Application of a Triasulfuron Enzyme Immunoassay to the Analysis of Incurred Residues in Soil and Water Samples

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An enzyme immunoassay (EIA) for triasulfuron produced by Millipore, Inc., was incorporated into an analytical method for the analysis of incurred residues in soil and water samples. All samples used in the validation study were obtained from triasulfuron field studies and were previously analyzed by either high-performance liquid chromatography (HPLC) or HPLC with mass spectrometric detection (LC/MS). Water samples were analyzed directly without any sample preparation. Soil samples were extracted in a methanol/phosphate buffer solvent system by vortex mixing and sonication. The soil extract was cleaned up by solid phase extraction (SPE). Residues in the SPE eluate were brought up in Tris-HCl buffer and analyzed directly in the buffer solution. Immunoassay results compared favorably with HPLC ($r = 0.93$, water samples) and LC/MS data ($r = 0.88$, soil samples). The immunoassay method has a lower limit of detection at 0.05 ppb than either of instrumental methods. The limits of quantitation for the EIA in water and soil are 0.05 and 0.10 ppb, respectively.

Keywords: *Triasulfuron; sulfonylurea; enzyme immunoassay; sample preparation*

INTRODUCTION

Triasulfuron [3-(6-methoxy-4-methyl-1,3,5-traizin-2-yl)-[2-(2-chloroethoxy)phenylsulfonyl]urea] is a selective sulfonylurea (SU) herbicide used for control of broadleaf weeds in wheat, barley, and fallow crop land. Like other sulfonylureas, triasulfuron is applied at very low use rates, ranging from 9.0 to 39.2 g of active ingredient/ha when formulated as the active ingredient of Amber herbicide. Analytical methods, therefore, must be correspondingly sensitive. Bioassay techniques are sufficiently sensitive but nonselective and require 2–21 days to complete (Hsiao and Smith, 1983; Walker and Brown, 1983; Iwanzik et al., 1988; Sunderland et al., 1991). Previous workers have applied high-performance liquid chromatography (HPLC) (Zahnow, 1982; Iwanzik and Egli, 1989), HPLC with mass spectrometric detection (LC/MS) (Shalaby and George, 1990; Shalaby et al., 1992), gas chromatography (Ahmad, 1987; Ahmad and Crawford, 1990), and capillary electrophoresis (Dinelli et al., 1993) to decrease time of analysis. These techniques require costly instrumentation and complex sample preparation and often lack adequate sensitivity. Several investigators have developed immunoassays to address these shortcomings and provide rapid, sensitive, and inexpensive analytical methods. Kelley et al. (1985) developed an assay that could detect 0.40 ppb of chlorsulfuron in soil. Schlaeppi et al. (1992) achieved a limit of detection (LOD) of 0.10 ppb of triasulfuron in soil utilizing extensive sample cleanup procedures. This work was refined in a recent paper (Schlaeppi et al., 1994) that lowered the LOD to 0.02 ppb in soil using a chemiluminescent detection system. This paper describes the application of a commercial triasulfuron immunoassay to the analysis of soil and water samples containing incurred residues.

MATERIALS AND METHODS

Materials. EnviroGard triasulfuron microtiter plate kits were obtained from Millipore Corp., Bedford, MA. C_8 bonded phase solid phase extraction cartridges and accessories were obtained from Varian Associates, Sunnyvale, CA. Polyprep columns and stack caps were purchased from Bio-Rad, Richmond, CA. A 12-position solid phase extraction manifold was obtained from Supelco, Bellefonte, PA. Soil extracts were centrifuged on a Sorvall RC-5B refrigerated superspeed centrifuge purchased from DuPont Instruments, Wilmington, DE. Absorbance readings were measured with a Multiskan MCC/340 MKII microtiter plate reader from ICN Biomedicals, Costa Mesa, CA. This instrument was controlled by a Gateway 2000 386DX/33 computer purchased from Gateway 2000, North Sioux City, SD.

Analytical standards were obtained from Ciba Production Technical Analytical Services or Ciba Chemical Synthesis, Greensboro, NC. Additional test substances were furnished by the U.S. EPA Pesticides and Industrial Chemical Repository in Research Triangle Park, NC.

