Quantification of Cyanazine in Water and Soil by a Magnetic Particle-Based ELISA

Timothy S. Lawruk,*,† Charles E. Lachman,† Scott W. Jourdan,† James R. Fleeker,‡ David P. Herzog,† and Fernando M. Rubio†

Ohmicron Corporation, 375 Pheasant Run, Newtown, Pennsylvania 18940, and Biochemistry Department, North Dakota State University, P.O. Box 5516, Fargo, North Dakota 58105

A competitive enzyme-linked immunosorbent assay (ELISA) for the quantitation of cyanazine in water and soil was developed using a novel magnetic particle solid phase. Cyanazine was covalently attached to a bovine serum albumin carrier, and the resulting herbicide–protein conjugate was used in rabbits to produce polyclonal antibodies specific for cyanazine. Specificity studies indicate that the antibody can distinguish cyanazine from other structurally similar triazine herbicides, including atrazine. This ELISA has a limit of detection of 0.035 parts per billion (ppb, ng/mL) in water and 3.5 ppb in soil. The standard curve allows quantitation up to 3.0 ppb in water and 300 ppb in soil. The ELISA compares favorably with GC measurements in the analysis of water samples ($r = 0.964$). Recoveries from fortified soils averaged 86% using a 24-h extraction with methanol/water (75:25 v/v).

INTRODUCTION

The interest in pesticide residue testing in the United States, Canada, and the European Community (EC) has increased due to concern over contamination of water, soil, and food. Because current testing methods are time-consuming and expensive and require specialized instrumentation such as liquid or gas chromatography, there is a growing need for faster, easier methods that allow screening of large numbers of samples, to identify those that require more extensive analysis. The emergence of enzyme immunoassays as a viable alternative to these traditional methods has shown them to be sensitive, reliable, cost-effective, and rapid (Van Emon and Lopez-Avila, 1992).

Cyanazine is a widely used selective systemic herbicide used for pre- and post-emergence control of most annual grasses and broad-leaved weeds in such crops as corn, soybeans, peas, field beans, onions, cotton, and cereals as well as fallow land and forestry. As a result of its usage, cyanazine residues may contaminate food, wells, and streams due to runoff, spills, and spraying. The U.S. EPA has classified cyanazine to be a systemic toxicant (U.S. EPA, 1984) and has set residue tolerances for cyanazine ranging from 20 to 200 ppb in or on raw agricultural commodities (U.S. EPA, 1988). In issuing a Lifetime Health Advisory at 10 ppb in drinking water, the U.S. EPA noted that cyanazine is a chloro-*s*-triazine that has a chemically analogous structure to atrazine, propazine, and simazine. These three analogs are classified as Group C oncogens, while cyanazine is classified in Group D, not classifiable, since inadequate animal evidence of carcinogenicity is available (U.S. EPA, 1989). Health and Welfare Canada has set an interim maximum acceptable concentration for drinking water at 10 ppb (Health and Welfare Canada, 1989). The European Community has set a maximum admissable concentration for cyanazine, as well as other pesticides, in drinking water at 0.1 ppb and at 0.5 ppb for the total of all pesticides (EC, 1980). A recent study (Roloff et al., 1992) on in vitro exposure of human lymphocytes to cyanazine concluded that cyanazine is clastogenic to human cells and could possibly be carcinogenic. In soil, the persistence of cyanazine could cause carry-over injury to sensitive succeeding crops such as oats or alfalfa (Libik and Romanoski, 1976), particularly in moderately acidic soils (Blumhorst and Weber, 1992).

The principles of enzyme-linked immunosorbent assay (ELISA) for pesticide residues have previously been described (Hammock and Mumma, 1980) and applied to triazine residue detection (Wittmann and Hock, 1989, 1991; Bushway et al., 1988; Schlaeppi et al., 1989; Dunbar et al., 1990; Goodrow et al., 1990). However, due to the lack of specificity of the antibody used, they have not been applied to the determination of cyanazine in environmental water and soil samples. In previously cited ELISAs, the solid phases employed are polystyrene wells, balls, or tubes on which antibody or hapten–protein conjugate is passively adsorbed. The desorption or leaching off of antibody or other proteins which have been passively adsorbed are major factors that adversely affect assay sensitivity and precision (Howell et al., 1981; Engvall, 1980; Lehtonen and Viljanen, 1980). Variability of wells within microtiter plates has been shown to be the greatest contributor to total assay imprecision (Harrison et al., 1989). Magnetic particle-based ELISAs have previously been described and applied to the detection of pesticide residues (Rubio et al., 1991; Itak et al., 1992; Lawruk et al., 1992). These ELISAs eliminate imprecision problems through the covalent coupling of antibody to the magnetic solid phase. The uniform dispersion of the particles throughout the reaction mixture allows for rapid reaction kinetics and precise addition of antibody. In the present work, we describe the development and evaluation of a competitive ELISA for the quantitation of cyanazine in groundwater and soil samples utilizing magnetic particles as a solid support and means of separation.

