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Development of a Magnetic Particle-Based Enzyme Immunoassay for the Determination of Penoxsulam in Water

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A competitive enzyme-linked immunoassay (ELISA) for the quantitation of Penoxsulam [2-(2,2-difluoroethoxy)-6-(trifluoromethyl-*N*-(5,8-dimethoxy[1,2,4]triazolo[1,5-*c*]pyrimidin-2-yl))benzenesulfonamide] in ground and surface waters was developed. This immunoassay utilizes magnetic particles as the solid phase to which polyclonal rabbit anti-Penoxsulam antibodies are attached. The ELISA has an estimated detection limit of 0.17 ppb (μg/mL) of Penoxsulam in water. Specificity studies indicate that the antibody can distinguish Penoxsulam from its major metabolites and structurally similar pesticides. Interference studies indicate that the ELISA has a wide tolerance of sample pH and salinity and for compounds commonly found in surface and ground waters. The ELISA was shown to compare favorably to LC-MS/MS on ground and surface water samples ($r^2 = 0.957$). The various studies performed demonstrate the usefulness of the ELISA technique as a rapid and high-throughput analytical method for the cost-effective monitoring of water samples.

KEYWORDS: ELISA; immunoassay; Penoxsulam; herbicide; water analysis; magnetic particles

INTRODUCTION

Penoxsulam [2-(2,2-difluoroethoxy)-6-(trifluoromethyl-*N*-(5,8-dimethoxy[1,2,4]triazolo[1,5-*c*]pyrimidin-2-yl))benzenesulfonamide] (**Figure 1**) is a herbicide used in the postemergent control of annual grasses, sedges, and broadleaf weeds in rice crops (*1*) and for aquatic weed control in various freshwater sites. Manufactured by Dow AgroSciences and licensed for aquatic use by SePRO Corporation, it is the active ingredient in granular and liquid formulations of both Grasp and Granite rice herbicides and the liquid herbicide formulation Galleon SC (*2*). It is a member of the triazolopyrimidine sulfonamide (sulfonanilide) class of herbicides, which affect both plant growth and reproduction through the inhibition of acetolactate synthase (ALS) (*3, 4*). ALS is an enzyme essential for the biosynthesis of branched-chain amino acids in plants, fungi, and bacteria, and is not found in animals; hence, Penoxsulam is expected to pose little threat to aquatic or terrestrial animal species including humans (*5*). For this reason, the U.S. Environmental Protection Agency has listed Penoxsulam as a reduced risk pesticide. Penoxsulam is highly mobile, but not very persistent in aqueous or terrestrial environments (*2, 5*). It is rapidly degraded through photolysis and microbial degradation to produce thirteen transformation products, several of which have a higher persistence in the environment than the parent compound (*1, 2*). The DT_{50} for Penoxsulam varies from 3 to 7 days depending on soil type and pH (*1, 6*).

Instrumental methods based on various GC-MS or LC-MS technologies are currently utilized to measure Penoxsulam and other triazolopyrimidine herbicides in environmental and food samples (*7–9*). However, these methods require sophisticated operating environments and are not conducive to the high-throughput analysis needed for environmental or food safety monitoring analysis. Immunochemical methods, due to their simplicity, ruggedness, and parallel sample analysis capability,

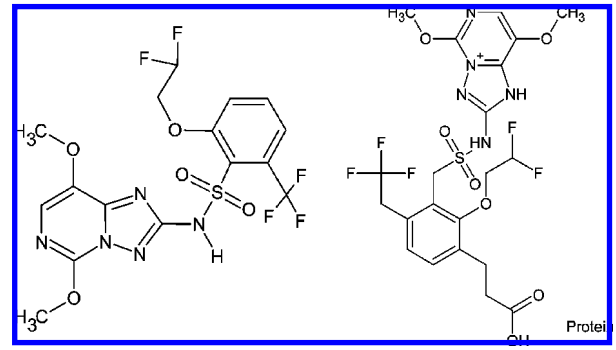

Figure 1. Molecular structures of Penoxsulam and Penoxsulam ligand used for immunoreagent development.

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are eminently suitable as high-throughput methods and retain the good sensitivity needed for this these applications. Enzyme immunoassays utilizing magnetic particle technology have been shown to be particularly well suited for the analysis of many environmental contaminants, producing assays that are field portable, user-friendly, and cost-effective (*10–12*). The Penoxsulam magnetic particle-based enzyme immunoassay described in this paper utilizes a polyclonal antibody specific for Penoxsulam with an enzyme-labeled Penoxsulam analogue. The assay takes 70 min to perform and requires no sample preparation for the analysis of water samples. To our knowledge this is the first reported ELISA for Penoxsulam.

