Development of an Enzyme-Linked Immunosorbent Assay for the Detection of Hydroxytriazines

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Hydroxytriazines are environmental decomposition products of *s*-triazine herbicides. Enzyme-linked immunosorbent assays (ELISAs) in various formats for the detection of hydroxypropazine, hydroxyatrazine, and hydroxysimazine were developed. 6-{4-Methoxy-6-[(1-methylethyl)amino]-1,3,5-triazin-2-yl}aminohexanoic acid was used as the immunizing hapten. One of the resulting ELISAs has IC_{50} values of 0.002, 0.004, and 0.017 μM (0.4, 0.8, and 3.1 ppb) for hydroxypropazine, hydroxyatrazine, and hydroxysimazine, respectively. This ELISA utilizes a coated antigen format with a simazine−mercaptopropanoic acid hapten conjugated to conalbumin as the coating antigen. The assay parameters are unchanged at pH values between 5.0 and 8.0 and at ionic strengths from 0.13 to 1.0 M phosphate-buffered saline. Methanol does not affect the assay up to 13%, whereas acetonitrile and acetone cause a decrease of sensitivity at 3.1%. Groundwater fortified with hydroxyatrazine was analyzed according to this method. Good correlations with spike levels were observed, making this assay potentially valuable for the screening of groundwater samples containing parts per trillion levels of hydroxytriazine contamination.

Keywords: *s-Triazine; hydroxytriazine; hydroxyatrazine; hydroxysimazine; hydroxypropazine; ELISA; enzyme immunoassay; herbicide metabolites*

INTRODUCTION

Hydroxypropazine, hydroxyatrazine, and hydroxysimazine are hydrolysis products of propazine, atrazine, and simazine, respectively (Esser et al., 1975). Members of the latter group are *s*-triazine herbicides, which are commonly used in agriculture. Specifically, in 1993, atrazine was the most widely used pesticide in U.S. agricultural crop production (Aspelin, 1994). Use estimates are 70−75 million pounds for atrazine and 3−6 million pounds for simazine. Since hydrolysis, along with dealkylation, is a significant mode of degradation of *s*-triazine herbicides in the environment (Jordan et al., 1970; Esser et al., 1975; Erickson and Lee, 1989), the analysis of *s*-triazine hydrolysis products is very important for the evaluation of total herbicide burden in the environment.

Instrumental methods that have been adapted for the analysis of hydroxytriazines include high-performance liquid chromatography (HPLC) (Berg et al., 1995; Coquart et al., 1993; Wenheng et al., 1991) and fast atom bombardment high-resolution mass spectrometry (FAB-HRMS) (Cai et al., 1994). Low limits of detection and high precision have been reported for these methods. However, they are expensive and cannot be readily deployed in the field or used to screen multiple samples simultaneously. Enzyme-linked immunosorbent assays (ELISAs) can complement these methods because they are inexpensive and portable and can be used to evaluate many samples at the same time. Lucas et al. (1993) developed and utilized an ELISA for hydroxyatrazine and hydroxysimazine using polyclonal antibodies derived from the immunogen *N*-[4-(ethylamino)-6-hydroxy-1,3,5-triazin-2-yl)]-β-alanine−KLH and *N*-[4-(ethylamino)-6-hydroxy-1,3,5-triazin-2-yl)]-β-alanine−AP as a heterologous tracer in a double-coated antibody format. IC_{50} values in the low parts per billion range were obtained, and the assay performed well in a series of matrices (water, soil, horse manure, urine, and fungal extracts). Schlaeppi et al. (1989) had earlier developed two monoclonal antibodies toward the immunogen 5-{4-hydroxy-6-[(1-methylethyl)amino]-1,3,5-triazin-2-yl}-aminopentanoic acid−KLH. Using a homologous hapten on BSA as the coating antigen, they developed a competitive ELISA for hydroxyatrazine with an IC_{50} of 0.002 μM (0.5 ppb). It displayed little recognition for the chlorotriazine herbicides but very strong recognition of similar hydroxytriazine structures. A second useful monoclonal antibody that they examined displayed an IC_{50} of 0.004 μM (1 ppb); it recognized hydroxyatrazine and hydroxysimazine equally well but had minimal recognition of other hydroxy and all chlorotriazines studied.

Because ELISA is a good complement to instrumental analytical methods and *s*-triazine herbicides are used worldwide, ELISAs to many members of this family of herbicides and their metabolites have been developed. Characterization of these has yielded valuable information about hapten design principles (Goodrow et al., 1990). The hapten used in this study (hapten **IIa**, Figure 1) was originally designed to elicit antibodies selective for prometon (Figure 2). Instead, all of the resulting assays studied were more selective for the hydroxytriazines examined, specifically, hydroxypropazine, hydroxyatrazine, and hydroxysimazine (Figure 2). Thus, further work focused on optimizing the assays for these analytes, rather than for prometon.

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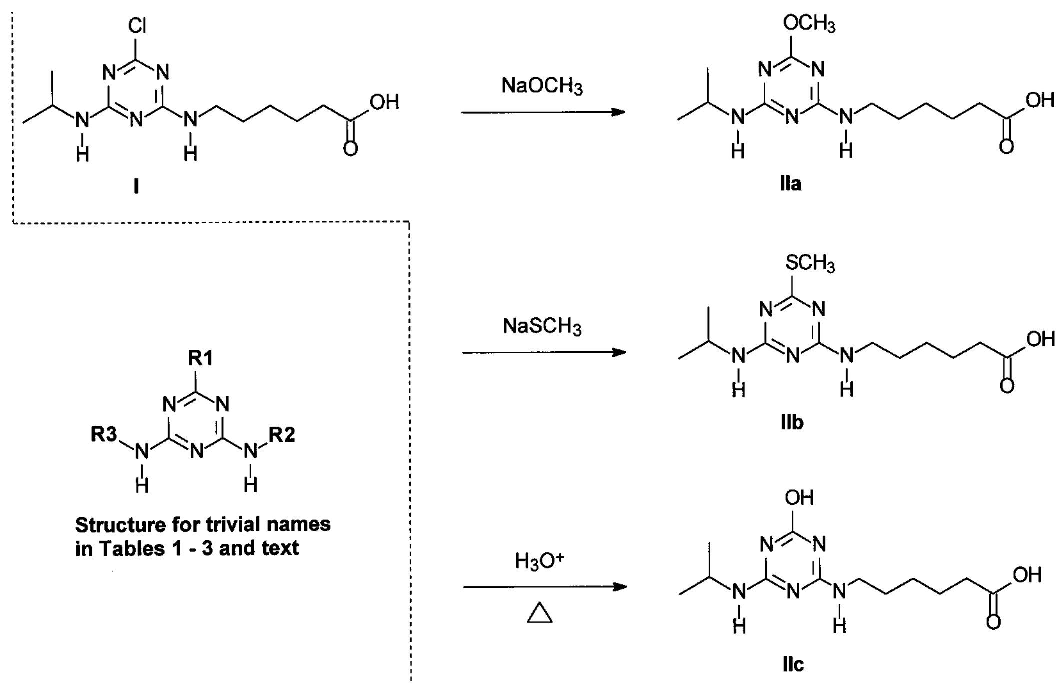

Figure 1. Synthetic routes and structures of *s*-triazine haptens for conjugation to proteins and key structure for use with Tables 1−3.

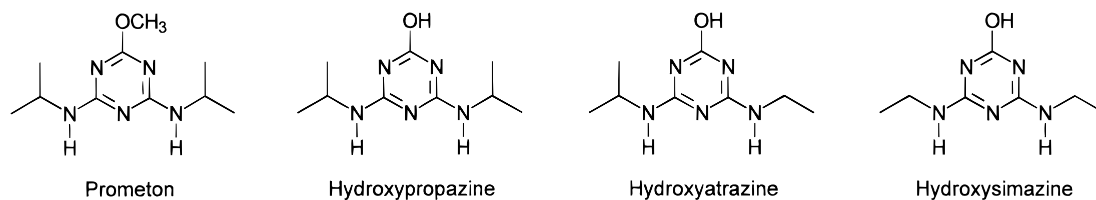

Figure 2. Structures of target *s*-triazines.

