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Atrazine Soil Extraction Techniques for Enzyme Immunoassay Microtiter Plate Analysis

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Atrazine (2-chloro-4-ethylamino-6-isopropylamino-s-triazine), a commonly used preemergent pesticide to control weeds in corn and sorghum has been detected in groundwater more often and at higher concentrations than any other pesticide (EPA, 1990, Ritter, 1990). Because of potential residual build-up and migration from the soil mantel into groundwater there is substantial interest in determining atrazine residues in soil using a rapid, inexpensive method. Commonly accepted methods and techniques (GC, HPLC and GC/MS) used for the determination and quantification of atrazine in soil are time consuming, expensive and require extensive clean-up. Enzyme immunoassay (EIA) determination of atrazine is a quick, inexpensive technique, but other triazine-based herbicides have been shown to cross-react (combine with the antibody to give a positive reading) and soil matrix effects have not been fully evaluated. Presently, EIA analysis is only being used as a screening method which requires confirmation by another analytical technique, such as GC/MS (Thurman et al. 1990).

Enzyme immunoassay (EIA) techniques using microtiter plates (Res-I-Quant™, Immunosystems, Inc, ME) have recently been developed for triazine screening. The low cost, high sensitivity and no cleanup or concentration requirements make EIA an attractive alternative to GC or HPLC analysis. Antibody-coated tubes (Res-I-Mune™, Immunosystems, Inc.) have been used to analyze atrazine in soil (Goh et al. 1991, Goh et al. 1990 and Bushway et al. 1988). Enzyme immunoassay detection of atrazine-fortified (2-80 ppb) soil samples using tubes had coefficients of variation ranging from 4-20% for 11 different soils (Bushway et al., 1988). When EIA was compared with HPLC analysis on extracted atrazine-treated soils, results were favorable except where simazine (a cross-reacting triazine) had also been applied. In another study using antibody-coated tubes, co-extracted soil compounds were thought to interfere with the EIA (Goh et al., 1990). In a follow-up study by Goh et al. (1991), EIA measured soil atrazine concentrations were slightly less than concentrations determined by GC at soil atrazine levels below 52 ppb and higher than GC determinations when the atrazine levels were above 52 ppb. Different extracting solvents were used for the GC and EIA analyses. Only a few analyses can be conducted simultaneously with the tube technique, whereas the microtiter plate method is designed to conduct 48 analyses in duplicate simultaneously. The purpose of this study was to evaluate the microtiter plate-based EIA method for quantification of soil-extracted atrazine using several extracting techniques and solvents.

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MATERIALS AND METHODS

Two experiments were conducted. Soils in Table 1 were used for the first experiment. The Baxter, Maury, and Iberia soils were sampled in 1965, and had no known history of triazine application. The Waynesboro soil was sampled in 1990, and had no prior record of triazine application.

Prior to extraction atrazine fortifying solutions were prepared at concentrations of 1000 and 100 ng/mL using reagent grade water and 99% pure reagent grade atrazine. Two 50 g samples of air-dried soil were fortified with 25 mL of each spiking solution resulting in soil samples containing 500 and 50 ppb of atrazine. Twenty-five mL of reagent grade water was added to a third air-dried soil sample to serve as a control (i.e. no atrazine added). The 500 ppb atrazine concentration represents a high level in the soil, which may injure crops, and the 50 ppb atrazine concentration is considered a threshold value at which there is less concern. The fortified soil slurry was thoroughly mixed and the following day air-dried on an evaporative dish. Soils were subsampled and extracted in triplicate 48 hr after spiking.

In the first experiment, two extraction techniques using two different solvents were compared. Extraction techniques, vortex mixing at room temperature and automated soxhlet extraction (Method 3541, 1990, EPA SW-846) were evaluated using acetonitrile:water (9:1) or 0.5 N ammonium acetate (NH_4OAC) solution buffered to pH 7. Mattson et al. (1970) reported that water was a necessary component of the extracting solvent. For the two extraction techniques, 4 g and 5 g of air-dried soil was used for the 500 ppb and 50 ppb fortification, respectively. Controls were similarly extracted. Automated soxhlet (soxtec) extraction procedure included weighing 4 or 5 g of soil into a 23 x 80 mm prerinsed cellulose thimble or sox and boiling with 50 mL of solvent for 60 minutes, followed by rinsing for 10 minutes. This was conducted with a Tecator Soxtec HT 4 extraction apparatus (Fisher Scientific, Pittsburg, PA). The solvent extract was concentrated to 10 mL for the 50 ppb fortified samples or diluted to 50 mL with reagent grade water for the 500 ppb fortified samples.

