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Development of an ELISA for the *N*-Dealkylated *s*-Triazines: Application to Environmental and Biological Samples

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A sensitive and rugged ELISA for the detection of the N-*dealkylated metabolites of atrazine and simazine was developed and applied to environmental and biological samples. The limits of detection were approximately 10 ng ml^{-1} for water samples, 40 ng ml^{-1} for human urine when analyzed without any sample preparation and approximately 45 ng g^{-1} for solvent-extracted soil. This assay was tolerant to changes in pH, salt and solvent effects. Comparison of the results from this ELISA with nominal values for a series of tap water samples spiked in a blind fashion resulted in a good correlation coefficient between the values found and those spiked. However, the ELISA systematically underestimated concentrations. Examination of the source of this error revealed that the dealkylated triazine concentrations of the samples declined with time. The assay was used to monitor the presence and loss of dealkylated triazines in a variety of samples. These data indicated that caution must be used in the analysis and interpretation of the presence of these* N-*dealkylated metabolites.*

Keywords: s-*Triazine,* N-*dealkylated triazines, ELISA, enzyme immunoassay, application, stability, metabolites*

INTRODUCTION

Atrazine and simazine are *s* triazine herbicides used extensively in agriculture for weed control. Both of these compounds are converted to *N*-dealkylated forms in the environment (Esser *et al.*, 1975; Pereia & Rostad, 1990; Winkelmann & Klaine, 1991) and in biological systems (Bakke *et al.*, 1972; Bradway & Moseman, 1982; Ikonen *et al.*, 1988). The large

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quantities of *s*-triazines used in the environment and the identification of atrazine as a major pesticide contaminant of ground water (Parsons & Witt, 1988; Eisler, 1989; Belluck *et al.*, 1991) necessitate extensive monitoring in order to comply with the recently enacted Safe Drinking Water Act. In addition, the *N*-dealkylated products of atrazine have been identified in human urine samples (Ikonen *et al.*, 1988; Lucas *et al.*, 1993), making them potential indicators of *s*-triazine exposure.

Chromatographic techniques for determination of the *N*-dealkylated metabolites of the *s*-triazines require sample preparation prior to analysis. Depending on the matrix, the extraction procedures for these metabolites can be relatively simple, such as liquid–liquid extraction for water (Pereia & Rostad, 1990) or more arduous multi-step preparations for soils (Winkelmann & Klaine, 1991). ELISAs are alternative analytical methods. They are rapid, because they often require less sample preparation, while still providing the desired sensitivity (Karu, 1993). ELISAs have been reported for the detection of *N*-dealkylated *s*-triazines in water (Wittmann & Hock, 1991) and in pesticide waste and rinsates (Muldoon *et al.*, 1994). Here, the development and application of an ELISA selective for the detection of the deethyl- and deisopropyl-*s*-triazines in environmental samples, such as water and soil, as well as in biological samples, such as urine, is described. In addition, loss of the mono-*N*-dealkylated *s*-triazines was observed under a variety of storage conditions. The use of these metabolites as environmental urinary biomarkers of exposure may be suspect unless immediate extraction and analysis is performed on fresh samples or the sample can be stabilized.

MATERIALS AND METHODS

Chemicals

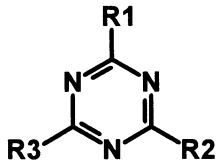
Alkaline phosphatase (AP), keyhole limpet hemocyanin (KLH), goat anti-rabbit–AP, bovine serum albumin (BSA), conalbumin (CONA) and *p*-nitrophenyl phosphate enzyme substrate tablets were obtained from Sigma Chemical Co. (St Louis, MO, USA). All solvents and salts used were purchased from Aldrich Chemical Co., Inc. (Milwaukee, WI, USA), J. T. Baker (Phillipsburg, NJ, USA) or Fisher Scientific (Pittsburgh, PA, USA). Solvents utilized in synthesis were of reagent grade from the same sources. A Sybron/Barnstead Nanopure II water system, set at 16.7 MΩ-cm, provided water for all aqueous solutions.

Technical atrazine was donated by Shell Agriculture Chemical Co. (Modesto, CA, USA). The analytical *s*-triazine standards were a gift from the Ciba-Geigy Corporation (Greensboro, NC, USA). The purity of the analytical standards used in this study were confirmed by observing a single UV-dense spot on a silica gel thin-layer chromatography (TLC) plate (0.25 mm pre-coated silica gel 60 F254 plastic sheet from E. Merck, Darmstadt, Germany) with two different solvent systems. Using a developing solvent of 2:13:35 tetrahydrofuran-ethyl acetate-hexane (v:v:v) plus 2% acetic acid, atrazine had an R_f of 0.57, deethylatrazine an R_f of 0.43 and deisopropylatrazine an R_f of 0.36. On development of the plate with ethyl acetate, atrazine had an R_f of 0.74, deethylatrazine an R_f of 0.73 and deisopropyl an R_f of 0.69. Details of the immunogen synthesized for this study (compound **I**) are listed in the section on synthesis, while the syntheses of the compounds **2a**, **2b**, **2c**, **4a** and **2e** are described in Goodrow *et al.* (1990; see Tables 1 and 2 for structural details). The hapten SBeAAT and BSA conjugates of SPrAAT and CAHeT (Table 1) were a generous gift from Mark Muldoon of the USDA Pesticide Degradation Laboratory (Beltsville, MD, USA).

