# Development of a Sensitive Enzyme-Linked Immunosorbent Assay for the Fungicide Fenpropimorph

F. Jung,[*,1] H. H. D. Meyer,[2] and R. T. Hamm[3]

A competitive enzyme-linked immunosorbent assay (ELISA) for the fungicide fenpropimorph was developed using its metabolite fenpropimorphic acid. The fenpropimorphic acid was coupled to bovine serum albumin (BSA) with use of N-hydroxysuccinimide, resulting in an active ester. Antibodies were raised in rabbits. The marker enzyme was prepared by linking fenpropimorphic acid to horseradish peroxidase by the mixed-anhydride procedure. The ELISA was performed with a new "second antibody technique" utilizing an immobilized affinity-purified sheep antibody to bind the hapten-specific rabbit antibody. With this technique antiserum dilutions down to 1:200 000 could be used. Further advantages are good reproducibility and high sensitivity. Unlike other assays with polyclonal antibodies, the antibody is not a limiting factor in this technique: 1 mL of antibody is sufficient for assays on 200 plates. To decrease relative binding by 50%, 6 pg/20 µL or 300 pg/mL of fenpropimorph was required. The antiserum was stereospecific and had very little cross-reactivity to structurally related compounds. Preliminary studies analyzing soil percolation water samples showed that this method can be used for screening of large numbers of samples without extraction or cleanup.

Fenpropimorph is a fungicide used extensively in the control of powdery mildew and rust in cereals (Saur et al., 1985). It also has high activity against fungi causing citrus decay (Tadeo and Lafuente, 1987). The fungitoxicity is due to the inhibition of ergosterol biosynthesis, which leads to the formation of abnormal mycelial membranes (Baloch et al., 1984; Rahier et al., 1986). Fenpropimorph is known to be metabolized to fenpropimorphic acid, which has a higher leaching potential in soil. At present, the conventional method for analysis of these compounds is gas chromatography (GLC) with an N-FID detector. The detection limit is 50 ng/L. However, this requires extensive sample preparation and cleanup steps, as well as a derivatization. Therefore, it is desirable to have an efficient and rapid assay system to estimate fenpropimorph and fenpropimorphic acid in soil samples and soil percolation water.
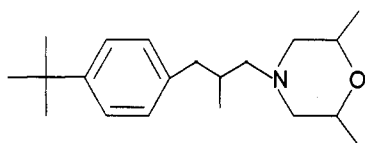
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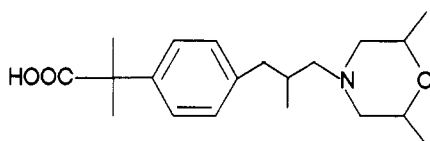
Fenpropimorph

4-[3-[4-(2-carboxypropyl)phenyl]-2-methyl]-propyl-2,6-(cis)-dimethylmorpholine

Fenpropimorphic acid

Lehrstuhl für Botanik der Technischen, Universität München-Weihenstephan, D-8050 Freising, West Germany.
[1] Current address: Department of Entomology, University of California, Davis, CA 95616.
[2] Current address: Institut für Physiologie der Südd. Versuchs und Forschungsanstalt für Milchwirtschaft, Technische Universität München, D-8050 Freising-Weihenstephan, West Germany.
[3] Current address: BASF AG, Landwirtschaftliche Versuchsstation Limburgerhof, D-6703 Limburgerhof, West Germany.

Immunological methods are becoming increasingly popular in pesticide residue analysis, because they are rapid and inexpensive and have several advantages over conventional analytical procedures (Wie et al., 1982; Wie and Hammock, 1982; Schwalbe et al., 1984; Huber and Hock, 1985; Kelley et al., 1985; Newsome, 1986). The high selectivity and sensitivity of the immunoassays make them very useful for detecting a wide variety of pesticides at nano- and picomole levels. The objective of this study was to develop a rapid and simple, but highly sensitive, enzyme-linked immunosorbent assay (ELISA) for the fungicide fenpropimorph as well as for the metabolite fenpropimorphic acid. A preliminary study demonstrating its application for screening is also reported.

