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Evaluation of an ELISA Kit for the Detection of Metribuzin in Stream Water

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A recently developed magnetic-based ELISA test kit for the herbicide metribuzin [4-amino-6-(1,1-dimethylethyl)-3-(methylthio)-1,2,4-triazin-5(4*H*)-one] was evaluated for its reproducibility, accuracy, and comparability to results from a gas chromatography/mass spectrometry (GC/MS) technique for the determination of metribuzin in stream water. Replicated metribuzin determination in stream water showed good daily reproducibility (CV < 10%) except at low concentrations ($\leq 0.68\ \mu g\ L^{-1}$). Although the assay correlated well ($r = 0.954$) with GC/MS results, the correlation was improved ($r = 0.990$) when concentrations $\leq 0.68\ \mu g\ L^{-1}$ were omitted. Predicted accuracy by the metribuzin assay was tested in two matrices [stream and deionized (DI) water]. Deionized water matrix exhibited excellent agreement between the theoretical and predicted metribuzin levels, indicating a high degree of accuracy. Predicted metribuzin concentrations were slightly elevated compared to theoretical concentrations in stream samples; however, no significant difference ($p < 0.05$) was found between the two sample matrices. This ELISA was found to tolerate an excessive amount of several chemical substances routinely found in environmental samples as well as a wide range of pH levels. Overall, the assay illustrated the ability to efficiently and accurately predict concentrations of metribuzin in stream water and demonstrated the utility of the ELISA technique as a screening tool for the determination of metribuzin in stream samples.

Introduction

Concern over maintaining high water quality in the United States and the European Community has created the need for efficient and reliable methods for the detection of pesticides in groundwater and surface water supplies. Current analytical methods for the determination of pesticides in water usually require an extraction procedure and measurement by gas chromatography (GC), GC/mass spectrometry (GC/MS), or high-pressure liquid chromatography. While these methods can be reliable, they have several potential drawbacks including the need for expensive instrumentation, large sample volume, extensive purification, experienced technicians, and a lengthy analysis. Due to these drawbacks, the analysis of a large number of samples may be both cost and time prohibitive. Over the last decade, enzyme-linked immunosorbent assays (ELISA) have been developed for the detection of pesticides in both soil extracts and water. Hammock and Mumma (*1*) presented an extensive report on ELISA theory and mechanisms for pesticide determination. The ELISA procedure presents an alternative to classical detection methods, particularly as a screening tool. It has the advantage of being relatively inexpensive and rapid.

Recently, a magnetic-based ELISA procedure for the determination of the asymmetrical triazine, metribuzin, was made commercially available. The ability to quickly and accurately screen for this pesticide is important since approximately 2×10^5 kg a.i. yr^{-1} of metribuzin was used for the control of grasses and broadleaf weeds in agronomic crops in the United States in 1990 (*2*). Additionally, it has been detected in both groundwater (*3*, *4*) and surface water (*5*).

Since the metribuzin ELISA kit has been made available recently, no reports documenting its performance exists in the literature. As part of an ongoing pesticide monitoring study, the metribuzin ELISA kit was evaluated for its reproducibility, accuracy, and comparability to results determined by a solid-phase extraction (SPE) and GC/MS method.

Procedures

Sampling. The samples used in this study were collected as part of an ongoing USDA monitoring project assessing surface water quality of an agricultural watershed on the North Carolina Coastal Plain (*6*). Nine field samples, collected from various points in the watershed, were used in the evaluation of this ELISA kit. After collection, samples were packed in ice and transported to the laboratory. Subsamples were shipped on ice to the USDA National Soil Tilth Laboratory for SPE and GC/MS analysis. The remaining portions of the samples were kept frozen (−5 °C) until the immunoassay analysis was conducted. The samples selected for ELISA analysis were chosen on the basis of previous GC/MS confirmation of metribuzin.

