Validation of a Monoclonal Immunoassay for Diuron in Groundwater†

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Diuron is one of the most heavily used herbicides in California, and well water and surface water are routinely monitored for diuron residues. In a previous paper, we described haptens and monoclonal antibodies suitable for quantitative competition enzyme immunoassay (EIA) of diuron. This paper documents the design and results of a study conducted with 49 blind-coded groundwater samples, analyzed by high-pressure liquid chromatography (HPLC) and EIA. Diuron residues were recovered using a C_{18} solid-phase extraction (SPE) protocol that also is applicable to the recovery of simazine, atrazine, prometon, and bromacil. To minimize analyst errors, all SPE and EIA steps were performed on automated equipment. Overall correlation of the EIA and HPLC results was $r^2 = 0.95$, with a low bias in the EIA (ppb estimated by EIA = 0.77 × ppb estimated by HPLC). The detection limits of the EIA and HPLC were approximately 0.07 and 0.1 ppb, respectively. There were no false positives or false negatives in the EIA. The results indicate that the residue extraction and EIA protocols are suitable for initial screening for compliance monitoring of diuron. The protocols are potentially adaptable to multiresidue screening for leachable herbicides in water samples.

INTRODUCTION

Diuron [3-(3,4-dichlorophenyl)-1,1-dimethylurea] is one of the most heavily applied herbicides in California, with usage on the order of 400 000 lb per year (California Department of Pesticide Regulation, 1984–1990). It is primarily used on rights-of-way, but substantial amounts are also employed for many other weed control applications. Diuron can leach into groundwater and surface water. Residues have been detected in a small number of wells in California (Cardozo et al., 1988; Miller et al., 1990), and low but significant amounts have been found in the Sacramento River delta and San Francisco Bay estuary (W. Pereira, U.S. Geological Survey, 1992, personal communication).

We developed a monoclonal enzyme immunoassay (EIA) for diuron as an intermediate goal in a project aimed at creating an immunochemical screening procedure for all five major leachable herbicides monitored in California groundwater: simazine, atrazine, prometon, bromacil, and diuron. To replace instrumental analyses for this purpose, the EIA methods would have to be able to quantify all five compounds. For EIA to be suitable for compliance monitoring, it should demonstrate a detection limit of 0.1 ppb or lower for the target analyte, with high precision and reproducibility and no ambiguous cross-reactivities or interference from the sample matrix. As for any method used in the regulatory environment, the EIA should give no false negatives, except for those samples very near the detection limit, where a small experimental error is likely to change the outcome. When EIA is used as an initial screening method, a low percentage of false positives can be tolerated because positives will be retested by a second analytical method. A few percent of false positives is, in fact, observed with conventional regulatory analytical methods such as high-pressure liquid chromatography (HPLC) and gas chromatography (GC). The EIA should be sufficiently specific for the regulated compound to ensure that there are few, if any, false positives from analogs or metabolites. The performance of the EIA must be evaluated on a representative set of samples with naturally incurred residues, as well as check samples prepared in the laboratory.

This paper describes an initial validation of the monoclonal EIA for diuron in groundwater. The replicates and controls were set up to provide maximum information about the performance criteria. In addition, the solid-phase extraction (SPE) and EIA protocols were adapted to work equally well for simazine, atrazine, prometon, and bromacil residues in the same sample. Finally, the procedures were designed to be automated. We modified the standard SPE method so that it would be EIA-compatible and could be performed on a Gilson ASPEC (automated solid-phase extraction) system. We also programmed a Beckman BioMek robotic pipetting workstation to perform all of the fluid-transfer steps in the EIA. The results of this evaluation indicate that the sample recovery procedure and EIA are sufficiently quantitative and reproducible to be used as an initial screening of groundwater for compliance monitoring. This study also suggests criteria for assessing performance of similar validations.

MATERIALS AND METHODS

The diuron I-bovine serum albumin conjugate described previously was generously provided for this study by M. H. Goodrow, University of California, Davis. Monoclonal antibody 481, also described previously, was used in the form of unpurified culture fluid (Karu et al., 1994). Diuron reference standard >99% pure (AccuStandard, Inc., New Haven, CT) was prepared as a 1 mg/mL solution in methanol, standardized by UV spectrophotometry, and stored at 4 °C in Teflon vials (Pierce Chemical Co.). Dilutions were made in PBS–Tween containing 12.5% methanol (0.01 M KH_2PO_4–K_2HPO_4, pH 7.4–0.15 M NaCl–0.05% Tween 20–12.5% methanol). Glass-distilled deionized water was used

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to prepare all reagents and procedure samples. Organic solvents used in this work were spectrograde, and all other chemicals were analytical reagent grade or better.

