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Quantification of Alachlor in Water by a Novel Magnetic Particle-Based ELISA

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Interest in pesticide residue testing in water has increased dramatically over the past few years. Current testing methods are time-consuming, expensive and require specialized instrumentation such as liquid or gas chromatography. The emergence of enzyme immunoassays as a viable alternative to these traditional methods has shown them to be sensitive, reliable, cost-effective and rapid (Hammock and Mumma, 1980).

Alachlor is a selective systemic herbicide used for pre- and post-emergence control of most annual grasses and broad-leaved weeds in such crops as corn, soybeans, peanuts, cotton and sugar cane. The active ingredient of Lasso® herbicide, alachlor is one of the most widely used in North America, an estimated 85 million pounds per year (National Research Council, 1987). As a result of its usage, alachlor residues may contaminate food, wells and streams due to runoff, spills and spraying. Alachlor has been classified in Group B2, a probable human carcinogen (U.S. EPA, 1989), by the EPA who have established a maximum contaminant level goal (MCLG) in drinking water at zero (U.S. EPA, 1991).

The principles of enzyme linked immunosorbent assay (ELISA) have previously been described and applied to the determination of alachlor in environmental water samples (Feng et al., 1990; Rittenberg et al., 1991). In the previously cited ELISAs, the solid-phase employed are polystyrene wells, balls or tubes, on which antibody or hapten-protein conjugate are 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). A magnetic particle-based ELISA eliminates these imprecision problems through covalent coupling of antibody to the solid-phase. The uniform dispersion of particles throughout the reaction mixture allows for rapid reaction kinetics and precise addition of antibody.

This paper describes the development and evaluation of an enzyme linked immunosorbent assay (ELISA) for the quantitation of alachlor in environmental water samples using a novel magnetic particle solid-phase. Water samples

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require no sample preparation and analysis is complete in less than one hour. Water samples and horseradish peroxidase (HRP) labeled alachlor are incubated for 30 minutes with the antibody coupled solid phase. A magnetic field is applied to the magnetic solid-phase to wash and remove unbound HRP-alachlor and eliminate any potential interfering substances. The enzyme subtrate (hydrogen peroxide) and chromogen (3,3',5,5'-tetramethylbenzidine [TMB]) are then added and incubated for 20 minutes. The reaction is stopped with the addition of acid and the final colored product is analyzed with a specially designed microprocessor controlled photometer with extensive data reduction capability (Rubio et al., 1991).

MATERIALS AND METHODS

Amine terminated superparamagnetic particles of approximately 1 um diameter were obtained from Advanced Magnetics (Cambridge, MA). Glutaraldehyde (Sigma Chemical, St. Louis, MO). Rabbit anti-alachlor serum (Ohmicron, Newtown, PA). Alachlor-HRP conjugate (Medix Biotech, Foster City, CA). Hydrogen peroxide and 3,3',5,5'-tetramethylbenzidine (TMB) solutions (Kirkegaard & Perry Labs, Gaithersburg, MD). Bovine serum albumin (BSA) (Miles, Kankakee, IL). Alachlor and related compounds as well as non-related crossreactants (Riedel-de-Haen, Hanover, FRG). All other reagents were reagent grade or chemically pure.

The following apparatus were utilized: Adjustable pipette, Gilson P-200 (Rainin, Woburn, MA). Repeating pipette (Eppendorf, Hamburg, Germany). RPA-1 Photometric Analyzer (Ohmicron, Newtown, PA). Magnetic separation rack (Ohmicron, Newtown, PA).

The anti-alachlor coupled magnetic particles were prepared by glutaraldehyde activation (Weston and Avrameas, 1971). The unbound glutaraldehyde was removed from the particles by magnetic separation and washing four times with 2-(N-morpholino)ethane sulfonic acid (MES) buffer. The alachlor antiserum was incubated overnight with agitation at room temperature with the glutaraldehyde activated particles. The unreacted glutaraldehyde was quenched with glycine buffer and the covalently coupled anti-alachlor particles were washed four times and diluted with 0.15M Tris/0.15M NaCl/0.1% BSA/1mM ethylenediaminetetraacetic acid preserved buffer.

All water samples were assayed by adding 200 uL of sample, 250 uL of conjugate and 500 uL of anti-alachlor magnetic particles to a test tube and incubating for 30 minutes at room temperature. A specially designed rack is used to magnetically separate the reaction mixture and wash twice with 1.0 mL of distilled water (Rubio et al., 1991). The colored product was developed for 20 minutes at room temperature by the addition of 500 uL of hydrogen peroxide/TMB solution. The color reaction was stopped with 500 uL of 2M sulfuric acid. The final concentrations of alachlor for each sample were determined using the RPA-1 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 5.0 ppb of alachlor.

