Hapten Synthesis and Immunoassay Development for the Analysis of Chlorodiamino-*s*-triazine in Treated Pesticide Waste and Rinsate

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A significant environmental degradation product of chloro-*s*-triazine herbicides is chlorodiamino-*s*-triazine. Current chromatographic analytical methods are limited by the water solubility and inefficient recovery of this analyte. A diamino-*s*-triazine hapten was synthesized for the production of polyclonal antibodies. Other haptens were synthesized which differed in bridging group and ring substitution for use in immunoassay development using both indirect and haptenated enzyme formats. In general, antibody recognition of substituted *s*-triazines decreased as a function of amino side chain substitution. The assays were sensitive in the low micromolar range. Two assays were optimized and validated by comparison with a HPLC method for the analysis of chlorodiamino-*s*-triazine in treated pesticide waste samples. The correlation coefficients found were 0.988 and 0.979. It is anticipated that this immunoassay will provide a useful monitoring technique for on-site sampling and remediation of pesticide-contaminated areas.

INTRODUCTION

The *s*-triazine class of herbicides is widely used in agriculture and in noncrop applications. A major degradative pathway for the 2-chloro-4,6-(alkylamino)-*s*-triazines, such as atrazine, simazine, and propazine, involves sequential N-dealkylation (Figure 1) resulting in the intermediate chlorodiamino-*s*-triazine (CAAT, Table 1). This route of metabolism has been shown to occur in microbial, plant, and animal systems (Bekhi and Khan, 1986; Wichman and Byrnes, 1975; Dauterman and Mueke, 1974). CAAT was an important intermediate in the two-step ozonation–microbial mineralization process used for the treatment of chloro-*s*-triazine herbicide-containing pesticide wastes and rinsates (Kearney et al., 1988). Recently, some *s*-triazines (atrazine, simazine, cyanazine) have been detected in a small percentage of groundwater samples at concentrations above health advisory levels (Aharonson et al., 1987; Parsons and Witt, 1988). The mono-N-dealkylated metabolites CIAT and CEAT have also been detected in groundwater (Isensee et al., 1990; Adams and Thurman, 1991), CIAT being the predominant degradate. The detection of N-dealkylated *s*-triazine degradates has recently been proposed for use in determining the source of environmental contamination, i.e., point source vs. non-point-source (Adams and Thurman, 1991). To date, few data have been reported on the detection of CAAT in environmental samples.

Current chromatographic methods for the analysis of the dealkylated metabolites are limited by their high water solubilities (>100 ppm) and inefficient recoveries from environmental matrices, typically less than 50% (Nash, 1990). In addition, these methods may be impractical in situations where large numbers of samples are generated.

Enzyme-linked immunosorbent assays (ELISAs) have been developed for the analysis of *s*-triazine herbicides (Huber, 1985; Bushway et al., 1988; Harrison et al., 1991a; Karu et al., 1991), hydroxyatrazine (Schlaeppi et al., 1989),

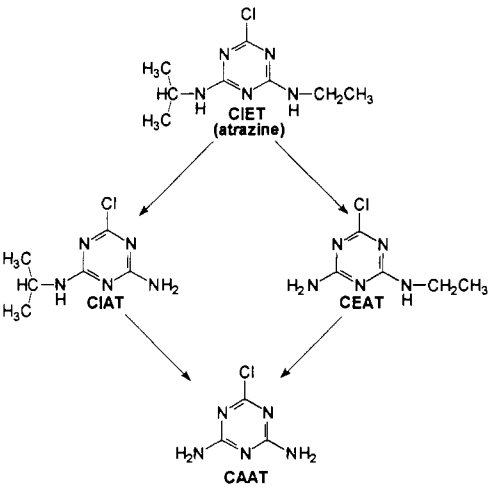

Figure 1. Degradation of atrazine by sequential N-dealkylation resulting in the formation of chlorodiamino-*s*-triazine (CAAT).

and the monodealkylated atrazine metabolites CEAT and CIAT (Wittman and Hock, 1991). ELISA has been shown to be very valuable for screening large numbers of samples prior to conventional analysis such as GC–MS (Thurman et al., 1990). An ELISA for the analysis of CAAT has not been reported. Although the distribution of CAAT in the environment is not fully understood, an ELISA for this analyte would nevertheless be useful for environmental and remediation monitoring. The purpose of the present study was to develop an ELISA for the analysis of CAAT in aqueous samples.

MATERIALS AND METHODS

Chemicals. Dichloroamino-*s*-triazine (CCAT) was purchased from Polysciences, Inc., Warrington, PA. 4-Mercaptobenzoic acid was purchased from Toronto Research Chemicals, Inc., Downview, ON. Chlorodiamino-*s*-triazine (CAAT), 3-mercaptopropanoic acid, 6-aminohexanoic acid, dicyclohexylcarbodiimide (DCC), and *N*,*N*-diisopropylethylamine were purchased from Aldrich Chemical Co., Milwaukee, WI. *N*-Hydroxysuccinimide (NHS), bovine serum albumin (BSA), keyhole limpet hemocyanin (KLH), alkaline phosphatase type VII-NT (AP), and all immu-

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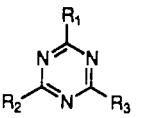
Table 1. Structure and Nomenclature of *s*-Triazines



common name	Cook system[a]	R_1	R_2	R_3
didealkylated atrazine	CAAT	Cl	NH_2	NH_2
	SPrAAT	SCH_2CH_2COOH	NH_2	NH_2
	SBeAAT	$S(C_6H_4)COOH$	NH_2	NH_2
	CAHeT	Cl	NH_2	$NH(CH_2)_5COOH$
	SAAT	SH	NH_2	NH_2
	CDAT	Cl	$NH[C(O)CH_3]$	NH_2
	CDET	Cl	$NH[C(O)CH_3]$	$NH(CH_2CH_3)$
	CDIT	Cl	$NH[C(O)CH_3]$	$NH[CH(CH_3)_2]$
	CDDT	Cl	$NH[C(O)CH_3]$	$NH[C(O)CH_3]$
deethylsimazine	CEAT	Cl	$NH(CH_2CH_3)$	NH_2
deethylatrazine	CIAT	Cl	$NH[CH(CH_3)_2]$	NH_2
atrazine	CIET	Cl	$NH[CH(CH_3)_2]$	$NH(CH_2CH_3)$
simazine	CEET	Cl	$NH(CH_2CH_3)$	$NH(CH_2CH_3)$
cyanazine	CENT	Cl	$NH(CH_2CH_3)$	$NH[C(CH_3)_2CN]$
ammeline	OAAT	OH	NH_2	NH_2
ammelide	OOAT	OH	OH	NH_2
cyanuric acid	OOOT	OH	OH	OH
melamine	AAAT	NH_2	NH_2	NH_2
N-isopropylammeline	OIAT	OH	$NH[CH(CH_3)_2]$	NH_2
N-ethylammeline	OEAT	OH	$NH(CH_2CH_3)$	NH_2
N-ethylammelide	OOET	OH	OH	$NH(CH_2CH_3)$
cyromazine	CyPAAT	$NH(C_3H_5)$	NH_2	NH_2
diamino-*s*-triazine	HAAT	H	NH_2	NH_2

