Development of Improved Immunoassay and HPLC Methods for the Analysis of Chlorodiamino-*s*-triazine in Environmental Samples†

Pedro L. Del Valle,‡ Mark T. Muldoon,§ Jeffrey S. Karns,⊥ Judd O. Nelson,‡ and Walter W. Mulbry*,⊥

Department of Entomology, University of Maryland, College Park, Maryland 20742, and Soil Microbial System Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Beltsville, Maryland 20705

An enzyme-linked immunosorbent assay (ELISA) for the analysis of chlorodiamino-*s*-triazine (CAAT) was developed using specific rabbit polyclonal antibodies. The assay was sensitive in the submicromolar range. The method was more sensitive and specific than previously reported mouse polyclonal antibody-based ELISAs. A competitive inhibition haptenated enzyme ELISA was optimized and validated which showed that the method was accurate, precise, and reproducible. A new HPLC method was developed to resolve polar *s*-triazine metabolites with improvements in run time and solvent use. The ELISA method was more sensitive than the HPLC method for detecting CAAT in soil and ground water samples. Solid-phase extraction was evaluated in an attempt to further increase the sensitivity of the ELISA method. However, nonspecific interferences were observed from various types of strong cation exchange columns from different manufacturers. This made it difficult to couple a solid-phase extraction technique with the ELISA.

Keywords: *Triazine; atrazine; herbicide; ELISA; HPLC; ground water; solid phase extraction*

INTRODUCTION

The *s*-triazine herbicides are among the most widely used pesticides. Atrazine, simazine, and propazine as well as the mono-N-dealkylated metabolites have been detected in ground water (EPA, 1990; Isensee et al., 1990; Adams and Thurman, 1991). Further N-dealkylation produces the intermediate chlorodiamino-*s*-triazine (CAAT) (Bekhi and Khan, 1986; Wichman and Byrnes, 1975; Dauterman and Mueke, 1974). To date, few data have been reported on the detection of CAAT in environmental samples. This is due, in part, because current chromatographic methods for the analysis of the polar *s*-triazine metabolites, such as cyanuric acid, ammelide, ammeline, and CAAT, are limited. These compounds are nonvolatile and highly water soluble (usually in acid or base) and are inefficiently recovered from environmental matrices (Nash, 1990). CAAT is particularly important because it may be regulated as a chlorinated metabolite of some *s*-triazine herbicides. Therefore, there is a need for improved analytical methods that can detect polar metabolites of *s*-triazine herbicides.

Enzyme-linked immunosorbent assays (ELISA) have been developed for the mono-N-dealkylated metabolites (deethyl- and deisopropylatrazine) and applied to environmental samples (Wittmann and Hock, 1991; Lucas et al., 1995). An ELISA using mouse polyclonal antibodies was reported for the analysis of CAAT (Muldoon et al., 1994). The assay was sensitive in the low micromole range, and it was used to detect CAAT in samples from a pesticide waste treatment process where relatively high concentrations were encountered (Muldoon and Nelson, 1994a). However, the usefulness of the mouse polyclonal-based ELISA for residue analysis in environmental samples was limited by the assay's sensitivity.

Our current objective was to improve ELISA methodology for the analysis of CAAT in environmental samples. Rabbit antibodies have been considered to be generally of higher affinity than mouse antibodies (Cheung et al., 1988), and we decided to pursue this route to generate better antibodies for use in an ELISA. In this research, an antigen used to produce specific antibodies in mice (Muldoon et al., 1994) was used for the production of rabbit polyclonal antibodies. A competitive inhibition haptenated enzyme ELISA was developed in which the rabbit specific antibodies were immobilized directly to the wells of microtiter plates. The previous method used anti-immunoglobulin antibodies for trapping the specific antibodies to the plate. This new assay demonstrated higher sensitivity than the previously reported assay for detecting CAAT in environmental samples.

A secondary objective of this work was to develop a high-performance liquid chromatography (HPLC) method that could resolve CAAT and related *s*-triazine compounds with improvements in sensitivity, run time, and solvent waste from previously reported techniques. An isocratic method using a phenyl matrix and phosphoric acid as elutant was developed and used to measure CAAT in environmental samples.

MATERIALS AND METHODS

Chemicals. CAAT was purchased from Aldrich Chemical Co., Milwaukee, WI. Bovine serum albumin (BSA), goat anti-rabbit IgG (H+L), goat anti-rabbit IgG (H+L) conjugated to alkaline phosphatase type VII-NT (AP), and enzyme substrate (*p*-nitrophenyl phospahte) were purchased from Sigma Chemical Co., St. Louis, MO. The syntheses of 3-(4,6-diamino-1,3,5-

* Author to whom correspondence should be addressed [fax (301) 504-7976; e-mail wmulbry@asrr.ars.usda.gov].

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‡ University of Maryland.

§ Present address: Food Animal Protection Research Laboratory, USDA-ARS, College Station, TX 77845.