The vortex procedure consisted of weighing soil into a 40 mL glass vial and adding 20 mL of extracting solvent. The soil solution was thoroughly mixed by vortexing three times for 2 minutes, allowed to sit overnight and the following morning vortexed four times for 10 seconds. The vials were centrifuged and the supernatant was pipetted into a glass vial. Appropriate dilutions (26, 67.7, and 201:1) of soil extract were prepared for EIA analysis in reagent grade water. Eighty μL of this solution was added to microtiter wells to determine recovery of atrazine. Atrazine standard solutions were prepared over a range of 0.1 to 0.8 ng/mL. The final atrazine soil concentration was calculated by multiplying by the solution:soil ratio (4 or 5:1) and the dilution factor (26, 67.7 or 201:1).

In the second experiment, Lexington silt loam soil (Table 2) was fortified with atrazine as described above. The Lexington soil samples were collected from cotton plots of different tillage and cover crop combinations from the West Tennessee Experiment Station in Jackson, Tennessee. The Lexington soil was treated in 1986 with prometryn, a cross-reacting triazine-based herbicide and sampled in June of 1986. These soils had been stored at room temperature for four years prior to this experimentation. Lexington soil was extracted by vortex mixing as described above. In addition to the solvents

acetonitrile:water (9:1) and 0.5 N NH_4OAC at pH 7, methanol:water (4:1) was also used.

In both experiments statistical analysis was conducted to test for differences in recoveries with extraction techniques, solvents, atrazine fortification levels, and soils using ANOVA and Duncan's Multiple Range Test.

Table 1. Characteristics of Soils used for the First Experiment

Soil name and texture	---Composition %---			
	O.M.*	Clay	pH	C.E.C.+
Baxter Silt Loam	1.3	12.5	4.8	5.0
Iberia Silty Clay	3.0	49.3	6.3	40.8
Maury Silt Loam	2.6	24.0	6.1	17.5
Waynesboro Silt Loam	2.8	21.0	6.2	15.6

*O.M. = soil organic matter
+C.E.C. = cation exchange capacity cmol/kg by ammonium acetate @ pH 7.

Table 2. Characteristics of Lexington Silt Loam Soil used for the Second Experiment

Lexington soil cropped in cotton with various tillage and cover	---Composition %---			
	O.M.*	Clay	pH	C.E.C.+
No Till, Clover	4.7	16.7	5.4	11.0
No Till, Rye	2.7	23.7	5.2	11.1
Till, No Cover	2.0	19.4	6.2	10.2
No Till, Vetch	3.3	23.1	4.8	10.2

*O.M. = soil organic matter
+C.E.C. = cation exchange capacity cmol/kg by ammonium acetate @ pH 7.

Standards were prepared and analyzed in the respective, diluted extracting solvents (0.5-3.8%). Solvent effects on the EIA analysis procedure were determined by examining atrazine concentrations (standards) in multiple-combinations of the solvent ratios (Table 3). In preliminary work, it was demonstrated that interference with EIA occurred with the solvents acetonitrile, methanol and 0.5 N NH_4OAC above 5%. Goh et al. (1990) also demonstrated interferences when acetonitrile was more than 5% of the solution added to the microtiter wells. The standard additions method was used to determine the cross-reactivity of prometryn in the Lexington control samples, based on atrazine standards. Atrazine (0.1, 0.2, and 0.3 ppb) was added to the diluted control acetonitrile:water (9:1) soil extract and the concentration in the control determined (Kolthoff et al., 1969).

The triazine EIA kit consisted of 96 antibody-coated wells in a microtiter plate, solutions of atrazine-enzyme conjugate, substrate, chromogen, 2.5 N H_2SO_4 and 0.1, 0.25, 1.0, and 2.0 ppb atrazine standards (Res-I-Quant™, ImmunoSystems, Inc., ME). Additional standards were also prepared as previously described. Principles of the EIA are based on competitive binding of atrazine and atrazine-enzyme conjugate to a limited number of antibody binding sites. An 80 μL aliquot of the diluted soil extract is mixed in the well with 80 μL of atrazine-enzyme conjugate on an orbital shaker at 200 rpm for 60 minutes. After incubation the plate wells were washed with water to remove unreacted reagent. Eighty μL each of substrate and chromogen was added to each well. The plate was covered and shaken on the orbital shaker for 30 minutes at 200 rpm. In the presence of bound enzyme-conjugate the substrate is converted to a compound which turns the chromogen blue. A sample extract or standard containing low atrazine concentration allows many enzyme conjugate molecules to be bound by the antibodies producing a darker blue solution. A sample having a high concentration of atrazine competes with the enzyme conjugate and fewer enzyme conjugate molecules are bound to the antibodies, and a lighter blue solution develops. The reaction is stopped by adding 40 μL of 2.5 N H_2SO_4 which changes the color from blue to yellow. The wells (color) in the plate were read within 15 minutes after the reaction was stopped on a microtiter plate reader at 450 nm. Standards were prepared in the extracting solvent and incubated and read on the same plate. Absorbance of samples was recorded and divided by the absorbance of the blank control solution ($\%B_o$) (Bushway et al., 1988). All atrazine standards and soil sample extracts were analyzed in duplicate on the microtiter plate. The microtiter plate reader (Flow Laboratories, Inc., McClean, VA) was programmed to read the plate in 25 seconds. The described EIA microtiter plate method of analyzing soil extracted atrazine required 2 hrs 30 min, including pipetting, incubation, development of color, and reading. Forty analyses and eight standards were conducted in duplicate.