MATERIALS AND METHODS

Fenpropimorphic acid, fenpropimorph, [³H]fenpropimorphic acid (specific activity 3.77 GBq/mM), and the compounds tested for cross-reactivity (*trans*-fenpropimorph, tridemorph, fenpropidin, 2,6-dimethylmorpholine, Wettol, Emulphor) were provided by the BASF AG. Bovine serum albumin (BSA), dextran, and Norit A charcoal were purchased from Serva (Heidelberg, FRG). Horseradish peroxidase E.C. 1.11.1.7. (No. 814393) was purchased from Boehringer Mannheim GmbH (FRG), polystyrene microtiter plates were from Nunc (Roskilde, DK; No. 439454), scintillation cocktail Rialuma was from Baker (Gross Gerau, FRG), *cis*-1,3-dimethylcyclohexane and 2,5-dimethyltetrahydrofuran were from Aldrich (Steinheim, FRG), and Freund's complete and incomplete adjuvant were purchased from Difco Laboratories (Detroit, MI). N,N'-Dicyclohexylcarbodiimide, N,N-dimethylformamide (DMF), N-hydroxysuccinimide, dimethyl sulfoxide (DMSO), isobutyl chloroformate, rabbit IgG agarose gel, and 3,3',5,5'-tetramethylbenzidine (TMB) were purchased from Sigma (München, FRG). All other chemicals used were obtained from Merck (Darmstadt, FRG). All spectrophotometric measurements were performed with a SLT Easy Reader (AU, Salzburg). The ³H counting was determined with a Philips PW 4700 scintillation counter (NL). Dilutions were carried out with a Hamilton Microlab 1000 diluter (bonaduz, CH). For GLC, a Perkin-Elmer Model 3920 B gas–liquid chromatograph with a N-FID detector and a 22-m SE 54 capillary column was used. Column, injector, and detector temperatures were 250, 300, and 300 °C. The fenpropimorph derivative had a retention time of 3.45 min.

Synthesis of the Fenpropimorphic Acid/BSA Conjugate. Fenpropimorphic acid was coupled to BSA by a procedure similar to that described by Bauminger and Wilchek (1980). The covalent linkage was performed with the aid of N,N'-dicyclohexylcarbodiimide and N-hydroxysuccinimide (Anderson et al., 1964; Goodfriend et al., 1964). N,N'-Dicyclohexylcarbodiimide (5.2 mg) and N-hydroxysuccinimide (5.74 mg) were added to a solution of fenpropimorphic acid (8.4 mg) in 1 mL of DMF which was

constantly stirred. The reaction was carried out at room temperature for 1 h, and the precipitated dicyclohexylurea was removed by centrifugation. BSA (16.0 mg) was dissolved in 2 mL of 0.2 N NaHCO$_3$ and the resultant mixture added slowly to the N-hydroxysuccinimide ester solution. Incubation was continued for 4 h at 4 °C. The solution was dialyzed against phosphate buffer (0.067 M, pH 7.2) and stored at -60 °C.

Immunization. Four female rabbits (German local breed), at least 12 weeks old, were immunized by intradermal injection of 0.5 mL of a 1:1 emulsion (0.5 mg/mL) of immunogen in saline and Freund's complete adjuvant (Vaitukaitis et al., 1971). The antigen was injected in small aliquots at 20–30 sites on shaven areas on the back. The animals were boosted once in 2-week intervals in the same manner. Subsequently, booster injections were given in 3-week intervals using the same antigen but emulsified in incomplete Freund's adjuvant. Rabbits were bled 1 week after the last injection; the serum was separated by centrifugation and stored at -60 °C.