[a] T, *s*-triazine ring; H, hydrogen; C, chlorine; A, amino; O, hydroxyl; S, thio; I, isopropylamino; E, ethylamino; D, acetoamido; N, cyanoisopropylamino; CyP, cyclopropylamino; SPr, thiopropanoic acid; SBe, thiobenzoic acid; He, aminohexanoic acid [adapted from Cook et al. (1987)].

nochemicals except anti-*s*-triazine antibodies were purchased from Sigma Chemical Co., St. Louis, MO. Compounds CDAT, CDET, CDIT, and CDDT were synthesized as previously described (Hapeman-Somich et al., 1992). Other *s*-triazines (except haptens) were gifts from Ciba-Geigy Corp. (Agricultural Division, Greensboro, NC).

Buffers. Phosphate-buffered saline (pH 7.5) containing Tween 20 and sodium azide (PBSTA) was used for dilution of immunoreagents and samples prior to immunoassay and for microtiter plate washing. Sodium carbonate buffer (pH 9.6) was used in coating microtiter plates with antibodies and hapten–BSA conjugates. Enzyme substrate buffer was diethanolamine, pH 9.8. The compositions of the various buffers used have been described in detail elsewhere (Karu et al., 1991).

Equipment. Melting points were determined using a Fisher-Johns melting point apparatus. High-performance liquid chromatography (HPLC) was performed using a Waters 4-μm, 8 mm × 10 cm, NVC-18 radial compression module. Two HPLC systems were used: (a) two Waters Model 6000 pumps with a Waters Model 990 photodiode array detector and accompanying NEC APC-III controller and software: (b) two Waters Model 510 pumps, a Waters Model 410 UV detector (210, 225 and 260 nm monitored), and a Waters Model 712 WISP sample injector. Infrared (IR) spectra (4000 to 700 cm^{-1}) were obtained by J. George Buta, Plant Hormone Laboratory, USDA/ARS, using a Nicolet Model 60-SX FT-IR spectrometer at a resolution of 4 cm^{-1}. The spectrometer used a globar source and a liquid nitrogen cooled Hg/Cd/Te (MCT-A) detector. Electron impact and chemical ionization mass spectra (methane, ammonia, and ammonia-d_3) were obtained by William R. Lusby, Insect Hormone Laboratory, USDA/ARS, using a Finnigan-MAT Model 4510 mass spectrometer equipped with a direct exposure probe. Electron impact mass spectra of SBeAAT (Table 1) was obtained on a Hewlett-Packard Model 5988A mass spectrometer with a particle beam HP Model 59980A interface. Two Waters Model 6000 pumps were used to deliver an acetonitrile/water solvent at 0.4 mL/min. ^1H NMR spectra were obtained by Rolland Waters, Environmental Chemistry Laboratory, USDA/ARS, on a General Electric QE-300 spectrometer using tetramethylsilane as an internal standard. Ultraviolet-visible (UV–vis) spectral measurements were made on a Beckman DU-7 spectrophotometer. ELISA procedures used 96-well U-bottom Nunc immunoplates (Nunc, no. 449824). A ThermoMax microplate reader with SoftMax software (Molecular Devices Corp., Menlo Park, CA) was used for ELISA optical density (OD) measurements and data calculations.

Hapten Synthesis. Haptens were synthesized using adaptations from Goodrow et al. (1990) and are illustrated in Figure 2. Syntheses were monitored by HPLC with UV diode array detection. Differences in retention time and UV spectra (Weber, 1967) were used for tentative product identification during synthesis. The typical HPLC solvent condition used a linear gradient of acetonitrile in aqueous phosphoric acid (pH 2) from 0 to 75% in 5 min. The latter condition was maintained for 10 min. The flow rate was 2 mL/min.

3-[(4,6-Diamino)-1,3,5-triazin-2-yl)thio]propanoic Acid Potassium Salt (SPrAAT). CAAT (0.725 g, 5 mmol) was suspended in 100 mL of absolute ethanol, and 0.574 g of 3-mercaptopropanoic acid (5.4 mmol) and 0.66 mL of 15.15 M KOH (10.8 mmol) in 10 mL of absolute ethanol were added. The reaction was refluxed for 12 h until CAAT was no longer detected by HPLC. The reaction mixture was filtered on No. 2 Whatman paper and the filter cake washed with absolute ethanol followed by distilled water (4 °C). The white solid was dried to obtain 0.3 g of SPrAAT (24% yield; >98% purity by HPLC), mp 220 °C (dec); IR (KBr) 3358, 3162 (m, NH), 1653 (s, C=O) cm^{-1}; ^1H NMR (CD_3OD) δ 2.63 (t, 2 H, J = 7.7 Hz), 1.95 (t, 2 H, J = 7.7 Hz); MS m/z (relative intensity) 213 (2, M − 2H), 198 (1, M − OH), 170 (1, M − CO_2H), 143 (36, M − CO_2 − CH_2CH_2), 72 (100, M − H − $C_3N_6SH_4$); MS (CI–CH_4) 216 (M + H); MS (CI–NH_3) 233 (M + NH_4), 216 (M + H); MS (CI–ND_3) 238 (M + ND_4), 218 (M + D); UV (H_2O) E_m (263 nm) 3218.