⊥ U.S. Department of Agriculture.

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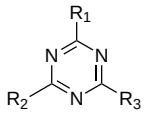
Table 1. Structure and Nomenclature of *s*-Triazines



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

[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). Data from Muldoon et al. (1994).

triazin-2-yl)thiopropanoic acid, potassium salt (SPrAAT), 4-(4,6-diamino-1,3,5-triazin-2-yl)thiobenzoic acid, potassium salt (SBeAAT), and 6-(4-chloro-6-amino-1,3,5-triazin-2-yl)amino-hexanoic acid (CAHeT) were previously described (Muldoon et al., 1994). Compounds CDAT, CDET, CDIT, and CDDT (Table 1) were synthesized as previously described (Hapeman-Somich et al., 1992). Other *s*-triazines were gifts from Ciba Plant Protection (Greensboro, NC).

Buffers. The compositions of the various buffers used have been described in detail elsewhere (Karu et al., 1991). 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).

Equipment. HPLC measurements were performed using a Waters Model 712 WISP sample injector, two Waters Model 510 pumps, a Waters Model 996 photodiode array detector, and a Gateway 2000 PC with Waters Millennium 2010 software. The column was a Waters 8 mm × 10 cm Phenyl NovaPak cartridge (4 μm) in a radial compression module. The solvent system was phosphoric acid in water (pH 2) at a flow rate of 1.5 mL/min.

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−Protein Conjugation and Antibody Production. Hapten−protein conjugation has been described in detail by Muldoon et al. (1994). Rabbit anti-SPrAAT-KLH polyclonal antibodies (primary antibodies) were prepared by Larry Tamarkin of Cytimmune Sciences Inc., College Park, MD. A specific pathogen-free rabbit was immunized (sc) with 500 μg of SPrAAT-KLH (antigen) in complete Freund's adjuvant (CFA) containing immune enhancer (Cytimmune Sciences). Two weeks later the rabbit was boosted (sc) with 250 μg of antigen in incomplete Freund's adjuvant (IFA) containing immune enhancer. At 6 weeks, the rabbit was again boosted and then bled 7 days after boosting. This was repeated at 1 month intervals. Five boosts and bleeds were made prior to exsanguination.

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 that produced 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 blocked with 100 μL/well of 0.5 mg/mL BSA in PBSTA for 60 min at room temperature and then frozen with the liquid remaining in the wells. When needed, the plates were brought to room temperature and 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; 50 μL aliquots of each mixture were applied to replicate wells of the BSA−hapten coated plate. This was incubated for 30 min at room temperature and then washed. Goat anti-rabbit IgG antibody conjugated to alkaline phosphatase diluted 1:500 in PBSTA was applied to the plate (100 μL/well), incubated for 30 min at room temperature, and washed. Enzyme substrate was added, and the plate OD measurement (405−650 nm) was made at 30 min.

Competitive Inhibition Haptenated Enzyme ELISA. Microtiter plates were coated with 100 μL of primary antibody diluted 1:800 in coating buffer, incubated for 18 h at 4 °C, and then washed. One hundred microliters of 0.5 mg/mL BSA in PBSTA was applied to the plate, incubated for 60 min at room temperature, and frozen with the liquid remaining in the wells. When needed, the plate was thawed and washed. One hundred twenty microliters of sample (+ *s*-triazine) and 120 μL of haptenated enzyme diluted 1:200 were mixed in a separate uncoated well, and 50 μL aliquots were applied to quadruplicate wells of the antibody-coated plate. This was

incubated for 60 min and then washed. Enzyme substrate was added, and the plate OD measurements were made at 60 min.

Antibody/Haptenated Enzyme Recognition and Development of the Competitive Inhibition Haptenated Enzyme ELISA. Antibodies from different bleeding days which were most sensitive and selective for CAAT using the indirect ELISA were tested for recognition of the haptenated-enzyme conjugate CAHeT−AP. This heterologous combination tested positive and was further evaluated.

Criteria for Assay Validation. Assay validation was performed as described in detail by Del Valle and Nelson (1994). Briefly, sensitivity was interpreted by the limit of quantification (LOQ) and the limit of detection (LOD). The former was defined by the working range of the standard curve at the lower limit. The LOD was defined as the lowest value outside the range of 2 standard deviations over background. Intraassay precision was set to a maximum coefficient of variation (CV) of 0.1. Quality control for ELISA was determined by the interassay variation measured by variabilities of the following standard curve working parameters mean values: IC_{50} (inhibitor concentration that produces 50% inhibition of the zero dose control), slope, and lower limit and upper limits of the working range. Accuracy was evaluated using tests for parallelism, recoveries, and specificity. The test of parallelism determines whether the assay value is independent of the sample size. Percent recoveries were calculated as the amount found minus the amount in the blank divided by the amount added multiplied by 100. The ELISA was characterized for cross-reactivity toward 24 *s*-triazine derivatives by assaying a zero-dose control and 10 concentrations of either *s*-triazine. Analyses were made in quadruplicate. IC_{50} values were calculated for each analyte.