Samples. The study included 49 well-water samples collected in October 1991 from domestic and municipal wells by staff of the Environmental Monitoring and Pest Management Division of the California Department of Pesticide Regulation [formerly the California Department of Food and Agriculture (CDFA)]. The samples were collected into residue-free brown glass sample bottles with Teflon-lined caps, using a Schrader sampling system, strictly following procedures documented by CDFA (Sava, 1986). Sample origin and dates and conditions of transportation and storage were recorded as chain-of-custody records. The samples were transported on ice to CDFA and between CDFA and University of California (UC) (Berkeley), and stored at 4 °C at both laboratories. Assays were conducted within 30 days after the samples were collected.

Six more samples were prepared as laboratory spikes of diuron and/or bromacil in well water that had no detectable residues. These samples and the well-water samples were coded by a CDFA staff person. The samples were extracted and analyzed by HPLC at CDFA. A separate set was extracted and analyzed by EIA at UC (Berkeley). The sample code was not made available to the analysts until after results were computed.

Sample Cleanup and Concentration. At CDFA, samples were prepared for HPLC from 500 g of each water sample, by a validated procedure that quantitatively recovers atrazine, simazine, prometon, bromacil, and diuron, using a 100-mg Sep-Pak C_{18} reversed-phase sorbent (Waters Division, Millipore Corp.) (Tran et al., 1990). The Sep-Pak cartridges were conditioned with methanol and distilled water, the samples were passed through at 3–5 mL/min, and the cartridges were centrifuged (1200 rpm, 1 min) to remove remaining water. Residues were eluted with 8 mL of methanol, which was concentrated under nitrogen to 1 mL. The concentrated eluate was then filtered through a 0.2-μm Nylaflo disk (Gelman, Inc.), and the filtrate was analyzed for diuron by HPLC.

HPLC was performed on a Perkin-Elmer Series 4 instrument with a Varian 2550 UV detector as follows: guard column, Beckman ODS, 5.0 μm, 4.6 mm × 4.5 cm; main column, Beckman ODS, 5.0 μm, 4.6 mm × 15.0 cm; sample injected, 60 μL; flow rate, 1.0 mL/min; mobile phase, 55% water, 45% acetonitrile; detection wavelength, 254 nm; retention time, 5.60 min. Each sample was run once *vs* diuron standards, and the practical quantitation limit was 0.1 ppb. In addition, an independent laboratory's HPLC analysis was available for 17 of the 49 field samples.

Residue recovery for EIA was performed at UC (Berkeley) as follows: 400 mL of each water sample was filtered through a 0.2-μm Gelman Nylaflo disk (47-mm diameter). A few of the samples contained suspended solids, but most left little or no visible residue on the filters. Two hundred milliliters of each filtrate was applied directly to a C_{18} SPE column and processed as described below. A "pre-extraction spike" of diuron was added to a final concentration of 0.2 ppb in the remaining 200 mL of each sample, and these were then processed on C_{18} SPE columns as described below. Thus, each sample was essentially extracted in duplicate, with a pre-extraction spike added to one replicate as a check on recovery and matrix effects.

C_{18} SPE columns (100 mg, Analytichem BondElut, Varian Corp.) were conditioned with 10 mL of methanol and glass-distilled water, consecutively. The sample (200 mL) was applied at 3–5 mL/min, the columns were washed with 10 mL of glass-distilled water, and residues were eluted into glass vials with 0.5 mL of methanol. The eluates were immediately diluted with an equal volume of PBS–Tween to dilute the methanol and reduce evaporation. The vials were sealed with tight polyethylene plugs and stored at −20 °C until they were analyzed.

Automation. SPE columns were conditioned with methanol and distilled water on a vacuum manifold. All other fluid transfers for SPE (sample loading, washing, and elution) were performed on a Gilson ASPEC 401 robotic system. The ASPEC program was prepared on an IBM PS/2 Model 50 and downloaded to the ASPEC's on-board memory for use.