ELISA Analysis. Metribuzin RaPID Assay kits (Ohmicron, Inc., Newtown, PA.) with a stated detection range of 0.04–3.00 $\mu g\ L^{-1}$ were used for ELISA analysis. Sample with a GC/MS concentration exceeding the linear range of the kit (3.0 $\mu g\ L^{-1}$) was diluted prior to analysis. All analyses were conducted according to instructions provided with the kits. Additionally, all necessary reagents were provided with the kit. Plastic 5-mL test tubes were placed into the upper half of a magnetic separation rack, which consisted of a test tube rack that held 60 test tubes and a magnetic base that extended 1 cm on either side of the test tube. A 250-μL aliquot of sample, standard, or control was pipetted into each test tube along with 250 μL of metribuzin enzyme conjugate and a 500-μL aliquot of metribuzin antibody coupled paramagnetic particles. Test tubes were vortexed for 1–2 s and incubated at room temperature (20–22 °C) for 15 min. The incubation period allowed for the competitive binding of either metribuzin or the metribuzin enzyme conjugate with the metribuzin antibody. At the end of the incubation period, the test tube rack was connected to its magnetic base allowing the paramagnetic particles and the enjoined sample and/or enzyme conjugate to adhere to the side of the test tube. Any unbound reagents were decanted, and the magnetically held particles were double rinsed with 1.0 mL of washing solution. The test tube rack and the magnetic base were separated, and 500 μL of coloring solution (hydrogen peroxide and 3,3′,5,5′-tetramethylbenzidine) was added to each tube. The tubes were vortexed for 1–2 s and incubated for an additional 20 min. The presence of the metribuzin enzyme conjugate

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catalyzed the conversion of the coloring solution to a colored product. Color intensity is inversely related to the amount of metribuzin present. At the end of the incubation period, the coloring procedure was stopped with 0.5% (v/v) sulfuric acid solution.

Each sample's absorbance was read spectrophotometrically at 450 nm with an Ohmicron RPA-1 RaPID analyzer. A four-point calibration curve was automatically calculated, and the analytes concentrations were calculated on the basis of a linear regression of ln/logit transformed data.

Reproducibility and Accuracy. Reproducibility of the metribuzin ELISA kit was measured by analyzing four subsamples of each of the nine stream sample on four different days. The relative variation between days was determined by a general linear model (GLM) using SAS (SAS Institute, Cary, NC). Accuracy of the metribuzin ELISA kit was tested using both stream and DI water as test matrices. Four replicates of each spike sample were analyzed. No residual metribuzin was detected in either stream or DI water. Additional analysis of the stream sample using GC (*7*) found no detectable amount of 10 other commonly used pesticides. The stream water sample used to test the kit's accuracy was not included in any of the other tested categories. Both matrices were spiked with metribuzin (0.00, 0.50, 1.00, 1.50, and 2.00 $\mu g\ L^{-1}$).

Extraction and GC/MS Analysis. Stream samples (100–150 mL) were filtered through glass-fiber filter paper (pore size = 1.5 μm) (Fisher G6, Pittsburgh, PA). A 100-g sample was placed in a beaker and spiked with propazine [6-chloro-*N*,*N*′-bis(1-methylethyl)-1,3,5-triazine-2,4-diamine] (1 $\mu g\ L^{-1}$) as a surrogate to monitor method performance.

Samples were extracted with an automated Zymate II robotic extraction system (Zymark, Hopkinton, MA) as outlined by Pfeiffer (*8*). The extraction procedure was determined to be 97.3% efficient at recovering metribuzin in samples spiked at the 2.0 $\mu g\ L^{-1}$ level. The extracts were subsequently analyzed by GC/MS using selective ion mode (SIM).

The GC/MS analysis of the eluates were performed on a Hewlett-Packard (HP) 5890 Series II gas chromatograph (Palo Alto, CA) equipped with an HP 5970B mass selective detector (MSD) reported in SIM mode. The conditions were as follows: ionization voltage, 70 eV; ion source temperature at 250 °C; electron multiplier, 400–600 V above auto tune (area counts of 10×10^6 for internal standard); direct capillary interface at 280 °C; and 50 ms dwell time per ion. An HP-1 (Hewlett-Packard, Wilmington, DE) fused silica column (0.33 μm film thickness × 12 m × 0.2 mm i.d.) was used for compound separation with helium as a carrier gas at approximately 1 mL min^{-1} with a head pressure of 35 kPa. Samples were autoinjected in a splitless mode. Column temperature was held at 50 °C for 1 min, programmed to 250 °C at 6 °C min^{-1}, and held for 10 min. Injector temperature was 280 °C.

