Fluroxypyr- and Triclopyr-Specific Enzyme-Linked Immunosorbent Assays: Development and Quantitation in Soil and Water

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Enzyme-linked immunosorbent assays (ELISAs) were developed to quantitate fluroxypyr [[(4-amino-3,5-dichloro-6-fluoro-2-pyridinyl)oxy]acetic acid] and triclopyr [[(3,5,6-trichloro-2-pyridinyl)oxy]acetic acid] in soil and water. The linear working range of the fluroxypyr ELISA was from 0.1 to 10 ng·mL^{-1} with a limit of detection (LOD) of 0.1 ng·mL^{-1} and an IC_{50} value of 1.6 ng·mL^{-1}. The linear working range of the triclopyr ELISA was from 0.1 to 5 ng·mL^{-1} with a LOD of 0.1 ng·mL^{-1} and an IC_{50} value of 1.7 ng·mL^{-1}. Cross-reactivity to selected pyridine metabolites and agrochemicals was determined. Significant cross-reactivity (within the linear working range of the ELISA) using the fluroxypyr ELISA was found only to the metabolite 4-amino-3,5-dichloro-6-fluoro-2-methoxypyridine. Significant cross-reactivity using the triclopyr ELISA was found only to the auxinic herbicide 2,4,5-trichlorophenoxyacetic acid (2,4,5-T). The ELISAs accurately estimated fluroxypyr and triclopyr in water at concentrations as low as 0.1 ng·mL^{-1}. Analysis of two different soil types with different textures (clay loam and sandy clay loam) required cleanup procedures using filtration and solid phase extraction to accurately estimate fluroxypyr and triclopyr concentrations.

Keywords: *ELISA; residue; pyridine; herbicide*

INTRODUCTION

Fluroxypyr [[(4-amino-3,5-dichloro-6-fluoro-2-pyridinyl)oxy]acetic acid] is registered for use in many European countries for the control of broadleaf weeds in small grains and pasture crops (DowElanco, 1987). It is applied as the methylheptyl ester (fluroxypyr-MHE) and quickly hydrolyzes in the plant and soil (half-life of 2−5 h) to the parent compound, fluroxypyr (Sanders and Pallet, 1987; Lehmann and Miller, 1989a). Fluroxypyr is degraded microbiologically (half-life of 1−3 weeks) to two primary metabolites, 4-amino-3,5-dichloro-6-fluoropyridin-2-ol and 4-amino-3,5-dichloro-6-fluoro-2-methoxypyridine, then to other secondary metabolites, and finally to carbon dioxide and water (Lehmann *et al.*, 1991). Fluroxypyr and its metabolites are currently analyzed using capillary column gas chromatography (GC), gas chromatography−mass spectroscopy (GC−MS) (Bergström *et al.*, 1990), or reversed-phase high-pressure liquid chromatography (HPLC) (Lehmann and Miller, 1989b).

Triclopyr [[(3,5,6-trichloro-2-pyridinyl)oxy]acetic acid] is registered in Europe and North America for post-emergence control of a wide range of annual and perennial broadleaf weeds and woody plant species in agriculture and forestry. Triclopyr can be applied as either the triethylamine salt or the butoxyethyl ester. These compounds are quickly transformed to triclopyr through photolysis and hydrolysis (McCall and Gavit, 1986; Woodburn *et al.*, 1993a). Solomon *et al.* (1988) constructed limnocorrals in a northern Ontario bog lake (50° N latitude) and observed an approximate 4 day summer half-life for triclopyr in natural water. Little triclopyr adsorption to particles was observed, and dissipation within the limnocorral was largely attributed to photodegradation. Woodburn *et al.* (1993a) reported an average first-order half-life of 1.2 days for midsummer photolysis of triclopyr in river water (40° N latitude, 25 °C), with oxamic acid being the principal photodegradation product. Triclopyr will also undergo biodegradation in soil environments, with an observed half-life of approximately 40 days in a darkened aerobic soil/water system (Norris *et al.*, 1987). The major degradation product was 3,5,6-trichloro-2-pyridinol. This compound is also the major metabolite produced by fish (Lickly and Murphy, 1987). In water, 3,5,6-trichloro-2-pyridinol is photolabile, with a measured half-life of less than 1 h in midsummer surface water at 40° N latitude (Dilling *et al.*, 1984).

Triclopyr is presently under development for use as an aquatic herbicide, formulated as the triethylamine salt. Studies to evaluate the environmental fate and distribution of triclopyr under aquatic field conditions will require analytical methods to follow its movement and dissipation. Triclopyr is quantified with a GC equipped with an electron capture detector (Woodburn *et al.*, 1993b; Solomon *et al.*, 1988). These methods require methylation, solvent transfer, and concentration for analysis.

Enzyme-linked immunosorbent assays (ELISAs) have a significant role to play in the analysis of environmental samples for detection of agrochemicals. Large sample loads and high analysis costs involved in registration of compounds such as fluroxpypyr and triclopyr for relatively small-market commodities (small grains, agroforestry, aquatic systems) could be cost prohibitive. Approximately 80% of all samples collected for environmental monitoring studies are below the limits of detection of current analytical methods (Hall *et al.*, 1993). Immunoassays developed for these small-market compounds could eliminate sample queues and reduce the cost of analysis when used as a qualitative prescreen prior to analysis by GC, GC−MS, or HPLC. Quantitative analysis of many pesticides in water matrices has been carried out with little matrix interference (Rubio *et al.*, 1991; Lawruk *et al.*, 1993a; Harrison *et al.*, 1989b); however, other sample matrices, such as foodstuffs and soil, have limited the usefulness of immunoassays as quantitative tools (Van Emon and Lopez-Avila, 1992).

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Assay format appears to have an effect on the ability of an immunoassay to overcome these matrix effects. For analysis of environmental samples, competitive ELISAs that use immobilized antibody and an enzyme conjugate (Rubio *et al.*, 1991; Schneider and Hammock, 1992; Wong and Ahmed, 1992; Lawruk *et al.*, 1993a) perform better than competitive ELISAs that use free antibody in solution and a coating conjugate (Bushway *et al.*, 1988; Feng *et al.*, 1990; Goh *et al.*, 1990).

The purpose of this paper is to describe the development of competitive ELISAs for the herbicides fluroxypyr and triclopyr and outline the application of these ELISAs for use in quantitating concentrations of fluroxypyr and triclopyr in water and soil.