MATERIALS AND METHODS

Materials. Penoxsulam reference standard and Penoxsulam metabolites were obtained from Dow AgroSciences LLC (Indianapolis, IN). Bovine serum albumin (BSA) was from Serologicals (Kankakee, IL). Horseradish peroxidase (HRP), humic acid, copper(II) chloride, calcium sulfate, magnesium sulfate, sodium chloride, magnesium chloride, sodium nitrate, sodium fluoride, potassium phosphate, calcium chloride, sodium thiosulfate, iron(II) sulfate, manganese sulfate, zinc sulfate, and 1-ethyl-3[3-dimethylaminopropyl]carbodiimide hydrochloride (EDAC) were obtained from Sigma-Aldrich (St. Louis, MO). Methanol, acetone, acetonitrile, and *N*,*N*-dimethylformamide (DMF) were obtained from EMD Chemicals Inc. (San Diego, CA). All reagents used were of >95% purity. All other chemicals were of reagent grade or chemically pure.

Buffers and Solutions. Tris-buffered saline (TBS) solution contains 150 mM Tris, 150 mM NaCl, and 0.1% BSA, adjusted to pH 7.4. Wash solution is preserved deionized water. Supermagnetic particles (1 μm) were obtained from Seradyn (Indianapolis, IN). The suspended antibody-coupled paramagnetic particles and the HRP conjugate were diluted in preserved Tris-buffered saline solution. The substrate solution was obtained from BioFX Laboratories (Owings Mills, MD). Enzymatic reactions were stopped with 0.2% sulfuric acid. Synthetic seawater (pH 7.82) contained 2.4% NaCl, 0.4% $NaSO_4$, 0.2% $CaCl_2 \cdot 6H_2O$, 0.07% KCl, 0.01% KBr, 0.03% H_3BO_3, and 0.0003% NaF.

Antibody Generation. The Penoxsulam ligand (**Figure 1**) was coupled to BSA as follows: 20.5 mg of the ligand was dissolved in 289 μL of DMF, and 8.5 mg of *N*-hydroxysuccinimide (NHS) and 12.6 mg of *N*,*N*′-dicyclohexylcarbodiimide in DMF were added, and the solution was mixed overnight in an ice−water bath. After removal of the precipitated urea, the supernatant was added dropwise to 26 mg of BSA (in 5 mL of 100 mM borax buffer, pH 9.4) and mixed overnight immersed in an ice−water bath. This was followed by extensive dialysis against 0.01 mM PBS, pH 7.4.

The Penoxsulam−BSA immunogen was diluted in sterile saline at a 4 mg/mL concentration and mixed with an equal amount of Freund's adjuvant and emulsified. Four female New Zealand white rabbits (no. 134−137) were used for the development of polyclonal antibodies against Penoxsulam. At day 1, the appropriate emulsion of the immunogen (0.5 mL) was injected into the hip muscle of each rabbit. On day 20, the back of the head of each animal was shaved and injected in six to eight sites with a total of 0.5 mL of the emulsion. Test bleeds were taken on day 30. Immunization was repeated, as on day 20, on day 45 to yield another test bleed drawn on day 55. After the test bleeds were taken, immunization was repeated at 30 day intervals using Freund's incomplete adjuvant. The rabbits were bled 7−10 days after each immunization, producing 15−25 mL of sera, which was stored at <−30 °C. Working aliquots are stored at 4 °C. Serum pulled after the fifth boost (July 18, 2006) from rabbit 134 was utilized for immunoassay development.

Enzyme Conjugate Synthesis and Magnetic Particle Activation. The conjugation procedure for the Penoxsulam ligand to horseradish peroxidase is similar to the procedure described above for the immunogen preparation. Goat antirabbit antibodies were coupled to magnetic particles using NHS/EDAC activation in the presence of 50 mM of 2-*N*-(morpholino)ethane sulfonic acid buffer. The rabbit anti-Penoxsulam was bound to the antirabbit particles by mixing the primary antibodies at a dilution of 1:8000 with the secondary magnetic particle coupled antirabbit antibodies and allowing it to incubate for at least 30 min at room temperature.

Instrumentation. The pH of all buffers and solutions were measured with a Corning pH-meter (pH 140) (Corning, NY). Samples were filtered using Whatman UNIPREP filters (Florham Park, NJ). Absorbances were read using a Photometric Analyzer (Abraxis, Warminster, PA) at a wavelength of 450 nm.