Characterization of Antisera. The titer of anti-fenpropimorphic acid antisera was first controlled by a binding test using ^{3}H-labeled fenpropimorphic acid (3.77 GBq/mM). The assays were performed in 60 × 50 mm glass tubes containing 500 μL of serum diluted with assay buffer solution (0.02 M Tris, 0.3 M NaCl, pH 7.5). A solution of ^{3}H-labeled fenpropimorphic acid (333 Bq/0.1 mL) in assay buffer was added to the diluted serum and incubated at 37 °C for 20 min and at 4 °C overnight. Unbound radioligand was precipitated by the addtion of 0.5 mL of charcoal–dextran suspension (0.4% activated Norit A charcoal and 0.4% dextran in H$_2$O), and the resultant mixture was incubated for 10 min at 0 °C and centrifuged (10 min, 4 °C, 3000 rpm). Radioactivity in the supernatant was determined by liquid scintillation counting of a 0.5-mL aliquot in 5 mL of Rialuma cocktail.

Coupling of Fenpropimorphic Acid to Horseradish Peroxidase. The enzyme tracer was prepared by a modified mixed-anhydride procedure (Liebermann et al., 1959) as follows: 6.25 μL of 4-methylmorpholine was added to a solution of 10 mg of fenpropimorphic acid in 0.5 mL of DMF and cooled to -15 °C. DMF (375 μL) was slowly added to a solution of horseradish peroxidase (10 mg diluted in 400 μL of H$_2$O) at 0 °C, with constant stirring. Isobutyl chloroformate (6.25 μL) was added to the hapten solution and the resultant mixture stirred for 3 min at -15 °C. Then, the enzyme solution was added and the pH adjusted immediately to 8.0 (20 μL of 1 N NaOH). Stirring was continued for 1 h at -15 °C and 2 h at 0 °C. Then, 10 mg of NaHCO$_3$ was added, and the product was dialyzed against 0.067 M phosphate buffer, pH 7.2, overnight. It was further purified by gel chromatography (Sephadex G 25 fine; column 1.6 cm × 50 cm) with 0.067 M phosphate buffer, pH 7.2. The purified enzyme tracer was stored at -60 °C.

Purification of the Sheep IgG by Affinity Chromatography. A small column containing 5 g of rabbit IgG agarose gel was prepared. Clear plasma (15–20 mL) containing 6 mM EDTA from sheep immunized with rabbit IgG was applied to the column. The gel was washed with 10 mL of 0.5 M NaSCN, pH 8.0, followed by 10 mL of 0.1 M glycine–HCl, pH 3.5, and eluted with 15 mL of 0.1 M glycine–HCl, pH 2.0 (all steps at room temperature). The eluate was immediately dialyzed against 66 mM NaH$_2$PO$_4$/Na$_2$HPO$_4$, pH 7.2, and the remaining IgG quantified by the biuret procedure.

Enzyme-Linked Immunosorbent Assay. The basic principle of a competitive solid-phase enzyme-linked immunosorbent assay (Voller et al., 1976) was extended by a second antibody technique (Meyer and Güven, 1986) by changing the basic conditions of the binding kinetics. Figure 1 is a schematic representation of the relationship of reagents after the completion of the immunoreactions. For all the immunoreactions, antiserum and enzyme tracer were diluted in 0.04 M phosphate buffer, pH 6.6, containing 0.9% NaCl and 0.1% BSA. All reactions were performed in polystyrene microtiter plates. The assay procedure is as follows:

1. Coat the affinity-purified anti-rabbit sheep IgG to the wells of the microtiter plate (1 μg/well in 100 μL of 0.05 M carbonate buffer, pH 9.6); incubation overnight at 0 °C.

