Immunoassay for Simazine and Atrazine with Low Cross-Reactivity for Propazine

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An antibody for simazine and atrazine has been developed that exhibits low cross-reactivity to propazine relative to most atrazine antibodies heretofore evaluated. The cross-reactivities obtained in an enzyme-linked immunosorbent assay were 100 ± 4% for simazine, 76 ± 9% for atrazine, and 12.6 ± 1.3% for propazine. This was achieved by immunizing rabbits with the hapten 6-[[[4-chloro-6-(methylamino)]-1,3,5-triazin-2-yl]amino]hexanoic acid coupled to keyhole limpet hemocyanin. The influence of tracer hapten structure on the assay sensitivity was investigated in two competitive formats. The performance of the assay with respect to pH differences and ionic strength was also examined. The lowest IC_{50} values achieved for simazine were in the 0.1 μg/L range, with the limit of quantitation being 50 ng/L. Spike−recovery studies in tap and ground water as well as analysis of crude ground water samples show the usefulness of this sensitive antibody for simazine detection.

Keywords: *Immunoassay; enzyme-linked immunosorbent assay (ELISA); simazine; atrazine; propazine; triazine herbicide*

INTRODUCTION

Triazine herbicides are used extensively for controlling broad-leaf weeds in agriculture or for road maintenance. Atrazine is the most extensively used pesticide in the United States (U.S. EPA, 1994). Simazine and atrazine are among the herbicides that have been detected in ground water in several areas of the United States for many years (Ritter, 1990; Aharonson et. al., 1987). In California, where approximately 20% of all pesticides used in the United States were applied as of 1986, simazine is used more extensively than atrazine (Domagalski and Dubrovsky, 1992).

Due to a growing need to monitor pesticide contamination, several immunoassays for atrazine have been developed as screening tools for environmental water and soil samples (Bushway et al., 1988; Wittmann and Hock, 1989; Schlaeppi et al., 1989; Karu et al., 1991; Harrison et al., 1991; Giersch, 1993). To immunochemically quantify simazine instead of atrazine, atrazine antibodies have been used (Goh et al., 1992; Lucas et al., 1991). However, most of these antibodies lack the ability to detect simazine sensitively as well. Only one antibody has been designed to detect simazine, but it detects atrazine equally well (Eremin, 1995). Most atrazine antibodies cross-react with simazine approximately 5−20% when atrazine is used as the standard (100%). In addition, antibodies produced specifically against atrazine usually exhibit a much higher cross-reactivity to propazine, which is the main analyte for these antibodies. This has been discussed previously by Giersch (1993). The structures of the three herbicides are shown in Figure 1.

Eremin (1995) obtained antibodies displaying equivalent cross-reactivity for both atrazine and simazine and 31% for propazine when immunizing with 6-[[[4-chloro-6-(ethylamino)]-1,3,5-triazin-2-yl]amino]hexanoic acid. Harrison et al. (1991), however, using the same immunogen, developed polyclonal antibodies that exhibited 40% cross-reactivity to simazine, 100% cross-reactivity to atrazine, and 72% cross-reactivity to propazine. Giersch (1993) obtained a monoclonal antibody with only 4% cross-reactivity to simazine, 100% cross-reactivity to atrazine, and 136% cross-reactivity to propazine. This was similar to the findings of Schlaeppi et al. (1989): 100% for atrazine, 90% for propazine, and 2.5% for simazine. Another monoclonal antibody described by Karu et al. (1991) was more specific for propazine (196%) than for atrazine (100%) or for simazine (31%). This monoclonal antibody was raised by immunization with a conjugate derived from 3-[[4-(ethylamino)-6-[(1-methylethyl)amino]-1,3,5-triazin-2-yl]thio]propanoic acid.

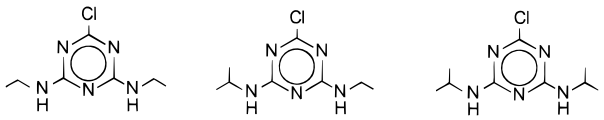


Figure 1. Structures of the three triazine herbicides simazine, atrazine, and propazine.

As immunoassays are used increasingly to distinguish among analytes in a single class of compounds, there is an increasing need for antibodies of high specificity. The key to the cross-reactivity characteristics lies in the immunizing hapten and, therefore, our goal was to find a better tailored immunogen to generate antibodies with higher binding to simazine than to atrazine or propazine. By introducing the smallest possible alkylamino group, methylamine, into the immunizing hapten side chain (Figure 2), we envisioned the generation of antibodies with at least enhanced selectivity for simazine. Our anticipation was that a small methylamine group would generate a small antibody pocket incapable of accommodating the much larger steric requirement of the isopropyl moieties of atrazine and propazine and hence provide selectivity for the ethylamino groups of simazine. This paper presents the results of this approach to produce simazine specific antibodies.

MATERIALS AND METHODS

Chemicals. All chemicals used for the hapten synthesis, dimethylformamide (DMF) and dimethyl sulfoxide (DMSO) of

S0021-8561(95)00583-8 CCC: $12.00

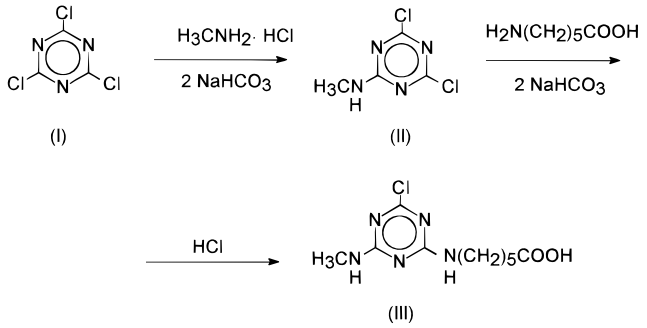


Figure 2. Synthetic route for the immunizing hapten.

liquid chromatography (LC) grade, tetramethylsilane (TMS), *N*-hydroxysuccinimide (NHS), and dicyclohexylcarbodiimide (DCC) were obtained from Aldrich (Milwaukee, WI). Silica gel 60 F_{254} plastic-backed thin layer chromatography plates of 0.25-mm thickness were purchased from E. Merck (Darmstadt, Germany). Triazine herbicide standards were obtained from Ciba-Geigy (Greensboro, NC). Horseradish peroxidase (HRP) conjugates of goat anti-rabbit IgG as well as goat anti-rabbit IgG, ovalbumin (ova) grade VI, crude ovalbumin, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC), and tetramethylbenzidine (TMB) were purchased from Sigma Chemical Co. (St. Louis, MO). Keyhole limpet hemocyanin (KLH) of 65% purity was obtained from Calbiochem (La Jolla, CA) and HRP from Boehringer (Mannheim, Germany). Buffer reagents of analytical grade were purchased from Fisher Scientific (Fair Lawn, NJ). For purification of hapten−protein conjugates, 5 and 10 mL Presto desalting columns (Pierce, Rockford, IL) were used. Microtiter plates were obtained from Nunc (Roskilde, Denmark).

