Chapter 6

Monoclonal Immunoassay of Triazine Herbicides

Development and Implementation

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This paper summarizes a three-laboratory effort to develop a sensitive, reliable enzyme immunoassay (EIA) for triazine herbicides using monoclonal antibodies (MAbs). Simazine and atrazine haptens with mercaptopropionic acid and aminohexanoic acid spacers were synthesized and conjugated to proteins via N-hydroxysuccinimide active esters. MAbs derived from mice immunized with these conjugates had I_{50} values of 3 ppb to 4 ppm for various triazines in standard and simazine-enzyme conjugate competition EIAs. The EIAs are compatible with simplified methods for triazine extraction and concentration from soil and water. The limit of detection for atrazine was approximately 0.05 to 0.1 ppb, similar to that obtained with gas chromatography. EIA and GC results agreed closely for 75 groundwater samples, with no "false negatives." Gas-liquid chromatography and EIA data for simazine in 48 soil extracts had a correlation of 0.97. The EIA has also been used to monitor groundwater from beneath a toxic waste pit and water from agricultural evaporation ponds.

The s-triazines, first developed in the early 1950s (1), are among the most effective and widely used herbicides known. They are of 3 major types, based on the substituent at R1 (Figure 1): the chlorotriazines, of which simazine and atrazine are the most-used, methoxytriazines, such as prometon, and the methylthio triazines, of which ametryne and prometryne are representative. Atrazine has been cited as the second most-used pesticide in the United States, with an estimated annual usage on the order of 79 million lbs (2). Roughly 3 million lbs. of triazines — mostly atrazine, simazine, and prometon — were applied in California from 1983 through 1987, with the largest percentages used in non-agricultural applications, such as industrial soil sterilization, landscape maintenance, and clearing of rights-of-way (3).

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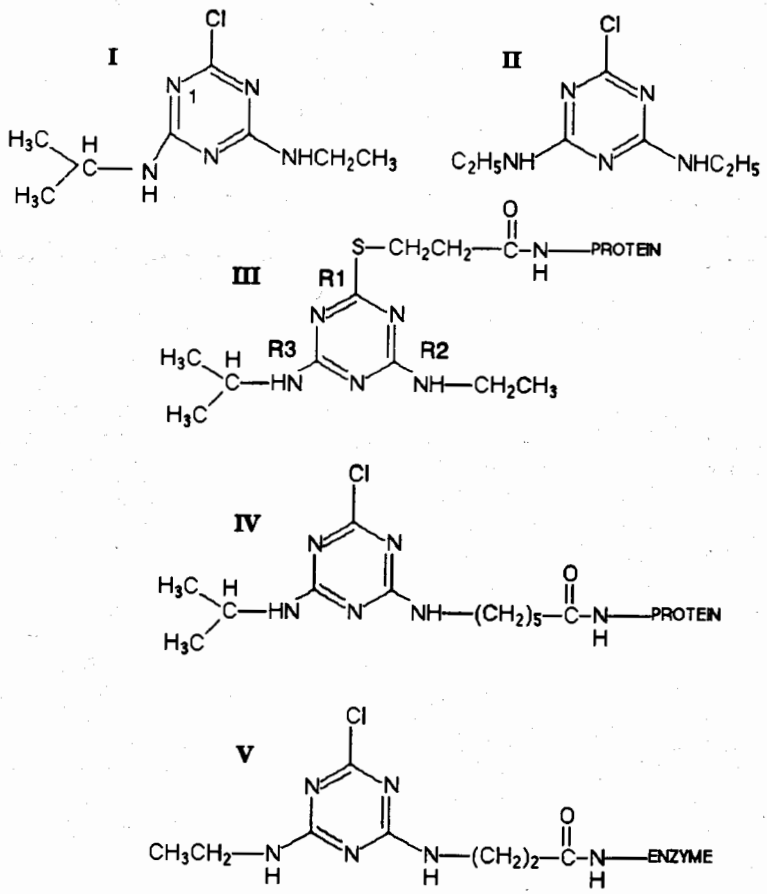

Figure 1. Atrazine (I), simazine (II), and haptens and conjugates used in this work. III — atrazine-mercaptopropionic acid hapten, and IV — atrazine aminohexanoic acid hapten, which were conjugated to BSA, CON, or KLH, and used as immunizing and EIA coating antigens. V — simazine-alkaline phosphatase "haptenated enzyme," used as the detector in competition EIAs where the monoclonal antibody was immobilized on the solid phase. Atoms on the triazine ring are numbered clockwise from N_1 shown in structure I.

Because of their different solubilities and modes of action, the triazines are selective to varying degrees in their effects on weeds and agriculturally important crops (4). Resistant plants dealkylate these compounds. Corn, sugarcane, and many other crops are naturally resistant, making triazines ideal for weed control on these crops. Various triazines can be used for pre- or post-emergence weed control, alone or in combination with other pesticides. Persistence of these compounds varies, and is a function of the soil properties and microbial ecology, and the climate. The triazines vary widely in their retention in various soils and their potential for leaching, and their mobility in groundwater is a good index of movement of other pesticides.

We undertook development of monoclonal antibodies and an immunoassay for triazines with sponsorship from the Environmental Monitoring and Pest Management Branch of the California Department of Food and Agriculture (CDFA), as part of a long-term plan to augment or replace more costly analytical methods with immunoassays, for regulatory purposes. The primary concern of the Environmental Monitoring and Pest Management Branch is groundwater. There are on the order of 40,000 domestic and municipal wells in California, and the state regulatory agencies analyze about 2,000 groundwater samples annually — primarily by gas chromatography (GC, 5). The number of wells CDFA must monitor will continue to increase, due to to recent legislation and increased public interest in water quality.

This report describes the initial results of a cooperative effort, in which haptens and conjugates were synthesized at UCD, monoclonal antibodies were derived and characterized at UCB, sample recovery methods were developed and initial feasibility tests with various types of field samples were conducted at UCD, UCB, and EMSL. The antibodies and assay methods were provided to the CDFA Analytical Laboratory in Sacramento, CA, in August 1989. Staff of that laboratory are in the process of validating the assay and acquiring data and experience that will be used to integrate the triazine EIA into their repertoire of tests for regulatory monitoring.