RESULTS AND DISCUSSION

Figure 1 illustrates the mean standard curve for the alachlor calibrators that was

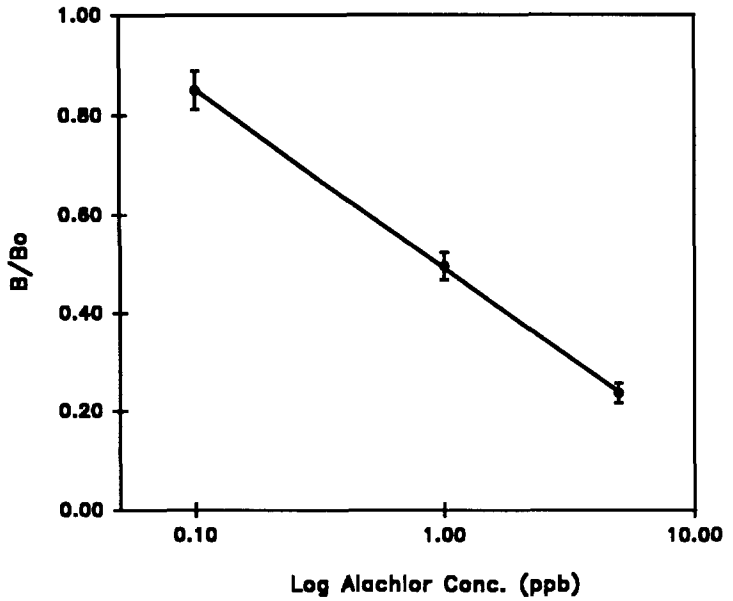

Figure 1. Dose response curve for alachlor. Each point represents the mean of 30 determinations. Vertical bars indicate +/- 2 SD about the mean.

Table 1. Precision of alachlor measurement

Sample	1	2	3	4
Replicates	5	5	5	5
Days	5	5	5	5
N	25	25	25	25
Mean (ppb)	0.27	0.50	2.11	3.91
% CV (within assay)	11.1	6.0	3.4	3.8
% CV (between assay)	5.5	5.9	1.9	3.3

collected over 30 runs. The displacement at the 0.1 ppb level is significant (85% B/Bo - where B/Bo is the absorbance at 450 nm observed for a sample or standard divided by the absorbance at the zero standard) and the assay sensitivity is estimated to be 50 ppt (90% B/Bo). This is consistent with the National Drinking Water Regulation Maximum Contaminant Level Goal (MCLG) of 0 ppb, Practical Quantitation Level (PQL) of 2 ppb and Method Detection Limit (MDL) of 200 ppt (U.S. EPA, 1991). Correlation of 15 groundwater samples obtained by the present ELISA method (y) and an established gas chromatography/mass spectroscopy (GC/MS) method are illustrated in Figure 2. The regression analysis yields a correlation of 0.984 and a slope of 1.04 between methods.

A precision study in which groundwater samples, spiked with alachlor at 4 concentrations, were each assayed 5 times in singlicate per assay on five different days is shown in Table 1. The within and between day variation was determined by the method of Bookbinder and Panosian (1986) using Statistical Analysis System (SAS) software (SAS Institute, NC). Coefficient of variation (%CV) within and between day were less than 12% and 6% respectively.

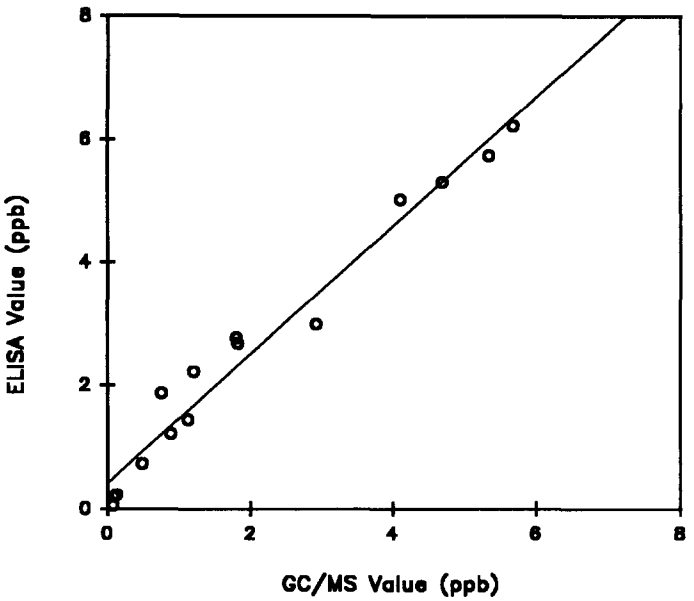

Figure 2. Correlation between alachlor concentrations as determined by ELISA and GC/MS methods. n = 15, r = 0.984, y = 1.04X + 0.42.