MATERIALS AND METHODS

Chemicals. Keyhole limpet hemocyanin (KLH), conalbumin (CONA), bovine serum albumin (BSA), 3,3′,5,5′-tetramethylbenzidine (TMB), and goat anti-rabbit horseradish peroxidase were obtained from Sigma Chemical Co. (St. Louis, MO). Horseradish peroxidase (HRP) and affinity-purified goat anti-rabbit antibodies were purchased from Boehringer Mannheim (Indianapolis, IN). All solvents and salts were purchased from J. T. Baker (Phillipsburg, NJ), Fisher Scientific (Pittsburgh, PA), or Aldrich Chemical Co., Inc. (Milwaukee, WI). A Sybron/Barnstead Nanopure II water system set at 16.7 MΩ-cm provided water for all aqueous solutions. Microtiter plates were purchased from Nunc (No. 4-42404, Roskilde, Denmark). Technical atrazine was donated by Shell Agriculture Chemical Co. (Modesto, CA), and the analytical *s*-triazine standards were a gift from Ciba-Geigy Corp. (Greensboro, NC). The apparent purity of these and the newly synthesized haptens herein was supported by observing a single UV dense spot on 0.25-mm thick precoated silica gel 60 F254 plastic-backed plates from E. Merck (Darmstadt, Germany) in several solvent systems. Compounds were detected first by viewing under UV light (254 nm) and then by staining with iodine vapors. Eluant solvent systems were as follows: (A) tetrahydrofuran (THF)/ethyl acetate/hexane (2:13:35 v/v/v) plus 2% acetic acid; (B) THF/ethyl acetate/hexane (10:1:39 v/v/v) plus 2% acetic acid; (C) hexane/ethyl acetate (2:1 v/v) plus 2% acetic acid; (D) hexane/ethyl acetate (1:1, v/v) plus 2% acetic acid. Details of the syntheses of haptens **IIa** and **IIb** (Figure 1) are described herein; the synthesis of hapten **IIc** is described by Lucas et al. (1993). Syntheses of haptens **I** and **III** (Table 2) are described by Goodrow et al. (1990).

Instruments. Melting points were determined with a Thomas Hoover apparatus (A. H. Thomas Co., Philadelphia, PA). Infrared (IR) spectra were recorded on a Mattson Galaxy Series FTIR 3000 spectrometer (Madison, WI). The ^{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 tetramethylsilane. FAB-HRMS spectra were obtained on a ZAB-HS-2F mass 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 to the matrix as a mass calibrant. The positive ion electrospray mass spectrum (PIES-MS) was obtained from a VG/Fisons BioQ triple quadrupole mass spectrometer (Fisons/VG, Manchester, U.K.). The sample was applied by direct injection; the mobile phase was a 50:50 (v/v) acetonitrile/H_2O plus 1% formic acid solution. Micellar electrokinetic capillary chromatography (MECC) utilized a Model 270A capillary electrophoresis system (Applied Biosystems, Inc., San Jose, CA) equipped with a 50 cm × 50 μm i.d. fused silica column. The electrolyte buffer, pH 8, consisted of 10 mM sodium phosphate and 25 mM sodium dodecyl sulfate. An applied voltage of 20 kV (resulting current 18 μA) was used with a UV detector (length to detector 22 cm) monitoring the effluent at 225 nm. The sample was vacuum-injected over 1.5 s. The temperature was 30 °C. Microtiter plates were read with a Spectramax 250 microplate reader (Molecular Devices, Menlo Park, CA). Inhibition curves used for data analyses were composed of a 12-point standard curve in quadruplicate, and the software package Softmax Pro (Molecular Devices) was used for fitting a sigmoidal curve based on the four-parameter logistic method of Rodbard (1981).

Synthesis of Haptens. *6-{4-Methoxy-6-[(1-methylethyl)-amino]-1,3,5-triazin-2-yl}aminohexanoic Acid (**IIa**, Figure 1).* To a sodium methoxide solution prepared in a nitrogen atmosphere from 0.56 g (24 mmol) of sodium metal and 50 mL of dry methanol was added 1.51 g (5.00 mmol) of chlorotriazine **I**. After 5 h of heating under reflux, a TLC of the

Table 1. cAg's and Results of 2-D Titer Experiments for cAg Assay Development

	structure			rabbit				
cAg	R1	R2	R3	4652	4653	4654	4655	4656
1	Cl	$CH(CH_3)_2$	$(CH_2)_5CONH(CONA)$	+++[a]	+++	+++	+++	+++
2	$S(CH_2)_2CONH(BSA)$	$CH(CH_3)_2$	CH_2CH_3	nt	nt	nt	++	-
3	$S(CH_2)_2CONH(CONA)$	$CH(CH_3)_2$	CH_2CH_3	++	+	++	+++	+++
4	OH	$CH(CH_3)_2$	$(CH_2)_5CONH(BSA)$	nt	nt	nt	+++	+++
5	Cl	CH_2CH_3	$(CH_2)_5CONH(BSA)$	nt	nt	nt	+++	+++
6	Cl	CH_2CH_3	$(CH_2)_5CONH(CONA)$	+++	+++	+++	+++	+++
7	$S(CH_2)_2CONH(CONA)$	CH_2CH_3	CH_2CH_3	++	+	+	+++	++
8	Cl	CH_2CH_3	$(CH_2)_2CONH(BSA)$	nt	nt	nt	+++	+++
9	Cl	CH_2CH_3	$(CH_2)_3CONH(BSA)$	nt	nt	nt	+++	+++
10	OH	$CH(CH_3)_2$	$(CH_2)_5CONH(KLH)$	+++	+++	+++	nt	nt
11	OCH_3	$CH(CH_3)_2$	$(CH_2)_5CONH(BSA)$	nt	nt	nt	+++	+++
12	OCH_3	$CH(CH_3)_2$	$(CH_2)_5CONH(KLH)$	+++	+++	+++	nt	nt

[a] Optical density levels at 0.625 μg/mL cAg and a 1/4000 dilution of serum: nt, not tested because rabbit immunized with the same protein as in cAg; −, 0.000−0.060; +, 0.061−0.300; ++, 0.301−0.999; +++, >0.999.

solution showed the absence of **I** at R_f 0.45 (eluant A). The mixture was concentrated by rotoevaporation and the residue dissolved in 25 mL of water. After filtration through Celite, the filtrate was acidified with 6 M HCl to pH 7. A viscous white semisolid, containing white crystals, precipitated. The combined solids were dissolved in chloroform and filtered through Celite to remove insoluble material. Concentration of the chloroform solution by rotoevaporation provided 1.76 g of a pale yellow viscous oil. The oil was dissolved in 20 mL of hot ethyl acetate from which was obtained an off-white solid on cooling. Recrystallization of this crude material from 2-butanone provided 1.17 g (79%) of **IIa** as a white powder: mp 87.0−89.0 °C; TLC R_f 0.20 (eluant A); IR (KBr) 3365 (m, NH), 3207 (m, NH), 3122 (m, NH), 1734 (vs, C=O), 1614 (vs, C=N), 1226 (m, C−O) cm^{-1}; ^1H NMR (DMSO-d_6) δ 11.9 (br, 1 H, OH), 8.70 (d, J = 7.2 Hz, 1 H, CHNH), 8.57 (t, J = 5.6 Hz, 1 H, CH_2NH), 4.1 (m, 1 H, CH), 3.58 (s, 3 H, CH_3O), 3.3 (m, 2 H, CH_2-6), 2.31 (t, J = 7.5 Hz, 2 H, CH_2-2), 1.5 (m, 4 H, CH_2-3,5), 1.3 (m, 2 H, CH_2-4), 1.20 (d, J = 6.5 Hz, 6 H, 2 CH_3) (with added D_2O the 11.9, 8.70, and 8.57 ppm peaks disappeared, the 4.1 multiplet resolved into a heptet at 4.11 ppm, J = 6.4 Hz, and the 3.4 multiplet became a triplet at 3.37 ppm, J = 6.7 Hz); ^{13}C NMR (DMSO-d_6) δ 173.3 (COOH), 156.4 (Ar-C6), 155.5 (Ar-C2), 146.9 (Ar-C4), 51.3 (CH_3O), 43.7 (CH), 40.6 (C6), 33.3 (C2), 28.2 (C5), 25.6 (C4), 24.1 (C3), 21.8 (2 CH_3); FAB-HRMS m/z calcd for $C_{13}H_{23}N_5O_3$ 298.1879, obsd 298.1874.