Methods

Details of the synthesis of haptens and conjugates, the production and characteristics of the MAbs, and optimization of the immunoassays, will be published separately (6; Schmidt et al., in preparation; Jung, et al., in preparation).

Synthesis of triazine haptens and hapten-protein conjugates. Simazine and atrazine were derivatized with mercaptopropionic acid (mpa) at R1, or aminohexanoic acid (aha) at R2, and these haptens were covalently linked to keyhole limpet hemocyanin (KLH), conalbumin (CON), or bovine serum albumin (BSA), by forming active esters with N-hydroxysuccinimide (6) (Figure 1, structures III and IV). This technique was also used to couple simazine-aminohexanoic acid to calf intestine alkaline phosphatase (Figure 1, structure V), for use as the "haptenated enzyme" in the EIA format described below.

Preparation of triazine-specific MAbs. Pairs of Swiss Webster, Biozzi, and B10.Q mice were immunized with 4 doses of one of the triazine-protein conjugates in Ribi adjuvant (MPL + TDM Emulsion, Ribi Immunochem Research, Hamilton, Montana) over 3 months. The sera showed wide variations in triazine-specific serum titers, limiting detectable dose and I_{50} (the dose giving half-maximal inhibition) in a competition EIA, using conjugates with a carrier and linker different from those of the immunizing antigen. Splenocytes from the four best-responding mice (two Swiss Webster and two B10.Q) were fused with P3X63AG8.653 myelomas, essentially as described by Fazekas de St. Groth and Scheidegger (7). 15,936 cultures were seeded (166 96-well culture plates), from which 3,156 colonies developed, and were screened for triazine-directed antibodies, again using conjugates with a carrier and linker different from those of the immunizing antigen. Of 232 triazine-specific antibodies, 74 were inhibited by free atrazine or simazine, and 36 of these proved to be genetically stable after several passages in culture. The 15 most sensitive MAbs had I_{50} values of 3 to 15 ppb for atrazine and 35 to 60 ppb for simazine, and all were of the IgGκ subclass. By contrast, the sera from the mice used to derive the hybridomas had I_{50} values of 100 to 200 ppb for atrazine and simazine.

The 5 most sensitive MAbs were subcloned by limiting dilution, and at least 12 clones of each cell line were frozen. Cultures were expanded to produce pools of 500 to 750 ml of antibody-containing culture fluid, which were used without purification in the assays. (Figure 2)

Enzyme Immunoassays. We carried out these studies with 3 variations of the competition EIA. Initial surveys of the responses in mice, screening and initial characterization of the hybridomas, and some of the method development studies were performed using a "classical" competition EIA, in which triazine in solution competed with atrazine-protein conjugate immobilized on the EIA plates, for binding a limiting amount of antibody, which was in solution. Most of the studies to optimize the quantitative EIA with soil and water extracts, and many of the specificity studies were carried out using a "haptenated enzyme" format, in which the MAb was immobilized on the EIA plate by trapping it with a goat anti-mouse antibody, and triazine in solution competed with a simazine-alkaline phosphatase conjugate for binding to the MAb. We recently perfected a more rapid and convenient version of this format, which was done as follows:

EIA wells (Immulon 2, Dynatech) were coated overnight at 4°C with 0.1 ml (approx. 200 ng) of affinity-purified goat anti-mouse IgG+IgM (Boehringer-Mannheim no. 605 24) 1:1,000 in "coating buffer" (0.015 M Na_2CO_3 — 0.035 M $NaHCO_3$ — 0.003 M NaN_3, pH 9.6). The wells were washed 3 times with "PBS-Tween" (0.01 M KH_2PO_4-K_2HPO_4, pH 7.4 — 0.15 M NaCl — 0.02% NaN_3 — 0.05% Tween 20), 0.1 ml of triazine MAb AM7B2 (hybridoma culture fluid, diluted 1:400 with PBS-Tween containing 0.5% bovine serum albumin) was then added to each well, the plates were incubated for 1 hr at room temperature, and then stored (with the fluid left in the wells) at -20°C in a sealed container to prevent evaporation until they were needed. At the time of assay, the EIA plates were thawed and washed 3 times with PBS-Tween. Standards and unknowns were diluted in PBS-Tween in microplates or polypropylene tubes, and mixed

with a limiting amount of simazine-N(C2)- alkaline phosphatase in PBS-Tween to give a final volume of 0.24 ml per well. Aliquots of 0.05 ml of these mixtures were then transferred to the EIA plates. The competition reaction was complete after 30 min at room temperature (Figure 3), at which time the plates were washed 3 times with PBS-Tween, and dried by rapping on lint-free paper towels. Substrate solution (1 mg/ml p-nitrophenyl phosphate in 10% (w/v) diethanolamine-HCl, pH 9.8 — 0.4 mM $MgCl_2$ — 3 mM NaN_3) was then added, and color development at 405 nm was monitored on an EIA reader.

Data Analysis. Standard curves (generally 11 dilutions in triplicate from a spectrophotometrically standardized stock solution) were fitted by iterative regression to the 4-parameter logistic equation (10) using Passage II™ (Passage Software, Inc., Fort Collins, CO) on a Macintosh computer, or Softmax™ software (Molecular Devices, Menlo Park, CA) on an IBM PC. Sample concentrations were determined by interpolation from the best-fit curves. Values that fell outside of the "working range," defined as 20% to 70% of the maximum normalized response, were not used.

Solid-phase Extraction of Atrazine from Water. Water samples of 100 to 220 ml were divided in two aliquots, one of which was spiked with atrazine standard to 0.2 ppb. C_{18} solid-phase extraction (SPE) columns (Analytichem "Bond-Elut") containing 100 mg or 300 mg resin were conditioned successively with 2 column volumes of pesticide analysis grade hexane, ethyl acetate, methanol, and glass-distilled water. The water samples were filtered through two layers of Whatman No. 4 paper to remove solids, and the filtrates were applied to the columns at 8 to 15 ml/min, followed by a wash with 2 column volumes of glass-distilled water. Triazines were eluted into glass tubes with a total of 2 ml of ethyl acetate. The eluates were evaporated to near-dryness under nitrogen, and dissolved in 1 ml of PBS-Tween. Generally, 5 dilutions of each sample were assayed in triplicate on each of two EIA plates, which also included atrazine standards in triplicate. This procedure shown schematically in Figure 4.

