtained from Floridin Co. Ltd. Both were heated overnight at 200°C to remove water. They were cooled in a vacuum dessicator and stored in a reagent jar to prevent extraneous water uptake. Water content was adjusted by adding the appropriate amount by weight of distilled water to the absorbent in a reagent jar and equilibrating it overnight. All solvents used were

nanograde and glass distilled.

(a) Preparation of Fenitrothion and Metabolites: Fenitrothion (0,0, dimethyl 0,3-methyl, 4-nitrophenol phosphorothioate) and fenitro-oxon (0,0, dimethyl 0,3-methyl, 4-nitrophenyl phosphate) were obtained from Sumitomo Chemical Co., Japan. Technical grade fenitrothion was purified by a modification of the method of Kovacicova et al. (1971). Ten grams of fenitrothion was added dropwise to the head of a stripping column at the bottom of which boiling toluene  $110^{\circ}$  C entered counter-current wise. Fenitrothion was collected free from some volatile impurities at the bottom of the stripping column. It was then taken up in 100 ml of benzene and washed with four 100 ml aliquots of 1% ammonium hydroxide to hydrolyze fenitro-oxon and S-methyl fenitrothion contaminants. This was followed by four washings with distilled  $H_2O$  to remove the hydrolysis products. It was then dried with anhydrous sodium sulphate. The solvent was removed by rotary vacuum evaporation at  $35^{\circ}$  C. Traces of solvent were removed under a stream of nitrogen.

3-methyl, 4-nitrophenol was a gift of Dr. J.R. Duffy (University of P.E.I.).

Aminofenitrothion (0,0 dimethyl, 0,3-methyl, 4-aminophenyl phosphorothioate) was prepared by the reduction of one gram of fenitrothion with one gram of tin and 40 ml 1 N HCl at  $30^{\circ}$  C for 1 hour. This gave approximately a 20% yield. The crude extract was extracted with chloroform once to remove residual fenitrothion. The acid was then neutralized with sodium carbonate and re-extracted with chloroform 3 times, giving pure aminofenitrothion.

Aminofenitro-oxon (0,0,dimethyl,0,3-methyl, 4-amino-phenyl phosphate) was prepared by the reduction of fenitro-oxon with chromous

chloride at 30° C (R. Greenhalgh, personal communication).

S-methyl fenitrothion (S-methyl,0-methyl,3-methyl, 4-nitro-phenyl phosphate) was prepared by adding an excess of methyl iodide to fenitrothion dissolved in benzene and re-fluxing this for 1/2 hour at 65° C. S-methyl fenitrothion was also formed by thermal isomerization of pure fenitrothion at 165° C for 1/2 hour.

Desmethylfenitrothion (0-methyl,0,3-methyl, 4-nitrophenyl phosphorothioate) was prepared by a modification of the method of Hutson et al (1972) with tetrachlorvinphos. Ten grams of fenitrothion in acetone was demethylated with triethylamine by refluxing for 3 hours. The solvent was removed by means of a rotary evaporator. The oily crystals were taken up in chloroform. The chloroform was partitioned with water to remove desmethyl-fenitrothion. Water was removed by rotary evaporation and the desmethylfenitrothion was taken up in the solvent appropriate for derivatization.

(b) Derivatization of Desmethylfenitrothion: A modification of the method of Schlenk and Gellerman (1960) was used. Diazoethane etylated, diazomethane methylated, and diazobutane butylated desmethylfenitrothion were prepared for analysis by gas chromatography.

(c) Derivatization of Aminofenitrothion by Trifluoroacylation: The method of Seiber (1972) was used to form trifluoroacetylfenitrothion from aminofenitrothion as a further confirmatory test for the presence of this compound.

(ii) Gas Chromatography:

A Pye model 104 gas chromatograph fitted with an alkali flame ionization detector (AFID), having a cesium bromide annulus was used. A 74.5 cm x 6.5 mm glass column which contained 4% SE30/6% QF-1 Chromsorb W

was used. Column temperature was 215<sup>0</sup> C, nitrogen flow 40 ml/min, air flow 500 ml/min, and hydrogen flow 35 ml/min. Duplicate determination were carried out, bracketed by injection of sample standards.

(iii) Thin Layer Chromatography:

The enzyme inhibition technique described by Mendoza (1972) using extract of steer liver homogenate as the spray reagent was found to be very sensitive to fenitrothion, fenitro-oxon, and S-methyl fenitrothion. A solvent system of 1:3 ethyl acetate and cyclohexane was used with silica gel plates (Yule and Duffy, 1972). A routine check was always made for the presence of these compounds in all eluant fractions. Due to its acidic nature desmethylfenitrothion was spotted on cellulose plates. These plates were developed with a mixture of 2 propanol: water: ammonium hydroxide, in a ratio of 75:24:1 (Hollingworth et al. 1967). A spray of 2,6,diazobromoquinonamine in glacial acetic acid which is specific for P-S bonds (Braithwaite, 1963) was used to detect desmethyl-fenitrothion.

Gas Chromatography Spectroscopy: A Pye Model 124 Gas Chromatograph fitted with a flame ionization detector FID was connected to a CEC Mass Spectrometer for analysis.

(iv) Column Separation:

Three types of columns were evaluated for the separations and recovery of fenitrothion, and fenitro-oxon and S-methyl fenitrothion. These were florisil, silica gel, and charcoal.

Florisil: A 25 by 450 mm glass column fitted with a glass scintered disc at the bottom was filled with Florisil to a depth of 65 mm and was dry packed by tapping. Anhydrous sodium sulfate was added to

a depth of 10 mm on top of the absorbant. Florisil was deactivated by adding 0, 5, 10, 15, and 20% distilled water by weight and these were checked for recovery of fenitrothion and fenitro-oxon.

(a) Silica gel: Columns of similar size were prepared as above with silica gel which had been similarly deactivated with 0, 5, 10, 15 and 20% distilled water as with florisil. The fine constituency of the adsorbant necessitated wet packing with hexane to prevent channeling of eluants. A slurry of silica gel was made in hexane, which was then poured into the column and allowed to pack while being continuously wet with hexane.

(b) Charcoal: A modification of the method of Watts et al. (1969) was used. A mixture of one part acid treated charcoal, two parts hydrated Seisorb 43, and four parts Celite (Johns-Manville) was added to a depth of 65 mm as above. The column was dry packed by tapping but was eluted under suction.

All columns were then eluted with 100 ml of hexane, followed by 100 mls of benzene, 100 ml of acetone-benzene 1:3, and 100 ml of methanol. The columns were never allowed to dry. Each eluant was flash evaporated to near dryness and taken up in 10 ml of hexane. They were then concentrated under forced air. 10% Florisil and 1% Silica gel columns were checked for recovery of S-methyl fenitrothion.

(c) Extraction from Water: Three times partitioning with chloroform gave 100% recoveries from water for fenitrothion, fenitro-oxon and S-methyl fenitrothion. Desmethylfenitrothion remained in the water fraction.

(v) Biological Application:

(a) Selection and Treatment of Seeds: White pine seeds (P. strobus) were extracted from fallen cones collected during the fall of 1972 in unsprayed regions of the Petawawa Forest, Ontario. Generally, for each single experiment, two groups of ten seeds were chilled at 10<sup>0</sup> C for 21 days in sterile 9 cm plastic petri plates containing 15 ml of  $1.5 \times 10^{-4}$  M solution of fenitrothion in distilled water. Population homogeneity was optimized by selecting seeds with air dried weight to within 5 mg. The seeds were then germinated in darkness with a diurnal temperature variation of 20<sup>0</sup> C nights (8 h) and 30<sup>0</sup> C days (16 h). A further set of seeds were germinated immediately in the fenitrothion solution without prior chilling (stratification).

Water (glass distilled) was not a limiting factor in germination. Fungal infection of the seeds was minimized by washing them for 15 minutes in a 1% solution of sodium hypochlorite and rinsing them for another 15 minutes in continuously running distilled water.

Following germination the seeds were dissected into three parts; the embryo, perisperm plus gametophyte and the seed coat. This permitted localization of the pesticide in the seed during germination, stratification and early seedling growth. Samples were taken after 21 days of stratification (S21), and at specified time periods during germination, namely 4, 8- (when the radicle emerged), 14- (when the hypocotyl developed) and 21 days (when the cotyledons emerged). These seeds have been designated g4, g8, g14, and g21 when not stratified and S21g4, S21g8, and S21g21 when stratified. The seeds parts were frozen in liquid nitrogen and stored at -70<sup>0</sup> C until the pesticide was extracted.

In most cases the whole sequence of germination stages was repeated at a later time period to provide duplicate data. The variation between replicates was generally within 10 per cent.

(b) Extraction: Ten seed parts were homogenized for 1 minute with a Polytron sonicator in 50 ml of acetonitrile. This homogenate was filtered through celite (Johns-Manville Co. Ltd.) to remove seed debris. Another 50 ml of acetonitrile was sonicated in the flask to remove any residual pesticide on the sonicator and on the walls of the flask. This was also filtered through the same celite and the volume made up to 100 ml.

(c) Cleanup: Oils and fats were removed by partitioning the acetonitrile with 50 ml of hexane 3 times (Getz, 1962). The acetonitrile was flash evaporated almost to dryness and taken up in 10 ml of hexane. This cleanup was sufficient to permit determination of fenitrothion by gas chromatography using an alkali-flame ionization detector, but the oxon at low levels was masked by interfering peaks on this GC column and had to be first separated by column chromatography.

(d) Column Separation: A 26 x 450 mm glass column fitted with a glass scintered disc at the bottom was filled with 65 mm of Florisil and dry-packed by tapping. On top of this, anhydrous  $\text{Na}_2\text{SO}_4$  was added to a depth of one half inch. The column was pre-wetted with 5 ml of hexane. Known amounts of standards or extracts were absorbed on the column in 10 ml of hexane. The column was then eluted with 100 ml of hexane followed by 100 ml of benzene, 100 ml of acetone-benzene 1:3, and 100 ml of methanol. The column was never allowed to dry out. The recoveries of fenitrothion from the benzene fraction and of fenitro-oxon from the acetone-

benzene fraction were both  $100 \pm 0.5$  per cent. Each eluant was flash evaporated to near dryness and taken up in 10 ml of hexane.

(e) Growth Studies: In parallel experiments, control and fenitrothion treated seds were planted in flats in loam soil and put in a controlled environment room with a monitored diurnal light and temperature regime. A 16 h day (10.1 lux light intensity) and an 8 h dark period at  $20^{\circ}$  C was maintained. The flats were watered daily to field capacity during the early stages of seedling growth and later three times weekly.

## RESULTS

### (i) Characterization of Fenitrothion and Metabolites:

Under our experimental conditions aminofenitrothion, fenitrothion, fenitro-oxon and S-methyl fenitrothion were clearly resolved and separated by gas chromatography using the SE30-QF1 column (Figure 1). The retention times and RF values are shown in Table 1. together with the RF values of these compounds using thin layer chromatography. With the enzyme inhibition spray, toxic compounds give a white spot on a blue background. This spray is especially sensitive to fenitro-oxon and S-methyl fenitrothion. Aminofenitrothion and aminofenitro-oxon proved to be toxic as well. 3-methyl, 4-nitrophenol was non-toxic but showed up as a yellow spot when applied in high concentrations. When sprayed with 2, 6, diazobromoquinonamine desmethyl-fenitrothion and fenitrothion show up as a reddish brown spot. Desmethylfenitrothion is non-toxic.