Enzyme Immunoassay. The Millipore assay was produced in the microtiter plate format. The assay was run in a similar fashion as previously described (Brady et al., 1995). The positions of all standard and sample solutions in the microtiter plate were recorded by the analyst on a plate layout sheet. All samples and standards were analyzed in duplicate. Standard concentrations ranged from 0.05 to 2.0 ppb. A standard solution, water sample, or soil extract (150 μL) was added to a well of an uncoated polystyrene microtiter plate (the reservoir plate). Using a reservoir plate enabled the analyst to transfer all solutions to their designated positions in the antibody-coated plate to incubate for an equal amount of time during the inhibition phase of the assay. The analyst transferred 100 μL of each solution with a multichannel pipetter. The same volume of enzyme conjugate was added, and the plate was incubated at room temperature with gentle shaking (about 90 oscillations/min) for 1 h. The shaker was covered with a cardboard box to protect the plate from drafts. The contents of the plate were then removed and each well was washed with three changes of distilled, deionized H_2O. After the final wash, the plate was inverted and blotted on a clean paper towel to remove remaining traces of liquid. Freshly prepared "color reagent" (2 parts of "substrate" combined with 1 part of "chromogen") was added to each well (150 μL), and the plate was incubated for approximately 30 min under conditions described above. Production of the colored signal was terminated by acidification (50 μL of 2 M H_2SO_4/well). The absorbance of the contents of each well was measured at 450 nm. A log/linear standard curve was generated by plotting the logarithm of the standard concentrations

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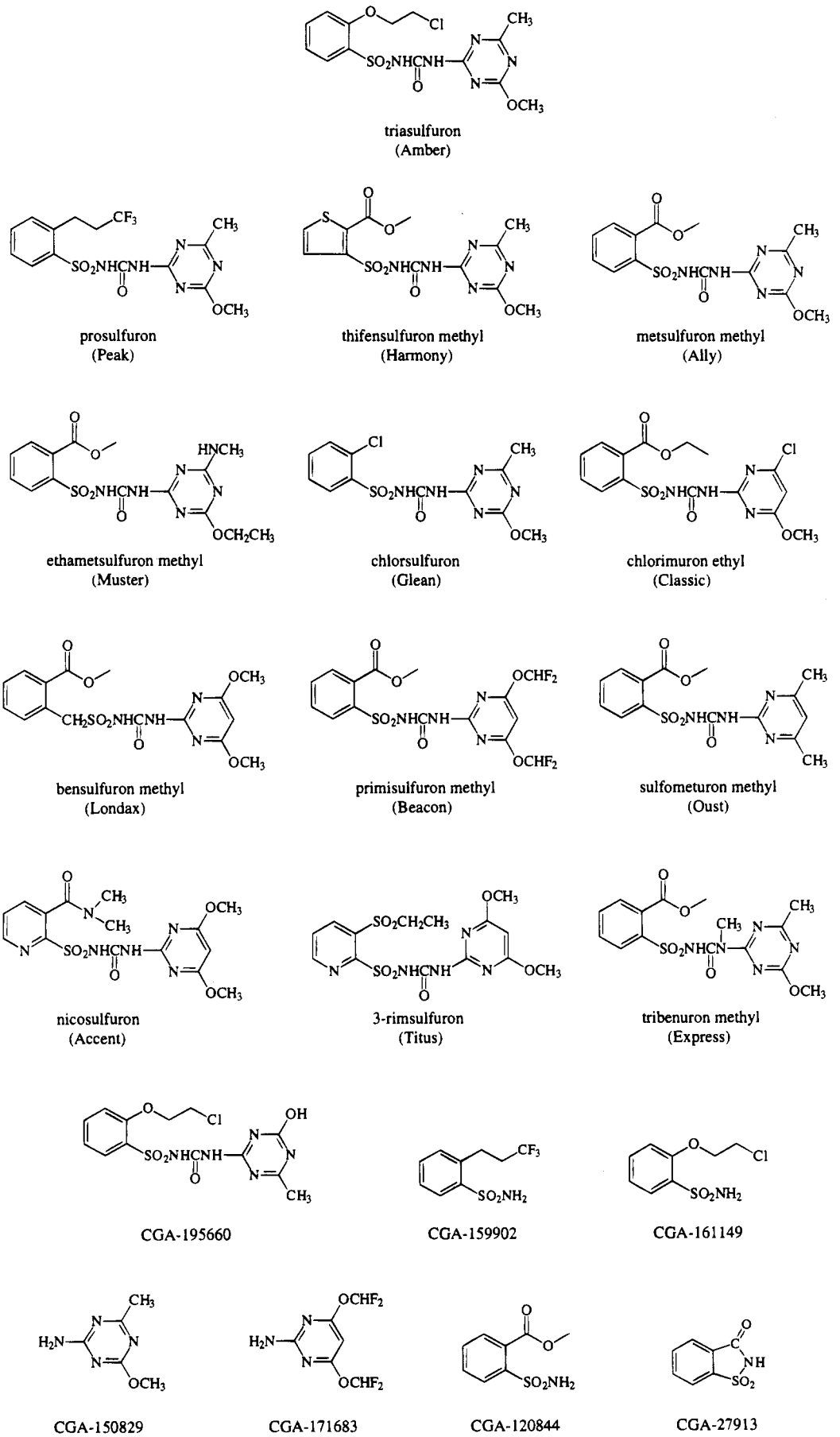