4-[(4,6-Diamino)-1,3,5-triazin-2-yl)thio]benzoic Acid Potassium Salt (SBeAAT). CAAT (0.725 g, 5 mmol) was suspended in 100 mL of absolute ethanol, and 0.924 g of 4-mercaptobenzoic acid (5 mmol) and 0.66 mL of 15.15 M KOH (10.8 mmol) in 10 mL of absolute ethanol were added. The reaction was refluxed for 24 h. At 16 h, 4-mercaptobenzoic acid was not apparent by HPLC. An additional 0.462 g of 4-mercaptobenzoic acid (2.5 mmol) and 0.33 mL of 15.15 M KOH (5.4 mmol) were added at 16 and 19 h. At 24 h, CAAT was no longer detected by HPLC. The reaction mixture was filtered on No. 2 Whatman paper and the filter cake washed successively with absolute ethanol, ethyl acetate, and again absolute ethanol. The

tan solid was dried, affording 1.17 g of SBeAAT (66% yield; >95% purity by HPLC), mp >300 °C; IR (KBr) 3326, 3162 (m, NH), 1682 (s, C=O) cm^{-1}; ^1H NMR (DMSO-d_6) δ 7.81 (d, 2 H, J = 8.1 Hz), 7.40 (d, 2 H, J = 8.1 Hz), 6.65 (br, 4 H); MS m/z (relative intensity) 263 (83, M^+), 262 (100, M - H), 220 (28, M - $NCNH_2$ - H), 137 (37, M - OH - $C_3H_3N_5$); UV (H_2O) E_m (263 nm) 10650.

6-[(4-Chloro-6-amino-1,3,5-triazin-2-yl-)amino]hexanoic Acid (CAHeT). To 0.820 g of CCAT (5 mmol) suspended in 15 mL of acetone was added 0.656 g of 6-aminohexanoic acid (5 mmol) followed by 1.29 g of *N,N*-diisopropylethylamine (10 mmol). The reaction was carried out at 40 °C until CCAT was no longer detected by HPLC (22 h). Substantial amounts of ammelide (insoluble) and 2-chloro-4-hydroxy-6-amino-1,3,5-triazine (COAT) were detected by HPLC/UV and HPLC/MS. The reaction mixture was filtered and the filtrate concentrated in vacuo, resulting in a tan oil. The material was reconstituted in 40 mL of 5% sodium carbonate and extracted with 10 mL of methylene chloride (2×). The aqueous phase was acidified (pH 1.5) and extracted with 25 mL of ethyl acetate (4×). The organic layer was dried over sodium sulfate and filtered, and the solvent was removed in vacuo. The resultant white solid was dried to obtain 0.08 g of CAHeT (6.6% yield, >98% purity by HPLC), mp 156–157 °C; IR (KBr) 3298 (b, NH, OH), 1755 (s, C=O) cm^{-1}; ^1H NMR (DMSO-d_6) δ 12.00 (s, 1 H), 7.67 (br, 1 H), 7.28 (s, 1 H), 7.10 (br, 1 H), 3.21–3.16 (m, 2H), 2.20 (t, 2 H, J = 7 Hz), 1.54–1.44 (m, 4 H), 1.29–1.25 (m, 2 H); MS m/z (relative intensity) 261 (1, M + 2), 259 (3, M^+), 214 (2, M - CO_2H), 200 (35, M - $C_2H_3O_2$), 186 (12, M - $C_3H_5O_2$), 172 (25, M - $C_4H_7O_2$), 158 (100, M - $C_5H_9O_2$), 145 (17, M - $C_6H_{10}O_2$); MS (CI-CH_4) 260 (M + H); MS (CI-NH_3) 260 (M + H); UV (H_2O) E_m (263 nm) 344, E_m (245 nm) 3114.

Hapten–Protein Conjugation. Haptens were conjugated to proteins (KLH, BSA, AP) via NHS-activated esters (Langone and Van Vunakis, 1975). Molar ratios of hapten/NHS/DCC used in active ester synthesis were typically 1:1:2. For hapten activation, 0.1 mmol of SBeAAT or 0.2 mmol of SPrAAT or CAHeT was dissolved or slurried in 0.5 (SBeAAT) or 0.8 mL (SPrAAT and CAHeT) of dry dimethylformamide. NHS was added followed by DCC. The activation reaction was carried out at ambient temperature for 5 h with stirring. The white dicyclohexylurea precipitate was removed from solution by centrifugation. For BSA or KLH conjugates, 0.3 mL of the supernatant was added to either 200 mg of BSA in 10 mL of distilled water, pH 9, or 30 mg of KLH in 3 mL of distilled water, pH 9. For AP conjugation, 20 μL of the supernatant was added to 3.8 mg of AP (2600 units/mg) in 3.0 M NaCl containing 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$, and 30 mM triethanolamine, pH 7.6, diluted 1:2 in 10 mM sodium tetraborate buffer, pH 9.0, containing 0.9% NaCl and 0.02% NaN_3. The conjugation reactions were carried out for 18 h at 4 °C with stirring. The protein solutions were exhaustively dialyzed against PBS containing 0.02% sodium azide and either dialyzed into distilled water, lyophilized, and stored frozen (BSA and KLH conjugates) or collected and stored as a solution in PBS–azide at 4 °C (AP conjugates). Hapten densities of the BSA conjugates were estimated by measuring the protein absorption at 280 nm and the additive absorbance of hapten and protein at 260 nm and calculating the relative absorbance contribution of the hapten moiety.

Immune Polyclonal Ascites Fluid Production. Immune polyclonal ascites fluid was produced by an adaptation of a previously described method (Lacy and Voss, 1986). Two hundred micrograms of immunogen (SPrAAT–KLH) was emulsified in 200 μL of complete Freund's adjuvant and injected intraperitoneally into BALB/c mice. On days 14 and 40, the mice were boosted with 50 μg of immunogen emulsified in complete adjuvant. Viable myeloma cells (10^6–10^7) were injected intraperitoneally 3–4 days after the last boost for inducing ascites fluid. Ascites fluid was collected 10–14 days later. The ascites fluid was centrifuged, titrated, aliquoted, and stored at −70 °C until used.

Initial Antibody Screening. Immunized animals were screened for the production of *s*-triazine-specific antibodies (primary antibodies) using the indirect ELISA (see below). Initial screening criteria considered the detection of antibody binding to the homologous SPrAAT–BSA conjugate. Secondary screening criteria evaluated inhibition of antibody binding by 0.5 mM CAAT. Antibodies that tested positive for each of these initial screens were further pursued.

ELISA Formats. For each of the assay formats, the amounts of the various immunochemical reagents used were determined by checkerboard titration (Gee et al., 1988; Jung et al., 1989). Optimal conditions were chosen to produce between 0.5 and 1.0 OD unit according to the procedures described below.

Competitive Inhibition Indirect ELISA. Microtiter plates were coated with 100 μL of a 1.25 μg/mL solution of BSA–hapten diluted in coating buffer, incubated for 18 h at 4 °C, and then washed with PBSTA. The plates were stored frozen. When needed, the plate was brought to room temperature and blocked with 100 μL/well of 0.5 mg/mL BSA in PBSTA for 60 min and then washed. For competitive inhibitions, 20 μL of a predetermined dilution of primary antibody was mixed with 100 μL of sample (± *s*-triazine) diluted in PBSTA and incubated for 30 min, and 50-μL aliquots of each mixture were applied to replicate wells of the BSA–hapten plate. This was incubated for 30 min and then washed. Goat anti-mouse IgG antibody conjugated to alkaline phosphatase diluted 1:500 in PBSTA was applied to the plate (100 μL/well), incubated for 30 min, and washed. Enzyme substrate (*p*-nitrophenyl phosphate) was added, and the plate OD measurement (405–650 nm) was made at 30 min.