MATERIALS AND METHODS

Chemicals and Instrumentation. The analytical standards of fluroxypyr and triclopyr as well as 2,6-^{14}C-radiolabeled fluroxypyr and 2,6-^{14}C-radiolabeled triclopyr were provided by the DowElanco Chemical Co., Indianapolis, IN. Horseradish peroxidase (HRP) (EC 1.11.1.7, 1280 units/mg), *N*-hydroxysuccinimide (NHS), 2,2-azinobis(3-ethylbenzothiazoline)-6-sulfonic acid diammonium (ABTS) substrate tablets, and urea hydrogen peroxide were obtained from Sigma Chemical Co., St. Louis, MO. Recombinant derived protein A, *N*,*N*′-dicyclohexylcarbodiimide (DCC), affinity-purified HRP-conjugated goat anti-rabbit (GaR) IgG, and 1-ethyl-3-[3-(dimethylamino)propyl]-carbodiimide·HCl (EDC) were obtained from Pierce Immunochemicals, Rockford, IL. Desalting columns (PD-10) were obtained from Pharmacia Fine Chemicals, Dorval, QU. C_{18} reversed-phase solid phase extraction columns were obtained from J. T. Baker Inc., Phillipsburg, NJ. Dynatech Immulon 4 flat-bottom microtitration plates were purchased from Fisher Scientific, Don Mills, ON. ELISAs were analyzed using a 3550-UV microplate reader from Bio-Rad Laboratories, Richmond, CA. All other chemicals were of reagent grade and obtained commercially.

Synthesis of Immunogen. Fluroxypyr was conjugated to porcine serum albumin (PSA) as described by Fleeker (1987) with the following modifications. Fluroxypyr (0.19 μmol) and an equal molar amount of NHS were solubilized in 2.0 mL of 1,4-dioxane. DCC (0.40 mmol) was solubilized in 500 μL of 1,4-dioxane. The two solutions were combined and incubated overnight at room temperature. PSA (200 mg) was added with slow stirring for 1 h. The fluroxypyr immunogen was dialyzed for 24 h against four 1 L changes of 10 mM phosphate buffer/15 mM saline, pH 7.5 (PBS).

Triclopyr was conjugated to both PSA and bovine serum albumin (BSA) as described by Fleeker (1987) with the following modifications. Triclopyr (0.38 μmol) and an equal molar amount of NHS were solubilized in 4 mL of dioxane. DCC (0.80 mmol) was solubilized in 1.0 mL of dioxane. The two solutions were combined and incubated overnight at room temperature. The solution was then divided into two 2.5 mL samples, and 200 mg of BSA or PSA was added to each with slow stirring for 1 h. Both immunogens were dialyzed for 24 h against four 1 L changes of PBS (pH 7.5).

Synthesis of Enzyme Conjugate. The enzyme conjugate for the competitive fluroxypyr ELISA and competitive triclopyr ELISA was prepared by covalently linking fluroxypyr to HRP using EDC and NHS. Fluroxypyr (4 μmol) was dissolved in 300 μL of pyridine. NHS (90 μmol) and EDC (210 μmol) were dissolved in 200 μL of pyridine. One hundred microliters of the NHS−EDC was then added to the fluroxypyr solution at room temperature. After a 1 h incubation, 200 μL of this solution was added to 1 mL of a 140 mM NaCl solution containing 10 mg of HRP. This mixture was stirred at room temperature for 5 h followed by removal of free herbicide (not conjugated) using a PD-10 column equilibrated with PBS. Aliquots of the fluroxypyr−HRP enzyme conjugate were stored at −20 °C until needed.

Polyclonal Antisera Production. For production of fluroxypyr-specific polyclonal antisera New Zealand white rabbits were injected intramuscularly with 500 μg of the fluroxypyr−PSA immunogen in 1 mL of a phosphate buffer/Freund's incomplete adjuvant (1:1 v/v) emulsion (500 μL immunogen in each of the left and right lumbar muscles). This primary immunization was repeated at weekly intervals for 3 weeks. Secondary immunizations of 200 μg of immunogen in 1 mL of PBS and Freund's incomplete adjuvant (1:1 v/v) were administered intramuscularly at monthly intervals following primary immunizations. The rabbits were bled from the central ear artery for anti-fluroxypyr antibody titer determinations 7 days after each secondary immunization.

For production of triclopyr-specific polyclonal antisera New Zealand white rabbits were injected intramuscularly with 500 μg of the triclopyr−BSA immunogen in 1 mL of a phosphate buffer/Freund's incomplete adjuvant (1:1 v/v) emulsion. This primary immunization was repeated at weekly intervals for 3 weeks. Secondary immunizations, composed of 200 μg of triclopyr−PSA immunogen dissolved in PBS and Freund's incomplete adjuvant (1:1 v/v) in a total of 1 mL, were administered at monthly intervals following primary immunizations. The rabbits were bled for anti-triclopyr antibody titer determinations 7 days after each secondary immunization.

Antisera Titer Determination. Fluroxypyr-specific and triclopyr-specific antisera titers were determined as described by Campbell (1984) with the following modifications. ELISA conditions were determined using the fluroxypyr−HRP enzyme conjugate for both the fluroxypyr antisera and triclopyr antisera. Optimal dilutions of fluroxypyr antisera, or triclopyr antisera, and fluroxypyr enzyme conjugate were determined by selecting reagent concentrations that resulted in an absorbance of approximately 1.0 at 405 nm after a 30 min incubation at room temperature.

Standard Curve and Sample Analysis. The competitive ELISA was performed as described by Deschamps and Hall (1991) with the following modifications. Immulon 4 flat-bottom microtiter plates were coated with protein A (0.2 μg·mL^{-1}, 100 μL·well^{-1}) and incubated overnight at 4 °C. Unbound Protein A was removed by washing three times using PBS with 0.05% (v/v) Tween 20. Unoccupied well sites were blocked with PBS containing 0.01% (w/v) gelatin for 20 min at room temperature. Fluroxypyr antisera or triclopyr antisera were diluted in PBS (100 μL·well^{-1}) and incubated overnight at 4 °C. Plates were washed as described above, and equal standard and/or sample volumes of free fluroxypyr or free triclopyr and appropriate concentrations of fluroxypyr−HRP enzyme conjugate were mixed and added (100 μL·well^{-1}) to the plates. This mixture was incubated for 1 h at room temperature. Unbound fluroxypyr or triclopyr and fluroxypyr−HRP enzyme conjugate were removed by washing, substrate (1 mg·mL^{-1} urea hydrogen peroxide with 1 mg·mL^{-1} ABTS) was added, and color was allowed to develop for 30 min. The intensity of color was inversely proportional to the concentration of free fluroxypyr or triclopyr.