Immunoassay Procedure. Amber, high density polyethylene (HDPE) bottles were used to collect the water samples. Standards and samples (250 μL), HRP−Penoxsulam conjugate (250 μL), and suspended anti-Penoxsulam magnetic particles (500 μL) were added into a 12 × 75 mm polystyrene test tubes, mixed by vortexing, and incubated at room temperature for 45 min. A magnetic field was then applied for 2 min to magnetically separate the reaction mixture, holding the magnetic particles and bound reagents in the tubes. The unbound reagents were then removed by decantation. The particles were then washed twice with 1.0 mL of wash solution, followed by the addition of TMB substrate solution (500 μL). After 20 min of incubation at room temperature, the reaction was stopped with 0.2% sulfuric acid solution (500 μL) and the absorbance read at 450 nm within 15 min. Sample concentration was determined utilizing a regression line constructed from a log−logit B/B_0 standard curve (where B/B_0 is the absorbance at 450 nm observed for a calibrator or sample divided by the absorbance at the zero calibrator) versus log concentration of calibrators containing 0, 0.25, 1.0, and 10.0 μg/L of Penoxsulam. Samples exhibiting Penoxulam concentrations >10 μg/L were diluted and reanalyzed. Final sample concentrations were calculated using the appropriate dilution factor.

Water samples for method comparison were surface and groundwater from various locations throughout the United States and were analyzed as received. Samples for the spike recovery study were prepared by adding 2.0 μg/L of Penoxsulam to ground and surface water samples from various states throughout the United States. The spike recovery of each sample was determined by analyzing the samples before and after the addition of Penoxsulam and then subtracting the value of Penoxsulam present in the unspiked sample.

RESULTS AND DISCUSSION

Assay Kinetics. Assay kinetics studies were performed to determine the shortest incubation time possible to achieve a minimum B_0 absorbance between 1.4 and 1.8 optical density units (OD) and to minimize assay drift. Studies were conducted following the general assay protocol; however, the first incubation was substituted with 20, 30, 45, and 60 min incubations. Data were analyzed by plotting incubation time versus absorbance (**Figure 2**). The data obtained indicates that the desired absorbance minimizing assay drift (see Drift) was obtained at a minimum incubation time of 45 min.

Drift. The incubation times used in the assay represent a nonequilibrium condition. In addition, there could be a significant lapse in time between the addition of reagents to the first samples and to the last samples. To determine the effect that these conditions have upon sample values, a drift study was performed. Two separate assays were run following the general assay protocol. Fifty-one samples containing no Penoxsulam and 51 samples containing 3.5 μg/L of Penoxsulam were assayed at a maximum assay preparation/pipetting time interval of 150 s. Analysis of the data was performed by plotting tube number versus absorbance for the 0.0 μg/L sample assay, and by plotting tube number versus concentration for the 3.5 μg/L spiked sample assay (**Figure 3**). Linear regression was utilized to determine change over time. The slope of the regression line was 0.0022 μg/L/s with a mean value of 3.386, which was calculated to be a 3.36% change across the assay preparation/pipetting time. The linear regression line of the zero standard had a slope of −0.0019