Standard Competitive Inhibition Haptenated Enzyme ELISA. Microtiter plates were coated with 100 μL of goat anti-mouse IgG antibody diluted 1:250 in coating buffer, incubated for 18 h at 4 °C, and then washed. One hundred microliters of a predetermined dilution of primary antibody diluted in 0.5 mg/mL BSA in PBSTA was applied to the plate, incubated for 60 min, and frozen with the liquid remaining in the wells. When needed, the plate was thawed and washed. One hundred microliters of sample (± *s*-triazine) and 100 μL a predetermined dilution of haptenated enzyme were mixed in a separate uncoated well and 50-μL aliquots applied in replicate in the antibody-coated plate. This was incubated for 30 min and then washed. Enzyme substrate was added, and the plate OD measurements were made at 60 min.

Determination of CAAT Sensitivities and Relative Reactivities Using Competitive Inhibition Indirect ELISA. Combinations of antibody and BSA–hapten were evaluated for CAAT sensitivity by assaying a zero-dose control and seven concentrations of CAAT and calculating the CAAT IC_{50} value (concentration of analyte which produces a 50% decrease in the maximum normalized response) generated from the four-parameter logistic curve fitting function in SoftMax (parameter C). The most sensitive antibody and BSA–hapten combinations were further evaluated for CAAT sensitivity and relative reactivity toward CIAT, CEAT, and CIET by assaying a zero-dose control and seven concentrations of either analyte. Analyses were made in triplicate. Relative reactivities were interpreted relative to CAAT (=100%) according to the formula

$$\% \text{ reactivity} = (IC_{50}\ \text{CAAT}/IC_{50}\ \text{class analog}) \times 100 \quad (1)$$

Antibody/Haptenated Enzyme Recognition and Development of the Competitive Inhibition Haptenated Enzyme ELISA. Antibodies that were most sensitive and selective for CAAT with the indirect ELISA were tested for recognition of haptenated enzyme conjugates SBeAAT– and CAHeT–AP. Combinations (preferably heterologous) that tested positive were further evaluated. Selected haptenated enzyme ELISAs were characterized for cross-reactivity toward 23 *s*-triazine derivatives (shown in Table 1) by assaying a zero-dose control and 11 concentrations of either *s*-triazine. Analyses were made in duplicate. IC_{50} values were calculated for each analyte.

Evaluation of Incubation Time and Haptenated Enzyme Dilution for Sensitivity with the Competitive Inhibition Haptenated Enzyme ELISA. The effect of incubation time of the haptenated enzyme and sample mixture on the antibody-coated plate on sensitivity was evaluated by incubating mixtures of CAAT standards (15 concentrations and a zero-dose control) and a 1:500 dilution of CAHeT–AP on the antibody-coated plate for either 30 min, 1 h, 4 h, 7 h, or 16 h (overnight). All other assay conditions were the same as the standard procedure. The effect of using a higher dilution of haptenated enzyme and either a longer incubation time of the haptenated enzyme and sample

mixture on the antibody-coated plate or a longer substrate development time for the assay on sensitivity was evaluated. For this, CAAT standards (15 concentrations and a zero-dose control) and a dilution of CAHeT–AP which gave an OD reading of 0.5 after either (a) 30 min of incubation on an antibody-coated plate and 7 h of substrate development time (1:8000 dilution of haptenated enzyme) or (b) 7 h of incubation on an antibody-coated plate and standard 60 min of substrate development time (1:2000 dilution of haptenated enzyme) were evaluated. Least-squares mean IC_{50} values derived from each protocol were tested for significant differences from the standard procedure ($\alpha = 0.01$).

Ozonation and HPLC Analysis of Pesticide Waste and Rinsate (PWR). PWR samples that were known to contain *s*-triazine herbicides were obtained from various collection facilities at the Beltsville Agricultural Research Center (BARC), ARS, USDA, Beltsville, MD, during the spring 1991 growing season. Sixty-milliliter aliquots of each of seven PWR samples were added to a 150-mL Erlenmeyer flask, and ozone gas (4.1% v/v) was bubbled at a flow rate of 1 L/min. The equipment used is described elsewhere (Hapeman-Somich et al., 1992). Samples were ozonated until parent *s*-triazine herbicides were no longer detectable by HPLC. Samples were diluted 1:2 in acetonitrile and were analyzed by direct injection reversed-phase HPLC. Ozonation product composition was determined on the basis of standard curves for the breakdown products CDAT, CEAT, CIAT, and CAAT (Hapeman-Somich et al., 1992) using authentic analytical standards. Subsamples of each ozonated PWR sample were spiked to either 1 or 2 mM CAAT above the original concentration to obtain a broader concentration range for analysis. All 21 samples were diluted 1:2 in acetonitrile and analyzed by HPLC.

Analysis of Ozonated PWR by the Standard Competitive Inhibition Haptenated Enzyme ELISA. The acetonitrile-diluted samples were further diluted in PBSTA and analyzed by competitive inhibition haptenated enzyme ELISAs using antibodies PAb 1 and PAb 5. Each microplate consisted of a CAAT standard curve (seven concentrations) and four dilutions each of six samples. Each sample dilution was assayed in triplicate.

Concentrations of *s*-triazine were calculated as micromolar CAAT equivalents (based on the standard curve for CAAT) using the lowest sample dilution that gave an OD value within the working range of the assay, defined as 70–20% of the normalized OD value of the zero-dose control. Individual ELISAs were evaluated by geometric mean regression (Sokal and Rohlf, 1981) of the amount found by ELISA on the expected response to total *s*-triazine product determined by HPLC utilizing the individual antibody–analyte reactivity coefficients.