MATERIALS AND METHODS

Reagents. Amine-terminated superparamagnetic particles of approximately 1-μm diameter were obtained from Advanced Magnetics (Cambridge, MA). Horseradish peroxidase and glutaraldehyde were purchased from Sigma Chemical Co. (St. Louis, MO). Cyanazine–HRP conjugate is available from Ohmicron Corp. (Newtown, PA). Cyanazine and related compounds as well as nonrelated cross-reactants were purchased from Riedel-

† Ohmicron Corp.
‡ North Dakota State University.

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de-Haen (Hanover, Germany). Hydrogen peroxide and 3,3',5,5'-tetramethylbendizine (TMB) were obtained from Kirkegaard and Perry (Gaithersburg, MD). All other reagents were of reagent grade or chemically pure.

Apparatus. The spectrophotometric measurements were determined using the RPA-I photometric analyzer (Ohmicron). The detailed functions of this instrument have previously been described by Rubio et al. (1991). A two-piece magnetic separation rack (Ohmicron) is required consisting of a test tube holder which fits over a magnetic unit containing permanently positioned rare earth magnets. The two-piece design allows for a 60-tube immunoassay to be set up, incubated, and magnetically separated without removal of the tubes from the holders (Itak et al., 1992). Gilson P-200 (Rainin, Woburn, MA) and Eppendorf repeating pipets (Eppendorf, Hamburg, Germany) were used for dispensing liquids.

Antibody Generation. The cyanazine ligand, 3-*s*-[2-(ethylamino)-4-[(1-cyano-1-methylethyl)amino]-1,3,5-triazin-6-yl]-thiopropionic acid, was coupled to bovine serum albumin (BSA) according to the mixed-anhydride procedure of Erlanger et al. (1957). The immunogen solution was brought to room temperature and dialyzed against sodium borate (pH 8.7) with two changes of water and freeze-dried. Such an approach leaves free the ring and side chains, ensuring maximal sensitivity to these functional groups and minimal cross-reactivity with other triazine herbicides. The cyanazine immunogen was dissolved in sterile saline to a concentration of 4 mg/mL. This solution was emulsified with an equal volume of Freund's complete adjuvant. A total of 0.5 mL of this emulsion was injected in the hip muscle of three rabbits. After 20 and 45 days, the rabbits were boosted with 0.5 mL of the emulsion and repeated at 30-day intervals using Freund's incomplete adjuvant. Whole blood (30–50 mL) was obtained 10 days after each boost, allowed to coagulate, and centrifuged to generate the antiserum which was stored at −70 °C.

Antibody Coupling Procedure. Rabbit anti-cyanazine coupled magnetic particles were prepared by glutaraldehyde activation of the magnetic solid phase according to the method of Rubio et al. (1991).

Immunoassay Procedure. All water samples and diluted soil extracts were assayed by adding 100 μL of sample, 250 μL of conjugate, and 500 μL of anti-cyanazine magnetic particles to a test tube and incubating for 15 min at room temperature. The magnetic rack is used to magnetically separate the reaction mixture. After separation, the magnetic particles are washed twice with 1.0 mL of deionized water. The colored product was developed for 20 min at room temperature by the addition of 500 μL of hydrogen peroxide/TMB solution. The color reaction was stopped with 500 μL of 2 M sulfuric acid. The final concentrations of cyanazine for each sample were determined using the RPA-I photometric analyzer by determining the absorbance at 450 nm. The observed sample results were compared to a linear regression line using a log/logit standard curve prepared from calibrators containing 0, 0.1, 1.0, and 3.0 ppb of cyanazine. Samples greater than 3.0 ppb are diluted in the zero standard (0.025 M Tris/0.15 M NaCl/0.1% BSA preserved solution) for analysis. Sample concentrations are calculated using the appropriate dilution factor.