All fluid transfers for the EIA were performed by a Beckman BioMek robotic pipetting workstation programmed with BioMek software version 2.1. Automated steps included plate coating, blocking, dilution of standards and samples in a 12 × 8 array of polypropylene tubes (1.4-mL TiterTube, Bio-Rad Laboratories), addition of MAb to standards and samples, and addition of antibody–sample mixtures and second antibody–enzyme conjugate to microplates. The EIA plates were washed manually three times after each incubation. For each wash, plates were emptied, flooded with PBST from a squirt bottle, emptied again, and rapped on lint-free paper towels to dry the wells.

Quantitative Indirect Competition EIA. The assay was performed essentially as described previously (Karu et al., 1994), but volumes were adjusted to 0.2 mL and the competition step was conducted in a final concentration of 12.5% methanol. Wells of Immulon II plates (Dynatech, Inc.) were coated with a subsaturating amount (250 ng) of diuron I–BSA in 0.2 mL. PBS–Tween containing 0.1% bovine γ-globulin (PBST–BGG) was the diluent for the MAb and the secondary antibody (alkaline phosphatase-conjugated goat anti-mouse IgG, Boehringer). Diuron MAb 481 was used at a limiting dilution of 1:1000, and the second antibody was diluted 1:5000. Standards and samples were incubated with the MAb in PBST–BGG–12.5% methanol overnight at 4 °C in sealed polypropylene tubes (TiterTube, Bio-Rad). Wells were blocked for 30 min at room temperature with PBST–BGG, the blocking solution was removed, and the standard and sample mixtures containing MAb were added to the wells for 2 h at room temperature. The plates were washed three times with PBST, incubated for another 2 h at room temperature with alkaline phosphatase-conjugated goat anti-mouse IgG (Boehringer), and washed again three times, and substrate solution (0.2 mL) was added as described previously. The rates of color development (ΔA_{405nm} per min) were determined from at least three readings of each plate on a Titertek Multiskan EIA reader interfaced with a Macintosh computer.

EIA Design. The objective was to obtain maximum information on reliability of the solid-phase extraction and the EIA. Each plate included 11 dilutions of diuron standard (0.05–200 ppb) in triplicate. There were two test samples on each plate (four dilutions in triplicate of the unspiked aliquot and four dilutions in triplicate of the aliquot with the 0.2 ppb pre-extraction spike). In addition, each plate had three wells each of two independently prepared 0.2 ppb check samples and four wells that received no analyte, as a negative control. Ten plates were run each day for 3 consecutive days, and eight plates were run on the fourth day.

Data Analysis. The EIA data were processed using AutoElisa, a program package that we recently developed specifically for large-volume, high-throughput quantitative EIA in regulatory laboratories (Karu et al., 1992). The data were fitted by a "parallel fitting" algorithm (a refinement of the classical four-parameter logistic equation) that uses the data for each unknown as well as the standards to compute parameters of the sigmoid response model. Data on all of the plates run each day were processed without editing by the analysts. All of the dilutions and replicates of each sample were used to calculate a mean and standard error of the diuron concentration in the original water sample. Standard error, which is the standard deviation of the mean, is also given as a measure of the confidence attributed to the mean value obtained for the estimated concentration of any one sample. The standard error includes the error contributed by the standards, as well as that due to replicates of the samples. Significant differences were identified by analysis of variance (ANOVA) and Tukey's post-hoc pairwise test for effects. ANOVA and other statistical results were computed using SYSTAT 5.2.1 on a Macintosh (Wilkinson et al., 1992). P values indicate the probability that the result could have occurred by chance. For example, $P < 0.01$ indicates less than 1% likelihood that a result is *not* significant.

RESULTS

Automated Fluid Handling. Although a direct EIA would have required less time to perform, diuron MAb 481 and the indirect EIA were used because this combination gave us the lowest limit of detection. The most error-prone steps in the assay—solid-phase extraction and the EIA—were performed on automated equipment. This was done primarily to ensure reproducibility, rather than to save time. The throughput of the ASPEC was limited by