RESULTS AND DISCUSSION

Hapten Synthesis. A panel of amino-*s*-triazine haptens were synthesized using adaptations from previously described chemistries (Figure 2) (Goodrow et al., 1990). Stepwise nucleophilic displacement of the chlorines of cyanuric chloride by amino- and mercapto-substituted organic acids has been well studied and is temperature dependent (Smolin and Rapoport, 1959). Replacement of the chlorine atom of CAAT with mercapto-organic acids at ethanolic reflux resulted in the formation of SPrAAT and SBeAAT. Substitution of a single chlorine atom of CCAT with an amino acid in acetone (to avoid ethoxylate formation with the use of ethanol) resulted in the formation of CAHeT. The long reaction times observed for SPrAAT and SBeAAT were probably due to a combination of the low CAAT solubility in the solvent and the low reactivity of the single chlorine atom of CAAT (Mosher and Whitmore, 1945). The stability of the mercapto structures themselves might have been enhanced with the use of nitrogen conditions in the synthesis but were not used here. A series of hydrolysis products (COAT, OOAT) were formed concurrently with the desired product when CAAT was used as the starting material. The low recovery of CAHeT probably resulted from a combination of these problems in addition to the zwitterionic character of the

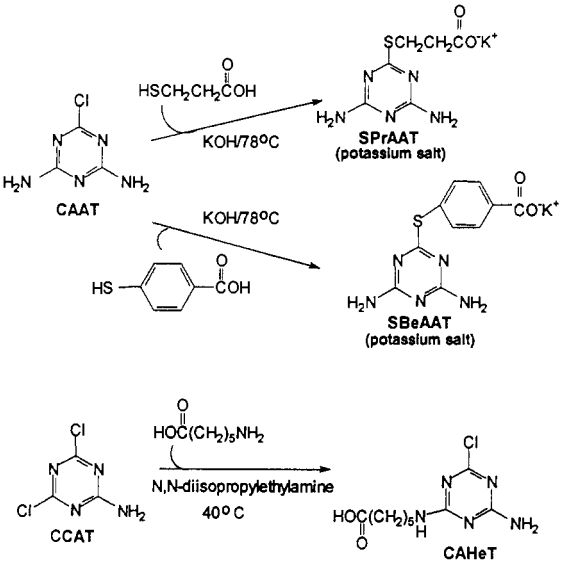


Figure 2. Routes used for hapten synthesis. Synthesis proceeded by nucleophilic substitution of a single chlorine atom of the *s*-triazine ring by either a mercapto- or amino-substituted carboxylic acid.

compound. No attempt was made to optimize the reaction conditions. Reaction starting materials, products, and byproducts were separated by HPLC, and characteristic differences in UV spectra (Weber, 1967) were utilized to tentatively identify structure during synthesis. NMR, IR, UV, and mass spectra confirmed product structures.

Goodrow et al. (1990) used a mercaptopropanoic acid to derivatize atrazine (CIET) through the chloro position. The antibodies generated using these haptens recognized the chlorodialkylamino-*s*-triazine herbicides to the exclusion of hydroxy and N-dealkylated metabolites. Their rationale, which was adapted in the present study, for using the sulfur-containing bridging group is due to the similarities between sulfur and chlorine in electron structure and molecular size. For the selective detection of CAAT by ELISA, it was desired to produce antibodies that recognized the diamino part of the molecule, with the chloro moiety being of secondary importance. SPrAAT was chosen as the immunizing hapten in an attempt to maximize antibody recognition of CAAT. The use of a diamino-*s*-triazine hapten with a thio linker was considered a rationale choice with the diamino moieties being distal to the bridging group. A discussion of hapten design can be found in Harrison et al. (1991b). The other haptens synthesized differed from the immunizing hapten in bridging group structure (SBeAAT) or both bridging group structure and ring substitution (CAHeT) for the purpose of designing a heterologous hapten ELISA format. The advantages of using heterologous haptens in ELISA development have been reported (Wie and Hammock, 1984) and were used for the design of an ELISA sensitive for *s*-triazine herbicides (Harrison et al., 1991a). Wittmann and Hock (1991) used CAHeT as the immunizing hapten for polyclonal antibody development. Their antibodies did not recognize CAAT but were highly selective toward the mono-N-dealkylated degradates CIAT and CEAT. This was evidence that the alkyl side chain of this hapten contributed to antibody recognition. However, a heterologous ELISA system was not used and could have possibly allowed for CAAT recognition by some antibodies in the polyclonal antibody preparation.

Hapten–Protein Conjugation. Haptens were conjugated to proteins via active NHS esters (Langone and Van Vunakis, 1975). Following exhaustive dialysis to remove

Table 2. Antibody CAAT IC_{50} (Micromolar) Values[a] of Various BSA–Hapten Conjugates Using Competitive Inhibition Indirect ELISA

	BSA hapten		
antibody	SPrAAT	SBeAAT	CAHeT
PAb 1	U[b]	U	16.1
PAb 2	U	202	38.8
PAb 3	U	114	ND[c]
PAb 4	U	224	44.6
PAb 5	143	10.4	4.08
PAb 6	U	U	27.8
PAb 7	U	U	64.2 (HB)[d]
PAb 8	225 (HB)	U	28.7 (HB)

[a] IC_{50} values were determined by assaying a zero-dose control (PBSTA) and seven concentrations of CAAT and deriving the value from the four-parameter logistic curve fitting function. Each concentration was assayed in duplicate wells of a BSA–hapten-coated plate. [b] U, undefined curve; four-parameter logistic curve did not fit, highest concentration of CAAT (1 mM) did not completely inhibit antibody binding. [c] ND, not determined; antibody did not recognize BSA–hapten conjugate. [d] HB, high background.

unconjugated hapten, hapten–protein conjugation was verified by observing changes in solubility and inhibitable antibody recognition of the conjugate. Hapten densities on the BSA–hapten conjugates were estimated spectrophotometrically by measuring the absorbance of the conjugate at 260 and 280 nm and determining the amount due to hapten at 260 nm by normalizing this for the contribution from protein at this wavelength. Hapten densities for SPrAAT–, SBeAAT–, and CAHeT–BSA were 4.4, 3.9, and 1.3 mol of hapten/mol of protein, respectively. The higher loading rate reported by Wittman and Hock (1991) for the conjugation of CAHeT to BSA may be accounted for by their use of a water-soluble carbodiimide and 2–4 times more hapten per mole of protein in the reaction.

Antibody and BSA–Hapten Screening by Indirect ELISA. All of the antibodies recognized homologous BSA–hapten conjugates SPrAAT and SBeAAT to a greater extent than the heterologous conjugate CAHeT. Antibody and BSA–hapten conjugate combinations that showed useful titers were evaluated for CAAT sensitivity. These results are presented in Table 2. Homologous conjugate ELISAs were either uninhibitable or showed high IC_{50} values and were not further pursued. CAHeT–BSA heterologous ELISAs were the most sensitive for CAAT quantitation and were further pursued.

