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Enzyme Immunoassay Microtiter Plate Response to Atrazine and Metolachlor in Potentially Interfering Matrices

G. K. Stearman and M. J. M. Wells

Center for the Management, Utilization, and Protection of Water Resources and Department of Agriculture, Tennessee Technological University, Box 5033, Cookeville, Tennessee 38505, USA

Enzyme immunoassay analysis (EIA) of pesticides has received much attention lately because it is simple, rapid, highly specific, and inexpensive compared to traditional techniques (Kaufman and Clower 1991; Jung et al. 1989; Vanderlaan et al. 1988). Immunoassays use antibodies for the detection of molecules that have a structure complementary to a specific region of the antibody molecule. The critical step in the procedure is the high affinity binding of antibody to analyte because of this complementarity. In competitive enzyme immunoassays for small molecules, such as pesticides, the assay is designed so that the binding of analyte to the specific antibody causes a reduction in the bound amount of enzyme-hapten conjugate (an enzyme labeled with a derivative of the analyte, called a hapten). After the addition of enzyme substrate and chromogen, the bound enzyme causes a color change which is inversely related to the concentration of analyte present. Advantages of immunoassays over conventional instrumental methods for the analysis of pesticide residues include cost, sensitivity, faster sample throughput, portability, simplicity, no cleanup or concentration step required on many water samples, and ease of use. However, as with all analytical procedures, there are also disadvantages to immunoassays. EIA techniques are limited by cross-reacting compounds, single analyte residue analyses, and matrix effects.

Interfering matrix effects derive from two sources. The interference can occur naturally in the sample itself and be co-extracted with the analyte-of-interest. Additionally, the interference may result from the chemicals used to process the sample during extraction and sample preparation. Immunoassays must deal realistically with matrix effects. Unexpected matrix effects may be overcome by recognizing that EIA methods require running control and spiked samples just as performed with conventional methods. The EIA has one built-in quality control check, that is, the degree of agreement when two dilutions of the sample give the expected result (Seiber et al. 1990). When interfering substances are absent, the standard curve in buffer is parallel to the curve obtained by diluting the sample (Jung et al. 1989).

Slope comparisons using linear regression of data provide a simple test for matrix effects. Alternatively, a sample may be divided and one part fortified with a known amount of analyte. Failure to show additivity is also evidence of an inhibitory effect, probably due to the matrix (Jung et al. 1989). Substances present in water and soil may affect the

Send reprint request to G.K. Stearman at the above address.

immunoassay technique. For example, chaotropic agents may be present in sufficient concentration to reduce the interaction between antibody and labelled enzyme and cause a false-positive response. Another source of false-positive value responses is synthetic or natural substances, including halogens or dissolved organic carbon (DOC) that weakly interact with the antibody compared to the strong interaction by the analyte but are present at a sufficiently high concentration to give a false-positive value with the assay. A substance that interferes with one immunoassay system may interfere to a much different degree in a different immunoassay. When diverse water sources are analyzed, the matrix variation limits an immunoassay to screening purposes rather than quantitation. However, for a given water source, the compositional variation may be small, and then an immunoassay may be useful for quantitation (Fleeker 1987). The objectives of this paper were to examine standard EIA atrazine and metolachlor responses at various dilutions of organic solvents and with humic acids.

MATERIALS AND METHODS

Enzyme immunoassay plate kits for triazines and alachlor were purchased from Immunosystems, Inc., Scarborough, ME, and currently from Millipore Corporation, Bedford, MA. The EIA plates were from the same lot number. Metolachlor cross-reacts with alachlor antibody and therefore alachlor plates were used for metolachlor analysis.