Instruments

ELISAs were carried out in 96-well plates (Nunc, Roskilde, Denmark, #442404) and read spectrophotometrically with a V_{max} microplate reader (Molecular Devices, Menlo Park, CA, USA). Standard curves were constructed using 10 concentrations and no analyte or antibody

TABLE 1. Structures of the haptens of interest. Structures indicated in the cross-reactivity data in Table 2 conform to the generic *s*-triazine structure depicted here



Compound	R1	R2	R3
I	Cl	NH_2	$NH(CH_2)_5COOH$
	Cl	NH_2	$NH(CH_2)_5$-Gly
	Cl	NH_2	$NH(CH_2)_5$ Trp
	Cl	NH_2	$NH(CH_2)_5$-Leu
2c	Cl	$NHCH_2CH_3$	$NH(CH_2)_3COOH$
2e	Cl	$NHCH_2CH_3$	$NH(CH_2)_5COOH$
4a	SCH_2CH_2COOH	$NHCH_2CH_3$	$NHCH_2CH_3$
SPRAAT	SCH_2CH_2COOH	NH_2	NH_2
CAHeT	Cl	NH_2	$NH(CH_2)_5COOH$
SBeAAT	SCH_6H_4COOH	NH_2	NH_2

controls (four well replicates each). Data were fitted to a sigmoidal curve based on the four-parameter logistic method of Rodbard (1981) using the software package Softmax (Molecular Devices). Micellar electrokinetic capillary chromatography (MECC) utilized a model 270A capillary electrophoresis system (Applied Biosystems, Inc., San Jose, CA, USA) equipped with a 70 cm × 50 μm internal diameter fused silica column. The electrolyte buffer, pH 9.33, consisted of 10 mM-sodium tetraborate, 50 mM-sodium dodecyl sulfate and 10 mM-β-cyclodextrin. An applied voltage of + 15 kV was used with a UV detector monitoring the effluent at 220 nm. The sample was vacuum-injected over 1.5 s.

Spectral Equipment

Infrared spectra were recorded on an IBM IR/32 FTIR spectrometer (Danbury, CT, USA). Nuclear magnetic resonance (NMR) spectra (^{1}H-NMR and ^{13}C-NMR) were obtained using a QE-300 spectrometer (General Electric NMR Instruments, Fremont, CA, USA) operating at 300.1 and 75.5 MHz respectively; chemical shifts were expressed in ppm down-field from tetramethylsilane. Fast atom bombardment low- and high-resolution mass spectra (FAB-MS, FAB-HRMS) and MS/MS were obtained on a ZAB-HS-2F mass spectrometer (VG Analytical, Wythenshawe, UK), 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 for FAB-HRMS.

Hapten I Synthesis: 6-[(4-Amino-6-chloro-1,3,5-triazin-2-yl)amino]hexanoic acid

A solution of 18.4 g (0.100 mol) of cyanuric chloride in 150 ml of acetone was maintained at 0°C by external cooling and vigorous stirring. To this, a solution of 13.4 g (0.10 mol) of 30% (w/w) aqueous sodium hydroxide and 6.7 g (0.11 mol) of 28% (w/w) aqueous ammonium hydroxide was added over 1 h. Stirring was continued at 0°C for another 2 h, whereafter the mixture was allowed to warm to ambient temperature while being purged with nitrogen to remove excess ammonia. After the effluent gas was no longer basic to moist pH paper, a quantity of 14.4 g (0.11 mol) of 6-aminohexanoic acid was added to the mixture. This was followed by the dropwise addition of 26.8 g (0.20 mol) of a 30% (w/w) aqueous sodium hydroxide solution over 0.5 h, while maintaining the reaction temperature at or

TABLE 2. Cross-reactivity of the optimized heterologous ELISA using antibody 2652 (immunized with **I**, Table 1) with the tracer SBeAAT–AP for a series of *s*-triazines. The values are presented relative to the IC_{50} value of deisopropylatrazine, which is assigned a cross-reactivity of 100%. Cross-reactivities are determined by expressing the ratio of the IC_{50} of the chemical assigned to be 100% to the IC_{50} of the other compounds and expressed as a percentage

	Structure			
	R1	R2	R3	Percentage cross-reactivity
Atrazine	Cl	$NHCH_2CH_3$	$NHCH(CH_3)_2$	0.2
Hydroxyatrazine	OH	$NHCH_2CH_3$	$NHCH(CH_3)_2$	<0.1
Simazine	Cl	$NHCH_2CH_3$	$NHCH_2CH_3$	0.2
Hydroxysimazine	OH	$NHCH_2CH_3$	$NHCH_2CH_3$	<0.1
Atrazine mercapturate	*N*-acetyl-L-cysteine	$NHCH_2CH_3$	$NHCH(CH_3)_2$	<0.1
Ametryne	SCH_3	$NHCH_2CH_3$	$NHCH(CH_3)_2$	<0.1
Simetryne	SCH_3	$NHCH_2CH_3$	$NHCH_2CH_3$	<0.1
Propazine	Cl	$NHCH(CH_3)_2$	$NHCH(CH_3)_2$	<0.1
Prometryne	SCH_3	$NHCH(CH_3)_2$	$NHCH(CH_3)_2$	<0.1
Prometon	OCH_3	$NHCH(CH_3)_2$	$NHCH(CH_3)_2$	<0.1
Terbutryne	SCH_3	$NHCH_2CH_3$	$NHC(CH_3)_3$	<0.1
Cyanazine	Cl	$NHCH_2CH_3$	$NHC(CH_3)_2CN$	0.1
Deethylatrazine	Cl	NH_2	$NHCH(CH_3)_2$	85.6
Deisopropylatrazine	Cl	NH_2	$NHCH_2CH_3$	100
Didealkylated atrazine	Cl	NH_2	NH_2	7.3
Cyromazine	*N*-cyclopropyl	NH_2	NH_2	2.4
Cyanuric acid	OH	OH	OH	<0.1
Ammeline	OH	NH_2	NH_2	<0.1
Ammelide	OH	OH	NH_2	<0.1
Melamine	NH_2	NH_2	NH_2	<0.1
2a	Cl	$NHCH_2CH_3$	$NHCH_2COOH$	<0.1
2b	Cl	$NHCH_2CH_3$	$NH(CH_2)_2COOH$	0.1
2c	Cl	$NHCH_2CH_3$	$NH(CH_2)_3COOH$	0.2
2d	Cl	$NHCH_2CH_3$	$NH(CH_2)_4COOH$	0.4
2e	Cl	$NHCH_2CH_3$	$NH(CH_2)_5COOH$	<0.1
4a	SCH_2CH_2COOH	$NHCH_2CH_3$	$NHCH_2CH_3$	<0.1
I	Cl	NH_2	$NH(CH_2)_5COOH$	4500
I-Trp	Cl	NH_2	$NH(CH_2)_5CO$-Trp	915
I-Leu	Cl	NH_2	$NH(CH_2)_5CO$-Leu	840
I-Gly	Cl	NH_2	$NH(CH_2)_5CO$-Gly	850