*6-{4-[(1-Methylethyl)amino]-6-(methylthio)-1,3,5-triazin-2-yl}aminohexanoic Acid (**IIb**, Figure 1).* A mixture of 1.51 g (5.00 mmol) of chlorotriazine **I**, 1.00 g (14.0 mmol) of sodium thiomethoxide, and 20 mL of ethylene glycol dimethyl ether was heated under reflux for 2.5 h in a N_2 atmosphere. A TLC of an acidified portion of the reaction mixture showed the total absence of **I** at this time and the presence of only one new component. The mixture was poured into 150 g of ice/water to provide a transparent alkaline solution. Acidification with concentrated HCl to pH 4.5 produced a white, flocculent precipitate. This was collected, washed with water, and dried to obtain 1.238 g of a white, powdery solid, mp 105.5−107.0 °C. An additional 0.225 g of product was obtained on extraction of the aqueous filtrate with $CHCl_3$. Recrystallization of the combined samples from acetonitrile gave 1.128 g (72%) of **IIb** as a white powder: mp 107.0−108.5 °C; TLC R_f 0.31 (eluant A), R_f 0.86 (eluant B), R_f 0.26 (eluant C), R_f 0.67 (eluant D); IR (KBr) 3273 (m, NH), 3126 (w, NH), 1704 (m, C=O), 1554 (vs, C=N) cm^{-1}; ^1H NMR (DMSO-d_6) δ 12.0 (br,1 H, OH), 7.2 (m, 1 H, CH_2NH), 7.1 (m, 1 H, CHNH), 4.0 (m, 1 H, CH), 3.2 (m, 2 H, CH_2-6), 2.34, 2.36 (two s, 3 H, CH_3S), 2.19 (t, J = 7.2 Hz, CH_2-2), 1.5 (m, 4 H, CH_2-3,5), 1.3 (m, 2 H, CH_2-4), 1.1 (m, 6 H, 2 CH_3) (with added D_2O the 12.0, 7.2, and 7.1 ppm peaks disappeared and the 3.2 multiplet became a triplet at 3.22 ppm, J = 6.8 Hz); ^{13}C NMR (DMSO-d_6) δ 180.0 (Ar-C6), 174.7 (COOH), 164.3 (Ar-2 or 4), 163.4, 163.2 (Ar-C2 or 4), 41.8, 41.3 (CH), 40.0 (C6), 33.8 (C2), 28.9 (C5), 26.2 (C4), 24.5 (C3), 22.6, 22.4 (2 CH_3), 12.3 (CH_3S); PIES-MS m/z (rel intensity) 315 (18, M + H^+ + 1), 314 (100, M + H^+), 115 [12, $(CH_2)_5CO_2H^+$], 74 (31); FAB-HRMS m/z calcd for $C_{13}H_{24}N_5O_2S$ 314.1651, obsd 314.1661.

Conjugation of Haptens to Proteins. Hapten **IIa** was conjugated to BSA (**11**, Table 1) and KLH (**12**, Table 1) in 0.05 M borate buffer (pH 8) using the active ester method of Bauminger and Wilchek (1980), and each conjugate was used for immunizing rabbits. The conjugates were purified by dialysis against 0.1 M PBS, pH 7.5 (PBS: 8 g/L NaCl, 1.15 g/L Na_2HPO_4, 0.2 g/L KH_2PO_4, 0.2 g/L KCl). The UV−vis spectrum (220−420 nm) of each hapten conjugate was compared to the spectrum of the corresponding native protein to confirm qualitatively the conjugation reaction. The slight shift in the λ_{max} to 276 nm and a large rise in optical density at this wavelength were used as evidence that coupling of the hapten to the protein had occurred. Analyses of the conjugates by ELISA using our antiserum 357 (Harrison et al., 1991), which had reasonable affinity for prometon, was performed to estimate the amount of hapten per milligram of protein. The hapten loading was estimated, using prometon as the standard, at 0.6 ng/mg of protein for the KLH conjugate **12** and 3 ng/mg for the BSA conjugate **11**. The syntheses of haptens and their conjugation to proteins to produce conjugates **1**−**3**, **5**−**7**, and **9** (Table 1) are described by Goodrow et al. (1990). Lucas et al. (1993) is the reference for conjugates **4**, **8**, and **10**. The conjugation of haptens **IIa** and **IIb** to HRP for use in competitive ELISA is described by Wortberg et al. (1995).

Immunization and Antiserum Collection. Five female New Zealand white rabbits (about 2.2 kg each) were immunized intradermally, three with conjugate **11** (rabbits 4652, 4653, and 4654) and two with conjugate **12** (rabbits 4655 and 4656). The hapten−protein conjugates for immunizing purposes were prepared by dissolving 100 μg of hapten−protein conjugate in 0.25 mL of PBS buffer and emulsifying this with an equal volume of Freund's complete adjuvant for each rabbit. After the initial immunization, subsequent boosts were administered every 4 weeks using the same hapten−protein but with Freund's incomplete adjuvant as a diluant. Rabbits were bled 7−10 days after each boost. The blood was allowed to clot overnight, whereafter the antisera were removed, separated from any remaining red blood cells by centrifugation, and stored at −20 °C. Each antiserum was tested for titer using the method outlined by Gee et al. (1988) and used to develop ELISAs as described below.

ELISA. In accordance with the principles of solid phase immunoassay (Voller et al., 1976), competitive ELISAs in the coated antigen, the coated antibody, and the double-coated antibody formats were evaluated (Figure 3). These formats represent the two possible approaches to competitive solid phase immunoassay (immobilized antibody and immobilized antigen) as described by Tijssen (1985). Prior to inhibition experiments, antiserum, enzyme tracer, and coating antigen dilutions were optimized by using the two-dimensional titration method as outlined by Gee et al. (1988). The specified incubation times are the ones necessary to achieve optical densities between 0.5 and 1.0 by using the minimum amount of immunoreagents. For the coated antigen format, which was eventually selected for further characterization on the basis of selectivity for hydroxytriazines, additional optimization of

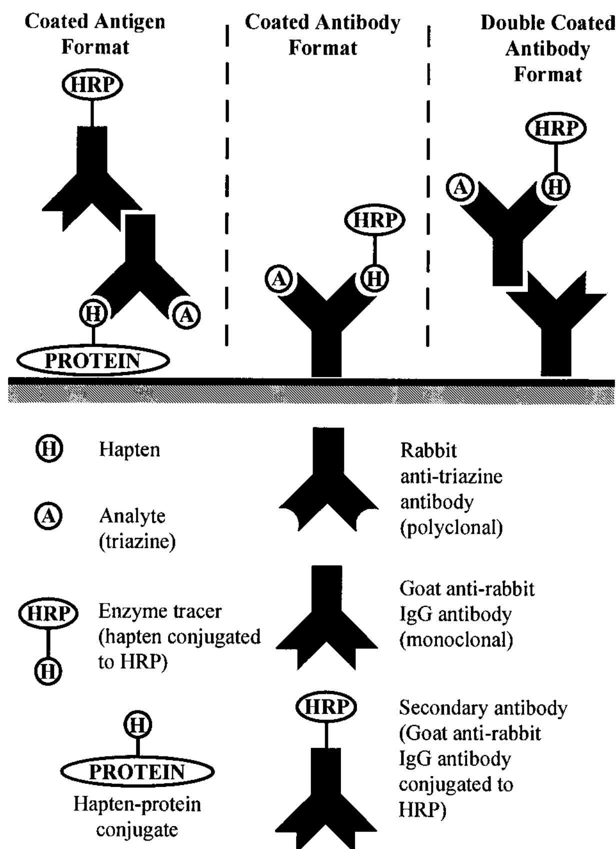

Figure 3. Conceptual representation of the three ELISA formats that were utilized in this study.

incubation parameters was undertaken as described below. For the coated antibody and double-coated antibody formats, no additional optimization was undertaken.

Coated Antigen (cAg) Format. Microtiter plates were incubated overnight at 4 °C with 100 μL/well of a hapten–protein conjugate in a carbonate/bicarbonate coating buffer (0.1 M, pH 9.6). The coated plates were washed with PBST [PBS at pH 7.5 with Tween 20: 8 g/L NaCl, 1.15 g/L Na_2HPO_4, 0.2 g/L KH_2PO_4, 0.2 g/L KCl, and 0.05% Tween 20 (v/v)]. Fifty microliters per well of antiserum solution in PBST and 50 μL/well of analyte solution in PBST were placed on the plates and incubated for 30 min at room temperature. After the plates were washed again with PBST to remove any unbound material, 100 μL/well of a commercially available goat anti-rabbit HRP conjugate in PBST was added. One hour later, the plates were washed and a substrate solution of 1% H_2O_2 (w/v)/0.6% TMB (w/v) in 100 mM sodium citrate (pH 5.5) was added to each well. Approximately 15 min thereafter, 50 μL of 4 M sulfuric acid solution was added to each well. The plates were then read in a dual wavelength mode at 450 nm minus 650 nm. The development of color was inversely proportional to the amount of *s*-triazine present.

Deviations from the previously described coated antigen format protocol were implemented for the purpose of evaluating the effects upon IC_{50} (concentration of analyte that will result in 50% inhibition of antibody binding to the coating antigen) and optical density by varying specific parameters of immunoreagent incubation. These parameters were time, temperature, pH, ionic strength, solvent, and groundwater matrix.