The triazine or alachlor EIA kit consisted of 96 antibody-coated wells in a microtiter plate; solutions of atrazine-enzyme conjugate or alachlor-enzyme conjugate; substrate; chromogen; 2.5 N H_2SO_4; and 0.1, 0.25, 1.0, and 2.0 ppb atrazine or alachlor standards (Res-I-Quant™, ImmunoSystems, Inc., ME). Temperature and time are important parameters that must be controlled for EIA to work properly. In all cases, enzyme immunoassay plates and solutions were allowed to warm to room temperature before using, and reaction times were consistent throughout the experiments. Principles of the EIA are based on competitive binding of atrazine and atrazine-enzyme conjugate to a limited number of antibody binding sites; the alachlor kit reacts in the same manner. An 80-μL aliquot of the standard was mixed in the well with 80 μL of atrazine-enzyme conjugate on an orbital shaker at 200 rpm for 60 min. After incubation the plate wells were washed with water to remove unreacted reagent, so that only the bound atrazine and atrazine-enzyme conjugate remain. Eighty μL each of substrate and chromogen was added to each well. The plate was covered and shaken on the orbital shaker for 30 min at 200 rpm. In the presence of bound enzyme-conjugate, the substrate is converted to a compound which turns the chromogen blue. A sample extract or standard containing low atrazine concentration allows many enzyme conjugate molecules to be bound by the antibodies and consequently a darker blue solution is produced. A sample having a high concentration of atrazine competes with the enzyme conjugate and fewer enzyme conjugate molecules are bound to the antibodies, and consequently a lighter blue solution develops. The reaction was stopped by adding 40 μL of 2.5 N H_2SO_4 which changes the color in the wells from blue to yellow. The wells (color) in the plate were read immediately after the reaction was stopped on a microtiter plate reader (Flow Laboratories, Inc., McClean, VA) at 450 nm (492 nm for metolachlor). Standards were prepared in the extracting solvent and incubated and read on the same plate. Absorbance of samples was recorded and divided by the absorbance of the blank control solution ($\%B_0$) (Bushway et al. 1988). All standards were analyzed in duplicate.

Atrazine standards were made in dilutions of acetonitrile, methanol, 0.5 M ammonium acetate, 0.01 M $CaCl_2$, and humic acid (Aldrich Chemical Co.). Metolachlor standards were made in reagent grade water and in solutions containing humic acid. Atrazine standards were plotted using both logarithm values of standards over a broad range (0.2-2 ng/mL) and linear values over the narrow, steeper range (0.1-0.4 ng/mL). Linear regression was computed with the Statistical Analysis System (SAS, Cary, NC). Slopes and intercepts of standard responses were compared using standard procedures for simple linear regression equations (Zar 1974). Coefficients of variation (CVs) were computed on duplicates of standards and averaged for each set of standard curves.

RESULTS AND DISCUSSION

The matrix effects of different organic solvents on enzyme immunoassays of a variety of analytes have been reported. Goh et al. (1990) using antibody coated tubes found that measurable changes in optical density could be obtained at all atrazine concentrations for solutions up to 3% acetonitrile and 10% methanol (highest level tested). Solutions of 4% acetonitrile interfered with the assay. Lucas et al. (1991) demonstrated that addition of methanol, acetonitrile, or acetone to 10% or tetrahydrofuran to 2.5% did not adversely affect atrazine assays. They reported, however, that single-coated antibody formats could withstand a larger percentage of modifier in the sample than double-coated antibody formats. Stearman and Adams (1992) demonstrated that interference with the EIA of atrazine occurred when acetonitrile was present above 5% concentration in the final dilutions. Gee et al. (1988) also observed that organic solvents can disrupt the antigen-antibody reaction. They examined the effect of solvents on the EIA of the herbicide molinate. Propylene glycol at 5% did not appreciably effect the standard curve while sensitivity decreased at concentrations of 12.5% and greater. Acetonitrile had no effect at 12.5%, but analyses at 5% acetonitrile demonstrated increased absorbances. They point out that enhanced absorbances in the presence of organic solvents have also been observed with other, but not all, immunoassays conducted in their laboratory. Harrison et al. (1989) studied the EIA of maleic hydrazide and determined that heterologous binding of antibodies was augmented by methanol with peaks at 10 to 25% methanol for different antibodies, while homologous binding was unaffected by 10 or 17.5% methanol for different antibodies. From studies of the EIA for the herbicide bentazon, Li et al. (1991) concluded that the effect of solvents on EIA was small if less than 10% acetonitrile or DMSO was added, and that solutions of acetonitrile up to 50% did not significantly effect the binding of coating antigen and antiserum/antibody. Li et al. (1991) also reported a very strong positive effect on the antibody-antigen binding when methanol was present. Similarly, during EIA of simazine, Goh et al. (1992) also found a maxima in optical density between 40-60% methanol corresponding to 3.4-5.1% methanol in the final volume analyzed by ELISA. Li et al. (1991) postulated that the phenomenon of enhanced or augmented absorbances in some assays may result from decreased interference from lipid micelles with complex matrices and from reduced binding of analyte to surfaces. Goh et al. (1992) attributed the decreased optical density at increased methanol levels to an effect on the enzyme conjugate which resulted in the suppression of hydrolysis of substrate to a colored product.