below 25°C by external cooling. This mixture was then stirred overnight at ambient temperature. Most of the acetone was then removed by vigorous stirring and purging with nitrogen. Thereafter, a quantity of 150 ml of water was added to dissolve the bulk of the remaining white solid. This pH 7 mixture was filtered through Celite, and the filtrate was acidified to pH 1 with concentrated hydrochloric acid. The resultant white precipitate was collected, washed thoroughly with ice water and dried in a vacuum desiccator to provide 25.4 g (98%) of crude **I**. A portion (2.00 g), recrystallized from 95% ethanol after filtering through Celite, had a melting point of 147.5–149.5°C. A second recrystallization from acetonitrile provided 1.01 g of **I** as a fine white powder, which was found to consist of a major component (98%) with a retention time of 30.9 min and two minor components of 1% each by MECC (see instruments section for conditions): IR (KBr) 3273 (m, N—H), 3174 (m, N—H), 3125 (m, N—H), 1706 (m, C=O, acid), 1570 (vs, C=N), 1254 (m, C—O) cm^{-1}; ^{1}H-NMR dimethyl sulphoxide (DMSO-d_6) (NMR spectra were complicated by the existence of isomers, as noted by more than one signal for the N—H moieties) δ 12.0 (br, 1 H, OH), 7.75, 7.59 (two t in a 5:2 ratio, J = 5.6 Hz, 5.7 Hz, 1 H, NH), 7.37, 7.27, 7.15, 7.05 (four br, s, 2 H, NH_2), 3.2 (m, 2 H, CH_2N), 2.20 (t, J = 7.3 Hz, 2 H, CH_2CO_2), 1.5 (m, 4 H,

CH_2—C—CH_2), 1.3 (m, 2 H, C—CH_2—C) (all the peaks from 12.0 to 7.07 ppm disappeared, and the 3.2 ppm multiplet became two triplets at 3.20 and 3.18 ppm with J = 6.8 Hz, 6.6 Hz respectively in an approximate 5:2 ratio with added D_2O); ^{13}C-NMR (DMSO-d_6) δ 174.7 (C=O, C_1), 167.9 (Ar—C_6), 166.7 (Ar—$C_{2\ or\ 4}$), 165.3 (Ar—$C_{2\ or\ 4}$), 40.4 (CH_2N, C_6), 33.4 (C_2), 28.2 (C_5), 25.7 (C_4), 24.0 (C_3). FAB-MS m/z (relative intensity) 262 (32, $M + H^+ + 2$), 260 (100, $M + H^+$); FAB-HRMS m/z calculated for $C_9H_{15}C1N_5O_2$ 260.0914, observed 260.0918.

Synthesis of Hapten–Protein and Hapten–Enzyme Conjugates

Hapten **I** was conjugated to BSA or KLH in 0.05 M-borate buffer, pH 8, using the active ester method of Bauminger and Wilchek (1980). Following dialysis against 0.1 M-phosphate-buffered saline (PBS), pH 7.5, the solutions were scanned spectrophotometrically from 220 to 600 nm. The hapten–protein conjugate spectra obtained were compared with those of the hapten and of the protein, but no clear evidence of coupling could be discerned using this method. Using a previously described ELISA (Harrison *et al.*, 1991, antibody 355) which possessed some sensitivity for the mono-dealkylated *s*-triazines, the hapten loading was estimated to be 1.3 μg mg^{-1} of protein for the KLH conjugate and 4.6 μg mg^{-1} for the BSA conjugate compared with a deisopropylatrazine standard. Only conjugates of hapten **I** were used as immunogens.

Using the active ester method cited above, the haptens illustrated in Table 1 were each conjugated to AP, BSA or CONA for use in a competitive ELISA. The series of haptens composed of **I** bonded to an amino acid and then to a protein was made by first using the active ester method to conjugate equimolar (0.2 mM) amounts of **I** in dry dimethylformamide (DMF) to the amino acid in 1:2 (v:v) DMF:borate buffer, pH 8.0. This product was stirred vigorously for 4 h, and then evaporated to near dryness under a gentle stream of nitrogen. The **I**–amino acid conjugates were dried overnight in a vacuum desiccator over Drierite, then dissolved in dry DMF. These solutions were used to prepare the active esters used subsequently for coupling to CONA or AP.

Immunization and Serum Collection

Six female New Zealand White rabbits were immunized intra-dermally, three with the **I**–BSA conjugate and three with the **I**–KLH conjugate. The hapten–protein conjugates were prepared by dissolving 100 μg of each immunizing hapten–protein conjugate in 0.25 ml of PBS and mixing 1:1 with Freund's complete adjuvant for each rabbit. Subsequent boosts were given to the animals every 4 weeks using the same immunizing preparation, with the exception that Freund's incomplete adjuvant was used. Rabbits were bled 7–10 days after each boost and the sera were tested for titer using the method outlined by Gee *et al.* (1988). The blood was allowed to clot overnight. On the following day, the sera were removed, separated from any remaining red blood cells by centrifugation and stored at − 20°C.