2. Complete coating of the wells by blocking with 1% BSA in 0.067 M phosphate buffer, pH 7.2 (300 μL/well); incubation for 60 min at 25 °C.
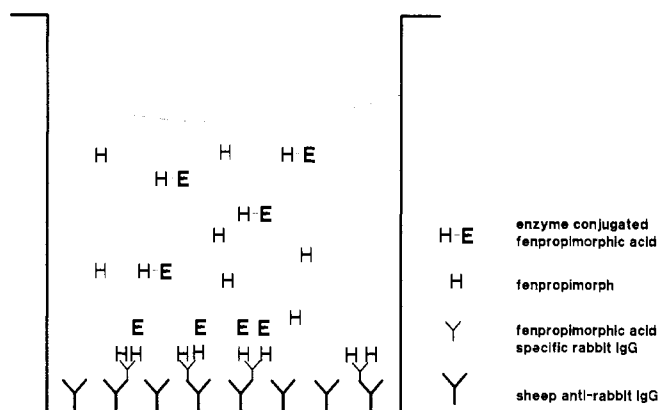

Figure 1. Schematic presentation of binding of reagents after completion of the immunoreaction. Microtiter plates were coated with sheep anti-rabbit IgG and then washed. A competitive immunoreaction with enzyme-conjugated fenpropimorphic acid, standard or sample of fenpropimorph, or fenpropimorphic acid and specific rabbit IgG occurred simultaneously.

3. Wash with 250 μL/well (0.05% Tween 20 in water).

4. Add 100 μL/well of enzyme-conjugated fenpropimorphic acid and 20 μL/well standard or sample (containing the unconjugated fenpropimorph) with the aid of a diluter dispenser.

5. Dispense 100 μL/well of fenpropimorphic acid antibody raised in rabbits.

6. Incubate overnight at 8 °C (fixation to the microtiter plate wells and competitive immunoreaction occur simultaneously).

7. Wash three times with 250 μL/well 0.05% Tween 20 in water.

8. Dispense 150 μL/well substrate solution consisting of 400 μL of TMB solution (6 mg/mL DMSO) and 100 μL of 1% H$_2$O$_2$ in 25 mL of 0.1 M sodium citrate buffer, pH 5.5; incubate for 40 min at 25 °C, in darkness.

9. Add 50 μL/well stopping reagent (4 N H$_2$SO$_4$).

10. Read absorbance at 450 nm.

To find the optimal concentrations of enzyme tracer and antibody dilution, a two-dimensional titer determination was performed with a decreasing concentration of enzyme–conjugate columnwise in the microtiter plate, usually ranging from 1:500 to 1:16 000 in two wells for each dilution. Specific antibody dilutions from 1:5000 to 1:320 000 are added rowwise. The optimum combination of antibody and enzyme–conjugate dilutions is chosen where both dilution curves were steep and gave an absorbance between 0.8 and 1.0.

Standard curves were prepared daily by diluting a stock solution of fenpropimorphic acid in ethanol (1 mg/mL) 1:2 in 0.04 M phosphate buffer, pH 6.6. The logarithm of the hapten concentrations plotted against the absorbance resulted in a sigmoidal curve. The detection limit was defined as the lowest concentraton of hapten showing a reduction of 3 standard deviations from the mean blank standard absorbance.

Several compounds structurally similar to fenpropimorph were examined for cross-reactivity in this immunoassay. Cross-reactivities were measured by determining the amount of binding in the presence of varying amounts of test compound. The amount of test compound required to inhibit the assay by 50% was used as a basis for comparison.