Water samples for method comparison were drinking water, surface water, and groundwaters from various locations throughout the United States and were analyzed as received. Samples for the spike recovery study were prepared by adding 0.5 ppb of cyanazine to 278 drinking water, surface water, and groundwaters collected from states throughout the United States including Florida, Idaho, Illinois, Minnesota, Pennsylvania, Rhode Island, Vermont, Washington, and Wisconsin. The recovery of each sample was determined by analyzing the samples before and after the addition of cyanazine and then subtracting the value of cyanazine present in the sample prior to spiking.

Soil Sample Analysis. Air-dried soils of known composition (Table I) were mixed for 2 h with cyanazine-spiked solutions prepared in water to yield soil concentrations of cyanazine from 20 to 200 ppb. Soils were then air-dried for 3 days and ground with a mortar and pestle. Ten grams of soil was extracted for 15 min–24 h by agitating in 20 mL of methanol/water (75:25 v/v). After settling for approximately 15 min, the extract supernatant is diluted 1:50 (20 μL in 980 μL) in the zero standard to eliminate solvent effects in the assay. The diluted soil extract is assayed as described above, and the results obtained are multiplied by the appropriate dilution factor to determine the soil cyanazine concentration (i.e., multiply by 100 for a 1:50 dilution to correct for the initial 1:2 dilution of soil with methanol/water).

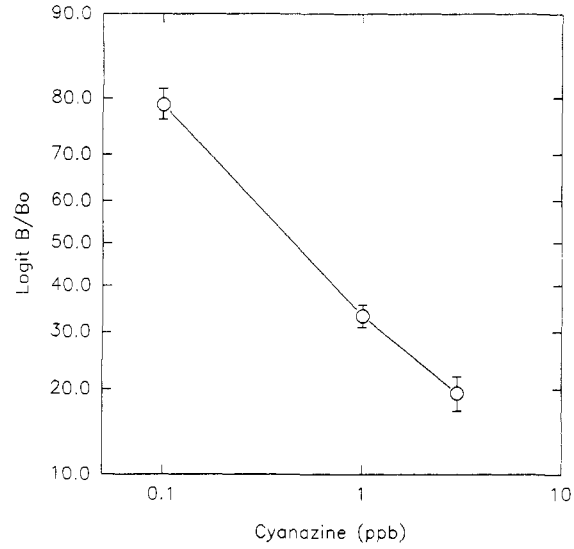


Figure 1. Dose–response curve for cyanazine. Each point represents the mean of 30 determinations. Vertical bars indicate ±2 SD about the mean.

Table I. Composition of Soils Fortified with Cyanazine

soil	% sand	% silt	% clay	% humus	pH
sassafras (New Jersey)	60	29	11	2	6.3
plano (Wisconsin)	38	48	26	5	6.1

RESULTS AND DISCUSSION

Dose–Response Curve and Sensitivity. Figure 1 illustrates the mean dose–response curve for the cyanazine calibrators that was collected over 30 runs. The results are linearly transformed using a log/logit curve fit. The error bars indicate the excellent reproducibility of the standard curve. The displacement at the 0.1 ppb level is significant (80% B/B_0). Immunoassay sensitivity is traditionally determined as the concentration corresponding to 90% B/B_0 (Midgley et al., 1969), where B/B_0 is the absorbance at 450 nm of a sample or standard divided by the absorbance of the zero standard. This ELISA's minimal detectable concentration was estimated to be 35 parts per trillion (ppt, pg/mL) using the 90% B/B_0 method. Sensitivity can also be defined as the mass equivalent of 2 or 3 times the standard deviation of the B_0 from its mean absorbance. The minimal detectable concentration is approximately 20 and 36 ppt, respectively, using this definition. This sensitivity exceeds the method detection limit reported by Nash (1990) of 0.1 ppb using gas chromatography with a nitrogen–phosphorus detector in combination with solid-phase C_{18} cartridge extraction and by the U.S. EPA high-performance liquid chromatography (HPLC) method applicable to the determination of cyanazine in water samples, Method 4, which has an estimated detection limit of 0.94 ppb (Munch et al., 1990).