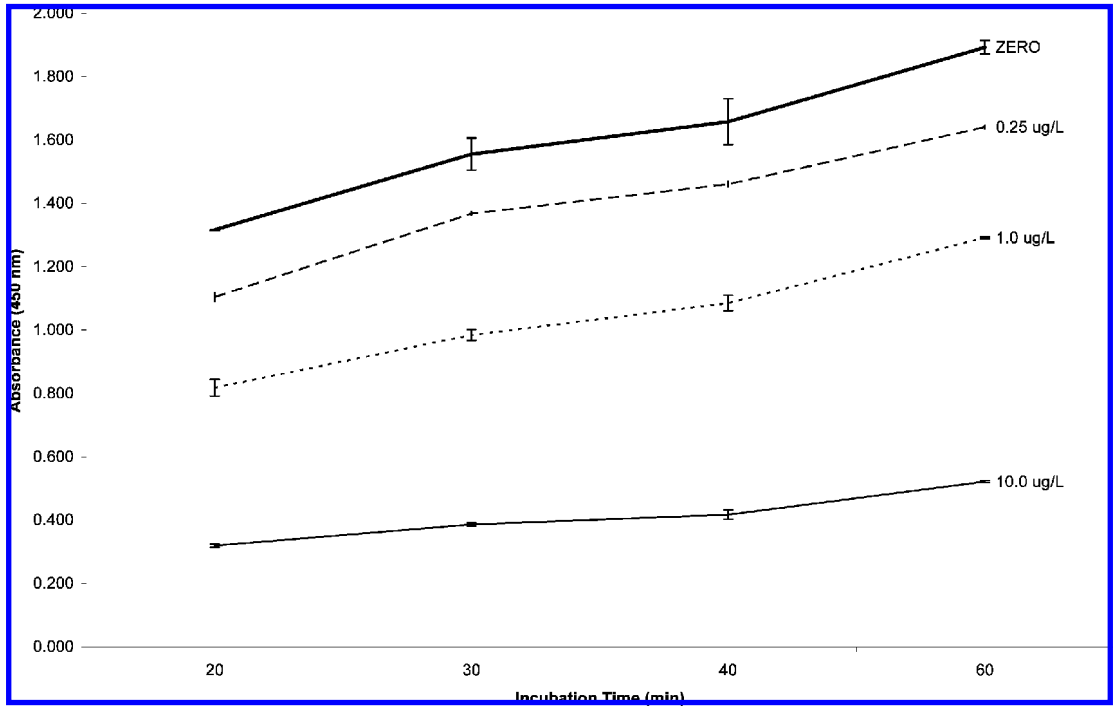

Figure 2. Assay kinetics study. Calibrators contained Penoxsulam at 0 μg/L (STD 0), 0.25 μg/L (STD 1), 0.75 μg/L (control), 1.0 μg/L (STD 2), 10.0 μg/L (STD 3).

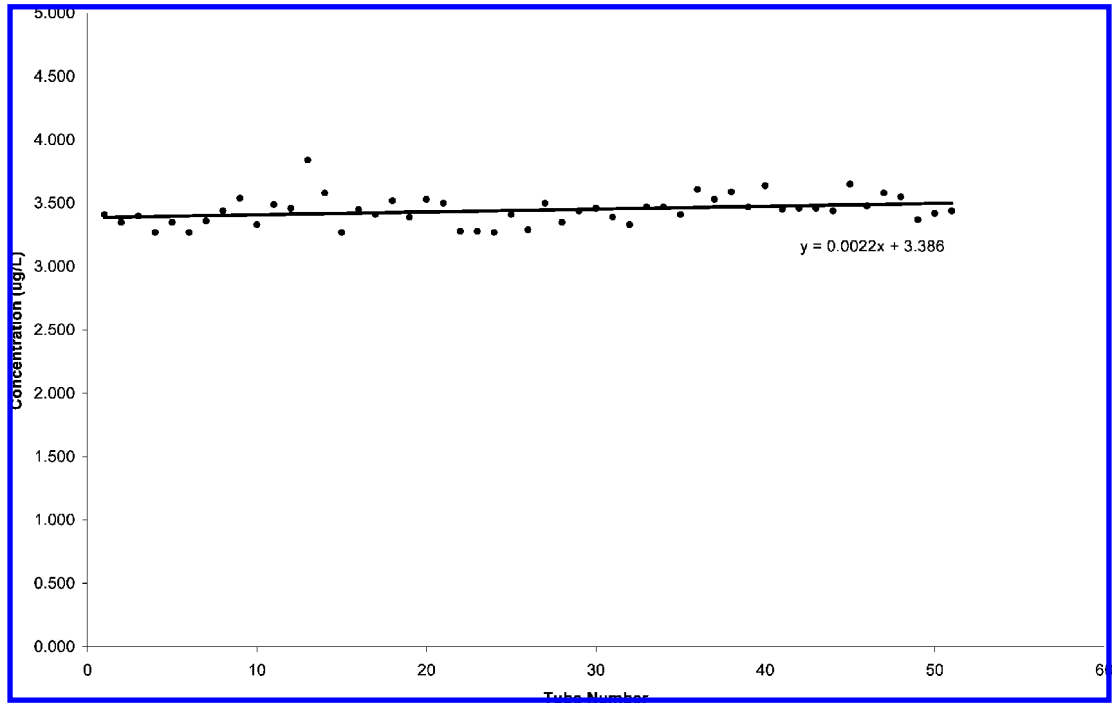

Figure 3. Drift study on a 3.5 μg/L Penoxsulam-spiked sample.

OD unit (data not shown). No zero sample was calculated to be a false positive (LOD > 0.167 μg/L), with the highest calculated concentration at 0.05 μg/L. The difference in recovery from samples at the beginning and the end of the assay was minimal; therefore, assay drift was considered to be negligible using a 45 min assay incubation period.

