Determination of Fenthion and Five of Its Metabolites in Corn, Grass, and Milk

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Fenthion {*O,O*-dimethyl *O*-[4-(methylthio)-*m*-tolyl] phosphorothioate} and five of its metabolites were determined in extracts of corn, grass, and milk. Extracts were separated by liquid chromatography into three fractions which then were concentrated and injected into a gas chromatograph equipped with a flame photometric detector sensitive to phosphorus. Only one of the three fractions from milk required an additional cleanup, a partitioning in a binary solvent system. Practically no interference from crop extracts was encountered. Recoveries from corn and grass at the 0.1-p.p.m. level and from milk at the 0.05-p.p.m. level were 95 to 100% with one exception; one metabolite was recovered from corn and grass in 75% yield. Response was linear with concentration, and sensitivity for each of the six compounds was 0.003 p.p.m. or better. Retention times are given for the six metabolites on four liquid phases; also, conditions for separating all six compounds by liquid chromatography on silica gel are described.

Fenthion, also called Baytex, Entex, Tiguvon, *O,O*-dimethyl *O*-[4-(methylthio)-*m*-tolyl] phosphorothioate, a product of the Chemagro Corp., Kansas City, Mo., has exhibited good insecticidal action against a variety of insect pests. A method was needed to determine residues of this chemical in corn and grass intended for use as feed for livestock and in the milk of cows fed forage containing residues of the insecticide. The need to determine metabolites of the parent compound is apparent from the work of several investigators who reported on metabolites formed from fenthion in plants and animals (Anderson *et al.*, 1966; Brady and Arthur, 1961; Niessen *et al.*, 1962).

Five metabolites that may form are shown in Figure 1 along with the parent compound. The method used to designate each compound is given in the legend. The six compounds differ from each other at two points: They contain either a P=S or P=O (O-analog) moiety, and the sulfur in the 4-position of the aromatic ring may exist as a sulfide, sulfoxide, or sulfone linkage.

In a method of determining fenthion residues recently reported (Anderson *et al.*, 1966), fenthion and the five metabolites are oxidized to the O-analog sulfone (VI) which is then hydrolyzed to the corresponding phenol. The phenol is brominated and acetylated and then analyzed by electron-capture gas chromatography. Sensitivity of the method is 0.1 p.p.m. The individual metabolites are not determined. Suffet *et al.* (1967) have described a gas chromatographic analysis of fenthion, its O-analog, and the phenolic hydrolysis product in water using either flame ionization or electron-capture detection. The method of Suffet *et al.* is not suitable for the present analysis without a cleanup, and apparently no procedure for separating the five metabolites was considered. Procedures for determining fenthion that are not applicable to residue analysis have also appeared (Frehse *et al.*, 1962; Gudzinowicz, 1965; Ibrahim and Cavagnol, 1966).

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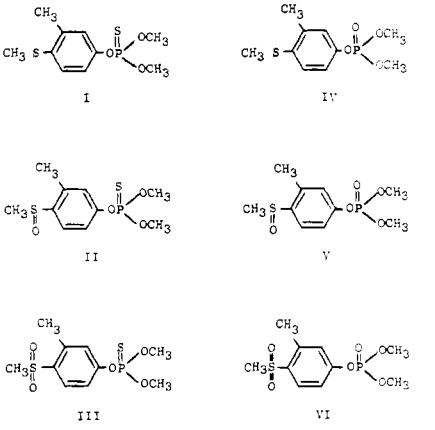

Figure 1. Fenthion (I) and five of its metabolites

I P=S,S; II P=S,SO; III P=S,SO_2; IV P=O,S; V P=O,SO; VI P=O,SO_2

A highly sensitive method has now been devised to determine all six compounds. Briefly, the foodstuff is extracted, the extract is separated into three fractions by liquid chromatography on a silica gel column, and an aliquot of each of the fractions is subjected to gas chromatography on an instrument equipped with the flame photometric detector of Brody and Chaney (1966) sensitive to phosphorus. In milk analyses, a hexane-acetonitrile partition on one fraction is also included. The method is sensitive to 0.003 p.p.m. or less of each metabolite. Recoveries are 95 to 100% for all but compound IV in corn or grass; it is recovered in 75% yield.