Base peak (198) and two other confirming ions (214 and 199) were chosen for the confirmation of metribuzin. Base peak ion current was measured for the quantification curve versus the response of the 214 ion of terbuthylazine (6-chloro-*N*-(1,1-dimethylethyl)-*N*′-ethyl-1,3,5-triazine-2,4-diamine). Confirmation was based on the presence of the molecular ion, two confirming ions (with area counts ± 20%), and a retention time match of ±0.2% relative to terbuthylazine. The detection limit of metribuzin was established at 0.2 $\mu g\ L^{-1}$ in unextracted samples.

Evaluation of Interfering Substances and pH Levels. In order to evaluate the possibility of naturally occurring substances interfering with the ELISA kit, 250 mg L^{-1} of various chemicals (calcium, copper, iron, magnesium, zinc, nitrate, and sulfate) were added individually to DI water samples that had been fortified with 2.00 $\mu g\ L^{-1}$ of metribuzin. The effect of 0.5 M sodium chloride on metribuzin determination was also evaluated. When necessary, the pH of each sample was adjusted to a range of 5–7 with sodium hydroxide. If interference was noted, subsequent dilutions (125 and 50 mg L^{-1}) were made and evaluated.

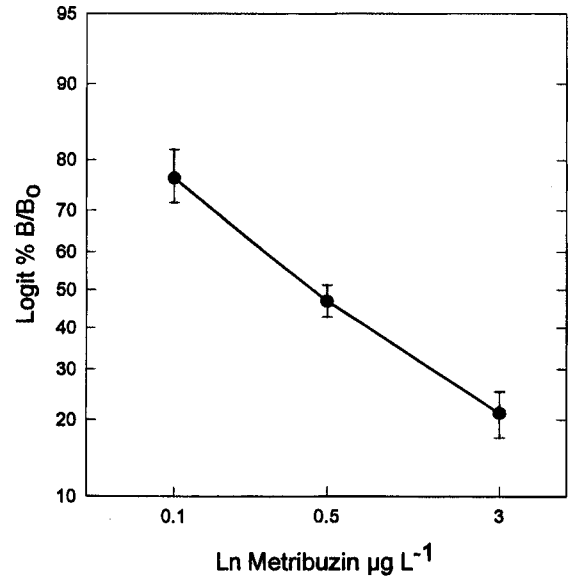

FIGURE 1. Mean dose–response curve (n = 16) for metribuzin calibrators. Error bars represent ±1 standard deviation about the mean.

The effects of pH levels on this ELISA were evaluated by adjusting the pH of DI water samples from 2 to 12 using either hydrochloric acid or sodium hydroxide. All samples were fortified with metribuzin at a concentration of 2.00 $\mu g\ L^{-1}$.

Determination of Dissolved Organic Carbon. Prior to carbon determination, stream samples were filtered through a Gelman membrane filter (0.45 μm) (Gelman Science, Ann Arbor, MI), and the pH was adjusted to <3.0. Dissolved organic carbon (DOC) content of each sample was determined using a Dohrman DC 190 high-temperature total organic carbon (TOC) analyzer (Emerson-Rosemount Analytical Inc., Santa Clara, CA) equipped with a Binos (Binos, Hanau, Germandy) non-dispersive infrared detector. Duplicate 400-μL injections of each sample were analyzed in the TOC mode. Minimum detection limit for the analyzer is 0.2 mg L^{-1} of C.

Results and Discussion

Dose–Response Curve. Dose–response data for metribuzin calibrators were collected from 16 calibrations performed during the course of this project. Figure 1 illustrates the mean standard curve, linearly transformed using a ln/logit curve fit. The error bars (±1 SD) at each standard point represent the small variation present between each run.

Quality Control. A four-point standard curve (0.0, 0.1, 0.5, and 3.0 $\mu g\ L^{-1}$) was calculated for each set of samples analyzed with an ELISA kit. The coefficient of correlation (r) for the standard curves ranged from 0.9949 to 0.9999. To ensure accuracy of the standard curve, a provided control sample (2.0 ± 0.4 $\mu g\ L^{-1}$) was analyzed with each set of samples. The mean value (n = 16) of the quality control samples was 2.03 ± 0.06 $\mu g\ L^{-1}$.