Absorbance values of the standards and the samples (A) were normalized by dividing by the absorbance values of the negative controls (wells containing 0 ng·mL^{-1} fluroxypyr or triclopyr; A_0). The A/A_0 values for standards were plotted against the log of fluroxypyr or triclopyr concentration to construct a standard curve. Concentrations of the samples in water and soil were determined by interpolating from a PBS standard curve.

Potential ELISA Interferences. The effect of selected organic solvents on the ELISAs to estimate fluroxypyr or triclopyr was determined. Known concentrations of fluroxypyr or triclopyr were prepared in 1−10% (v/v) final concentration of methanol, acetone, or acetonitrile diluted in PBS. Results were compared to the same concentrations of fluroxypyr or triclopyr prepared in PBS.

The effect of sample pH on the ELISAs to accurately estimate fluroxypyr or triclopyr concentrations was also studied. Known concentrations of fluroxypyr or triclopyr were prepared in PBS with pH values ranging from 2.0 to 10.0. Results were compared to the same concentrations of fluroxypyr or triclopyr prepared in PBS.

Buffer strength was examined to determine the effect of increased ionic strength on the two ELISAs. Fluroxypyr or triclopyr concentrations were prepared in PBS ranging from 20 mM phosphate and 30 mM saline to 100 mM phosphate and 150 mM saline. Results were compared to the same concentrations of fluroxypyr or triclopyr prepared in PBS.

Water Sample Analysis. Water from the Speed River, Guelph, ON, was collected in 1 L Nalgene bottles and stored frozen until required. For sample analysis, 10 mL of water was spiked with known concentrations of fluroxypyr or triclopyr and filtered through a 0.45 μm nylon filter, and the pH was adjusted to 7.5. A 500 μL aliquot of the sample was then mixed 1:1 with fluroxypyr−HRP for analysis, and concentrations were interpolated from a PBS standard curve.

Soil Sample Analysis. Air-dried samples of either Saskatchewan sandy clay loam or Manitoba clay loam were used. For sample analysis, 5 g of soil was spiked with a known concentration of fluroxypyr or triclopyr dissolved in 1 mL of acetone. For each sample run, the following controls were added to each experiment: duplicate blanks (to ensure no matrix effect was present), known concentrations of ^{14}C-radiolabeled fluroxypyr or ^{14}C-radiolabeled triclopyr (to determine extraction efficiency), fluroxypyr or triclopyr with concentrations lying below the linear working range of the ELISA (to check for false positives), fluroxypyr or triclopyr with concentrations lying within the linear working range of the ELISA (for quantitation), and fluroxypyr or triclopyr with concentrations lying above the linear working range of the ELISA (to check for false negatives). All vials were incubated for 24 h to allow acetone evaporation and fluroxypyr binding to soil colloids. After incubation, vials were shaken with 20 mL of 90% methanol/10% 0.1 N HCl for 2 h on an orbital shaker (180 rpm). Vials were centrifuged at 3000*g* (Sorval SS-34 rotor) for 10 min, and the supernatant was decanted into a 50 mL Erlenmeyer flask. A second 20 mL aliquot of 90% methanol/10% 0.1 N HCl was added, and vials were shaken for 2 h. Each soil slurry was filtered through a Whatman No. 1 qualitative filter paper, and the supernatant from the first extraction was used to rinse the centrifuge tube and passed through the same filter as described in the washing step. A further 10 mL of the 90% methanol/10% 0.1 N HCl aliquot was passed through the soil on the filter paper as a final rinsing step.

For soil extracts containing fluroxypyr, each sample was filtered through a 0.45 μm nylon filter, and the final sample volume was brought to 50 mL with 90% methanol/10% 0.1 N HCl (v/v). A 10 mL aliquot of the soil extract was evaporated to dryness, using an analytical evaporator, and reconstituted with 10 mL of PBS. Each sample was filtered through a 0.45 μm nylon filter and mixed in a 1:1 (v/v) ratio with fluroxypyr−HRP for analysis. Fluroxypyr concentrations were interpolated from a PBS standard curve.

For soil extracts containing triclopyr, each sample was filtered through a 0.45 μm nylon filter. Samples were evaporated to less than 5 mL, but not to dryness, using an analytical evaporator at 45 °C, diluted to 50 mL with H_2O, and acidified to pH 2.0 with concentrated H_2SO_4. Triclopyr was extracted from these acidified aqueous samples using C_{18} reversed-phase columns as described by Wells and Michaels (1987) with the following modifications. Columns were conditioned with 5.0 mL of methanol followed by 10 mL of 4% acetic acid (v/v) under vacuum at a flow rate of 10−12 mL·min^{-1}. Before the column could dry, samples were passed through the column followed by 2.0 mL of 4% acetic acid (v/v). Samples were eluted three times with 1 mL aliquots of methanol and diluted to 5.0 mL with PBS in a volumetric flask and then diluted at least a further 10-fold. All samples were mixed in a 1:1 (v/v) ratio with fluroxypyr−HRP for analysis. Triclopyr concentrations were interpolated from a PBS standard curve.

Cross-Reactivity. Selected agrochemicals and metabolites of the respective herbicide were tested for cross-reactivity to the fluroxypyr and triclopyr antisera. Standards were prepared in PBS and IC_{50} values calculated. IC_{50} values were considered to be compound concentrations that inhibited 50% of the color development of the A_0 negative control. Percent cross-reactivity was determined to be the IC_{50} for fluroxypyr or triclopyr divided by the IC_{50} for the respective compound multiplied by 100. The least detectable dose (LDD) was determined to be the compound concentration required to inhibit 10% of the color development of the A_0 negative control.