Precision. A precision study in which groundwater samples, spiked with cyanazine at four concentrations, were each assayed five times in singlicate per assay on five different days is shown in Table II. The within- and between-day variation was determined by analysis of variance (ANOVA) (Bookbinder and Panosian, 1986) using

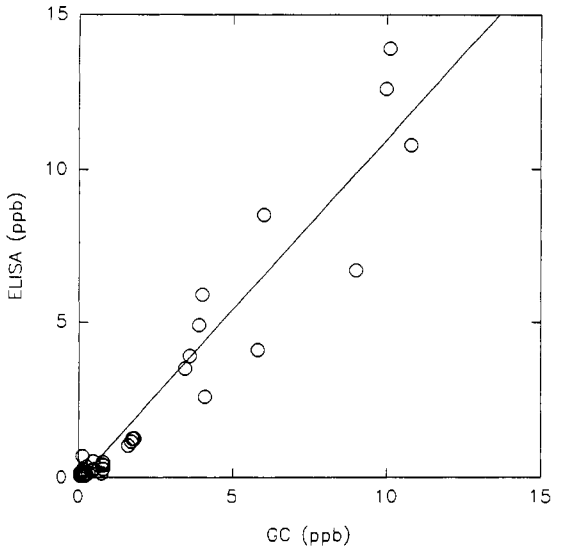


Figure 2. Correlation between cyanazine concentrations as determined by ELISA and GC methods. $n = 55$, $r = 0.964$, $y = 1.11x - 0.12$.

Table II. Precision of Cyanazine Measurement

	sample			
	1	2	3	4
replicates	5	5	5	5
days	5	5	5	5
N	25	25	25	25
mean, ppb	0.25	0.54	1.11	1.97
% CV (within assay)	14.6	9.2	8.7	8.1
% CV (between assay)	0.1	1.0	3.1	5.3

SAS software (SAS Institute, 1988). Coefficient of variation (% CV) within and between day were less than 15% and 6%, respectively.

Method Comparison. Comparison of results of 55 groundwater samples obtained by the present ELISA method (y) and an established GC method (x) are illustrated in Figure 2. The regression analysis yields a correlation of 0.964 and a slope of 1.11 between methods. For the GC method, the water samples were extracted with hexane/methylene chloride, dried with sodium sulfate, and concentrated in an evaporator. A flame photometric detector is utilized with this GC method based on U.S. EPA Method 608 (U.S. EPA, 1980). The 11% higher cyanazine results obtained by the ELISA are probably due to the loss of pesticide in the sample preparation in the GC method (U.S. EPA, 1992) which ranges from 78% to 100% recovery. The GC results were not corrected for procedural recoveries. Higher cyanazine values obtained by the ELISA could result from the cross-reactivity of the antibody with metabolites or other triazines, but this is unlikely given the high specificity of the antibody used.

Accuracy. In addition to the accurate recovery of cyanazine from buffer and distilled water (average recovery 107%), the accuracy of the assay was analyzed by adding known amounts of cyanazine to four water samples obtained locally. The samples included drinking water from a well, a municipal water source, a small creek, and the Delaware River. The accuracy was evaluated by analyzing the samples before and after the addition of cyanazine and subtracting the concentration of cyanazine before spiking. Table III summarizes the accuracy of the cyanazine ELISA. Added amounts of cyanazine were recovered correctly in all cases with an average assay recovery of 103%. The excellent recovery of spiked samples indicates that no sample matrix problems were present and the accuracy of the ELISA is linear across the range of the assay.

Table III. Accuracy of Cyanazine ELISA

amt of cyanazine added, ppb	cyanazine recovered			
	mean, ppb	n	SD, ppb	%
0.25	0.25	8	0.03	100
0.50	0.52	8	0.04	104
1.00	1.08	8	0.07	108
2.00	1.99	8	0.19	100
av				103

Table IV. Sample Dilution

sample ID	neat	1:2	1:4	1:8
sample 1				
assayed, ppb	1.97	1.08	0.51	0.23
expected, ppb	1.97	0.99	0.49	0.25
recovery, %		109	104	92
sample 2				
assayed, ppb	2.13	1.17	0.57	0.30
expected, ppb	2.13	1.07	0.53	0.27
recovery, %		109	108	111
sample 3				
assayed, ppb	2.43	1.24	0.67	0.30
expected, ppb	2.43	1.22	0.61	0.30
recovery, %		102	110	100