Dose−Response Curve and Sensitivity. The immunoassay described in this work uses a competitive assay format. Since the enzyme labeled Penoxsulam tracer competes with the analyte in the samples for the available antibody binding sites, the color developed is inversely proportional to the concentration of Penoxsulam. It is common to report displacement in terms of B/B_0 measurement to describe color inhibition. B/B_0 is defined as the absorbance observed for a sample or standard divided by the absorbance at zero analyte concentration. To construct a calibration curve for the ELISA, the B/B_0 values for the Penoxsulam calibrators were linearly transformed using a log/logit curve (**Figure 4**). The assay sensitivity, established as the lowest concentration (LOD) that can be distinguished from zero, was calculated by either the 90% B/B_0 (*13*) or the mean of 20 replicate blanks (B_0) plus 4 standard deviations. The mean LOD determined by

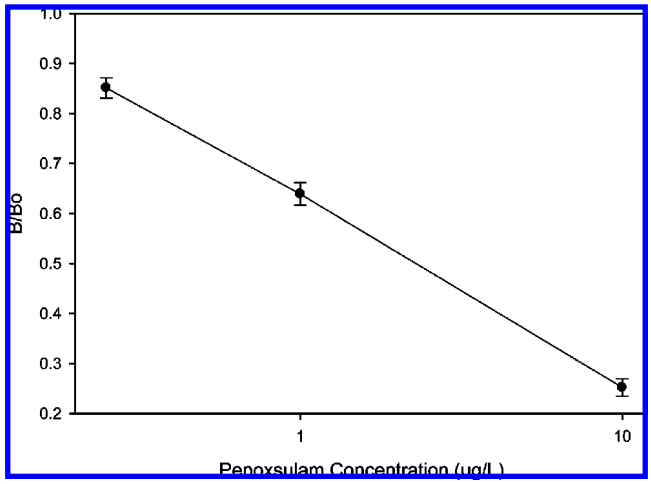

Figure 4. Dose−response curve for Penoxsulam. Each point represents the mean of 77 determinations. Vertical bars indicate ±2 SD about the mean.

Table 1. Precision Study: Four Penoxsulam-Fortified Samples Analyzed over 5 Days, Five Assays per Day

control	1	2	3	4
replicates	5	5	5	5
days	5	5	5	5
n	25	25	25	25
mean (µg/L) %CV	0.86	2.78	5.60	7.60
within assay %CV	5.2	3.9	3.6	3.9
between assay %CV	8.6	5.7	6.3	5.7

the 4 SD from the B_0 method from three assay runs was 0.163 µg/L. The calculated LOD using the 90% B/B_0 method was 0.167 µg/L (data not shown). Results indicated that either method was acceptable in determining assay sensitivity.

Precision Studies. Precision studies using spiked samples were performed to measure the percent coefficient of variation (%CV) within and between assays. Following the general assay protocol, four surface and ground water samples spiked with 0.75, 2.5, 5, and 7.5 µg/L of Penoxsulam, numbered 1, 2, 3, and 4, respectively, were analyzed over a 5 day period with five assays per day, in singlicate. Data were analyzed using an ANOVA statistical model (*14*). The mean concentration of the 25 assays for control 1, 2, 3, and 4 were 0.86, 2.78, 5.60, and 7.60 µg/L, respectively, with percent coefficients of variation below 10% for all concentrations tested (**Table 1**).

Accuracy in Water. The accuracy of the immunoassay was assessed by evaluating four surface water samples each fortified with Penoxsulam at 0.5, 1.0, 2.0, 4.0, and 8.0 µg/L. Water samples were obtained from Neshaminy Creek (Doylestown, PA), Peace Valley Lake (New Britain, PA), Delaware River (New Hope, PA), and municipal tap water (Doylestown, PA). Each sample was analyzed three times in duplicate following the assay protocol. The average of each spiked sample over three assays was used to calculate the average percent recovered. The average value for the 0.5, 1.0, 2.0, 4.0, and 8.0 µg/L spiked water samples were 102, 108, 112, 109, and 100%, respectively. The average assay recovery was 106% (**Table 2**). The accurate recoveries of the spiked water samples suggest that there are no sample matrix problems or interferences present in the samples tested and that the immunoassay is accurate across the assay range.

Specificity. The IC_{50}, defined as the inhibitory concentration estimated at 50% B/B_0, was determined by estimating the concentration of Penoxsulam necessary to displace 50% of the

Table 2. Accuracy Study: Water Samples from Four Different Locations Were Fortified with Penoxsulam at Five Different Concentrations and Analyzed by the ELISA Method over Three Assays

spiked concn (µg/L)	determined value (µg/L)	recovery (%)
0.0	<LOD	NA
0.5	0.51	101.8
1.0	1.08	107.8
2.0	2.24	111.8
4.0	4.37	109.2
8.0	8.02	100.3
		av = 106.2