ELISA

Following the principles of solid-phase immunoassay (Voller *et al.*, 1976), the ELISA was conducted using a competitive format. Two different formats were used in screening for the best serum and in optimizing assay conditions. Prior to using any format for inhibition studies, antibody, enzyme tracer and coating antigen dilutions were optimized by using the two-dimensional titration method as outlined by Gee *et al.* (1988).

Coated antigen format. Plates were coated with 100 μl/well of a hapten–protein conjugate in a carbonate buffer (1.59 g l^{-1} Na_2CO_3, 2.93 g l^{-1} $NaHCO_3$, 0.2 g l^{-1} NaN_3) and allowed to stand overnight at 4°C. On the following day, the plates were washed with PBSTA (PBS with Tween 20 and sodium azide: 8 g l^{-1} NaCl, 1.15 g l^{-1} Na_2HPO_4, 0.2 g l^{-1} KH_2PO_4,

0.2 g l^{-1} KCl, 0.05% Tween 20 and 0.02% NaN_3). Then, 50 μl/well of the anti-triazine antibody and 50 μl/well of the analyte were placed on the plate and incubated for 2 h. After washing again with PBSTA to remove any unbound material, a quantity of 100 μl/well of a commercially available goat anti-rabbit–AP conjugate was added. After 2 h the plate was washed, and a substrate solution of 1 mg ml^{-1} *p*-nitrophenyl phosphate in 10% diethanolamine was added to each well. Approximately 1 h thereafter, the plates were read spectrophotometrically in a dual wavelength mode at 405 nm minus 650 nm. The development of a yellow color was inversely proportional to the amount of triazine present.

Coated antibody format. For this format, plates were coated with the anti-triazine antibody in carbonate buffer overnight at 4°C. On the following day, the plates were washed with PBSTA and 50 μl/well of analyte and 50 μl/well of enzyme tracer were added. After 30 min the wells were washed, removing any unbound reactants, then 1 mg ml^{-1} of *p*-nitrophenyl phosphate was added. This yielded color development, the absorbance of which was inversely proportional to the amount of analyte present.

Sample Extraction and Preparation Methods

Soil. Yolo silty loam containing 1.74% organic matter (from Yolo County, CA, USA) was extracted and analyzed for assay interference according to the method described by Winklemann and Klain (1991). Briefly, a quantity of 100 g of blank soil (not treated with *s*-triazines) was placed in a flask with 250 ml of acetonitrile:methanol:water (7:1.5:1.5, v:v:v), shaken for 0.5 h and allowed to settle overnight. An aliquot of the extract was analyzed by ELISA, but dilutions of 50-fold or more were needed to obtain an acceptable background level (a reading equivalent to less than 2.5 ng ml^{-1}). The remaining supernatant was evaporated to near dryness and then reconstituted with PBSTA buffer as a means of further reducing background noise and matrix effects.

Water. Water from three different sources was used directly to evaluate any potential matrix effects. Tap water from Davis, CA, USA (ground water source), distilled water and water from Putah Creek (a small local oxbow lake in Davis with minimal flow and turbid with algae) were compared with the standard assay buffer of 0.1 M-PBSTA, pH 7.5.

In addition to the evaluation of field waters, the utility of this assay to detect the decrease of deethylatrazine as a function of the pH in water over time was examined. A total of 50 μM of deethylatrazine was spiked to distilled water at pH 5.1, 7.2 or 9.5. Both plastic and glass containers, kept at room temperature, were used to examine any adsorption of the analyte that occurred over time. Aliquots of these water samples were taken over time and analyzed for deethylatrazine.

Urine. The ability of this assay to detect the mono-*N*-dealkylated *s*-triazines was determined by spiking human urine with known quantities of deethyl- and deisopropylatrazine, and then assaying various dilutions of the raw urine. Samples with less than 25% urine were generally tolerated quite well; however, for some individual specimens, matrix effects were observed at sample dilutions as low as a 10% dilution. Unless the effect of urine on the assay performance was well characterized, dilution of human urine to 10% or less prior to analysis was necessary to minimize this matrix effect.

As the *N*-dealkylated products of atrazine are major biological and environmental metabolites, the stability of deethylatrazine in urine was examined. Six sets of urine samples (all at pH 7) were prepared. Each set comprised 10 ml aliquots of urine spiked with 0, 5, 25 or 100 μM of deethylatrazine. To 50% of these sets, sodium azide was added to a final concentration of 0.02%. One set of spiked urine which was azide-free and

one set with azide were left at room temperature. A second set was refrigerated (4°C) and the final set was frozen (− 20°C). Over a period of 1 month, the concentration of deethylatrazine in these samples, as determined by the ELISA, was used to assess the stability of this analyte.

RESULTS AND DISCUSSION

Hapten Selection and Synthesis

In an immunoassay, it is desirable that the immunizing hapten exposes all of the unique functional groups of the target analyte and resembles it as closely as possible. With this in mind, the hydrocarbon handle in hapten **I** was designed to resemble the corresponding aliphatic substitution of an *N*-dealkylated *s*-triazine. Such a six carbon handle has resulted in excellent triazine antibodies in studies from numerous laboratories. This structure also left the critical chloro and amino groups exposed and distal to the handle location. Two classes of conjugates were developed for use in a competitive assay against antibodies generated by hapten **I** (Table 1). The homologous group, which possessed both the chloro and amino groups, differed in the composition of the aliphatic side chain (the first six structures in Table 1). Of the haptens used for development of a heterologous assay, SPrAAT, the mercaptopropionic acid of simazine, and SBeAAT, the thioether, were selected to resemble the 2-chloro moiety (R1, Table 1) of the analyte. In each case, heterology with the structure of the handle and/or heterology with its position is possible. Often, the best combination for generating a sensitive assay is obtained by altering only the handle; however, in many cases it is also necessary to have position heterology.