In the experiment to evaluate incubation time effects, the incubation time of the solution containing antiserum and analyte in the coated plate was varied. Incubation times of 30, 60, and 120 min were tested with all other assay parameters remaining constant.

The temperature control feature of the Spectramax 250 microtiter plate reader was used to characterize the effects of temperature control during incubation (plate washing was conducted at room temperature) upon assay performance. In the first set of experiments, the temperature of both incubation steps (antiserum/analyte incubation and the goat anti-rabbit HRP incubation) were set at one of the following temperatures: 25, 30, 35, 40, and 45 °C. In all, five assays were performed, one for each temperature. In the second set of experiments, four assays were conducted. In two of the assays, within-assay incubation temperatures were varied. One assay was conducted with the antiserum/analyte incubation at 25 °C and the goat anti-rabbit HRP incubation at 40 °C. The other assay was conducted with the opposite temperature control arrangement. In the other two assays, the within-assay incubation temperatures were held constant at 25 and 40 °C, respectively. To characterize the effects of varying pH, both the analyte and antiserum were dissolved in PBST buffer at the specified pH and added to coated plate in equal volumes (50 μL of each per well). pH values of 5, 6, 7, and 8 were tested in this incubation step with all other parameters being held constant.

Ionic strength effects were determined in the same manner as previously mentioned except that, instead of pH, PBST concentration was varied. PBST concentrations of 0, 0.063, 0.13, 0.25, 0.50, and 1.0 M were tested.

The effects of various solvents were tested by dissolving the analyte in a mixture of 0.1 M PBST and solvent in various proportions (0, 3.1, 13, and 50% solvent) and incubating these with antiserum in 0.1 M PBST on the coated plate. Methanol, acetonitrile, and acetone were tested in this way.

Groundwater matrix effects were evaluated by dissolving analyte in the appropriate groundwater and adding 50 μL of this solution to every well of the coated plate which already contained 50 μL/well of antiserum in 0.1 M PBST. In an additional experiment, each groundwater was diluted with 1 M PBST (9 parts groundwater plus 1 part 1 M PBST) before spiking with analyte and using this in the same manner as before. Other parameters remained the same as in the original protocol.

Coated Antibody (cAb) Format. For this format, 100 μL of a 1:4000 dilution of anti-triazine antibody in coating buffer was dispensed into each well and incubated overnight at 4 °C. After washing with PBST, 50 μL/well of analyte in PBST and 50 μL/well of enzyme tracer (**IIb**–HRP or **IIa**–HRP) in PBST were added. After 2 h at room temperature, the wells were washed to remove any unbound components. TMB substrate solution was added, and after 15 min, 50 μL/well of 4 M sulfuric acid solution was added to each well and the plates were read in a dual wavelength mode at 450 nm minus 650 nm.

Double-Coated Antibody (dcAb) Format. One hundred microliters per well of a 1:2000 dilution of goat anti-rabbit trapping antibody in coating buffer was dispensed into plate wells and incubated overnight at 4 °C. After any unbound materials were washed away, the plates were recoated (100 μL/well) with antiserum (1:16000) in coating buffer for 3 h at room temperature. After washing, 50 μL/well of analyte in PBST and 50 μL/well of enzyme tracer (**IIb**–HRP or **IIa**–HRP) in PBST were added. Three hours later the wells were washed to remove any unbound components. TMB substrate solution was added to each well. Fifteen minutes thereafter, 50 μL/well of 4 M sulfuric acid solution was added to the plates and these were read in a dual wavelength mode at 450 nm minus 650 nm.

RESULTS AND DISCUSSION

Hapten Syntheses. The immunizing hapten 6-{4-methoxy-6-[(1-methylethyl)amino]-1,3,5-triazin-2-yl}-aminohexanoic acid (**IIa**) and tracer haptens **IIb** and **IIc** were prepared by the nucleophilic displacement of the chlorine in compound **I** with sodium methoxide, sodium thiomethoxide, and water, respectively, as illustrated in Figure 1. These were definitively characterized by IR, extensive ^1H- and ^{13}C-NMR, and mass spectral (FAB-MS or PIES-MS) studies to confirm their structures. The purity of each was qualitatively evaluated by TLC and by melting points. Of particular note

were the assignments confirmed by $^1H-^1H$ two-dimensional (2-D) correlated spectroscopy (COSY), the attached proton test (APT) 2-D spectroscopy, and $^1H-^{13}C$ heteronuclear correlation spectroscopy (HETCOR). The $^1H-^1H$ COSY spectrum for the methoxy hapten (**IIa**) permitted a definitive assignment of the 8.70 ppm doublet to the isopropyl side chain and the 8.57 ppm triplet to the hexanoic acid side chain. For the methylthio hapten (**IIb**), this order was reversed. In all three compounds, **IIa**−**c**, the 3.3 ppm CH_2 multiplets (CH_2-NH) and the 2.1 ppm CH_2 triplets (CH_2CO_2) of the aminohexanoic acid moiety were coupled to the 1.5 ppm multiplet (CH_2-3,5: two CH_2 groups) as was the 1.3 ppm CH_2-4 multiplet (one CH_2 group). The assignments found as given under Materials and Methods are thus substantiated. The APT and $^1H-^{13}C$-HETCOR spectra confirmed both proton and carbon-13 assignments as originally ascribed to the conventional one-dimensional spectra. Assignments for the aromatic carbons were made in accordance with our former triazine spectral assignments (Goodrow et al., 1990) and should be considered tentative only.

Production, Screening, and Selection of Antisera. Antisera were screened using the 2-D titration method with the coated antigen, the coated antibody, and the double-coated antibody formats. In the coated antigen format, each rabbit antiserum was tested with all available hapten−protein conjugates (Table 1), except those that contained the same proteins with which the rabbit had been immunized. The objective was to find the coated antigen to which the antiserum being tested had the highest affinity but could still be displaced by the analyte of interest. On the basis of observations from the 2-D titration experiments, combinations of coated antigen and antiserum that resulted in the highest optical densities were selected (++ and +++ in Table 1) for further development. It is interesting to note that in several cases, heterologous assays showed the same titer as homologous assays. This result suggests that in the conjugated form the polyclonal antibodies recognized the chloro, hydroxy, and methoxy haptens.

To evaluate the relative selectivity of each of the above selected combinations, competitive inhibition experiments utilizing each combination were conducted with prometon, prometryne, propazine, and hydroxypropazine, in the coated antigen format. These inhibitors were chosen to help evaluate the selectivity of each antiserum for the methoxy, thiomethoxy, chlorine, and hydroxy components of the corresponding triazine structures, with all else remaining the same. Since all rabbits were immunized with hapten **IIa**, which contains a methoxy moiety, it was expected that prometon would be the strongest inhibitor of antibody binding to the coated antigen. However, hydroxypropazine was observed to be the best inhibitor in every combination of antibody and coated antigen tested. (In an additional effort to find a prometon-specific system, combinations marked + in Table 1 were also tested in the competitive inhibition format.) Since the antisera demonstrated higher affinity for hydroxypropazine relative to prometon, it was decided to utilize them for the development of hydroxytriazine-selective assays.

The search for systems that would express the highest sensitivity for hydroxytriazines proceeded through several iterations of optimization of antiserum and coated antigen concentrations. The goal of this process was to find the reagent concentrations that would result in optical densities of approximately 1 and the lowest IC_{50} values. For the target analytes (hydroxytriazines) the lowest possible IC_{50} is desirable because this parameter is positively correlated with the limit of detection for the target analyte. After optimization, the combinations of coating antigen **7** with antiserum 4653 (hereafter designated **7**/4653) and antiserum 4652 (hereafter designated **7**/4652) gave the lowest IC_{50} values when using hydroxytriazines as the inhibitors. These two systems were therefore selected for further development and characterization.

Two-dimensional titrations with the coated antibody as well as the double-coated antibody formats were conducted using hapten **IIa** conjugated to HRP (**IIa**−HRP) and hapten **IIb** conjugated to HRP (**IIb**−HRP) as enzyme tracers. After several optimization iterations of coated antibody and enzyme tracer concentrations, the combination of antiserum 4656 and **IIa**−HRP [hereafter designated 4656(cAb)/**IIa**−HRP] resulted in the lowest IC_{50} values for the hydroxytriazines. With the double-coated antibody format, antiserum 4655 and **IIb**−HRP [hereafter designated 4655(dcAb)/**IIb**−HRP] was the most sensitive combination for hydroxytriazines.