Solvent Extraction of Simazine from Soil. Soil samples of 10 grams (sandy loam with low organic carbon content) were dried at 80 °C, suspended in 10 ml of ethyl acetate, and shaken or sonicated at low power for 30 min. Solids were allowed to settle, and the extract was decanted. The soil was resuspended in 10 ml of ethyl acetate, and this second extract was added to the first one, and filtered through Na_2SO_4. These extracts were used directly for gas chromatography. For EIA, the ethyl acetate was evaporated to dryness, the eluate was reconstituted in 1 ml PBS-Tween, and aliquots were taken directly into the EIA.

Solid-phase Extraction of Atrazine from Soil. This procedure was modified from the method described by Hill and Stobbe (8). For studies involving spiked samples, atrazine standards in methanol were added to give the desired ng of atrazine per gram of dry soil, and the samples were dried again before extraction. Samples of 5 grams of "U.S. Army Standard Soil" were suspended in 10 ml of acetonitrile: water :: 9:1, and the slurry was sonicated (30 min,

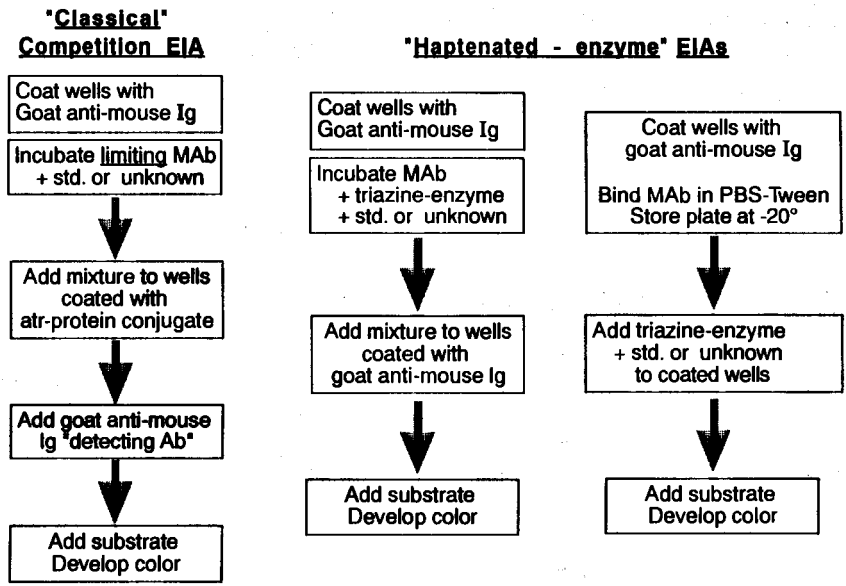

Figure 2. Flow diagrams of 3 competition EIA procedures. The "classical" competition EIA (left panel) was used to monitor the immunizations, select the hybridomas, and for several of the demonstration projects described in this paper. The haptenated-enzyme EIA in the center panel was used for most of the method development. This is the assay that is presently being evaluated by the CDFA analytical laboratory. The simplified haptenated-enzyme EIA in the right panel is described in Methods.

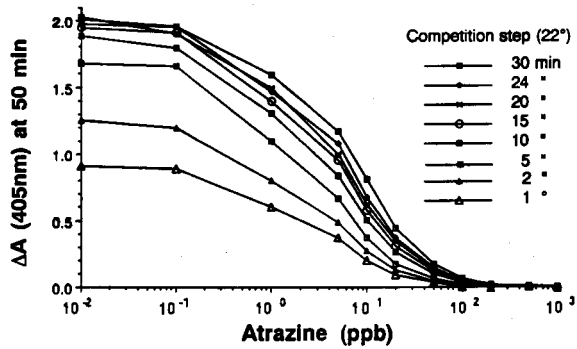

Figure 3. Kinetics of the competition step in the simplified "haptenated-enzyme" EIA. The assay diagrammed in the rightmost panel of Fig. 2 was conducted at room temperature as described in Methods. Mixtures of atrazine standards and simazine-alkaline phosphatase conjugate in PBS-Tween were added to rows of EIA wells coated with MAb AM7B2, which was "trapped" on the wells by affinity-purified goat anti-mouse IgG. At the times indicated, the wells were rinsed, substrate solution was added, and the absorbance was read 50 min later.

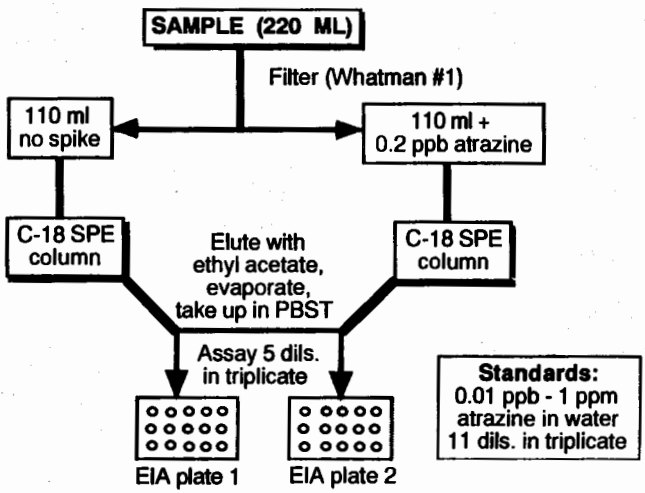

Figure 4. Flow chart for recovery and EIA measurement of atrazine in water.

Branson B12 sonic bath). The extracts were decanted, centrifuged (10 min, 10,000 x g) to remove particulate material, and 0.01 volume of glacial acetic acid was added. These solutions were applied to SCX aromatic sulfonic acid SPE columns (Analytichem) containing 300 mg of resin. The columns were washed with 5 ml of 1 M K_2HPO_4, and atrazine was then eluted with 2 ml of acetonitrile: 0.1 M K_2HPO_4 :: 1:1. The eluates were diluted to 5 ml with PBS-Tween, and dilutions were analyzed by EIA.