Comparison with Gas Chromatography. Tap water and soil percolation water samples were spiked with 200 ng/L fenpropimorphic acid and split for analysis by ELISA and GLC. Preliminary studies on percolation water from treated soil were also done. These water samples were collected with suction lysimeters. The pH of these samples ranged from 7.0 to 7.4, and the samples exhibited a variety of yellow or red colors. The ELISA analyses were run without any prior sample workup. The results were based on two replicates of each sample calculated from a buffered standard curve and were repeated on two separate days. For each GLC analysis a 500-mL water sample was extracted with dichlormethane. The fenpropimorph metabolite in the organic phase was methylated with diazomethane followed by separation by C$_8$ silica gel chromatography. GLC conditions were described above. The amount of fenpropimorphic acid in the extracts was
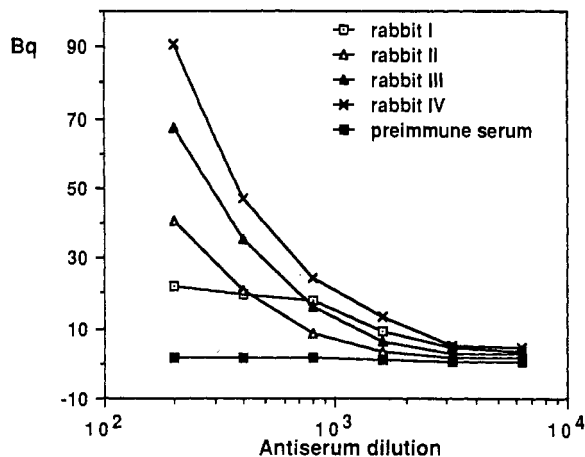
Figure 2. Radioimmunoassay of serum from four rabbits (I–IV) and preimmune serum at several dilutions. The radioactivity (Bq) bound by serum is plotted against the serum concentration used.
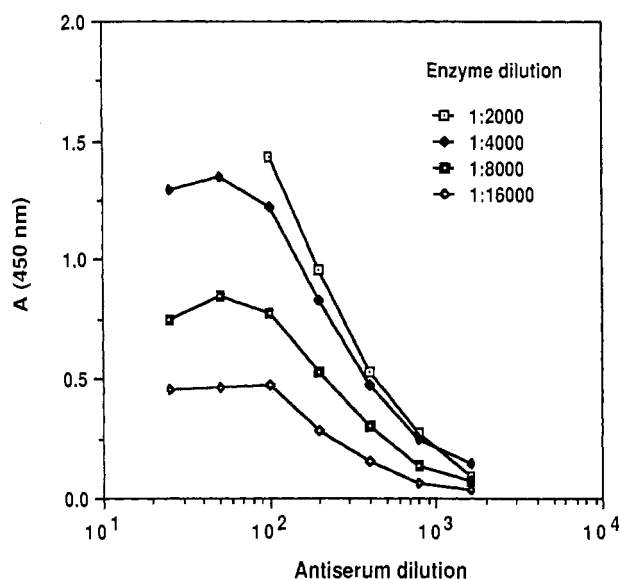

Figure 3. Two-dimensional titer determination for anti-fenpropimorph antibody. The antibody was raised in rabbit IV (2.titer) against an acid derivative of fenpropimorph conjugated to BSA by the active ester method. Horseradish peroxidase was conjugated to the same acid derivative by a mixed-anhydride method followed by purification on a Sephadex G-25 column. The optimum combination was 1:200 000 for the specific antibody and 1:5000 for the enzyme conjugate.

determined by comparing the peak height with that of the standard curve obtained from pure methylated fenpropimorphic acid. The mean recovery of the standards determined on four replicates was about 86.5 ± 7.8 at the detection limit of 50 ng/L.

RESULTS

Characterization of the Antisera. The antisera were first tested for their ability to bind [^{3}H]fenpropimorphic acid. Figure 2 summarizes the results from screening the sera of the four rabbits for anti-fenpropimorph antibodies after the third boost. The individual rabbits responded quite differently. After the sixth boost, repeated immunization of the rabbits with the same antigen had little positive effect on the specific antibody titer.

