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Atrazine Soil Residue Analysis by Enzyme Immunoassay: Solvent Effect and Extraction Efficiency

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Atrazine, a triazine herbicide, is used in California to control weeds in utility right-of-ways, corn, landscape areas, sorghum, industrial areas and conifers. In 1987, according to the Pesticide Use Report (1989), 133,607 lb of atrazine (active ingredient) were applied in California. It was concluded that atrazine had contaminated ground waters when it was found in drinking-water wells sampled in California. To mitigate further contamination of groundwaters, studies are being conducted to characterize the transport of atrazine and other triazines through soil under different irrigation methods and regimes, so that the best herbicide management practice can be recommended. These studies generate hundreds of soil samples that require costly residue analyses. Enzyme immunoassay (EIA) as reported by Bushway et al. (1988) looks promising as a more rapid and cheaper alternative to HPLC for analysis of atrazine in soil (Ferris and Haigh 1987). The detection limit in earlier EIA of atrazine residues using acetonitrile and water as extracting solvents was comparable to HPLC analysis (Bushway et al. 1988). We report i) the effects of methanol and acetonitrile on the EIA system, and ii) the selection of the best solvent level to optimize extraction efficiency and quantification of atrazine in fortified soil.

MATERIALS AND METHODS

The triazine EIA kits consist of antibody-coated test-tubes, atrazine-enzyme conjugate, substrate, and chromogen solutions (Res-I-Mune™, ImmunoSystems, Inc., ME) (Bushway et al. 1988). Test principles are based on competitive binding of atrazine and atrazine-enzyme conjugate to a limited number of antibody binding sites. A sample containing atrazine is incubated in the tube with a fixed amount of the atrazine-enzyme conjugate. After incubation the unreacted molecules are washed away. The substrate and chromogen are then added to the tube. In the presence of bound enzyme

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conjugate the substrate is converted to a compound which turns the chromogen blue. A sample containing low concentration of atrazine allows many enzyme conjugate molecules to be bound by the antibodies giving rise to a darker blue solution. Conversely, a high concentration of atrazine will allow fewer enzyme conjugate molecules to be bound to the antibodies, resulting in a lighter blue solution.

In the first experiment, atrazine solutions at 0.5, 2.0, and 5.0 ng/mL were prepared in 0 (water only), 2, 3, and 4% acetonitrile-water (v/v) and in 0, 5, 7, and 10% methanol-water (v/v). Assays were run in sets of eight tubes: four samples (four solvent levels at a single level of atrazine) and four control tubes (four solvent levels with no atrazine). Differential optical density (ΔOD) was read between each sample tube and a control tube of the same percent solvent using a portable differential photometer set at 450 nm (Artel, ME). Each set was replicated four times. The (ΔOD) were analyzed using the SAS General Linear Models Program with a confidence level of $p<0.05$ (Statistical Analysis System, SAS Institute, Cary, NC) to determine if there were i) significant interactions between percent solvent and atrazine levels, and ii) significant differences between solvent levels.

In the second experiment, acetonitrile, methanol, and water were used to extract soil fortified with atrazine. The Hanford sandy loam, known to be free of triazines, was obtained from the California State University, Fresno Farm Laboratory (Field 15). Prior to fortification, the soil was sieved through a 2-mm screen. For the acetonitrile extraction, five 25.0-g aliquots were measured into 500-mL glass bottles. The soils were fortified at 15, 25, 50, 100, and 400 ppb (w/w) using stock solutions prepared from 98.2% technical atrazine (U.S. EPA). The soils were then thoroughly mixed. The levels of atrazine were selected so that the final concentrations after dilution would lie within the linear range of 0.5-5.0 ppb for the EIA (a conservative estimate; Bushway et al. (1989) reported a linear range of 0.5-10 ppb). To perform the extraction, 25 mL of acetonitrile-water (90:10, v/v), prepared from 99.99% high purity solvent (American Scientific Products) was added to each bottle. The bottle and contents were shaken for 10 min at 150 rpm on a platform shaker. Soil was allowed to settle for 2 min before the supernatant was filtered through a Whatman #1 filter paper. The filtrate was diluted 1:50 with water making the final solution 1.8% acetonitrile (this concentration was shown to be compatible with the EIA). To obtain the calibration curves, control (0.0) and standards of 0.5, 1.0, and 5.0 ng/mL atrazine were prepared in 1.8% acetonitrile. The calibration curve, calculated for each set using SAS PROC REGRESSION, was the linear regression of ΔOD on the logarithm of ng/mL atrazine for the three standard

tubes. The final atrazine concentration in each soil sample was obtained by multiplying the estimated value from the calibration curve by the dilution factor of 50.

For the methanol extraction, six 25.0-g soil aliquots were fortified at 5, 6, 8, 10, 20, and 40 ppb (w/w) of atrazine. Each soil was mixed thoroughly. Twenty-five mL of 99.98% methanol were added to each bottle and processed as for acetonitrile. The filtrate was diluted 1:10 with water which gave atrazine in 10% methanol. Control (water only) and atrazine standards of 0.5, 2.0, and 5.0 ng/mL were prepared in 10% methanol from a working stock solution of 100 ng/mL atrazine. The soil atrazine concentration was estimated from the calibration curve and multiplied by 10 (the dilution factor).

