none of the feeding studies at 0.2, 0.6, 3, or 5.8 Mrad showed any adverse effects. Clearly, if there is no effect at a higher dose, there should be none at a lower dose.

This rationale is very important in consideration of meeting standards for acceptance of irradiated food products, for many of the products now undergoing feeding tests are being irradiated at higher doses than may be used later. It is with these problems in mind that the current studies have been undertaken to elucidate the behavior of the formation of the various radiolytic products in meat.

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Trace Analysis of Etrimfos and Two Degradation Products in Corn and Alfalfa

Malcolm C. Bowman,* Claude L. Holder, and Larry G. Rushing

A method was devised for determining residues of etrimfos [*O,O*-dimethyl *O*-(6-ethoxy-2-ethyl-4-pyrimidinyl)phosphorothioate], its O analogue, and its hydrolysis product in corn and alfalfa at levels of about 10 ppb or less. Salient elements of the procedure include Soxhlet extraction of the residues overnight with dichloromethane–10% methanol, preliminary cleanup on a column of Sephadex LH-20, separation of the three compounds on a silica gel column, and further cleanup of the hydrolysis product by liquid–liquid partitioning. The parent and O analogue fractions are separately analyzed by using gas chromatography with either a flame photometric detector sensitive to phosphorus or a rubidium-sensitized nitrogen–phosphorus detector. The hydrolysis product is analyzed directly by high-pressure liquid chromatography or converted to a pentafluorobenzoate derivative and assayed by electron-capture gas chromatography. Ancillary analytical chemical data and information concerning the efficiency of extracting field-weathered residues from corn were obtained and anticholinesterase activity and lethality data for etrimfos and the O analogue are also presented.

Etrimfos [formula I, *O,O*-dimethyl *O*-(6-ethoxy-2-ethyl-4-pyrimidinyl)phosphorothioate (also known as SAN 197, ENT 29126, and Ekamet), Sandoz, Inc., Homestead, Fla.] is a new nonsystemic, nonpersistent insecticide with a relatively low mammalian toxicity (acute oral LD_{50} for male rat is 1800 mg/kg). It is recommended for control of chewing, sucking, and biting pests on most crops (Knutti and Reisser, 1975).

Analytical methodology for the compound was required in our studies concerning relationships between chemical

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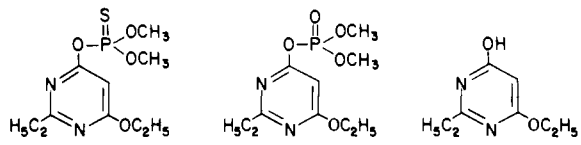

I II III

Figure 1. Formulas of etrimfos (I), its O analogue (II), and its hydrolysis product, EEHP (III).

structures of pesticides and their biological activity. Additionally, such methodology would also be useful for determining residues in crops, feed, and other products; however, no method could be found in the literature. Since residues may consist of the parent insecticide itself, its oxygen analogue (formula II, dimethyl 6-ethoxy-2-ethyl-4-pyrimidinyl phosphate, hereafter O analogue) and the hydrolysis product (formula III, 6-ethoxy-2-ethyl-4-hydroxypyrimidine, hereafter EEHP), all three compounds should be determined. Formulas of these compounds are presented in Figure 1.

This paper describes a procedure for determining residues of the three compounds in corn and alfalfa at levels of about 10 ppb or less. Additional analytical information and data concerning efficiency of extracting field-weathered residues were obtained; anticholinesterase activity and lethality data for etrimfos and the O analogue are presented.

EXPERIMENTAL SECTION

Apparatus. Three instruments were used for gas chromatography (GC). One was a Hewlett-Packard (Avondale, Pa.) Model 5710A instrument equipped with a linear ^{63}Ni electron-capture (EC) detector. Another was a Hewlett-Packard Model 5750B equipped with the phosphorus-sensing flame photometric detector (FPD) of Brody and Chaney (1966) (Tracor, Inc., Austin, Tex.), and the third was a Perkin-Elmer (Norwalk, Conn.) Model 3920 instrument equipped with a rubidium-sensitized nitrogen-phosphorus detector (NPD).

A Waters Associates, Inc. (Milford, Mass.) instrument equipped with a Model 6000A solvent delivery system, a Model U6K septumless injector, and a Model 440 multiple wavelength detector operated at 254 nm was used for the high-pressure liquid chromatography (HPLC).

Reagents. The silica gel (No. 3405, J. T. Baker Chemical Co., Phillipsburg, N.J.) was heated overnight in an oven at 130 °C and stored in a desiccator prior to use. The silica gel was then deactivated for use in the analytical procedures by adding 40 g of the dry material to a glass-stoppered bottle containing 10 mL of 0.05 M phosphate buffer (pH 7, No. SO-B-108, Fisher Scientific Co., St. Louis, Mo.); the contents were mixed well and allowed to stand for 24 h with occasional shaking prior to use.

The Sephadex LH-20 was from Pharmacia Fine Chemicals, Inc., Piscataway, N.J.

The "keeper" solution was paraffin oil (No. 01762, Applied Science Lab., State College, Pa.), 1 mg/mL of benzene.

Pentafluorobenzoyl chloride (No. 270571) was obtained from Regis Chemical Co., Morton Grove, Ill. A solution containing 5 μL of the reagent/mL of hexane–25% benzene was prepared just prior to use.

The bovine erythrocyte acetylcholinesterase (No. C2379) and the acetylcholine chloride (No. 420-750) were purchased from Sigma Chemical Co., St. Louis, Mo.

Boric acid solution was freshly prepared daily by dissolving 3.36 g of the reagent in distilled water and adjusting the volume to 100 mL; the pH was adjusted to exactly 4.0. Also a mixture containing 7 mL of this solution and 15 mL of a 0.1 N sodium hydroxide solution must be within the pH range of 7.8–8.0 prior to use.

Analytical standards of etrimfos, the O analogue, and EEHP, samples of untreated corn and alfalfa, and field-treated corn were furnished by Sandoz, Inc.