Results

Derivation of MAbs We used 3 strategies to obtain MAbs with the greatest sensitivity and specificity: First, to maximize the chances of evoking different repertoires of antibodies, we tested simazine and atrazine haptens with two different linker groups (aha or mpa) on each of 3 different carriers (BSA, CON, and KLH) as immunizing antigens in pairs of 3 strains of mice (Swiss Webster, Biozzi, and B10.Q). The responses to the triazine were quantified by EIA on wells coated with a conjugate that had a linker and carrier different from the immunizing antigen. Second, for hybridoma production we selected only the best responding mice, with respect to serum titer, lowest detectable dose, and I_{50} for atrazine and simazine. Third, we prepared and screened a large number of hybridomas. Although all of the immunizing antigens evoked good triazine-directed responses in most of the mice, the statistics cited in the Methods section demonstrate that the most sensitive MAbs were only a small percentage of all of the triazine-directed MAbs.

Specificity of the MAbs. At UCB we compared the specificity of the MAbs using the "classical" competition EIA, which measured the ability of various triazines to compete with atrazine conjugates (immobilized on the EIA wells) for binding the MAbs. A similar set of experiments at UCD was done using the "haptenated enzyme" EIA format, with simazine-N(C2)-alkaline phosphatase as the competitor.

Table I summarizes the relative recognition of 37 triazine analogs and haptens, by MAbs AM7B2 and AM5D1. The results using the two different EIA formats and simazine-N(C2)-alkaline phosphatase were essentially the same for 7 of the most-used triazines. These results can be summarized as follows: (a) Propazine, procyazine, and cyanazine were recognized better than atrazine. Atrazine-mercaptopropionic acid, which was the hapten used to elicit the antibodies, was also recognized better than atrazine by both MAbs. This indicated that the MAbs bound better to analogs with isopropyl, cyclopropyl, or cyanoisopropyl groups at R2 or R3. (b) Both MAbs were much less reactive with prometon, which is used in substantial amounts in California and elsewhere, than they were for atrazine and simazine. (c) Hydroxyatrazine and hydroxysimazine reacted only 1% to 5% as well as atrazine. (d) The mono-dealkylated triazines reacted 0.1% to 0.2% as well as atrazine, and (e) these MAbs did not measurably (< 0.2%) recognize di-dealkylated triazines. Thus, the MAbs are not effective probes for these triazine metabolites.

Table I. Relative reactivity of triazine MAbs AM7B2 and AM5D1 with various triazines and triazine haptens.

	Compound	R1	R2	R3	% cross-reactivity AM7B2	AM5D1
1	procyazine	Cl	$NHCH(CH_2)_2$[a]	$NHCCN(CH_3)_2$	526	583
2	atrazine-mpa	$S(CH_2)_2COOH$	$NHCH_2CH_3$	$NHCH(CH_3)_2$	261	181
3	propazine	Cl	$NHCH(CH_3)_2$	$NHCH(CH_3)_2$	196	161
4	cyanazine	Cl	$NHCH_2CH_3$	$NHCCN(CH_3)_2$	106	116
5	atrazine	Cl	$NHCH_2CH_3$	$NHCH(CH_3)_2$	100	100
6	dipropetryne	SCH_2CH_3	$NHCH(CH_3)_2$	$NHCH(CH_3)_2$	95	68
7	simazine-mpa	$S(CH_2)_2COOH$	$NHCH_2CH_3$	$NHCH_2CH_3$	66	76
8	simazine	Cl	$NHCH_2CH_3$	$NHCH_2CH_3$	31	31
9	prometryne	SCH_3	$NHCH(CH_3)_2$	$NHCH(CH_3)_2$	30	16
10	tertbutylazine	Cl	$NHCH_2CH_3$	$NHC(CH_3)_3$	23	22
11	terbutryne	SCH_3	$NHCH_2CH_3$	$NHC(CH_3)_3$	21	17
12	atr-N(C5)-COOH	Cl	$NH(CH_2)_5COOH$	$NHCH(CH_3)_2$	21	24
13	sim-N(C5)-COOH	Cl	$NH(CH_2)_5COOH$	$NHCH_2CH_3$	16	19
14	ametryne	SCH_3	$NHCH_2CH_3$	$NHCH(CH_3)_2$	14	14
15	sim-N(C4)-COOH	Cl	$NH(CH_2)_4COOH$	$NHCH_2CH_3$	8.2	12
16	cyanazine amide	Cl	$NHCH_2CH_3$	$NHCCONH_2(CH_3)_2$	6.5	6.2
17	hydroxyatrazine	OH	$NHCH_2CH_3$	NHCH(CH3)2	5.7	4.1
18	prometon	OCH_3	$NHCH(CH_3)_2$	NHCH(CH3)2	5.1	3.3
19	terbumeton	OCH_3	$NHCH_2CH_3$	$NHC(CH_3)_3$	5	4
20	simetryne	SCH_3	$NHCH_2CH_3$	$NHCH_2CH_3$	4.4	4.7
21	sim-N(C3)-COOH	Cl	$NH(CH_2)_3COOH$	$NHCH_2CH_3$	3.8	4
22	atratone	OCH_3	$NHCH_2CH_3$	$NHCH(CH_3)_2$	2.3	2.3
23	trietazine	Cl	$NHCH_2CH_3$	$N(CH_2CH_3)_2$	1.8	1.7
24	atr-N(C2)-COOH	Cl	$NH(CH_2)_2COOH$	$NHCH(CH_3)_2$	1.5	1.1
25	hydroxysimazine	OH	$NHCH_2CH_3$	$NHCH_2CH_3$	1.3	1.1
26	desmetryne	SCH_3	$NHCH_3$	$NHCH(CH_3)_2$	1.2	1.1
27	sim-N(C2)-COOH	Cl	$NH(CH_2)_2COOH$	$NHCH_2CH_3$	1.2	1.5
28	desethyl simazine	Cl	NH_2	$NHCH_2CH_3$	0.9	1
29	desethyl atrazine	Cl	NH_2	$NHCH(CH_3)_2$	0.7	0.8
30	desethyl simetryne	SCH_3	NH_2	$NHCH_2CH_3$	0.2	0.3
31	atr-N(C1)-COOH	Cl	$NHCH_2COOH$	$NHCH(CH_3)_2$	<0.2	<0.2
32	sim-N(C1)-COOH	Cl	$NHCH_2COOH$	$NHCH_2CH_3$	<0.2	<0.2
33	didesethyl simazine	Cl	NH_2	NH_2	<0.2	<0.2
34	ammelide	NH_2	OH	OH	<0.2	<0.2
35	ammeline	NH_2	NH_2	OH	<0.2	<0.2
36	melamine	NH_2	NH_2	NH_2	<0.2	<0.2
37	cyanuric acid	OH	OH	OH	<0.2	<0.2

NOTE: The assays were conducted using the haptenated-enzyme competition EIA shown in the middle panel of Figure 2, with simazine N(C2)-alkaline phosphatase as the competitor. Stocks of each analog were prepared by weight, and their molar concentrations were calculated from the molecular weight. The concentrations of each analog giving half-maximal inhibition (I_{50}) were calculated from multi-point dose-response curves, and the "percent cross-reactivity" is the ratio of the I_{50} for the analyte to the I_{50} of atrazine.