A means of separating all of the metabolites by liquid chromatography is also presented, and retention times of fenthion and the five metabolites on four gas chromatographic liquid phases are given.

EXPERIMENTAL

Reagents and Solvents. Silica gel, J. T. Baker Chemical Co., No. 3405, was used as received. When heated overnight at 110° C., it lost 3.52% of its weight.

Fenthion and its metabolites were analytical grade chemicals kindly supplied by the Chemagro Corp.

Acetone, benzene, hexane, acetonitrile, and methylene chloride were C.P. grade solvents redistilled. Sodium sulfate was the anhydrous reagent grade chemical.

Equipment. An F & M Scientific Corp. (Avondale, Pa.) Model 700 gas chromatograph was equipped with the Melpar flame photometric detector (MicroTek Instruments, Inc., Baton Rouge, La.) (Brody and Chaney, 1966) and a 526-mμ interference filter, which detects phosphorus.

Sample Preparation and Extraction of Corn and Grass. Transfer 20 grams of the finely chopped and well mixed plant material to a Soxhlet extraction apparatus (Fisher Scientific Co., No. 9-556 B) containing a plug of glass wool to prevent insoluble plant material from siphoning over during solvent exchanges. Extract the sample under nitrogen for 2 hours with 150 ml. of chloroform-methanol, 9 to 1 by volume, at the rate of about six solvent exchanges per hour. Allow the extract to cool and percolate it through a plug of anhydrous sodium sulfate, about 25 mm. in diameter by 30 mm. thick; then wash the container and plug with 10 ml. of chloroform. Evaporate the extract to dryness on a 50° C. water bath under water pump vacuum (ca. 35 mm. of Hg). (Presence of chloroform, water, and methanol can cause difficulty in the subsequent liquid chromatography.) Take up the residue in 10 ml. of benzene for the liquid chromatography.

Extraction of Milk. Shake the sample to disperse the cream uniformly and add 100 grams to a Waring Blendor. Add 300 ml. of acetone, blend for 3 minutes, and filter through Whatman No. 1 paper on a Büchner funnel. Wash the blender and filter funnel with an additional 25 ml. of acetone. Extract the filtrate with 200 and then 100 ml. of methylene chloride, and percolate each methylene chloride extract successively through a plug of sodium sulfate about 4 cm. in diameter and 5 cm. thick. Evaporate the percolate almost to dryness under a Snyder column on a steam bath and then just to dryness at room temperature under water pump vacuum. Add 10 ml. of benzene to dissolve the fatty residue, and reserve for the liquid chromatographic separation.

Liquid Chromatographic Separation. Prepare a silica gel column by adding successively in a 12-mm. I.D. glass column (Kontes No. K-42000) a plug of glass wool, 2 grams of sodium sulfate, 4 grams of silica gel, and 2 grams of sodium sulfate. Prewash the column with 20 ml. of benzene and discard the filtrate. Add the corn or grass extract (10 ml., equivalent to 20 grams of product) or the milk extract in benzene (10 ml., equivalent to 100 grams of milk). Allow the extract to percolate into the column, and reserve all the eluate. Wash the vessel and column with 5 ml. of 1% acetone in benzene; then elute with 45 ml. more of the same solvent. The eluate (fraction A) contains fenthion (I) and its sulfone (III).

Change receivers and elute with 50 ml. of 10% acetone in benzene. This eluate (fraction B) contains the sulfide of the O-analog (IV), the sulfoxide of fenthion (II), and the sulfone of the O-analog (VI).

Finally, elute with 50 ml. of acetone. The eluate (fraction C) contains the sulfoxide of the O-analog (V).

A summary of these liquid chromatography operations is given in Figure 2.

Preparation of Fractions for Gas Chromatography. CORN AND GRASS. Evaporate all fractions to near dryness by using water pump vacuum and a 50° C. water bath. Transfer the residue to a calibrated tube and adjust the volume to exactly 2 ml. with benzene (5 μl. equivalent to 50 mg. of corn or grass) for gas chromatographic analysis.

MILK. Evaporate fractions B and C as described for the corn and grass fractions but adjust volume to exactly 5 ml. with benzene (5 μl., equivalent to 100 mg. of milk).