RESULTS AND DISCUSSION

Assay Performance. The linear working range of the fluroxypyr competitive ELISA was 0.1−10 ng·mL^{-1}. The linear working range of the triclopyr competitive ELISA was 0.1−5 ng·mL^{-1}. Fleeker (1987) reported the limit of detection (LOD) of an assay to be 3 times the standard deviation of the A_0 from its mean absorbance, whereas Midgely *et al.* (1969) calculated the LOD as the concentration that corresponds to 90% of the A/A_0. Though the former LOD description is used by the American Association of Official Analytical Chemists (AOAC), the latter LOD description may be a more accurate assessment of the true LOD because the LOD is a function of the ability of a compound to inhibit antibody−hapten binding rather than a function of A_0 precision. Using both criteria, the LOD values of both ELISAs were 0.1 ng·mL^{-1}.

The limit of quantitation (LOQ) of an assay has been reported as 10 times the standard deviation of the A_0 from its mean absorbance (Fleeker, 1987). According to this method, the LOQ for the fluroxypyr ELISA was 0.9 ng·mL^{-1} and that for the triclopyr ELISA was 0.5 ng·mL^{-1}. Soil samples required a 50-fold dilution for accurate analysis of fluroxypyr, which increased the LOD to 5 ng·mL^{-1} and the LOQ to 45 ng·mL^{-1}. These limits are comparable to those reported by Bergström *et al.* (1990), who reported fluroxypyr detection limits of 0.1 ng·mL^{-1} for water and 5.0 ng·mL^{-1} for soil using GC−MS. For soil analysis of triclopyr, a 10-fold (v/v) dilution in PBS was required for accurate analysis, which increased the LOD and LOQ to 1 and 5 ng·mL^{-1}, respectively.

In the described ELISA format, microtiter wells were precoated with protein A. Wittmann and Hock (1989, 1990) reported excellent quantitation of atrazine in soil and water by using an ELISA system that employed direct immobilization of antibodies to microtiter plates without a protein A precoating. However, direct immobilization of fluroxypyr and triclopyr antibodies to polystyrene wells resulted in limited ELISA color development. Random orientation of antibodies passively adsorbed to polystyrene wells may limit the variable region from taking part in antibody−hapten interaction and could explain limited color development. Another possible reason for this limited color development could be antibody denaturation upon passive adsorption, which may inhibit antibody−hapten interaction. This latter possibility is reinforced by the work of Butler *et al.* (1993), who found 90% of monoclonal antibodies and 75% of polyclonal antibodies were denatured by passive adsorption. Protein denaturation or poor antibody orientation could explain the high antisera concentrations required for adequate color development when passive binding is employed. Protein A binds the F_c region of selected immunoglobulins with a greater affinity than that of the antibody to the support matrix (Deisenhofer, 1981). The use of protein A reduced antisera required for analysis by 1000-fold.

Assay precision for the fluroxypyr competitive ELISA was determined by preparing three replicate sets of standard concentrations of herbicide (standards). Each set of standards was run on each of five plates per day.

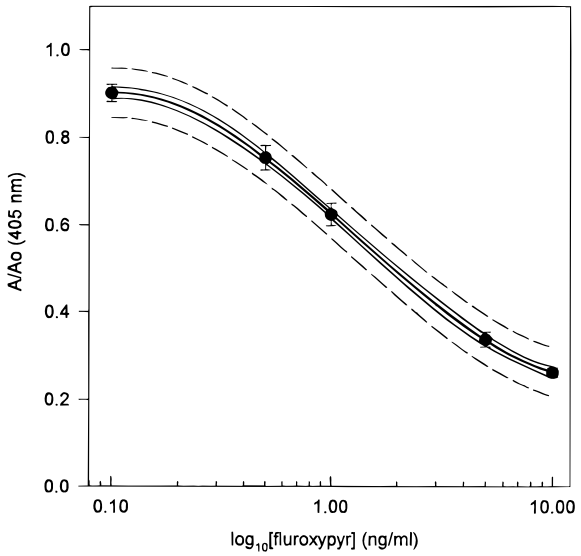

Figure 1. Cumulative standard curve for the fluroxypyr ELISA (read at 405 nm) illustrating 99% confidence intervals for standards and 99% confidence intervals (—) and prediction intervals (– –) for the standard curve ($n = 45$).

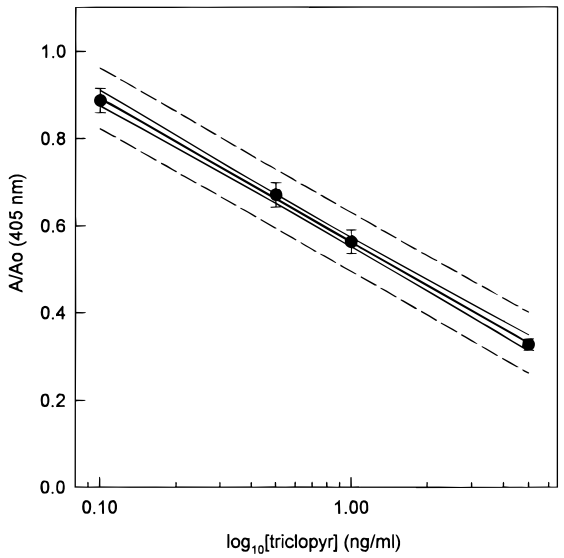

Figure 2. Cumulative standard curve for the triclopyr ELISA (read at 405 nm) illustrating 99% confidence intervals for standards and 99% confidence intervals (—) and prediction intervals (– –) for the standard curve ($n = 30$).

This experiment was repeated on each of 3 days. Therefore, the overall design consisted of three replicate standards repeated over 3 days on each of five plates per day with the total number of plates equal to 45. Variability between standards, between days within standards, and between plates within days was analyzed using a statistical analysis package (SAS, 1989). Harrison *et al.* (1989b) utilized the negative control, or A_0, to test for ELISA variability and found variability between wells on one plate were responsible for 91–95% of the error of an ELISA for the herbicide molinate. In our study, between-well variation was not examined because the average of three wells was used for standards and samples, thereby accounting for intraplate variability. However, within a plate, the absorbance coefficient of variation of the average of three wells for both standards and spikes was consistently below 5% (data not shown). The intercept (B_0) and slope (B_1) of fluroxypyr standard curves were used to determine contribution to variability because they take into account variability of each herbicide concentration within a standard curve. Both variables were analyzed separately and compared to ensure both had a normal distribution and similar variability (data not shown). Variability was primarily found between standard preparations ($p < 0.05$). Variability between days within standards was not found to be significant at the 95% confidence level; however, it was significant at the 99% confidence level. Goh *et al.* (1990) also tested for standard curve variability of an atrazine ELISA using B_0, B_1, and goodness of fit and found variability was primarily due to between-standard preparations of ELISA kits. On the basis of their results, they suggested a calibration curve was required for each replicate of their immunoassay. Our results indicate variability is due to differences between the preparations of sets of standards, which can be reduced by preparing standards in large batches at one time.