[a] (cyclopropyl)

Quantitative EIA for triazines. The MAbs were compatible with the 3 variations of the competition EIA shown in Figure 2. For the "haptenated-enzyme" format a simazine-enzyme conjugate proved to be better than an atrazine-enzyme conjugate for detection of atrazine. Simazine is recognized only about 30% to 40% as well as atrazine. This enables free atrazine to compete better than the simazine-enzyme detecting conjugate, making the assay more sensitive. The optimized "haptenated-enzyme" EIA done with simazine-alkaline phosphatase conjugate proved to be about 5-fold more sensitive than the conventional competition EIA using atrazine-protein conjugates as competitor.

The working range of the EIA standard curves was generally from 0.7 to 70 ppb, with an I_{50} of about 13 ppb. Thus, using a sample concentration step of about 100-fold for the EIA brought the limit of detection (an inhibition of 2 standard deviations from the signal with zero analyte) down to, or below that of gas chromatography, i.e., *0.01 to 0.02 ppb. Regardless of format, the EIA is very economical; it requires less than 50 ng of triazine conjugate and less than 1 µl* of MAb culture fluid (which could be used as filtered hybridoma culture fluid without additional processing) per well. The culture fluid could be freeze-dried and reconstituted with no significant loss of triazine binding capacity, and EIA plates coated with captured MAb could be stored frozen until they were needed. The maximum response of the EIA in these was lower, but the I_{50} and slope values were the same as with plates prepared the night before use. These properties lengthen shelf-life and improve quality control. Furthermore, the MAbs developed for this study tolerated at least 20% (v/v) methanol in the PBS-Tween buffer used for the competition step. This makes it easier to use solvent- and solid-phase extracts in the assay, and may reduce sequestration of analytes in lipid micelles from various sample matrices.

Variability between assays is an important consideration for regulatory applications of EIA. Figure 5 is a plot of the I_{50} values and slopes of the standard curves for 17 consecutive "classical" competition EIAs performed at UCB during June and July 1989, using plates coated with atrazine-aha-BSA. The I_{50} values remained in the same range through December 1989, indicating that there was no apparent deterioration of this conjugate.

Variation when the same assay is done by different analysts is also a concern. At UCD, a study was conducted in which an immunochemist with several years' experience and a graduate student newly trained in EIA each analyzed 56 well water samples containing 0 to 0.25 ppb, using the haptenated enzyme EIA shown in Figure 2 (center panel). The results these persons obtained correlated with a slope of 1.08 and r = 0.98 (data not shown).

Sample Extraction Methods. Solid-phase recovery of atrazine and simazine on C_{18} columns proved to be convenient and efficient, using the method described above. Recovery of atrazine as a function of sample volume was assessed by gas chromatography. As Table II indicates, quantitative recovery of a 200 ng spike was achieved for samples of up to 1 liter. Efficiency was measured by recovery of [^{14}C]atrazine, as well as by gas chromatography (using a nitrogen-phosphorus detector). The results, summarized in Table III, indicate that recovery is nearly 100% from 0.1 to 100 ppb.

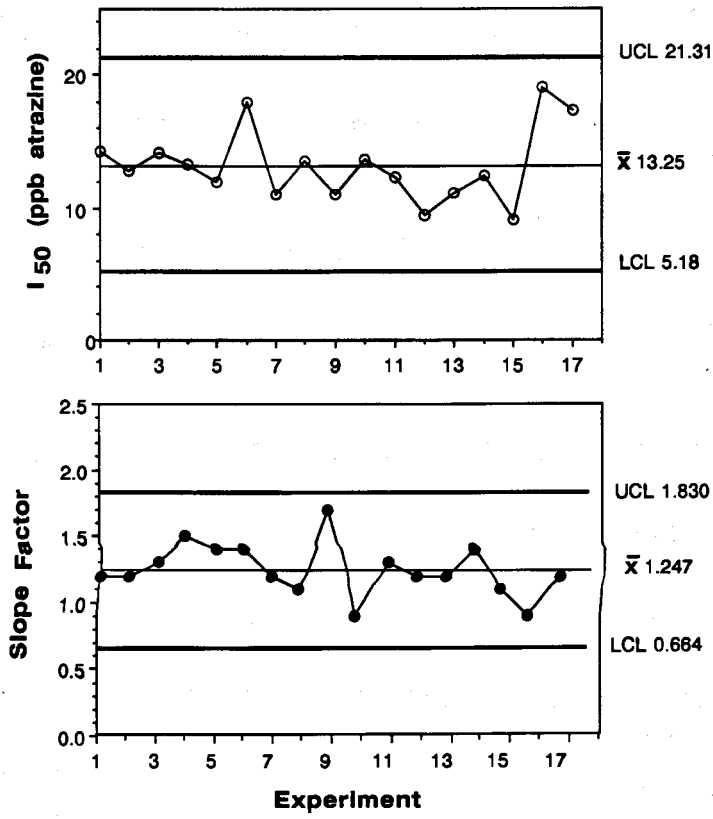

Figure 5. Quality control charts for 17 atrazine EIAs conducted by the "classical" method (Fig. 2, left panel) at U.C. Berkeley. Wells were coated overnight at 4° with 50 ng of atrazine-aha-BSA. Mixtures of standards and MAb AM7B2 in PBS-Tween were incubated overnight at room temperature, and then applied in 0.1 ml to the coated wells after they were rinsed 3 times with PBS-Tween. After 2 hr at room temperature, the wells were rinsed 3 times, and 0.1 ml of a 1:1,000 dilution of alkaline phosphatase-conjugated goat anti-mouse immunoglobulin (Sigma) was added. The plates were incubated for 2 hr at room temperature, rinsed again, substrate solution was added, and the color development was read on an EIA reader. The standard curves were fitted as described in Methods, to derive the I_{50} and slope values. The upper and lower confidence limits are one standard deviation from the mean.