Preparation of Gravity-Flow Clean-Up Columns. The Sephadex LH-20 columns (15 mm i.d.) containing 5 g of dry Sephadex powder were prepared, operated, and regenerated for repeated use exactly as described by King et al. (1977).

The silica gel columns (12 mm i.d., No. 420,000, Kontes Glass Co., Vineland, N.J.) for separation and cleanup of the three residues were prepared by successively adding a plug of glass wool, 2 g of sodium sulfate, 5 g of silica gel (20% buffer, pH 7), and 2 g of sodium sulfate. The columns were prepared just prior to use and washed with 15 mL of benzene which was discarded.

The silica gel column for cleaning up the pentafluorobenzoate (PFB) derivative of EEHP was prepared as described above except that 1 g of the silica gel was used and the sodium sulfate was omitted.

Extraction and Cleanup of Samples. The chopped sample (25 g) was placed in a Soxhlet extraction apparatus (No. 9-551C, Fisher Scientific Co.) containing a plug of glass wool and a layer of sodium sulfate (40 g) to prevent insoluble plant material and water from siphoning over during the solvent exchanges. The sample was then extracted under a gentle stream of carbon dioxide for 16 h (overnight) by using 230 mL of dichloromethane–10% methanol (9 to 1 by volume) at the rate of about four solvent exchanges per hour. The extract was percolated through a plug of sodium sulfate (ca. 25 mm diameter × 30 mm thick) and 10 mL of dichloromethane was used to wash the container and plug. The combined extract and washings were then evaporated to dryness by using a 60 °C water bath and water pump vacuum.

The dry residue was transferred to a Sephadex column by using five successive 5-mL portions of benzene–10% methanol, allowing each portion to percolate into the bed; then the column was eluted with an additional 25-mL portion of the solvent. The column eluate was evaporated to dryness as described for subsequent cleanup and separation of the residues on silica gel.

The dry residue from the Sephadex column was transferred to a silica gel column (5 g), prepared as described, by using four 5-mL portions of benzene. The column was eluted with an additional 30-mL portion of benzene and the effluent collected in a 100-mL round-bottom flask; this eluate contains the parent etrimfos (P=S). The receiver was then changed and the O analogue (P=O) was eluted from the column by using 50 mL of benzene–5% acetone. Next, the column was eluted with 50 mL of benzene–10% acetone which was discarded and finally the EEHP was eluted with 50 mL of benzene–25% acetone. The separate fractions were evaporated to dryness as described and the etrimfos and O analogue fractions were transferred to calibrated graduated tubes by using benzene or acetone, respectively; the volume was adjusted to 1 mL or more for GC analysis employing the FPD.

The dry residue from the EEHP fraction was transferred to a 30-mL culture tube containing 8 mL of 0.1 N sodium hydroxide by using 10 mL of chloroform [Note: All culture tubes were equipped with Teflon-lined screw caps.] The contents of the tube were shaken and centrifuged for 5 min at 1000 rpm, and the chloroform layer was transferred to

a second culture tube containing 7 mL of 0.1 N sodium hydroxide by using a syringe and cannula. The contents of the second tube were shaken and centrifuged as described, and the chloroform layer was discarded. The flask and the aqueous sodium hydroxide phase in both tubes were again sequentially washed and extracted in the same manner by using two additional 10-mL portions of chloroform which were also discarded. The aqueous phases in both tubes were then transferred to a 50-mL culture tube by using 7 mL of boric acid solution and the mixture was extracted with three 10-mL portions of chloroform which were sequentially percolated through a plug of sodium sulfate and collected in a 50-mL round-bottom flask. The combined extracts were evaporated to dryness and the residue dissolved in 2 mL of methanol for direct analysis by HPLC or for derivatization to PFB–EEHP and analysis by EC–GC.

Preparation and Cleanup of PFB Derivative of EEHP. An appropriate aliquot (e.g., 1 mL) of the methanol solution (2 mL) containing the cleaned-up residue of EEHP was transferred to an 8-mL culture tube and evaporated to dryness by using a tube heater (No. 720,000, Kontes Glass Co.) set at 45 °C and a gentle stream of dry nitrogen. One milliliter of the pentafluorobenzoyl chloride solution was added to the residue and the tube was sealed and heated for 2 h in a tube heater set at 60 °C. After the tube had cooled, the contents were added to a silica gel column (1 g) prepared as described, and 5 mL of hexane–25% benzene was used to wash the tube and column. The column effluent (6 mL) was discarded (this contains the excess derivatizing reagent which would interfere with EC–GC assays); then the PFB derivative of EEHP was eluted with 25 mL of hexane–25% benzene. After the addition of 1 mL of "keeper" solution, the eluate was evaporated to dryness as described, the residue dissolved in an appropriate volume of benzene (1 mL or more) and immediately analyzed by EC–GC. Standards of EEHP were also carried through the derivatization and clean-up procedure for use in quantitating residues in the sample extracts.

GC Analysis. The following conditions were used for GC employing the FPD for residue analysis of etrimfos and its O analogue. Column: glass, 100-cm × 4-mm i.d. (6 mm o.d.); packing: OV-101, 5% (w/w) on 80–100 mesh Gas-Chrom Q (Applied Science Labs); gases: nitrogen (carrier) at 160 mL/min; oxygen at 40 mL/min; hydrogen at 200 mL/min; and temperature: column 160 °C, injection port 200 °C, detector 230 °C. The column was conditioned at 250 °C overnight prior to use, and under the specified conditions the retention times (t_R) of etrimfos and the O analogue were 3.45 and 3.10 min, respectively.