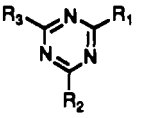
Sample Dilution. A well-validated ELISA should demonstrate acceptable comparison of various concentrations of analyte in the sample to the assay standards; i.e., the standard curve should be parallel to the curve obtained by diluting a sample (Jung et al., 1989). Values obtained from three groundwater samples diluted (1:2, 1:4, 1:8) in the zero standard (0.025 M Tris/0.15 M NaCl/0.1% BSA stabilized solution) showed agreement between measured and expected values (Table IV). The expected values were derived from the cyanazine concentration in the undiluted sample.

Specificity. The 50% inhibition concentration (I_{50}) was determined by estimating the amount of triazine analog necessary to displace 50% of the cyanazine–enzyme conjugate. The least detectable dose (LDD) was determined as the amount of analog required to achieve 90% B/B_0. At the 90% B/B_0 concentration, each related compound would yield an apparent cyanazine concentration greater than the LDD of cyanazine. Table V summarizes the specificity data using a variety of triazine analogs and their structures, as well as many nonstructurally related agricultural compounds. The antiserum used in the present ELISA is very specific for cyanazine as exhibited by the low cross-reactivity to related triazine compounds.

Drift. An optimized assay should exhibit little or no variation in sample values from the beginning to the end of a run due to timing. The time needed to complete all manipulations in the protocol depends on the number of samples being assayed. To test for drift, a sample containing 1.5 ppb of cyanazine was assayed in 50 replicates, or 60 tubes total including standards and controls, approximately 2 s/tube. Figure 3 illustrates the insignificant drift of sample concentrations in this ELISA. The slope of the regression line (−0.00097 ppb/s) suggests that for a 60-tube assay the analyte concentration difference from beginning to end would be minimal, 6.3% at the 1.5 ppb of cyanazine level.

Sample Spike Recovery. Two hundred seventy-eight groundwater samples obtained from throughout the United States were fortified with a known concentration of cyanazine as a test for sample matrix interference. Figure 4 shows an acceptable recovery when these samples were

Table V. Specificity (Cross-Reactivity)[a]



inhibitor	R_1	R_2	R_3	LDD, ppb	I_{50}, ppb
cyanazine	$NHCCN(CH_3)_2$	Cl	$NHCH_2CH_3$	0.035	0.43
terbuthylazine	$NHC(CH_3)_3$	Cl	$NHCH_2CH_3$	0.05	12.0
terbutyrn	$NHC(CH_3)_3$	SCH3	$NHCH_2CH_3$	0.11	15.0
ametryn	$NHCH(CH_3)_2$	SCH3	$NHCH_2CH_3$	0.50	80.0
prometryn	$NHCH(CH_3)_2$	SCH3	$NHCH(CH_3)_2$	1.50	640
simazine	$NHCH_2CH_3$	Cl	$NHCH_2CH_3$	1.60	200
propazine	$NHCH(CH_3)_2$	Cl	$NHCH(CH_3)_2$	3.50	390
prometon	$NHCH(CH_3)_2$	OCH_3	$NHCH(CH_3)_2$	82.0	1900
atrazine	$NHCH(CH_3)_2$	Cl	$NHCH_2CH_3$	200	>10000

[a] The following pesticides were assayed at 10 000 ppb and found to have no reactivity in the assay: alachlor, aldicarb, aldicarb sulfone, aldicarb sulfoxide, benomyl, butachlor, butylate, captan, captafol, carbaryl, carbendazim, carbofuran, 2,4-D, 1,3-dichloropropene, dinoseb, metolachlor, MCPA, pentachlorophenol, picloram, propachlor, terbufos, thiophanat-methyl, and thiabendazol.