Determination of CAAT Sensitivities and Relative Cross-Reactivities Using Competitive Inhibition Indirect ELISA. The most sensitive CAHeT–BSA ELISAs were evaluated for CAAT sensitivity and relative cross-reactivity toward CAAT, CIAT, CEAT, and CIET. These results are presented in Table 3. Differences in CAAT IC_{50} values reported were different from those reported in Table 2, and this was probably due to variability in batches of antigen-coated plates. All of the antibodies except PAb 4 recognized CAAT more than the other s-triazines analyzed and showed considerable recognition of CEAT. Little or no recognition was observed for CIAT or CIET with any of the antibodies. Since the antibodies recognized CEAT, it can be hypothesized that a population of the antibodies recognized that part of the analyte molecule distal to the chlorine, the ethylamino side chain of CEAT mimicking the bridging group of the immunizing hapten. The antibodies did not recognize CIAT, perhaps due to the larger size of the isopropylamino side chain.

Development of the Haptenated Enzyme ELISA. Antibodies PAb 1 and PAb 5 were evaluated for recognition of haptenated enzymes SBeAAT– and CAHeT–AP. Rec-

Table 3. Selected Antibody Reactivities for CAAT, CIAT, CEAT, and CIET (Atrazine) Using BSA–CAHeT with Competitive Inhibition Indirect ELISA[a]

	% reactivity			
antibody	CAAT (IC_{50} μM)	CIAT	CEAT	CIET
PAb 1	100 (29.0)	2.9 (E)[b]	42.4	NI[c]
PAb 2	100 (75.6)	0.8 (E)	31.0	NI
PAb 4	100 (136)	13.6 (E)	152.8	NI
PAb 5	100 (5.6)	7.1	87.1	NI
PAb 6	100 (29.4)	6.9	40.9	NI

[a] IC_{50} values were determined by assaying a zero-dose control (PBSTA) and seven concentrations of inhibitor and deriving the value from the four-parameter logistic curve fitting function. Each concentration was assayed in duplicate wells of a BSA–CAHeT-coated plate. [b] E, IC_{50} values were extrapolated from an undefined curve; highest concentration of inhibitor (1 mM) did not completely inhibit antibody binding. [c] NI, no inhibition at 1 mM.

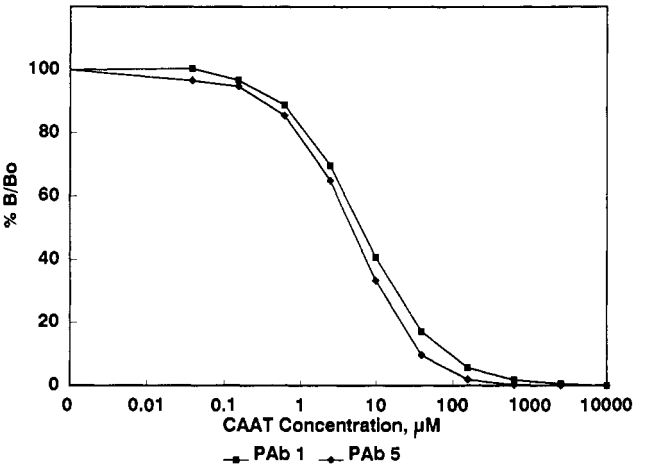


Figure 3. CAAT competitive inhibition haptenated enzyme ELISA curves using PAb 1 or PAb 5. Mixture of sample and 1:500 dilution of CAHeT-AP were incubated for 30 min on an antibody-coated plate followed by 60 min of substrate development time. ELISA OD measurement were normalized by conversion to $\%B/B_0$ values according to the formula

$$\%B/B_0 = [(OD_{sample} - OD_{excess})/(OD_{zero\ dose} - OD_{excess})] \times 100$$

ognition of the pseudohomologous tracer SBeAAT–AP by PAb 1 and PAb 5 was 3 and 5 times more, respectively, than that of the heterologous tracer CAHeT–AP. CAHeT–AP ELISAs were chosen for further characterization since these would most likely result in more sensitive assays.

Typical CAAT competitive inhibition curves using PAb 1 and PAb 5 with CAHeT–AP and the standard assay are shown in Figure 3. Curve parameters are presented in Table 4 and were obtained from ELISAs performed over a 6-month period. Both gave steep slopes in the working range of the assays and low background absorbances. The IC_{50} values reported for PAb 1 and PAb 5 correspond to 838.1 and 656.9 ppb, respectively. Working ranges for the assays averaged 2.19–37.10 μM for PAb 1 and 2.15–21.55 μM for PAb 5. Limits of detection were 0.47 μM for PAb 1 and 0.55 μM for PAb 5. The low %CVs for both the slope and CAAT IC_{50} values indicate highly reproducible assays. IC_{50} %CVs for individual experiments ranged from 2.2 to 9.4% and from 1.6 to 3.5% for PAb 1 and PAb 5, respectively.

Modifications of the standard haptenated enzyme ELISA protocol were investigated in an attempt to improve the sensitivity of the assay. The effects of incubation time of the sample and haptenated enzyme mixture on the antibody-coated plate and haptenated enzyme dilution

Table 4. CAAT Competitive Inhibition Curve Data for Haptenated Enzyme ELISAs[a]

antibody	max absorbance (OD units)	slope (OD/μM)	CV (%)	IC_{50} (μM)	CV (%)	min absorbance (OD units)	R^2
PAb 1	0.792	0.844	(7.99)	5.78	(7.00)	0.0196	0.998
PAb 5	0.647	1.064	(7.07)	4.53	(10.13)	0.0261	0.995

[a] Mixtures of sample and 1:500 dilution of CAHeT-AP were incubated for 30 min on an antibody-coated plate followed by 60 min of substrate development time. The values shown are the mean from ELISAs performed on several days over a 6-month period. The numbers of ELISAs utilizing PAb 1 and PAb 5 were 13 and 12, respectively.