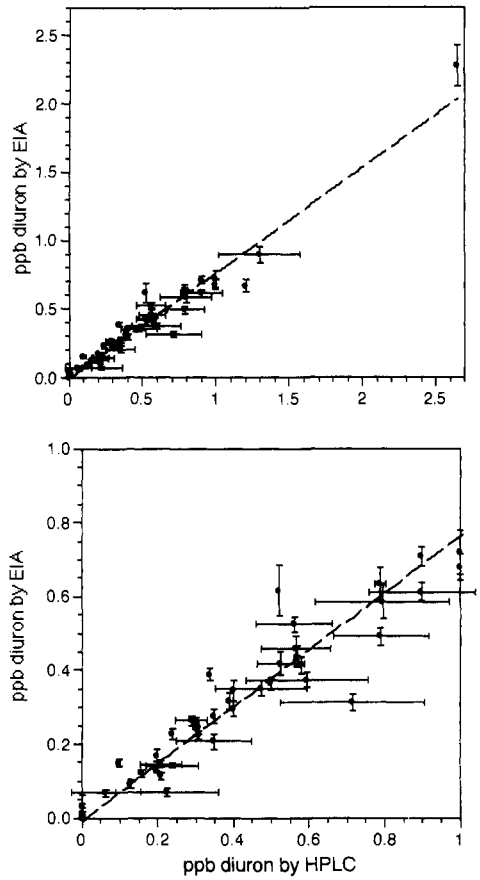

Figure 1. Correlation of estimates obtained by EIA and HPLC for the 49 blind-coded samples. HPLC estimates without estimated errors were single values obtained at CDFA. Bars on 17 of the samples are ± standard deviation of the mean of one HPLC determination at CDFA and one made at an independent laboratory. The range of 0–1 ppb is expanded in the bottom panel.

the maximum volumes it could transfer and the number of columns it could process at one time. SPE took several times longer on the ASPEC than it would have if done manually—more than 12 h to process 110 columns. However, the reproducibility was excellent, as demonstrated by comparison of standard curves, color development rates, and values obtained for the 0.2 ppb check samples (n = 5; mean = 0.188 ppb; SD = 0.03 ppb). This may be due in part to the fact that fluid transfers and column drying steps are accomplished by pressure, rather than vacuum.

Similarly, fluid transfers to set up the EIA were done more slowly by the BioMek system, but at least as accurately as manual pipetting, and with much less risk of error. The BioMek required about 10 min to set up each matrix of 96 tubes containing MAb and dilutions of samples and standards. The transfer of these samples to EIA plates, as well as the later additions of second antibody and substrate, required about 1 min per plate.

Data Analysis and Error Estimation. The standard curves were fitted and refined by a unique variation of the four-parameter logistic model which we refer to as "parallel fitting" (Karu et al., 1992). The standard errors of the diuron concentrations include a term to account for the concentration errors in replicates of the standards, as well as errors in replicates of the test sample. The reported values for the diuron concentration in each original sample was calculated from all dilutions and all replicates. Each sample's concentration was thus computed from 12 data points. The standard error of the mean concentration was approximately 5.8% (r^2 = 0.85) for the 55 test samples.

Table 1. Correlations between EIA and HPLC Determinations of Diuron

correlates	n	Pearson r^2
HPLC estimates (lab 1 *vs* lab 2)	17	0.904
EIA *vs* av HPLC est (both labs)	17	0.931
EIA *vs* HPLC est from lab 1	49	0.963
EIA *vs* HPLC est from lab 2	17	0.906

Performance of the EIA. The correlation between the EIA and HPLC results for all 49 samples is shown in Figure 1. The HPLC determinations were not done in replicate, so no comparable error bars could be drawn on the HPLC estimates. However, independent HPLC determinations were run by a third laboratory on 17 of the samples. The mean ± standard deviation for these samples is shown as a rough estimate of HPLC error in Figure 1. The linear least-squares regression fit of these data had a slope of 0.77, an intercept of −0.007 ppb, and a correlation coefficient (r^2) of 0.946. The slope indicates that the EIA estimates are biased approximately 23% below the HPLC estimates. The cause of this consistent bias in the estimates of the two methods was not identified, but it may have been at least partly due to differences in reference standards used to calibrate the EIA and HPLC. Table 1 lists the Pearson correlation coefficients between the HPLC and EIA estimates. The data indicate that the correlations between EIA and HPLC were at least as good as the correlation between the independent HPLC values. Because there were many more observations for the EIA *vs* HPLC estimate for lab 1 and we could not estimate confidence limits of the correlation coefficients, we cannot say that the differences in correlation were significant.