### (ii) Gas Chromatography-Mass Spectroscopy:

Analysis of fenitrothion showed a parent peak at 227 m/e

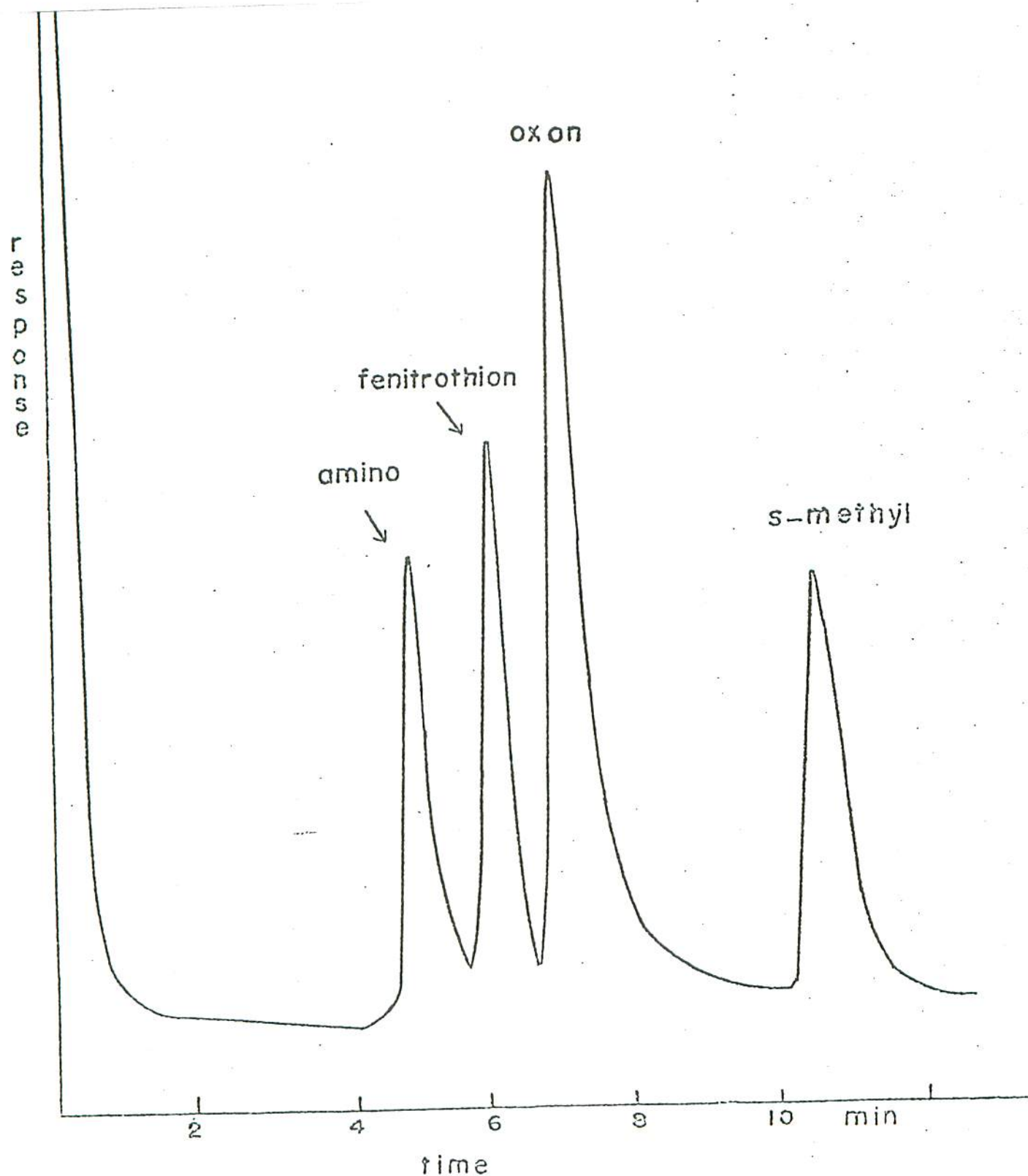


Fig. 1. Gas chromatogram of fenitrothion, fenitro-oxon, S-methyl fenitrothion, and aminofenitrothion.  
G.C. conditions are the same as those for Table 1.

TABLE 1  
Rf Values for Gas and Thin Layer Chromatography of  
Fenitrothion and its Metabolites

Compound	<u>Gas Chromatography<sup>1</sup></u>		<u>Thin Layer Chromatography<sup>2</sup></u>	
	RF	RRT (fenitrothion)	RF	RRF (fenitrothion)
Fenitrothion	5.375	1	0.58	1
Fenitro-oxon	6.375	1.186	0.09	0.155
3-methyl-4-nitrophenol	1.313	0.244	0.38	0.552
S-methyl fenitrothion	10.0	1.860	0.149	0.295
amino fenitrothion	4.125	0.767	0.214	0.437
amino fenitro-oxon	5.375	1	--	--
TFA amino fenitrothion	5.375	1	--	--
desmethyl fenitrothion*	--	--	0.713	0.713
butylated desmethyl- fenitrothion	10.0	1.860	--	--

<sup>1</sup> Conditions: 74.5 x 6.5 mm in od. 4% SE30/6% QF-1 on Chromsorb W (80-100 mesh) temp. 215° C.

<sup>2</sup> Conditions: on Silica gel, elution mixture 25% Ethyl acetate, 75% Cyclohexane

\* Conditions: on cellulose, elution mixture 75%, 2 propanol, 25% H<sub>2</sub>O, 1% NH<sub>4</sub>OH

together with peaks at 152, 125, 109, 93, and 79, with the relative amounts of these fragments: 3, 100, 61, 28, and 41% (Figure 2 a,b). 3-methyl, 4-nitrophenol showed a parent peak at 153 m/e with peaks at 136, 108, 107, and 77. The relative amounts of these fragments was 100, 15, 15, and 19% (Figure 3 a,b). This would indicate that the fragment at 125 m/e of the parent compound is the reactive phosphorous group which when added to the cresol compound would give the 277 m/e of the parent compound. This is evidence of a split of the P-O bond between the reactive group and 3-methyl, 4-nitrophenol which is characteristic of fenitrothion and its derivatives.

Aminofenitrothion showed a fragmentation pattern with a parent peak at 247 m/e indicating that an oxygen  $O_2$  molecule was reduced from the fenitrothion molecule which has a parent peak of 277 m/e. Further peaks were found at 150, 138, 125, 122, 106, 93, and 77. The relative amounts of these fragments were 100, 22, 56, 13, 24, and 26% (Figure 4 a, b). The peak at 125 m/e is indicative of an intact fragment of the reactive phosphorous group of fenitrothion. The fragment at 122 m/e indicates a fragment of 3-methyl, 4-aminophenol suggesting that the molecule is indeed aminofenitrothion in the parent peak.

Fenitro-oxon showed a parent peak at 261 m/e with peaks at 244, 127, 109, 79, and 63, with the relative amounts of these fragments 43, 11, 100, 20, and 12% (Figure 5 a, b). The loss of sulphur and its replacement by oxygen on the reactive phosphorous group is reflected by the change in the parent peak from 277 m/e in fenitrothion to 261 m/e in fenitro-oxon and in the relative amounts of fragments at 109 m/e in fenitro-oxon rather than 125 m/e as in fenitrothion.

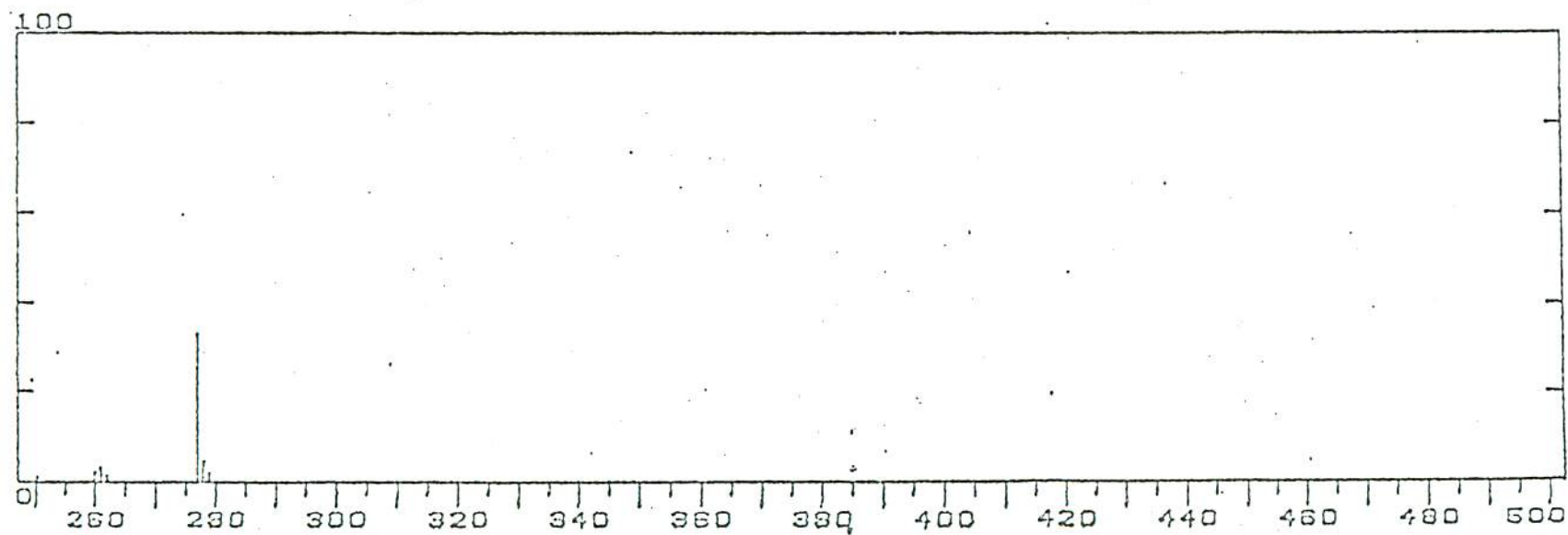
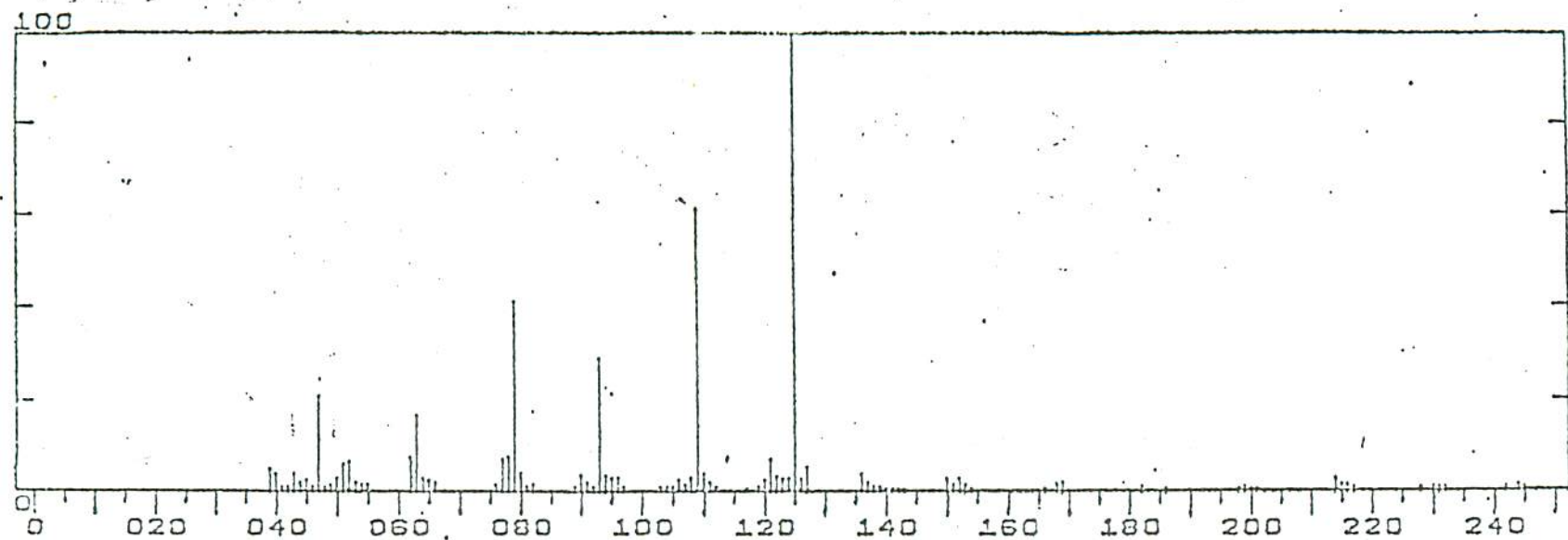