Titration of Enzyme Tracer and Antibody. Figure 3 shows the two-dimensional titration of fenpropimorphic acid antibody of rabbit IV and horseradish peroxidase–fenpropimorphic acid conjugate dilution. The decrease in absorbance at higher antibody concentrations (1:25 000) is explained by insufficient binding capacity of the sheep
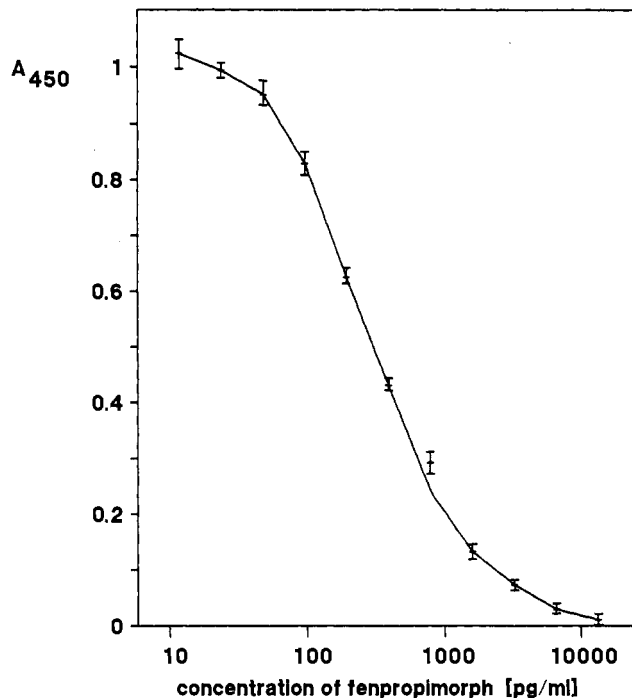

Figure 4. Standard curve for the determination of fenpropimorph and fenpropimorphic acid by ELISA (antiserum of rabbit IV, second titer, 1:200 000 dilution; enzyme tracer dilution 1:5000). Absorptions are corrected for nonspecific binding and are the means ± SD of 10 replicates. The curve shows a linear range at concentrations between 100 and 800 pg/mL.

IgG. The absorbance follows a normal titration curve at lower antibody concentrations. The optimized ELISA test in the microtiter plate was performed with a working dilution of 1:5000 for the enzyme tracer and 1:200 000 for the antibody (rabbit IV) at an appropriate absorbance range between 0.7 and 1.0.

Sensitivity and Specificity of the ELISA. Each standard curve was performed in plates after coating with 1 μg/well affinity purified sheep IgG anti-rabbit IgG to the microtiter plate. Batches of about 50 plates were coated in advance and stored at -20 °C. Standard curves made after 2 weeks and 1, 2, 3, and 4 months of storage were similar. Blocking with 1% BSA prevented nonspecific binding of the antibody to the carrier protein BSA. Furthermore, reproducibility was enhanced by maintaining constant temperature during the immunoreaction and the wash steps. The fenpropimorphic acid standard curve shown in Figure 4 reflects the high sensitivity of the test system with a linear range for hapten concentrations between 100 and 800 pg/mL. The absorbance of each fenpropimorphic acid dilution was corrected for nonspecific binding and represents the means and standard deviation of 10 assay conducted on different days. The limit of detection was about 13 pg/mL. The concentration of fenpropimorph required to inhibit the assay by 50% was 300 pg/mL.

The specificity of the antiserum is shown by the data in Figure 5. In a large number of tests checking structurally related pesticides, detergents, and other chemicals, no cross-reactivity could be detected except for tridemorph (2%). Apparently, the antiserum is highly specific and can distinguish cis-fenpropimorph and trans-fenpropimorph (8% cross-reactivity). Fenpropimorph and fenpropimorphic acid are recognized equally because of the chosen coupling site. Comparison of the cross-reactivity of the antibody to 2,6-dimethylmorpholine with that of tridemorph suggests that the phenyl group is an important factor in structural recognition. Replacement of the 2,6-

1186 J. Agric. Food Chem., Vol. 37, No. 4, 1989

Jung et al.