The top section of Table 2 summarizes the mean, range, and variation of the standard curve midpoints (I_{50}) and the values obtained for the 0.2 ppb check samples on each of the four days the assay was run. The bottom of Table 2 is a summary of the range and variation of the I_{50}s and slopes of the standard curves, the standard deviation of the standards about the fitted curves, and the values obtained for spikes of 0.2 ppb on each plate. The MAb culture fluids and Diuron I–BSA coating antigen appear to be stable indefinitely when stored in aliquots at −70 °C.

Statistical analysis of the results of an assay such as this one depends upon whether the results were independent and approximate a normal distribution (Massart et al., 1988). Probability plots (actual distribution of the values *vs* those expected from a normal distribution) of the I_{50} and slope values of all standard curves, the standard deviation of the standards about the fitted curves, and the values obtained for the two 0.2 ppb check samples included on each plate were all roughly linear, indicating that this criterion was met.

Table 3 summarizes an analysis of variance (ANOVA) performed on these data and on the standard curve slope and scatter of the standards about the fitted curves. Over the entire assay (38 plates) between-plate differences proved not to be significant for any of these parameters. Small but statistically significant differences in the I_{50} between 3 days, and in the estimates of the check samples between 2 days, were revealed by the ANOVA, as described in Table 3. Only two statistically significant differences occurred. The standard curve I_{50} values differed on days 2–4, and the values for 0.2 ppb check samples differed between days 1 and 2. Figure 2 summarizes the daily variations in the I_{50} of the standard curves and estimates of the 0.2 ppb check samples on each plate.

Recovery of the precolumn spikes gave an indication of the efficiency and reproducibility of the entire assay, including the SPE steps. Excluding one sample (for which no precolumn spike was run because of insufficient

Table 2. Summary Statistics for Variations in Standard Curves and Check Samples

	std curve I_{50} (ppb)				0.2 ppb check samples			
day:	1	2	3	4	1	2	3	4
no. of plates:	10	10	10	8	10	10	10	8
min	3.94	4.05	3.45	3.48	0.168	0.159	0.152	0.142
max	5.45	5.69	5.5	5.64	0.251	0.200	0.248	0.208
mean	4.47	5.07	4.35	4.29	0.211	0.176	0.186	0.180
±SD	0.47	0.50	0.61	0.74	0.03	0.01	0.03	0.02
±SE	0.15	0.16	0.19	0.26	0.008	0.004	0.011	0.008
% RSD	10.5	9.9	14.0	17.2	14.2	5.7	16.1	11.1

	I_{50}[a] (ppb)	slope[a]	SD of standards (ΔA_{405nm}/min)	0.2 ppb spikes[c] (ppb)
no. of plates:	38	38	38	37
min	3.45	1.04	0.008	0.142
max	5.69	1.81	0.954	0.251
mean	4.56	1.38	0.440	0.189
±SD	0.64	0.22	0.29	0.03
±SE	0.1	0.04	0.05	0.004
% RSD	14.0	15.9	65.9	15.9

[a] Parameters of the standard curve fitted by the four-parameter logistic equation. [b] Standard deviation of the standards about the fitted curve. [c] Data for two spike samples per plate, except plate 1 of day 3, from which the spikes were inadvertently omitted.

Table 3. Summary of ANOVA Test for Differences between Days and between Plates

variable	factor	significant variation?	P[a]
std curve I_{50}	days	yes	0.021[b]
	plates	no	0.732
std curve slope	days	no	0.117
	plates	no	0.408
SD of standards from fitted curve	days	slight	0.065
	plates	no	0.406
0.2 ppb check samples	days	yes	0.012[c]
	plates	no	0.902

[a] Probability that the variation is *not* significant. $P < 0.05$ signifies 95% confidence that the variation did *not* occur by chance. [b] Significant differences only between days 2 and 3 ($P = 0.041$) and days 2 and 4 ($P = 0.035$). [c] Significant differences only between days 1 and 2 ($P = 0.012$).