With the above selected systems, chosen on the basis of high sensitivity for hydroxytriazines, the next step in the assay development process was the comprehensive determination of cross-reactivity profiles for each. In this stage, the high selectivity of a system for hydroxytriazines relative to other triazines was the criterion used to select a system for further characterization.

Cross-Reactivities (CR). For the purpose of describing selectivity, CR is determined by dividing the IC_{50} of the chemical assigned to be 100% by the IC_{50} of another compound and multiplying by 100 to obtain a percent figure. Thus, the process of obtaining a good assay becomes a function of developing an assay with the lowest possible IC_{50} and the best CR profile (CR figures for analytes other than the target are as small as possible).

Coated Antibody Format. ELISA system 4656(cAb)/**IIa**−HRP expressed potential for the detection of hydroxyatrazine ($IC_{50} = 0.011$ μM, 2.2 ppb), hydroxypropazine ($IC_{50} = 0.028$ μM, 5.9 ppb), and hydroxysimazine ($IC_{50} = 0.038$ μM, 7.0 ppb) (Table 2). Sensitivity for prometon was low ($IC_{50} = 1$ μM, 225 ppb). As expected, with this homologous format (hapten used for immunization was the same as that used in the ELISA) absolute sensitivity was highest for hapten **IIa**.

Double-Coated Antibody Format. An ELISA system with this format was developed, optimized, and designated 4655(dcAb)/**IIb**−HRP. Although this system did have high sensitivity for hydroxyatrazine ($IC_{50} = 0.053$ μM, 10 ppb), sensitivities for hydroxypropazine ($IC_{50} =$ 0.073 μM, 17 ppb), and hydroxysimazine ($IC_{50} = 0.86$ μM, 160 ppb) were not as good. In fact, the highest sensitivity obtained with this assay was for atrazine ($IC_{50} = 0.024$ μM, 5.2 ppb). However, selectivity for any single *s*-triazine examined was not high enough to make this system useful for the detection of specific *s*-triazines. Thus, this system was not chosen for further development (Table 2). A recognition pattern did become apparent with the concurrent presence of both an isopropylamino moiety and an aminohexanoic acid moiety attached to the triazine ring being the most important determinants for antibody recognition (Table 3). Of the 19 inhibitors tested, 4 met this criteria,

Table 2. CR Profiles (Ascending Order of IC_{50} of System D)

inhibitor	structure R1	structure R2	structure R3	system A[a] IC_{50} (μM)	system A[a] CR[e] (%)	system B[b] IC_{50} (μM)	system B[b] CR[e] (%)	system C[c] IC_{50} (μM)	system C[c] CR[e] (%)	system D[d] IC_{50} (μM)	system D[d] CR[e] (%)
hydroxypropazine	OH	$CH(CH_3)_2$	$CH(CH_3)_2$	0.028	40	0.073	70	0.0050	200	0.0020	200
IIa	OCH_3	$CH(CH_3)_2$	$(CH_2)_5COOH$	0.00016	7000	0.010	500	0.0090	90	0.0020	200
IIc	OH	$CH(CH_3)_2$	$(CH_2)_5COOH$	0.00019	6000	0.0080	700	0.087	9	0.0030	100
hydroxyatrazine	OH	CH_2CH_3	$CH(CH_3)_2$	0.011	**100**	0.053	**100**	0.0080	**100**	0.0040	**100**
hydroxysimazine	OH	CH_2CH_3	CH_2CH_3	0.038	30	0.86	6	0.045	20	0.017	20
I	Cl	$CH(CH_3)_2$	$(CH_2)_5COOH$	0.052	20	0.0040	1000	0.23	4	0.049	8
propazine	Cl	$CH(CH_3)_2$	$CH(CH_3)_2$	8.0	0.1	0.038	100	0.76	1	0.14	3
III	Cl	$CH(CH_3)_2$	$(CH_2)_2COOH$	0.25	4	0.13	40	0.27	3	0.15	3
prometon	OCH_3	$CH(CH_3)_2$	$CH(CH_3)_2$	1.0	1	0.054	100	0.088	9	0.15	3
IIb	SCH_3	$CH(CH_3)_2$	$(CH_2)_5COOH$	0.14	8	0.012	400	0.054	10	0.23	2
atrazine	Cl	CH_2CH_3	$CH(CH_3)_2$	57	<0.1	0.024	200	0.48	2	0.27	2
prometryne	SCH_3	$CH(CH_3)_2$	$CH(CH_3)_2$	2.2	1	0.060	90	0.19	4	0.45	1
procyazine	Cl	$CH(CH_3)_2$	$CCN(CH_3)_2$	1.5	1	1.7	3	1.3	1	0.50	1
terbumeton	OCH_3	CH_2CH_3	$C(CH_3)_3$	18	0.1	1.3	4	0.33	2	0.58	1
terbutryne	SCH_3	CH_2CH_3	$C(CH_3)_3$	69	<0.1	0.97	6	0.30	3	1.2	0.3
ametryne	SCH_3	CH_2CH_3	$CH(CH_3)_2$	2.3	1	0.071	70	0.11	7	1.5	0.3
simazine	Cl	CH_2CH_3	CH_2CH_3	0.90	1	0.46	10	1.4	1	1.6	0.3
cyanazine	Cl	CH_2CH_3	$CCN(CH_3)_2$	0.16	7	2.0	3	>100	<0.1	2.9	0.1
simetryne	SCH_3	CH_2CH_3	CH_2CH_3	2.4	1	0.83	6	0.57	1	3.1	0.1

[a] System A: ELISA system [designated 4656(cAb)/**IIa**−HRP in text] in which serum 4656 is used as coating antibody and hapten−enzyme conjugate **IIa**−HRP is used as tracer. [b] System B: ELISA system [designated 4655(dcAb)/**IIb**−HRP in text] in which anti-rabbit antibodies are coated onto plate followed by incubation with serum 4655. Serum 4655 antibodies are trapped by the anti-rabbit antibodies, and hapten−enzyme conjugate **IIb**−HRP is then used as tracer. [c] System C: ELISA system (designated **7**/4653 in text) in which hapten−protein conjugate **7** is used as coating antigen and serum 4653 is used in the subsequent competitive inhibition step. [d] System D: ELISA system (designated **7**/4652 in text) in which hapten−protein conjugate **7** is used as coating antigen and serum 4652 is used in the subsequent competitive inhibition step. [e] CR numbers are expressed as a percent relative to the IC_{50} for hydroxyatrazine in a specific system. The assay parameters are detailed in the text. CR is determined by expressing the ratio of the IC_{50} of the chemical assigned to be 100% (hydroxyatrazine) to the IC_{50} of the other compounds and expressed as a percent.

Table 3. Inhibitor Ranking Table for ELISA System 4655(dcAb)/IIb−HRP

inhibition rank	structure R1	structure R2	structure R3	no. of inhibitors
1	−[a]	$CH(CH_3)_2$	$(CH_2)_5COOH$	4
2	−	−	$CH(CH_3)_2$	7
3	−	$CH(CH_3)_2$	−[b]	1
4	−	CH_2CH_3	CH_2CH_3	3
5	−	−	−	4

[a] −, functional group in the corresponding column of Table 2 that, when input, will result in a structure which matches that of an inhibitor represented in Table 2. [b] Does not include $CCN(CH_3)_2$.

resulting in the 4 lowest IC_{50} values. In the absence of both of these determinants, the next most important determinant was the presence of at least one isopropylamino moiety attached to the triazine ring. The 9 inhibitors with this appendage expressed the second lowest IC_{50} values. The only exception was cyanazine, which proved to be a weak inhibitor with an IC_{50} of 2.0 μM. The next identifiable group with the next lowest IC_{50} values was that with two ethylamino moieties attached to the triazine ring. Three inhibitors fell into this category and expressed the third lowest IC_{50} values as a class. Because hapten IIa was used as immunogen, one could have reasonably predicted that both the isopropylamino and aminohexanoic acid moieties would be strongly recognized by the induced antibodies.

This system could be useful to distinguish between triazines that possess isopropylamino side chains from those that contain ethylamino side chains. The relatively large difference in CR between compounds differing by a single methyl group could prove especially useful in multianalyte systems (Wortberg et al., 1995).

Coated Antigen Format. ELISA systems **7**/4653 (coating antigen **7** with antiserum 4653) and **7**/4652 expressed the highest sensitivity to hydroxypropazine, followed by hydroxyatrazine and hydroxysimazine (Table 2). The order of affinity for these hydroxytriazines is indicative of the expected predominant recognition by both systems of isopropylamino groups attached to the triazine ring. As expected, since hapten **IIa** was used for immunization, it was more strongly recognized by both systems than the other haptens tested.