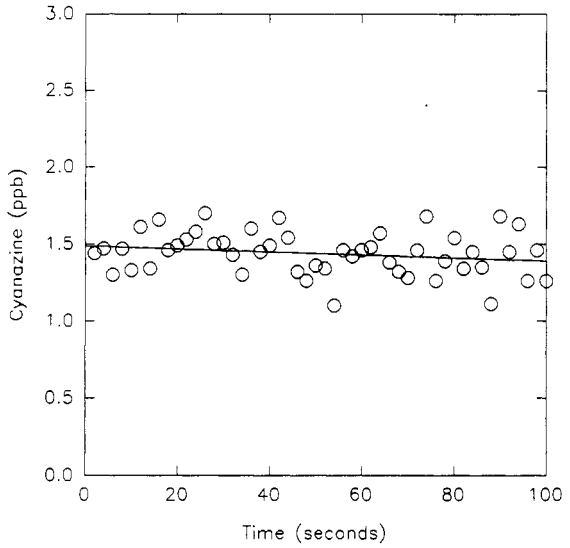


Figure 3. Assay drift: plot of 50 consecutive determinations of a single sample containing cyanazine.

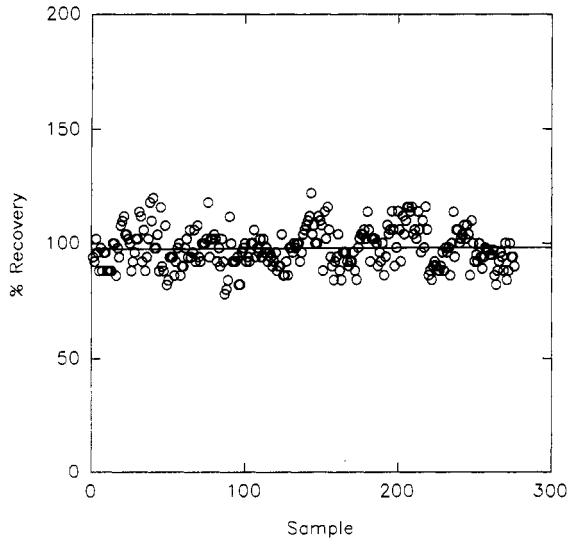


Figure 4. Interference testing: recovery (percent) after fortification of 278 groundwater samples with 0.5 ppb of cyanazine.

spiked with 0.5 ppb of cyanazine (range 78–122%), indicating that no sample matrix effects are present in this ELISA. The mean recovery of all samples was 98% (SD = 9%).

The same 278 water samples were also tested for their ability to catalyze the conversion of substrate and chromogen in the assay and therefore give a false negative result (Rubio et al., 1991). The acceptable recovery for all samples suggests that any substances which may catalyze the substrate are sufficiently removed from the assay during the wash steps.

Table VI. Effect of Possible Interfering Substances

compd	max concn of compd tested, ppm	0 ppb of cyanazine sample	0.5 ppb of cyanazine sample
iron	250	0.00	0.42
humic acid	100	0.00	0.57
nitrate	250	0.00	0.55
thiosulfate	250	0.00	0.59
sulfide	250	0.00	0.55
magnesium	250	0.00	0.59
calcium	250	0.00	0.52
copper	250	0.00	0.51
nickel	250	0.00	0.57
sulfate	10000	0.00	0.56
NaCl	1.0[a]	0.00	0.50
silicates	1000	0.00	0.53

[a] Molar.

Interferences. The following compounds were added to water samples at 250 parts per million (ppm, μg/mL) and tested for possible interference in the immunoassay: nitrate, iron, magnesium, calcium, copper, nickel, sulfide, and thiosulfate (a water preservative). The results obtained are listed in Table VI and indicate no interferences up to the tested levels. In addition, sulfate to 10 000 ppm, NaCl to 1.0 M, silicates to 1000 ppm, and humic acid to 100 ppm exhibited no interferences. The concentrations of the compounds chosen are those that would most likely exceed those found in groundwater samples (American Public Health Association, 1989).

To test for pH interference, the pH of the zero standard was adjusted with 6 N HCl or 6 N NaOH to obtain pH 1–12. Samples were assayed neat and spiked with 0.60 ppb of cyanazine in the ELISA. Figure 5 illustrates that no adverse effect in the assay due to sample pH was seen from pH 2 to 12. Therefore, environmental water samples with a wide range of pH can be assayed with this ELISA without neutralization or pH interference.