Soil and Ground Water Samples. Soil samples used in this study were taken from a field site at Beltsville, MD, in 1993 and stored at −20 °C until needed. This site is being used in a long-term study of the effects of conventional and no-till agricultural practices on the movement of pesticides into shallow ground water and of atrazine distribution in the soil profile (Isensee et al., 1990; Sadeghi and Isensee, 1992, 1994). Samples were thawed, a subsample (25 g) was extracted with 100 mL of methanol/water 4:1 (v/v) on a wrist-action shaker for 1 h, and the extract was filtered through a glass-fiber filter paper. The methanolic extract was concentrated *in vacuo*. The residual water was mixed with sodium phosphate buffer to a final volume of 5 mL. Aliquots were assayed by ELISA and HPLC, and the results were analyzed by geometric mean regression (GMR) as described in Del Valle and Nelson (1994).

Ground water (GW) samples were taken in October 1994 and January 1995 from the same field site described above. Sodium azide was added to a final concentration of 0.02% in 1 L of water to inhibit any biological activity. Samples were analyzed directly by ELISA and HPLC. In addition, aliquots (25 mL) from four different samples were fortified at three different levels with CAAT (5.0, 1.0, and 0.05 ppm) and analyzed by HPLC and ELISA.

Solid-Phase Extraction (SPE) of GW Samples. Strong cation exchange (SCX) columns (0.5 g) from different manufacturers were evaluated as a CAAT concentration technique to lower the ELISA method LOD. The columns tested were as follows: BakerBond-spe sulfonic acid (J. T. Baker, Phillipsburg, NJ); SCX benzenesulfonic acid, propanesulfonic acid (PRS), and carboxylic acid (CBA) from Phenomenex (Torrance, CA); SCX sulfonic acid column (Whatman, Hillsboro, OR); and SCX column resin base (Burdick & Jackson, Muskegon, MI). C_{18} Environmental-Plus Sep-Pak cartridges (Waters, Inc., Medford, MA) were considered as control columns since they were not SCX, they have been shown to give poor recoveries with CAAT (Muldoon, 1993), and they have been used with other ELISA systems (Thurman et al., 1990; Bushway et al., 1991; Del Valle and Nelson, 1994). Various column washing and conditioning procedures using organic solvents and/or buffers were performed according to the manufacturers' recommendations. Optimization in SPE protocol development considered flow rate, loading capacity, and solvent and sample pH as described in Bouvier (1994). Sodium phosphate buffer (50 mM, pH 8 or 10) was used for analyte elution from the SPE column since this was compatible with the ELISA.

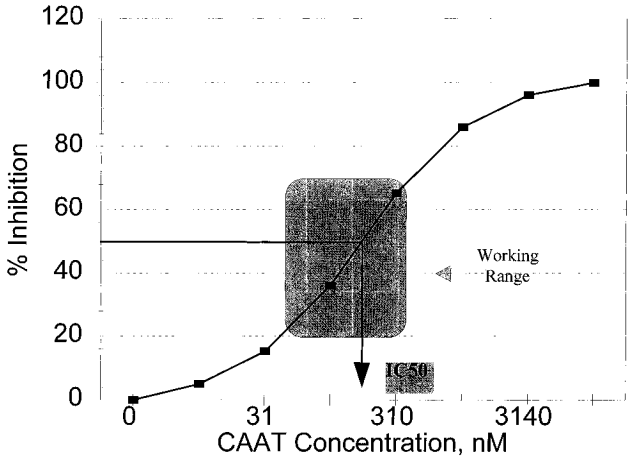


Figure 1. CAAT competitive inhibition curve using the haptenated enzyme ELISA with ARI108 rabbit polyclonal antibodies and haptenated enzyme CAHeT-AP. Absorbance measurements were normalized by conversion to percent inhibition (%*I*) values according to the formula $B/B_0 = [(A_{sample} - A_{blank})/(A_{zero\ dose} - A_{blank})]$; $\%I = (1 - B/B_0) \times 100$. CAAT concentrations are in nanomolar.

RESULTS AND DISCUSSION

Antibody and BSA−Hapten Screening by Indirect ELISA. Rabbit SPrAAT-KLH antisera recognized homologous BSA−hapten conjugates SPrAAT and SBeAAT as well as the heterologous conjugate CAHeT, which is very different in *s*-triazine ring substitution (Table 1). Competitive inhibition ELISAs that used homologous conjugates showed high IC_{50} values (>10 mM) and were not further pursued. The heterologous ELISA was the most sensitive for CAAT (IC_{50} = 67.9 μM), and this format was used to titrate the antisera from the different bleeding days. Antisera from two bleeding days from one rabbit (10-8-1 and 10-8-2) showed the highest titers and were combined and designated ARI108.