Fluroxypyr ELISA precision is illustrated by one replicate of 15 fluroxypyr ELISAs (Figure 1). Confidence intervals for the standard curve reached a minimum at 30 assays. Analysis of the final 15 ELISAs was not required to optimize the validation of the fluroxypyr ELISA.

As a result of the fluroxypyr ELISA precision study, assay precision for the triclopyr ELISA was determined by preparing three sets of standard concentrations. Each set of standard concentrations was run on each of five plates per day. This experiment was repeated on each of 2 days. Therefore, the overall design consisted of three replicate standards repeated over 2 days on each of five plates per day with the total number of plates equal to 30. Analysis of more than 30 assays for the fluroxypyr ELISA did not reduce the confidence intervals of the standard curve; therefore, the number of plates for the triclopyr validation study was reduced from 45 to 30. The majority of the variability found was associated with between-standards at the 95% confidence level. Between-days within standards variability was not found to be significant at the 95% confidence level. This variability allocation is consistent with the findings of the fluroxypyr ELISA precision study. Figure 2 illustrates the final standard curve produced from one set of 10 ELISAs.

Soil and Water Analysis. A sandy clay loam and a clay loam were chosen for this study because these soil types represent diverse soil textures (Table 1). All soil spikes were air-dried for 24 h prior to analysis. Acetone was chosen as the herbicide spike carrier because it rapidly evaporates, thereby ensuring maximum herbicide binding to soil colloids. Initially, an indirect competitive fluroxypyr ELISA was developed which used a picloram coating conjugate prepared as described by Deschamps *et al.* (1990). However, the direct ELISA that utilized immobilized antibodies and the fluroxypyr enzyme conjugate was chosen for further assay development because the ELISA which used the picloram coating conjugate could not overcome matrix effects of water and soil (data not shown) and required more time to complete the assay (180 min compared to 90 min). Difficulty in dealing with matrix effects using an indirect competitive ELISA was also reported by Feng *et al.* (1990) and Li *et al.* (1989). The direct ELISA in which antibodies were immobilized to the polystyrene wells was able to overcome matrix effects in water without sample dilution with PBS. Lawruk *et al.* (1993b, 1994) also found no detectable matrix interferences when using similar direct ELISA formats to

Table 1. Composition and Physical Characteristics of Soils Used for ELISA Analysis

		% composition			physical characteristics					
location	soil type	sand	silt	clay	cec[a]	pH[b]	EC[c]	OM[d]	IC[e]	FC[f]
Bradwell, SK	sandy clay loam	53	26	21	17.3	6.4	0.1	3.3	0.06	18.6
Minto, MB	clay loam	28	39	33	31.5	7.9	0.6	6.6	0.18	34.8

[a] Cation exchange capacity (mequiv/100 g). [b] 1:2 soil/water (w/v). [c] Electrical conductivity (mS/cm). [d] % organic matter. [e] % inorganic carbon. [f] Field moisture capacity (0.33 bar).

Table 2. Determination of Fluroxypyr in River Water and Two Soil Types with Different Textures Using a Fluroxypyr Polyclonal-Based ELISA

		determined		
	added ($ng \cdot mL^{-1}$)	mean ($ng \cdot mL^{-1}$)	recovery (%)	coefficient of variation (%)
river water	0.1	0.2	200	15
	0.5	0.4	80	10
	1	1	100	10
	5	6	120	2
$n = 45$	10	11	110	6
sandy clay loam	5	8	160	38
	25	25	100	20
	50	40	80	23
	250	260	105	15
$n = 45$	500	540	110	16
clay loam	5	5	100	60
	25	30	120	33
	50	70	140	31
	250	310	125	11
$n = 45$	500	450	90	9

measure concentrations of agrochemical in environmental water samples.

A 1:50 (v/v) soil extract:PBS dilution was required to eliminate matrix effects and achieve consistent quantitation of fluroxypyr in both soil types (Table 2). Diluting 50-fold with PBS reduced the effects of coextractants in the clay loam soil, whereas the fluroxypyr ELISA gave accurate determinations of fluroxypyr concentrations in the sandy clay loam soil samples at 1:25 (v/v) dilutions; however, to ensure accuracy and consistency for estimations of fluroxypyr concentrations in most soils, 1:50 dilutions were used for this study.

The soil extraction method for fluroxypyr resulted in mean recoveries of 96% for the Saskatchewan sandy clay loam and 95% for the Manitoba clay loam over three replications using ^{14}C-radiolabeled fluroxypyr. Recoveries decreased to 72 and 70%, respectively, when 15 mL of extraction solvent was used instead of 20 mL and when samples were shaken only once for 2 h instead of two times as described above.

Initially, a triclopyr ELISA was developed using triclopyr as the ligand for the enzyme conjugate. However, the ELISA had a LOD of 5 $ng \cdot mL^{-1}$, which was not sensitive enough for assay application. As a result, development efforts focused on the production of a heterologous assay since sensitivity can be improved by promoting an environment with a "biased equilibrium" in which the antisera's affinity for the compound to be detected is higher than the affinity of the antisera for the hapten conjugate (Wie and Hammock, 1984; Harrison *et al*., 1989a). Desired sensitivity was achieved by using fluroxypyr as the enzyme conjugate ligand. However, reduced antibody−hapten affinity may have resulted in reduced binding stability and, consequently, limited herbicide quantitation in soil matrices. To overcome this matrix effect in soil, solid phase extraction using C_{18} reversed-phase columns was required to achieve accurate triclopyr estimations.