Conditions employed for the NPD–GC analysis were: column: glass, 90-cm × 2-mm i.d. (6 mm o.d.); packing: same as above; gases: helium (carrier) at 24 mL/min; hydrogen, 2.2 mL/min; air, 100 mL/min; temperatures: column, 170 °C; injection port, 220 °C; detector, 250 °C; and the temperature controller for the rubidium bead was set at 575. Under these conditions the t_R's for etrimfos and its O analogue were 4.55 and 4.05 min, respectively. Atratone [2-(ethylamino)-4-(isopropylamino)-6-methoxy-*s*-triazine], used as a nitrogen-containing reference standard, had a t_R of 3.05 min.

Operating conditions for EC–GC assays were: column: glass, 90-cm × 4-mm i.d. (6 mm o.d.); packing: OV-101, 10% (w/w) on 80–100 mesh Gas-Chrom Q, conditioned overnight at 250 °C prior to use; gas: argon–5% methane (carrier) at 50 mL/min; and temperatures: column, 190 °C; injection port, 250 °C; detector, 250 °C. Under these conditions t_R's for PFB–EEHP and a lindane standard were 4.15 and 3.20 min, respectively.

All injections were 5 μL and quantification was based on peak height. Atratone and lindane were used as reference standards to monitor the performance of the NPD– and EC–GC systems, respectively.

HPLC Analysis. The following conditions were used to assay residues of EEHP in the cleaned-up samples. Column: stainless steel, 30-cm × 4-mm i.d. (6-mm o.d.) μBondapak C_{18} (reverse phase, Waters No. 27324); mobile phase: 60% water–40% methanol at 1.0 mL/min (1800 psi). Under these conditions the t_R of EEHP was 7.20 min.

For the analysis of etrimfos, its O analogue, and EEHP in admixture, the following conditions were used: column: same as above; mobile phase: 70% methanol–30% water at 1.0 mL/min (1400 psi). Under the specified conditions t_R's for etrimfos, the O analogue, and EEHP were 7.10, 4.80, and 3.55 min, respectively.

All injections were 5 μL and quantification was based on peak height.

Extraction and Recovery Experiments. Corn treated with etrimfos and weathered in the field was used in studies concerning the efficiency of extracting residues. Corn plants in a plot located at Napoleon, Henry County, Ohio, were treated with a 5% granular formulation at the rate of 1.12 kg (active ingredient)/hectare on June 25, 1976 and again on August 17, 1976. On August 24, 1976, the whole plants were cut at ground level, chopped, and stored in a freezer. Rainfall totaling 18.1 cm occurred between the first and second application; none fell between the second application and harvest.

Samples (25 g) of the treated corn were thoroughly mixed and subjected to Soxhlet extractions with either dichloromethane or dichloromethane–10% methanol for 60 h. The reflux flasks containing fresh solvent were replaced at various intervals and the amount of residues extracted during each interval was determined.

The field-treated corn was also subjected to extraction by blending for comparison with the Soxhlet procedure. Samples (25 g) were blended at medium speed for 3 min in a Waring blender with 40 g of sodium sulfate and 125 mL of dichloromethane–10% methanol and filtered through Whatman No. 1 paper. The filter paper and residue were extracted and filtered twice again as described; the combined filtrates were evaporated to dryness as described for the Soxhlet extracts and subjected to cleanup and analysis.

Triplicate samples (25 g) of untreated corn and alfalfa unspiked, spiked with 5.0 ppm of etrimfos, or 0.50 and 0.050 ppm of all three compounds were extracted for 16 h in a Soxhlet as described to investigate any possible oxidation or hydrolysis of etrimfos to the O analogue or EEHP during the extraction or clean-up procedures and to determine the accuracy and precision of the method.

Acetylcholinesterase Inhibition (I_{50}) and Lethality (LD_{50}) Experiments. Tests to determine the anticholinesterase activity of etrimfos, its O analogue, and EEHP were performed by a modification of the method of Michel (1961). Briefly, a fixed amount of acetylcholinesterase was incubated in phosphate buffer at 37 ± 0.5 °C with each of the three compounds for 30 min and the substrate (acetylcholine chloride) was then added. After exactly 30 min of reaction time, the pH of treated, untreated, and blank (no enzyme added) reaction systems was measured by using an expanded-scale pH meter and the amount of acid produced in each system was determined from a calibration curve. Five levels of inhibition were employed in triplicate tests for each compound. The

Table I. Residues of Etrimfos and Degradation Products from Field-Treated and Weathered Corn Plants after Various Intervals of Soxhlet Extraction with Two Solvents

Intervals of extraction, h	Residues of etrimfos and rate of extraction for interval and solvent indicated[a]			
	Dichloromethane		Dichloromethane–10% methanol	
	ppb	ppb/h	ppb	ppb/h
0–1	1735 (2, 20)	1735	2055 (4, 20)	2055
1–2	330	330	709 (1, 6)	709
2–4	297	148	387	193
4–8	520	130	354	87
8–12	121	30	70	18
12–20	230	29	70	9
20–28	171	21	25	3
28–44	62	4	4	<1
44–60	30	2	4	<1
Total	3496 (2, 20)		3678 (5, 26)	

[a] Per 25-g sample. Values in parentheses denote residues of the O analogue and EEHP, respectively; no residues of these compounds were detected in other fractions.

I_{50} values and 95% confidence limits were calculated by the method of inverse prediction from linear regression analysis (Ostle, 1963).

The mice, male, CD-1 strain (Charles River Laboratories, Wilmington, Mass.), 25–30 g, were housed at 25 ± 1 °C with a 12-h light-dark cycle and had free access to food and water. Suspensions of etrimfos (30 mg/mL) and its O analogue (5 mg/mL) were prepared in an aqueous solution containing 20% polysorbate 80 by using an ultrasonic dismembrator for 15 min. Ten mice were used for each of five dose levels of each compound. For the etrimfos, appropriate volumes of suspension were used to administer doses of 800, 850, 900, 950, and 1000 mg/kg; for the O analogue, appropriate volumes were used to administer 150, 160, 180, 190, and 200 mg/kg. The chemical suspensions were administered orally via a ball-tipped syringe needle, and the animals were observed for 24 h. The results were analyzed by a computer programmed maximum likelihood probit method (Finney, 1971).