Development of the Competitive Inhibition Haptenated Enzyme ELISA. Previous studies with mouse anti-SPrAAT−KLH polyclonal antibodies showed that use of the heterologous tracer CAHeT-AP, as opposed to homologous ones, resulted in more sensitive assays (Muldoon et al., 1994). This was consistent with many other competitive ELISAs for low molecular weight haptens (Wie and Hammock, 1984; Harrison et al., 1991). Therefore, CAHeT-AP was chosen for further characterization of ARI108. Initial studies showed that higher antibody titers (less amount of antibody) were obtained when plates were coated directly with primary antibody in coating buffer, as opposed to using an intermediate anti-immunoglobulin antibody. This was not the case with the mouse polyclonal antibodies developed previously, which had prohibitively low titers (high amount of antibody) when coated directly to the plate (Muldoon et al., 1994). In the latter case, plates were first coated with anti-iimmunoglobulin antibodies to "trap" the primary antibodies onto the surface. Higher serum specific antibody concentration, higher affinity, or higher nonspecific binding of the rabbit antibodies could have accounted for these differences which allowed for the use of a direct coating method. Stored plates were stable for up to 1 month.

Validation of Competitive Inhibition Haptenated Enzyme ELISA. *Sensitivity.* Figure 1 shows

Table 2. Precision of the Haptenated Enzyme ELISA Standard Curve Working Parameters

working parameter	mean[a] (μM)	SD (μM)	CV (%)
IC_{50}	0.165	0.014	8.4
slope[b]	0.998	0.037	3.7
lower limit (LL)[c]	0.072	0.007	9.8
upper limit (UL)[d]	0.675	0.055	8.1

[a] Mean value for 74 experiments. An experiment consisted of 1 ELISA plate which contained 1 standard curve and 15 sample determinations in quadruplicate. [b] Slope values are in μM^{-1}. [c] 70% of the zero concentration OD measurements. [d] 20% of the zero concentration OD measurements.

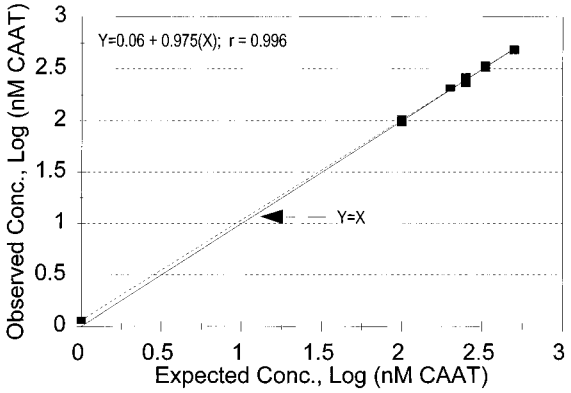


Figure 2. Test for accuracy of the ARI108 ELISA: parallelism. Known amounts of CAAT standard were added to 11 samples from different matrices (ozonated pesticide waste, ground water, and soil samples), and dilutions that fell in the working range were analyzed using the haptenated enzyme ELISA.

a typical CAAT competitive inhibition curve using polyclonal antisera ARI108 with CAHeT-AP in the competitive haptenated enzyme ELISA. Curve parameters are presented in Table 2 and were obtained from 74 experiments performed over an 8-month period. The assay IC_{50} value was 23.9 ppb, which is 27 and 35 times more sensitive than the values reported previously using PAb 1 and PAb 5, respectively (Muldoon et al., 1994). The working range for the assay averaged 10.4−97.9 ppb, and the limit of detection averaged 1 ppb (6.9 nM). Samples with a concentration of CAAT equivalents higher than 100 ppb (675 nM) must be diluted to be quantitated, and samples with concentrations less than 10 ppb (71.7 nM) must be concentrated to be accurately quantitated.

Precision and Reproducibility. The competitive inhibition haptenated enzyme ELISA was very precise and reproducible. Variation of the working parameters is shown in Table 2. The coefficients of variation for all parameters were below the limit of 0.1, which indicated an interassay variation less than 10%. With regard to precision, CV values between wells at all concentrations used to fit the 74 standard curves were also below 0.1. In addition, few of the total number of samples determined by ELISA had to be repeated due to high CV values.

Accuracy. 1. Parallelism. Known amounts of CAAT were added to 11 samples from different matrices (ozonated pesticide waste and rinsate, ground water, and soil samples), and dilutions that fell in the working range were analyzed using the competitive inhibition haptenated enzyme ELISA. Figure 2 shows a plot of the log of the expected value versus the log of the observed value. The slope of this plot was not significantly different from 1.0 ($P > 0.2$; df = 9), and the intercept was not significantly different from 0 ($P = 0.1$; df = 9). This test suggested that the true analyte concentration in any sample dilution would be determined accurately by using the ELISA.

