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Enzyme-Linked Immunosorbant Assay (ELISA) of Simazine for Delhi and Yolo Soils in California

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Enzyme immunoassays to determine atrazine residues in soil using a commercially available polyclonal-antibody test-tube kit (Bushway et al. 1988, Goh et al. 1991), and a monoclonal-antibody, 96-well microtiter-plate assay (Schlaeppi et al. 1989) have been reported. Recently, a monoclonal-antibody ELISA developed by the University of California (UC) at Davis and Berkeley campuses sponsored by the California Department of Pesticide Regulation for s-triazine herbicides (mainly atrazine and simazine) has been characterized and optimized (Goodrow et al. 1990, Harrison et al. 1991, Karu et al. 1991). This ELISA has been shown to be comparable to gas chromatographic (GC) analysis for determining simazine and atrazine concentrations in water and soil (Lucas et al. 1991).

Ethyl acetate was used in extraction of soils for GC analysis. However, since ethyl acetate interfers with the ELISA, this extraction process required a subsequent evaporation step to eliminate the solvent. The analyte was redissolved in an ELISA-compatible buffer (Lucas et al. 1991). This procedure is more time consuming compared to a methanol and water extraction, which is more compatible with ELISA and does not require a evaporation step (Goh et al. 1990, Schlaeppi et al. 1989). A previous study has also shown that methanol and water is an efficient mixture for atrazine extraction from soil (Huang and Pignatello 1990).

This study was conducted to evaluate the ELISA for quantifying simazine residues in two soil types using methanol and water as the extraction solvent by 1) determining the effect of methanol on the ELISA; 2) characterizing standard curves generated in different diluents; 3) optimizing the methanol level in terms of extraction efficiency; and 4) comparing ELISA to (GC) for spiked soil samples.

MATERIALS AND METHODS

The double-antibody, haptenated enzyme, competitive inhibition ELISA is schematically represented in Fig. 1. The syntheses of triazine haptens and hapten-protein conjugates (Goodrow et al. 1990) and mouse anti-triazine antibodies (monoclonal AM7B2.1) (Karu et al. 1991) have been described. Microtiter plates (Nunc #4-42404, Intermountain Scientific; Bountiful, UT) were coated with 100 uL/well of affinity-purified goat anti-mouse antibody (IgG+IgM, Boehringer-Mannheim #60524; Indianapolis, IN) 1:2,000 in "coating buffer" (1.59 g/L Na_2CO_3, 2.93 g/L $NaHCO_3$, and 0.2 g/L NaN_3; pH 9.6), sealed with a acetate plate sealer (Dynatech; Chantilly, VA) and incubated overnight at 4°C (Fig.1a).

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ELISA plates were either immediately used after washing 5 times with PBSTA (0.2M phosphate buffer with 0.8% NaCl, 0.05% Tween 20, 0.02% NaN_3, pH=7.5) using a 12-channel manual washer (Immuno Wash 12, Nunc) or were stored at -20°C until needed. Pipetting was performed using a 12-channel (50-300 uL) digital multichannel micropipette (Flow; McLean, VA). In a separate plate (Dynatech #001-012-9200), a competitive inhibition reaction between the mouse anti-triazine monoclonal antibody (100 uL/well of 1:200 dilution in PBSTA), a standard or soil extract (40 uL/well), and an "enzyme tracer", simazine hapten conjugated to an alkaline phosphatase (SIM N-(C2)-AP) (100 uL/well of 1:3,000 dilution in PBSTA), was sealed and incubated for 1 h (Fig. 1b). The ELISA plate was blocked with 100 uL/well of 0.5% mg/mL bovine serum albumin (Sigma) in PBSTA after one PBSTA wash of the coated plate. After 1 h the ELISA plate was washed 5 times with PBSTA, and then 50 uL/well of the mixture from the competitive inhibition plate was added (Fig. 1c). The ELISA plate was then sealed and incubated for 60 min and washed 5 times with PBSTA. A 100 uL/well of 1 mg/mL substrate solution [freshly prepared by dissolving one 5-mg p-nitrophenyl phosphate tablet (Sigma) in 5 mL of 10% diethanolamine buffer (97 mL diethanolamine, 0.2g NaN_3, 0.1 g $MgCl_2.6H_2O$ per L of distilled water; pH 9.8)] was added and the plate sealed. Plates were read after 30-60 min in a Vmax® microplate reader (Molecular Devices; Palo Alto, CA) using the dual wavelength endpoint mode (450-650 nm). The plate reader was interfaced with a personal computer, and data were analyzed using the Softmax® 2.01 software package (Molecular Devices).