Apparatus. Melting points were determined with a Thomas-Hoover apparatus (A. H. Thomas Co., Philadelphia, PA) and are uncorrected. Infrared (IR) spectra were recorded on a Mattson Galaxy Series FTIR 3000 spectrometer (Madison, WI). ^{1}H- and ^{13}C-NMR spectra were measured on a General Electric QE-300 spectrometer (Bruker NMR, Billerica, MA) operating at 300.1 and 75.5 MHz, respectively. Chemical shifts (δ) are expressed in parts per million downfield from internal TMS. Fast atom bombardment low- and high-resolution mass spectra (FAB-MS and -HRMS) were obtained on a ZAB-HS-2F spectrometer (VG Analytical, Wythenshawe, U.K.) using xenon (8 keV, 1 mA) for ionization and 3-nitrobenzyl alcohol as the matrix. Polyethylene glycol 300 was added as a mass calibrant. Optical densities were read on a Molecular Devices UVMax reader (Sunnyvale, CA) equipped with the standard ELISA software Softmax (Molecular Devices). The software package for fitting sigmoidal calibration curves and for calculating concentrations of samples was based on the log−logistic four-parameter fit.

Hapten Synthesis: 6-[[[4-Chloro-6-(methylamino)]-1,3,5-triazin-2-yl]amino]hexanoic Acid (III) (Figure 2). To a vigorously stirred solution of 7.38 g (40.0 mmol) of cyanuric chloride (**I**) in 100 mL of acetone maintained at −5 to −3 °C was added dropwise over 0.5 h a solution of 2.70 g (40.0 mmol) of methylamine hydrochloride in 10 mL of water. This was followed by the dropwise addition of a slurry of 6.72 g (80.0 mmol) of $NaHCO_3$ and 20 mL of water over 0.5 h at the same temperature range. After an additional 1 h of stirring at this temperature, the mixture was allowed to warm slowly to 15 °C. Then 5.51 g (42.0 mmol) of solid 6-aminohexanoic acid was added followed by a slurry of 7.06 g (84.0 mmol) of $NaHCO_3$ in 20 mL of water. After stirring overnight at room temperature, 30% NaOH was added until the pH reached 11. The mixture was filtered through Celite, and the residue was thoroughly washed with 200 mL of water. The filtrate and washes were combined and rotoevaporated to remove the acetone and then acidified to pH 1 with 6 M HCl. The resultant precipitate, a pasty, white solid, was collected and dried in a vacuum desiccator over Drierite to constant weight, 3.73 g (34%, crude yield), mp 146.5−148.0 °C (dec). Compounds were detected on TLC first by viewing under UV light (254 nm) and then by staining in an iodine chamber. TLC R_f 0.27 [hexane−ethyl acetate (2:1 v/v) plus 2% acetic acid], 0.81 [acetonitrile−water−acetic acid (88:12:2 v/v/v)]; IR (KBr) 3267 (m, NH), 3121 (w, NH), 1701 (m, C=O), 1572 (vs, C=N), 1228 (m, C−O) cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 12.0 (s, 1 H, OH), 7.8 (m, 1 H, CH_2NH), 7.6 (m, 1 H, CH_3NH), 3.2 (m, 2 H, CH_2-6), 2.76, 2.72 (two d, J = 4.6 Hz, 3 H, CH_3), 2.19 (t, J = 7.4 Hz, 2 H, CH_2-2), 1.5 (m, 4 H, CH_2-3,5), 1.3 (m, 2 H, CH_2-4) (with added D_2O the 12.0, 7.8, and 7.6 ppm peaks disappeared, the 3.2 ppm multiplet became a triplet at 3.27 ppm with J = 6.6 Hz, and the two doublets centered at 2.76 and 2.72 ppm became two singlets); ^{13}C NMR (DMSO-d_6) δ 174.9 (COOH), 168.5, 168.4, 167.7 (Ar, C-4), 166.3, 166.1, 165.9 (Ar, C-2 or -6), 165.8, 165.7, 165.2 (Ar, C-2 or -6), 40.3, 40.2 (C-6), 34.0 (C-2), 29.0, 28.7 (C-5), 27.6, 27.4 (CH_3), 26.2 (C-4), 24.5 (C-3); FAB-MS, *m*/*z* (relative intensity) 276 (35, M + H$^+$ + 2), 275 (26, M + H$^+$ +1), 274 (100, M + H$^+$); FAB-HRMS calcd for $C_{10}H_{16}ClN_5O_2$ 274.1071, obsd 274.1049.

Immunogen Synthesis. Hapten **III** was coupled to KLH via a modified activated NHS ester method using a water soluble carbodiimide (Tijssen, 1985). For coupling, a 20 mg/mL stock solution of NHS in DMF, a 5 mg/mL stock solution of EDC in DMF, and a 10 mg/mL hapten stock solution in DMF were prepared. Five hundred microliters (17 μmol) of the hapten solution was mixed with 794 μL (21 μmol) of the EDC solution and 159 μL (28 μmol) of the NHS stock and stirred for 7 h at room temperature. Fifty milligrams of 65% pure KLH was dissolved in a 1:1 mixture of PBS buffer (phosphate-buffered saline) and 0.13 M $NaHCO_3$ to a final concentration of 4 mg/mL and cooled to 4 °C. The organic solution was added in 50 μL aliquots to 5 mL of the protein solution under constant stirring; 0.75 mg (2.6 μmol) of hapten/mg of KLH was used, which equals addition of a total volume of 1226 μL of the organic solution to 5 mL of the aqueous solution. The solution was stirred for 30 min at 4 °C and then overnight at room temperature. The turbid solution was then centrifuged to remove precipitated protein. Subsequently, the KLH−conjugate was passed through gel filtration columns equilibrated in PBS and the fractions containing the conjugate were pooled. The protein content of the pooled column fractions was tested by spectrophotometry ($E_{280}-E_{260}$, Kalckar method) and determined to approximately 2 mg/mL. Note that the pooled column fractions precipitated shortly after they were collected. Therefore, it is recommended to apply the centrifuged supernatant immediately to the column to prevent formation of a precipitate in the column.

Immunization Protocol. Three 6-month-old female New Zealand White rabbits (no. 2281, 2282, and 2283) were immunized every 4 weeks and bled 10 days after each injection. The immunogen for the three rabbits consisted of 200 μL of the KLH−hapten **III** conjugate (400 μg of protein) diluted in 1.6 mL of PBS mixed with 1.6 mL Freund's adjuvant. For the initial immunization Freund's complete adjuvant was used; for all subsequent immunizations the incomplete adjuvant was used. The components were emulsified, and 1 mL of the mixture was injected into each rabbit subcutaneously to deliver 125 μg of KLH per rabbit. For the first three bleeds, 5 mL of blood was collected each time, bleeds four and five provided 15 mL each. Rabbit 2282 was exsanguinated during the fifth bleed.

Coating Hapten Synthesis. Ovalbumin conjugates of a variety of triazine haptens were synthesized according to an adapted standard NHS ester method as described elsewhere (Wortberg et al., 1995). For synthesis of coating hapten conjugates the hapten to protein ratio was as low as 5:1 or 10:1 to ensure a sufficiently low avidity desirable for competitive immunoassay.