The use of C_{18} reversed-phase columns performed a dual purpose for the study. First, triclopyr was concentrated from a 50 mL sample volume to a 3 mL sample volume and, second, soil coextractants that interfere with ELISA performance were removed, which allowed for accurate triclopyr determinations from a PBS standard curve using a heterologous assay format. The soil extraction method for triclopyr resulted in mean recoveries of 95% for the sandy clay loam and 92% for the clay loam using ^{14}C-radiolabeled triclopyr. Recoveries of ^{14}C-radiolabeled triclopyr from the solid phase extraction system were 95% for both soil types, which resulted in combined recoveries of 90% for the sandy clay loam and 87% for the clay loam soil. Data from the determination of triclopyr concentrations in soil using the triclopyr ELISA and C_{18} reversed-phase columns are shown in Table 3.

Table 3. Determination of Triclopyr in River Water and Two Soil Types with Different Soil Textures Using a Triclopyr Polyclonal-Based ELISA

		determined		
	added ($ng \cdot mL^{-1}$)	mean ($ng \cdot mL^{-1}$)	recovery (%)	coefficient of variation (%)
river water	0.1	0.2	200	10
	0.5	0.5	100	20
	1	1.3	130	15
$n = 30$	5	5.3	105	11
sandy clay loam	5	6	120	15
	25	28	110	11
	50	55	110	13
$n = 30$	250	245	98	10
clay loam	5	8	160	12
	25	26	105	11
	50	48	96	10
$n = 30$	250	255	102	8

Direct analysis of river water samples (i.e. without dilution or solid phase extraction) resulted in accurate determinations of fluroxypyr and triclopyr concentrations. Because an extraction was not required for water analysis, ^{14}C-radiolabeled fluroxypyr or ^{14}C-radiolabeled-triclopyr was not used as a tracer to determine sample recovery. Fluroxypyr and triclopyr quantitation in river water illustrates the accuracy of this ELISA for determination of these herbicides in water (Tables 2 and 3, respectively).

Potential ELISA Interferences. Bhat *et al.* (1994) observed that water molecules occupy holes and channels created by the association of antigen and antibody and thus contribute to the stabilization of the complex. This result is reinforced by the work of Ysern *et al.* (1994), who observed that two water molecules partially filled a cavity created by a site-directed mutant of FvD1.3 (variable domains of the heavy and light polypeptide chains). In aqueous environments water acts as a mediator in antibody−antigen interactions. However, the presence of organic solvent may alter the organization of water and disrupt the antibody−antigen complex, which could result in either an increase or decrease in binding, depending on the concentration of the solvent. The presence of organic solvents in an ELISA system

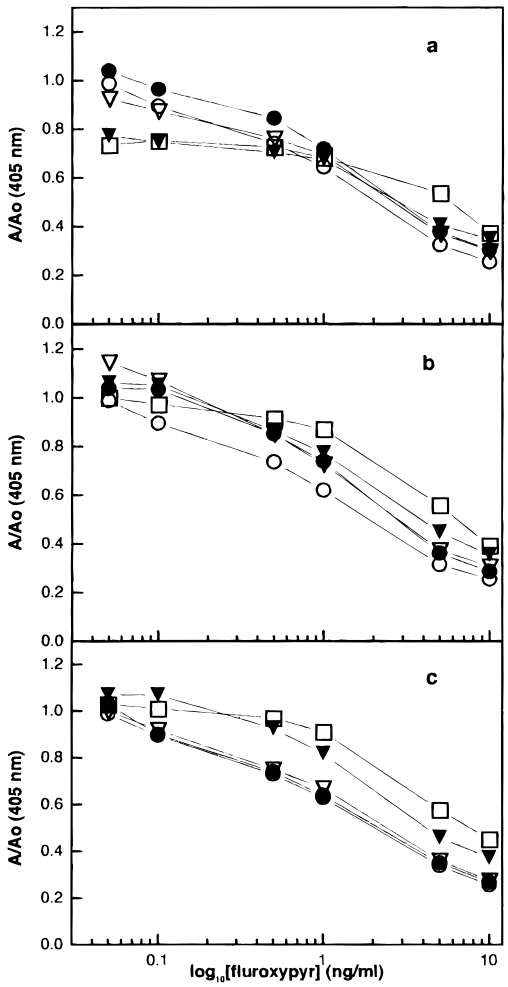

Figure 3. Effect of organic solvent on the fluroxypyr ELISA standard curve, (a) acetonitrile, (b) acetone, and (c) methanol, read at 405 nm. Organic solvent concentrations are (○) 0% (v/v), (●) 1% (v/v), (▽) 2% (v/v), (▼) 5% (v/v), and (□) 10% (v/v) in PBS at pH 7.5.

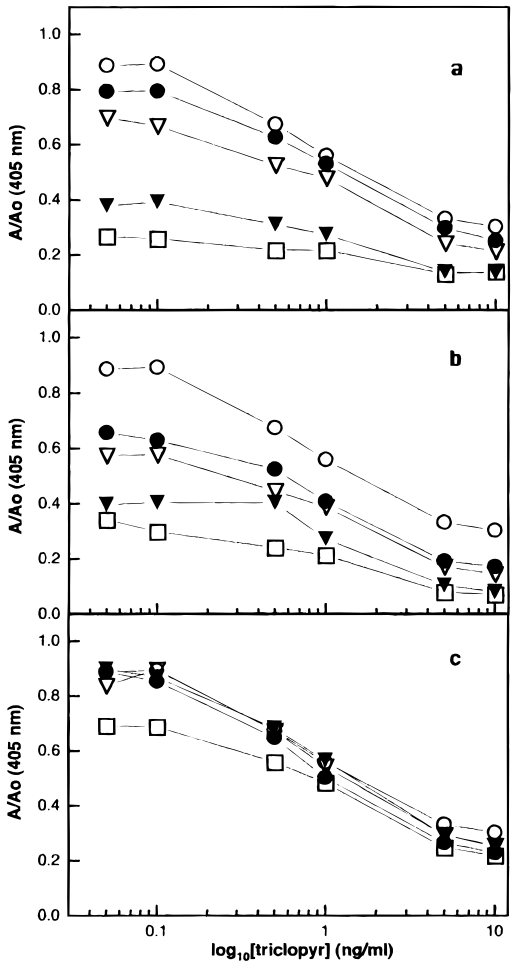

Figure 4. Effect of organic solvent on the triclopyr ELISA standard curve, (a) acetonitrile, (b) acetone, and (c) methanol, read at 405 nm. Organic solvent concentrations are (○) 0% (v/v), (●) 1% (v/v), (▽) 2% (v/v), (▼) 5% (v/v), and (□) 10% (v/v) in PBS at pH 7.5.