For the water extraction, six 25.0-g soil aliquots were fortified with 0.5, 0.7, 1, 2, 4, and 5 ppb atrazine. Atrazine standards and control were prepared in distilled water. Twenty-five mL of distilled water were used to extract each soil sample. The extraction procedure was similar to that for methanol and acetonitrile. Because water extract contained fine suspended particles that tended to clog the filter paper, a syringe-type, 25-mm, 0.2-um, nylon cartridge filter (Alltech Assoc., Inc., IL) was used instead. The filtrate was introduced directly into the EIA tube without dilution, and hence the atrazine concentration in soil could be estimated directly from the calibration curve, which was based on atrazine standards of 0.5, 2.0, and 5.0 ng/mL.

All reagents of the EIA kit were allowed to warm to room temperature before proceeding with the assay. For each solvent, two sets of ten antibody-coated tubes were run, each set being a complete replicate for that solvent. Each set consisted of a control (0), three standards (0.5, 2.0, and 5.0 ng/mL atrazine), and six acetonitrile-, methanol- or water-extracted samples, one at each fortified level of atrazine. A 160-uL sample or standard was introduced into each tube followed immediately with enzyme conjugate (160 uL); the tube contents were gently swirled and mixed for 2-3 sec. After the tubes were left to incubate for 5 min, the contents of each tube were shaken out; the emptied tube was filled with cool distilled water and then shaken out. The wash step was repeated four more times with as much water removed as possible on the last wash step. Four drops (160 uL) of substrate (hydrogen peroxide) were added, followed immediately with four drops of chromogen (tetramethylbenzidine), both provided in dropper bottles. Tubes were swirled gently for a few seconds and incubated for 2 min, then a drop of stop solution (2.5 N sulfuric acid) was added to each tube, and the tube swirled. This arrested the blue color development and turned the reaction solution yellow. Water (0.5 mL) was added and gently mixed in each

"stopped" tube. The ΔOD for each sample or standard tube was read against the control tube. In order to run 10 tubes per set, two people were needed, following the time sequence shown in Table 1.

Table 1. The immunoassay steps and time sequence for running ten tubes.

Tube #	1	2	3	4	5	6	7	8	9	10
	Time in min-sec (00'00")									
Sample & Enzyme	0000	0030	0100	0130	0200	0230	0300	0330	0400	0430
Washing (Person A)	0500	0530	0600	0630	0700	0730	0800	0830	0900	0930
Substrate & Chromogen (Person B)	0520	0550	0620	0650	0720	0750	0820	0850	0920	0950
Stop (Person A)	0720	0750	0820	0850	0920	0950	1020	1050	1120	1150

RESULTS AND DISCUSSION

Figures 1a and b show the results of the first test: the response of the EIA to atrazine concentrations of 0.5, 2, and 5 ng/mL in 2, 3, and 4% acetonitrile and 5, 7, and 10% methanol, respectively, in comparison to atrazine in water (0% organic solvent). There were no significant interactions of atrazine with percent acetonitrile ($F=1.17$; $df=4, 27$; $p>0.34$) or with percent methanol ($F=0.28$; $df=6, 36$; $p>0.94$), i.e., the slopes of the response curves were not significantly different for the solvents and the water samples. Although the responses for acetonitrile ($F=19.88$, $df=2, 27$; $p<0.000$) and methanol ($F=48.06$; $df=3, 36$; $p<0.0001$) were significantly different from the water controls, measurable ΔOD could be obtained at all atrazine concentrations for up to 3% acetonitrile and 10% methanol (highest level tested). The 4% acetonitrile interfered with the assay as indicated by the negative ΔOD at 0.5 ng/mL atrazine (Fig. 1a). The manufacturer's recommendation of diluting the 90% acetonitrile extraction-solvent to the final concentration of 1.8% was justified and, to be conservative, was chosen for our subsequent soil extraction study. The 10% methanol was selected as the dilution upper limit for the final EIA procedure when methanol was used as a solvent.

The percent of atrazine recovered at various fortified levels and estimated atrazine concentrations for fortified soil samples extracted