Evaporate fraction A as described for the corn and grass fractions, but take to dryness. Transfer the fatty residue to a glass-stoppered tube with 5 ml. each of pre-equilibrated hexane and acetonitrile, and shake the contents of the tube for 1 minute. When the layers separate, withdraw the acetonitrile layer and extract the remaining hexane phase once more with an additional 5 ml. of acetonitrile. Discard the hexane layer and combine the acetonitrile layers, adjusting them to 10 ml. if necessary. The acetonitrile layer (5 μl., equivalent to 50 mg. of milk) is used in the gas chromatographic analysis.

Gas Chromatographic Analysis. Use the following conditions:

Column. Glass, 90 cm. × 4-mm. I.D. (6-mm. O.D.) for fractions A and B; 45-cm. long column for fraction C.

Packing. DC 200, 10% (w./w.) on 80- to 100-mesh Gas-Chrom Q (Applied Science Laboratories, State College, Pa.).

Carrier Gas. Nitrogen at 160 ml. per minute.

Other Gases. Oxygen at 40 ml. per minute; hydrogen at 200 ml. per minute.

Temperatures. Column 210° C.; injection port 225° C.; detector (external) 200° C.

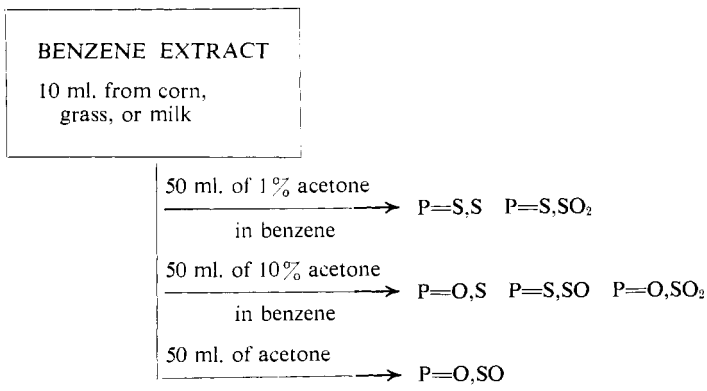

Figure 2. Summary of operations in liquid chromatography on silica gel

See legend of Figure 1 for compound designations

After conditioning the column overnight at 230° C., condition the column further with the gas chromatograph operating as described above by injecting 250-ng. amounts of insecticide in extract equivalent to 50 or 100 mg. of plant or milk until several successive injections of 5-ng. amounts of insecticide in plant extract (0.1 p.p.m.) produce a constant response. Conditioning the column for the oxygen analogs was a much slower process than conditioning it for the thionophosphates.

With the stated conditions, inject on the conditioned column 5 μl. of the extracts as prepared for gas chromatographic analysis or diluted as appropriate. Base response on peak height.

Recovery Experiments. Fortify corn and grass samples with 1-ml. solutions of the six compounds in benzene at the 0.1-p.p.m. level prior to extraction. Fortify milk samples with 1-ml. acetone solutions of the six compounds at the 0.05-p.p.m. level, and blend for 1 minute prior to adding the acetone for the extraction. Fortifications at these levels were made with each of the six compounds and with a mixture of all six compounds.

RESULTS AND DISCUSSION

Samples of corn, grass, and milk fortified with the six compounds individually and together as described were carried through the entire analytical procedure. Recoveries were between 95 and 100% except for the O-analog of fenthion (P=O,S) in corn and grass, for which recoveries were about 75%. (Recovery of fenthion in milk was corrected for loss in the hexane-acetonitrile partition.) Samples fortified with a single compound were carefully analyzed to check for possible conversion to any of the other compounds during the course of analysis, but no evidence of such conversion was found.

Chromatograms of fractions A, B, and C obtained in the liquid chromatographic separation on silica gel are shown as solid lines in Figure 3. Since nothing in the grass, corn, or milk interfered with the analysis of the six compounds, only the chromatograms of the standards are shown. The broken line shown in Figure 3*A* was inserted to permit a comparison of the response of the O-analog sulfoxide (P=O,SO) on a 90-cm. long column with that obtained on a column half as long (Figure 3*C*). The shorter column was used because the response (peak height) of the compound was much greater.