Fig. 2 (a) Mass spectrum of fenitrothion; abscissa represents mass to charge ratio ( $m/e$ ) and ordinate represents base percentage.

ABBREVIATED SPECTRUM

M/E	PEAK HGT	% OF BASE
47	36.	21.1765
39	5.1	4.7647
52	10.8	6.35224
51	10.1	2.94117
63	28	16.4706
62	13	7.64706
79	20	41.1765
78	12.6	7.41176
93	49	28.8235
94	6.2	3.64706
109	104	61.1765
110	7.1	4.17647
125	170	100
121	11.6	6.82353
136	6.4	3.76471
137	3.4	2
150	5	2.94118
152	4.7	2.76471
169	3.5	2.05882
163	2.6	1.52941
162	1.5	.682353
165	1	.588235
199	1.7	1
198	1.2	.705882
214	5	2.94118
215	2.2	1.29412
216	2.7	1.58823
217	1.3	.764706
242	1.7	1
231	1.6	.941176
244	2.7	1.58823
245	1.5	.682353
261	5.1	2
260	3.2	1.68235
277	56	32.9412
278	7.6	4.47059

Fig. 2 (b) Abbreviated spectrum of fenitrothion.

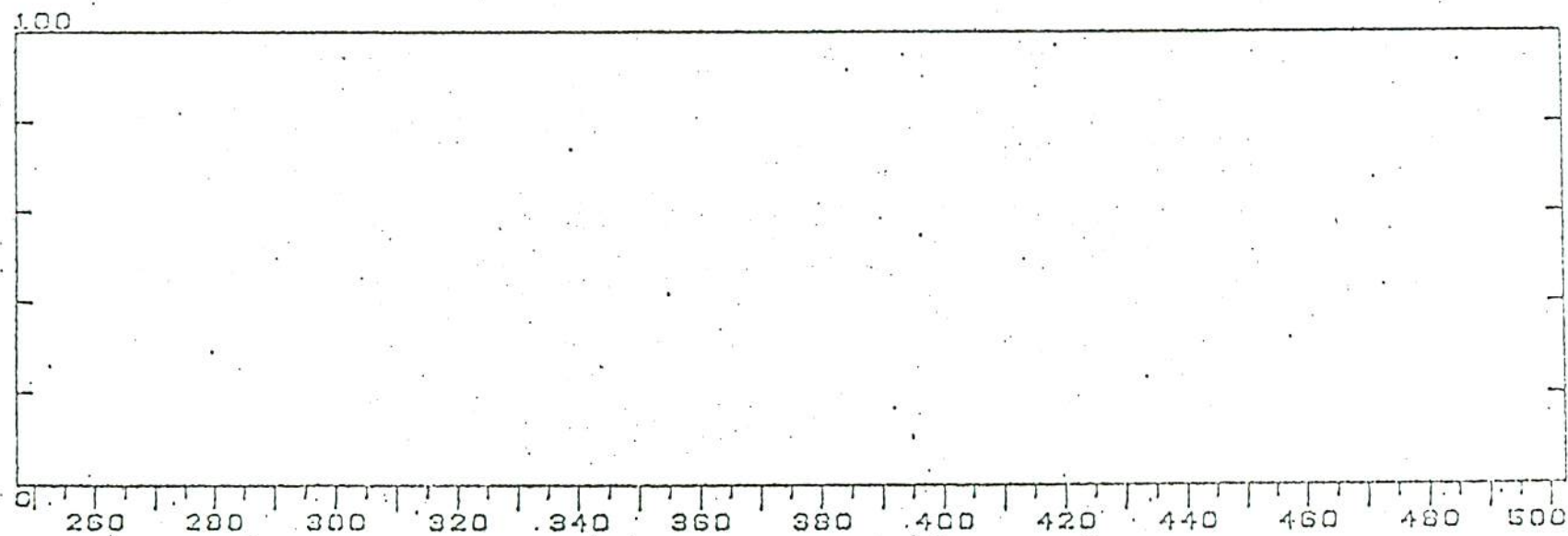
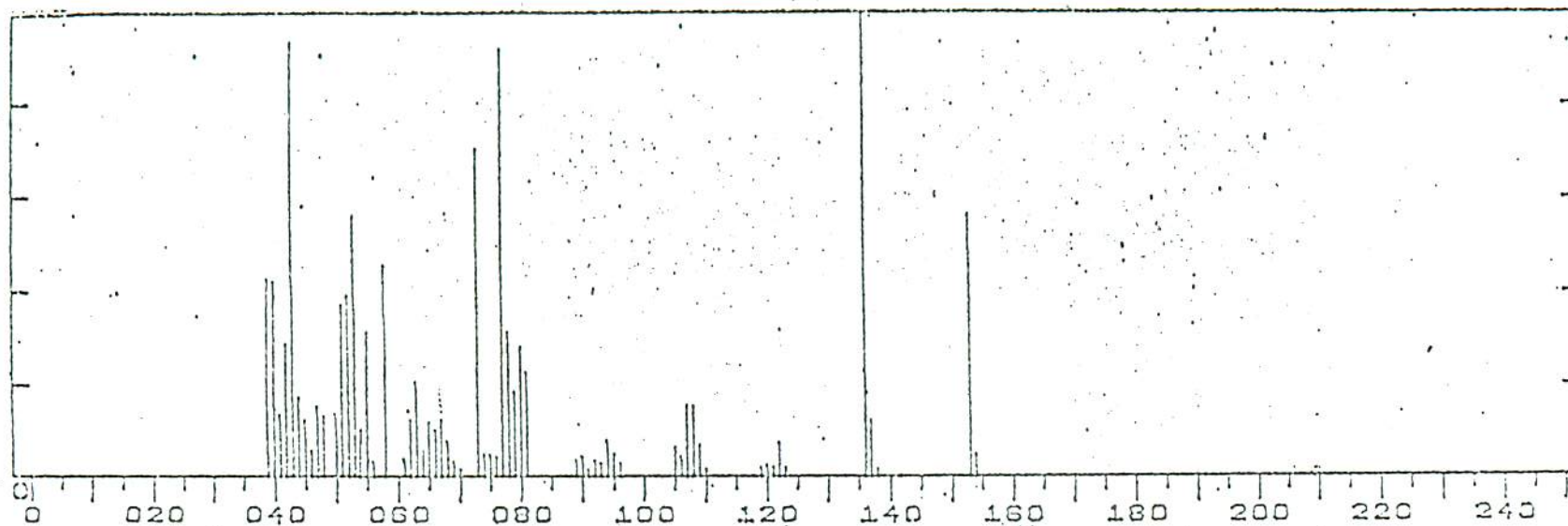


Fig. 3 (a) Mass spectrum of 3-methyl, 4-nitrophenol, abscissa represents mass to charge ratio ( $m/e$ ) and ordinate represents base percentage.

ABBREVIATED SPECTRUM

M/E	PEAK HGT	% OF BASE
43	132	92.9577
59	61	42.9577
53	60	56.238
58	45	45.7746
73	100	70.4225
63	29	42.4225
77	130	91.5492
78	45	31.6901
94	11.5	8.09859
95	7	4.92955
107	22	15.493
108	22	15.493
122	10.6	7.46472
120	3.6	2.53521
136	142	100
137	18	12.6761
153	60	56.238
154	7.4	5.21127

Fig. 3 (b) Abbreviated spectrum of 3-methyl, 4-nitrophenol.