Penoxsulam−HRP conjugate. The least detectable dose (LDD) was calculated as the concentration of Penoxsulam required to achieve 90% B/B_0. The cross-reactivity of eight Penoxsulam metabolites was determined by testing at 1, 10, 100, 1000, and 10000 µg/L concentrations. Metabolite dilutions were prepared in deionized water. Penoxsulam metabolites tested were TSN 102471, TSN 102354, TSN 105514, TSN 101837, TSN 105480, TSN 103594, TSN 104889, and TSN 101824 (**Figure 5**). Data analysis was performed by calculating the IC_{50} and the LDD for Penoxsulam and the eight metabolites (**Table 3**). The LDD for Penoxsulam was 0.17 µg/L with an IC_{50} of 2.2 µg/L. The antiserum used in this work is very selective for Penoxsulam as illustrated by the low cross-reactivity of Penoxsulam metabolites.

Determination of Interferences. Compounds commonly found in water samples were tested to determine if their presence negatively affects assay performance. All compounds were diluted in deionized water. Humic acid was analyzed at 0.1, 1, 10, 100, and 1000 mg/L concentrations, whereas $CuCl_2$, $CaSO_4$, $MgSO_4$, NaCl, $MgCl_2$, $NaNO_3$, NaF, PO_4, $CaCl_2$, $FeSO_4$, $MnSO_4$, $ZnSO_4$, and sodium thiosulfate were analyzed at 1, 10, 100, 1000, and 10000 mg/L. Interference, at a specified concentration was defined as a false-positive result if their calculated concentrations were equal to or greater than the concentration of standard 1 (0.25 µg/L). Data were analyzed by observing the maximum tolerated concentration of each of the compounds. Humic acid was found to interfere at a concentration of 10 mg/L; sodium fluoride at a concentration of 1000 mg/L; copper(II) chloride, calcium sulfate, magnesium sulfate, magnesium chloride, sodium nitrate, phosphate, calcium chloride, sodium thiosulfate, iron(II) sulfate, manganese sulfate, and zinc sulfate at a concentration of 10000 mg/L or greater; and sodium chloride at a concentration of 100000 mg/L.

Solvent Tolerance. Four solvents, methanol, acetone, acetonitrile, and DMF, were tested to determine their effects on assay performance. Solvents were diluted in preserved deionized water. DMF and acetonitrile were diluted at 0.1, 0.25, 0.5, 1, and 2.5% (v/v). Acetone and methanol were diluted at 0.5, 1, 2.5, 5, and 10% (v/v). This study included the evaluation of both neat and spiked (2 µg/L Penoxsulam) solvent samples. Interferences were defined as a false positive result equal to or greater than the concentration of standard 1 (0.25 µg/L) and/or the calculated percent recovered of the sample was found to be <80% or >120% of theoretical values. Data were analyzed by observing the maximum tolerated concentrations of the tested solvents. The maximum tolerated concentration of methanol, acetone, acetonitrile, and DMF were 5.0, 5.0, 1.0, and 1.0%, respectively. Other solvents and/or higher solvent concentrations may be used with additional modification of the assay reagents and/or protocol.