Tracer Synthesis with Horseradish Peroxidase. HRP tracers were synthesized according to a modified version of Schneider and Hammock (1991) using the NHS ester method via DCC. A 35:1 molar ratio of hapten to enzyme was used. Stock solutions in dry DMF containing 10 mg/mL hapten were prepared. For synthesis of **XIV**−HRP, as an example, 72 μL (0.72 mg, 2.4 μmol) of the hapten **XIV** stock solution was mixed with 32.5 μL (6 μmol) of a 20 mg/mL solution of NHS in DMF. Then 3.1 mg (12 μmol) of DCC was added. The organic mixture was stirred for 20 h at room temperature and

centrifuged to separate the precipitated urea. Two milligrams (4.4×10^{-2} μmol) of HRP was dissolved in 3 mL of 0.13 M $NaHCO_3$ and cooled to 4 °C. Of the organic solution, 73 μL (35-fold molar excess) was added in 10 μL aliquots to the stirred HRP solution over several minutes. The solution became turbid and a precipitate formed. After stirring overnight at 4 °C, precipitated protein was removed by centrifugation. The supernatant was passed through a gel filtration column equilibrated with PBS. Fractions containing the HRP conjugate were pooled and frozen.

Coating Hapten Format Enzyme-Linked Immunosorbent Assay (ELISA). Optimal concentrations of the hapten−ovalbumin conjugates were determined by two-dimensional titration. A working dilution of the conjugates of 1:10000 in PBS was used, unless otherwise indicated. The coating hapten solution (150 μL) was incubated in wells of microtiter plates overnight at 4 °C. Alternatively, coating was performed at room temperature over a 2 h incubation period. The wells were then emptied and incubated for 30 min with 150 μL of a 0.5% (w/v) ova−PBS solution (blocking solution) without intermediate rinsing. The plates were then washed four times with PBS containing 0.05% Tween 20 (PBST) and were ready to use or were stored for up to 5 weeks at 4 °C in PBS containing 0.02% NaN_3. Triazine stock solutions were prepared in DMSO (1.00 mg/mL). Starting from the organic stock solution, triazine standards were diluted with PBS or other buffers, tap water, or ground water. The optimal antibody titer of serum 2282 was determined to be 1:10000 in a two-dimensional titration. For competition, 50 μL of diluted antiserum solution and 100 μL of sample or standard were incubated together for 1 h. For all assays, samples and standards were pipetted in triplicates. The wells were washed four times with PBST and then incubated with 100 μL of a goat anti-rabbit IgG−HRP conjugate (diluted 1:12500 in PBS) for 1 h. After four washing steps, 100 μL of substrate solution was added (2.4 mg of TMB dissolved in 400 μL of DMSO plus 100 μL of 1% H_2O_2 in 25 mL of 0.1 M sodium acetate buffer of pH 5.5). The reaction was stopped after 15−30 min by adding 50 μL of 2 M H_2SO_4.

Tracer Format ELISA. Wells were coated by incubating with 100 μL of goat anti-rabbit IgG diluted 1:2000 in PBS at 4 °C overnight. The wells were then washed four times with PBST and incubated for 1 h with 100 μL of specific antibody 2282, diluted 1:10000 in PBS. The wells were emptied without a washing step, and 150 μL of the ovalbumin blocking solution was added. After 30 min, the plates were rinsed four times and were then used immediately or stored in PBS containing 0.02% NaN_3. For competition studies, 100 μL of standard or sample was incubated together with 50 μL of the HRP tracer (in triplicates). Optimal HRP tracer dilutions were determined to be 1:1000−1:2000 (in PBS) by two-dimensional titration. Standards were pipetted first, and then the tracer was added. After 1 h of incubation, the wells were rinsed four times and subsequently incubated with 100 μL of substrate solution. The substrate conversion was stopped by adding 50 μL of 2 M H_2SO_4.

RESULTS AND DISCUSSION

Hapten Synthesis. The immunizing hapten, 6-[[4-chloro-6-(methylamino)]-1,3,5-triazin-2-yl]amino]hexanoic acid (**III**) (Figure 2), was synthesized by treating cyanuric chloride (**I**) in acetone with 1 equiv of methylamine hydrochloride and 2 equiv of aqueous $NaHCO_3$ while maintaining the reaction mixture at −5 to −3 °C. One equivalent of solid 6-aminohexanoic acid was then added followed by 2 equiv of aqueous $NaHCO_3$ at room temperature to replace the second chlorine of **II**. Acidification provided crude hapten **III**, which required recrystallizations from three different solvents to obtain a pure product.

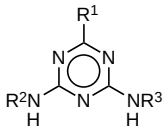
Structural confirmation was accomplished by ^1H and ^{13}C NMR, HRMS, MS, and IR. All of these were consistent with the assigned structure for the product. Both the ^1H- and ^{13}C-NMR spectra determined at room temperature displayed multiple peaks for those atoms in or adjacent to the ring. At 80 °C the number of ^1H peaks collapsed to that expected for one structure except for the *N*-methyl doublet of doublets, which appeared as two singlets. At ambient temperature, a correlated spectroscopy (COSY) spectrum permitted definitive assignments of each set of peaks and was especially useful for assigning the two N−H peaks. The lower field (7.8 ppm) multiplet displayed coupling to the $N-CH_2$ appendage (3.2 ppm), whereas the higher field (7.6 ppm) multiplet was coupled to the $N-CH_3$ (2.76, 2.72 ppm) peaks. A ^{13}C distortionless enhanced by polarization transfer (DEPT) experiment confirmed the assignment of the 27.6 and 27.4 ppm peaks to the $N-CH_3$ moiety as well as substantiating the presence of the 40.3 and 40.2 ppm C-6 peaks masked, but surmised due to the unsymmetrical appearance of the DMSO solvent peaks in the normal ^{13}C-NMR spectrum. Finally, $^1H-^{13}C$ heteronuclear correlation spectroscopy (HETCOR) confirmed the assignment of the four protons at 1.5 ppm to the C-3 (24.5 ppm) and C-5 (29.0, 28.7 ppm) carbon atoms and the two protons at 1.3 ppm to the C-4 (26.2 ppm) carbon atom of the hexanoic acid side chain.

Antisera Screening. Initially, the titer of each bleed was tested in a two-dimensional titration against a homologous system; i.e., the immunizing hapten coupled to ovalbumin instead of KLH was used as a coating hapten. This ensured that increase in titer after subsequent boost injections was due to specific recognition of the hapten and not to binding to the KLH moiety of the conjugate. Incorporation of blanks using ova as coating alone showed that the binding was specific to the hapten. After the third bleed (second boost), no significant increase of the titers was observed for any of the three antisera.

The affinity of the antisera to heterologous systems (structurally different coating haptens) was qualitatively tested in a comparative binding study, using the second bleed. For this experiment coating haptens were diluted 1:1500, the antisera 1:2000. Coating haptens exhibiting a sufficiently high antibody affinity (OD $>0.2-0.3$) but a lower affinity than the immunogen (OD <0.9) were further investigated. We have found that coating haptens that bind with titers more like the immunogen result in assays that are poorly inhibited by free analyte (Goodrow et al., 1995). The triazine haptens used in this study and the results of the qualitative binding assessment are presented in Table 1. These coating haptens were then tested in a competitive assay using a series of simazine standards and a suitable antibody concentration of later bleeds. Qualitative examination of coating hapten binding with later bleeds revealed no major differences to the data shown here.