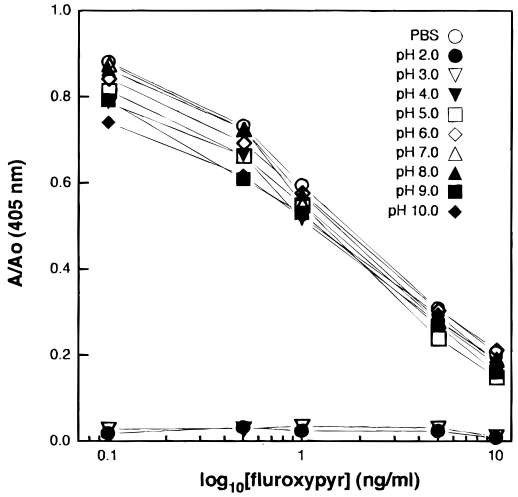

Figure 5. Effect of pH (from 2.0 to 10.0) on the fluroxypyr ELISA PBS standard curve read at 405 nm.

may also cause a change in the conformational structure of the antibody and decrease antibody−antigen binding and the subsequent enzyme reaction. Our research results and those of others (Gee *et al.*, 1988; Bushway *et al.*, 1989; Goh *et al.*, 1990) show that there is a decrease in antibody binding at higher organic solvent concentrations. For example, with the fluroxypyr ELISA, acetonitrile caused a change in the slope of the sample curves (Figure 3a), while acetone caused increased color development at low solvent concentrations and decreased color development at high solvent concentrations (Figure 3b). Methanol had limited effects on ELISA performance at concentrations up to 2% (v/v) (Figure 3c). However, methanol concentrations above 2%, combined with the effect of soil coextractants, caused a shift in the ELISA curve which resulted in overestimations of fluroxypyr concentrations in soil samples. On the basis of these results, methanol was chosen as the extraction solvent and was reduced to a concentration of less than 2% using an analytical evaporator prior to analysis to eliminate any effects it may have at higher concentrations. For the triclopyr ELISA, acetonitrile caused decreased color development (Figure 4a), as did acetone (Figure 4b), whereas methanol had limited effects on ELISA performance at concentrations up to 5% (v/v) (Figure 4c). Goh *et al.* (1990) and Bushway *et al.* (1989) found methanol concentrations as high as 10% were tolerated by their ELISA for analysis of soil and foodstuffs.

Tests were performed to determine the stability of the fluroxypyr and triclopyr ELISAs over a range of buffer strengths and sample pH (Figures 5 and 6, respectively). Increasing PBS buffer strength up to 100 mM phosphate buffer/150 mM saline did not significantly ($p < 0.05$) affect the determination of fluroxypyr or triclopyr

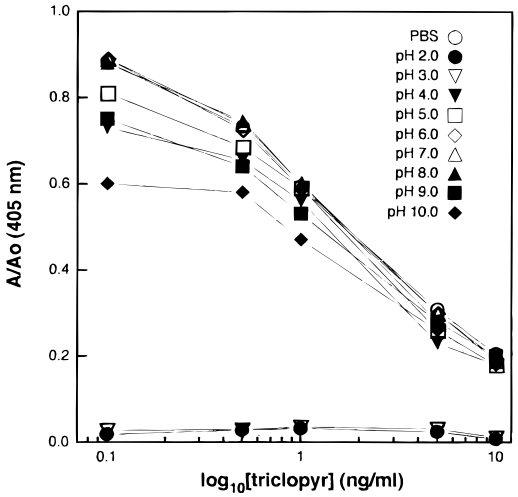

Figure 6. Effect of pH (from 2.0 to 10.0) on the triclopyr ELISA PBS standard curve read at 405 nm.

concentrations (data not shown). However, maintenance of sample pH is essential for accurate estimations of fluroxypyr and triclopyr in environmental samples. The purpose of the pH stability study was to imitate conditions experienced in routine water analysis and to determine if water samples could be directly analyzed or if sample pH adjustment would be required. Fluroxypyr or triclopyr standards were prepared in PBS, and then the pH was adjusted to the respective value (pH 2.0−10.0). Buffers with buffering capacity at the desired pH were not used because isolation of a possible pH effect was desired, rather than an effect due to different buffer types. Therefore, when the pH stability study was performed, final pH was not necessarily sample pH because the addition of enzyme conjugate in PBS to the pH-adjusted standard may have shifted the pH closer to 7.5. However, the shift of pH closer to 7.5 by adding enzyme conjugate more closely simulated an environmental sample analysis situation. Although two-tailed Student *t* tests comparing B_0 and B_1 showed the ELISA standard curve was not affected by sample pH values of 6.0 and 8.0, outside this pH range there was a significant ($p < 0.05$) difference between fluroxypyr and triclopyr standards when compared to a PBS standard curve at pH 7.5. When these samples are adjusted to pH 7.5, accurate concentrations were interpolated.

Cross-Reactivity. For the fluroxypyr ELISA, greater than 20% cross-reactivity was found to 4-amino-3,5-dichloro-6-fluoro-2-methoxypyridine with a LDD and IC_{50} within the linear working range of the ELISA; however, the other primary fluroxypyr metabolite, 4-amino-3,5-dichloro-6-fluoro-2-pyridinol, was not significantly cross-reactive (Table 4). Triclopyr was only 4% cross-reactive with IC_{50} and LDD values lying outside the linear working range of the ELISA curve. Cross-reactivity to other agrochemicals was below 1%. It would be advantageous to detect the two major metabolites of fluroxypyr (e.g. 4-amino-3,5-dichloro-6-fluoro-2-methoxypyridine and 4-amino-3,5-dichloro-6-fluoro-2-pyridinol) to follow their movement and degradation in the environment. Efforts to improve the cross-reactivity of the pyridinol metabolite (increased temperature and time) were unsuccessful. Although a two-dimensional structural representation of these metabolites would suggest that they are structurally similar to fluroxypyr, upon investigation using three-dimensional models it is apparent that the pyridinol metabolite is electronically and hydrophobically dissimilar to both the methoxypyridine metabolite and fluroxypyr. This is due to differences in the degree of electron donation and hydrophobicity of the hydroxyl group relative to the methoxy or oxyacetic acid groups at the 2-position of the pyridine ring.