Figure 1. Chemical structures, chemical names, and trade names (where available) of compounds screened for cross-reactivity in the triasulfuron enzyme immunoassay.

against their responses (absorbances). EIA results were expressed as parts per billion of triasulfuron equivalents (TE).

Cross-Reactivity Analysis. Distilled, deionized H_2O was fortified with potentially reactive test substances (Figure 1)

Table 1. Characteristics of Representative North Dakota Soils Analyzed by the Triasulfuron Enzyme Immunoassay

soil texture	% sand	% silt	% clay	% organic matter	pH
sandy clay loam	67	11	22	0.6	8.0
loamy sand	83	6	11	0.3	8.4
sandy loam	74	10	16	2.0	7.3
sand	88	5	7	0.1	8.0

at concentrations ranging from 1000 to 0.10 ppb. Aliquots of each fortification were analyzed as described above. The mean absorbance for each compound at each concentration (B) was normalized to the mean of the zero standard response (B_0) on a percentage basis (% B/B_0). These values were plotted on a linear scale against the logarithm of the range of concentrations tested to generate dose–response curves for each test substance. The amount of each test substance that yields half the response of the zero dose (I_{50}) was determined from these curves. The reactivity of each test substance relative to that of triasulfuron was determined by dividing the I_{50} of triasulfuron by the I_{50} of each compound tested and multiplying that ratio by 100%. This determination arbitrarily assigns triasulfuron 100% reactivity. The LOD of each test substance was determined according to a modified version of Rodbard's method (Brady, 1995).

Sample Preparation and Analysis. Sample collection and analysis were conducted in compliance with good laboratory practice guidelines. Samples were obtained from triasulfuron field studies. Water samples from a Kansas ground water study were received and stored under refrigerated conditions (4 °C) until brought to room temperature for analysis. The pH of each sample was measured prior to analysis. Only samples with a pH value between 5.5 and 9.0 were suitable for this EIA. Water samples were analyzed without further preparation.

Soil samples were obtained from a North Dakota study site. The soil series at the test site was classified as a Hecla fine sandy loam (Table 1). Samples were received and stored under frozen conditions. Samples were thawed overnight under refrigeration or warmed to room temperature immediately prior to use. Aliquots of each (2.0 g) were removed for analysis. If a sample had not been previously prepared for analysis, the analyst passed about 50 g through a No. 7 USA standard testing sieve (2.80 mm opening; Fisher Scientific, Pittsburgh, PA) to remove rocks, twigs, and other debris. If a sample was clumped and did not pass through the sieve, it was ground into finer particles with a mortar and pestle. The samples were collected in stainless steel mixing bowls. Analysts thoroughly mixed each sample with a stainless steel wire whisk for 1 min. Subsamples were then collected for analysis.