RESULTS AND DISCUSSION

The importance of efficiently extracting residues from field-weathered crops as well as some of the pitfalls that may lead to erroneously low results with various extraction procedures have been previously documented (Bowman et al., 1968; Bowman, 1975). Extraction experiments were therefore initiated by subjecting samples of whole corn plants, treated with etrimfos, and weathered in the field, to Soxhlet extractions for periods up to 60 h employing either dichloromethane or dichloromethane–10% methanol to determine appropriate parameters for efficiently extracting the residues without chemically altering them. The use of higher boiling solvents was avoided to prevent possible thermal degradation of the residues. Results of these tests are presented in Table I. These data demonstrate that the added polarity of 10% methanol in the dichloromethane enhances the total amount of residues recovered and also the speed of extraction; nevertheless, extensive extraction periods are required. Both solvents continued to remove traces of etrimfos at the 60-h interval, whereas all detectable amounts of the O analogue and EEHP were recovered within 2 h. Soxhlet extraction with dichloromethane–10% methanol was therefore selected as the procedure of choice, and since the extraction period

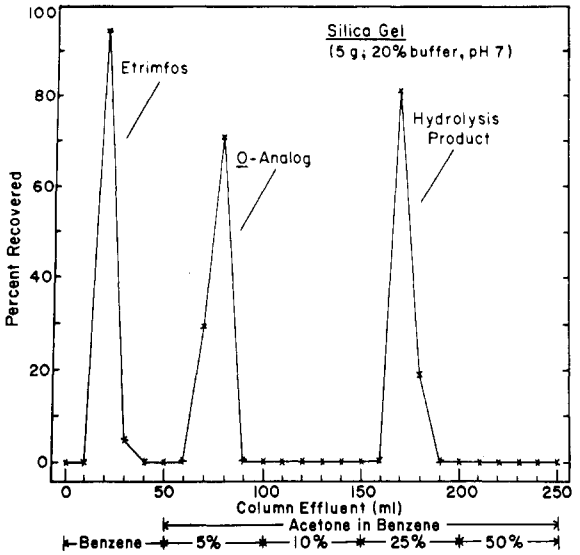

Figure 2. Elution profiles of etrimfos, its O analogue, and EEHP from gravity-flow clean-up column of silica gel.

of 60 h was considered impractical, 16 h (overnight), which yields about 98% of the residues extractable by the procedure, was considered adequate. On the other hand, residues from samples of untreated crop spiked with the three compounds in an organic solvent (e.g., 1 mL of chloroform), which was then allowed to dry for 1 h, were completely recovered after 1 h of Soxhlet extraction. This wide difference between the extractability of residues from laboratory-spiked vs. field-treated and weathered samples, again, emphasizes the importance of efficient extraction procedures devised by using authentic field-treated and weathered samples. Separate tests with 1-mg amounts of each compound carried through the 16-h extraction (plant material absent) indicated that no detectable oxidation or hydrolysis and no appreciable loss of the compounds occurred.

Exhaustive extraction by blending the field-treated sample with dichloromethane–10% methanol gave recoveries about 25% lower than the Soxhlet procedure, and had the insecticide been applied as an emulsifiable concentrate instead of a granular formulation, an even wider difference in recovery would be expected.

Initial studies concerning cleanup and separation of the three compounds, performed in the absence of crop extractives, were based on elution of the etrimfos and O analogue from Sephadex LH-20 with benzene, followed by elution of EEHP with benzene–10% methanol. The fraction containing etrimfos and O analogue was separated on silica gel and analyzed while the fraction containing EEHP was subjected to liquid–liquid partitioning, then cleaned up further on silica gel prior to analysis. However, this procedure failed in the presence of plant extractives which caused the EEHP to partially elute with the etrimfos and O analogue; it therefore became necessary to revise the procedure as described in the Experimental Section.

The cleanup achieved by eluting all three compounds from Sephadex with benzene–10% methanol was sufficient to permit subsequent cleanup, separation, and quantitative recovery of all three compounds on silica gel; the elution profiles of the three compounds from silica gel are presented in Figure 2.

Although none of the fractions from silica gel were sufficiently clean to allow residue analysis at low ppm levels via EC–GC or HPLC, satisfactory analyses of etrimfos and the O analogue could be performed by FPD–GC. A liquid–liquid partitioning cleanup, based on

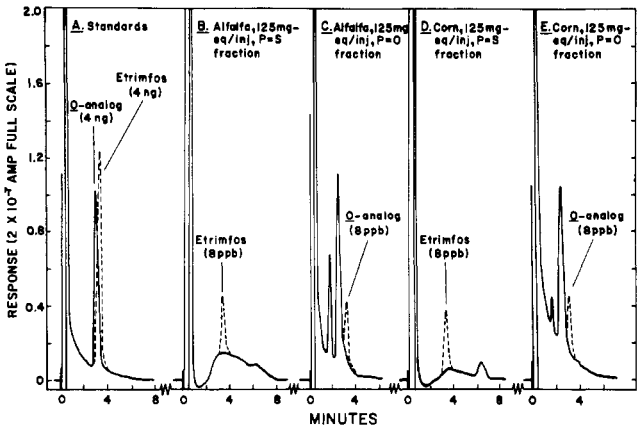

Figure 3. Gas chromatograms via flame photometric detection. A is standard of etrimfos and its O analogue. In B and D, solid lines are etrimfos fractions from untreated crops; broken lines (superimposed) illustrate responses of etrimfos spiked into the extracts; in C and E, solid lines are O analogue fractions from untreated crops; broken lines illustrate responses of the O analogue spiked into the extracts.

the acidic property of EEHP, in chloroform vs. aqueous solutions of different pH values, was required prior to analysis by HPLC or derivatization and analysis by EC–GC. The following p values (Beroza and Bowman, 1965; Bowman and Beroza, 1966) for EEHP, determined during the development of the procedure, may be useful to the analyst: Chloroform vs. water and aqueous buffers pH 2, 8, 12 were 0.644, 0.687, 0.870, and 0.000, respectively; benzene vs. water and aqueous buffers pH 2 and 12 were 0.090, 0.096, and 0.000, respectively. With hexane–acetonitrile, p values for etrimfos, the O analogue, and EEHP were 0.200, 0.048, and 0.014, respectively.