Table 3. Characterization of the Haptenated Enzyme ELISA with Selected *s*-Triazines[a]

	% reactivity (CAAT = 100%)	
inhibitor	ARI108	PAb 1[b]
CAAT (IC_{50}, nM)	100.0 (160)	100.0 (5780)
SPrAAT[c]	295.0	140.5
SBeAAT[c]	921.0	242.1
CAHeT	0.08	7.7
SAAT	24.7	35.8
CEAT	38.4	92.8
CIAT	3.8	2.2
CIET	0.06	0.2 (E)[d]
CEET	0.02	0.2 (E)
CENT	0.02	0.2 (E)
CDAT	2.1	2.3
CDET	2.5	1.1
CDIT	0.3	1.1
CDDT	0.2	1.1
OAAT	0.1	0.8
OOAT	0.03 (E)	0.1 (E)
OOOT	0.03 (E)	NI[e]
AAAT	0.2	1.0
OIAT	<0.02	<0.06
OOET	0.05 (E)	0.6
CyPrAAT	2.1	1.0
HAAT	0.03 (E)	1.1
OIET	0.2	ND[f]
COAT	<0.02	ND

[a] Mixture of samples and 1:200 dilution of CAHeT-AP were incubated for 60 min on an ARI108 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 calculating the value using the four parameter logistic curve function. Analyses were made in quadruplicate. [b] Data from Muldoon et al. (1994). [c] Carboxylate anion equivalent. [d] E, highest concentration of inhibitor (10 mM) did not completely inhibit antibody binding; IC_{50} values were extrapolated from an undefined curve. [e] NI, no inhibition at 10 mM. [f] ND, not determined. The convention for naming triazines is from Cook (1987) and modified by Hapeman-Somich et al. (1992).

2. Recoveries. Samples from different matrices were fortified at three different levels and dilutions were determined by ELISA. Recoveries ranged from 92.2 to 106.9. A *t*-test indicated that recoveries were not significantly different from 100% ($P > 0.4$; df = 10). This test suggested that a known increment of the target analyte added to a sample can be measured quantitatively by the assay.

3. Specificity. Characterization results of ARI108 using CAHeT-AP and 24 *s*-triazines derivatives are presented in Table 3. Corresponding results for the mouse polyclonal antibody PAb 1 (Muldoon et al., 1994) were included for reference. The convention for naming triazines is from Cook (1987) and modified by Hapeman-Somich et al. (1992). The IC_{50} values were calculated in nanomolar. ARI108 showed highly significant recognition of CAAT. The most important improvement in specificity was diminished recognition of CEAT in comparison to the previous ELISAs. Like PAb 1, substitution of the bridging group with either hydroxyl (OAAT) or amino (AAAT) (Table 1) resulted in a near loss of recognition. In addition, the antibodies recognized the thiol SAAT, which was not surprising since it was very similiar to the immunizing hapten SPrAAT (Table 1). Standard curves for all 24 environmentally relevant structurally related analytes tested in this study were parallel. This is important to note because if the standard curves for analyte and the cross-reactant are not parallel, no single numerical value can ad-