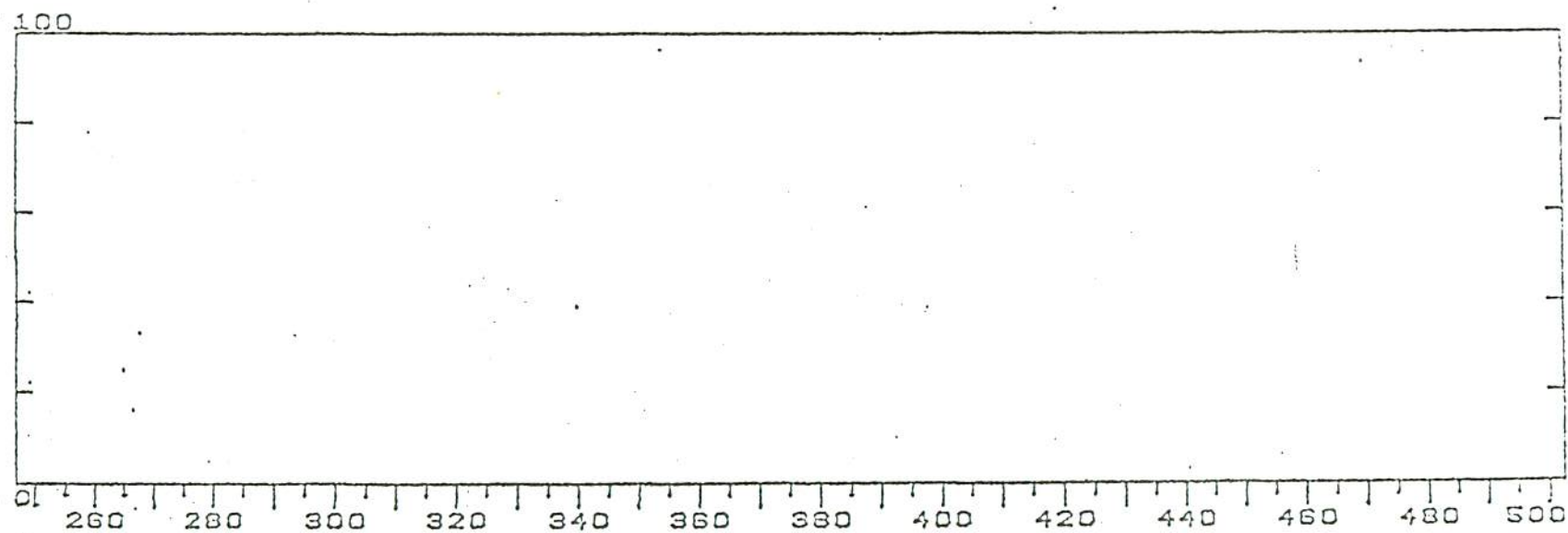
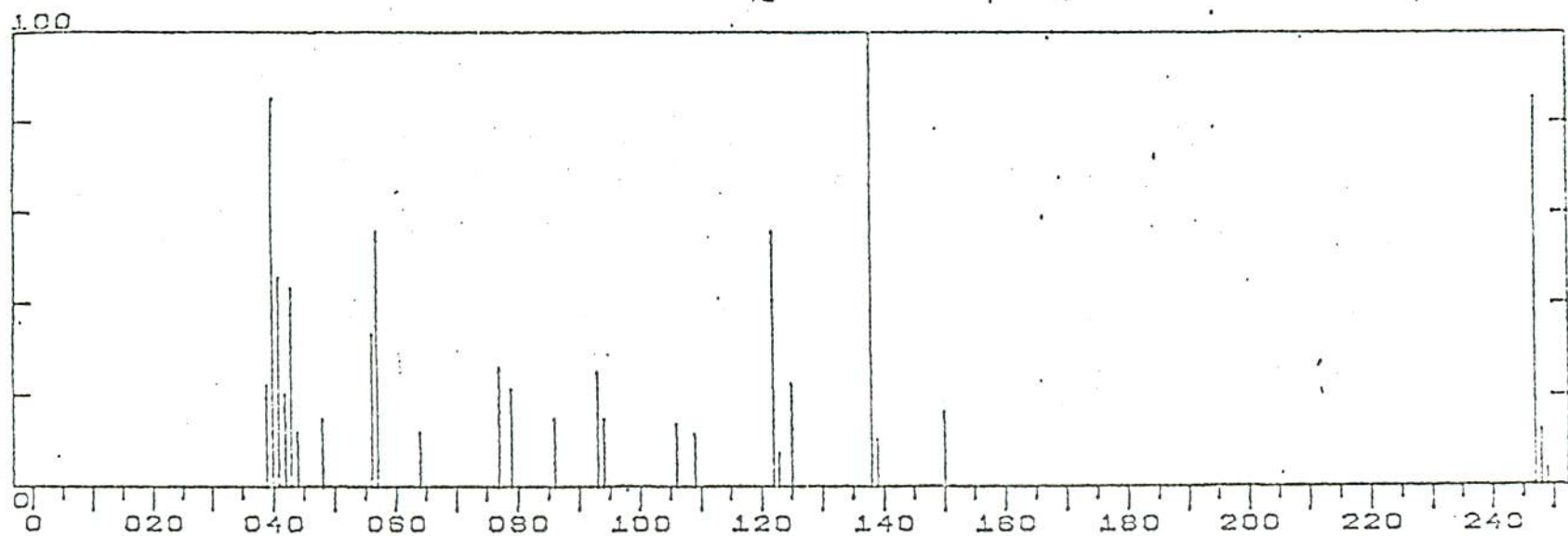


Fig. 4 (a) Mass spectrum of aminofenitrothion; abscissa represents mass to charge ratio ( $m/e$ ) and ordinate represents base percentage.

ABBREVIATED SPECTRUM

M/E	PEAK HGT	% OF BASE
41	22	45.8333
43	21	43.75
57	27	56.25
56	16	33.3333
77	12.5	26.0417
79	10.4	21.6667
93	11.9	24.7917
94	7.1	14.7917
106	6.7	13.9583
109	5.5	11.4583
122	27	56.25
125	10.7	22.2917
138	48	100
139	5	10.4167
150	8	16.6667
247	41	85.4167
248	6	12.5

Fig. 4 (b) Abbreviated spectrum of aminofenitrothion.

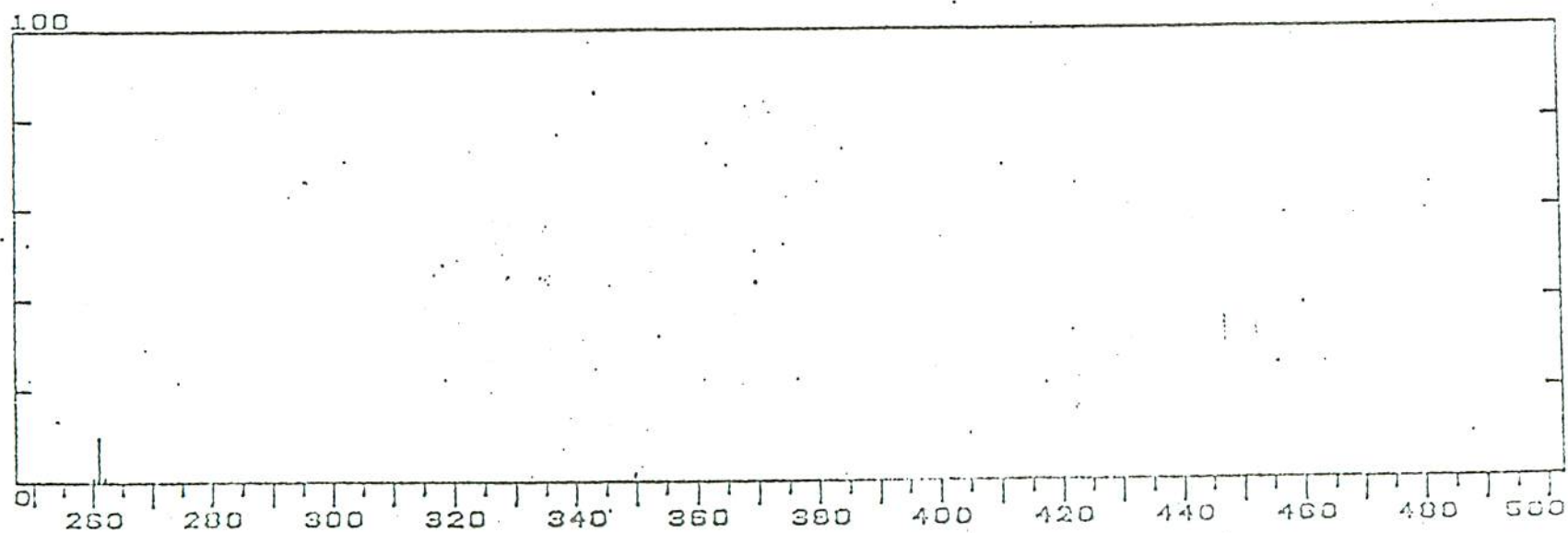
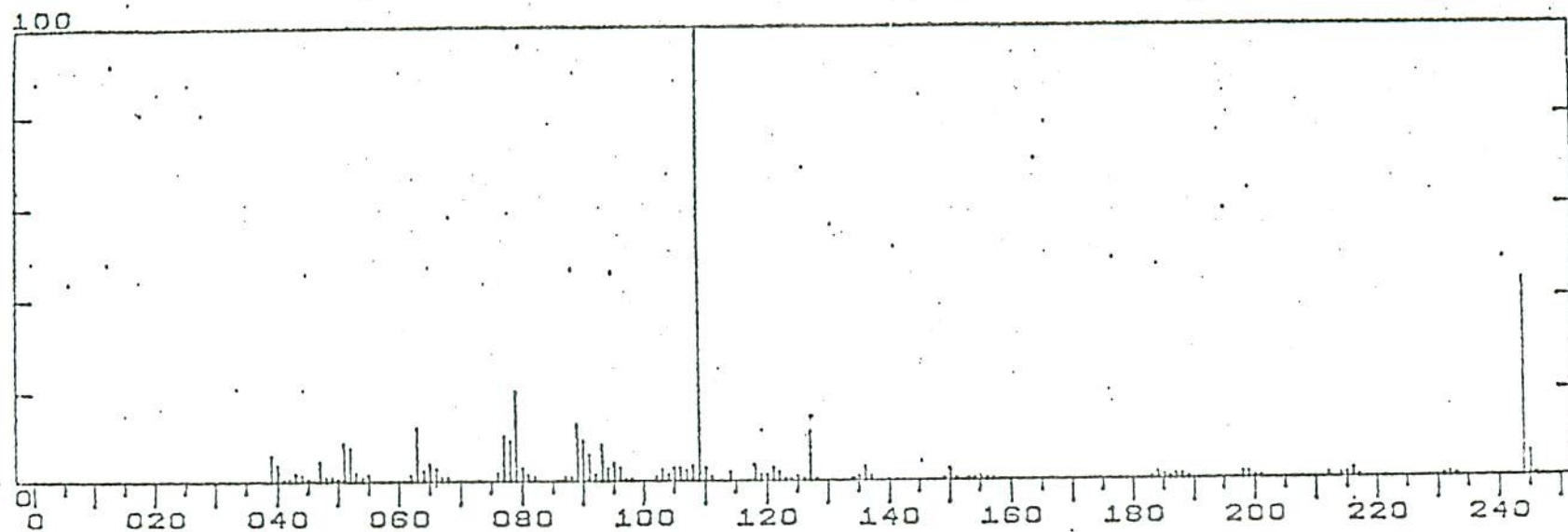


Fig. 5 (a) Mass spectrum of fenitro-oxon; abscissa represents mass to charge ratio (m/e) and ordinate represents base percentage.

ABBREVIATED SPECTRUM

N/E	PEAK HGT	% OF BASE
39	35	6.14035
47	25	4.36596
51	47	8.59649
52	43	7.54386
63	69	12.1053
65	23	4.03509
79	116	20.3509
89	70	12.2507
90	51	8.94737
93	45	7.69474
109	570	100
109	20	2.50877
127	64	11.2281
116	20	3.50877
136	17	2.98246
135	4.8	0.842105
150	14	2.45614
155	5.4	0.947363
184	8.2	1.4386
185	4.7	0.824561
198	8.8	1.54386
199	7.6	1.33333
214	7	1.22807
215	5.1	0.894737
216	11.1	1.94737
217	3	0.526316
232	5.7	1
231	3.2	0.561403
244	250	43.8526
245	30	5.26316
261	54	9.47363
262	6.8	1.19298

Fig. 5 (b) Abbreviated spectrum of fenitro-oxon.