Table II. Recovery of atrazine by C_{18} solid-phase extraction, as a function of sample size.

Sample vol. (ml)	atrazine recovered (ng)	%
100	220	110
250	210	105
500	200	100
1,000	200	100

NOTE: Duplicate samples of distilled water as indicated were spiked with 200 ng of atrazine from a reference standard in methanol, and applied to C_{18} columns (Analytichem Bond-Elut, 100 mg resin) at 8 to 15 ml/min. Columns were washed and eluted as described in Methods, and the eluates in ethyl acetate were analyzed by gas chromatography, using a nitrogen-phosphorus detector. Data were quantified as peak areas, relative to reference standards.

Table III. Recovery of atrazine from water using Analytichem C_{18} Bond-Elut™ columns.

Atrazine spike (ppb)	[^{14}C]atrazine recovered (cpm ± s.d.)	%	Recovered (GC analysis) (ng)	%
0	25 ± 2	—	nd	nd
0.01	2,172 ± 67	96.6	nd	nd
0.1	2,321 ± 60	103.3	nd	nd
1	2,307 ± 43	102.7	45	60.2
10	2,265 ± 55	100.8	746	99.5
100			7,350	98.0
1,000			74,100	98.6

NOTE: Samples of 75 ml of distilled water were spiked with the indicated amounts of atrazine, from reference standards in methanol. Where indicated, 2,266 ± 67 cpm of ring-labeled [^{14}C]atrazine was added to each spike. Samples were applied and recovered from 3 separate C_{18} columns as described in Methods, and the eluates were counted by liquid scintillation, or analyzed by gas chromatography (GC) using a nitrogen-phosphorus detector. Because of co-eluting contaminants, the limits of detection and quantification for GC were 2 ppb (3 std. dev.) and 7 ppb (10 std. dev.) respectively. The low (60.2%) recovery determined by GC for a 1 ppb spike appeared to be due to detector suppression by co-extracted material.

(nd = not determined)

EIA and GC results were compared for the analysis of simazine in soil, using ethyl acetate for extraction of dried samples of sandy loam from a site contaminated by an experimental simazine spill. For 24 samples that had simazine content between 0 and 350 ppb by GC analysis, the results by EIA correlated with r = 0.93 and a slope of 1.26 (Figure 6). For an extended data set of 48 samples containing simazine from 0 to 3 ppm, the correlation between EIA and GC determinations was 0.97, with a slope of 0.81 (A. Lucas, unpublished data). To ensure solubilization of the simazine recovered from the most heavily contaminated samples, methanol was added to the PBS-Tween to 5% (v/v) as cosolvent. Atrazine residues were recovered by extraction with 90% acetonitrile and concentration on SCX solid phase columns. For samples spiked with 10, 25, 50, and 100 ppb (ng atrazine per gram of soil) recoveries of 80%, 82%, 79%, and 93%, respectively, were obtained.

Demonstration Projects. During 1989 we conducted several studies to determine the accuracy, precision, and robustness of the EIA for quantifying triazines in various sample matrices. The U.C. Davis and U.C. Berkeley laboratories collaborated in EIA tests of well water samples that had been analyzed for triazine by gas chromatography at CDFA. Figure 7 is a bar chart of the triazine content of 75 of these samples, determined by the haptenated enzyme EIA. Three major results were evident from this study. First, the limit of detection of the EIA (the SPE blank in Figure 7) was below the limit of approx. 0.05 ppb for GC. *Second, all of the samples that showed detectable amounts of triazine by* GC also registered positive by EIA; in other words, there were no "false negatives" in the survey by EIA. Thirty-six of these samples were also analyzed at UCB, using the conventional EIA. Again, there were no "false negatives," and the results obtained by the two laboratories using different EIA methods correlated with a coefficient of 0.87. Third, the precision of the EIA was slightly better than that of the GC method, as shown by the coefficients of variance for the paired samples in Figure 7.

The UCB group also conducted a survey of groundwater from test wells sunk to different levels in and around a toxic waste site contaminated with atrazine. The data from this study are summarized in Table IV. Two groundwater samples from test wells contained high concentrations of triazines. Confirmatory values for these samples were obtained by the remediation site contractor using EPA Method 619. Two points can be made from these data. First, measurements obtained by recovering the triazines on C_{18} SPE columns were higher that those obtained when the samples were assayed directly after filtration through Whatman #1 paper. Second, the remediation contractor's gas chromatographic analysis of the samples from wells 4 and 5a revealed ppb to ppm levels of other contaminants, including 2,4,-D, 2,4,5,-T, MCPA, and xylene. These did not interfere appreciably with the triazine immunoassay.

The UCB group also performed triazine EIAs on samples from agricultural evaporation ponds in the San Joaquin Valley. These ponds collect drainage from fields where triazines may be used, and thus have the potential for accumulating these, and other pesticides. Metal ions, salts, and suspended solids accumulate in amounts up to 20 times those found in sea water, and various species of bacteria, algae and even brine shrimp may propagate in water from these

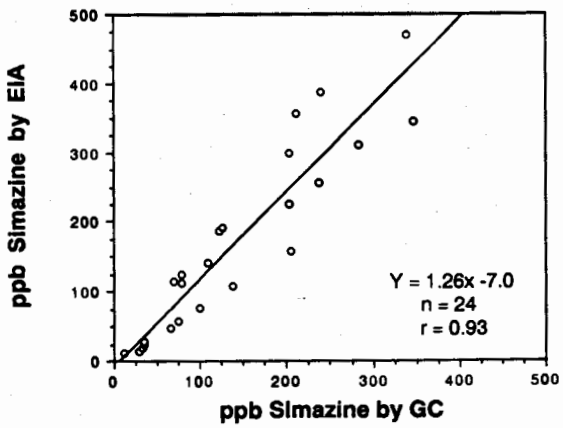

Figure 6. Comparison of soil analyses by gas chromatography and monoclonal EIA. Simazine-contaminated soil samples were extracted with methanol as described in Methods. Portions of the extract were analyzed by gas-liquid chromatography (GLC) using a nitrogen-phosphorus detector. The remainder of each extract was analyzed using the haptenated-enzyme EIA diagrammed in the center panel of Figure 2. The solid line was obtained by linear regression.