In this laboratory, solutions containing less than 3.5% acetonitrile had no adverse effect on atrazine standard curves based on similarity of slopes of standard lines, even though intercepts were different, therefore indicating parallelism. At acetonitrile concentrations of 0.2, 0.5, 1.1, 1.8, and 3.5%, slopes of standard lines were not different at the 95%

level using atrazine standards from 0.1, 0.2, 0.3 and 0.4 ng/mL (F(slope) = 2.02) (Table 1). Intercepts were different indicating that although lines were parallel, they were not equivalent. The coefficients of determination (r^2) for the standard lines ranged from 0.93 to 0.99. Coefficient of variation (CV) for duplicate standards averaged 2.82% with a range of 0 to 10.78%. At atrazine concentrations of 0.1 to 0.3 ng/mL in 0.25, 1.0, and 2.2%, acetonitrile slopes from standard curves were not different at the 99% level (F(slope) = 0.61) (Table 1). The coefficient of determination was 0.96 for each of the three standard lines. The mean coefficient of variation of the standards was 2.52%. Both these data sets were plotted as linear functions, because the logarithmic function did not increase the coefficient of determination. In research applications where quantitative determination necessitates working with the steepest portion of the standard response, it is best to use atrazine standards in the low range (0.1-0.4 ng/mL). At the low range of atrazine standards slopes are steeper, and a linear instead of a logarithmic function can be used to compute results.

Table 1. Atrazine enzyme immunoassay responses of standard solutions in dilute acetonitrile using a linear scale from 0.1 to 0.4 or 0.1 to 0.3 ng/mL atrazine.

Range 0.1 to 0.4 ng/mL			
% Acetonitrile	Slope	Intercept	Coefficient of determination (r^2)
0.2	-1.01	0.866	0.93
0.5	-1.18	0.879	0.99
1.1	-1.01	0.862	0.95
1.8	-1.02	0.849	0.96
3.5	-1.08	0.864	0.95
Range 0.1 to 0.3 ng/mL			
0.25	-1.20	0.905	0.96
1.0	-1.11	0.809	0.96
2.2	-1.27	0.907	0.96

At broad ranges of atrazine standards a logarithmic scale best fits the standard response. Table 2 includes six standard lines of atrazine in dilutions of acetonitrile, plotted as a logarithm function. Slopes of lines were not significantly different at the 95% level of probability (F(slope) = 1.61). Atrazine standards were 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7 ng/mL. Coefficient of variation for standards averaged 2.99%.