After **IV**−ova was chosen as a suitable coating hapten for all antisera and optimizing dilutions, cross-reactivities of the third bleeds to an array of triazine herbicides were tested in a preliminary fashion. As was predicted from the structure of the immunizing hapten, all three antisera showed lower cross-reactivities to propazine than to atrazine or simazine. Antiserum 2281 showed a preference for atrazine (100%) with a high simazine cross-reactivity (68%). The slope of all calibration curves was low, however (*B* ranged from 0.5 to 0.75). Antiserum 2283 responded equally well to simazine and atrazine (both 100%) with an acceptable slope of 0.7−0.9, whereas antiserum 2282 clearly favored simazine

Table 1. Triazine Haptens Used for Coating Hapten or HRP Tracer Synthesis[a]



no.	R^1	R^2	R^3	2281	2282	2283	ref
III	Cl	CH_3	$(CH_2)_5-COOH$	+++	+++	+++	
IV	Cl	CH_2CH_3	$(CH_2)-COOH$	+	+	+	Goodrow et al., 1990
V	Cl	CH_2CH_3	$(CH_2)_2-COOH$	+++	+++	+++	Goodrow et al., 1990
VI	Cl	CH_2CH_3	$(CH_2)_3-COOH$	+++	+++	+++	Goodrow et al., 1990
VII	Cl	CH_2CH_3	$(CH_2)_4-COOH$	+++	+++	++	Goodrow et al., 1990
VIII	Cl	CH_2CH_3	$(CH_2)_5-COOH$	+++	+++	+++	Goodrow et al., 1990
IX	Cl	$CH(CH_3)_2$	$(CH_2)-COOH$	nt	nt	nt	Goodrow et al., 1990
X	Cl	$CH(CH_3)_2$	$(CH_2)_2-COOH$	nt	nt	nt	Goodrow et al., 1990
XI	Cl	$CH(CH_3)_2$	$(CH_2)_5-COOH$	+++	+++	+++	Goodrow et al., 1990
XII	Cl	H	$(CH_2)_5-COOH$	+++	+++	+++	Lucas et al., 1995
XIII	OCH_3	$CH(CH_3)_2$	$(CH_2)_5-COOH$	−	−	0	Kido et al., 1996
XIV	SCH_3	$CH(CH_3)_2$	$(CH_2)_5-COOH$	++	++	++	Kido et al., 1996
XV	OH	H	$(CH_2)_5-COOH$	0	0	+	Goodrow, unpublished
XVI	OH	CH_2CH_3	$(CH_2)_2-COOH$	++	+	+	Lucas et al., 1993a
XVII	$SOCH_3$	$CH(CH_3)_2$	CH_2CH_3	+	−	−	Huber and Hock, 1986
XVIII	*N*-acetylcysteine	CH_2CH_3	$CH(CH_3)_2$	+	−	−	Lucas et al., 1993b
XIX	$S(CH_2)_2-COOH$	$CH(CH_3)_2$	CH_2CH_3	++	+	0	Goodrow et al., 1990
XX	$S(C_6H_6)-COOH$	H	H	+	−	−	Muldoon et al., 1994

[a] The degree of binding of the antisera 2281−3 to these haptens when used as coating haptens is indicated qualitatively, using the absolute OD as a measure of affinity. A KLH−**III** conjugate was the immunogen. Key: − = OD <0.2; 0 = OD 0.2−0.4; + = OD 0.4−0.6; ++ = OD 0.6−0.8; +++ = OD >0.8; nt, not tested in the initial screen.

(100%) over atrazine (83%) and had slope parameters *B* between 0.7 and 1.1. Further studies were conducted with 2282 only since it had the highest preference for simazine and a sufficient slope. Bleed five of antibody 2282 was somewhat more cross-reactive to atrazine than bleed four, while showing no change in sensitivity for simazine; thus, bleed four was chosen for optimization. Although 2283 may be of practical use as a generic triazine antibody since it detects atrazine and simazine equally well, the goal of this study was the development of a simazine selective antibody. If screening for atrazine and simazine was desired, 2283 would be preferred. Since we are interested in distinguishing structurally related compounds from each other (Wortberg et al., 1996) and/or quantitation of mixtures (Jones et al., 1994; Wortberg et al., 1995) by using an array of antibodies with differing specificities and multivariate statistics, the "simazine" antibody 2282 was used.

After the preliminary experiments showed that antibody 2282 was the antibody displaying the most desirable properties, it was further tested against all hapten conjugates that seemed useful according to the qualitative binding experiment. All coating haptens were then diluted 1:7500, the antiserum was diluted 1:10000, and the simazine standard concentrations were chosen according to the location of the curve midpoints. These midpoints were estimated in an initial assay. The dose−response curves for simazine against an array of different coating haptens are shown in Figure 3. The IC_{50} values obtained with different hapten structures cover a range of almost 4 orders of magnitude, thus showing that hapten structure is very crucial for assay sensitivity. The best haptens have extremely short handles with just one carbon atom between the triazine ring and the functional group. Additionally, they have a Cl atom and the ethylamino or an isopropylamino group.

Cross-Reactivity Studies. A list of the triazines tested for cross-reactivity and their respective structures is given in Table 2. The cross-reactivities of those triazine herbicides that bind significantly (here defined as cross-reactivity >0.2%) to antibody 2282 (fourth bleed) are shown in Figure 4. Since all cross-reactivities are relative to the simazine curves, the IC_{50} and the standard error for simazine were determined from six replicate curves to give a statistically useful standard deviation. All other IC_{50} values and the resulting cross-reactivities were determined in replicates of four curves each. The errors of the cross-reactivities were calculated by adding the individual CVs for the averaged IC_{50} values of simazine and the respective cross-reactant.

As described above, the antiserum 2282 showed lower cross-reactivity to propazine than to atrazine or simazine. Virtually all atrazine antibodies reported in the literature to date are actually propazine antibodies, a fact that is usually ignored because this compound is not a commonly used herbicide. Most research groups have designed atrazine immunogens by using an ethylamino group or an isopropylamino group and a five-carbon handle terminated with a functional group, which mimics an "extended" ethylamino group. The result usually is an antibody that detects propazine best (two isopropyl groups), followed by atrazine (one isopropyl group) and simazine (no isopropyl substituent). The affinity is observed to be higher for an analyte that has one carbon atom more than the immunogen or is more branched than the immunogen at the same position. In this study, we maintained the handle location and length but reduced the size of the remaining alkylamino group by one carbon to methylamine. The result is an antibody that again favorably binds analytes with one carbon more than the immunogen. Consequently, we obtained antibodies that detect simazine and atrazine very well, but none is specific for simazine. Since the methylamine substituent of the immunogen is distinctly smaller than an isopropyl group, the isopropyl groups of propazine do not fit very well into the binding pocket of the antibody. As a consequence of the ethylamine moiety in position R^2 (see Table 2), deisopropylatrazine has a relatively high cross-reactivity. Deethylatrazine has a lower cross-reactivity