Minimum detectable levels of each of the six compounds (based on twice the noise level) are given in Table I; they show that the method has unusually high sensitivity. Response (peak height) is linear with concentration to at least 250 ng. Retention times of the compounds are given in Table II for the 90-cm. long DC 200 column and for three other columns: QF-1, Carbowax 20M, and diethylene glycol succinate (DEGS). The retention times on the three additional columns may prove useful for confirming identity of peaks should unexpected interference occur in the analysis of products other than those described here; it is suggested that comparisons be based on the retention times or relative retention times of known standards.

The ancillary techniques of ultraviolet, infrared, and NMR are not generally applicable for confirming the identity of a metabolite because they require micrograms of compounds, amounts not normally present in the gas chromatography of pesticide residues. *p*-Values, which are useful at the nanogram level (Beroza and Bowman, 1965), were therefore determined in several solvent sys-

Table I. Minimum Detectable Levels of Fenthion and Five of Its Metabolites

Chemical	In Grass or Corn, P.P.M., 50 Mg. Equiv./Analysis	In Milk, P.P.M., 100 Mg. Equiv./Analysis
P=S,S (I)	0.001	0.0005
P=S,SO (II)	0.003	0.002
P=S,SO_2 (III)	0.002	0.001
P=O,S (IV)	0.001	0.0005
P=O,SO (V)	0.003	0.002
P=O,SO_2 (VI)	0.003	0.002

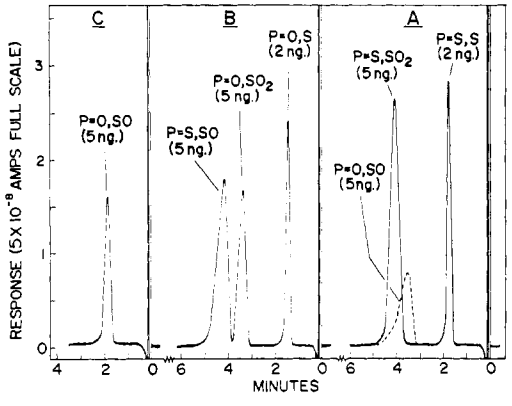

Figure 3. Gas chromatograms of fenthion and five of its metabolites in fractions A, B, and C obtained by liquid chromatography on silica gel (solid lines only)

Column length 90 cm. in gas chromatography of fractions A and B, 45 cm. for fraction C. Broken-line peak in chromatogram A and peak in chromatogram C show chromatograms of 5 ng. of the same compound (P=O,SO) on columns 90 and 45 cm. long, respectively (P=O,SO is not found in fraction A)

Table II. Retention Times of Fenthion and Five of Its Metabolites on Four Gas Chromatographic Columns

Gas Chromatographic Column[a]	Retention Time, Min., for Indicated Compound					
	P=S,S	P=S,SO	P=S,SO_2	P=O,S	P=O,SO	P=O,SO_2
DC 200 (10%), 90 cm.	1.75	4.20	4.05	1.45	3.60	3.40
QF-1 (10%), 50 cm.	0.40	2.50	2.75	0.60	3.90	4.10
Carbowax 20M (1%), 50 cm.	0.30	1.70	2.25	0.35	1.80	2.35
DEGS (5%), 50 cm.	<0.30	3.05	3.70	<0.30	4.10	5.40

[a] Borosilicate glass, 4-mm. I.D.; support was 80- to 100-mesh Gas-Chrom Q; columns conditioned overnight at 230° C. and operated at 210° C. Other parameters were as described in the method.

tems. These data are given in Table III with the suggestion that they may be useful for confirming identities.

Fraction A of the milk extract contained a large amount of butterfat which was easily removed by partitioning the fraction between the hexane-acetonitrile solvent pair. A second extraction of the hexane layer with acetonitrile was desirable because about 8% of the fenthion remained in the hexane phase after the initial distribution. (Less than 1% of the sulfone of fenthion remained in the hexane phase of the initial distribution.) The double-extraction procedure halved the sensitivity of the analysis for fenthion and its sulfone, but the sensitivity (0.001 and 0.002 p.p.m. per 50 mg. equivalent per analysis of fenthion and its sulfone, respectively) was more than adequate (Table I).