Typical gas chromatograms from the analysis of standards and the parent and O analogue fractions from both crops by using FPD–GC are presented in Figure 3. The FPD–GC system, highly specific for phosphorus, permits the detection and measurement of both residues in corn and alfalfa at levels of about 4 ppb or less (based on twice background) and an analysis can be completed within about 5 min under the specified conditions.

Standards and similar fractions from corn and alfalfa were also analyzed by using the NPD–GC system; chromatograms are presented in Figure 4. It is interesting to note that etrimfos ($C_{10}H_{17}N_2O_4PS$, mol wt 292, 9.6% N) gives about ten times the response of atratone ($C_9H_{17}N_5O_2$, mol wt 211, 33.2% N) with the NPD–GC system operated in the nitrogen mode; the presence of phosphorus in the etrimfos and O analogue molecules undoubtedly contributes to this enhancement. Figure 4 illustrates that etrimfos can be assayed in the parent fraction of both crops with about the same sensitivity as the FPD–GC system; however, periods of about 30 min are required between injections to allow extraneous peaks to emerge. The analysis of O analogue fractions is much less sensitive than FPD–GC because of background interference. The FPD–GC system was therefore the method of choice for etrimfos and the O analogue because it provided high sensitivity for the compounds in both crops, was less temperamental, and permitted the assays to be performed more rapidly. Assays of the compounds were not attempted with the NPD–GC system operated in the phosphorus mode because at that time we were also attempting to analyze residues of EEHP which contains no phosphorus.

Typical chromatograms from HPLC assays are presented in Figure 5. Chromatogram A is included to illustrate the separation and relative responses of etrimfos, the O analogue, and EEHP. Although the system may be used for preparative work, assays of formulations, etc., the fractions containing etrimfos and the O analogue from corn and alfalfa are not sufficiently clean to permit residue analysis; also, the mobile phase using 60% water–40% methanol is recommended for residue analysis of EEHP. Chromatograms of a standard of EEHP and the EEHP fractions from corn and alfalfa are also presented in Figure 5. Although the backgrounds for corn and alfalfa amount to about 100 and 70 ppb, respectively, good reproducibility

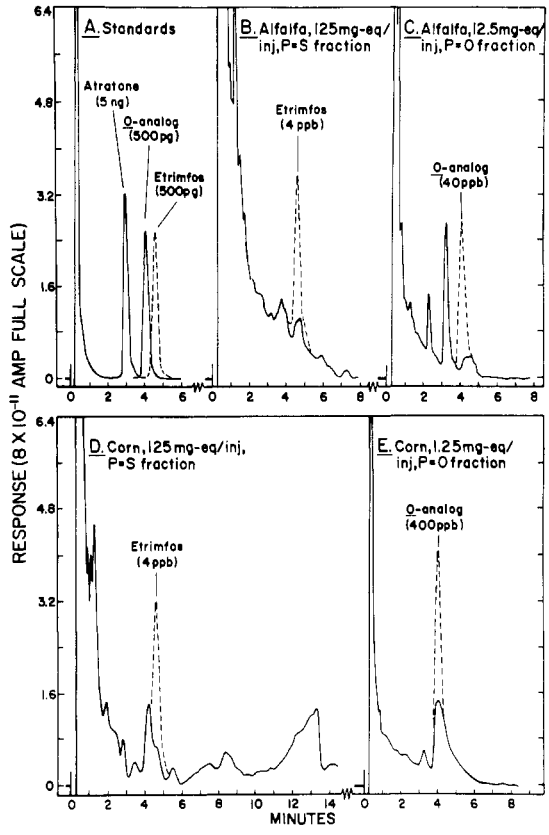

Figure 4. Gas chromatograms via the nitrogen–phosphorus rubidium-sensitized detector. A is standards of etrimfos, its O analogue, and atratone. In B and D, solid lines are etrimfos fractions from untreated crops, broken lines (superimposed) illustrate responses of etrimfos spiked into the extracts; in C and E, solid lines are O analogue fractions from untreated crops; broken lines illustrate responses of the O analogue spiked into the extracts.

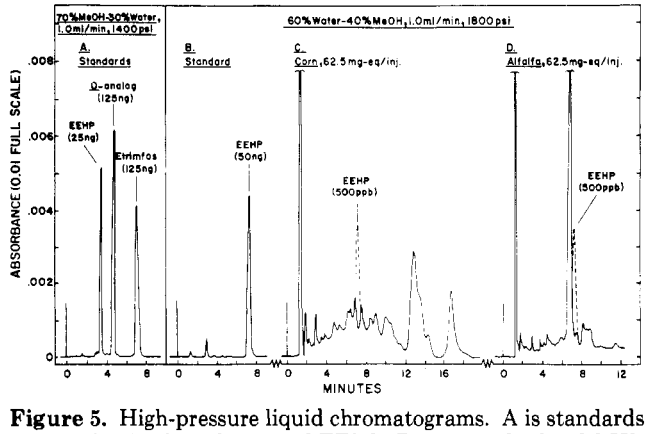

Figure 5. High-pressure liquid chromatograms. A is standards of etrimfos, its O analogue and EEHP; B is a standard of EEHP under conditions for residue analysis; in C and D, solid lines are EEHP fractions of untreated crops, broken lines (superimposed) illustrate responses of the EEHP spiked into the extracts.