The pyridinol metabolite forms and degrades readily in soil, with maximum concentrations of 5−20% of the amount of parent compound applied occurring after 2−4 weeks of incubation (Lehmann *et al.*, 1990, 1991). The 4-amino-3,5-dichloro-6-fluoro-2-methoxypyridine metabolite accumulates to 16% of the amount of parent compound 120 days after application to soil (Lehmann *et al.*, 1991). Furthermore, Lehmann *et al.* (1990) reported 4-amino-3,5-dichloro-6-fluoro-2-methoxypyridine was present in soil during the following growing season. However, neither metabolite was present in large enough quantity to have phytotoxic effects on susceptible species (Lehmann *et al.*, 1991). The methoxypyridine metabolite is the most persistent and

Table 4. IC_{50}, Percent Cross-Reactivity, and Least Detectable Dose for Selected Agrochemicals and Metabolites Using the Fluroxypyr Polyclonal Antisera

compound	IC_{50} (ng·mL^{-1})	cross-reactivity[a] (%)	least detectable dose[b] (ng·mL^{-1})
fluroxypyr	1.6	100	0.1
4-amino-3,5-dichloro-6-fluoro-2-methoxypyridine	7.7	21	0.5
triclopyr	37	4	15
2,4,5-trichlorophenoxyacetic acid	176	0.9	12
2,4-dichlorophenoxyacetic acid	492	0.3	25
MCPA	1488	0.1	129
2,4-dichlorophenoxybutanoic acid	2591	<0.1	206
clopyralid	>10000	<0.1	ND[c]
picloram	>10000	<0.1	ND
mecoprop	>10000	<0.1	ND
dichlorprop	>10000	<0.1	ND
chlorpyrifos	>10000	<0.1	ND
dicamba	>10000	<0.1	ND
tralkoxydim	>10000	<0.1	ND
metolachlor	>10000	<0.1	ND
atrazine	>10000	<0.1	ND
4-amino-3,5-dichloro-6-fluoro-2-pyridinol	>10000	<0.1	ND
2-methoxy-3,5,6-trichloropyridinol	>10000	<0.1	ND
3,5,6-trichloro-2-pyridinol	>10000	<0.1	ND
4-amino-5-chloro-6-fluoropyridin-2-ol	>10000	<0.1	ND
4-amino-3-chloro-6-fluoropyridin-2-ol	>10000	<0.1	ND

[a] Cross-reactivity is IC_{50} for fluroxypyr/IC_{50} for respective compound × 100. [b] Least detectable dose is the concentration of compound that inhibits 10% of the negative control. [c] Not detectable.

Table 5. IC_{50}, Percent Cross-Reactivity, and Least Detectable Dose for Selected Agrochemicals and Metabolites Using the Triclopyr Polyclonal Antisera

compound	IC_{50} (ng·mL^{-1})	cross-reactivity[a] (%)	least detectable dose[b] (ng·mL^{-1})
triclopyr	1.7	100	0.1
2,4,5-trichlorophenoxyacetic acid	2.0	85	0.04
4-amino-3,5-dichloro-6-fluoro-2-pyridinol	70	2	5.0
2,4-dichlorophenoxyacetic acid	192	0.1	0.8
fluroxypyr	223	0.1	28
4-amino-3,5-dichloro-6-fluoro-2-methoxypyridine	237	0.1	54
MCPA	555	<0.1	10
2,4-dichlorophenoxybutanoic acid	825	<0.1	25
3,5,6-trichloro-2-pyridinol	3144	<0.1	43
dichlorprop	6590	<0.1	67
2-methoxy-3,5,6-trichloropyridine	6650	<0.1	100
clopyralid	>10000	<0.1	ND[c]
picloram	>10000	<0.1	ND
mecoprop	>10000	<0.1	ND
chlorpyrifos	>10000	<0.1	ND
dicamba	>10000	<0.1	ND
MCPP	>10000	<0.1	ND
tralkoxydim	>10000	<0.1	ND
metolachlor	>10000	<0.1	ND
atrazine	>10000	<0.1	ND
4-amino-3-chloro-6-fluoropyridin-2-ol	>10000	<0.1	ND
4-amino-5-chloro-6-fluoropyridin-2-ol	>10000	<0.1	ND

[a] Cross-reactivity is IC_{50} for fluroxypyr/IC_{50} for respective compound × 100. [b] Least detectable dose is the concentration of compound that inhibits 10% of the negative control. [c] Not detectable.

significant fluroxypyr metabolite found in soil and is readily detected using the ELISA.

For the triclopyr ELISA, the cross-reactivities were 85 and 2% to 2,4,5-trichlorophenoxyacetic acid and 4-amino-3,5-dichloro-6-fluoro-2-pyridinol (Table 5), respectively. It would be advantageous to detect the major triclopyr metabolite, 3,5,6-trichloro-2-pyridinol; however, 3,5,6-trichloro-2-pyridinol exhibited little cross-reactivity. The use of fluroxypyr as an enzyme conjugate may have restricted optimal conditions for metabolite detection. No other cross-reactivity (>1%) was reported to any other compound tested.

Conclusions. The fluroxypyr ELISA accurately estimates fluroxypyr concentrations in the ranges of 0.1−10.0 ng·mL^{-1} in river water and 5−500 ng·mL^{-1} in soil. The triclopyr ELISA accurately estimates triclopyr concentrations in the ranges of 0.1−5.0 ng·mL^{-1} in river water and 1−50 ng·mL^{-1} in soil. The ELISAs were affected by organic solvents, sample pH, and soil coextractants; however, steps were taken to eliminate these interferences. Significant cross-reactivity (within the linear working range of the ELISA) with the fluroxypyr ELISA was found only to the metabolite 4-amino-3,5-dichloro-6-fluoro-2-methoxypyridine, while minor (greater than 0.1%) cross-reactivity was found to the auxinic herbicides triclopyr, 2,4,5-T, MCPA, 2,4-D, and 2,4-DB. Significant cross-reactivity with the triclopyr ELISA was found only to the auxinic herbicide 2,4,5-T, while some cross-reactivity was found to 4-amino-3,5-dichloro-6-fluoro-2-pyridinol, 2,4-D, fluroxypyr, 4-amino-3,5-dichloro-6-fluoro-2-methoxypyridine, MCPA, 2,4-DB, 3,5,6-trichloro-2-pyridinol, dichlorprop, and 2-methoxy-3,5,6-trichloropyridine.

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