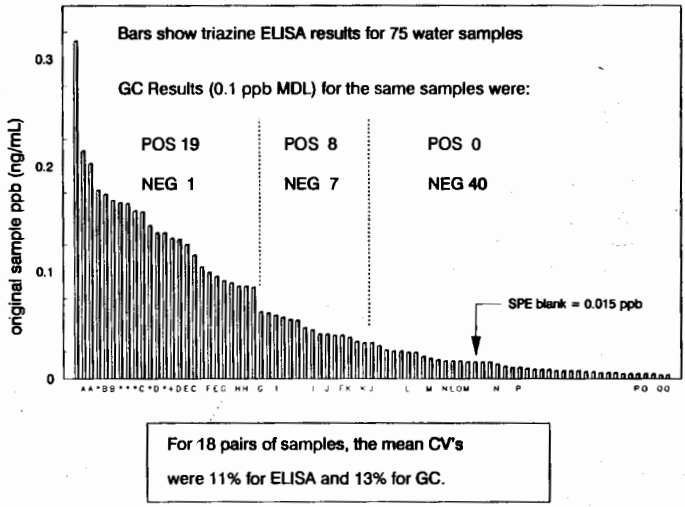

Figure 7. Summary of EIA results for 75 samples of well water. These samples were analyzed by gas chromatography (GC) at the CDFA analytical laboratory, and by the haptenated enzyme EIA (Figure 2, center panel) at U.C. Davis. For the EIA, triazine was recovered from the water samples essentially as diagrammed in Figure 4. Samples indicated by the same letter were quadruplicates taken at the wellhead, and analyzed in duplicate by EIA and GC. These samples are the basis for the precision comparison noted. Samples marked (*) are replicate determinations of one sample (mean ± SD, 0.16 ± 0.01 ppb; CV = 8.8%). Its duplicate sample is marked (+).

Table IV. Application of the triazine EIA to analysis of contaminated groundwater at a toxic waste pit.

Description	Appearance of C_{18} extract	Atrazine Spike (0.2 ppb)	Assay A	C_{18} Column Extracts Assay B Plate 1	Assay B Plate 2	Assay C Plate 1	Assay C Plate 2	EPA Method 619†
			ppb triazine (atrazine equivalent, mean ± std. error) *					
Groundwater, test well approx. 100 ft east of toxic site	clear	-		≤ 0.08				
		+		0.18±0.03	0.29±0.05			
Groundwater, well MA-4	turbid	-	18.3	> 7.1		< 45	> 7, < 34	29
		+				< 45	> 7, < 34	
Groundwater, well MA-5a	yellow, with odor	-	465±36	> 7.1		633±74	541±58	510
		+				536±13	513±28	
Groundwater, domestic well about 150 ft west of toxic site	clear	-		< 0.08				
		+		0.19±0.03	0.26±0.01			
glass-distilled H_2O		-	< 0.1	< 0.08		< 0.1		
"		+ 0.2 ppb[a]		0.23±0.02	0.28±0.01			
"		+ 0.2 ppb[b]		0.19±0.01	0.23±0.03			
"		+ 100 ppb	104±18					

NOTE: Samples were collected at a remediation site in Northern California, concurrent with a scheduled quarterly sampling by the EPA-certified remediation contractor. Groundwater samples were obtained from two 3 inch test wells, MA-4 (150 feet deep) and MA-5a, (49 feet deep) at the edge of the toxic waste pit. To ensure that groundwater seepage was being measured accurately, ten well volumes were pumped from these pipes into a waste tank before the test samples were taken. The samples were transported to UCB in foil-covered glass bottles with foil-lined caps, on ice. Three assays were performed. In each case, half of the sample volume was unspiked, and the other half was spiked to a concentration of 0.2 ppb with atrazine before concentration or cleanup. In Assay A, the samples were filtered through 2 layers of Whatman #1 paper and analyzed directly. For Assays B and C, the filtrates were applied to C_{18} solid-phase columns (Fisher Prep-Sep) and the triazine was recovered and analyzed as described in Methods. After assay B was run, it was apparent that some samples exceeded the anticipated range. Assay C was then run using greater dilutions of the samples recovered from the C_{18} columns. Controls consisted of 0.2 or 100 ppb of atrazine in glass-distilled water. Four or 5 dilutions of each sample were measured by EIA with MAb AM7B2, and only the values falling between 0.2 and 0.8 of the full range of the standard curve were used. The data are the mean ± standard error for triplicate samples — i.e., 3 separate C18 column eluates, in Assays B and C.

* Values preceded by < are below the indicated minimum detection limit.
Values preceded by > are above the indicated maximum amount measurable in this assay.
Data without standard errors represent only one value in the working range.

† Data reported by EPA contractor to Calif. Regional Water Quality Control Board, July 1989

[a] Data from Assay B, plates processed 6/30/89 [b] Data from Assay C, plates processed 7/5/89

ponds. Table V shows results of EIAs on water from 3 evaporation ponds that were potential accumulators of triazines, and samples of sump and canal water from an area not subject to triazine application. Unspiked and spiked samples of the evaporation pond water showed an extreme matrix effect when they were added directly to the EIA. This effect was greatly reduced when the samples were subjected to the C_{18} solid-phase extraction protocol described in Methods. However, a significant matrix effect remained, as was evident from the values obtained for spikes of 0.5 ppb recovered from these samples. We speculate that the high metal and salt content in these samples may create an inhibitory "matrix effect" that could account for all of the "triazine" estimated in the unspiked samples. Additional studies are under way to determine and eliminate the cause of this bias in samples of this type.

Summary and Conclusions

The thiocarbamate herbicides described by Gee, et al., (this volume) and the triazines discussed in this paper are the first of several herbicides for which we plan to develop monoclonal antibodies and sensitive immunoassays for CDFA. Monoclonal antibodies offer the advantages of defined affinity and specificity, adaptibility to virtually any immunoassay format, and potentially unlimited supply. These advantages are of particular importance to agencies such as CDFA, that intend to configure and validate the immunoassays for regulatory purposes.