The immunizing hapten, **I**, was prepared by the amination of cyanuric chloride at 0°C followed by the introduction of the 6-aminohexanoic acid handle moiety at room temperature. Purification of the crude product by recrystallization from two different solvents resulted in a product assaying 98% by capillary electrophoresis. TLC displayed one major component accompanied by barely detectable trace impurities. **I** was characterized by its infrared spectrum, ^{1}H- and ^{13}C-NMR spectra and low- and high-resolution mass spectrometry. The NMR spectra warrant comment in that both the ^{1}H- and ^{13}C-NMR spectra displayed multiple peaks for some of the nuclei, due to multiple slowly equilibrating tautomeric structures. These result from restricted rotation of the amino and alkylamino side chains. They are attributable to delocalization of the nitrogen lone pair electrons creating double bond character between the ring carbons and the appended external nitrogen atoms, with accompanying transfer of hydrogens from external nitrogens to ring nitrogens. This phenomenon has been observed and studied extensively by Welhouse and Bleam (1993a,b) for the atrazine ^{1}H-NMR. Multiple ^{13}C-NMR signals have also been observed for a triazine compound similar to **I**, 6-[{4-chloro-6-[(1-methylethyl)amino]-1,3,5,-triazin-2-yl}amino] hexanoic acid (Ulrich *et al.*, 1991).

Hapten **I** was conjugated to BSA, KLH, CONA and AP proteins via an *N*-hydroxysuccinimide ester. Rabbits were immunized with the resulting **I**–BSA or **I**–KLH conjugates.

Screening and Selection of Antisera

Antisera were screened using the two-dimensional titration method with a coated antigen format (Gee *et al.*, 1988). Those rabbits immunized with **I**–KLH (2653, 2655 and 2656) showed titers equivalent to those immunized with **I**–BSA (2584, 2586 and 2652) when examined against a homologous coating antigen. Attempts to design a homologous system (using the same ELISA hapten as immunizing hapten) using **I** conjugated to AP or CONA yielded poor inhibition curves for **I**–AP and no inhibition curves in the coated antigen format when either deethylatrazine or deisopropylatrazine were used as the competitor. Even pre-incubating the analyte with the coated antibody and then adding **I**–AP did not

TABLE 3. The IC_{50}, slope and fit of the curves generated when antibody 2652 was evaluated in a coated antibody format with SBeAAT–AP (Table 1) as the enzyme tracer

Analyte	IC_{50} (μM)	Slope	Curve fit (r)
Deethylatrazine	0.167	1.06	0.932
Deisopropylatrazine	0.117	1.03	0.964
Atrazine	50.6	0.767	0.610
Didealkylated triazine	1.62	1.41	0.906

improve assay performance; however, it did improve curve fit. Insertion of the common amino acids glycine, leucine or tryptophan between the enzyme and the six carbon spacer of the homologous hapten **I** (Table 2) was attempted in order to create a tracer with a significantly different handle to allow the target analyte to compete and bind for sites on the antibody. None of these amino acid modified enzyme tracers provided any improvement in assay sensitivity. Subsequent analysis of these hapten–amino acid compounds, in the final optimized assay, generated cross-reactivity values similar to those of the immunizing hapten (Table 2). These numbers indicated that the antibodies recognized these homologous haptens, including the amino acid modified series (see Table 1), at least an order of magnitude better than the target analytes. This is in contrast to the results of Wittmann and Hock (1991), who developed an extremely sensitive homologous assay for the *N*-dealkylated *s*-triazines. These apparently conflicting results emphasize the need to optimize each unique pool of antibody with the haptens used for coating antigens or enzyme tracers.

In evaluating a heterologous system (utilizing a different ELISA hapten than that used for immunization), a series of haptens was tested against the antisera (Table 1). Of these haptens, SBeAAT (Table 1; Muldoon *et al.*, 1994) generated a reasonably sensitive assay when used as an enzyme tracer in the coated antibody format with antibody 2652 (Table 3) or in a coated antigen format with 2653 (Table 4) when the analyte was pre-incubated with the antisera. The remaining haptens were insufficiently recognized by any of the antibodies using a competitive format to obtain a useful assay. Since the coated antibody

TABLE 4. The IC_{50}, slope and fit of the curves generated when antibody 2653 was pre-incubated with deethylatrazine and deisopropylatrazine overnight at room temperature and then this mixture placed on a coated antigen plate (SBeAAT–BSA) for various lengths of time

Time (h)	Analyte	IC_{50} (μM)	Slope	Curve fit (r)
1	Deethyl	0.158	1.09	0.949
	Deisopropyl	0.397	1.38	0.991
3	Deethyl	0.105	1.15	0.920
	Deisopropyl	0.367	1.50	0.923
5	Deethyl	0.124	1.43	0.971
	Deisopropyl	0.452	1.50	0.968
7	Deethyl	0.106	1.33	0.984
	Deisopropyl	0.327	1.52	0.931
24	Deethyl	0.0418	1.71	0.925
	Deisopropyl	0.0362	1.58	0.943
48	Deethyl	0.0754	1.34	0.976
	Deisopropyl	0.0759	1.41	0.962