Table 4. Diuron Concentrations and Pre-extraction Spike Recoveries for 15 Samples Analyzed on Different Days

	est ppb ± SE[a]		% recovery of 0.2 ppb pre-SPE spike	
sample	1st EIA	2nd EIA	1st EIA	2nd EIA
179	1.901 ± 0.224	2.274 ± 0.148	217	102
239[b,c]	0.686 ± 0.038	0.541 ± 0.047	207	55
333[b,c]	0.333 ± 0.023	0.283 ± 0.022	138	88
361[b]	0.536 ± 0.022	0.463 ± 0.028	195	127
369[b]	0.153 ± 0.022	0.334 ± 0.022	47	83
373[b]	0.392 ± 0.046	0.482 ± 0.032	296	124
401	1.089 ± 0.061	0.856 ± 0.058	21	62
414	0.800 ± 0.044	0.692 ± 0.047	153	125
447[b]	−0.04 ± 0.002	0.0 ± 0.004	52	87
480[b]	0.499 ± 0.067	0.623 ± 0.044	−12	91
612[b]	0.183 ± 0.018	0.291 ± 0.019	39	116
625	0.822 ± 0.049	0.722 ± 0.036	149	114
627	0.729 ± 0.04	0.627 ± 0.03	115	105
628	1.252 ± 0.044	0.956 ± 0.053	81	72
629	1.319 ± 0.093	0.869 ± 0.052	113	60

[a] Values are averages of the determinations for the unspiked aliquot and the spiked aliquot with the spike subtracted. [b] For these samples, a second SPE was performed for the second EIA. [c] These samples contained sediment removed by the filtration step before SPE.

volume), the mean precolumn spike recovery was 96.4% (median = 94%; $n = 54$). The standard deviation was large (±43.5%). Recovery of the precolumn spike was less than 75% for 13 samples, of which 2 left deposits of sediment on the Nylaflo filters, and 10 samples had recoveries >125%, of which 3 left sediment on the filters. However, there was no significant correlation between the diuron concentration estimated by EIA and the percent of precolumn spike recovered, nor did the spike recovery correlate significantly with the date of assay.

Fifteen samples that had unacceptably low (<75%) or high (>125%) recoveries of the 0.2 ppb pre-SPE spike were subjected to a second EIA. The entire extraction was repeated for eight of these samples for which there were insufficient amounts of the first solid-phase extract to do the reanalysis. Table 4 summarizes these results. All but one of these samples contained more than 0.1 ppb of diuron, and the results of the first and second EIA were sufficiently close that the original classification of the samples as "positive" or "negative" remained unchanged. The spike recovery was improved for nearly all of the samples in the second assay. Although we speculate that the anomalous spike recoveries are likely to be due to matrix effects, no other information was available for these samples (such as pH or the presence of filterable sediment) to suggest a cause. We noted, however, that most of the changes in the diuron concentration estimates in the second assay were in the same direction as the changes in the spike recoveries.

Limit of Detection. Four types of data were available to estimate the detection limit. Each plate included four replicates of a negative "plate control" prepared with PBST–12.5% methanol. Second, five "procedure check samples" were prepared by subjecting glass-distilled water to SPE with and without the 0.2 ppb precolumn spike. Two or more of these were included essentially at random in the groups of samples assayed on each day. Third, 4 of the 49 well-water samples were identified to us as "known blanks" (diuron-free) prior to the study. Two more samples were identified as having less than 0.1 ppb of diuron by HPLC after the study. Finally, nine of the blind samples and all five procedure check samples gave values between 0 and 0.1 ppb in the EIA. Statistics for these groups of samples are summarized in Table 5.

The 38 negative "plate controls" had EIA values ranging from 0 to 0.073 ppb; 12 of these were zero. On the basis of these samples, the mean ± 3 SD (a common definition of the detection limit) was 0.067 ppb. Assuming the 5.8% standard error of the concentration estimate described above, the detection limit would be 0.074 ppb. Another definition, when the standard deviation of the estimate may vary with concentration of the sample, is that the detection limit is the concentration below which the standard deviation is no longer a function of the amount of sample (Liteanu and Rîca, 1980). A plot of the standard error of the mean *vs* concentration for these 38 samples