Table II. Analysis of Etrimfos, Its Oxygen Analogue, and Hydrolysis Product in Whole Corn Plants

Trial	Compounds	Added[a]		Method of analysis	Recovered ($\bar{x}$ ± SE)[b]		
		μg	ppm		μg	ppm	%
1	Etrimfos	0	0	FPD-GC	<0.032 ± 0.018	<0.001 ± 0.001	
	O analogue	0	0	FPD-GC	<0.042 ± 0.005	<0.002 ± 0.000	
	EEHP	0	0	EC-GC	<0.075 ± 0.025	<0.003 ± 0.001	
				HPLC	<2.50 ± 0.42	<0.100 ± 0.017	
2	Etrimfos	125	5.0	FPD-GC	120. ± 0.8	4.78 ± 0.03	96.0 ± 0.6
	O analogue	0	0	FPD-GC	0.030 ± 0.008	0.001 ± 0.000	
	EEHP	0	0	EC-GC	0.500 ± 0.225	0.020 ± 0.009	
				HPLC	0.275 ± 0.200	0.011 ± 0.008	
3	Etrimfos	12.5	0.50	FPD-GC	11.2 ± 0.05	0.448 ± 0.002	89.6 ± 0.4
	O analogue	12.5	0.50	FPD-GC	11.0 ± 0.15	0.440 ± 0.006	88.0 ± 1.2
	EEHP	12.5	0.50	EC-GC	9.33 ± 3.00	0.373 ± 0.120	74.6 ± 24.0
				HPLC	8.50 ± 0.95	0.340 ± 0.038	68.0 ± 7.6
4	Etrimfos	1.25	0.050	FPD-GC	1.09 ± 0.015	0.044 ± 0.001	87.2 ± 1.2
	O analogue	1.25	0.050	FPD-GC	1.02 ± 0.025	0.041 ± 0.001	81.6 ± 2.0
	EEHP	1.25	0.050	EC-GC	1.18 ± 0.72	0.047 ± 0.029	94. ± 58.
				HPLC	0.875 ± 0.375	0.035 ± 0.015	70. ± 30.
Field-treated (extracted by Soxhlet)	Etrimfos			FPD-GC	81.2 ± 8.45	3.25 ± 0.34	
	O analogue			FPD-GC	0.090 ± 0.010	0.004 ± 0.000	
	EEHP			EC-GC	5.68 ± 0.30	0.227 ± 0.012	
				HPLC	5.50 ± 0.30	0.220 ± 0.012	
Field-treated (extracted) by blending)	Etrimfos			FPD-GC	61.8 ± 5.00	2.47 ± 0.20	
	O analogue			FPD-GC	0.050 ± 0.010	0.002 ± 0.000	
	EEHP			EC-GC	5.12 ± 0.12	0.205 ± 0.005	
				HPLC	4.00 ± 0.32	0.160 ± 0.013	

[a] Per 25 g sample. [b] Per 25 g sample. Mean and standard error from triplicate assays; spiked and treated samples are corrected for background of unspiked samples.

and the absence of interference peaks at the t_R of EEHP in untreated samples permits estimations of residues as low as 50 ppb. Nevertheless, the relatively low sensitivity of the HPLC procedure was recognized and alternate methods for determining residues of EEHP were sought.

The gas chromatographic properties of EEHP using the NPD–GC system were found to be very poor on columns of low (OV-101, methyl silicone) and medium (AN-600, cyanoethyl silicone) polarities; i.e., while 500-ng amounts responded strongly and tailed badly, minimum detectable amounts were about 50 ng, and repeated injections to condition the column failed to improve the performance. Experiments were, therefore, undertaken to derivatize the EEHP prior to analysis via NPD–GC. Diazomethane was found to completely derivatize EEHP at ambient temperature within 1 h, yielding one major product and two minor products that chromatographed well. Unfortunately, derivatization of the EEHP fraction from an untreated sample of corn with diazomethane yielded backgrounds on 5% OV-101 and 5% AN-600 columns equivalent to 11 000 and 180 ppb of EEHP, respectively. BSA reagent [*N,O*-bis(trimethylsilyl)acetamide], 100 μL, in 2 mL of hexane was found to completely derivatize 200 μg of EEHP at ambient temperature in 15 min yielding a single peak. However, injection of the derivatized solution containing excess BSA reagent into the NPD–GC system severely diminished the sensitivity of the detector (e.g., more than 50% per injection). Two washings of the derivatized solution with equal volumes of 0.001 N HCl greatly reduced the detrimental effect on the detector; however, where high sensitivity was sought by injecting the concentrated washed solution, each injection continued to diminish detector response by about 30%. Attempts to analyze derivatives of EEHP via NPD–GC were therefore abandoned and electron-capturing derivatives were sought.

Pentafluoropropionic acid anhydride or heptafluorobutyric acid anhydride used with trimethylamine catalyst, heptafluorobutyryl imidazole, and pentafluorobenzyl bromide alone or used with tetrabutyl ammonium hydrogen sulfate catalyst failed to derivatize EEHP. Finally, pentafluorobenzoyl chloride did in fact form a derivative of EEHP. The excess derivatizing reagent resulted in a broad injection peak that interfered with the quantitation of 1-μg amounts or less of the EEHP. The excess reagent was not sufficiently removed or the derivative was partially destroyed by washing the derivatized solution with water, phosphate buffer (pH 6), 5% aqueous sodium bicarbonate, 0.1 N sodium hydroxide, or hydrazine sulfate; chemical reaction of the excess reagent with methanol, 5 to 20% ammonium carbonate, or hydrazine also gave unsatisfactory results. However, by limiting the amount of derivatizing reagent to 5 μL, a sufficient amount of the excess reagent could be separated on a column of silica gel and discarded, thus permitting the analysis of submicrogram amounts of EEHP. It should be pointed out that the cleaned-up PFB derivative of EEHP is not completely stable; 25-μg amounts stored in the dry state or in hexane–25% benzene at 0 °C for 2 days gave only 60 and 69% of their initial response. Analysis should therefore be performed immediately; we found it convenient to derivatize samples at 30-min intervals to allow sufficient time for cleanup and analysis before the next sample was ready.