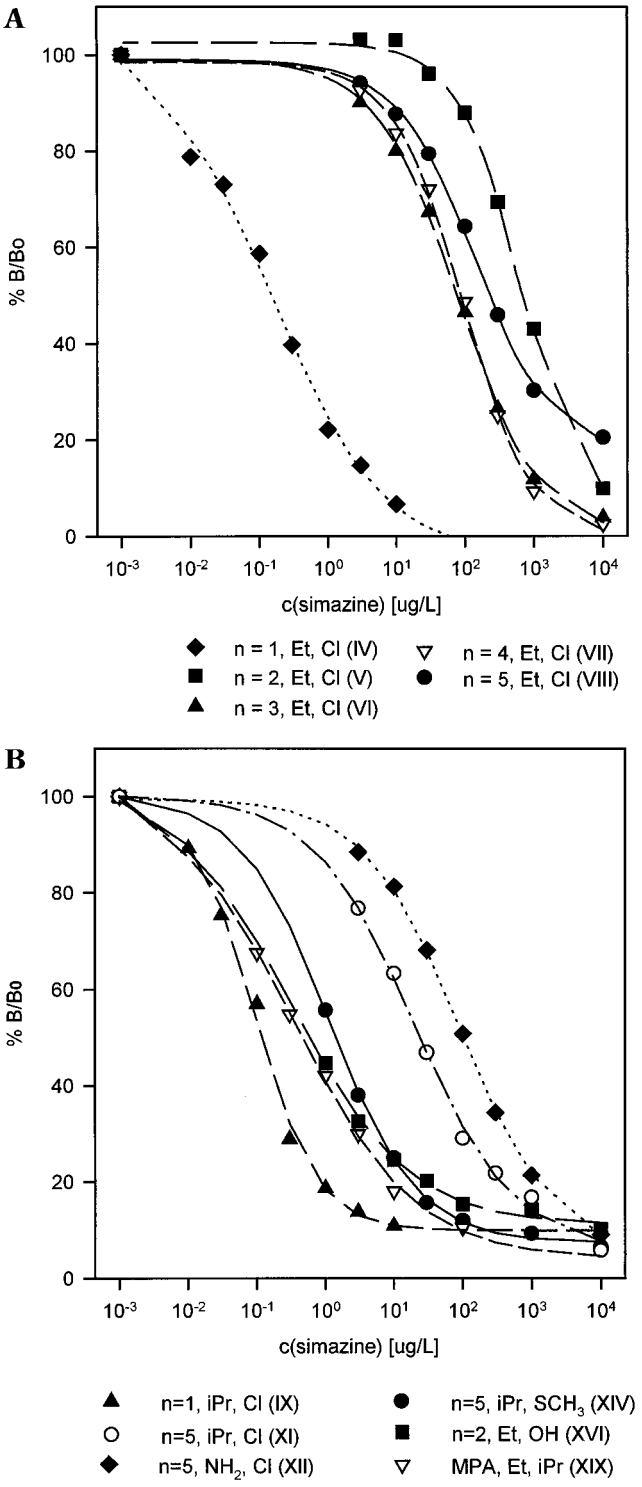


Figure 3. Dose−response curves for simazine using an array of different coating haptens. n = number of carbon atoms in the handle in R^3; Et, ethylamino; iPr, isopropylamino; OH, hydroxy; MPA, mercaptopropanoic acid in R^1. The structure of the hapten largely determines the location of the IC_{50} and therefore the assay sensitivity. Haptens with short spacers ("handles") generally result in more sensitive assays. No curves were obtained with haptens **XIII**, **XV**, **XVII**, **XVIII**, and **XX**. The homologous system **III** was not tested. Three replicates of each standard were analyzed. The simazine concentrations for each calibration curve were chosen after the approximate location of the IC_{50} was known from previous experiments.

than deisopropylatrazine because its remaining side chain is an isopropyl group. Triazines with a methoxy or a methylthio substituent exhibit very little or no cross-reactivity at all; the same is true for hydroxy metabolites, 2- or 3-fold dealkylated metabolites, and triazine precursors. Terbutryn with its methylthio substituent cross-reacts only negligibly (<0.2%), but terbutylazine exhibits a cross-reactivity of as much as 11%. Surprisingly, there is no significant difference in cross-reactivity between propazine and terbutylazine. One would expect terbutylazine to cross-react less than propazine because of its even bulkier tertiary butyl substituent. But in contrast to propazine, terbutylazine also bears one less bulky ethylamino group instead of only isopropylamino groups. These findings indicate that besides the alkylamino group in position R^2, the Cl atom plays a key role in the recognition process. If both of the features are present, the third substituent on the triazine has less influence on the cross-reactivity. As a consequence of this preference, antibody 2282 is class-specific for chloro-*s*-triazines and exhibits a high selectivity for simazine and atrazine.

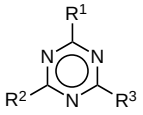
Enzyme Tracer Assays. Figure 5 shows an array of simazine calibration curves obtained with different HRP tracers. Note that the range of curve midpoints is only spread out over 2 orders of magnitude as opposed to almost 4 orders of magnitude in the coating hapten format. Several different approaches to couple hapten **IV** to HRP (all based on variations of NHS ester activations) did not produce a tracer that exhibited any affinity to the antibody, although all showed high enzyme activity. The coupling ratio was not confirmed, but when we used the same batch of hapten−NHS ester in a parallel experiment to synthesize an ovalbumin conjugate, we obtained a functioning coating hapten. That indicates that the triazine hapten was sufficiently activated for coupling. Also, earlier coupling experiments with a similar triazine derivative (**XI**), which has a five-carbon spacer instead of a one-carbon spacer, yielded an HRP tracer with sufficient affinity to the antibody as well as a functioning ovalbumin-based coating hapten. This indicates that generally coupling of NHS-activated carboxylic acid triazine derivatives to HRP is a functioning method.

Our conclusion is that the conjugation of **IV**−HRP was most likely successful, but the tracer would not bind to the antibody. It appears likely that the optimal spacer length for an HRP tracer is different from the optimal spacer length for an ovalbumin conjugate. We speculate that in case of the unsuccessful tracer with the short handle, the entire triazine moiety merely "disappeared" inside some pocket in the enzyme, thus rendering antibody binding sterically hindered. This possibility is currently under investigation.

The same effect would then explain another interesting finding. Hapten **VIII** with its long five-carbon handle yielded a very insensitive assay in the coating hapten format (Figure 3A), supposedly due to the antibodies' high affinity to the conjugate (quasi-homologous hapten), but when the same hapten was used as an HRP tracer, the affinity was lower than expected (Figure 5A), which was indicated by the relatively low curve midpoint of the tracer format assay. Still, this hapten resulted in one of the "poorest" tracer format assay compared to haptens with shorter handles.

Comparison of the Two Formats. Since spacer length is apparently more crucial in the coating hapten format than in the tracer format assay, with a very short length yielding the best assays, we must assume that the concept of an "apparent handle length" should be considered. With the HRP tracer, differences in handle length seem to be less pronounced, as can be seen in