Atrazine standards in dilute solutions of methanol were plotted, and no difference in slope was observed for standards in 0.4, 1.6, and 3.5% methanol solutions (F(slope) = 1.66) (Table 3). Coefficient of variation for standards averaged 4.07%. In Tables 3, 4, and 5 atrazine was plotted over an expanded range of standards. The x-axis was plotted with logarithmically transformed data because of the improved fit of the standard line, based on the higher coefficient of determination compared to a linear scale.

Table 2. Atrazine enzyme immunoassay responses of standard solutions in dilute acetonitrile using a logarithmic scale from 0.1 to 0.7 ng/mL atrazine.

Range 0.1 to 0.7 ng/mL			
%Acetonitrile	Slope	Intercept	Coefficient of determination (r^2)
0.5	-0.459	0.279	0.94
0.5	-0.528	0.195	0.99
0.5	-0.435	0.233	0.98
0.5	-0.481	0.220	0.96
1.1	-0.508	0.269	0.97
3.5	-0.545	0.249	0.84

Table 3. Atrazine enzyme immunoassay responses of standard solutions in dilute methanol using a logarithmic scale from 0.1 to 0.8 ng/mL atrazine.

Range 0.1 to 0.8 ng/mL			
% Methanol	Slope	Intercept	Coefficient of determination (r^2)
0.4	-0.495	0.275	0.85
0.4	-0.580	0.242	0.94
1.6	-0.487	0.269	0.94
3.6	-0.591	0.280	0.96

Ionic matrix effects upon the enzyme immunoassay of various pesticides have also been demonstrated. Harrison et al. (1989) examined the effects of pH, ionic strength, Mg^{2+} and Ca^{2+}, on the EIA of maleic hydrazide. They found that Mg^{2+} effects were negligible at levels below 3 mM while 3 mM Ca^{2+} effects were significant but partially blocked by EDTA. Stearman and Adams (1992) showed that ammonium acetate (0.5 N) had a small effect at 3.8% dilution and recorded positive readings when used at 5% strength or greater. (*Baum, personal communication*) found that Zn^{2+} interferes with the atrazine ELISA at concentrations greater than 250 ppm, and Hg^{2+} interferes with alachlor, metolachlor, and 2,4-D assays at concentrations greater than 2000 ppm. No interferences were found from Ca^{2+}, Cd^{2+}, Cr^{3+}, Cu^{2+}, Fe^{3+}, Mg^{2+}, Mn^{2+}, Na^{+}, NH_4^{+}, Ni^{+}, Cl^{-}, CN^{-}, F^{-}, I^{-}, NO_3^{-}, PO_4^{3-}, S^{2-}, and SO_4^{2-} at levels below 250 ppm. Lucas et al. (1991) reported that the presence of trifluoroacetic acid to 0.05% was tolerated by atrazine assays, while a trend to lower IC_{50} values was observed with increased concentrations of the salts $CaCl_2$, $(NH_4)_2SO_4$, KCl, and Na_2HPO_4 in the sample. Increased sensitivity was observed with both single- and double-coated antibody formats.

Table 4. Atrazine enzyme immunoassay responses in standard solutions in dilute 0.5N NH_4OAc, and 0.01M $CaCl_2$, as compared to that for reagent grade water.

Matrix	Slope	Intercept	Coefficient of determination (r^2)
0.5N NH_4OAc (0.5%)	-0.511	0.653	0.65
0.5 N NH_4OAc (3.8%)	-0.707	0.755	0.93
0.01M $CaCl_2$	-0.482	0.251	0.98
Reagent Grade H_2O	-0.312	0.641	0.94

Table 4 includes responses of standard atrazine solutions in 0.5 and 3.8% dilutions of 0.5 N ammonium acetate, in 0.01 M $CaCl_2$, and in reagent grade water. Ammonium acetate solution produced no deviation of slope for standard curves in 0.5% and 3.8% solutions of 0.5 N ammonium acetate, although r^2 for standard lines were lower than with acetonitrile and methanol. Slopes of lines were significantly different at 90% level of probability (F(slope) = 3.02). Coefficient of variation for standards averaged 3.54%. Intercepts of the standard responses were different. The log transformed standard curve in 0.01 M $CaCl_2$ was linear to 2 ppb, while at 10 ppb the values did not follow a linear pattern (not shown).