Although we can not draw many conclusions about structure-activity relationships from these data, the antibodies we generated showed a preference for binding to triazines that have isopropyl groups at R2 and R3. The substituents are clearly the major determinants of specificity, as shown by the very poor recognition of the mono- and di-dealkylated triazines and the hydroxytriazine metabolites. Detection of hydroxytriazines may be important for some environmental monitoring applications, because they are indicators of exposure of plants and soil microorganisms to the parent compounds. However, the hydroxytriazine metabolites are not herbicides, and they are not defined as hazardous pollutants. A recent paper by Schlaeppi, et al. (9) described the production of MAbs that were specific for hydroxyatrazine, using hydroxyatrazine conjugates as immunizing antigens.

Results with the three EIA formats used in this study were very similar. However, use of the simazine N(C2)-enzyme conjugate as the competitor in the haptenated enzyme format gave this method a more sensitive limit of detection than the classical competition EIA. The specificity of the EIA is primarily characteristic of the MAb that is used, although it may vary slightly with different EIA formats. The major advantage of the haptenated enzyme format was that its lower detection limit enabled one to work with smaller amounts of environmental samples. However, this format was also more sensitive than the classical competition EIA to inhibition by organic solvent in the incubation solution.

Tables II and III demonstrate that recovery of atrazine was quantitative from the small C_{18} solid-phase columns used for the analysis of groundwater and soil samples. Quantitative recovery was obtained from up to 1 liter of water,

Table V. Analysis of triazines in water from agricultural evaporation ponds.

Sample	Atrazine spike (ppb) *		
	0	1	10
Evap. pond A	8.7	10.8	NT
Evap. pond B	6.2	9.8	19
Sump T4	<0.2	NT	9.6
San Luis Canal	<0.2	NT	7.4

NOTE: Samples of water from 3 agricultural evaporation ponds in the San Joaquin Valley, and from a drain sump (T4) and the San Luis water delivery canal, were analyzed without concentration or cleanup. The samples and analyses of their ionic content were provided to us by the California Central Valley Regional Water Quality Control Board.

*NT = not tested

Sample	Matrix	Atrazine spike (0.5ppb)	ppb triazine *(atrazine equiv., mean ± std. error)* *	
			plate 1	plate 2
Evap. pond A	Mo > 6 mg/l	-	0.20 ± 0.03	0.13
	As > 1 mg/l	+	0.92 ± 0.06	0.82 ± 0.02
Evap. pond B	Se > 1.5 mg/l	-	0.13 ± 0.03	≤ 0.09
	SO_4 ~ 18 g/l	+	0.83 ± 0.08	0.74 ± 0.01
Evap. pond C	Cl^- = 16 g/l	-	0.09	≤ 0.09
	SO_4 ~ 21 g/l	+	0.87 ± 0.08	0.82 ± 0.02
glass-distilled water		-	≤ 0.09	≤ 0.09
		+	0.66 ± 0.07	0.64 ± 0.03

NOTE: Residues recovered from the samples described above, and one other evaporation pond, using the SPE procedure for groundwater described in Methods.

* Values preceded by ≤ are below the indicated minimum detection limit.

Data without standard errors represent only one value in the working range.

and the detection limit of the assay was less than 0.1 ppb. To date, we have not examined the efficiency with which other triazines can be recovered, primarily because of the preference of the MAbs for atrazine. We speculate that if a method could be identified for selectively recovering triazines other than atrazine, it could be interfaced with the EIA using our MAbs, for single-analyte analysis.

The methods we adapted for recovery of atrazine and simazine from water and soil are faster and less involved than the recovery and cleanup procedures used for GC analysis. The recovery study in Table III demonstrated that our protocol for C_{18} solid-phase extraction recovered 100% of the atrazine from ordinary groundwater samples. In this experiment, data for spikes less than 1 ppb could not be obtained by GC, due to limitations of the detector and interference from co-extracted material. However, in other experiments, such as our studies on the 75 well water samples, the EIA was able to precisely quantify levels over 0.1 ppb. For example, 6 replicates of one sample in Figure 7 gave 0.16 ppb with a coefficient of variation of 8.8% (data not shown). Thus, the minimum detection limit of the EIA for atrazine appeared to be lower than that of GC. The recovery of atrazine from methanol extracts of soil was similarly efficient for the experiment of Figure 6. These results and the results of the toxic site groundwater study (Table IV) demonstrate that the monoclonal EIA is useful for surveys of highly contaminated soil and water, as well as for surveys of groundwater containing atrazine or simazine at the limit of detectability.

Our studies with solid-phase extraction also revealed differences in the types of errors it can introduce to GC or EIA analysis. The extreme metal and salt content of agricultural evaporation water is one example of a matrix that may interfere with the triazine EIA, and necessitate additional sample preparation steps. EIA and GC are likely to be sensitive to different sets of interfering factors, so EIA may not be as sensitive as GC to differences between manufacturers and different lots of SPE columns. However, material from improperly conditioned columns can interfere with EIA, and we found that this inhibitory effect was manifested as a bias toward higher estimates of the analyte. We found that to avoid this effect, the C_{18} columns must be scrupulously washed with hexane, ethyl acetate, methanol, and water before the sample is applied.

To facilitate the development of methods and test the immunoassay on the widest variety of samples, it has been our policy to distribute our antibodies and conjugates to all investigators who request them. We believe that this will help to more quickly reveal any shortcomings of the assay. It is enabling some investigators to conduct projects for which the cost and time for instrumental analysis would be prohibitive, and it is encouraging evaluation of the MAbs in new formats, such as sensors and field-portable kits. The availability of these MAbs should give more environmental chemists experience with immunoassay, help to establish the usefulness of the EIA as a screening method, and its validity as a quantitative research tool.

In summary, a coordinated effort between our three laboratories and the CDFA has resulted in development of MAbs , a sensitive, economical EIA, and simple, efficient residue recovery methods that CDFA analytical chemists are now validating, for integration into their repertoire of tests for regulatory

monitoring of groundwater on a large scale in California. The assay and sample recovery protocols are sensitive, reproducible, fast, inexpensive, and amenable to automation. The limit of detection is comparable to that of gas chromatography. The monoclonal antibodies will provide a continuing source of the critical immunoprobe, with assured quality. These methods will initially be used as screening tools to reduce the number of samples submitted for instrumental analysis. However, future work at CDFA and in our laboratories will focus on identifying and eliminating sample matrix effects, and rigorously validating the entire procedure, so that it will be highly reliable, fully quantitative, and certifiable for regulatory purposes.

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