Table 2. Structure of the Triazine Compounds Tested for Cross-Reactivity



triazine herbicide	R^1	R^2	R^3
atrazine	Cl	$NHCH_2CH_3$	$NHCH(CH_3)_2$
cyanazine	Cl	$NHCH_2CH_3$	$NHCCN(CH_3)_2$
procyazine	Cl	NHcyclopropyl	$NHCCN(CH_3)_2$
propazine	Cl	$NHCH(CH_3)_2$	$NHCH(CH_3)_2$
simazine	Cl	$NHCH_2CH_3$	$NHCH_2CH_3$
terbutylazine	Cl	$NHCH_2CH_3$	$NHC(CH_3)_3$
ametryn	SCH_3	$NHCH_2CH_3$	$NHCH(CH_3)_2$
prometryn	SCH_3	$NHCH(CH_3)_2$	$NHCH(CH_3)_2$
simetryn	SCH_3	$NHCH_2CH_3$	$NHCH_2CH_3$
terbutryn	SCH_3	$NHCH_2CH_3$	$NHC(CH_3)_3$
prometon	OCH_3	$NHCH(CH_3)_2$	$NHCH(CH_3)_2$
terbumeton	OCH_3	$NHCH_2CH_3$	$NHC(CH_3)_3$
hydroxyatrazine	OH	$NHCH_2CH_3$	$NHCH(CH_3)_2$
hydroxysimazine	OH	$NHCH_2CH_3$	$NHCH_2CH_3$
deethylatrazine	Cl	NH_2	$NHCH(CH_3)_2$
deisopropylatrazine	Cl	NH_2	$NHCH_2CH_3$
didealkylated atrazine	Cl	NH_2	NH_2
cyanuric acid	OH	OH	OH
ammelide	NH_2	OH	OH
ammeline	NH_2	NH_2	OH
melamine	NH_2	NH_2	NH_2
atrazine mercapturate	*N*-acetylcysteine	$NHCH_2CH_3$	$NHCH(CH_3)_2$

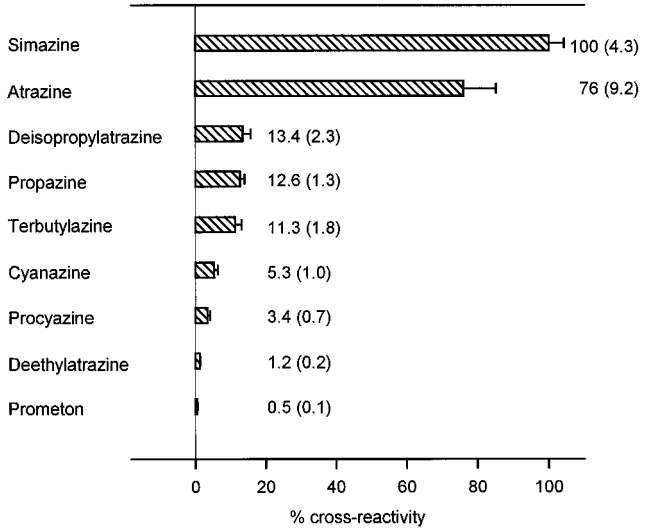


Figure 4. Cross-reactivity pattern of antibody 2282 against a panel of triazine herbicides. The error bars represent the standard deviation calculated from replicate calibration curves which were obtained with the same set of standards ($n = 6$ curves for simazine, $n = 4$ for all others). All other triazines listed in Table 1 showed a cross-reactivity of less than 0.2%.

Figure 5 compared to Figure 3. This indicates that the apparent handle length of a given hapten is different in both assay formats and is not necessarily constant as one would assume from the triazine hapten structure alone. Thus, discussion of the optimal handle length should be modified by discussing an apparent handle length, which may differ from protein to protein. This idea would help explain the findings of Schneider and Hammock (1991) and Eremin (1995), from which it was observed that in a heterologous system a shorter handle yielded a better assay in an HRP tracer format, but in an example with an alkaline phosphatase (AP) tracer, the reverse appeared to be true (Schneider and Hammock, 1991). The idea that a hapten behaves differently in terms of affinity when coupled to different proteins may also explain why some groups developed sensitive assays despite using a homologous tracer. One would assume that using a homologous hapten system in an assay for a small molecule automatically results in an insensitive assay. This was indeed observed in our study with ovalbumin conjugates of the immunogen **III** (data not shown). Investigators using the homologous system either used monoclonal antibodies (Giersch, 1993; Schlaeppi et al., 1989) that had been screened to result in a sensitive assay or polyclonal antibodies in combination with an HRP tracer (Wittmann and Hock, 1989; Eremin, 1995). There is no evidence in the literature of a successful polyclonal (triazine) assay employing a homologous albumin conjugate.

For quantitative comparison of curve midpoints the exact hapten density has to be determined, e.g. by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) as was demonstrated by Wengatz et al. (1992). Coupling ratios may differ from protein to protein even when using the same excess of reagents for coupling, and different coupling densities lead to different affinities (avidity concept) and thus different IC_{50} values.

Influence of Ionic Strength and pH on the Assay. To evaluate the variability of the coating hapten format assay due to matrix effects, we prepared simazine standards in several different buffer systems. One approach was to vary the pH, another was changing the ionic strength but maintaining constant pH. We also investigated the influence of an organic solvent. For the pH variation study we used several 0.01 M buffers ranging from pH 4.0 to 10.0, which all contained 0.125 M NaCl. The NaCl served as an "ion buffer" to level out differences between buffer systems and to adjust the total ionic strength to the physiological saline concentration of 0.135 M. Buffers of pH 4.0, 5.0, 6.0, 7.0, and 8.0 were prepared by adjusting the pH of 0.01 M NaH_2PO_4 and Na_2HPO_4 solutions with 1 M NaOH and 1 M HCl, respectively. The buffer of pH 9.0 contained Tris-HCl; the buffer of pH 10.0 was made from glycine–NaOH. The effect of pH variation on the calibration curve with antibody 2282 is shown in Figure 6A.

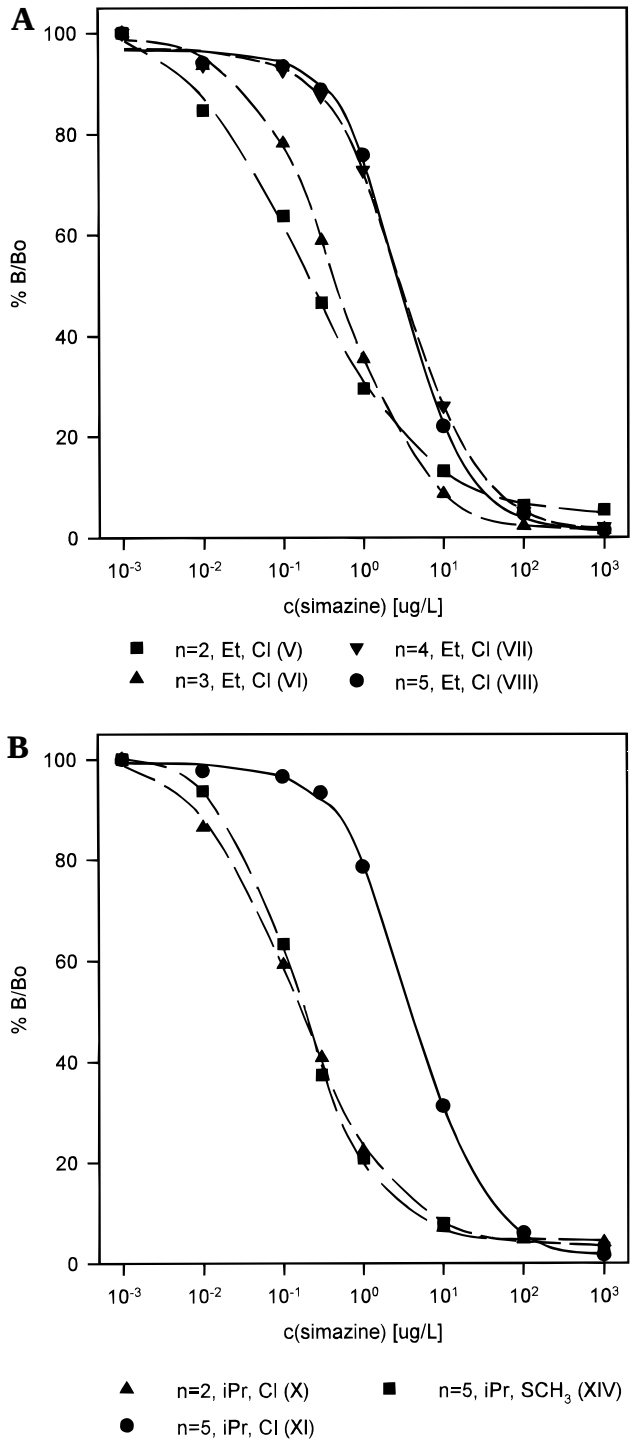


Figure 5. Dose−response curves for simazine using an array of different HRP−hapten tracers. Again, the handle length determines the assay sensitivity. In the tracer format differences in IC_{50} values are less pronounced with the same haptens than in the coating hapten format. No binding was observed to haptens **IV** and **IX**. All other haptens not shown have not been investigated. (For abbreviations, see Figure 3.)