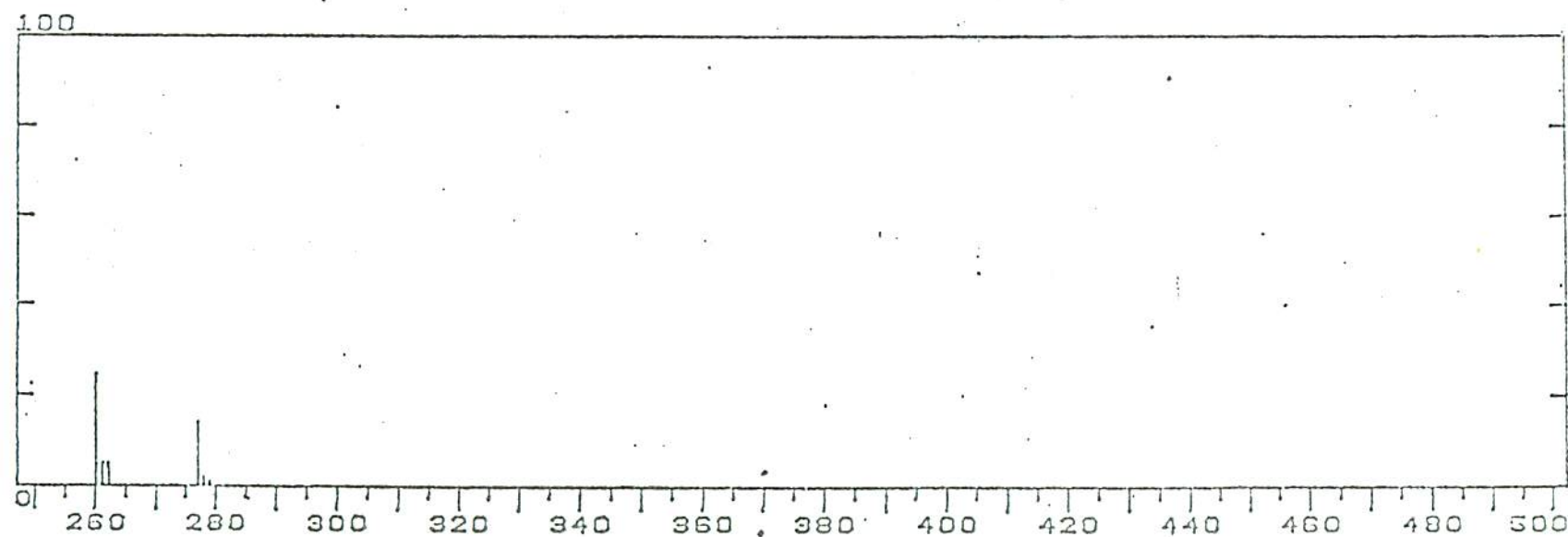
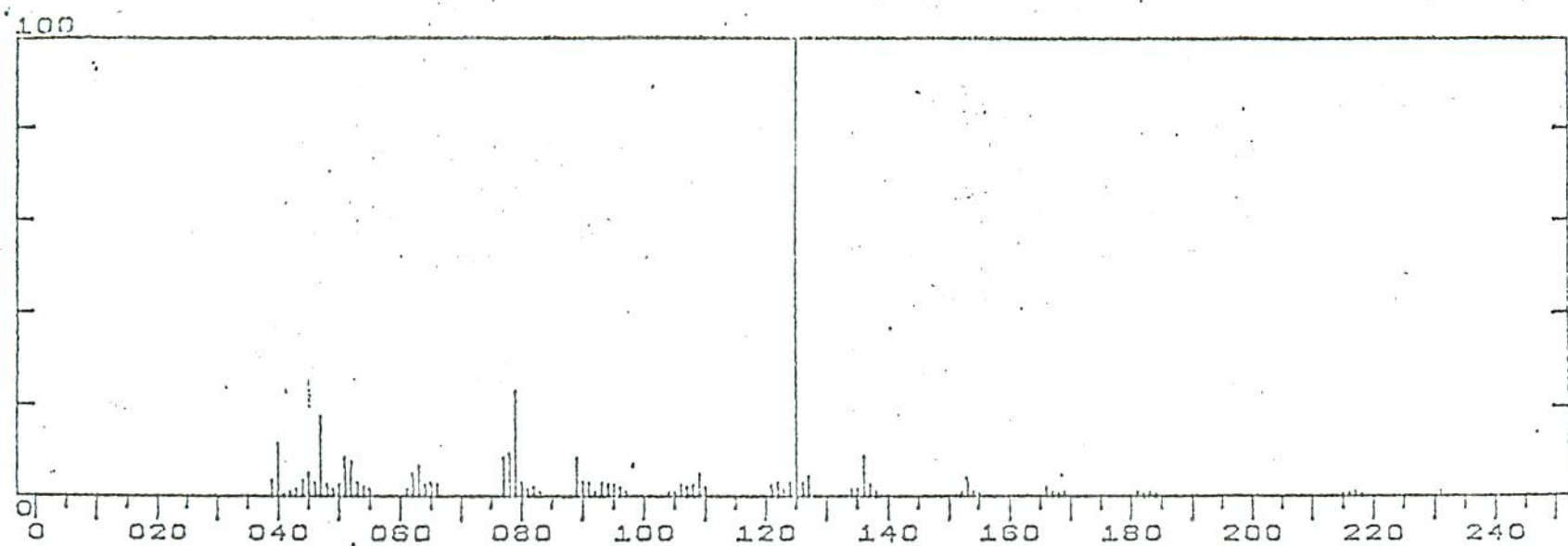


Fig. 6 (a) Mass spectrum of S-methyl fenitrothion; abscissa represents mass to charge ratio (m/e) and ordinate represents base percentage.

ABBREVIATED SPECTRUM		
M/E	PEAK HGT	% OF BASE
47	33	17.3634
49	22	11.5739
51	16	8.42105
52	14	7.36342
63	12.4	6.52631
62	9.4	4.94737
79	44	23.1579
78	18.	4.47365
90	6.3	3.21579
91	6	3.15789
109	9.8	5.15789
106	4.6	2.42105
125	190	100 ...
127	8.6	4.63158
136	17	6.94737
137	4.4	2.31579
153	8.9	4.63421
152	2.3	1.21053
165	4	2.10526
167	1.6	0.842105
181	2.3	1.21053
183	2.1	1.10526
217	1.6	0.842105
218	1.4	0.736342
231	1.6	0.847368
260	47	24.7365
262	9.9	5.21053
277	27	14.2105
278	4	2.10526

Fig. 6 (b) Abbreviated spectrum of S-methyl fenitrothion.

S-methylfenitrothion, an isomer of fenitrothion, showed a parent peak of 277 m/e. Further peaks were found at 260, 153, 136, 125, 77, 47, and 40. These were found in relative amounts of 29, 6, 11, 100, 8, 16, and 12% (Fig 6 a,b). In this isomer the fragment at 127 m/e indicates that the sulphur atom is kept in the reactive phosphorous group of the molecule as in fenitrothion.

Ethylation of desmethylfenitrothion (Figure 7) gave four peaks indicating the production of four derivatives of fenitrothion. Methylation gave two distinct peaks which were fenitrothion and S-methyl fenitrothion (Figure 8). Butylation gave one main peak of O-butyl, O-methyl, 3-methyl, 4-nitrophenyl phosphorothioate (Figure 9). This is the best derivatization for determining amounts of desmethylfenitrothion by gas chromatography.

(iii) Column Separation:

Table 2 shows that 100% recoveries of fenitro-oxon were obtained with florisil columns containing 10% or more water by weight, and with silica gel columns containing 10% or less water by weight. 1.6 ug of fenitro-oxon was applied to these columns. Florisil columns deactivated with 10% by weight water (used in the pine seed study) gave a 25% recovery of S-methyl fenitrothion. Activated silica gel containing 0% water was found to give 100% recovery of this isomer. The carbon mixture gave good recoveries of fenitrothion and fenitro-oxon but was a more tedious method. Carbon is, however, an excellent decolourizing agent and may be more useful with samples containing more chlorophyll and other pigments.

(iv) Biological accumulation and degradation:

The amounts of fenitrothion and fenitro-oxon found in the

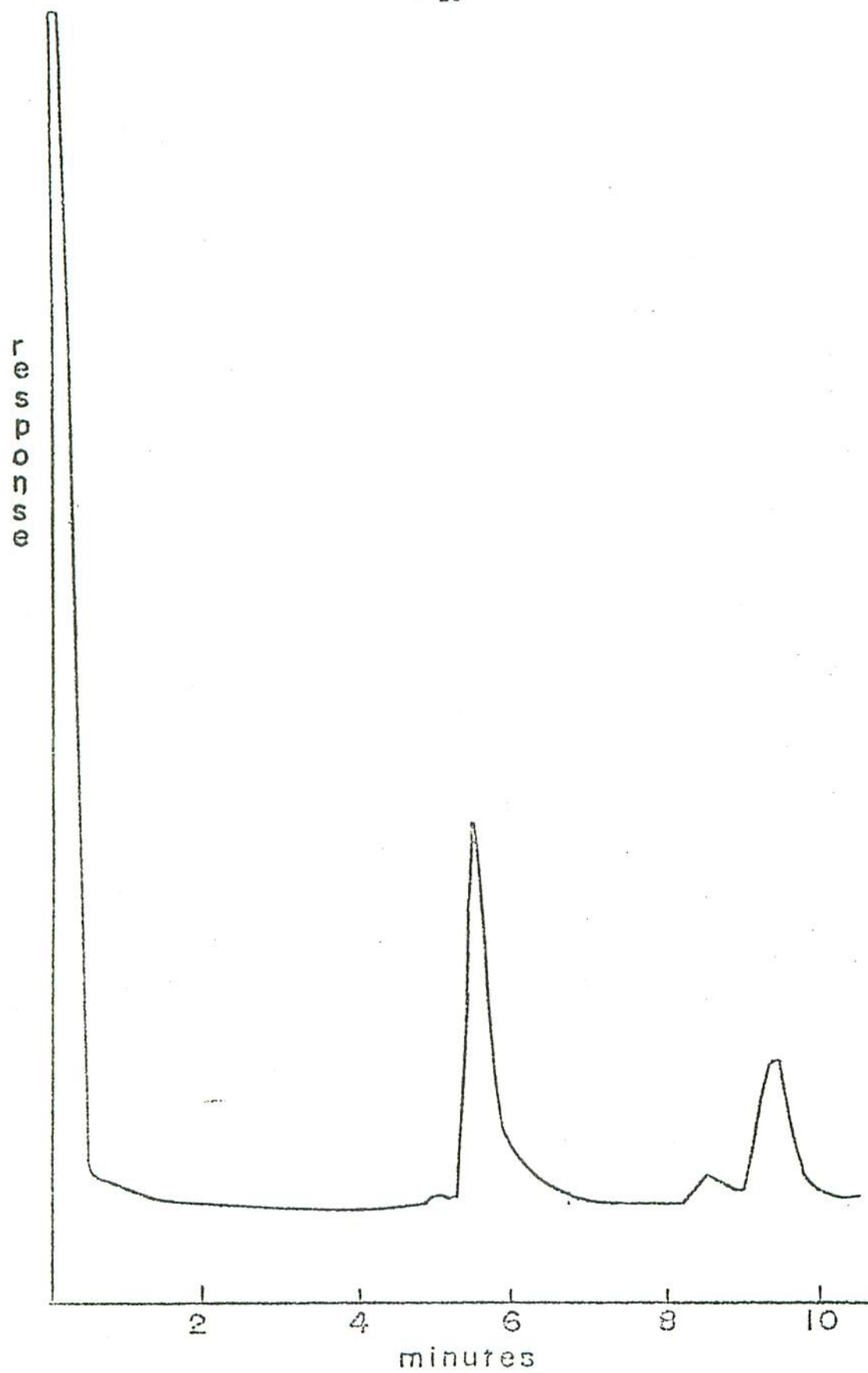


Fig. 7. Gas chromatogram of ethylated desmethyl-fenitrothion.