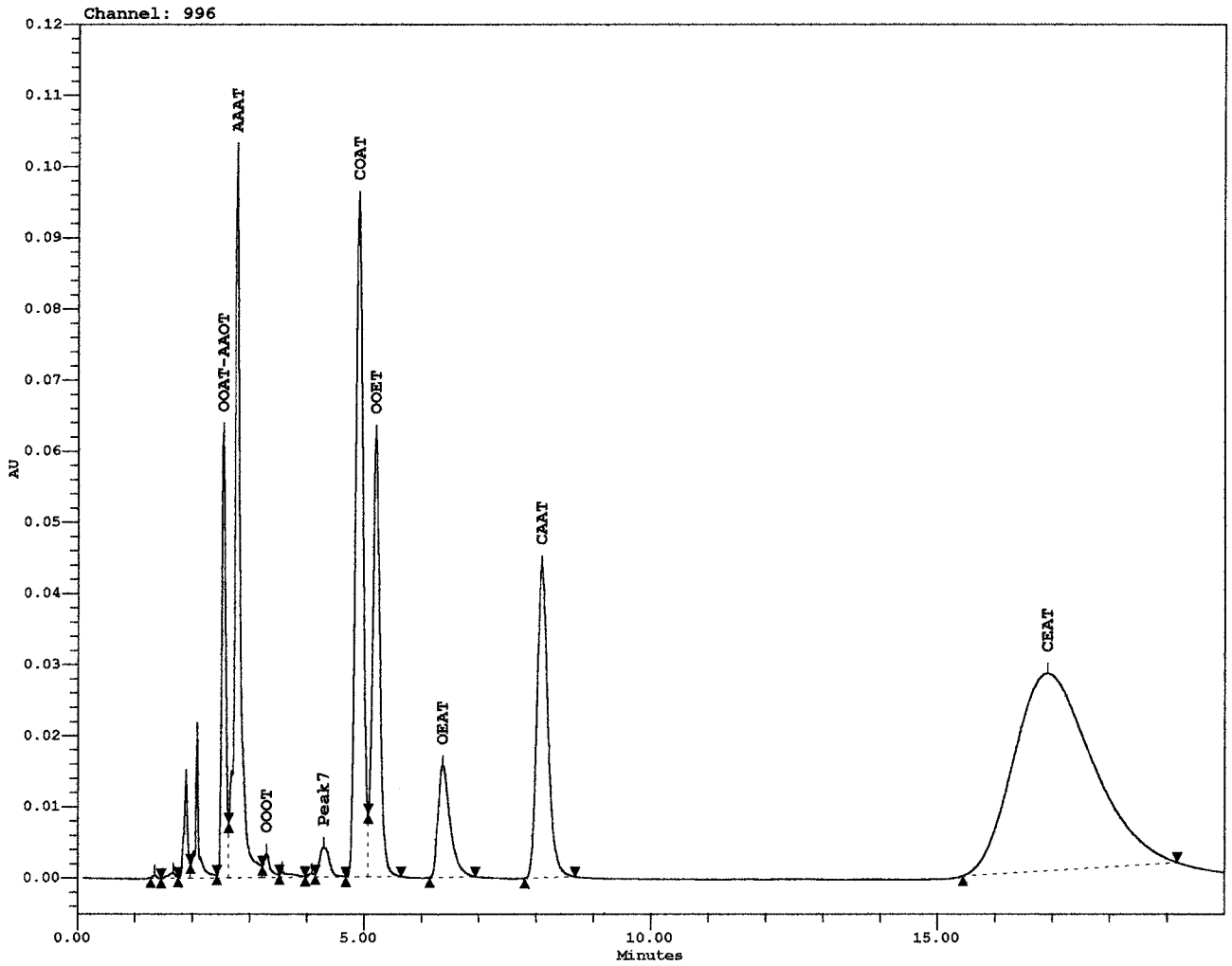


Figure 3. Chromatogram for a nine-compound standard *s*-triazine metabolites mixture. Compound names are described in the text.

equately represent cross-reactivity (Abdul-Ahad and Gosling, 1994).

HPLC Analysis. A new method was developed to resolve polar *s*-triazine metabolites with improvements in run time, solvent waste, and elimination of the use of ion pairing reagents and solvent gradients (Hapeman-Somich et al., 1992; Muldoon and Nelson, 1994a,b). In addition, this method was compatible with buffers used with the ELISA. Figure 3 shows a chromatographic separation of nine polar *s*-triazine metabolites. The separation was nearly baseline for all compounds. The retention time of CAAT was 8.1 min. The response was linear in the range from 10 to 0.05 ppm. HPLC results were precise with a CV < 0.1. However, a test for accuracy performed during sample determination runs showed a 20−40% overestimation of standards at 0.01 and 0.05 ppm. Accordingly, HPLC LOQ was considered at 0.5 ppm and the HPLC LOD was considered at 0.05 ppm.

The use of phosphoric acid (pH 2) allowed the separation of melamine (AAAT), cyanuric acid (OOOT), chlorohydroxyamino-*s*-triazine (COAT), *N*-ethylammelide (OOET), *N*-ethylammeline (OEAT), CAAT, and chloroethylamino-*s*-triazine (CEAT). However, ammeline (OAAT) and ammelide (OOAT) could not be separated (Figure 3). Separation of OAAT and OOAT was not important in this study since these two compounds have very low cross-reactivity with the primary antibody and they were not present in the samples analyzed. CEAT is the most important cross-reactant species with ARI108. The retention time of CEAT was 16.9 min, making the run time much longer than desired (20 min). The advantages of this method are the use of a nonorganic solvent and the separation of CAAT from CEAT. If CEAT is not the compound of interest, the run time may be reduced in half. However, it would be necessary to run a gradient with an organic solvent to resolve triazines such atrazine (CIET), simazine (CEET), and cyanazine (CENT) (Hapeman-Somich et al., 1992).

Soil Analysis. Six soil sample extracts were analyzed by ELISA and HPLC. Concentration of the methanolic extract *in vacuo* should retain water-soluble compounds extracted from the soil in the extract. When the residual water (1 mL) was applied to a SPE column (C_{18}) for the purpose of cleaning up the sample, water-soluble compounds were frequently washed away (Del Valle and Nelson, 1994). However, dilution of the residual water with sodium phosphate buffer provided a suitable matrix for detection of the analytes by ELISA. GMR data of the amount found by the ELISA method on the amount found by the HPLC method are shown in Figure 4. The slope of the GMR indicated the presence in ELISA of 12% proportional error, i.e., a small overestimation of the analyte concentration in the samples. This may be due to errors involving the accuracy of the HPLC method. The bias of the ELISA results with respect to the HPLC result was 0.02 μM, and the *Y*-intercept suggested a constant error of 0.04

Table 4. Results from ELISA Analysis of Blanks Obtained with Different Types of Strong Cation Exchange Columns (SCX) Using Separate Solid Phase Extraction Protocols (SPE)