R
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N

1 (R = i-C$_{13}$H$_{27}$)   2   4   5

3   6
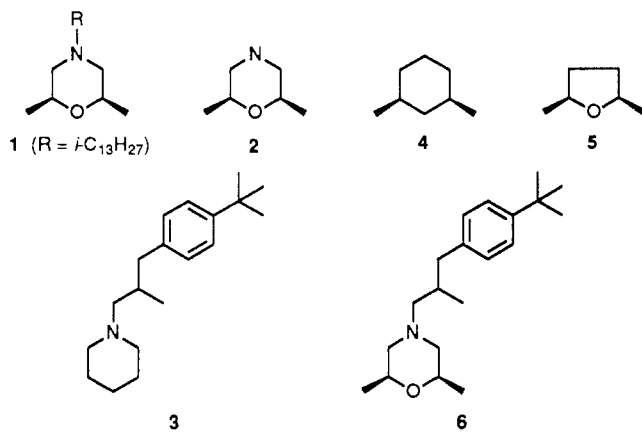
name	no.	cross-reactivity, %
fenpropimorph	6	100.0
tridemorph	1	2.0
2,6-dimethylmorpholine	2	0.1
fenpropidin	3	0.01
cis-1,3-dimethylcyclohexane	4	0
2,5-dimethyltetrahydrofuran	5	0

Figure 5. Structures of several compounds tested for their cross-reactivity. The five compounds shown here are a representative sample of 10 different compounds tested. Significant cross-reactivity was found only for trans-fenpropimorph (8%) and tridemorph (2%).

dimethylmorpholine group by a piperidine as demonstrated by fenpropidine resulted in a decrease in cross-reactivity.

Soil Percolation Water Analysis. The ELISA and GLC determinations of spiked tap and soil percolation water were in good agreement. The difference between ELISA and GLC of 9.5% was within the coefficient for variation of both test systems. Recoveries for spiked tap water were 160 ± 19 and 170 ng/L for ELISA and GLC, respectively. Soil percolation water gave higher results with both methods, 230 ± 26 and 220 ng/L by ELISA and GLC, respectively. The ELISA and GLC results for fenpropimorph and fenpropimorphic acid determination in soil percolation water are given in Table I. For ELISA, the relative standard deviations of the results from three independent assays were determined to be 13.3% and 3.6% for the estimates in soil percolation water and in buffer, respectively. Control samples devoid of fungicide gave same results in ELISA and GLC. For positive samples, the concentration determined by ELISA were higher.

DISCUSSION

The high sensitivity and specificity of the ELISA described in this study are the result of a combination of several techniques. The use of N-hydroxysuccinimide, a better activator of fenpropimorphic acid, resulted in an epitope density of 17, which seems to be optimal for raising high-affinity antibodies (Erlanger, 1980). Although radioimmunoassay using [³H]fenpropimorphic acid as a marker was shown to be useful for the detection of anti-fenpropimorph antibodies, its use in analysis was found to be impractical due to the low specific activity of the [³H]fenpropimorphic acid. The activity of the horseradish peroxidase remains high after coupling to fenpropimorphic acid since this enzyme contains relatively few unblocked amino groups and only a few fenpropimorph molecules per enzyme molecule are bound, even in an excess of fenpropimorphic acid (Welinder, 1979; Nakane and Kawaoi, 1974). The mixed-anhydride procedure resulted in a high-activity enzyme tracer that maintained its activity in 1:50 dilutions while stored in aliquots in the refrigerator,

Table I. Comparison between ELISA and GLC Analysis of Soil Percolation Water

samples	ELISA,[a] ng/L	GLC,[b] ng/L
untreated field	<13	<50
fungicide-treated field	140 ± 5.6	161
	100 ± 7.0	76
	260 ± 7.8	166
	100 ± 4.0	55
	220 ± 6.6	83
	180 ± 9.5	93
	140 ± 10.6	96
	150 ± 7.5	74
	330 ± 12.21	195
	70 ± 4.3	76
	200 ± 2.0	84