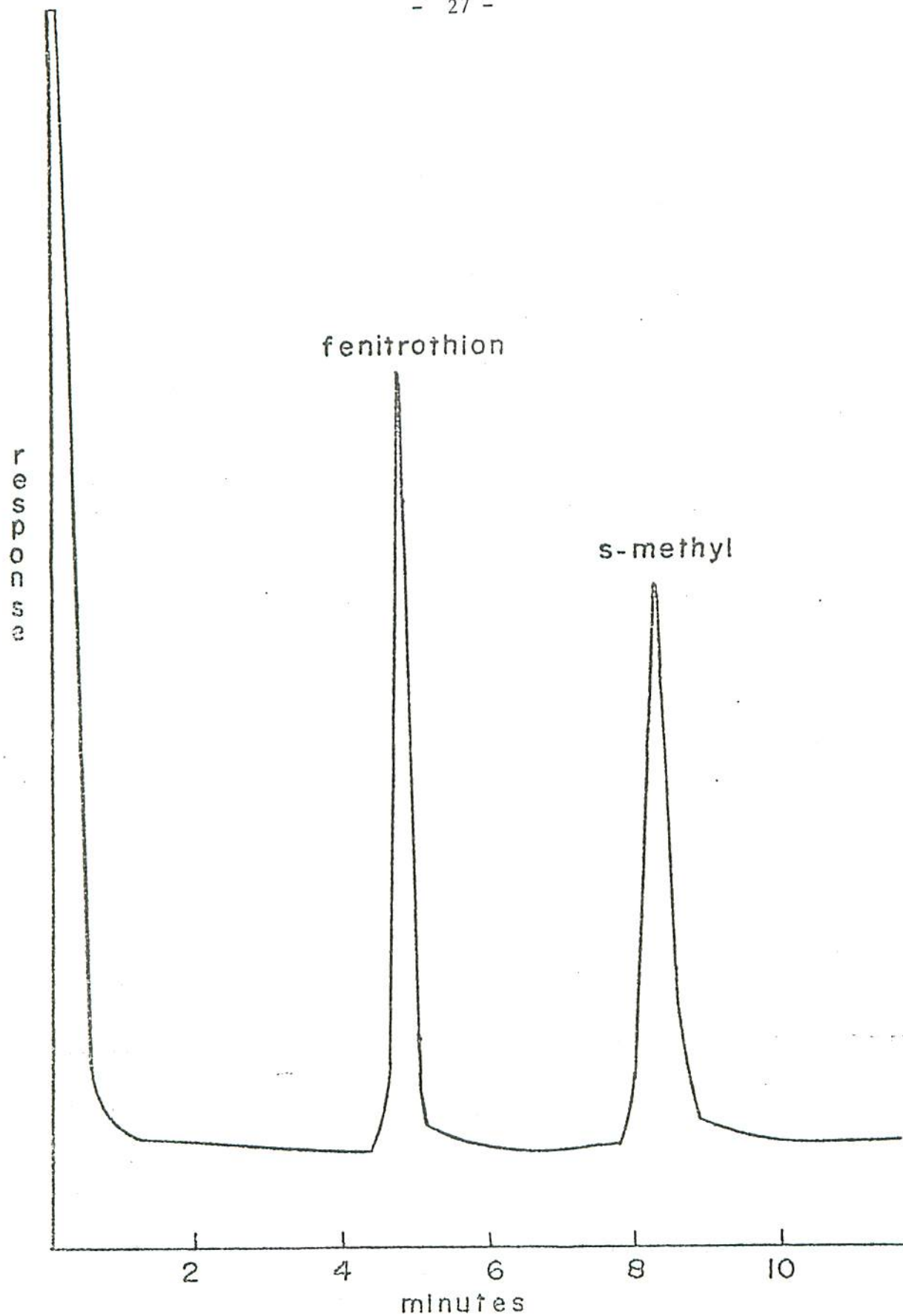


Fig. 8. Gas chromatogram of methylated desmethyl fenitrothion.

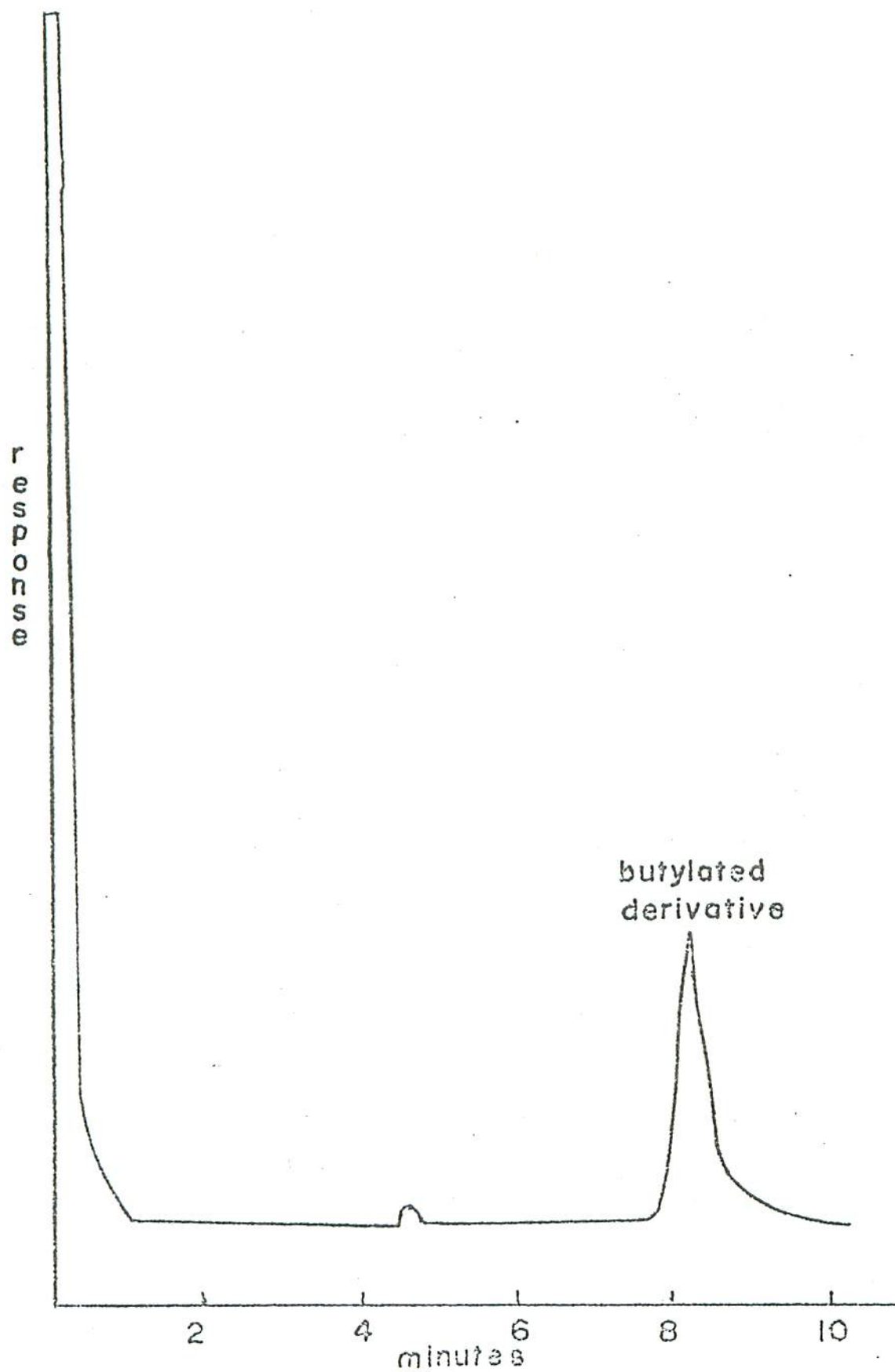


Fig. 9. Gas chromatogram of butylated desmethyl fenitrothion.

TABLE 2

Recovery of fenitro-oxon on florisisil, silica gel, and  
charcoal columns

Activation (% H <sub>2</sub> O)	Percent Recovery (of 1.7 ug fenitro-oxon)		
	<u>Florisisil</u>	<u>Silica Gel</u>	<u>Carbon</u>
0	0, 0,	100, 100**	100, 100
5	50, 52, 58	100, 100	
10	100, 100*	100, 100*	
15	100, 100**	64, 66	
20	100, 100	64, 66, 67, 69	

---

\* Checked with 0.16 ug fenitro-oxon

\*\* 25% recovery of S-methyl fenitrothion

\*\*\* 100% recovery of S-methyl fenitrothion

separated seed parts are shown in Table 3 and 4. The results are expressed in ug per seed part rather than ppm as this shows the accumulation of the compounds in the growing seed parts more clearly.

The total amount of pesticide absorbed by the seeds increased during germination (Table 3). This accumulation of the fenitrothion can be correlated with duration of exposure and to seed preconditioning (stratification). A large amount of the fenitrothion; 7.5 ug or 51.5% of the total, was initially absorbed in the seed coat after 4 days of germination. This amount remained relatively constant through 21 days of germination, but represents only 22.9% of the total pesticide absorbed after 12 days of germination. The amount of fenitrothion absorbed into the perisperm increased 2.5 times between 4 and 21 days of germination from 6.82 to 16.69 ug. This is an increase from 48.5 to 71.9% of the total amount of pesticide absorbed. The fenitrothion was therefore absorbed and transported from the seed coat to the perisperm where the pesticide accumulated. The dynamic accumulation and transport is also substantiated by increased levels of pesticide found in the embryo (Table 3). Seeds pre-conditioned by stratification accumulated more fenitrothion in the embryo during 21 days germination (9.00 ug) than non-stratified seeds (1.19 ug). This represented 40.0% of the total pesticide absorbed by the preconditioned seeds vs. 5.1% by the non-stratified seeds. The total amount of pesticide absorbed by the whole seed was however similar; 22.46 ug vs. 23.19 ug.