TABLE 5. The minimum and maximum absorbance (405–650 nm), IC_{50}, slope and fit of the curves generated when the antibody dilution of 2652 was kept constant (1:1000) and the dilution of the enzyme tracer SBeAAT–AP was varied from 1:40 000 to 1:1000

Reciprocal dilution	Maximum absorbance	Slope	IC_{50} (μM)	Minimum absorbance	Curve fit (r)
40 000	0.0431	0.885	0.154	0.0027	0.995
20 000	0.0811	0.772	0.188	0.0039	0.996
10 000	0.119	0.789	0.245	0.0073	0.995
8 000	0.135	0.798	0.297	0.0105	0.995
5 000	0.189	0.854	0.307	0.0193	0.986
4 000	0.214	0.773	0.401	0.0171	0.999
2 000	0.361	0.666	0.648	0.0250	0.983
1 000	0.608	0.649	0.774	0.0618	0.993

format required no pre-incubation and minimal time was needed for actual analysis, this system was selected for further study.

A two-dimensional titration of antibody 2652 and the enzyme tracer SBeAAT–AP was performed in order to choose an appropriate dilution of these two reagents for inhibition studies with the *N*-dealkylated *s*-triazines. After selecting an optimal dilution of the antibody and enzyme tracer (1:1000 and 1:2000 respectively), reasonable curves for the mono-*N*-dealkylated metabolites were obtained (Table 3). However, the curves for the *N*-dealkylated *s*-triazines were quite different when a second batch of SBeAAT–AP was used in the assay, even after repeating two-dimensional titration experiments for this new tracer. Although some experimental error in the conjugation and dilution could very well be involved in the difference between these two batches of enzyme tracer, it could not account for the disparity between the performance of these reagents. Because two-dimensional titrations generate a performance range of antibody and enzyme tracer, further study on the dilutions of these two components was conducted to fine-tune the signal-to-noise ratio of this assay. When dilutions of this second batch of enzyme tracer were varied while keeping the dilution of antibody 2652 constant (1:1000), a decrease in both the background and maximum absorbance and an increase in sensitivity and slope were observed as the enzyme tracer was diluted (see Table 5). These data clearly demonstrate that a balance between sensitivity (low IC_{50} value, high enzyme tracer dilution) and the amount of time for running the assay (low absorbance values) needs to be optimized for each assay application.

Cross-reactivity. Cross-reactivity values were calculated as the ratio of the IC_{50} of parent compound (deisopropylatrazine) to the IC_{50} of the test compound, and expressed as a percentage. This system was remarkably selective for the *N*-dealkylated *s*-triazines. The only compounds which demonstrated any inhibition in this assay possessed one amino group or two amino groups attached to the *s*-triazine ring (Table 2). The limited solubility of ammelide, ammeline and melamine in the assay buffer probably prevented an accurate measurement of their cross-reactivities. The cross-reactivity spectrum observed was similar to that obtained by Wittmann and Hock (1991). This was expected as the same immunizing hapten was used.

Salt, solvent and pH effects. Standard curves prepared in 0.1–1.0 M-PBS showed no significant deviation from curves using the standard assay buffer of 0.1 M-PBS. Additionally, this assay proved to be resistant to sample pH, although a loss of sensitivity (above 10%) outside a pH range of 7–8 was seen. Several dilutions of selected solvents were tested in the ELISA because solvents are often used in the extraction of analytes from various

matrices. This assay showed no changes to assay parameters at concentrations of acetonitrile and methanol of up to 12.5% of the sample volume. To test for potential effects of hydrophobic solvents, ethyl acetate was taken to dryness, then the residue was dissolved in PBSTA and examined for matrix effects. A minimal signal (less than 2.5 ppb) was observed, even when the ethyl acetate was reconstituted to one-tenth of the initial solvent volume, a 10-fold concentration factor.

It was noted that subtle effects occur when solvents or salt concentrations were varied in this assay. A decrease in the maximum absorbance and an increase in the background absorbance resulted, with a tendency to poorer curve fit. For this reason, a corresponding amount of modifier, such as the extraction solvent for the unknown soil samples being analyzed, was added to the standards used to generate a standard curve.

Soil and water matrix effects. Different sources of water, used in constructing standard curves of deethyl- or deisopropylatrazine, had no significant matrix effect on assay performance. A series of 33 tap water samples was spiked with deisopropylatrazine in a blind fashion in order to evaluate this assay as a means of routine analysis. A good correlation was obtained between the amount spiked and the amount determined by ELISA (Figure 1). Although the assay underestimated the true value of the spike in water, with a slope of 0.63, this appeared to be a systematic error in the analytical procedure because buffer samples spiked in an identical fashion yielded a similar slope (0.64). In order to identify the source of this systematic error, triplicate samples of 0 μM-, 10 μM- and 50 μM-deisopropylatrazine solutions in PBSTA were analyzed over time. There was a decrease in the average amount of deisopropylatrazine immunoreactivity seen over 24 h, but the value was not statistically significant until 24 h (Table 6). A similar experiment using atrazine, analyzed using an assay selective for atrazine (Karu *et al.*, 1990), showed no detectable decrease in immunoreactivity over this time period. Because the blind tap water and known buffer-fortified samples were prepared the evening before analysis (12–18 h), it was reasonable to assume that the underestimation of the true spiked values by ELISA was due to analyte loss from the prepared solutions while awaiting analysis. Standards were unaffected by water or buffer, because dilutions of stock solutions in DMSO were prepared just prior to use. In a subsequent study, where 10 different concentrations of deisopropylatrazine were added to tap water samples and analyzed immediately, the slope was 1.19.