There were several differences between the two systems on which the selection of a single system could be based. These were differences in IC_{50}, relative selectivity, signal to background ratio, and reproducibility. IC_{50} values of hydroxytriazines achieved with **7**/4652 were slightly lower than those achieved with **7**/4653 (0.002, 0.004, and 0.017 μM with **7**/4652 vs 0.005, 0.008, and 0.045 μM with **7**/4653). The relative selectivity of **7**/4652 for hydroxypropazine, hydroxyatrazine, and hydroxysimazine relative to the triazine herbicides is better than that of **7**/4653. In addition, the signal to background ratio is much higher with **7**/4652 than with **7**/4653 (data not shown). Finally, since additional experiments indicated that combination **7**/4652 gave more reproducible results than **7**/4653, the former system was selected for further assay development and characterization. The coated antibody format 4656(cAb)/**IIa**−HRP was not characterized further because the IC_{50} values for hydroxytriazines were higher than those achieved with both coated antigen formats tested.

The antisera's unexpected selectivity for hydroxy over methoxy groups brought up the possibility that the immunizing hapten may have hydrolyzed either before or during the antibody development process. In light of this, interesting systems to compare with each other were the ones utilizing coating antigens containing hydroxy and methoxy groups, respectively. This is why the performances of conjugates **10** and **12** were evaluated in the coating antigen format with antiserum 4653. The same dilution of 4653 was used for both cAg's. This experiment would help exclude the possibility that hydrolysis of hapten **IIa** on conjugate **12** occurred if the CR profiles of these systems were to significantly differ

Table 4. CR Profiles of Systems 10/4653 and 12/4653[a]

inhibitor	structure			10/4653		12/4653	
	R1	R2	R3	IC_{50} (μM)	CR[b] (%)	IC_{50} (μM)	CR[b] (%)
hydroxypropazine	OH	$CH(CH_3)_2$	$CH(CH_3)_2$	0.0090	**100**	0.00040	**100**
prometon	OCH_3	$CH(CH_3)_2$	$CH(CH_3)_2$	1.3	0.7	0.098	0.4
propazine	Cl	$CH(CH_3)_2$	$CH(CH_3)_2$	2.2	0.4	0.45	0.09
prometryne	SCH_3	$CH(CH_3)_2$	$CH(CH_3)_2$	2.6	0.3	1.3	0.03

[a] **10**/4653: ELISA system in which hapten–protein conjugate **10** is used as coating antigen and serum 4653 is used in the subsequent competitive inhibition step. **12**/4653: ELISA system in which hapten–protein conjugate **12** is used as coating antigen and serum 4653 is used in the subsequent competitive inhibition step. [b] CR numbers are expressed as a percent relative to the IC_{50} for hydroxypropazine in a specific system. The assay parameters are detailed in the text. CR is determined by expressing the ratio of the IC_{50} of the chemical assigned to be 100% (hydroxypropazine) to the IC_{50} of the other compounds and expressed as a percent.

from each other. Since conjugate **10** contains a hydroxy moiety, it was expected that system **10**/4653 (hapten–protein conjugate **10** and antiserum 4653 used in coated antigen format) would express higher selectivity for hydroxypropazine than for prometon. In principle, since hapten **IIa** was used to immunize rabbits, assay system **12**/4653 should have been more selective for prometon than for hydroxypropazine. CR profiles of coated antigen ELISA formats **10**/4653 and **12**/4653 were determined with prometryne, prometon, propazine, and hydroxypropazine as model inhibitors for this purpose. Both systems tested were more selective for hydroxypropazine, and their CR profiles did not differ significantly from each other (Table 4). In general, the IC_{50} values with system **12**/4653 were lower than the corresponding ones with **10**/4653. Along with both systems' selectivity for hydroxypropazine, this suggests that antiserum 4653 recognizes the hydroxy appendage more strongly than the corresponding methoxy one. Thus, 4653 is more easily displaced from conjugate **12** than from **10**. However, these results cannot exclude the possibility that the methoxy group of conjugate **12** may have hydrolyzed to a hydroxy group. This would also account for the similar CR profiles of the two systems tested. The question of the hydrolytic stability of hapten **IIa** was explored further as described below.

Hapten Stability. Selectivity of the antisera for hydroxytriazines suggested that some hydrolysis of hapten **IIa** may have occurred either before and or after its injection into the rabbits. Thus, the stability of hapten **IIa** to hydrolysis at various pH values was tested. Fifty parts per million solutions of **IIa** were made in buffers of various pH values: 2 (100 mM sodium phosphate), 4 (100 mM sodium acetate), 6 and 7 (100 mM sodium phosphate), 8 and 9 (100 mM sodium borate), and 10 (100 mM borax/NaOH). These solutions were then analyzed with capillary electrophoresis both immediately and after incubation at 37 °C for up to 8 days. After 1 day, significant reductions in the **IIa** occurred in the pH values of 2, 9, and 10 (Figure 4). After 8 days at pH values of 7 and 8, approximately 80% of the original **IIa** concentration remained. With the exception of pH 7, in all cases of observed **IIa** concentration reductions, corresponding increases of **IIc** concentrations were also detected. At pH 7, an increase in the concentration of **IIc** was not evident even though a reduction in the concentration of **IIa** was. Hapten **IIa** appeared to remain stable at pH values of 4 and 6 after 8 days of incubation. These results suggest that **IIa** is subject to slow hydrolysis at physiological pH and temperature as well as under the pH conditions used for coupling. There may also be a pH effect on tautomerization of the triazine structure, which may affect antibody recognition (Weber, 1967).

Whatever the means may have been, the end result was an assay system (**7**/4652) with high sensitivity and

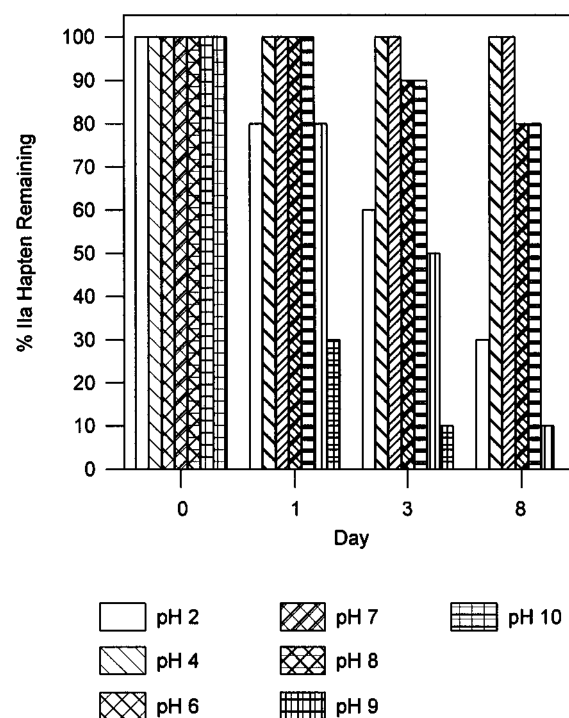

Figure 4. Effect of varying pH upon stability of hapten **IIa**. The percentage of **IIa** remaining was calculated as a ratio of **IIa** peak area relative to the peak area of a hydroxypropazine internal standard. The only decomposition product observed was the corresponding 2-hydroxy compound.

selectivity for hydroxytriazines. To develop this ELISA into a practical analytical tool, some characterization of its performance under simulated field conditions was necessary. For this purpose, hydroxyatrazine was selected to serve as a model inhibitor since atrazine is used in much greater quantities than either simazine or prometon (Aspelin, 1994); it is more important to detect its major degradation product (hydroxyatrazine) than the degradation products of the other herbicides (hydroxysimazine and hydroxypropazine). Thus, further characterization was undertaken with hydroxyatrazine as the model inhibitor.