SPE protocols				column types				
no.	column wash[a]	column condition	sample elution[b]	BakerBond	Whatman ELISA	Phenomenex[c] response	B&Jackson	C_{18} Env+
1		10 mL of MeOH 10 mL of water	50 mM sodium buffer	+	+	+	+	−
2		20 mL of MeOH 10 mL of water	50 mM sodium buffer	+	+	+	+	−
3	10 mL of buffer 20 mL of water	10 mL of MeOH 10 mL of water	50 mM sodium buffer	+	+	+	+	−
4	20 mL of buffer 30 mL of water	10 mL of MeOH 10 mL of water	50 mM sodium buffer	+	+	+	+	−
5	40 mL of buffer 40 mL of water	10 mL of MeOH 10 mL of water	50 mM sodium buffer	+	−	NP	−	−
6	100 mL of water	10 mL MeOH 10 mL of water	50 mM sodium buffer	+	+	NP	NP	NP

[a] Sodium phosphate buffer, 200 mM (pH 8). [b] 10 mL of didistilled water was passed through the column to simulate sample loading. Sodium buffer (pH 8 or 10). [c] Includes SCX benzenesulfonic acid, propanesulfonic acid, and carboxylic acid columns; +, false positive reaction; −, no reaction; NP, not performed.

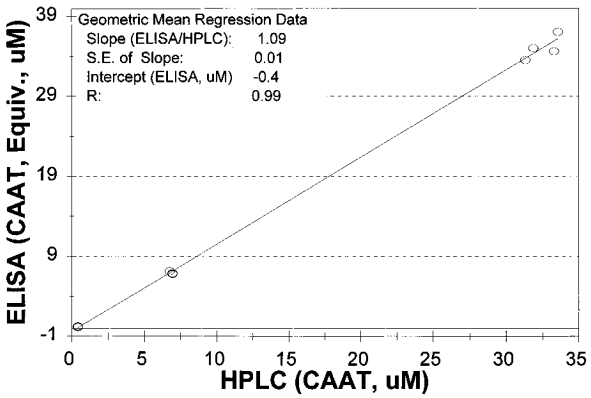


Figure 4. Comparison of ELISA and HPLC results from analysis of soil samples. Twenty five grams of soil was extracted for 1 h with 100 mL of methanol/water 4:1 (v/v), filtered, and concentrated *in vacuo*. The residual water (1 mL) was diluted with 50 mM sodium phosphate buffer (pH 8) to a final volume of 5 mL.

μM between the two methods. Both bias and the *Y*-intercept reflect systematic errors in concentration units. The correlation coefficient indicated there was good agreement between soil sample determinations from both methods.

SPE for GW Samples. Preliminary work using radiolabeled CAAT with the BakerBond-spe sulfonic acid and Whatman SCX columns indicated recoveries higher than 90% (Muldoon, 1993). GW samples taken in 1994 were loaded on BakerBond columns after being conditioned with 10 mL of methanol followed by 10 mL of distilled water. Comparison of the haptenated enzyme ELISA determinations of GW samples before and after passage through the BakerBond SCX column showed the presence of false positives in both samples and the 10 mL sodium phosphate elution buffer. An unidentified substance apparently leached from the columns, producing a false positive response with the ELISA. After this was observed, various columns were conditioned as described and eluted with sodium phosphate buffer (50 mM). Table 4 presents ELISA results using different protocols performed in an effort to remove the interferent. The resin-based Burdick & Jackson column was compatible with the use of pH 10 buffer. All other columns were silica-based, which are stable only at pH 8. A washout step with 40 mL of high ionic strength buffer (200 mM sodium phosphate buffer, pH 8) applied to the column before the condition step (protocol 5; Table 4) was sufficient to remove the leacher from all of the columns tested except the BakerBond. The SCX column resin base (Burdick & Jackson) was further evaluated; however, recoveries determined with the haptenated enzyme ELISA were poor (<30%). Blank elutants from the C_{18} Environmental-Plus Sep-Pak cartridges (Waters) (controls) gave no false positives. The sodium phosphate buffer (50 mM, pH 8 or 10) used for the elution step showed no effects on the ELISA; however, the high ionic strength sodium phosphate buffer (200 mM, pH 8) produced a reduction in OD of 20%.

The nonspecific interaction seen with the SCX columns was also observed using other haptenated enzyme ELISA systems. We observed similar results using monoclonal antibody AM7B2.1 in an ELISA for atrazine (Karu et al., 1990) and mouse polyclonal antibodies PAb 1 and PAb 5 in ELISAs for CAAT (data not shown). Although it is possible that the nonspecific effect is unique for *s*-triazine ELISAs, the atrazine ELISA and the CAAT ELISAs do not recognize common *s*-triazines and may be treated as very different assays.

Interfering compounds from silica base SPE C_{18} columns have been identified as alkanes, alkenes, plasticizers, and antioxidants (Junk et al., 1988). Thurman et al. (1990) also identified some of these compounds during an evaluation of commercially available ELISA kits and GC/MS for analysis of triazine herbicides in ground water, but no interferences with the ELISA system were reported.