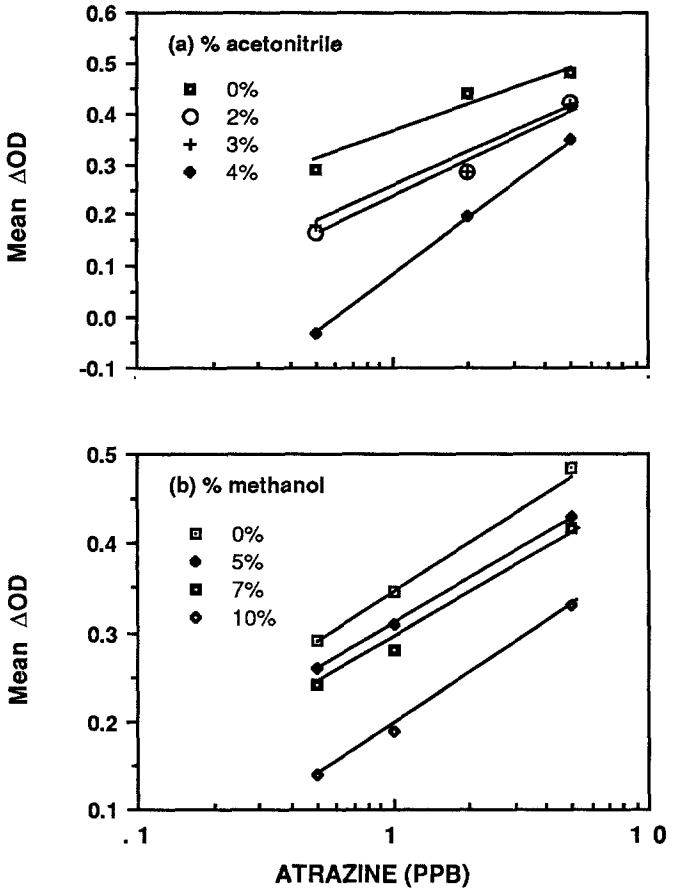

Figure 1. The response curves (mean ΔOD, n=4) of the enzyme immunoassay for various percentages of (a) acetonitrile and (b) methanol at levels of atrazine concentration.

with acetonitrile, methanol, and water are shown in Figure 2 and Table 2, respectively. All cases gave over 100% recovery except for water solvent at fortified levels of 4 ppb (79% recovery) and 5 ppb (71%), and acetonitrile at 400 ppb (86%). The over-estimates are probably from interference of soil components co-extracted with atrazine. It appears that the soil has substances that interfere with the enzyme-conjugate antibody reaction thus giving falsely high concentration estimates. This interference has also been reported by Bushway et al. (1989) in the determination of atrazine in food using the same EIA. Hence the control and calibration standards need to be prepared by adding atrazine to the background soil extract instead of in normal solvents or by standard addition if no untreated background soil is available. Figure 2 suggests that the extraction efficiency for each solvent is different and dependent on the atrazine levels. Acetonitrile (10% water) seems to be the most efficient for the

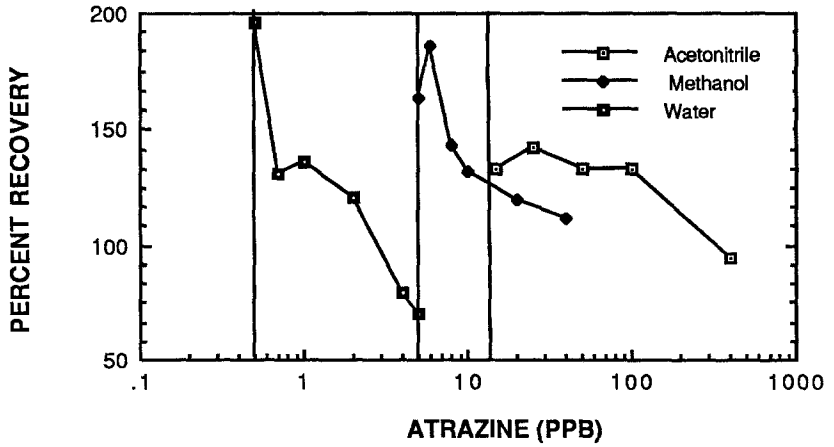

Figure 2. Mean percent recovery (n=2) for levels of fortified atrazine in soil using acetonitrile, methanol, and water as extracting solvents (vertical lines are probable lowest levels of reliable measurement for corresponding solvent).

higher atrazine levels from 15 to 100 ppb as tested. Because of the limitation of the maximum levels of acetonitrile (3%) and methanol (10%) tolerated by the EIA, the lowest atrazine levels that can be detected with acetonitrile and methanol are approximately 15 and 5 ppb, respectively (Fig. 2), if the EIA is to be amenable to field use, i.e., without needing facilities and equipment for concentrating extracts. The dependencies of the recovery rate on atrazine concentration and

Table 2. Estimated atrazine levels of extracted fortified soil using acetonitrile (10% water), methanol, and water as solvent and analyzed by enzyme immunoassay.

Solvent		Atrazine (ppb)					
Acetonitrile[a] (n=2)	Fortified	15	25	50	100	400	
	Estimated	21[b]	37	66	128	345[a]	
Methanol (n=2)	Fortified	5	6	8	10	20	40
	Estimated	8	11	11	13	24	45
Water (n=2)	Fortified	0.5	0.7	1	2	4	5
	Estimated	0.9	0.9	1	2	3	4

[a]Contains 10% water

[b]Estimates obtained by extrapolating beyond linear range.

the choice of a solvent on the expected predominant field concentration present challenges for quantification in the field.

Further studies will be needed to characterize soil substances interference, select the solvent type and level and the series of dilutions needed to bring the final extract solution to within linear range of the EIA. A scheme can then be constructed so that all field soil samples of wide-range of possible atrazine concentration can be routinely processed.

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