Time Effects. Because minimization of analysis time is important for any field analysis method, for system **7**/4652, the effect of incubation time of hydroxyatrazine and antiserum 4652 on the coated plate was tested. Incubation times of 30, 60, and 120 min were evaluated. As illustrated in Figure 5, the 30 min incubation time yielded the lowest IC_{50} (0.007 μM), 60 min yielded the intermediate IC_{50} (0.018 μM), and 120 min of incubation time yielded the highest IC_{50} (0.054 μM). Absolute maximum absorbencies observed at zero concentration of hydroxyatrazine increased slightly from 0.229 optical density (OD) unit at 30 min to 0.239 OD unit at 120 min. Since increased incubation time did

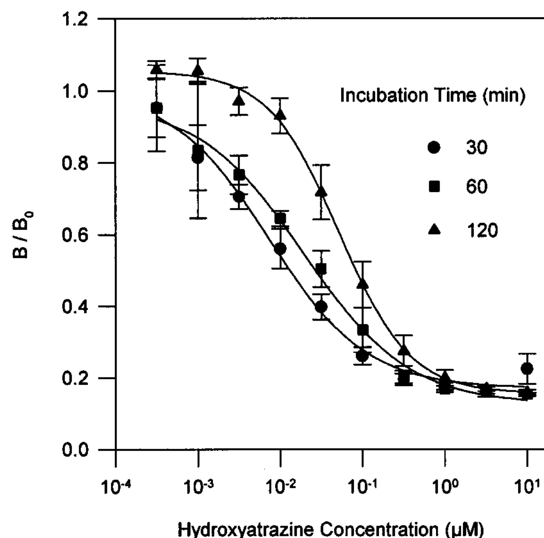

Figure 5. Effect of varying incubation time of a microtiter plate coated with conjugate **7** with antiserum 4652 and hydroxyatrazine (hereafter referred to as **7**/4652). Other incubation steps were held constant as described under Materials and Methods. Dose–response curves are plotted relative to response of assay to 0 M concentration of hydroxyatrazine (control).

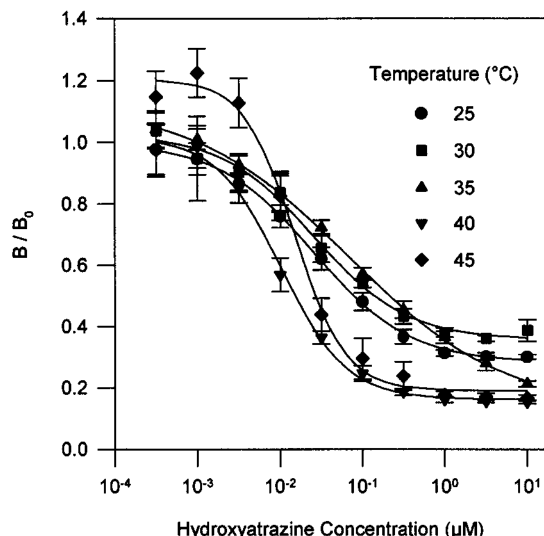

Figure 6. Effect of varying incubation temperature of ELISA **7**/4652.

not improve assay performance, the 30 min incubation time was used in subsequent assay characterizations.

Temperature Effects. Field analysis methods are often subjected to a wide range of temperatures. To determine the effect of various temperatures of incubation upon assay **7**/4652, a temperature control experiment involving both immunoreagent incubations steps of the assay was undertaken. It was determined that no significant change in IC_{50} occurred in the range from 25 to 35 °C (Figure 6). However, at 40 and 45 °C, significant increases in the signal to noise ratio were evident. To identify the incubation step in which temperature control resulted in the greatest improvement, additional experiments, in which the first incubation was done at 25 °C with the second at 40 °C and vice versa, were conducted. The IC_{50} of the former combination was lower than that of the latter but greater than that obtained when both incubation steps were run at 40 °C. These results imply that the greatest benefit of temperature control is realized when the

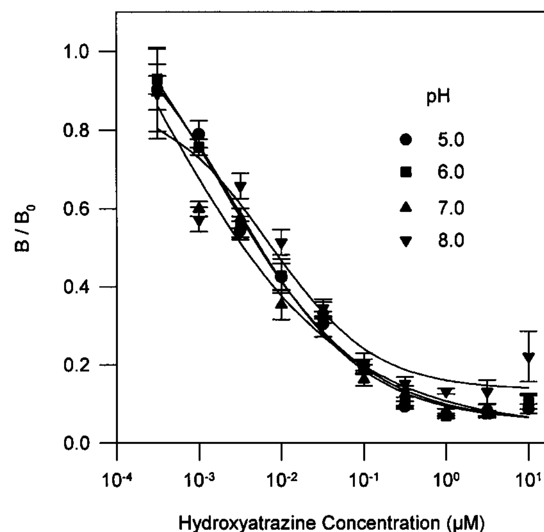

Figure 7. Effect of varying pH upon ELISA **7**/4652. Values refer to actual pH values in the well of the microtiter plate, after antiserum and hydroxyatrazine standard solutions have been added.

temperatures of both incubation steps are controlled. Temperature control during the second incubation alone results in lower IC_{50} than when it is exercised during the first incubation alone.

Even though the above results indicate significant effects by elevated incubation temperatures, effective detection of hydroxyatrazine will still occur as long as standard and unknown samples are analyzed at the same ambient temperature. In cases when the preservation of between-assay parameters is important, constant temperature control would be indicated. However, since assay parameters vary only slightly over a wide range of temperatures (25–35 °C), the use of laboratory temperatures would probably be sufficient to maintain constant assay parameters.

Salt, Solvent, and pH Effects. The ionic strengths and pH values of aqueous environmental samples may interfere with ELISA analysis. The same applies to extraction solvents used for environmental soil analysis. To address these possibilities, assay performance under various ionic strengths, pH values, and selected solvent concentrations was evaluated. At the concentrations of hydroxytriazines tested, no precipitation was detected. In system **7**/4652, when both analyte and antiserum were dissolved and incubated in buffer at various pH values, no significant effect upon IC_{50} was noted. Thus, this assay was able to effectively detect hydroxyatrazine at pH values ranging from 5.0 to 8.0 (Figure 7).

When analyte and antiserum were dissolved and incubated in PBST buffers of various salt concentrations but constant Tween 20 concentration, significant increases in optical density as well as IC_{50} values resulted at the lowest PBST concentrations (Figure 8). However, from 0.13 to 1.0 M PBST, assay parameters remained virtually unchanged. The maintenance of a minimal ionic strength appears to be very important.

The assay can accurately detect hydroxyatrazine in up to 13% methanol (Figure 9). At concentrations up to 50% methanol, hydroxyatrazine might be quantified effectively (albeit at lower sensitivity) by making sure that standard dilutions contain the same concentration of methanol as in unknown samples.

The assay is subject to strong interference from both acetonitrile (Figure 10) and acetone (Figure 11). In the case of acetonitrile, the IC_{50} of hydroxyatrazine doubles

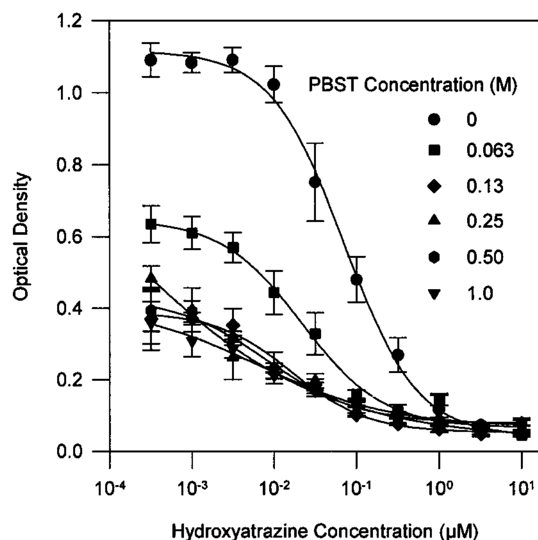

Figure 8. Effect of varying ionic strength upon ELISA **7**/4652. Values refer to final PBST concentrations in each well, after antiserum 4652 and hydroxyatrazine solutions have been added.

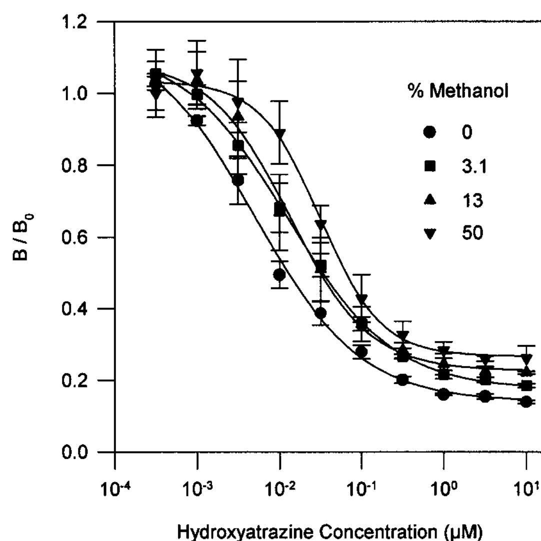

Figure 9. Effect of varying methanol concentration upon ELISA **7**/4652. Values refer only to methanol v/v concentrations in hydroxyatrazine standard solutions.

at 13% acetonitrile relative to that at 0% acetonitrile. For acetone, the IC_{50} doubles at only 3.1% acetone. In cases when these solvents must be used for extraction of solid environmental matrices, detection of hydroxytriazines can still be determined (at lower sensitivity) as long as standard dilutions contain the same amounts of solvents as the unknowns. However, a solvent exchange protocol in which the extracting solvent is evaporated onto a small volume of nonvolatile trap solvent may be warranted to increase assay sensitivity.