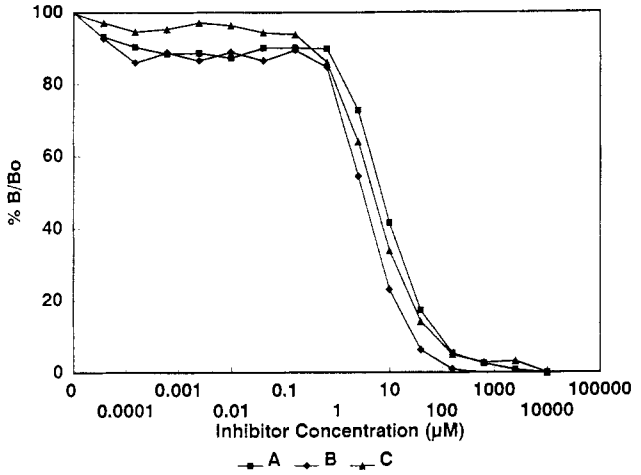


Figure 4. CAAT competitive inhibition curves using PAb 1 and various dilutions of haptenade enzyme and either a longer incubation time of sample and haptenated enzyme mixture on the antibody-coated plate or a longer substrate development time. CAAT standards and a dilution of CAHeT-AP gave an OD reading of 0.5 after either (A) a mixture of sample and 1:500 dilution of CAHeT-AP incubated for 30 min on an antibody-coated plate and 60 min of substrate development time, (B) a mixture of sample and 1:8000 dilution of CAHeT-AP incubated for 30 min on an antibody-coated plate and 7 h of substrate development time, or (C) a mixture of sample and 1:2000 dilution of CAHeT-AP incubated for 7 h on an antibody-coated plate and 60 min of substrate development time. ELISA OD measurements were normalized by conversion to $\%B/B_0$ values according to the formula given in the legend of Figure 3. The IC_{50} values for PAb 1 for A, B, and C were 8.08, 3.81, and 4.94 μM CAAT, respectively. The IC_{50} values for B and C were significantly different from that for A ($p \leq 0.01$).

(and subsequent incubation time of sample and haptenated enzyme mixture or enzyme substrate) were investigated. No differences in IC_{50} values were observed when sample and haptenated enzyme (1:500 dilution) mixtures were incubated for various times on the antibody-coated plate for both antibodies ($p > 0.05$). Karu et al. (1991) used a similar haptenated enzyme ELISA format for the detection of atrazine and showed the competition step to be compete at 30 min; however, longer incubation times were not reported. The results seen in the present study indicate a similar trend. When lower amounts of haptenated enzyme (1:2000 to 1:8000 dilutions) were used (Figure 4), a significant improvement in sensitivity was made ($p < 0.01$) but was in conjunction with longer incubation times of either sample and haptenated enzyme mixture or enzyme substrate. IC_{50} values for PAb 1 are given in Figure 4. IC_{50} values for PAb 5 for protocols A, B, and C were 6.95, 3.74, and 3.46 μM CAAT, respectively. From these results it can be suggested that the use of an enzyme tracer with higher substrate turnover [such as horseradish peroxidase (HRP)] should enhance the sensitivity of the assay since less tracer can be used in the competition step. Schneider and Hammock (1992) showed that sensitivity could be improved 100-fold when an HRP–hapten conjugate was used in place of an AP–hapten conjugate with an *s*-triazine herbicide-sensitive haptenated enzyme ELISA.

Table 5. Characterization of Haptenated Enzyme ELISAs with Selected *s*-Triazines[a]

inhibitor	% reactivity (CAAT = 100%) PAb 1	PAb 5
CAAT (IC_{50}, μM)	100.0 (5.78)	100.0 (4.40)
SPrAAT[b]	140.5	723.8
SBeAAT[b]	242.1	345.1
CAHeT	7.7	5.7
SAAT	35.8	103.6
CEAT	92.8	92.0
CIAT	2.2	3.7
CIET	0.2 (E)[c]	0.2 (E)
CEET	0.2 (E)	0.2 (E)
CENT	0.2 (E)	0.3 (E)
CDAT	2.3	15.7
CDET	1.1	2.0
CDIT	1.1	1.0
CDDT	1.1	1.0
OAAT	0.8	0.5
OOAT	0.1 (E)	<0.04
OOOT	NI[d]	0.04 (E)
AAAT	1.0	1.2
OIAT	<0.06	0.2 (E)
OEAT	<0.06	0.1 (E)
OOET	0.6	0.3 (E)
CyPrAAT	1.0	0.6
HAAT	1.1	0.6 (E)

[a] Mixtures of sample and 1:500 dilution of CAHeT-AP were incubated for 30 min on an antibody-coated plate followed by 60 min of substrate development time. IC_{50} values were determined by assaying a zero-dose control (PBSTA) and 10 concentrations of CAAT and deriving the value from the four-parameter logistic curve fitting function. Each concentration was assayed in duplicate or triplicate wells of an antibody-coated plate. [b] Carboxylate anion equivalent. [c] E, IC_{50} values were extrapolated from an undefined curve; highest concentration of inhibitor (10 mM) did not completely inhibit antibody binding. [d] NI, no inhibition at 10 mM. Structures of the inhibitors are shown in Table 1.

Antibody Characterization Using the Haptenated Enzyme ELISA. PAb 1 and PAb 5 were characterized using CAHeT–AP and 23 *s*-triazines derivatives. These results are presented in Table 5. The two antibodies showed significant recognition of CAAT with very similar cross-reactivity patterns toward *s*-triazine analogs. Cross-reactivities of both antibodies were greatest with the homologous haptens SPrAAT and SBeAAT. With respect to the immunizing hapten SPrAAT, substitution of the entire bridging group with an alkyl group (CyPrAAT) or hydrogen (HAAT) resulted in nearly complete loss of recognition, whereas substitution of the carbon structure [$(CH_2)_2COOH$ or C_6H_4COOH] of the bridging group with hydrogen (SAAT) had less of an effect. This suggests that the thio moiety of the bridging structure is a significant binding epitope for the polyclonal antibodies. For both antibodies, substitution of the bridging group with either hydroxyl (OAAT) or amino (AAAT) resulted in a near loss of recognition. The extent of alkylation of the amino groups in the 4- and 6-positions had a negative effect on recognition. CEAT was recognized to the same extent as CAAT, more than that seen in the indirect ELISA (Table 3), whereas CIAT and the herbicides CIET, CEET, and CENT were not recognized. In addition, PAb 5 recognized CDAT among the acetoamino *s*-triazine ozonation prod-

Table 6. Concentrations of Ozonation Products in Unspiked Pesticide Waste and Rinsate (PWR) As Determined by HPLC

	analyte (μM)			
sample	CAAT	CDAT	CEAT	CIAT
BW2B	105.4	85.7	nd[a]	98.1
BW2T	157.8	83.6	nd	41.4
BE1B	137.8	24.7	nd	nd
BE2M	119.8	44.0	nd	nd
HF1M	114.6	35.6	nd	50.6
HF2M	106.5	79.7	nd	70.6
HF3B	122.5	81.6	nd	38.9

[a] nd, not detected at a concentration of approximately 5 μM.

ucts CDAT, CDET, CDIT, and CDDT. These data indicates that amino side chain "bulkiness" and degree of N-alkylation both contribute to antibody recognition. Both PAb 1 and PAb 5 possess high selectivity for CAAT and CEAT among the environmentally relevant herbicides and degradates.