Each subsample was combined with 12 mL of methanol/0.07 M phosphate (1:3) buffer, pH 7.1, in a 25 mL glass centrifuge tube. The tube was inserted into a foam pad fitted on a recessed metal platform attached to a vortex mixer. Tube contents were mixed by vortexing at the highest setting for 10 min. Each tube was then placed in a sonicating bath for an additional 10 min. Samples were centrifuged for 20 min at approximately 17000g at 4 °C. Before the sample could warm to room temperature, a 1 g equivalent of the supernatant (6 mL) was transferred to a reservoir connected to a C_8 solid phase extraction cartridge (SPE) (Figure 2). The cartridge was preconditioned with 5 mL of methanol and 5 mL of a solution consisting of extraction solvent, H_2O, and phosphoric acid (20:30:1). The sample extract was diluted with 9 mL of H_2O and 250 μL of phosphoric acid and slowly drawn through the cartridge (vacuum adjusted to 3–5 in. of Hg). The SPE was washed with 2 mL of H_2O adjusted to pH 2 with phosphoric acid followed by 10 mL of distilled, deionized H_2O. Ambient air was subsequently drawn through the packing for 20 min. Triasulfuron residues were eluted in 4 mL of methylene chloride, which was passed through a column of anhydrous sodium sulfate to remove traces of moisture. The eluate was reduced to dryness under a stream of nitrogen. Residues were brought up in 1.0 mL of 0.01 M Tris-HCl buffer, pH 7.2, for immunochemical analysis.

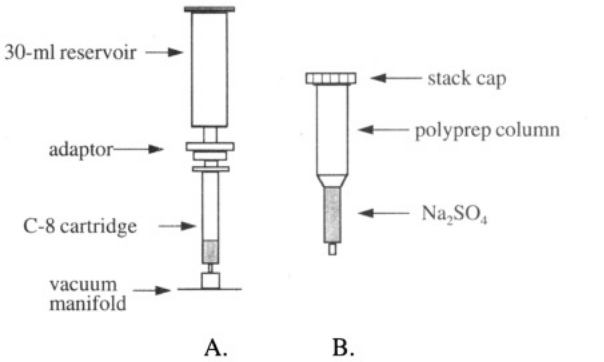

Figure 2. Apparatus used for SPE cleanup of soil extracts. (A) A 1-g equivalent of the extract is added to the reservior, diluted to 10% organic content with water, and acidified before being drawn through the cartridge. (B) After the column was washed and air-dried, a column of sodium sulfate was inserted between the cartridge and manifold to remove water from the eluate.

Analytical results for soil samples were corrected for the amount of moisture in each sample. Moisture determination was carried out gravimetrically by weighing an aliquot of the sample (usually 1.0 g) before and after overnight incubation in an oven set to 100 °C. The percent moisture in the sample (m, expressed as a decimal) was inserted into the expression

$$g_d = g_w V_a / [V_s + (m g_w)]$$

in which g_d is the calculated dry weight in grams of the sample, g_w is the wet weight of the sample in grams, V_a is the volume (milliliters) of the aliquot transferred to the SPE, and V_s is the total volume (milliliters) of extraction solvent. The amount of TE found in the 1.0 g (wet weight) sample aliquot was divided by g_d to adjust for the true amount of soil analyzed.

The concentration of TE in a sample may have been further adjusted if the mean percent recovered of the procedural recovery samples included in the same analytical set were less than 100%. Analyses of soil samples were always accompanied by procedural recovery samples to assess the efficiency of the method. Recovery samples (control soils) were usually fortified at 0.10 and 1.0 ppb of triasulfuron, but higher levels were used when field residues were shown to lie outside this range. Sample values were divided by the mean results of all procedural recoveries if those results were less than 100%. Sample data were never adjusted if the average procedural recoveries were greater than 100%, since so doing would minimize the residues found. If any residues were found in the control sample, that amount was subtracted from each procedural recovery result before the mean percent recovered was determined.

Accuracy and Precision. The accuracy and precision of this method were determined by conducting standard addition experiments with water and soil samples obtained from field test locations. Kansas water samples were fortified with 0.05, 0.10, and 1.0 ppb of triasulfuron and concurrently analyzed pre- and postfortification. The mean net differences between neat and fortified samples were used to determine the percent recoveries at each level. Soil samples from seven states where triasulfuron is registered (Colorado, Kansas, Nebraska, North Dakota, South Dakota, Texas, and Washington) as well as from Mississippi were fortified with 0.10 and 1.0 ppb of triasulfuron and analyzed as described above. The LOQ for each matrix was determined from these data.