Subsequent studies on the decrease of deethylatrazine in water at different pH values over time further support a set of conditions which promote analyte instability. Acidic and basic hydrolysis of *s*-triazines has been reported (Skipper *et al.*, 1976; Plust *et al.*, 1981). Within 7 days, a significant decrease in assay response could be seen relative to neutral pH when all samples were stored in glass containers (Figure 2). After 29 days, the quantity of deethylatrazine detected in the sample at neutral pH had decreased by about 10%, while those samples at pH 5.1 or 9.5 had decreased to 50% of the initial immunoreactive response. It must be noted that these samples were not aseptically prepared and were allowed to stand at ambient temperature. Therefore, microbial contamination (and potentially microbial metabolism) and fluctuation of temperature cannot be ruled out as factors that decreased the analyte concentration over time. When polypropylene test-tubes were used, a rapid and similar decline at all pH levels was observed (data not shown). This result points to adsorption of the analyte on to the container surface, because no significant change of deethylatrazine at pH 7 was expected based on the previous experiment.

The analysis of the acetonitrile:methanol:water soil extract required large dilutions (50-fold) in order to minimize the matrix and solvent effects on the assay. After evaporating and reconstituting the soil extract, there was no significant assay perturbation when four-fold dilutions were used; however, some insoluble material was present upon reconstitution of the soil extract. This would suggest that some co-extracted material was possibly

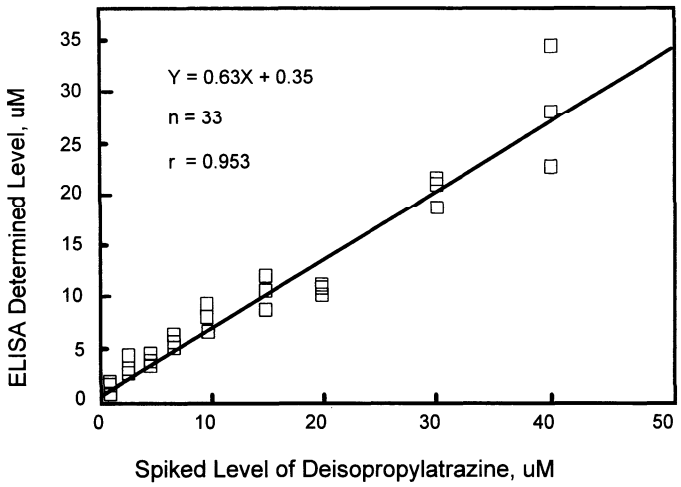


FIG. 1. A series of 33 tap water samples was spiked with deisopropylatrazine in a blind fashion. A good correlation was obtained between the amount spiked and the amount determined by ELISA. Although the assay underestimated the true value of the water, with a slope of 0.63, this appeared to be a systematic error due to analyte hydrolysis. In a subsequent study, where 10 different concentrations of deisopropylatrazine were spiked into tap water samples and then immediately analyzed, the slope was 1.19.

responsible for the large matrix effect in the initial organic extract of the soil. The limits of detection in the evaporated and reconstituted soil extracts were approximately 20 ng ml^{-1} of soil extract or 45 ng mg^{-1} of soil.

Analysis of urine. Analysis of urine samples from five individuals who were not exposed to *s*-triazines indicated that this assay could tolerate up to 25% of urine in the sample volume. However, dilutions as low as 10% of the sample volume were occasionally required. For this reason, all subsequent urine samples analyzed were diluted to 10% with PBSTA prior to analysis.

In order to assess the value of the mono-*N*-dealkylated *s*-triazines as a urinary biomarker of exposure, urine (pH 7) was spiked at concentrations from 100 μM to 5 μM with deethylatrazine and kept at -20°C, 4°C and room temperature, in sets with and without sodium azide. After approximately 1 week, a detectable decrease in the amount of deethylatrazine was observed at all spiking levels and under all storage conditions. Figure 3 depicts the percentage of deethylatrazine remaining over time in urine stored at room temperature with sodium azide as a preservative. The solid line represents the average for

TABLE 6. The amount of deisopropylatrazine detected in spiked PBSTA buffer over time. Triplicate samples of 0, 10 and 50 μM solutions of deisopropylatrazine were prepared in 0.1 M-PBSTA pH 7.4 and analyzed after 0, 16, 24 and 40 h

	10 μM		5 μM	
Time (h)	Mean	SD	Mean	SD
0	9.93	1.48	48.11	7.48
16	7.88	1.40	41.72	2.08
24	6.00	0.46	29.6	3.10
40	4.42	0.55	22.99	1.25

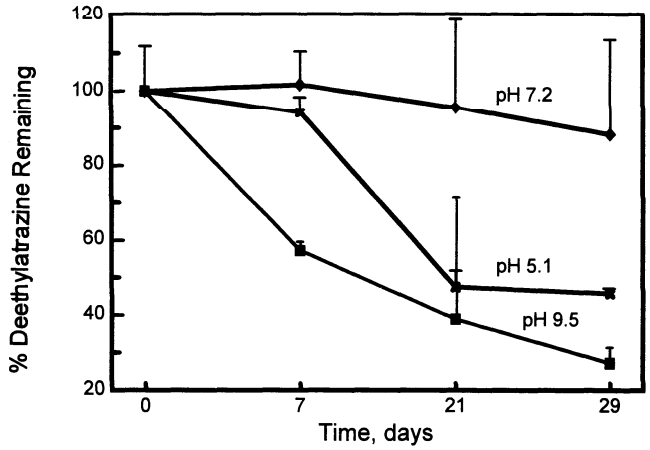


FIG. 2. The percentage of immunoreactive deethylatrazine equivalents remaining in water at pH 5.1, 7.2 and 9.5 over time. Error bars indicate one standard deviation from the mean.

the mean for the composite value generated by averaging the three spiking levels (100, 25 and 5 μM). Samples stored under different conditions showed no significant differences from the data presented here. After 1 month, approximately one-half of the initial amount of analyte remained. There was no apparent difference among samples stored under different storage conditions or between those with or without sodium azide regarding the apparent stability of deethylatrazine over time. These results reflect the stability of deethylatrazine in the urine of only one human volunteer; this may vary widely among individuals.