It would appear that P. strobus seeds contain multi-function oxidases in the perisperm and embryo, which actively oxidize fenitrothion to fenitro-oxon. Comparatively high levels of fenitro-oxon, approximately

TABLE 3

Fenitrothion uptake in P. strobus seeds, ugm per part and  
per cent distribution per seed part\*

Days	Seed Coat		Perisperm		Embryo		Total	
set	g**	S21**	g	S21	g	S21	g	S21
0	0	4.70 (17.7%)	0	2.34 (42.1%)	0	0.03 (40.0%)	0	7.07
4	7.40 (51.5%)	3.60 (39.4%)	6.82 (48.4%)	6.98 (63.3%)	0.11 (0.8%)	0.44 (1.7%)	14.30	11.02
8	4.62 (31.4%)	3.01 (21.7%)	9.84 (66.0%)	9.27 (61.5%)	0.36 (2.5%)	1.92 (16.6%)	14.82	14.20
14	6.62 (36.7%)	7.86 (34.1%)	11.29 (58.7%)	11.16 (48.4%)	0.83 (4.5%)	4.01 (17.4%)	18.73	23.03
21	5.31 (22.9%)	3.97 (17.1%)	16.69 (71.9%)	9.48 (42.1%)	1.19 (5.1%)	9.00 (40.0%)	23.19	22.46

\* estimated from 10 combined seed parts

\*\* germinated

\*\*\* S21 stratified 21 days prior to germination.

TABLE 4

Distribution of fenitro-oxon in P. strobus seeds, ugm per seed  
part and per cent distribution per seed part\*

Days	Seed Coat		Perisperm		Embryo		Total	
	g***	S21****	g	S21	g	S21	g	S21
0	**	**	**	**	**	**	**	**
4	**	0.003 (5.5%)	**	0.035 (65.5%)	**	**	**	0.053
8	**	0.003 (0.8%)	**	0.262 (88.5%)	**	0.082 (10.5%)	**	0.114
14	0.003 (0.1%)	0.065 (27.7%)	0.046 (16.0%)	0.080 (34.1%)	0.104 (83.9%)	0.089 (38.0%)	0.153	0.234
21	0.001 (0.2%)	0.073 (49.5%)	0.190 (42.5%)	0.014 (9.6%)	0.256 (57.2%)	0.060 (40.9%)	0.447	0.147

\* estimated from 10 combined seed parts

\*\* detectable limit 0.001 ugm

\*\*\* germinated

\*\*\*\* S21 stratified 21 days prior to germination.

# THIN LAYER CHROMATOGRAM OF Sg8 SEEDS

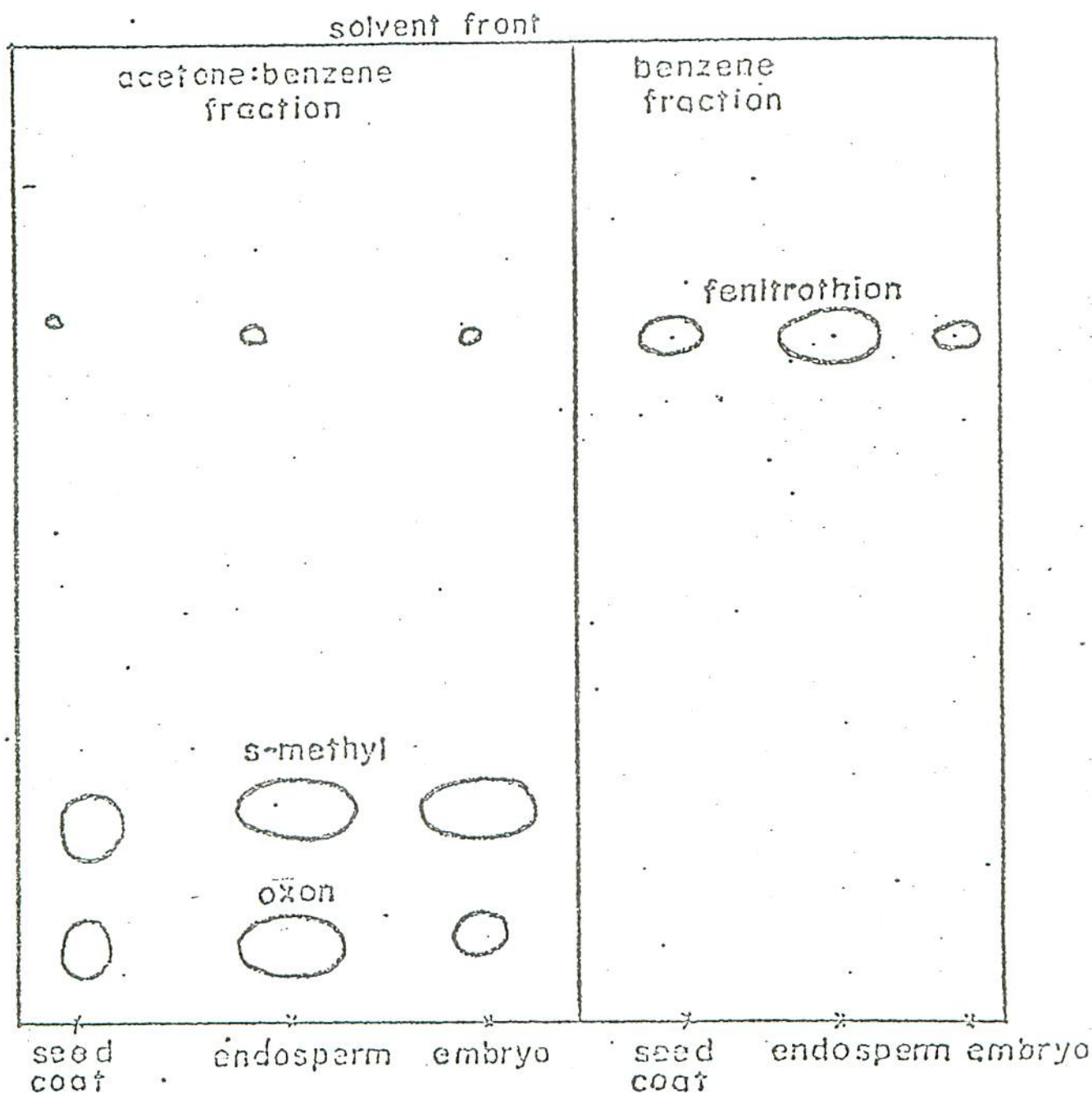


Fig. 10. Thin layer chromatogram of seed extract showing fenitrothion, fenitro-oxon and S-methyl fenitrothion.

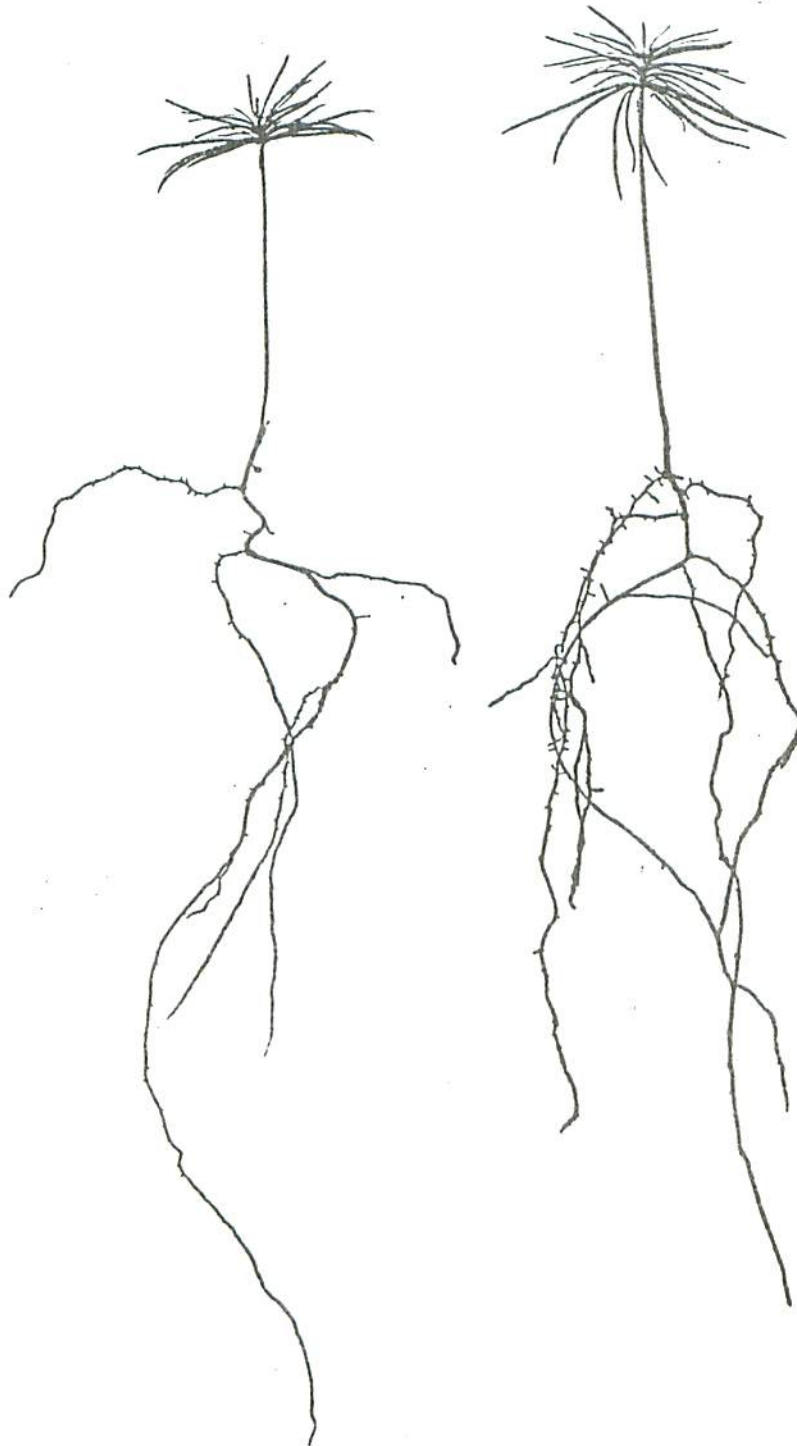


Plate 1. Six month old seedlings grown under identical conditions derived from control seeds (left) and seeds germinated in  $1.5 \times 10^{-4}$  M fenitrothion solution

0.2 ug per seed part, were found in the perisperm of 8 day old (stratified) seedlings and in the 21 day old embryos of non-stratified seeds (Table 4). These amounts represented 88.5 and 57.2% of the total detectable fenitro-oxon in the seed.

S-methyl fenitrothion was detected by TLC in the seed parts containing fenitro-oxon, Figure 10. It was estimated to be present in amounts twice as high, relative to fenitro-oxon, as the oxon and S-methyl isomer have approximately equal sensitivities to the enzyme inhibition spray. The S-methyl isomer and 3-methyl-4-nitrophenol were also detected by gas chromatography.

A growth study was also carried out to determine the effects of the insecticide on the seed. Plate 1 shows six month old seedlings derived from fenitrothion treated seeds together with a non-treated control, no developmental deviations are apparent.

#### DISCUSSION

It is evident that fenitrothion in an aqueous solution is absorbed through the seed coat of P. strobus seeds and transported through the perisperm to the embryo. During the stages of early seed morphogenesis fenitrothion was shown to accumulate preferentially in seeds preconditioned by stratification. A small amount of the fenitrothion was metabolized to fenitro-oxon in all the seed parts and this was principally evident in the endosperm and embryo. The transport and metabolism of fenitrothion, to the oxon form and the S-methyl isomer in the perisperm and embryo of P. strobus seeds has not been noted previously.