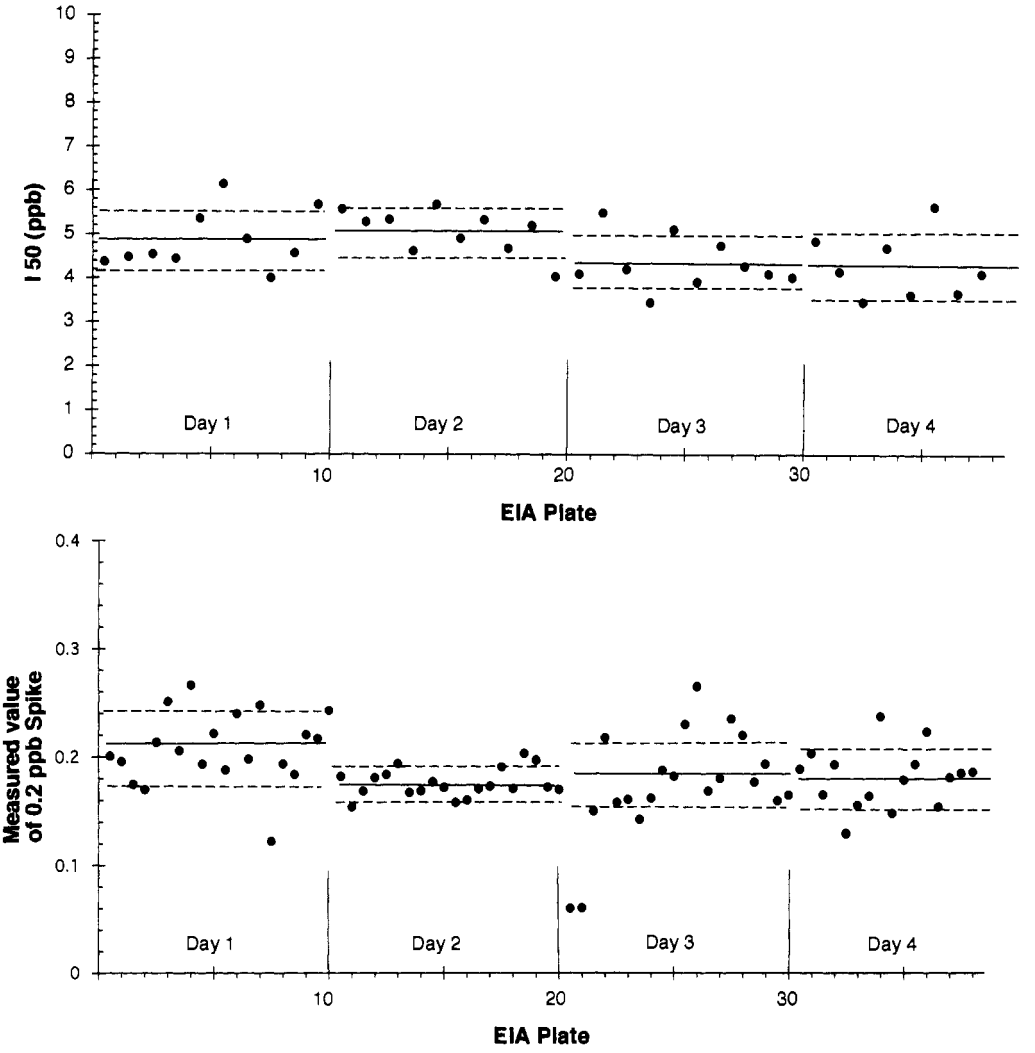

Figure 2. (Top) Day-to-day variation in the I_{50} of the EIA standard curves. (Bottom) Day-to-day variation in the EIA estimates of the 0.2 ppb check samples on each plate. Solid and dashed lines are the daily mean ± 1 SD, respectively. The two points less than 0.1 ppb in this panel were actually wells that did not receive the check samples, due to pipetting error. They were omitted in computation of the means for day 3.

Table 5. Samples Used To Estimate the Detection Limit

	EIA negative controls	procedure check samples	samples known to be <0.1 ppb before EIA	all samples <0.1 ppb in EIA[a]
no. of samples	38	5	4	9
ppb min	0	0	0	0
ppb max	0.073	0.015	0.034	0.092
ppb mean	0.013	0.006	0.020	0.035
ppb SD	0.018	0.006	0.016	0.034
SE of mean	0.003	0.003	0.008	0.011
median ppb	0.007	0.006	0.023	0.031
mean % recovery of 0.2 ppb pre-col spike	98.6	91.2	88.8	96.6

[a] Includes the four samples known to be <0.1 ppb before the EIA (previous column).

Table 6. Diuron Estimated by EIA in Blind-Coded Samples in the Presence and Absence of Bromacil

sample no.	diuron spike (ppb)	bromacil spike (ppb)	EIA est ppb ± SE[a]	diuron recovery[b] (%)
626	0.2	0	0.17 ± 0.02	85
624	0.2	0.4	0.17 ± 0.01	85
625	0.9	0	0.71 ± 0.04	79
627	0.9	1	0.71 ± 0.03	79
628	1.4	0	1.27 ± 0.04	91
629	1.4	1	1.31 ± 0.09	94

[a] Results computed from three replicates of four dilutions of each sample. Standard errors were calculated by the AutoElisa program. [b] Estimated from unspiked samples. Recovery of 0.2 ppb spikes added before SPE was between 81% and 114% for these samples.

showed that this point occurred around 0.035 ppb. All of the known blanks and the five procedure check samples were well below this value. On the basis of all of these results, it appeared reasonable to define the detection limit as 0.07 ppb.