RESULTS AND DISCUSSION

Linear regression atrazine data using diluted acetonitrile, methanol or 0.5 N NH_4OAC are given in Table 3. Standards were run in a narrow range at the lower end of the linear scale (0.1-0.8 ng/mL atrazine), which corresponded to the steepest portion of the curve.

Table 3. Linear Regression for Atrazine Standards in Dilute Solutions of Acetonitrile, Methanol and 0.5 N NH_4OAC

	Acetonitrile			Methanol		0.5N NH_4OAC	
	0.5%	1.3%	3.5%	0.4%	3.1%	0.5%	3.8%
Slope	0.58	0.57	0.57	0.57	0.51	0.49	0.71
Intercept	0.24	0.23	0.19	0.28	0.25	0.36	0.24
r^2	0.93	0.91	0.97	0.91	0.85	0.92	0.98

where y=difference in optical density between controls and standards and x=ppb atrazine

Acetonitrile and methanol had no effect on the EIA atrazine analysis for solvent dilution up to 3.5 and 3.1%, respectively. The 0.5 N NH_4OAC solvent showed a slight difference in slope. Atrazine detection limits for this procedure were 0.1 ng/mL in water. The soil

detection limits were computed by multiplying by the 4:1 solution to soil ratio and the 26 extract dilution to give a detection limit of 10.4 ppb atrazine. This could be further improved by decreasing the extraction solution:soil ratio from 4:1 to 3 or 2:1 or concentrating the soil extract before dilution.

There was a difference between acetonitrile and 0.5 N NH_4OAC extracting solvents in recovery of soil atrazine in the first study (Table 4). Since methanol extractions were not used in this study, no comparisons could be made. A significant difference in extraction efficiency was observed, with the vortex procedural recoveries greater than the soxtec, except with the 50 ppb acetonitrile extraction where no significant difference occurred at the 95% level. Mean atrazine recoveries for the acetonitrile:water (9:1) vortex, acetonitrile:water (9:1) soxtec, 0.5 N NH_4OAC vortex and 0.5 N NH_4OAC soxtec methods were 104, 90, 54 and 44%, respectively. The 0.5 N NH_4OAC extraction was effective on some soils (it efficiently extracted atrazine in a preliminary study using two soils), although there was no pattern with the four soils used. Atrazine degradation appeared to occur when the Baxter soil was heated with 0.5 N NH_4OAC (soxtec method). It was concluded from this study that vortex mixing at room temperature using acetonitrile:water (9:1) extracting solvent and allowing the soil solution to sit overnight was the most efficient soil atrazine extraction method. Therefore, vortex mixing was the extracting method of choice in the second experiment.

Table 4. Atrazine Soil Extraction Recovery using Two Methods and Two Solvents

		% Recovery ± Standard Deviation			
Solvent and Method	Atrazine Rate (ppb)	Baxter Soil	Iberia Soil	Maury Soil	Waynesboro Soil
Acetonitrile:H_2O(9:1) Vortex	500	100±3	110±3	95±3	110±3
	50	122±6	101±4	101±8	96±18
Acetonitrile:H_2O(9:1) Soxtec	500	76±7	81±11	81±16	90±28
	50	97±6	96±16	93±10	107±9
0.5 N NH_4OAC Vortex	500	40±11	31±14	89±10	63±17
	50	49±3	46±5	61±3	51±23
0.5 N NH_4OAC Soxtec	500	11±1	73±22	51±1	59±9
	50	36±12	35±5	46±10	48±19

Results from the second experiment using Lexington soil demonstrated that more atrazine (93% average recovery) was extracted with acetonitrile:water (9:1) than with methanol (82.5% average recovery), or 0.5 N NH_4OAC (65% average recovery) (Table 5). The significant difference (99% level) in solvent recovery of atrazine agrees with some previous studies (Mattson et al., 1970), while other studies have demonstrated no difference between methanol and acetonitrile extracting efficiencies (Huang and Pignatello, 1990).

Standard deviations for soil extractions and EIA analysis as reported in Tables 4 and 5 ranged from 1-28%. In both tables EIA analysis of atrazine recovery was used to compare soils.