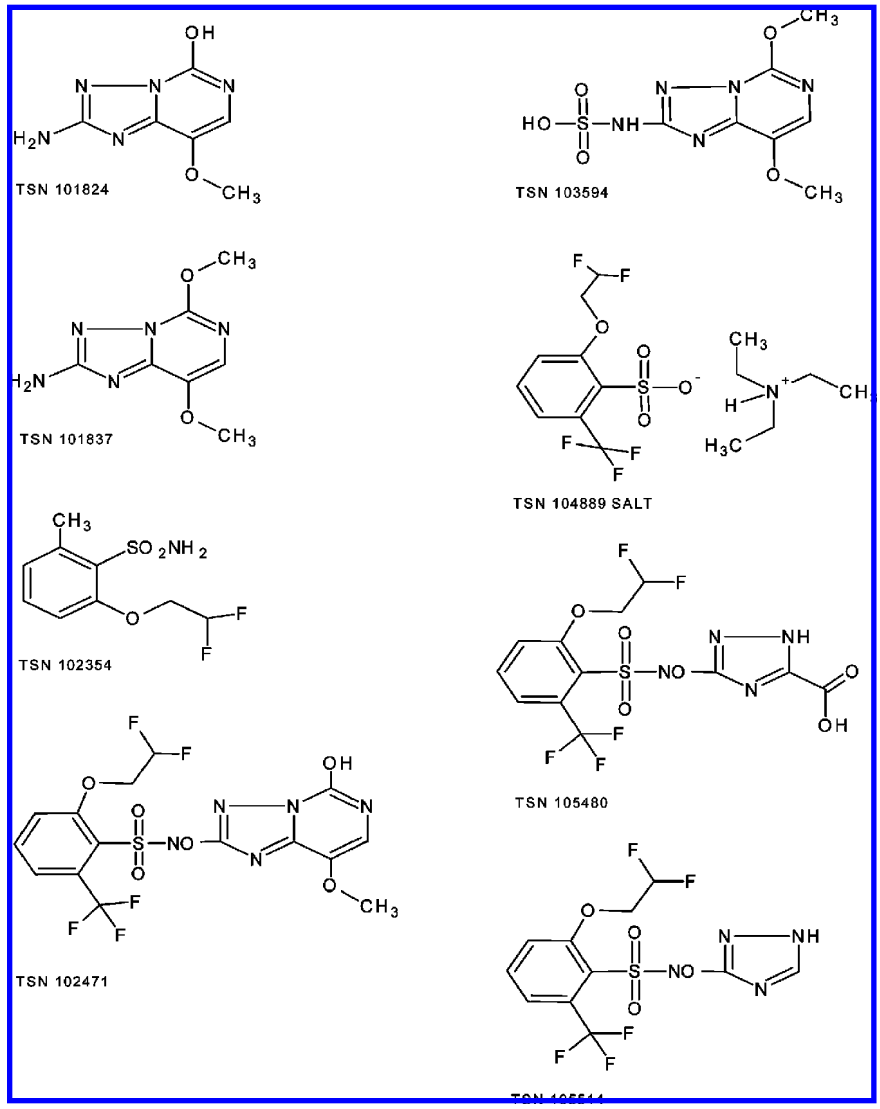

Figure 5. Molecular structures of Penoxsulam metabolites.

Table 3. Assay Sensitivity and Selectivity toward Penoxsulam and Metabolites

B/B_0 compd	LDD (μg/L)	IC_{50} (μg/L)
Penoxsulam	0.17	2.2
TSN 102471	10	470
TSN 102354	2500	>10000
TSN 105514	3700	>10000
TSN 101837	4200	>10000
TSN 105480	7700	>10000
TSN 103594	8500	>10000
TSN 104889	>10000	>10000
TSN 101824	>10000	>10000

Table 4. pH Tolerance of the Penoxsulam ELISA

sample pH	0 μg/L	4 μg/L	recovery (%)
2	1.38	12.36	309
3	nd	4.38	110
4	nd	4.48	112
5	nd	4.40	110
6	nd	4.39	110
7	nd	4.39	110
8	nd	4.20	105
9	nd	4.47	112
10	nd	4.35	109
11	nd	4.32	108
12	nd	4.06	102

pH Tolerance. The pH of the zero standard and a 4 μg/L Penoxsulam control was adjusted across a pH range of 2−12 to determine the effect of sample pH on the assay. Interfering pH was defined as a level that caused the calculated sample concentration of the zero to be equal to or greater than the concentration of standard 1 (0.25 μg/L) and/or the calculated percent recovery of the control was found to be <80% or >120% of the theoretical value. The percent recovery at pH 2 was 309%. The percent recovery at all other pH levels was approximately 110% (**Table 4**). Therefore, environmental water samples with a wide range of pH can be assayed with this ELISA without neutralization.

Salinity Interference Study. Studies were conducted to determine the effects of salinity on the assay. Samples were prepared by making 1:1 serial dilutions starting with 100% synthetic seawater down to a concentration of 1.56%. Spiked samples contained 6.7 μg/L Penoxsulam. Data were analyzed by comparing the spiked salt water samples with the spiked sample 5 μg/L Penoxsulam (**Table 5**). At seawater concentrations below 50%, the recoveries of the neat samples were below the LOD (<0.17 μg/L) and the spiked samples were within 20% of the theoretical concentration, indicating that the maximum tolerated seawater concentration in the assay is 50%.

Table 5. Salinity Tolerance of the Penoxsulam ELISA

condition	blank (μg/L)	determined (ug/L)	recovery (%)
100% synthetic sea water	0.24	8.08	120.6
50% synthetic sea water	<LOD	7.08	105.67
25% synthetic sea water	<LOD	6.79	101.34
12.5% synthetic sea water	<LOD	6.92	103.28
6.25% synthetic sea water	<LOD	6.18	92.24
3.125% synthetic sea water	<LOD	7.34	109.55
1.5625% synthetic sea water	<LOD	6.6	98.51
control (0% synthetic sea water)	<LOD	6.7	

Temperature Tolerance. The ability of the assay to perform under a range of temperatures was evaluated by running the assay at 4, 22, and 37 °C following the described assay procedure. Penoxsulam was spiked into preserved deionized water at 0.5, 1, 2.5, and 7.5 μg/L concentrations. Each assay was run at their respective temperature with the exception of pipetting and washing steps. Data were analyzed by comparing B_0 and B/B_0. At 4 °C, the calculated concentrations of the 0.5 and 1.0 ppb spiked solutions were significantly higher than their theoretical concentrations, with calculated values above 120%. At room temperature (22 °C) and 37 °C, concentrations were within the acceptable range (80−120%), indicating that the assay can accurately recover within the range of 22−37 °C, whereas excessively cold temperatures result in higher recoveries.