Water Matrix Effect. Since the potential for groundwater contamination by *s*-triazine herbicides is high and hydrolytic degradation to hydroxytriazines is prevalent, characterization of matrix effects from groundwaters is especially important. Slight increases in IC_{50} values were noted when blank groundwater samples taken from three separate locations (Wortberg et al., 1995) were fortified with hydroxyatrazine and analyzed with ELISA system **7**/4652. This discrepancy was most likely due to the lower ionic strengths of the groundwaters. It was eliminated when 10 mL of 1.0 M PBST was added to 90 mL of groundwater before hydroxyatrazine fortification and testing (Figure 12). As an alternative to dilution, future implementations might use blank groundwaters for standard curve preparation.

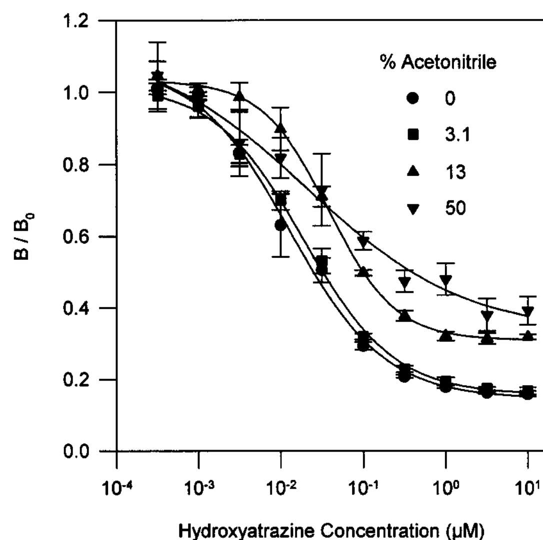

Figure 10. Effect of varying acetonitrile concentration upon ELISA **7**/4652. Acetonitrile percentages were determined on a v/v basis.

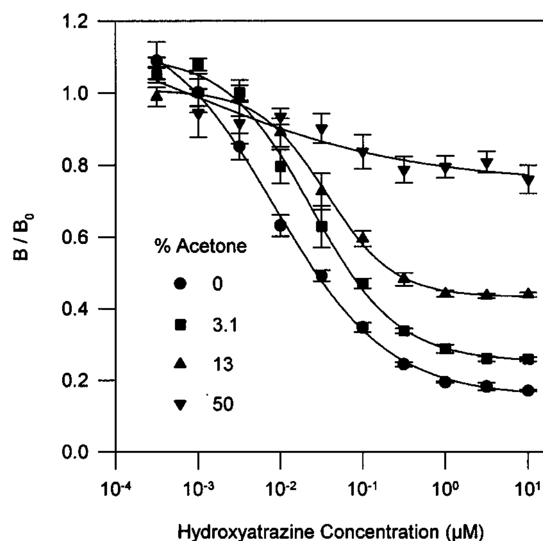

Figure 11. Effect of varying acetone concentration upon ELISA **7**/4652.

CONCLUSIONS

Several ELISAs with high selectivity and sensitivity for hydroxypropazine, hydroxyatrazine, and hydroxysimazine have been developed by using **IIa** as the immunizing hapten. ELISA **7**/4652, which has the lowest IC_{50} values, also exhibits good performance characteristics at various temperature, pH, and solvent levels. Even though it does demonstrate some sensitivity to low ionic strength, with the implementation of dilution, this assay may be used for screening groundwater.

The assays' unexpected selectivity for hydroxytriazines over prometon has the possible explanation that hapten **IIa** is hydrolytically unstable, with partial degradation to **IIc** occurring during the process of conjugation and immunization. If **IIc** is more antigenic than **IIa**, one would see selectivity for hydroxytriazines. To help assess whether or not hydrolysis of **IIa** may have occurred during conjugation, the CR profiles of

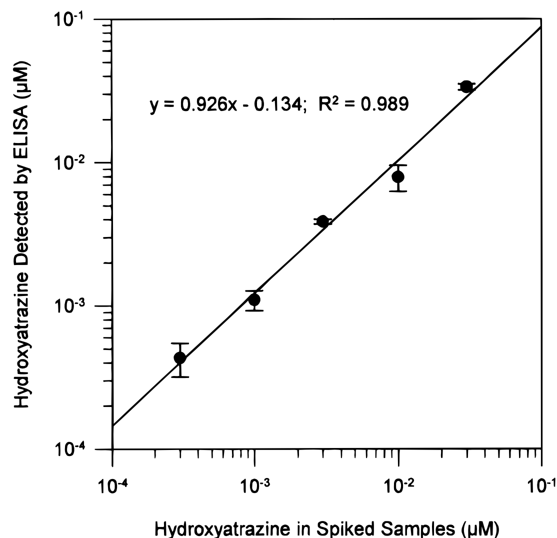

Figure 12. Correlation among five different hydroxyatrazine spike levels of three different groundwater samples and levels detected with ELISA **7**/4652. A buffer dose−response curve was used as control. Samples were diluted 10% with 10× PBS buffer.

systems **10**/4653 and **12**/4653 (conjugates **10** and **12** contain haptens **IIc** and **IIa**, respectively) were evaluated. The results could not rule out the possibility that the methoxy on conjugate **12** was partially hydrolyzed, especially since the two systems' CR profiles were very similar to each other.

To obtain more definitive results regarding the hydrolytic stability of **IIa**, the effects of various pH values were tested. Evidence of degradation of **IIa** at pH values of 2, 7, 8, 9, and 10 was confirmed but not at pH values 4 and 6. Even though an increase in the concentration of **IIc** was not detected at pH 7, physiological pH is approximately 7.4 and hapten−protein conjugation was conducted at pH 8. Thus, it appears likely that some hapten **IIc** was presented to the rabbit's immune system in addition to hapten **IIa**.

Besides hapten degradation, another possibility which cannot be excluded is that hapten **IIa** was successfully presented to the rabbits' immune system. The higher affinity of the antisera for hydroxytriazines relative to prometon may be due to the fact that hydroxytriazines have tautomeric forms that can serve as both H-bond donor and acceptor (Pearlman and Banks, 1948). This hydrogen bonding could result in greater recognition of the antigen from hapten **IIc** relative to **IIa** or could enhance the affinity of the resulting antibodies to *s*-triazines. The methoxy triazines do not undergo such tautomerism and hence the OCH_3 can only act as a H-bond acceptor. Thus, it is possible that antibodies raised to a methoxy hapten could bind more tightly to the corresponding hydroxy derivative. This possibility is further supported by the fact that the methoxytriazine herbicides prometon and atraton are more stable than the corresponding chlorotriazine herbicides propazine and atrazine (Sheets, 1970). Yet, anti-atrazine antibodies have been successfully developed (Harrison et al., 1991) under conditions identical to those used in this paper.

A third possibility is that partial hydrolysis of **IIa** occurred, resulting in the production of antibodies to both **IIa** and **IIc**, with antibodies to the latter having higher affinity for that analyte.

The results point to three possible explanations: (a) all of hapten **IIa** degraded to hapten **IIc**, resulting in antibodies specific for hapten **IIc**; (b) antibodies to hapten **IIa** were successfully developed, but they have a higher affinity for hydroxytriazines; or (c) some of hapten **IIa** was converted to **IIc**, which induced hydroxytriazine-specific antibodies with affinities for hydroxytriazines higher than those of methoxytriazine-specific antibodies for methoxytriazines.

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Registry Numbers Supplied by the Author (M.H.G.): Atrazine, 1912-24-9; hydroxyatrazine, 2163-68-0; simazine, 122-34-9; hydroxysimazine, 2599-11-3; cyanazine, 21725-46-2; prometon, 1610-18-0; hydroxypropazine, 7374-53-0; propazine, 139-40-2; ametryne, 834-12-8; prometryn, 7287-19-6; procyazine, 32889-48-8; terbutryne, 886-50-0; terbumeton, 33693-04-8; simetryn, 1014-70-6; **I**, 98849-84-4; **III**, 125454-29-7; KLH, 9013-72-3; CONA, 139-06-6; BSA, 9048-46-8; HRP, 9003-99-0; disodium 4-nitrophenylphosphate hexahydrate, 4268-83-9; sodium methoxide, 124-41-4; sodium thiomethoxide, 5188-07-8; ethylene glycol dimethyl ether, 110-71-4; 3-nitrobenzyl alcohol, 619-25-0; polyethylene glycol 300, 24322-68-3.

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