Differences in Lexington soil organic matter and pH (Table 2) did not result in different extraction efficiencies except with tilled no cover treatment, spiked to a level of 500 ppb atrazine using methanol extraction. This soil had the lowest amount of soil organic matter and the largest methanol extraction recovery (97%). For the 50 ppb fortified soils there was no difference among soils using methanol extraction.

Table 5. Comparison of Three Atrazine Extraction Solvents Using Lexington Soil

	Extracting Solvent and Atrazine Fortifications					
	% Recoveries ± Standard Deviation					
Lexington Soil	Acetonitrile		Methanol		0.5 N NH_4OAC	
Tillage and Cover	50 ppb	500 ppb	50 ppb	500 ppb	50 ppb	500 ppb
No Till, Clover	91±4b+	90±3a	77±1a	74±3b	59±12a	66±9a
No Till, Rye	91±4b	91±3a	85±6a	81±1b	65±3a	68±16a
Till, No Cover	95±6ab	95±3a	80±1a	97±3a	67±2a	68±5a
No Till, Vetch	100±5a	92±6a	84±7a	78±6b	67±9a	59±3a
MEAN	94	92	82	83	65	65

LSD 0.05 = (7) (8) (11) (12) (17) (15); respectively
+Numbers with the same letter are not significantly different at the 0.05 level

Excellent mean recoveries (99%) were obtained using acetonitrile:water (9:1) and vortex mixing on all eight soils fortified at two atrazine levels (Tables 4 and 5). Standard deviations for these soil extractions were relatively low except for the Waynesboro soil at the 50 ppb atrazine level 96% ± 18. Mean standard deviation for the eight soils grouped together were 12% and 8% for the 50 and 500 ppb fortification, respectively, for the acetonitrile:water (9:1), vortex mixing method.

A positive atrazine concentration (ppb) determined in the Lexington control soil samples as a result of the prometryn residue was subtracted from the 50 ppb fortified soil results. Since these were surface soils sampled at 0-3.8 cm, and organic matter-coated soil particles dominated surfaces (Greenland, 1965), it may be that hydrophobic organic reactions were important in sorption of atrazine. Acetonitrile was more effective in extracting this type of bound atrazine than methanol or 0.5 N NH_4OAC (Huang and Pignatello, 1990).

Atrazine recoveries for the Lexington control soils using the three different solvents and the standard addition method for determining residual triazine-based prometryn in soil are given in Table 6. The standard addition method (see materials and methods) used to

compute triazine level in soil was very close to the acetonitrile-extracted triazine concentration. The methanol and NH_4OAC solvents extracted much lower but similar amounts of atrazine from the Lexington control soils. This would be similar to extracting a field-weathered soil, since prometryn was applied prior to sampling the soils in 1986. Prometryn, a triazine-based compound, cross-reacts with the atrazine-antibody at a 5 ppb prometryn to 4 ppb atrazine ratio (Thurman et al., 1990). Other triazine compounds also cross-react with the atrazine EIA antibody. Therefore it is imperative to work with known fortified samples or known soil pesticide application history in order to use EIA as a quantitative technique. If the above criteria are not met, EIA may be used as a screening technique, eliminating negatively responding samples from further instrumental analysis (Thurman, et al., 1990).

Table 6. Extraction of Lexington Silt Loam Soil Control

	From Standard Add. Computation	--------Solvent--------		
		Acetonitrile	Methanol	0.5 N NH_4OAC
Soil Tillage and Cover Crops	----------Triazines ppb----------			
No Till, Clover	28	26	13	11
No Till, Rye	54	52	17	17
Till No Cover	49	46	24	21
No Till, Vetch	97	83	32	34

Results indicate that vortex mixing using acetonitrile:water (9:1) and standing overnight achieved highest procedural extraction efficiencies. Acetonitrile and methanol did not effect the EIA at concentrations of less than 3.5%, while the 0.5 N NH_4OAC solvent had a small effect at the 3.8% dilution. All solvents recorded positive readings when used at 5% strength or greater. Therefore, the microtiter-based assays must be conducted in diluted extracting solvents, which may limit detection for soil-extracted compounds.

The EIA technique is a sensitive, quick technique requiring no clean up or concentration step at these concentrations. However, soil matrix effects have not been fully examined over a broad range of soils and other triazine-based compounds cross-react at various ratios. The EIA microtiter plate analysis is rapid and reproducible and may be used for quantitative analyses when it is known what compounds have been added to soil. Evaluation of field-weathered atrazine-fortified soils is necessary to develop wide application of soil atrazine extraction using EIA techniques. Results from this study indicate EIA microtiter plate analysis of pesticides has promise as an analytical tool. The major advantages are no cleanup and 80 duplicate samples can be analyzed after soil extraction in an 8 hr day.

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