Chromatograms from EC–GC assays of standards of lindane and the PFB derivative of EEHP along with derivatized EEHP fractions from corn and alfalfa are presented in Figure 6. The PFB derivative of EEHP, which exhibits about the same electron-capturing properties as lindane, may be detected at extremely low levels. Although several extraneous peaks of high magnitude are obtained in chromatograms of cleaned-up corn and alfalfa extracts, most of them are attributed to the reagent blank (derivatization system) and none appreciably interfere with the analysis. Analysis via EC–GC of the PFB derivative is therefore the method of choice for determining low-level residues of EEHP.

Results from assays of corn and alfalfa unspiked or spiked with etrimfos or various amounts of the three compounds in admixture along with residues found in the sample of field-treated corn as determined by Soxhlet extraction and blending are presented in Tables II and III. Recoveries of etrimfos and the O analogue are better than

Table III. Analysis of Etrimfos, Its Oxygen Analog, and Hydrolysis Product in Alfalfa

Trial	Compound	Added[a]		Method of analysis	Recovered ($\bar{x}$ ± SE)[b]		
		μg	ppm		μg	ppm	%
1	Etrimfos	0	0	FPD-GC	<0.000 ± 0.000	<0.000 ± 0.000	
	O analogue	0	0	FPD-GC	<0.050 ± 0.000	<0.002 ± 0.000	
	EEHP	0	0	EC-GC	<0.075 ± 0.000	<0.003 ± 0.000	
				HPLC	<1.75 ± 0.28	<0.070 ± 0.011	
2	Etrimfos	125	5.0	FPD-GC	116. ± 1.8	4.64 ± 0.072	92.8 ± 1.4
	O analogue	0	0	FPD-GC	0.050 ± 0.000	0.002 ± 0.000	
	EEHP	0	0	EC-GC	1.00 ± 0.90	0.040 ± 0.036	
				HPLC	0.50 ± 0.50	0.020 ± 0.020	
3	Etrimfos	12.5	0.50	FPD-GC	10.9 ± 0.05	0.436 ± 0.002	87.2 ± 0.4
	O analogue	12.5	0.50	FPD-GC	11.0 ± 0.08	0.440 ± 0.003	88.0 ± 0.6
	EEHP	12.5	0.50	EC-GC	8.75 ± 0.42	0.350 ± 0.017	70.0 ± 3.4
				HPLC	9.00 ± 0.30	0.360 ± 0.012	72.0 ± 2.4
4	Etrimfos	1.25	0.050	FPD-GC	1.03 ± 0.08	0.041 ± 0.003	82.4 ± 6.4
	O analogue	1.25	0.050	FPD-GC	1.00 ± 0.02	0.040 ± 0.001	80.0 ± 1.6
	EEHP	1.25	0.050	EC-GC	1.03 ± 0.08	0.041 ± 0.003	82.4 ± 6.4
				HPLC	0.985 ± 0.250	0.039 ± 0.010	78.8 ± 20.0

[a] Per 25 g sample. [b] Per 25 g sample. Mean and standard error from triplicate assays; spiked samples are corrected for background of unspiked samples.

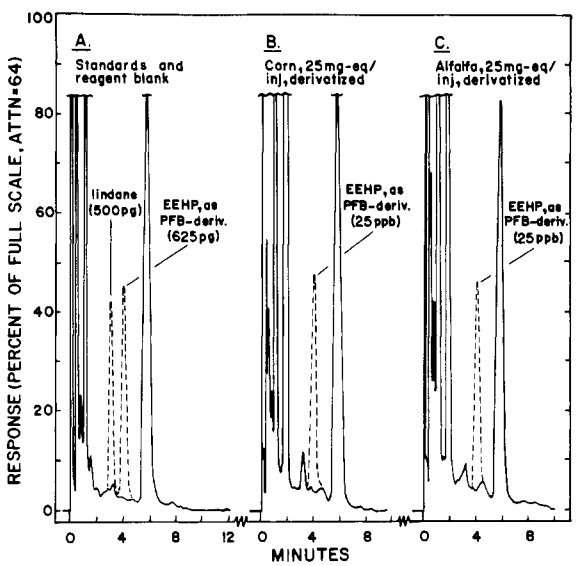

Figure 6. Electron-capture gas chromatograms. In A, solid line is the reagent blank from the derivatization procedure, broken lines are standards of lindane and the PFB derivative of EEHP. In B and C, solid lines are derivatized untreated crop extracts, broken lines (superimposed) illustrate responses of EEHP spiked into an extract and derivatized prior to use.

80% even in the samples spiked at the 50 ppb level; precision is excellent. Also, there is no indication that any of the etrimfos is converted to O analogue during the extraction of either crop. A comparison of the residues of the three compounds found in the field-treated samples of corn by using Soxhlet and blending extractions, again, demonstrates the superiority of the Soxhlet procedure. Results from the EC-GC assays of EEHP in corn and alfalfa unspiked and spiked with 5 ppm of etrimfos suggest that a small amount (ca. 1% or less) of the parent insecticide may be hydrolyzed to EEHP during the extraction and cleanup of the plant material; however, hydrolysis of this magnitude is not regarded as a serious deficiency in the method. Recoveries of EEHP from crops spiked at all levels are satisfactory (70% or better), and the precision in assays of alfalfa and field-treated corn was generally good. Wider variations in results were obtained from samples of corn spiked at the 500 and 50 ppb level.

Etrimfos inhibited acetylcholinesterase activity by 50% (I_{50}) at a concentration of 3.5×10^{-5} M [CL_{95} (2.4×10^{-5})–(5.3×10^{-5}) M] and the O analogue had an I_{50} of 3.6×10^{-8} M [CL_{95} (2.5×10^{-8})–(5.8×10^{-8}) M]. As expected, EEHP failed to produce significant inhibition at the highest concentration tested (1.1×10^{-3} M).