Groundwater samples were fortified with a known concentration of alachlor, as a test for sample matrix interference, and their recoveries calculated by subtracting the alachlor value of the neat sample from the spiked sample. Figure 3 shows an acceptable recovery when 272 groundwater samples obtained from around the U.S. were spiked with 1 ppb of alachlor (range 80% to 127%). The mean recovery of all samples was 107% (+/-9%). The 272 groundwater samples were also tested for their ability to catalyze the conversion of substrate and thus interfere in the assay (false negative result). Sample (100 uL) and TMB/Peroxide (500 uL) were reacted for 20 minutes, the reaction stopped and the absorbance determined at 450 nm. The mean absorbance at 450 nm was 0.014 (SD = 0.006). Of those samples, only 2 had absorbances greater than 0.040, however, the recoveries with these samples were 108% and 92%. The acceptable recoveries suggests that whatever substance(s) that is catalyzing the substrate is sufficiently removed from the assay during the wash step.

The accuracy of the assay was evaluated by adding known amounts of alachlor to five groundwater samples and analyzing the samples before and after the addition of alachlor and subtracting the concentration of alachlor before spiking. Table 2 summarizes the accuracy of the alachlor ELISA. Added amounts of alachlor were recovered correctly in all cases with the average assay recovery of 94%.

A well validated ELISA must demonstrate acceptable comparison of various concentrations of analyte in the sample to the standards used in the assay. The standard curve should be parallel to the curve obtained by diluting a sample, in the absence of interfering substances (Jung, et al., 1989). Values obtained from three groundwater samples diluted (3:4, 1:2, 1:4, 1:8) in the Zero standard/diluent (0.025M Tris/0.15M NaCl stabilized solution) showed

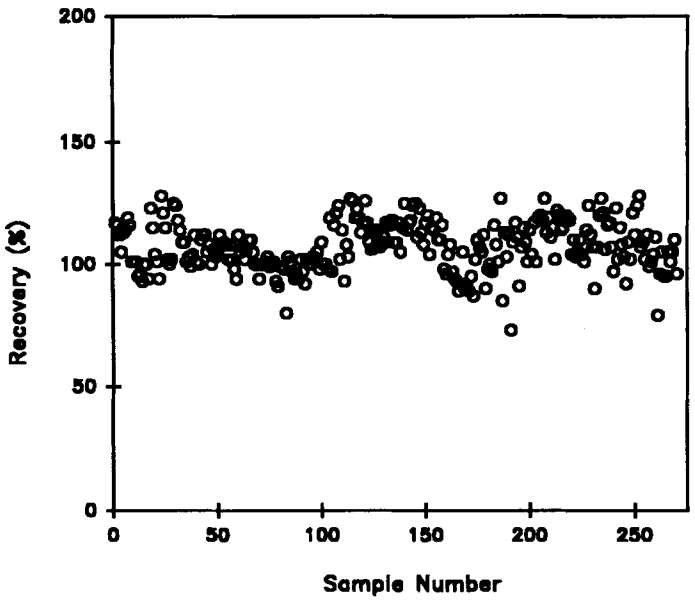

Figure 3. Interference testing, Recovery (%) after fortification of groundwater samples with 1 ppb of alachlor.

Table 2. Accuracy of alachlor ELISA

Amount of Alachlor Added (ppb)	Mean (ppb)	S.D. (ppb)	%
0.5	0.49	0.03	98
1.5	1.40	0.07	93
3.0	2.70	0.11	90
Average			94

agreement between measured and expected values (Table 3). The expected values were derived from the alachlor concentration in the undiluted sample.

The percent cross-reactivity was determined by estimating the amount of analogue necessary to displace 50% of the enzyme conjugate, compared to the 50% displacement of alachlor. The least detectable dose (LDD) was determined as the amount of analogue required to achieve 90% B/Bo. Table 4 summarizes the cross-reactivity data using a variety of chloroacetanilide analogues, as well as many non-structurally related agricultural compounds. The antiserum used in the present ELISA is very specific for alachlor as exhibited by the low cross-reactivity to related compounds.