Soil Fortification Study. A reproducibility and recovery study was performed with two soils of known composition. Soils fortified with 20–200 ppb of cyanazine were extracted and analyzed by ELISA. As shown in Table VII, recoveries for a 15-min extraction ranged from 50% to 80%. A 24-h extraction (Table VIII) gave higher recoveries of 59–92%. Coefficients of variation for three determinations show reproducibility in the extraction

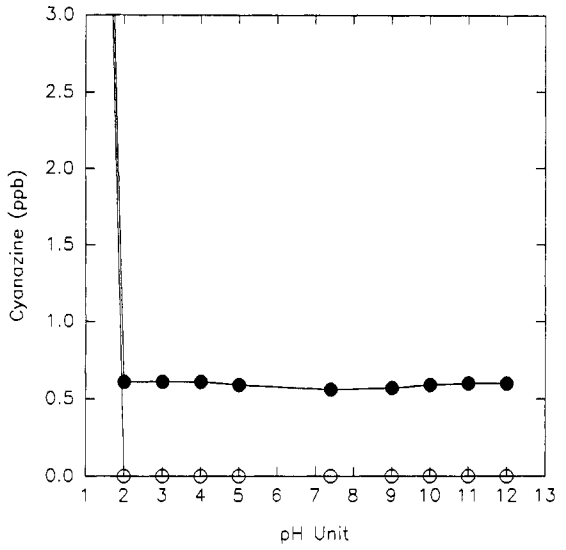


Figure 5. Effect of sample pH on apparent cyanazine concentration. (○) Neat sample; (●) 0.6 ppb sample.

Table VII. Soil Reproducibility and Recovery (15-min Extraction)

soil	cyanazine spike, ppb	% recovery	cyanazine result: mean, ppb	SD, ppb	% CV
sassafras	20	50	10.0	0.8	8.2
	50	61	30.3	2.1	6.8
	100	77	77.3	3.1	4.0
plano	50	67	33.3	4.8	14.4
	100	75	75.0	3.6	4.7
	200	80	160.7	13.0	8.1

Table VIII. Soil Reproducibility and Recovery (24-h Extraction)

soil	cyanazine spike, ppb	% recovery	cyanazine result: mean, ppb	SD, ppb	% CV
sassafras	20	59	11.7	1.7	14.6
	50	67	33.3	0.5	1.4
	100	84	84.0	1.6	1.9
plano	50	77	38.7	1.7	4.4
	100	92	92.3	9.0	9.7
	200	90	180.3	10.5	5.8

Table IX. Methanol Tolerance

methanol concn, %	0 ppb of cyanazine sample	0.5 ppb of cyanazine sample
0.0	0.00	0.53
0.2	0.00	0.60
0.5	0.00	0.54
1.0	0.00	0.57
2.0	0.00	0.55
5.0	0.00	0.55
10.0	0.00	0.54
50.0	0.00	0.38

procedure and ELISA analysis (1.9–14.6%) (Tables VII and VIII). Diluting the soil extract in the diluent eliminates the need for solvent evaporation and reduces any possible matrix or solvent interference in the assayed sample. At least a 1:10 dilution of the soil extract into the diluent is necessary to be at the 10% maximum methanol tolerance for this ELISA (Table IX). Since 2% methanol has no effect in the assay, preparing standards containing methanol is unnecessary. Using a 1:50 extract dilution provides a detection range of 3.5–300 ppb of cyanazine in soil. The recovery of cyanazine is also affected by the breakdown of the pesticide in different soil types and binding to soil constituents. As with any analytical method, the extraction efficiency may vary with soil type and substances that interfere with the analysis may be extracted (Schneider and Hammock, 1992).

Conclusion. This work describes a magnetic particle-based immunoassay for pesticide residues and its performance characteristics in the quantitation of cyanazine in groundwater and soil samples. The assay compares favorably to GC determinations and exhibits excellent precision and accuracy, which guarantee consistent monitoring of environmental samples. The assay sensitivity of 35 ppt (90% B/B_0) in water exceeds the U.S. EPA and Health and Welfare Canada health advisory limits of 10 ppb, the EC maximum contaminant level of 0.1 ppb, and the U.S. EPA HPLC method detection limit of 0.94 ppb. The highly specific antibody employed allows for the detection of cyanazine in the presence of other pesticides. The ELISA is also free from interferences from commonly found groundwater components.

The current magnetic particle-based immunoassay for cyanazine provides results in less than 45 min without the problems of variability encountered with coated tubes, beads, and microtiter plates (e.g., coating variability, antibody leaching, etc.). The assay is ideally suited for adaptation to on-site monitoring of low levels of cyanazine in water and soil samples and could be applied to residue detection in foodstuffs given the solvent tolerance of the system.

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