Efficiency of Sample Classification. The correct classification of samples is a key index of the assay's reliability. False positives are defined as samples above the EIA detection limit but below the HPLC detection limit. False negatives are samples below the EIA detection limit but above the HPLC detection limit. With detection limits of 0.07 and 0.1 ppb, respectively, for the EIA and HPLC, there were no false positives and no false negatives among the 49 samples. If the detection limit for both assays is defined as 0.1 ppb, there would have been three false negatives by EIA (all ≥0.07 ppb).

Effect of Bromacil. After the EIA was completed, six of the unknowns were revealed to be laboratory preparations, of which three contained bromacil as well as diuron. The results for these are shown in Table 6. The estimates of diuron concentration were 79–94% of the actual diuron spikes. Furthermore, the recoveries of pre-extraction diuron spikes of 0.2 ppb that we added to each sample were between 81% and 114%, with no apparent relation to the amount of bromacil. These data indicate that the

presence of up to 1 ppb of bromacil had no detectable effect on the EIA determination of diuron.

DISCUSSION

The data from this collaborative study indicate that the solid-phase extraction and monoclonal EIA are sufficiently sensitive, accurate, and reproducible to be used in place of HPLC as an initial screening method for diuron in groundwater. The HPLC detection limit of 0.1 ppb required extraction of 500 mL of water, while only 200 mL was required for EIA with a detection limit between 0.07 and 0.1 ppb. The variation in the EIA between plates and from day to day could be followed by monitoring the I_{50}, standard deviation of the standards from the fitted curves, and recovery of check samples and method spikes. The fluctuations we observed were not greater than those that may be expected in HPLC determinations.

This study was conducted with more replicates and dilutions of samples and standards than would be practical for routine compliance monitoring. The HPLC data were individual values estimated from a standard curve that had only one value per concentration. This illustrates a common dilemma encountered in validation of EIAs *vs* an instrumental method which is assumed to be the "gold standard". Cost and time limitations prevented us from obtaining HPLC data with as many replicates and check samples as we used in the EIA. Nevertheless, the HPLC data used in this study meet the present CDFA criteria for accuracy, sensitivity, and reproducibility as a primary determinative method for compliance monitoring.

EIAs can be as rigorously quantitative as instrumental methods, and performance can be evaluated by most of the same criteria. Although pre-SPE spike recoveries were valuable indicators of the reliability of the entire method, most anomalous spike recoveries occurred with samples that had high diuron content (>0.5 ppb). Anomalous spike recovery appeared to be a random event because it did not correlate with errors in the estimated amount of diuron in the unspiked portions of the same samples. A low or high bias *vs* the instrumental results is frequently observed with EIAs. In this study the bias appeared not to be due to matrix effects or random interference, as it was proportional to sample concentration, and the Y-intercept of the regression line in Figure 1 is essentially zero in the absence of diuron.

The analysis of variance is valuable for identifying sources of error and parameters that are good indicators of deviation for quality assurance of EIA. In this study, fluctuations in the I_{50} of the fitted standards and the estimates of check samples on each plate were small, but they detected trends from day to day. In no case were any of the variables listed in Table 3 or any of the concentration estimates as great as 3 SD from the mean.

Automation reduces random analyst errors and improves reproducibility, but it can also introduce consistent errors. In one instance, a poorly fitting socket on a multitip pipet tool caused errors in the same well on all 10 plates that were run on a particular day. The logistics (such as volumes, time required for extraction, storage conditions), as well as the chemistry of residue recovery (solvents used, extraction method), must be adapted and scaled to available automated equipment, as well as to the EIA. Because of the number of replicates we used, automated SPE required substantially more time than the EIA. Consequently, we extracted all of the samples before we began the assays.

The results of this validation represent the performance of this EIA in routine practice, using the methods we describe. Changes in any of the major variables—extraction method, plate coating conjugate, incubation times, purity of MAb, etc.—would be expected to affect the correlation with HPLC.

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