To determine the effect of methanol on (a) enzyme tracer (ET) alone: optical densities (OD) of ELISA in methanol (v:v) at 0, 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100% were measured; (b) analyte competitive binding: the complete ELISA was run generating standard curves with simazine (99.7% technical, Ciba-Geigy Corp.) in 0-100% methanol.

To generate standard curves in a) PBSTA, b) methanol (40%), and c) Delhi and Yolo soil extracts (extracted with 40% methanol): each diluent was spiked at 11 levels of simazine (0-2,304 ppb, dilution factor=2.5). Four-parameter calibration curves were generated as discussed in Lucas et al. (1991). The soil extracts were from Delhi loamy sand (Typic Xeropsamment; 89.3% sand, 5.2% silt, 5.4% clay, and 0.1% organic carbon) and Yolo silt loam (Typic Xerorthents; 46.2% silt, 24.7% clay, 28.4% sand, 0.7% organic carbon).

To determine the method detection limit (MDL) for Delhi and Yolo soils: untreated soils were extracted with 40% methanol and read against standards generated in corresponding soil extracts.

To determine the extraction efficiency of methanol: Delhi and Yolo soils were spiked at 100 ppb simazine and extracted with 20, 40, 60, or 80% methanol. The extraction procedure was described in Goh et al. (1991). Briefly, 25-g soil were extracted with two 25-mL portions of appropriate methanol and water mixtures. The soil sample was first thoroughly wetted with methanol (HPLC grade); water was then added and the soil was extracted for 10 min with vigorous shaking on a platform shaker. The extract solvent mixture was decanted, and the extraction was repeated. The combined extract was filtered through a 25-mm 0.2-um syringe-filter (Gelman Sciences), and the filtrate was used directly for ELISA. Simazine recovery was determined by reading against a standard curve made in the corresponding % methanol.