Aquatic macromolecular dissolved organic carbon (DOC) is found in the microgram per liter range in groundwater and in the milligram per liter range in surface freshwater (Aiken 1985). Terrestrial DOC is solvent extracted from soil/sediment matrices during analytical sample preparation. The potential interference with enzyme immunoassay from DOC has not been well characterized.

Gee et al. (1988) studied the effect of the source of water on the enzyme immunoassay of molinate. Standard curves prepared from distilled, creek, or rice field water were parallel to those run in phosphate-buffered saline containing 0.05% Tween 20 and 0.02% sodium azide. The curves were shifted to the right, resulting in higher limits of quantitation. Subsequent normalization of the analyses by the addition of ten times the concentration of PBS-Tween produced data for the field water samples that were indistinguishable from standards. (*Baum, personal communication*) did not observe any interferences from fulvic and humic acids in surface waters.

Goh et al. (1990) reported greater than 100% recovery of atrazine residue extracted from soil when analyzed by enzyme immunoassay. Over-estimation of concentration was attributed to the potential interference of co-extracted soil components with the enzyme-conjugate antibody reaction. They suggest that standard-addition experiments or addition of atrazine to background soil extracts for control and calibration standards may be necessary to overcome this problem. Stearman and Adams (1992) did not observe over-estimation of atrazine concentrations by EIA from various soils containing 4.7% organic matter or less.

Herbicide responses in concentrations of humic acid (HA) are reported in Table 5 for atrazine and Table 6 for metolachlor. Slopes of lines for atrazine were not significantly different at the 95% level of probability (F(slope) = 1.99). Coefficient of variation of standards averaged 3.55%. Humic acid at 4 or 40 ppm did not interfere with atrazine or metolachlor except at very low levels of atrazine (0.2 ng/mL). At 0.2 ng/mL atrazine, humic acids (HAs) interfered with the enzyme immunoassay response at (4, 40, and 100 ppm HA), perhaps because the small amount of atrazine was bound to HA and could not attach to the antibody. At HA concentrations equal or greater than 100 ppm, interferences were observed with responses of atrazine standard solutions. At 100 ppm HA, response to atrazine could not be predicted (data not shown), perhaps due to HA physically preventing atrazine from attaching to the antibody-coated wells. Atrazine detection limits were 0.1 ppb as determined by %Bo (i.e., percent of blank absorbance).

Table 5. Atrazine enzyme immunoassay responses of standard solutions in humic acid, and reagent grade water using a logarithmic scale 0.2 to 2.0 ng/mL.

Solution	Slope	Intercept	Coefficient of determination (r^2)
Reagent grade water	-0.312	0.641	0.94
Humic acid 4 ppm	-0.491	0.674	0.94
Humic acid 40 ppm	-0.613	0.541	0.74

Table 6. Comparison of metolachlor enzyme immunoassay responses of standard solutions in humic acid, and reagent grade water using a logarithmic scale 1.0 to 40.0 ng/mL metolachlor.

Solution	Slope	Intercept	Coefficient of determination (r^2)
Reagent grade water	-0.28	0.755	0.76
Humic acid 4 ppm	-0.31	0.935	0.94
Humic acid 40 ppm	-0.25	0.927	0.68

Metolachlor standards were used to determine effects of HA on response (Table 6). At 0.6 ng/mL metolachlor, HAs interfered with the response as they did with the low concentration of atrazine. This point was not included in the data presented in Table 6. Slopes of lines with HA concentration of 4 and 40 ppm were not significantly different (F(slope) = 0.377), indicating no interference of HA. The mean CV for atrazine standards was 2.99%, while mean CV for metolachlor standards was 9.7%.

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