RESULTS

Cross-Reactivity. The cross-reactivity analyses show the antibodies used in this assay react primarily with triasulfuron and to a lesser extent with prosulfuron, thifensulfuron methyl, metsulfuron methyl, and ethametsulfuron methyl (Table 2). The assay did not react with the sulfonamide (CGA-161149) or triazine (CGA-150829)

Table 2. Cross-Reactivity Parameters of the Triasulfuron Enzyme Immunoassay

test substance	LOD[a]	I_{50}[a]	% reactivity
triasulfuron	0.05	0.48	100
prosulfuron	0.08	13	3.2
thifensulfuron methyl	0.09	50	1.0
metsulfuron methyl	0.28	50	1.0
ethametsulfuron methyl	0.64	660	<1.0
chlorsulfuron	2.1	50	1.0
chlorimuron ethyl	3.5	720	<1.0
bensulfuron methyl	NR[b]	NR	NR
primisulfuron methyl	NR	NR	NR
sulfometuron methyl	NR	NR	NR
nicosulfuron	NR	NR	NR
3-rimsulfuron	NR	NR	NR
tribenuron	NR	NR	NR
CGA-195660	NR	NR	NR
CGA-161149	NR	NR	NR
CGA-150829	NR	NR	NR
CGA-159902	NR	NR	NR
CGA-171683	NR	NR	NR
CGA-27913	NR	NR	NR
CGA-120844	NR	NR	NR

[a] In units of ppb. If the LOD was determined to be less than the smallest standard, it assumed the higher value. [b] NR, not reactive.

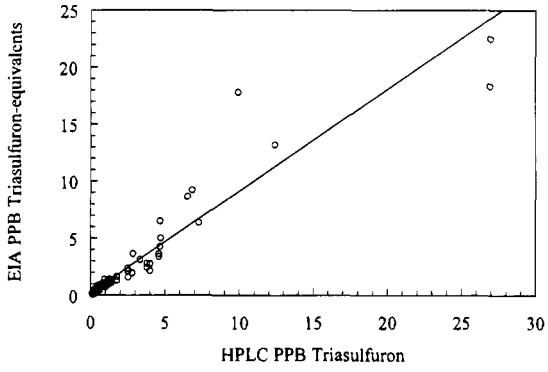

Figure 3. Comparison of analytical results obtained by HPLC (Ourisson, 1989) and EIA for 89 water samples (116 analyses). The LOQ of the HPLC method was 0.10 ppb.

degradation products of triasulfuron (Figure 1), both of which lack phytotoxic properties. Only prosulfuron was found to have a relative reactivity greater than 1% compared to triasulfuron. However, the LODs of the compounds bound by antibody lie within the range of realistic residue concentrations. For example, residues of Harmony and Ally, competitive products in the wheat market, might occur in samples collected for triasulfuron monitoring. Analysts must recognize, therefore, this EIA is a screening method and confirm the concentration and identity of detections by an alternative analytical technique.

Sample Analysis. Eighty-nine water samples from a Kansas ground water study previously analyzed by HPLC (Ourisson, 1989) were reanalyzed by EIA. The best fit regression line of the mean immunoassay results versus the HPLC data determined the relationship between the two data sets to be ppb of TE = 0.89 HPLC + 0.19 (r = 0.93, Figure 3). A slope less than 1 indicates the values obtained by HPLC are generally greater. The apparent positive bias by HPLC is the result of two outlying points (duplicate analyses of the same sample, TE greater than 25 ppb). When these outliers are removed, the EIA results exhibit a slight positive bias (ppb of TE = 1.26 HPLC − 0.25, r = 0.94), which is a typical immunochemical response.

Twenty-one soil samples from a North Dakota field plot were also analyzed by the EIA methodology described above. Regression analysis of the average of immunoassay duplicate analyses and the LC/MS results collected by A. D. Little personnel (Kyranos, 1993) calculated a relationship of ppb of TE = 0.54 LC/MS + 0.93 (r = 0.88, Figure 4).

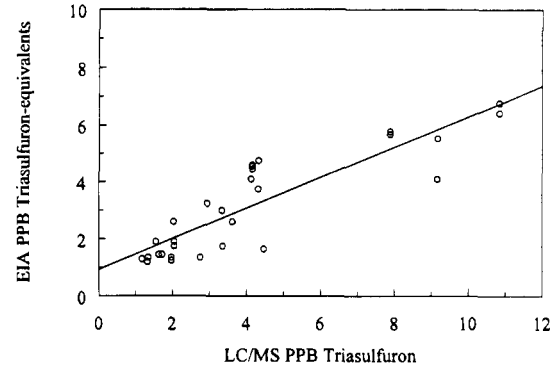

Figure 4. Comparison of analytical results obtained by LC/MS (Kyranos, 1993) and EIA for 21 soil samples (29 analyses). The LOQ of the LC/MS method was 1.0 ppb.