To evaluate the changes in curve parameters due to ionic strength variation, we used 10 times concentrated PBS, PBS, and 10 times diluted PBS, all pH 7.5. In addition, we spiked a batch of PBS buffer with 10% methanol and another batch with 0.5 M Na_2SO_4. We also used tap water and ground water for comparison. Calibration curves obtained with simazine standards in dilutions of different ionic strength are shown in Figure 6B.

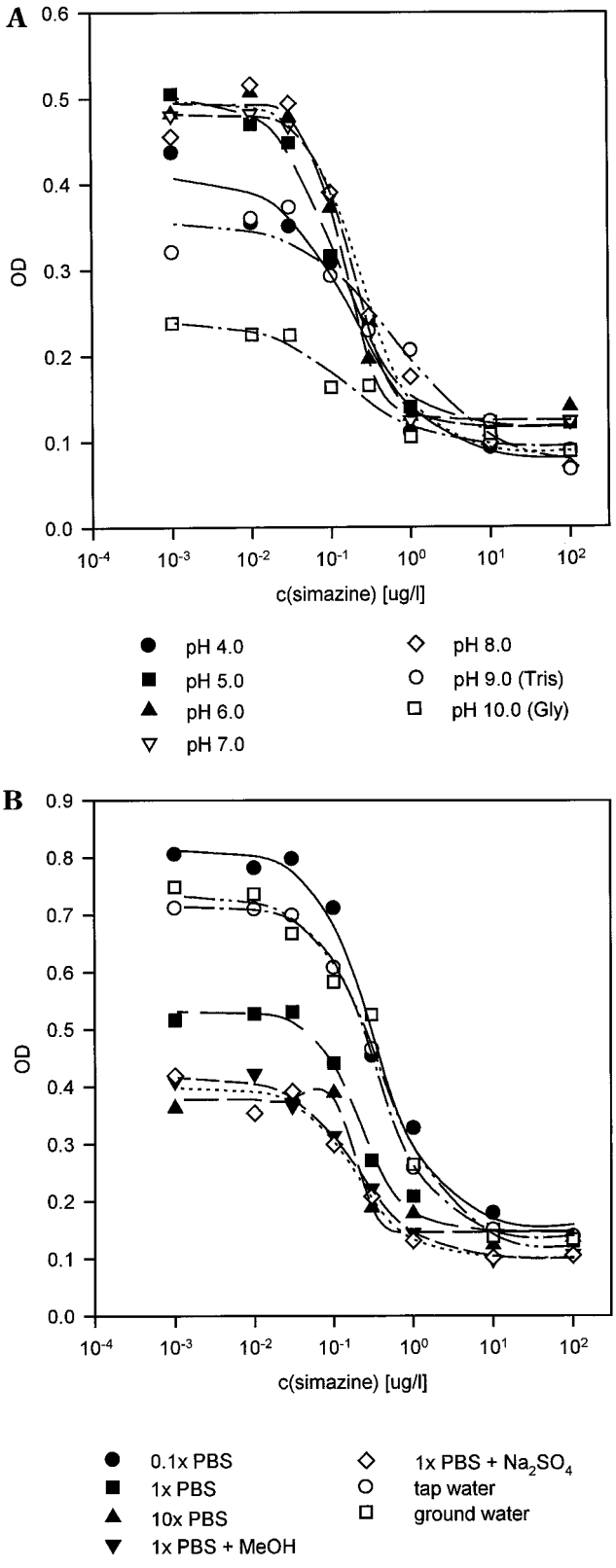


Figure 6. (A) Influence of varying pH on the simazine calibration curve. The ionic strength was constant in all buffers. (B) Influence of the ionic strength on the simazine calibration curve. Low ionic strength results in higher optical density but also in higher IC_{50}.

The curves generated in phosphate buffers pH 5.0−8.0 show little variation. At pH 4.0 the OD_{max} drops slightly, at pH 10.0, it decreases very sharply. Curve quality in terms of correlation coefficient and sufficient OD_{max} (>0.5) deteriorates at pH 9.0 and above as well as at pH 4.0. The IC_{50} values obtained at pH 5.0−8.0

range from 0.13 to 0.26 μg/L, but there is no correlation observed between the direction of the pH change and the increase or decrease of the IC_{50}. In general, it seems important that the sample pH is between 5.0 and 8.0−9.0 to ensure good curve quality. However, to level out differences between samples of different pH values, one could easily use a PBS buffer with a higher buffering capacity for diluting the antiserum. Also, one does not expect to observe extreme pH differences in drinking water samples. The pH range of the ground water samples (see below) was only from 6.9 to 7.7. In general, we observed an increase in IC_{50} with a decrease in ionic strength. The nature of the ions present is not as important as the ion concentration (more specifically, ionic strength). The highest IC_{50} was obtained with tap water (0.35 μg/L) and ground water (0.40 μg/L), the lowest (0.15 μg/L) with PBS. High ionic strengths reduced the overall binding and resulted in lower maximum optical densities. The OD_{max} dropped from 0.8 to 0.4 when the buffer concentration was increased from 1 times PBS (0.135 M solution) to 10 times PBS (1.35 M solution) or when the PBS buffer contained 0.5 M Na_2SO_4. Both resulted in less precise curves in terms of curve fit (Figure 6B). These observations are in qualitative agreement with the earlier findings of Lucas et al. (1991) and Muldoon and Nelson (1994). They also indicate that the ion concentration of the matrix is crucial for the accurate analysis of real samples. When environmental samples are analyzed, the matrix effect has to be minimized by diluting the sample, "buffered out" by adding a sufficiently concentrated buffer (ionic strength adjustment), or the standards must be prepared in a blank matrix that is as similar to the environmental samples as possible. Since ground water samples, as well as tap water, may have a lower ionic strength than PBS buffer, as was observed herein, the overall ionic strength in the assay should be increased by preparing antibody or tracer solution in more concentrated PBS. This will ensure an ionic buffering as well as a pH effect and also will result in a low IC_{50}. We used 3 or 10 times PBS for the following experiments.

Analysis of Spiked Water Samples. To examine the spike recovery, tap water (source: Davis, CA) and ground water (source: San Joaquin Valley, CA) samples were spiked with different levels of simazine. In one study, tap water was spiked over the entire range of the assay from 0.05 to 0.5 μg/L, using four replicates at each spike level (individually prepared). The results are shown in Figure 7. The regression data for the recovery study are $R^2 = 0.943$, $b = 0.994$, and $a = 0.041$ (52 data points).