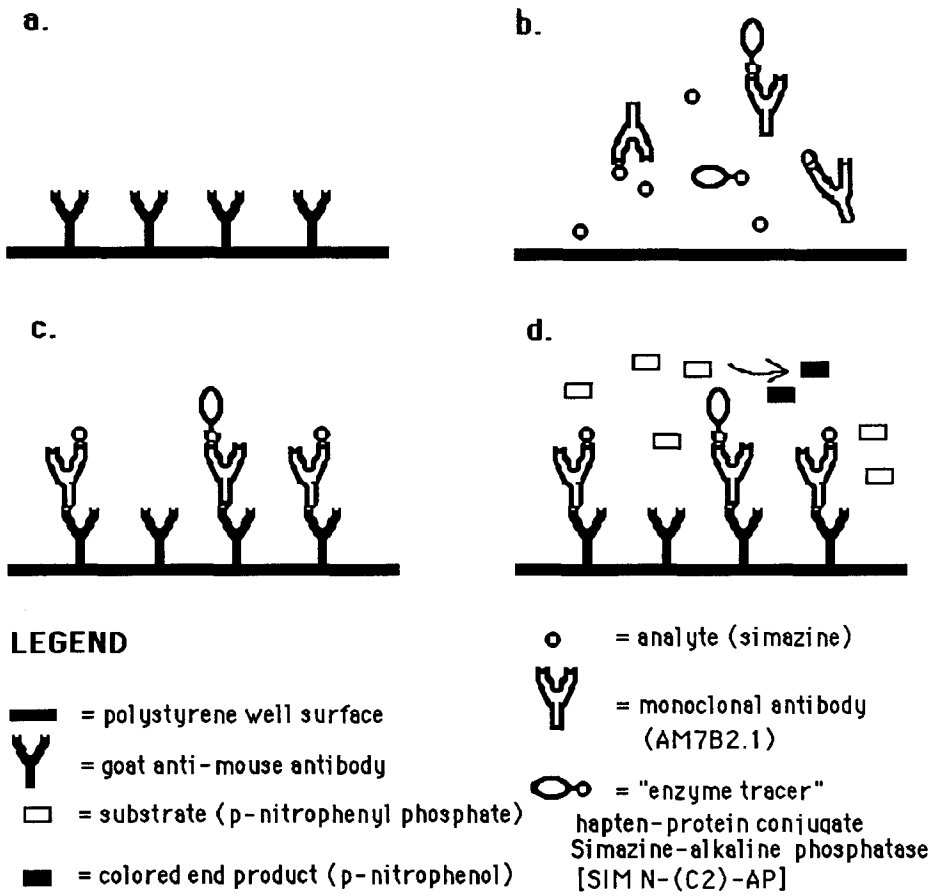

Figure 1. Double-antibody, competitive ELISA. a) Microtiter plate is coated with goat anti-mouse antibody. b) Analyte competes with hapten-enzyme conjugate for binding to monoclonal antibody. c) An aliquot of the mixture from (b) is added to the (a). Competition of analyte and haptenated-enzyme bound monoclonal antibody for goat anti-mouse antibody. d) Substrate is converted by the enzyme to yellow end-product.

To compare ELISA and GC analysis: Delhi and Yolo soils were spiked with simazine (in 25-250 uL methanol) at 10, 30, 50, 90, 100, 200, 300, 400, 500, 600, 700, 800, 900, and 1,000 ppb in 5 replicates, and samples were split for extraction and analysis within 24 h. Extraction and analytical conditions for GC were as follows: ethyl acetate (1 mL:1 g soil) was added to air-dried soil samples that were then shaken on a platform shaker for 15 min followed by ultrasonication for 5 min. The slurry was filtered through ca. 10 g of anhydrous sodium sulfate in a glass funnel. The funnel stem was packed with glass wool. The clear extract was analyzed within 48 h. Analysis was performed using capillary gas chromatography with a wide-bore, 30-m, 0.75-mm id, SPB-35 bonded phase column with nitrogen carrier gas. Inlet, oven, and detector temperatures were 240, 220, and 270°C, respectively. The detector used was a thermionic detector optimized for nitrogen

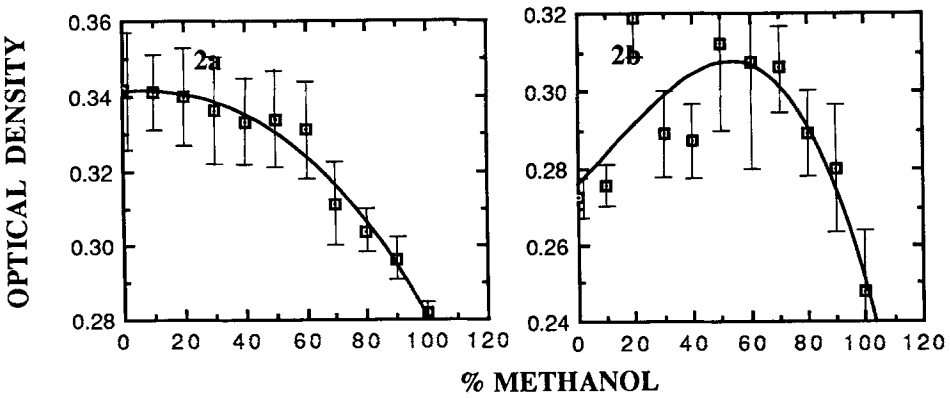

Figure 2. Effects of methanol (mean OD±SD) on a) enzyme tracer and b) complete ELISA.

response. Quantification was based on triplicate injections. For the ELISA analysis, as determined by prior experiment, 25 g of Delhi soil were extracted with 2 aliquots of 40% solvent: methanol+water (10+15 mL). Yolo soil was extracted with 60% methanol (methanol+water=15+10 mL). All statistical analyses were performed using the SAS® system (SAS 1987).

RESULTS AND DISCUSSION

Increasing methanol levels had an increasing effect on the enzyme tracer resulting in the suppression of hydrolysis of substrate to a color product (p-nitrophenol). This relationship was significantly fitted by a quadratic regression as shown in Fig. 2a ($F=48.52$; $df=2,41$; $p<0.0001$, $R^2=0.70$). In the complete ELISA with addition of simazine, there was a statistically significant quadratic relationship between percent methanol and OD (Fig. 2b; $F=25.96$; $df=2,80$; $p<0.0001$; $R^2=0.39$). The maximum OD was between 40-60% methanol, i.e., 3.4-5.1% methanol in the final ELISA volume. The enhanced signal obtained at 5% methanol was previously described for the antigen/antibodies interaction in the molinate assay (Gee et al. 1988). A moderate amount of methanol was reported to increase positive antigen/antibodies binding, decrease matrix effect, and reduce binding of analyte to interfering surfaces (Li et al. 1991).