Filtration Study. A filtration study was conducted to evaluate the feasibility of filtering samples with suspended solids contaminated with Penoxsulam. Samples (same samples an in accuracy studies) were spiked with Penoxsulam at 0.5, 1, 2.5, and 7.5 μg/L concentrations. To determine whether there was a significant loss of analyte during the filtering step, an initial analysis was performed prior to filtering, followed by a second analysis after filtration. Data were analyzed by comparing recoveries before and after filtration. The average percent recovery of the four spiked samples was 96%, suggesting that water samples can be filtered, if necessary without affecting Penoxsulam recovery.

Sample Spiked Recovery. Twenty-three surface and groundwater samples obtained from various locations across the United States were analyzed, spiked and unspiked to evaluate matrix effects. Spiked samples were fortified with 2 μg/L Penoxsulam. The mean concentration recovered in spiked samples was 2.20 μg/L (110%), with a standard deviation of 0.10; all neat samples showed concentrations below the LOD of 0.17 μg/L, indicating that the assay accurately detects Penoxsulam in natural water samples.

Method Comparison. A comparison of 34 groundwater samples analyzed using the ELISA method and Dow AgroSciences (Indianapolis, IN) analytical method GRM 01.30 liquid chromatography with positive-ion electrospray tandem mass spectrometry (LC-MS/MS) (*1*) showed good correlation. Data were analyzed by plotting the concentration determined by the LC-MS/MS (*x*-axis) versus the concentration determined by the ELISA method (*y*-axis) (**Figure 6**). Correlation was determined using linear regression. The slope of the linear regression line was 1.033 with a *y*-intercept of 1.676 μg/L. The slope of the linear regression line is approximately equal to the theoretical desired slope of 1, which would indicate a 1:1 relationship between the results of the two quantitative methods. The r^2 value of 0.957 also suggests that there was very good correlation between the two sets of data.

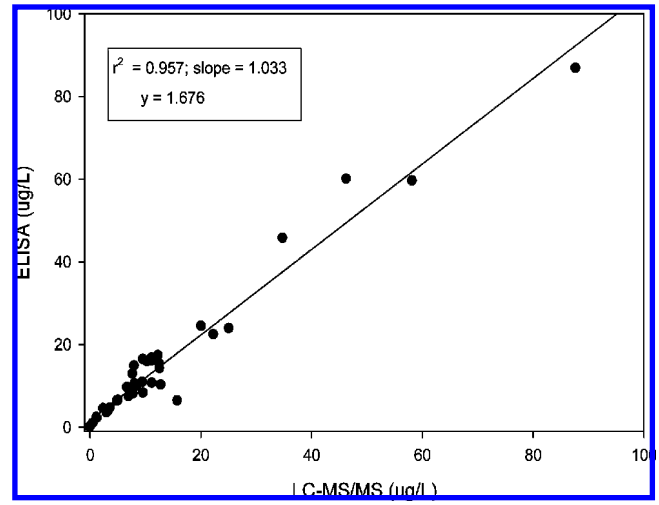

Figure 6. Correlation between LC-MS/MS and ELISA method.

In conclusion, this work describes a magnetic particle-based ELISA for pesticide residues and its performance characteristics in the quantitation of Penoxsulam in surface and ground water samples. The assay compares favorably to LC-MS/MS and exhibits good precision and accuracy, which guarantees consistent monitoring of environmental samples. The highly specific antibody employed allows for the detection of Penoxsulam in the presence of other pesticides. No adverse effect in the assay due to sample pH were seen from pH 3 to 12; therefore, samples with a wide range of pH can be analyzed with the ELISA without pH adjustment. The ELISA is also free from interferences from commonly found surface and ground water components and exhibits a wide tolerance of salinity.

The ELISA for Penoxsulam provides up to 45 results (in duplicate) in approximately 3 h without any sample concentration or cleanup steps; the same number of samples can be analyzed by LC-MS/MS in 8 h. The assay is ideally suited for adaptation to on-site monitoring of low levels of Penoxsulam in water samples and could be applied to residue detection in soils and foods given the solvent tolerance of the assay system.

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