Hapten **I** also proved to be unstable following storage at room temperature at pH 9.33. When the hapten was prepared in electrolytic buffer for MECC analysis and run immediately, a single peak at 30.9 min accounted for 98% of the UV-absorbing material. After 1 week at this pH and room temperature, a series of peaks was observed. These peaks

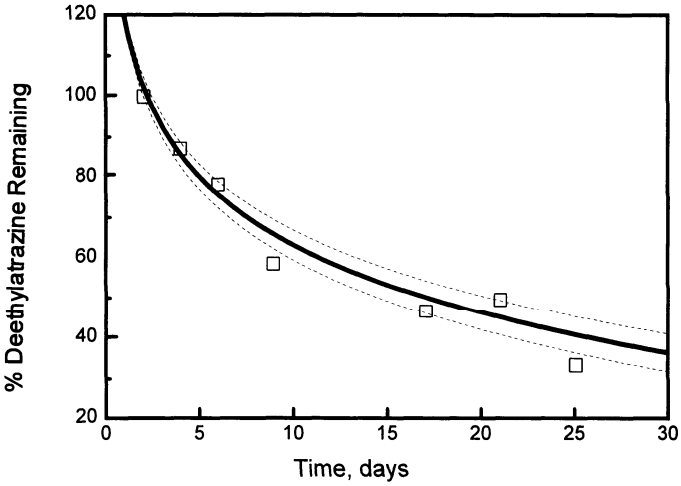


FIG. 3. The percentage of immunoreactive deethylatrazine equivalents remaining over time in urine stored at room temperature with sodium azide as a preservative. The solid line represents the average for the mean for the composite value generated by averaging the three spiking levels (100, 25 and 5 μM) left at room temperature. The dashed lines indicate plus and minus the SEM. Samples stored under different conditions showed no significant difference from the data presented here.

displayed not only molecular ions $M + H^+ + 2$ and $M + H^+$ (260.0918 atomic mass units) in the FAB-HRMS with the correct isotopic ration of 1:2.9 characteristic of hapten **I**, but also the molecular ion for 2-hydroxy-*s*-triazine at 242.1266 atomic mass units ($M + H^+$). The intensities of the molecular ion peaks indicated a hapten **I**/2-hydroxy ratio of 3:2. This is consistent with work describing both the acidic and basic hydrolysis of chloro-*s*-triazines (Skipper *et al.*, 1976; Plust *et al.*, 1981).

CONCLUSIONS

The analysis of metabolites, in addition to the parent compound, is important for a more complete understanding of the fate, distribution and impact of agrochemicals on ecosystems. In order to analyze samples more efficiently for the presence of the mono-*N*-dealkylated *s*-triazines, an ELISA was developed, characterized and validated. This assay showed remarkable selectivity for 2-chloro-4-amino-*s*-triazine structures. Also, the ability of this assay to be operated quickly and efficiently in tap water was examined by conducting a blind spiking study of a series of water samples. A good correlation between the fortified levels and concentration of *N*-dealkylated triazine found was obtained in these experiments. However, the analyte stability in buffer after 24 h demonstrated a significant decrease in the immunoreactivity of the *N*-dealkylated *s*-triazine, indicating that this compound was unstable in buffered solutions. Although this ELISA did demonstrate some matrix effects from soil (above 50 ng ml^{-1} of extract or 20 ng g^{-1}) and organic solvents (above 12.5% of the sample volume), it proved to be a reliable and efficient means of analysis.

The use of the ELISA in evaluating the stability of deethylatrazine provided information on the possible use and limitations of the *N*-dealkylated *s*-triazines as environmental or urinary biomarkers of exposure. These storage stability experiments in water and urine indicated that analysis of the *N*-dealkylated triazines should be completed within 1 week. It should be noted that these results were generated using the urine from one individual and one set of water samples. The stability of these triazine metabolites in other human urine and environmental samples may differ significantly.

Additional evidence of analyte instability was obtained using a sample of the immunizing hapten. After analysis by MECC, the sample was allowed to stand at room temperature for 1 week at pH 9.33, and then reanalyzed by MS. Approximately one-third of the sample was hydrolyzed. For biological samples, analysis of the mercapturic acid conjugate of the *s*-triazines would provide additional information on exposure, as well as more reliable results for samples not analyzed quickly. Immediate extraction or analysis of environmental samples is needed to generate accurate information on the presence of *N*-dealkylated *s*-triazines.

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APPENDIX A

Registry No. **I**, 153635–76–8; atrazine, 1912–24–9; hydroxyatrazine, 2163–68–0; simazine, 122–34–9; hydroxysimazine, 2599–11–3; atrazine mercapturate, 138722–96–0; ametryne, 834–12–8; simetryn, 1014–70–6; propazine, 139–40–2; prometryn, 7287–19–6; prometon, 1610–18–0; terbutryne, 886–50–0; cyanazine, 21725–46–2; cryomazine, 36576–43–9; cyanuric acid, 108–80–5; cyanuric chloride, 108–7–0; cyanazine acid, 36576–43–9; deethylatrazine, 6190–65–4; deisopropylatrazine, 4147–58–4; didealkylated atrazine, 3397–62–4; melamine, 108–78–1; ammelide, 645–93–2; ammeline, 654–92–1; **2a**, 68228–19–3; **2b**, 82784–46–1; **2c**, 125454–26–4; **2d**, 125454–27–5; **2e**, 125454–28–6; **4a**, 125454–30–0.