Table 3. Results of Immunoassay Soil Procedural Recovery Experiments

fortification level (ppb of triasulfuron)	N	mean ppb of TE found ± SD	mean % recovery
0.10	16	0.10 ± 0.02	99
1.0	14	1.0 ± 0.16	103
10.0	2	10.3 ± 1.1	103

Table 4. Results of Immunoassay Standard Addition Experiments

substrate	fortification level[a]	N	mean net difference[b]	SD	mean % recovery	% CV
soil	0.10	18	0.09	0.02	89	22
	1.0	18	1.1	0.15	105	14
water	0.05	19	0.07	0.02	135	29
	0.10	17	0.14	0.03	137	21
	1.0	18	1.1	0.33	109	30

[a] Concentration units of ppb of triasulfuron. [b] Concentration units of ppb of TE.

Results of immunoassay soil procedural recovery samples were nearly quantitative (Table 3). The mean percent recovery of 32 spiked samples, half of which were fortified to 0.10 ppb of triasulfuron, was 102%.

Accuracy and Precision. Three groups of Kansas water samples were fortified with various concentrations of triasulfuron and analyzed before and after fortification. The initial levels of triasulfuron in these samples ranged from approximately 0.10 to 1.5 ppb.

The mean net increases in triasulfuron levels are summarized in Table 4. Average percent recoveries at all levels of fortification indicate the method may have a positive bias for the analysis of water samples, especially at lower concentrations of triasulfuron. This bias is consistent with that observed in the analytical results of the Kansas water samples. These fortification experiments can be used to support an LOQ of 0.05 ppb in water.

Soils were fortified with 0.10 and 1.0 ppb of triasulfuron and analyzed in a similar fashion as described for water samples. The mean percent recoveries of 89 and 105%, respectively, indicate the methodology can be successfully applied to soil analyses with an LOQ of 0.10 ppb (Table 4).

The recovery data suggest the EIA is less precise in water samples than in soils as indicated by the standard deviation and coefficient of variation data (Tables 3 and

4). The SD and CV of water analyses range from 0.02 to 0.33 ppb of TE and from 21 to 30%, respectively. Soil results, by contrast, had SDs of 0.02 and 0.15 ppb of TE and CVs of 14 and 22%, respectively.

DISCUSSION

Given concerns about low-level residual activity of SUs, analytical methods should be able to quantitate extremely low concentrations of active ingredient. To be practical techniques, these methods should not require the purchase of extremely expensive instrumentation. LC/MS techniques, for example, can measure as low as 1 ppb in soil (Kyranos, 1993) but require instrumentation to which many investigators may lack access. Contracting LC/MS analyses to third parties may not be satisfactory because costs are nearly $500.00/analysis. A capillary gas chromatography method can quantitate in the sub parts per billion range from water samples after liquid–liquid extraction of a 1 L sample followed by a derivatization step with diazomethane (Ahmad, 1987). Analytical costs for this approach generally fall in the range of $200.00–$250.00/sample.

A more economical and efficient way to address these problems is through application of immunochemical techniques. In the microtiter plate format described above, an analyst can run up to 48 water samples in 4 h at a cost of approximately $20.00/sample. A single analyst can generate more water data in one day by EIA than can typically be produced in a week by a team using chromatographic techniques.

The full utility of immunoassays will not be reached, however, until EIAs have been successfully applied to a variety of agrichemical matrices. Immunochemical analysis of water samples, while a valuable contribution to the residue chemist's arsenal, represents a limited application of the technology. Other substrates, such as soil and crop tissue, require the test substance to be extracted and isolated from the sample matrix prior to analysis. Soils have proven to be problematic as the extractibility of test substances varies with soil types (Goh et al., 1990). Nonspecific interferences coextracted with the test substance and solvent effects have often produced false positive results (Kelley et al., 1985; Schlaeppi et al., 1989, 1992; Goh et al., 1990; Stearman and Adams, 1992).