Reproducibility. Mean, standard deviation, and coefficient of variation (% CV) for each sample are shown in Table 1. Reproducibility of repeated metribuzin measurement was good since the majority of the samples had % CV values of less than 10%. Samples with mean metribuzin concentrations of ≤0.68 $\mu g\ L^{-1}$ had the highest % CV (>10%). The GLM showed that two samples (nos. 1 and 2) had significant variation (p > 0.1) at the 90% confidence level. The source

TABLE 1. Reproducibility of Repeated ELISA Measurements of Metribuzin in Stream Samples[a]

sample	mean[b] (μg L^{-1})	SD[c]	% CV (%)
1	0.37[d]	0.09	16.21
2	0.59[d]	0.09	10.95
3	0.68	0.12	14.33
4	1.35	0.14	8.85
5	1.57	0.11	6.95
6	2.10	0.16	6.37
7	2.20	0.14	5.97
8	2.68	0.18	6.88
9	3.98	0.27	6.51

[a] Significant difference between repeated ELISA measurements per sample was compared using a general linear model (GLM). [b] Mean of 16 replicated measurements. [c] SD, standard deviation. [d] Indicates significant difference at the 0.01 level of rejection.

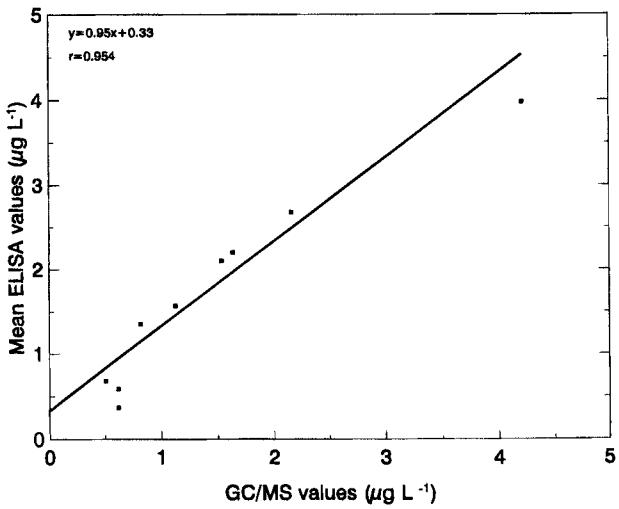

FIGURE 2. Regression comparison of stream water metribuzin concentrations (■) as determined by ELISA and GC/MS.

of this variation was probably related either to the kit's performance at lower concentrations or to non-homogeneous samples.

Method Comparison. Prior to the comparison tests, GC/MS values were corrected for recovery. A conducted *t*-test (95% confidence level) found no statistically significant difference between the two sets of data. The correlation of GC/MS values and mean values obtained from the ELISA tests for each sample, in general, was good ($r = 0.954$, $n = 9$) (Figure 2). The correlation improved when the three samples with GC/MS concentration of ≤ 0.68 μg L^{-1} were eliminated from the comparison ($r = 0.990$, $n = 6$).

Accuracy. The accuracy of the spiked samples are summarized in Table 2. The DI water matrix showed excellent agreement between the expected and predicted concentrations. Determination of metribuzin in the stream water matrix was elevated at all levels, particularly at the 0.50 μg L^{-1} level; however, a two tail *t*-test (Sigmastat, Jandel Scientific; San Rafael, CA) failed to show significant difference (95% confidence level) between the two matrices. The observed differences between the two different sample matrices could be related to the environmental nature of the field collected samples; however, the source of an environmental interference is unclear. Concern has been expressed about the presence of DOC causing false positive or overestimated responses with ELISA test kits. A Pearson Product Moment Correlation analysis comparing the concentration of metribuzin and DOC (1.50–8.58 mg L^{-1} of C) present in the stream samples used in the method comparison study found no significant relationship between the two compounds. Overestimation of predicted pesticide concentrations has been reported with several different types of ELISAs, with various matrices (*9–11*) and therefore may be a problem inherent to the ELISA technology and not to this particular assay.