The four-parameters (A, B, C, D) of the standard equation $y=A-D/\{1+[x/C]^B\}+D$ (Rodbard 1981) were not found to be significantly different for curves generated in PBSTA, methanol, Delhi, or Yolo blank-soil extract (multivariate analysis of variance, $p>0.34$). The equation relates concentration (x) to optical density (y), where A is the absorbance at zero dose, B is the curvature parameter, C is the concentration giving 50% inhibition, and D is the absorbance at infinite dose. Desirable mean parameter values comparable to Lucas et al. (1991) were obtained: A=0.46, B=1.21, C=25.21, and D=0.002. This indicated that the standard curve need not be generated using the blank-soil extracts as was shown necessary in a previous atrazine kit study (Goh et al. 1991). The Method Detection Limit (MDL) for Delhi was 5.3 ppb and Yolo 2.4 ppb. MDL was defined as 4.65 standard deviation (s) of blank responses (Kirchmer 1988).

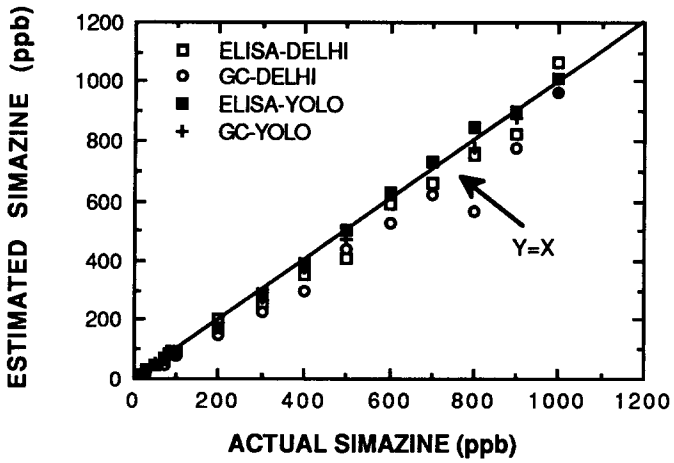

Figure 3. Agreement between estimated and simazine fortifications for ELISA and GC analyses of Dehli and Yolo soils.

In the extraction efficiency study only the percent methanol, and not the spiked simazine level, showed statistically significant effect on recovery for Delhi ($F=7.1$, $df=2,47$; $p<0.002$) and Yolo ($F=119.5$, $df=3,11$; $p<0.0001$) soils. The optimum levels of methanol for extraction were approximately 40 and 60% for Delhi and Yolo, respectively. These different optimum levels indicated the difference in soil types and pesticide interactions (mainly adsorption) and the resulting ease of extraction with solvents. Hence, for each soil type and pesticide species combination, an extraction efficiency and possible interference of co-extracts with ELISA should be evaluated.

Figure 3 shows the linear regressions for nominal spike level versus estimated level by ELISA and GC for Yolo and Delhi soils. The homogeneity of slopes test was run using weighted least squares estimation, with the inverse of estimated variance used as the weight because the variance was heterogeneous, i.e., dependent on spiked level. In this case, the variance increases with increasing spiked concentration. Both soil types and analytical methods gave different slopes of spike recovery ($F=19.7$, $df=1,54$, $p<0.001$, and $F=10.1$, $p<0.003$, respectively). The ELISA gave 93±11% (mean±SD) and 95±9% recoveries for spiked Delhi and Yolo soils, respectively. The GC method gave significantly poorer recovery for Delhi soil than Yolo soil at 85.4±12.5% and 100.8±11.8%, respectively. This is probably because the laboratory's GC extraction procedure and instrument conditions were previously optimized for routine analyses of Yolo soil samples; the ELISA, however, was individually optimized for Delhi (40% methanol extraction) and Yolo (60% methanol) soils. The recoveries with methanol extraction were comparable to that reported for atrazine at corresponding percentage methanol extraction, i.e., 85 and 94% recoveries for 40 and 60% methanol extractions, respectively (Huang and Pignatello 1990).

The results of sample analysis using ELISA were similar to GC analysis for two different soil types. The ELISA method used the more direct soil extraction method of methanol+water as extracting solvent.

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