The IC_{50} values reported here for the target analyte CAAT were considerably higher than those reported for target analytes in other s-triazine ELISAs (Huber, 1985; Bushway et al., 1988; Harrison et al., 1991a; Karu et al., 1991; Wittman and Hock, 1991). CAAT, which is symmetrical and nearly planar, possesses fewer distinct antibody recognition sites compared to the chloroaminoalkyl-s-triazines targeted in the previous studies. This simplicity would tend to make the bridging group structure of the immunizing hapten an important antibody recognition site and present difficulties in assay development given the limitations in designing recognizable heterologous haptens.

Analysis of Ozonated PWR by Competitive Inhibition Haptenated Enzyme ELISA. PWR samples were treated with ozone until the s-atrazine herbicides were no longer detected by HPLC at a detection limit of approximately 5 μM (1 ppm of atrazine). The concentrations of the atrazine ozonation products CAAT, CDAT, CEAT, and CIAT, which are cross-reactive toward PAb 1 and PAb 5, were determined, and these results are presented in Table 6. CEAT, which is a significant ELISA cross-reactant, was not detected in the treated PWR samples. This was not surprising since CEET (simazine) was present in only two of the samples (BE1B and BE2M) and represented less than 10% of the total s-triazine herbicides in these samples (Muldoon et al., 1993). In addition, CEAT does not accumulate during the ozonation of other s-triazines to CAAT (Hapeman, 1993). To obtain a wider concentration range for analysis, each sample was spiked to an additional 1 and 2 mM CAAT, resulting in a total of 21 samples. The samples were analyzed by HPLC and the standard competitive inhibition haptenated enzyme ELISA. The results from the analysis using PAb 1 are shown in Figure 5. Geometric mean regression data of the amount found by ELISA on the amount found by HPLC for PAb 1 are given in Figure 5. For PAb 5, the slope was 1.36 with a standard error of 0.06. The y-intercept was −226.73. The correlation coefficient was 0.98. The slopes for geometric mean regression indicate that the ELISAs gave higher estimates of cross-reactive analytes in the samples than those found by the HPLC method. This can be due to the presence of unknown materials in the ozonated mixture which cross-react with the ELISAs. Cyanazine was present in the untreated sample and may produce an as yet unidentified cross-reactive product. However, the HPLC method showed high background absorbance readings in the region where the analytes CDAT, CEAT, and CIAT elute, and this may have resulted

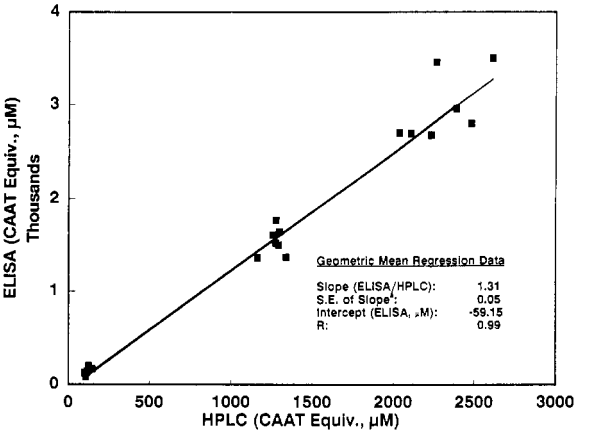


Figure 5. Comparison of results from analysis of ozonated pesticide waste and rinsate (PWR) by HPLC and competitive inhibition haptenated enzyme ELISA using PAb 1. PWR samples were ozonated and then spiked to either 1 or 2 mM CAAT above the original concentration. Unspiked and spiked samples were diluted 1:2 in acetonitrile and analyzed by HPLC and further diluted in buffer and assayed by the standard competitive inhibition haptenated enzyme ELISA. Mixtures of sample and 1:500 dilution of CAHeT–AP were incubated for 30 min on an antibody-coated plate followed by 60 min of substrate development time. S.E., standard error approximation from least-squares estimates.

in an underestimation of CDAT, CEAT, and CIAT. This would also explain the negative intercept values reported here. The very high correlation coefficients for the regressions indicate that the ELISAs were precise for CAAT quantitation in ozonated PWR.

CONCLUSIONS

A diamino-s-triazine hapten was synthesized and an immunogenic hapten–protein conjugate made for the purpose of eliciting antibodies which specifically bound the chlorodiamino-s-triazine metabolite (CAAT) over the parent s-triazine herbicides and monodealkylated metabolites. For the purpose of developing a heterologous ELISA format, haptens were synthesized that differed from the immunizing hapten in bridging group structure and ring substitution. The haptens were synthesized using adaptations of procedures previously reported for the synthesis of related structures (Goodrow et al., 1990).

The immunizing hapten SPrAAT was effective for the production of polyclonal antibodies that could be used in both indirect ELISA and haptenated enzyme ELISA formats for the detection of CAAT. Two antibodies were used for the design of a haptenated enzyme ELISA. This format offers several advantages over the indirect ELISA such as conservation of antibody, increased sensitivity, and decreased time of analysis (Jung et al., 1989). In addition, this ELISA format can be more readily adapted to tube-based assays for analysis in the field (Bushway et al., 1988). These haptenated enzyme ELISAs were characterized using a panel of 23 s-triazines. The assays were shown to be highly selective for CAAT and the monodealkylated metabolite CEAT among the environmentally relevant analytes. The IC_{50} values for the standard competitive inhibition haptenated enzyme protocol were in the low micromolar range and could be improved by using lower amounts of tracer with longer enzyme substrate incubation times. This suggested that some improvement in sensitivity may be made with the use of an enzyme tracer with a higher substrate turnover rate. We are currently developing rabbit polyclonal antibodies using the same immunogen. This should provide much more

antibody for future use and improve the sensitivity of the assay as these have been considered to be generally of higher affinities than mouse antibodies (Cheung et al., 1988).

The standard haptenated enzyme format was shown to be effective in detecting the target analyte in ozonated pesticide waste samples. This is particularly interesting to our group as it will allow for remediation monitoring by ELISA. The assay can be used in combination with an ELISA developed for mixed *s*-triazine waste analysis (Muldoon et al., 1993) for monitoring the complete disposal process of *s*-triazine-contaminated materials. The assay should be useful in applications where an extremely sensitive ELISA may not be required or preferred. In situations where analyte concentrations are relatively high, such as waste disposal and remediation monitoring (Seiber, 1987), large sample dilution may be an important source of analytical error in ELISA. Solid-phase extraction methodology is also being developed for coupling with the ELISA for the purpose of separating CAAT from the cross-reactant CEAT and for increasing the sensitivity of the method through concentration.

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