The low recovery (75%) of the O-analog of fenthion (P=O,S) from corn and grass seemed odd in the face of 95 to 100% recoveries in all other instances, and it was investigated further. Direct gas chromatographic analysis of the benzene extract of the untreated crop fortified only with the compound showed that the compound was completely extracted from the crop. However, in the presence of the crop extract, only 75% of the compound would pass the silica gel column even though 100% passed when the crop extract was absent. The 25% of compound remaining on the column was not eluted with acetone (fraction C), nor was it found in fraction A. Possibly, this analog formed a stable conjugate with some ingredient(s) of corn or grass.

As part of this investigation, a complete separation of all six compounds was attempted and achieved (Figure 4) by using liquid chromatography with the silica gel column described. A 1-ml. solution containing 100 μg. of each of the six compounds was placed on the column, and the solution was washed into the adsorbent with a few milliliters of benzene. The following operations were performed sequentially, and each 5 ml. of eluate was analyzed gas chromatographically: 50 ml. of benzene completely eluted the fenthion (P=S,S); 50 ml. of 1% acetone in benzene-eluted fenthion sulfone ($P{=}S,SO_2$) (benzene will also elute this compound but more solvent is required); 40 ml. of 5% acetone in benzene removed the fenthion O-analog (P=O,S); fenthion sulfoxide (P=S,SO) was eluted with 35 ml. of 7.5% acetone in benzene; less than 50 ml. of 10% acetone in benzene was required to elute the O-analog sulfone ($P{=}O,SO_2$) (this compound may also be removed with 7.5% acetone in benzene but it tails badly); and finally, pure acetone was used to elute the O-analog sulfoxide (P=O,SO) (this peak may also be eluted with 50% acetone in benzene, but pure acetone was preferred because it is easier to evaporate).

The foregoing separation of the six compounds is not intended for use in the usual residue analysis. It may be useful for metabolic studies or for separating the parent and five metabolites of similar thionophosphates—i.e., those with a sulfide group (as is present in fenthion). It also provides a means of separating each of the six compounds if additional confirmation of identities by means requiring more material is needed.

Table III. *p*-Values of Fenthion and Metabolites

Compound	Hexane–Water	Hexane–20% Acetonitrile[a]	Hexane–40% Acetonitrile[a]	Benzene–Water[b]
P=S,S	1.00	0.98	0.92	...
P=S,SO	0.50	0.18	0.03	...
$P{=}S,SO_2$	0.94	0.61	0.12	...
P=O,S	0.92	0.65	0.18	...
P=O,SO	0.00	0.00	0.00	0.35
$P{=}O,SO_2$	0.01	0.00	0.00	1.00

[a] 20% acetonitrile = 1 + 4 by volume acetonitrile-water; 40% acetonitrile = 2 + 3 acetonitrile-water.
[b] This solvent system was used for P=O,SO and $P{=}O,SO_2$ because they were not distinguished in the other systems.

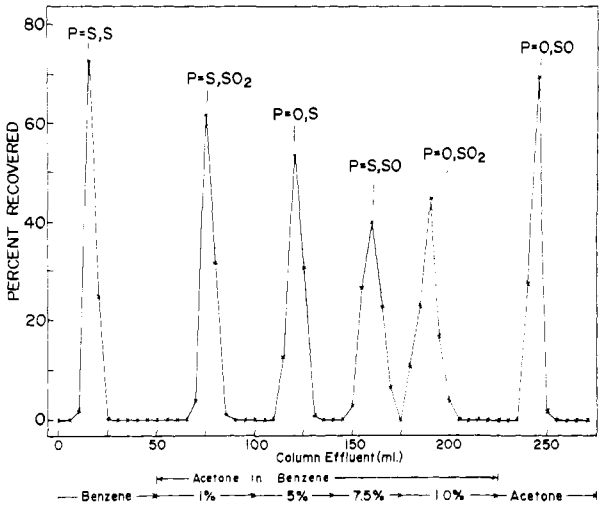

Figure 4. Separation of fenthion and five of its metabolites by liquid chromatography on silica gel

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