[a] ELISA detected fenpropimorph and fenpropimorphic acid equally and was the mean of three replications without any sample workup. The detection limit was 13 ng/L. [b] GLC data were obtained on one determination for fenpropimorphic acid after sample cleanup and derivatization. The detection limit is 50 ng/L.

and allowed the use of a working dilution of 1:5000, corresponding to an absolute dilution of 0.5 × 10⁻⁶. TMB is a better alternative to the chromogen o-phenylenediamine (OPD), because it is not mutagen (Bos et al., 1981). The same author found that the rapidity and intensity of color development with TMB are better than that of OPD. Stopping the reaction with 4 N sulfuric acid resulted in a color change from blue to bright yellow. This color has a more intense peak in the spectrum at 450 nm, and this contributed to improved sensitivity of the ELISA.

The new second antibody technique was a significant improvement. The results clearly point out that it is the antibody that determines the specificity and sensitivity, although prudent selection of assay format can improve sensitivity. In former techniques, the specific antibody was directly coated to the wells (Weiler et al., 1981). A further purification was also needed for the specific antibody to reduce background binding. This purification and the direct coating procedure often resulted in decreased titer and increased variability (Meyer, 1986).

A phenomenon known as the time-dependent drift (Munro and Stabenfeldt, 1984) is caused by different incubation times within the plate due to the time required for pipetting different samples or standards into 96 wells. To overcome this difficulty, the second antibody technique was used. In this method the microtiter well was first coated with an excess of anti-rabbit IgG raised in sheep, followed by samples and enzyme tracer. Following rapid dispensation of fenpropimorphic acid specific antibody, the immunoreaction starts almost simultaneously in the 96 wells. This resulted in very good reproducibility between replicates, a high titer of the specific antiserum, as well as a high test sensitivity. Since storage of precoated plates did not have any affect on the ELISA, precoating had the advantage of being a one-step rapid coating and guaranteed a uniform solid phase for the immunoreactants decreasing the plate to plate variability.

Compared with other enzyme immunoassay formats the number of hapten-binding antibodies required per well is significantly lower, as there is no inactivation during the binding to sheep IgG. The experiments in this study showed that an antibody dilution of only 1:200 000 was needed in the second antibody technique in contrast to a 1:17 000 dilution needed for the direct coating technique.

This greatly conserves a valuable reagent, the specific antibody. As described above, this method required only 20 μL of sample. Samples were analyzed directly without any adjustment of pH or ionic strength before dilution in the assay buffer. Assays that are very high in sensitivity

mean that the sample can be greatly diluted, minimizing the interferences while maintaining sensitivity for the specific compound.

Preliminary studies with soil percolation water showed that this ELISA is particularly useful for screening samples. Both methods showed concentrations below their limit of detection for the untreated field sample, and all positive samples by GLC were positive by ELISA. A reason for the higher concentrations in positive samples by ELISA may be due to the differences between the two detection systems. Because of the derivatization procedure GLC detects only fenpropimorphic acid, while the ELISA detects fenpropimorph as well as fenpropimorphic acid. As mentioned before, fenpropimorph is metabolized rapidly to fenpropimorphic acid, and the ability to analyze the two compounds together in environmental samples may be useful since they have the same biological activity.

The preliminary field studies show the potential of the ELISA method for analyzing small sample volumes without derivatization or sample cleanup. It is capable of excluding false negative results and can make screening by ELISA a very fast and cost-effective method.

ABBREVIATIONS USED

BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent immunoassay; DMF, N,N-dimethylformamide; DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; Tween 20, polyoxyethylene sorbitan monolaurate; N-FID, nitrogen flame ionization detector; TMB, 3,3′,5,5′-tetramethylbenzidine; OPD, o-phenylenediamine; Bq, becquerel; GLC, gas chromatography.

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Registry No. H₂O, 7732-18-5; fenpropimorph, 67306-03-0; fenpropimorphic acid, 121098-45-1.

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