TABLE 2. Agreement between Theoretical and Predicted Concentration of Metribuzin in DI and Stream Water

spiked level (μg L^{-1})	mean[a] (μg L^{-1})	SD[b]	agreement (%)
	DI Water		
0.50	0.48	0.04	96
1.00	0.98	0.08	98
1.50	1.48	0.06	99
2.00	2.01	0.02	100
			mean 98
	Stream Water		
0.50	0.65	0.13	130
1.00	1.13	0.06	113
1.50	1.58	0.13	106
2.00	2.25	0.16	113
			mean 115

[a] Mean of four replicated measurements. [b] SD, standard deviation.

TABLE 3. Effect of Possible Interfering Substances on Determination of 2.00 μg L^{-1} Metribuzin Solution

chemical species	concn (mg L^{-1})	metribuzin determination (μg L^{-1})
calcium	250	2.00
copper	250	2.08
iron	50	1.97
magnesium	250	2.08
zinc	250	2.00
sulfate	250	1.94
nitrate	250	2.04
sodium chloride	0.5 M	2.06

TABLE 4. Effects of High Concentrations (500 μg L^{-1}) of Selected Pesticides on Metribuzin Determination

	metribuzin determination (μg L^{-1})	
pesticide	0 μg L^{-1}	0.2 μg L^{-1}
alachlor	ND[a]	0.18
atrazine	ND	0.17
metolachlor	ND	0.17

[a] ND, nondetected, (<0.04 μg L^{-1}).

Interferences. The effects of possible interfering substances are summarized in Table 3. The only substance found to interfere with this ELISA at the 250 mg L^{-1} level was iron. Overestimation of the metribuzin concentration was noted with the presence of 250 and 125 mg L^{-1} of iron; however, no effect was noted with 50 mg L^{-1} iron. Additionally, the presence of 0.5 M sodium chloride did not have an effect on the kit's performance (Table 3).

The determination of metribuzin was not effected by pH values ranging from 4 to 12 (data not shown). At pH levels lower than 4, metribuzin determinations were off-scale. This indicates that the metribuzin kit can be used to analyze samples from a wide range of pH values without experiencing interference due to the pH level of the samples.

Cross-Reactivity. Meulenberg et al. (*12*) reported that cross-reactivity between antibodies and compounds that are structurally similar to the target compound is an inherent problem with immunoassay technology. This cross-reaction can affect test results by either indicating the target compound is present when it is not (false positive) or by elevating the predicted concentration of the target compound when both the target and another structurally similar compound are present. Cross-reactivity has been reported for several

different magnetic particle-based ELISA kits (*13*, *14*). According to the manufacturer's information, the metribuzin ELISA kit has limited cross-reactivity with other pesticides (*15*). The compound listed with the highest cross-reactivity is the deaminated metribuzin metabolite [6-(1,1-dimethylethyl)-3-(methylthio)-1,2,4-triazin-5(4*H*)-one]. According to the provided information (*15*), this ELISA is 87.5 times less sensitive to the deaminated metabolite than it is to the parent compound. In this study, eight of the nine samples used contained one or more commonly used pesticides other than metribuzin. These additional pesticides detected included alachlor (2-chloro-*N*-(2,6-diethylphenyl)-*N*-(methoxymethyl)acetamide) (1.7–14.2 $\mu g\ L^{-1}$), atrazine (6-chloro-*N*-ethyl-*N'*-(1-methylethyl)-1,3,5-triazine-2,4-diamine) (0.3–19.6 $\mu g\ L^{-1}$), and metolachlor (2-chloro-*N*-(2-ethyl-6-methylphenyl)-*N*-(2-methoxy-1-methylethyl)acetamide) (0.4–54.8 $\mu g\ L^{-1}$). To evaluate if these three pesticides had an effect on the metribuzin ELISA kit, 500 $mg\ L^{-1}$ of each pesticides was added to blank and spiked (0.20 $\mu g\ L^{-1}$) metribuzin water samples. Table 4 indicates that the presence of these additional pesticides would not have reacted with the metribuzin antibodies to cause an overestimation of metribuzin concentrations.

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