Sample matrix interferences with the ELISA system may occur at two targets: the antibody itself or the haptenated enzyme. A main difference between the indirect ELISA and the haptenated enzyme ELISA formats is that samples being determined by the indirect ELISA are in contact only with the antibody. Blank elutants from all columns were determined using the indirect ELISA format and gave no false positives. Therefore, the interfering compounds affect the enzymatic reaction performed by the alkaline phosphatase enzyme bound to the hapten and used during the haptenated enzyme ELISA format. Thus, it may be possible to use SPE for sample concentration and the indirect ELISA for determination of the elutants.

Ground Water Analysis. Eight GW samples were analyzed directly using both HPLC and ELISA methods. CAAT was not detected either by ELISA (LOD = 1 ppb) or by HPLC (LOD = 50 ppb). This result was not surprising since the samples were taken during the winter, 5 months after agricultural activities had ended.

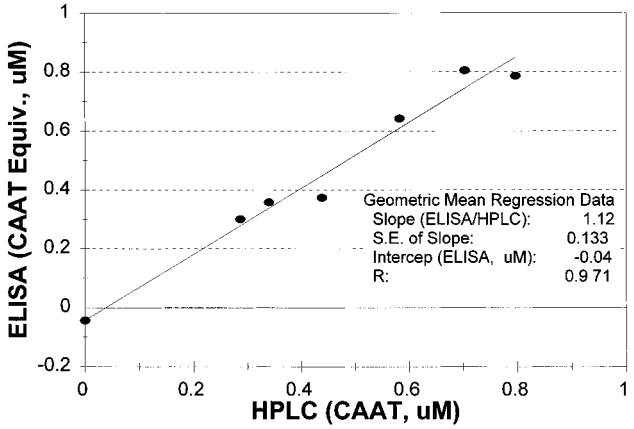


Figure 5. Comparison of ELISA and HPLC results from analysis of shallow ground water samples. Twenty-five milliliter aliquots were centrifuged to remove particulate material and fortified to 5.0, 1.0, and 0.05 ppm of CAAT. Dilutions were made for ELISA analysis.

In addition, CAAT is rapidly metabolized by soil microorganisms (Cook, 1987). To determine if CAAT reaches ground water, it would be important to use the assay during the regular agricultural season to monitor for the presence of CAAT in GW samples.

To verify the ability of the ELISA to detect the target analyte in the ground water matrix, four of the samples were fortified with CAAT at 5.0, 1.0, and 0.05 ppm. The results from the analysis using the ELISA are shown in Figure 5. GMR mean regression data indicated that ELISA had a 9% proportional error, a bias of 0.115 ppm, and a constant error of 0.058 ppm. The correlation coefficient was 0.99, which indicated a good agreement for the method.

The HPLC method overestimated analyte concentration at the lower fortification level by 30% as was expected according to the accuracy test results. ELISA underestimated the analyte at the lower fortification level due to matrix interferences. Ground water samples were centrifuged prior to fortification to remove particulate material that could interfere with the analysis. Since this was the only sample preparation step, it is possible that interferences observed in the analysis of GW samples using ELISA could have been due to the presence of metals or salts (present in GW and not in methanolic extracts) which interfered with the antibody directly or possibly associated with CAAT and rendered it unrecognizable by the antibody. A survey of potential interferents on ELISA was recently reported which showed that magnesium chloride can produce false negative results and possibly underestimate sample concentrations when present in the matrix (Muldoon and Nelson, 1994b).

Conclusions. The immunizing hapten SPrAAT was effective for the production of rabbit polyclonal antibodies that could be used in both an indirect ELISA and a competitive inhibition haptenated enzyme ELISA for CAAT. These rabbit polyclonal antibodies showed 35 times more sensitivity than mouse polyclonal antibodies developed with the same immunogen (Muldoon et al., 1994) as well as an improved specificity determined by cross-reactivity studies with 24 *s*-triazines. The IC_{50} values for the haptenated enzyme protocol were in the submicromolar range. The direct-coating ELISA format offers advantages over the indirect ELISA such as decreased time of analysis, increased sensitivity, and conservation of primary antibody.

Results from the validation study showed that the haptenated enzyme ELISA was sensitive, accurate, precise, and reproducible. The ELISA was shown to be as effective as and more sensitive than HPLC in detecting CAAT in soil and ground water samples. The assay should be useful in applications for which a sensitive ELISA may applicable. In situations of relatively high analyte concentrations, the mouse polyclonal antibody assay may be preferred.

The presence of an interferent associated with various strong cation exchange columns from different manufacturers made it difficult to couple solid-phase extraction methodology with the ELISA. The evidence that eluant from a C_{18} column did not affect the ELISA may indicate the need for special requirements during SCX column manufacture to prevent leaching of this interferent.

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