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 ppb of alachlor was assayed in 50 replicates, or 60 tubes total including standards and controls. Figure 4

Table 3. Sample dilution

Sample ID	Neat	3:4	1:2	1:4	1:8
Sample 1					
assayed (ppb)	2.47	1.97	1.22	0.69	0.38
expected (ppb)	2.47	1.85	1.23	0.62	0.31
recovery (%)	-	106	99	111	121
Sample 2					
assayed (ppb)	3.52	2.46	1.63	0.83	0.39
expected (ppb)	3.52	2.64	1.76	0.88	0.44
recovery (%)	-	94	93	94	89
Sample 3					
assayed (ppb)	4.11	3.07	2.11	1.14	0.59
expected (ppb)	4.11	3.08	2.06	1.03	0.51
recovery (%)	-	100	102	110	115

Table 4. Specificity (Cross-Reactivity)

Compound	% Cross-reactivity	LDD (ppb)
Alachlor	100	0.05
Butachlor	1.0	6.0
Metolachlor	1.3	5.6
Propachlor	<0.02	NR

NR = No reactivity up to 10,000 ppb.
The following demonstrated no reactivity up to 10,000 ppb: Aldicarb, Aldicarb Sulfate, Aldicarb Sulfoxide, Atrazine, Benomyl, Butylate, Captan, Captofol, Carbaryl, Carbendazim, Carbofuran, Cyanazine, 2,4-D, 1,3-Dichloropropene, Dinoseb, MCPA, Metribuzin, Pentachlorophenol, Picloram, Simazine, Terbufos, Thiophanat-methyl, and Thiabendazol.

illustrates that insignificant drift of sample concentrations in this ELISA. The slope of the regression line (-0.001) suggests that on a 60 tube assay the analyte concentration difference from beginning to end would be <5.0%.

The following compounds were added to water samples and tested in the immunoassay to determine if they interfered: Nitrate, Magnesium, Calcium, Copper, Nickel, Sulfide and Thiosulfate (a water preservative) to 250 ppm. Table 5 summarizes that no interferences are present up to the tested levels. In addition, Sulfate to 10,000 ppb, NaCl to 1.0M and Iron and Humic Acid to 100 ppm exhibited no interferences. The concentrations of the compounds chosen are those that would most likely exceed levels found in groundwater samples (American Public Health Association, 1989). Water samples containing 0 ppb and 1 ppb alachlor were assayed at various pH's. 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 a pH interference.

This ELISA demonstrates both the feasibility of using magnetic particles as a solid-phase in an immunoassay for pesticide residues and its performance characteristics in the quantitation of alachlor in water samples. The assay compares favorably to GC/MS determinations and exhibits excellent precision and accuracy which guarantees consistent monitoring of environmental water

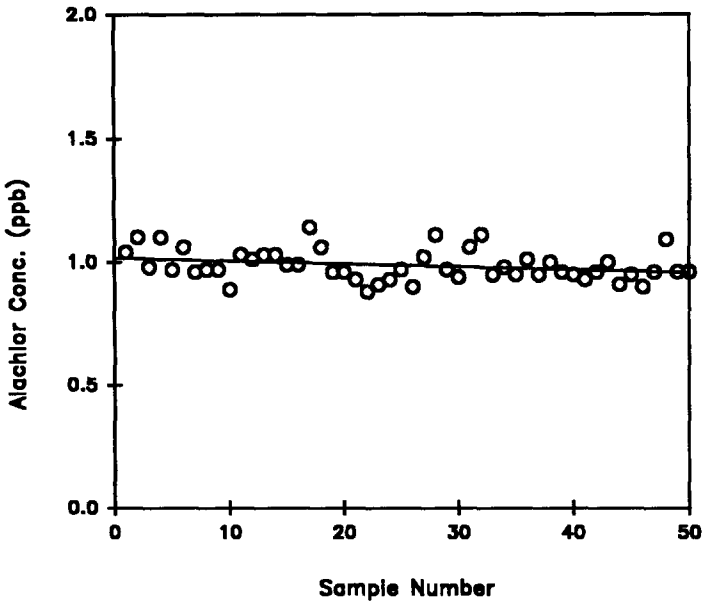

Figure 4. Assay Drift: Plot of 50 consecutive determination of a single sample containing alachlor.

Table 5. Effect of possible interfering substances

Compound	Max. Conc. Tested (ppm)	0 ppb Sample	1 ppb Sample
Iron	100	0.00	0.96
Humic acid	100	0.00	1.18
Nitrate	250	0.00	1.04
Thiosulfate	250	0.00	1.05
Sulfide	250	0.00	1.10
Magnesium	250	0.00	0.97
Calcium	250	0.00	0.95
Copper	250	0.00	0.92
Nickel	250	0.00	1.06
Sulfate	10000	0.00	1.08
NaCl	1.0M	0.00	1.07

samples. The assay sensitivity of 50 ppt (90% B/Bo) exceeds the U.S. EPA Practical Quantitation Limit (PQL) of 2 ppb and the Method Detection Limit (MDL) of 200 ppt. The highly specific antibody employed allows for the detection of alachlor in the presence of other pesticides. This ELISA is also free from interferences from commonly found groundwater components. The current magnetic particle-based immunoassay for alachlor provides results in less than 60 minutes 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 alachlor in water samples. Future efforts will extend to the development of ELISAs for other environmentally important compounds and the application of the alachlor immunoassay to soil and food samples.

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