The I_{50} value obtained for etrimfos (3.5×10^{-5} M) is in good agreement with that of parathion determined in this laboratory (3.5×10^{-5} M) and to a value reported in the literature (2.5×10^{-5} M) determined with acetylcholinesterase from horse serum (Holmstedt, 1963). However, it has been long recognized that inhibition values obtained with parent phosphorothioates may be questionable because of possible contamination with oxidation products or isomers (Aldridge and Davison, 1952b). Although no impurity of oxidation products could be found in our sample of etrimfos, the possibility that our value could be biased cannot be disregarded. The O analogue was highly active as an anticholinesterase agent ($I_{50} = 3.5 \times 10^{-8}$ M) and compares favorably with the value (2.0×10^{-8} M) of Aldridge and Davison (1952a) for erythrocyte cholinesterase inhibition by paraoxon.

The LD_{50}'s of etrimfos and the O analogue were 921 (CL_{95} 828–1001) and 170 (CL_{95} 119–197) mg per kg, respectively. The lethal potencies of these compounds were analogous to those of fenitrothion [*O,O*-dimethyl *O*-(4-nitro-*m*-tolyl)phosphorothioate] and fenitrooxon (dimethyl 4-nitro-*m*-tolyl phosphate) which were also determined at this laboratory to be 988 (CL_{95} 920–1056) and 120 (CL_{95} 102–138) mg per kg, respectively.

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Uptake of Ethoprop (Mocap) by Ten Vegetables Grown in Soil Treated for Control of Nematodes

Robert J. Argauer* and Julius Feldmesser

An analytical method was developed for the analysis of the nematocide ethoprop (*O*-ethyl *S*,*S*-dipropyl phosphorodithioate) in vegetable crops. The method was rapid and efficient with recoveries nearly 100% for samples fortified at levels as low as 0.01 ppm. Ethoprop was found at harvest at levels above 0.01 ppm in onion (0.12, 0.52, 1.34 ppm), carrot (0.14, 0.34, 0.81 ppm), radish (0.12, 0.33, 0.66 ppm), and eggplant (0.027, 0.044, 0.086 ppm) which were grown in soil treated 1 week before planting with 30, 60, and 120 lb of ethoprop 10% granules/acre (3.4, 6.7, 13.4 kg/ha active ingredient). Ethoprop was not detected (<0.01 ppm) in beet, cabbage, cantaloupe, pea, and tomato. Skin, core, or roots of certain selected vegetables sampled had higher concentrations of ethoprop than had the whole vegetable.

Ethoprop (*O*-ethyl *S*,*S*-dipropyl phosphorodithioate), a nematocide–insecticide, is currently registered for use on sugarcane, soybean, corn, banana, plantain, peanut, sweet and white potatoes, pineapple, snap and lima beans, cabbage, and cucumber with a tolerance level at harvest set at 0.02 ppm. The objective of this study was to develop an efficient and rapid method suitable for analysis of this chemical in various vegetable crops in order to accumulate the chemical data required to support minor use pesticide registration requirements for the establishment of appropriate tolerances for this chemical in vegetables.

RESIDUE ANALYSIS

Preparation of Samples. All samples were analyzed within 2 weeks after freezing. The frozen samples were washed by hand under cold tap water to help remove traces of soil. All samples except tomato and cantaloupe were chopped in a food mill into small pieces. The greens 3 cm above the onion bulbs were discarded and the bulbs chopped. Both pea and pod were chopped. Tomatoes were thawed at 25 °C and slurried in a Vitamix 3600 blender. Cantaloupes were thawed and halved, seeds were discarded, and the pulp was slurried.

Sample Analysis. For sample analysis, 100-g portions of the chopped or slurried vegetable, 10 mL of 10% sulfuric acid, and 250 mL of methylene chloride were blended together for 3 min in a Waring Blendor. The blend was filtered by gravity through filter paper into a flask that contained anhydrous granular sodium sulfate. A 83-mL portion of the filtrate was concentrated to near dryness on a Rinco evaporator at about 20 °C under a water aspirator vacuum. Five milliliters of ethyl acetate was added to the concentrate to dissolve the residue, the ethyl acetate solution was centrifuged to remove any insoluble material, and 5 μL of the supernatant was injected into a gas chromatograph (see Figure 1 for GC conditions). The response was compared with that of ethoprop standards. Standard solutions were prepared from analytical grade ethoprop (Mocap) supplied by Mobil Oil Corp. at 95.8% purity. All ethoprop concentrations are reported with the phosphorus filter positioned in the flame photometric detector.

Extraction Efficiency of Method. For the determination of the efficiency of extraction, 100-g portions of chopped or slurried vegetable grown in untreated plots were fortified at levels between 0.01 and 0.2 ppm by adding 1 to 20 μg of ethoprop standards before blending and analyzed as were the samples.

Confirmation by Mass Spectrometry. A methylene chloride extract of a 200-g portion of chopped onions (replicate 4; 6 pounds/acre treatment level) (0.89 ppm) was concentrated under vacuum, diluted to 10 mL with methylene chloride, and rinsed into a 10 mm i.d. glass column that contained 14 g of 60–200 mesh silica gel 45 cm deep. The column was eluted with methylene chloride, and 25-mL fractions were collected. The fifth through seventh fractions were combined, evaporated to near dryness, taken up in 0.5 mL of ethyl acetate, and 2 μL injected into a Hewlett-Packard Model 5930A GC mass spectrometer with 5932A Data System that contained a 5% OV-17 gas chromatographic column held at a temperature of 220 °C. This represented an injection into the GC–MS estimated at approximately 700 ng of ethoprop.

Plot Size, Soil Treatment, Planting, and Sampling Dates. Vegetables were grown in light sandy soil with irrigation as needed in plots located at the University of Maryland Experimental Farm, Salisbury, Md. Each plot measured 30 ft by 33.33 ft. Ethoprop, except in the untreated control plots, was dispersed at the soil surface as a 10% granular formulation and worked into the soil to a depth of 1–2 in. with a spiked tooth harrow; vegetables

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