Traces of the S-alkyl and S-aryl isomers of parathion have been found on cotton leaves, two weeks after treatment. Joiner and Baetcke (1973) attributed their formation to photolysis. Recently, S-methyl fenitrothion, which is a potent cholinesterase inhibitor has been reported as a contaminant of technical grade fenitrothion (Kovacicova et al. 1973). Since the pine seeds were germinated in the dark and the fenitrothion used in this work contained only 0.2% of the S-methyl isomer, the S-methyl fenitrothion detected in the seeds is thought to arise from metabolism of the parent compound by seed enzymes.

From the growth study, it does not appear that fenitrothion, fenitro-oxon and S-methyl fenitrothion have any long term effects on seedling growth.

S-methyl fenitrothion probably is formed from the intermediary metabolite desmethylfenitrothion. Desmethylfenitrothion has been shown by Miyamoto to occur in both plant and animal tissues (Miyamoto et al. 1965, 1969). Hollingworth et al. (1967, 1973) has described dealkylation of fenitrothion by mouse liver enzymes to form S-methyl glutathione and desmethylfenitrothion, both in vivo and in vitro. Hutson et al. (1972) have isolated a phosphoric acid triester-glutathione alkyl-transferase from mouse, rat, rabbit, and pig liver. Dealkylation is, therefore, a major pathway of metabolism of fenitrothion.

As fenitrothion is a strong alkylating agent it is likely that S-methylfenitrothion is a product of the realkylation of desmethylfenitrothion. Kovacicova et al. (1972) has shown that like fenitro-oxon, S-methylfenitrothion is more subject to hydrolysis than the parent compound. The hydrolysis rate is nearly 3 orders greater than fenitrothion. This results from the higher electrophilic effect of the

phosphoryl rather than the thiophosphoryl group.

It is possible that just as glutathione is alkylated by fenitrothion to form S-alkyl glutathione and desmethylfenitrothion (Hollingworth et al. 1967, 1973) other biological molecules such as the amino acid cysteine and the nucleic acid guanine may also be alkylated. These moities have been shown to be alkylated by other carcinogenic and pesticide agents (Lawley and Shah, 1972). Alkylation of these sites would have mutagenic effects on the cells affected; cysteine is essential to the secondary structure of protein molecules and if alkyl guanine was incorporated into DNA and RNA, genetic codons would be misread. The extent of alkylation of these molecules will be investigated using  $C^{14}$  labelled pesticide. Labelled fenitrothion has been kindly supplied by Dr. J.R. Duffy for this purpose.

Premium grade fenitrothion used in the seed study was found to contain 0.2% of S-methyl fenitrothion. A second aliquot obtained for an analytical standard contained 1.0%. Purification of technical grade fenitrothion gives 0.1% of the S-methyl derivative remaining which can be used as a starting point in metabolic studies.

New, quick gas chromatographic methods have been obtained to determine amounts of desmethylfenitrothion by derivatization. These methods provide more accurate measures of the molecule than by thin layer chromatography, and do not necessitate the use of labelled pesticide in experimental work.

The silica gel column separations indicated lower recoveries of oxon with 20% water to deactivate the adsorbent than was previously reported (Bowman and Beroza, 1969; Yule and Duffy, 1972). The maximum amounts of oxon recovered from pine seeds were 0.2 ug. Therefore lower

amounts of oxon were used in this study; 1.6 to 0.16 ug, closer to those recovered from treated material. Previous methods had used 5 to 50 ug of oxon with fortified samples. The amount of hydrolysis due to water in the case of silica gel, or other hydrolysing agents in the case of florisil per molecule of oxon would be much higher with fewer molecules of oxon on the column. This hydrolysis would account for the lower recoveries of oxon. Silica gel containing from 0 to 10% water by weight is the best absorbent for column separation of fenitrothion from fenitro-oxon and S-methyl fenitrothion.

Residue levels of these metabolites will be quantitated in our ongoing study in plants exposed to fenitrothion and then consumed by animals in the food chain.

#### SUMMARY

- 1) A method has been established for the extraction, column clean-up and quantitation by GLC of fenitrothion, fenitro-oxon, and S-methyl fenitrothion from white pine (Pinus strobus L.) seeds and seedlings.
- 2) Fenitrothion has been shown to be absorbed through the seed coat of P. strobus seeds and transported through the perisperm to the embryo where it accumulates during germination.
- 3) Fenitrothion was shown to be metabolized to fenitro-oxon, and S-methyl fenitrothion in the perisperm and embryo of germinating seeds. Fenitro-oxon exhibits up to 2 logs, and S-methyl fenitrothion up to 3 logs higher anticholinesterase activity than fenitrothion. Fenitrothion and these metabolic products do not appear to have long term effects on seedling growth.

- 4) Methods for synthesizing; amino fenitrothion, amino fenitro-oxon, S-methyl fenitrothion, and desmethylfenitrothion are described. These molecules were characterized by gas and thin layer chromatography for quantitation as metabolites of fenitrothion.
- 5) All methods developed can be probably adapted to detect residues and metabolites in other biological material including animal tissues.

#### CONCLUSIONS

Residues of fenitrothion, fenitro-oxon, S-methyl fenitrothion, and desmethyl-fenitrothion should be monitored closely in the forest environment. S-methyl fenitrothion has not been previously considered as a biological metabolite or a break-down product of fenitrothion. If only 1 percent is converted to S-methyl fenitrothion the anticholinesterase activity of the residues will be up to 10 X higher than the parent compound.

Fenitrothion does not appear to show long term phytotoxicity to white pine seedlings.

#### ACKNOWLEDGMENTS

The authors wish to thank Ms. M. Wilson (Canada Agriculture) for her help in developing gas chromatographic methods, Mr. S. Skinner for mass spectroscopy and Mr. L. Pomber for his help in the growth studies. They are grateful to Dr. J. J. Fettes, Director, Chemical Control Research Institute, Dept. of the Environment, for a grant which made this work possible and to Mssrs. Dal Travnick and B. Wang for collecting seed samples used in this study.

#### REFERENCES

- Bowman, M.C. and Beroza, M. 1969. Determination of Accothion, its oxygen analogue, and its creson in corn, grass and milk by gas chromatography. *J. Agr. Food Chem.* 17: 271-6.
- Fettes, J.J. 1968. Chemical control of forest insects by aircraft. *Pulp and Paper Mag. of Canada* 69: 99.
- Getz, M.E. 1962. Six phosphate pesticide residues in green leafy vegetables: cleanup method and paper chromatographic identification, *J. of the A.O.A.C.*, 45: 393.
- Hollingworth, R.M., Metcalf, R.L., Fukuto, T.R. 1967. The selectivity of sumithion compared with methyl parathion. Metabolism in the White Mouse. *J. Agr. Food Chem.* 15: 242-249.
- Hollingworth, R.M., Alstott, R.L. and Litzenberg, R.D. 1973. Glutathion S-Aryl transferase in the metabolism of parathion and its analogs. *Life Sciences.* 13:191-199.
- Hutson, D.H., Pickering, B.A. and Donniger, C. 1972. Phosphoric acid triester-glutathione alkyltransferase. A mechanism for the detoxification of dimethyl phosphate triesters. *Biochemical Journal* 127: 285-293.
- Joiner, R.L., and Baetcke, K.P. 1973. Parathion: Persistence on cotton and identification of its photoalteration products. *J. Agr. Food Chem.*, 21: 391.
- Kovacikova, J., Masud, Z., Batora, V., Kovac, J. and Truchlik, S. 1971. Laboratory purification of fenitrothion. *Pesticide Science*, 2: 101-102.

- Kovacícova, J., Batora, V. and Truchlík, S. 1973. Hydrolysis rate and in vitro anticholinesterase activity of fenitrothion and S-methyl fenitrothion Pesticide Science, 4:
- Kovacícova, J. and Greenhalgh, R. 1974. Determination of the S-methyl isomer in technical grade fenitrothion samples, in press.
- Lawley, P. and Shah, S. 1972. Reaction of alkylating mutagens and carcinogens with nucleic acids: detection and estimation of a small extent of methylation at O-6 of guanine in DNA by methyl methanesulphonate in vitro. Chem.-Biol. Interactions, 5:268-288.
- Mendoza, C.E. 1972. Analysis of pesticides by the thin-layer chromatographic-enzyme inhibition technique. Residue Reviews, 43: 105-142.
- Miyamoto, J., Sato, Y., Kadota, T., Fujinami, A., and Endo, M. 1963. Studies on the mode of action of organophosphorus compounds. Part I. Metabolic fate of  $P^{32}$  labelled sumithion and methylparathion in guinea pigs and white rats. Agricultural Biol. Chem., 27:381.
- Miyamoto, J., Sato, Y., Kadota, T., Fujinami, A., and Endo, M. 1964. Studies on the mode of action of organophosphorus compounds. Part III. Activation and degradation of Sumithion and methylparathion in mammals in vivo. Agr. Bio. Chem. (Tokyo) 28:411.
- Miyamoto, J., Kawaguchi, Y., Sato, Y. 1965. Determination of insecticide residue in animal and plant tissues. II. Metabolic fate of Sumithion in rice plants applied at the preheading stage and its residue in harvested grains. Botyo-Kagaky (Scientific Pest Control), 30: 45-9.

- Miyamoto, J., Kitagawa, K., Sato, Y. 1966. Metabolism of organophosphorus insecticides by Bacillus subtilis, with special emphasis on Sumithion. Japanese Journal of Experimental Medicine, 36: 211-225.
- Miyamoto, J., Sato, Y., Suzuki, S. 1967. Determination of insecticide residues in animal and plant tissue. IV. Determination of Sumithion and some of its metabolites in fresh milk. Botyukagaku, 32: 95-100.
- Miyamoto, J. 1969. Mechanism of low toxicity of Sumithion toward mammals. Residue Reviews, 25: 251-64.
- Schlenk, H. and Gellerman, J.L. 1960. Esterification of fatty acids with diazomethane on a small scale. Analytical Chemistry, 32: 1412-1414.
- Seiber, J.N. 1972. N-perfluoroacyl derivatives for methyl-carbamate analysis by gas chromatography. J. Agr. Food Chem., 20:443-446.
- Sumitomo Chemical Company Ltd., Osaka, Japan. 1969. Sumithion, its toxicity, metabolism and residues. Unpub. rept.
- Sundaram, and Sundaram, K.M.S. 1969. Studies on the translocation pattern, persistence characteristics and metabolic pathway of Sumithion in the cocoa tree Theobroma cacao L., Ghana Journal of Science, 9: 96-110.
- Watts, R.R., Storherr, R.W., Pardue, J.R. and Osgood, T. 1969. Charcoal column cleanup method for many organophosphorus pesticide residues in crop extracts. Journal of the AOAC, 52: 522-526.
- Yule, W.N. and Duffy, J.R. 1972. The persistence and fate of fenitrothion insecticide in a forest environment. Bull. of Environ. Contamination and Toxicology. 8: 10-18.