Under ideal conditions, EIA sample extraction techniques should be rapid, not require specialized equipment, minimize use of organic solvents, and reduce overall generation of hazardous waste. SUs may be excellent candidates for such metholodogies because of their solubility characteristics. SUs are soluble, on a percentage basis, in water-miscible organic solvents such as methanol or acetonitrile. Water solubility is pH dependent. Most SUs have a pK_a around 3–5 and are readily soluble at neutral or slightly alkaline pH (pH 7–8) (Hay, 1990) but can be easily partitioned out of the aqueous phase by acidifying the extract (phosphoric acid is the reagent of choice because SUs are rapidly hydrolyzed by other mineral acids).

The methodology described in the current study utilizes a similar solvent system [methanol/0.07 M phosphate buffer (1:3), pH 7.1] as that described by Schlaeppi et al. (1992), but the mechanical breakdown of the sample by vortex mixing and sonication reduced extraction time from 2 h to 20 min. Centrifugation was used in lieu of filtration to produce a clean extract. The analyte was subsequently isolated by partitioning on a C_8 SPE.

This step was beneficial in several ways. First, the eluate produced was free of inhibitory substances to the extent false positive results were not observed. Second, minimal use of organic solvents was required to achieve these results. Only 5 mL of methanol (cartridge conditioning) and 4 mL of methylene chloride (elution solvent/sample) were used. Third, a 1 g equivalent of extract (6 mL) was added to the SPE. By concentrating this volume to 1.0 mL, the sensitivity of the EIA was maintained. Investigators have conventionally diluted soil extracts to yield results in the parts per million range. The approach used in this study permits quantitation in the sub parts per billion range. Fourth, the SPE cleanup contributed to the overall economy of the method. Soil samples were extracted and analyzed at an estimated cost of $50.00 each.

EIA results of soil analyses (Figure 4) do not correlate as highly with chromatographic data as the water results. The disparity between methods may be attributed to several causes. First, subsamples of each sample were analyzed by different laboratories using different analytical techniques. Immunoassays are typically not evaluted in this fashion. Instead, some authors have made comparisons between techniques using a common extract (Bushway et al., 1988; Schlaeppi et al., 1989; Lucas et al., 1991). This may yield closer results between methods but does not reflect the way each methodology will actually be applied. Confirmatory analyses performed to verify residue detected by an immunoassay screen will probably not use the screening extract since sample sizes, solvent systems, and solvent volumes are often different. Extraction conditions for conventional analyses are also frequently harsher than those used for a screening technique. As a result, employing a single extract for comparing different methods reduces the variability between results but may yield an unrealistic comparison.

Low procedural recoveries often obtained by the LC/MS soil method also contributed to the disparity of results. Three samples containing the greatest concentration of triasulfuron by LC/MS (Figure 4) were corrected upward for procedural recoveries of 66% (Kyranos, 1993). Immunoassay soil procedural recoveries, on the other hand, averaged approximately 102% (Table 3), so sample data were usually not affected by the results of the recovery samples.

Finally, soils used in this work contained incurred residues. This is in sharp contrast to the common practice of conducting immunoassay validations with freshly fortified samples (Schwalbe et al., 1984; Kelley et al., 1985; Bushway et al., 1988; Schlaeppi et al., 1989, 1992; Goh et al., 1990; Wittmann and Hock, 1990; Lucas et al., 1991; Schneider and Hammock, 1992). Typically, soil samples are fortified and immediately extracted. The extracts are then analyzed by the method of choice. This technique may provide information about the suitability of a method for analysis of freshly fortified samples but not about its utility when applied to weathered soils. Residues may be bound to soil particles, rendering facile extractions useless. Analytical results in this study were generated using real-world samples under conditions similar to those under which the technique is intended to be applied. Thus, this study presents a realistic assessment of the EIAs utility.

CONCLUSION

A commercial immunoassay for triasulfuron has been incorporated into an analytical method for quantifying

residues in soil and water. The EIA is cost efficient compared to HPLC and LC/MS techniques. Water samples are measured directly without sample preparation. Soil samples require 20 min for extraction in a methanol/phosphate buffer solvent system. An aliquot of the extract is partitioned on a C_8 SPE. This approach uses very little organic solvent and permits quantitation of soil residues in the sub parts per billion range. The EIA has an LOD of 0.05 ppb. The LOQs of the method in water and soil are 0.05 and 0.10 ppb, respectively. EIA results correlated well with chromatographic analyses of water and soil samples collected from triasulfuron field studies.

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