In a second study, tap and ground water samples were each spiked with four concentration levels (0, 0.05, 0.1, and 0.5 μg/L) and analyzed. To not simply evaluate the repetition error of the assay, the six replicate samples of each concentration were prepared individually. The data are shown in Table 3. Spike−recoveries in both assay formats were compared. The recoveries at the lowest simazine concentration of 50 ng/L, which we define as the limit of quantitation, were 74 ± 10% in the coating hapten format and 138 ± 15% in the tracer format. The best recoveries, as expected, were around the IC_{50} concentration of 0.1 μg/L (113 ± 22% and 107 ± 5%, respectively), because here the curve has the highest slope. Since different ground or tap waters might result in different recoveries, these data are interpreted as a general indicator of the performance of the assay in ground and tap water.

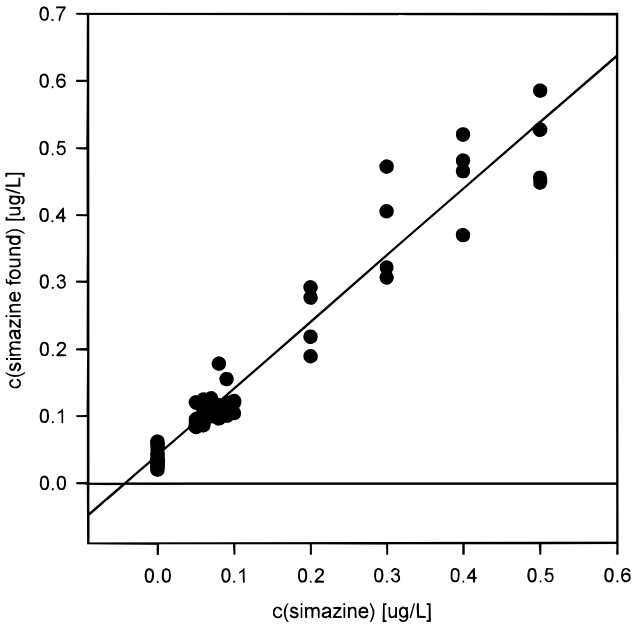


Figure 7. Spike recovery for simazine in tap water at various concentration levels. The regression data for the recovery study are $R^2 = 0.943$, $b = 0.994$, and $a = 0.041$ (52 points).

Table 3. Spike Recoveries in Ground Water for both the Coating Hapten and the Tracer Format[a]

spike level (μg/L)	recovery (μg/L)	std error (μg/L)	recovery (%)	std error (%)
IV−ovalbumin				
0	0.007	0.006		
0.05	0.037	0.004	74	9.8
0.1	0.113	0.025	113	22
0.5	0.531	0.039	106	7.4
XI−HRP				
0	0.015	0.004		
0.05	0.069	0.010	138	15
0.1	0.107	0.006	107	5
0.5	0.398	0.041	80	10

[a] $n = 6$ samples were tested at each concentration level.

Analysis of Ground Water Samples. We obtained 28 ground water samples from the California Department of Pesticide Regulation, CalEPA. The samples were collected throughout the San Joaquin Valley of California in the fall of 1994 and had been analyzed for simazine and a few other herbicides. Data were obtained by GC; positives were confirmed by HPLC. ELISA analyses were conducted without prior knowledge of the GC results.

Initially, raw ground water samples were analyzed by using calibration curves in PBS to identify negatives. Then we repeated the assay using a standard curve in a blank (negative) matrix, adding reagents in 3 times PBS buffer. The results obtained from a coating hapten format ELISA were correlated to the data provided by CalEPA as plotted in Figure 8.

The ELISA results compared favorably with the GC data. Since the immunoassay tends to slightly overestimate concentrations, as can be seen from spike−recovery data, false positives are likely to occur. Since the limit of quantitation was defined as 50 ng/L, all concentrations found below that level were set automatically to zero; this corresponds to the practice of the reference GC method. Since these GC negative samples are not necessarily simazine-free, there can be a bias between both methods. The immunoassay might estimate a concentration greater than the limit of its quantitation, whereas the GC analysis still detects it

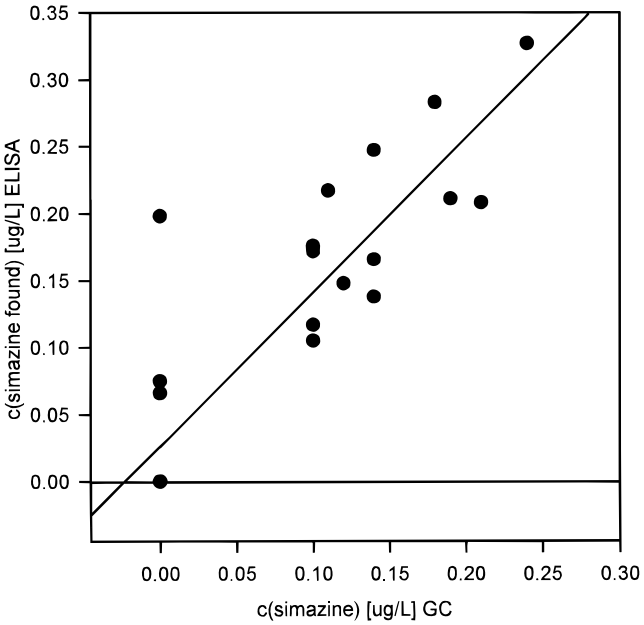


Figure 8. Analysis of ground water samples. Simazine detected by ELISA vs simazine measured by GC. Regression data are $R^2 = 0.674$, $b = 1.321$, and $a = 0.024$ (28 points).

as below that limit. Thus, a "false positive" is inevitably generated. However, no false negatives were observed and only one sample gave a strong false positive (0.2 μg/L instead of <0.05 μg/L).

Immunoassay data were generated using untreated, crude samples, whereas the GC analyses required time-consuming and costly solid-phase or liquid−liquid extraction procedures. Thus, the immunoassay developed here is a means of quickly and inexpensively assessing the degree of water contamination by simazine.

Conclusions. The development of an antibody with highest cross-reactivity toward the triazine herbicide simazine was achieved by choosing a tailored immunogen with a methylamine substituent on the triazine ring instead of an ethylamine substituent. Implementation of this antibody in our multianalyte immunoassay system (MELISA; Jones et al., 1994; Wortberg et al., 1995) should improve the performance by better discriminating simazine from atrazine than in the initial MELISA system.

Another means to obtaining an antibody with a higher simazine over atrazine selectivity may be the use of an immunogen with one or two methylamine groups with attachment to the protein through the R^1-position. The most common handle used at R^1 has been the mercaptoalkanoic acid. The resulting antibody, however, would likely be mainly selective for simetryn and possibly ametryn, whereas simazine and atrazine would only be minor analytes as predicted from the report of Harrison et al. (1991). If, however, the presence of simetryn and ametryn is not expected in a sample, this approach might be one solution to the atrazine−simazine problem. An antibody of such specificity would prove very valuable in discriminating subclasses of triazine herbicides in the MELISA system. To achieve generation of an antibody with *specificity* for simazine remains a challenge since the structures of atrazine and simazine are very closely related.

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