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ELISA Regulatory Application: Compliance Monitoring of Simazine and Atrazine in California Soils

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Residues of atrazine and simazine have been found in California groundwater as a result of legal agricultural use. The continued use of these pesticides are reviewed under the authority of the Pesticide Contamination Prevention Act (State of California 1985). As part of the Department of Pesticide Regulation's (DPR) groundwater protection program, all uses of atrazine and non-crop uses of simazine have been prohibited in Pesticide Management Zones which are one square mile areas that are considered sensitive to groundwater pollution (State of California 1991). In order to monitor compliance with these prohibitions, a routine systematic soil sampling program has been implemented. This sampling program generates a large number of soil samples that could be rapidly and economically analyzed using ELISA (enzyme-linked immunosorbent assays). ELISAs have been used successfully for the analysis of atrazine (Goh et al. 1991) and simazine residues in California soils (Goh et al. 1992 a, b). This study was conducted to evaluate ELISA as compared to GC (gas chromatography) for the analysis of atrazine and simazine residues in soil samples taken for routine compliance monitoring.

MATERIALS AND METHODS

During 1991-92, 90 samples were randomly selected for split GC and ELISA analyses of atrazine and simazine. These samples were collected for compliance monitoring from Pesticide Management Zones of seven counties in California (Fresno, Glenn, Los Angeles, Merced, Orange, Riverside, and Tulare). Sites of varying soil types and uses were monitored (Table 1). At each site five samples of soil were collected at approximately 3-m intervals along a 16-m length marked-off with a measuring tape. Each sample consisted of a

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Table 1. Characteristics of soils from representative counties analyzed for atrazine and simazine for compliance monitoring in California, 1991-92.

County	Site	Composition (%)					Moisture[3] (%)	pH[3]
		Gravel[1]	Sand	Silt	Clay	Carbon[2]		
Los Angeles	Fence	15	64	14	7	0.2	10.7	8.0
	Rail	46	44	8	2	0.9	3.3	6.7
Fresno	Canal	0	66	26	8	0.6	2.3	5.7
	Roadside	5	76	16	3	0.9	0.7	5.6
Tulare	Rail	14	59	13	14	0.3	2.4	6.6
	Roadside	30	38	15	17	0.8	2.2	7.4
	Canal	9	70	14	7	0.4	2.6	5.5
	Fence	7	74	11	8	0.2	2.3	6.5
Glenn	Canal	31	15	35	19	0.4	7.4	7.6
	Roadside	51	35	8	6	0.7	1.9	7.4
	Fence	33	15	25	27	1.5	12.9	7.0

[1] Gravel >2.0 mm, sand 0.05-2.0 mm, silt 0.002-0.05 mm, clay <0.002 mm; Bouyoucos G.J. (1962).

[2] Organic carbon; Calif Fertilizer Assoc (1980)

[3] Hausenbuiller, RL (1972)

composite of three subsamples taken approximately 0.3-m apart in a triangular pattern. Subsamples were collected with a stainless steel cylinder (15.2-cm long and 5.3-cm id) at approximately 10 cm depth. If the soil was too compacted or gravelly for sampling with the cylinder, a clean steel-pick was used to loosen the soil in an area approximately the same diameter and depth as that collected with the tube. Then a clean scoop was used to remove the equivalent amount of soil. The three subsamples were combined in a large plastic bag, thoroughly mixed, weighed, poured into a 0.5-pint jar, capped and refrigerated until analyzed. A clean set of sampling instruments was used at each site to avoid cross contamination with triazine residues.

Procedures for extracting s-triazine from soil for ELISA were described previously (Goh et al. 1991). Briefly, 25 g of soil was extracted with 10 mL of methanol and 15 mL of water. Soil and solvents were shaken for 10 min at 200 rpm. Extraction was repeated once. The combined extract was filtered through a 0.2-µm nylon cartridge syringe filter (Gelman Sciences) using the Baker® 10 (J.T. Baker) vacuum system. The filtered extract was diluted ten-fold and thereby reduced the methanol content to less than 4% (Schneider and Hammock 1992).

The double-antibody, haptenated enzyme, competitive inhibition

ELISA is schematically represented in Fig. 1. The assay was run according to Format II of Schneider and Hammock (1992). Briefly, microtiter plates (Nunc #4-42404; Intermountain Scientific; Bountiful, UT) were coated with 100 µL/well of affinity-purified goat anti-mouse antibody (IgG and IgM; 1 mg/mL, Boehringer Mannheim #60524; Indianapolis, IN) diluted 1:2,000 in 0.5 M carbonate buffer (pH 9.6), sealed with an acetate plate sealer (Dynatech; Chantilly, VA) and incubated overnight at 4°C (Fig. 1a). After washing 5 times with PBSTA (0.2 M 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), the plates were then tapped dry and coated with 100 µL/ well of hybridoma culture fluid with monoclonal mouse anti-atrazine antibodies (AM7B2.1; 1:3,000 in PBSTA), sealed and incubated overnight at 4°C or 4 h at room temperature (Fig. 1b). After being washed 5 times with PBSTA, the plates were tapped dry and either used immediately or stored for several weeks at -20°C.

Fifty µL of standard or sample were added into each well followed with 50 µL of enzyme tracer (horseradish peroxidase-simazine hapten conjugate) (Schneider and Hammock 1992) and incubated for 15 min at room temperature. Plates were washed 5 times to removed the unbound immunoreactives. Color development was obtained by adding 100 µL aliqout of substrate buffer per well. The substrate buffer consisted of 200 µL of chromogen tetramethylbenzidine (TMB) (6 mg TMB in 1 mL of dimethyl sulfoxide) and 50 µL of 1% H_2O_2 in 12.5 mL of 0.1 M sodium acetate buffer (pH 5.5.). The color reaction was stopped after 25 min by adding 50 µL of 4N H_2SO_4. The plates were formatted with a 7-point standard curve, blanks, positive and negative controls, and 20 samples in triplicate (Fig. 2). The absorbances were measured at 450 - 650 nm in a Vmax® reader (Molecular Devices; Palo Alto, CA), and data were analyzed using Softmax® 2.01 software (Molecular Devices). The method detection level (MDL) for ELISA was 15 ppb and average recovery was determined to be greater than 90% (Goh et al. 1992b). The ELISA has the following cross reactivities to the s-triazines commonly used in California: 100% atrazine, 32% simazine, and 3% prometon. To be conservative, that is, to maximize the chances of detecting a chemical that has been illegally applied, ELISA are run using simazine standards, so that all simazine will be measured and atrazine overpredicted. Atrazine and simazine as well as prometon and breakdown products if present are reported as triazine and compared to the total atrazine and simazine detected by GC.

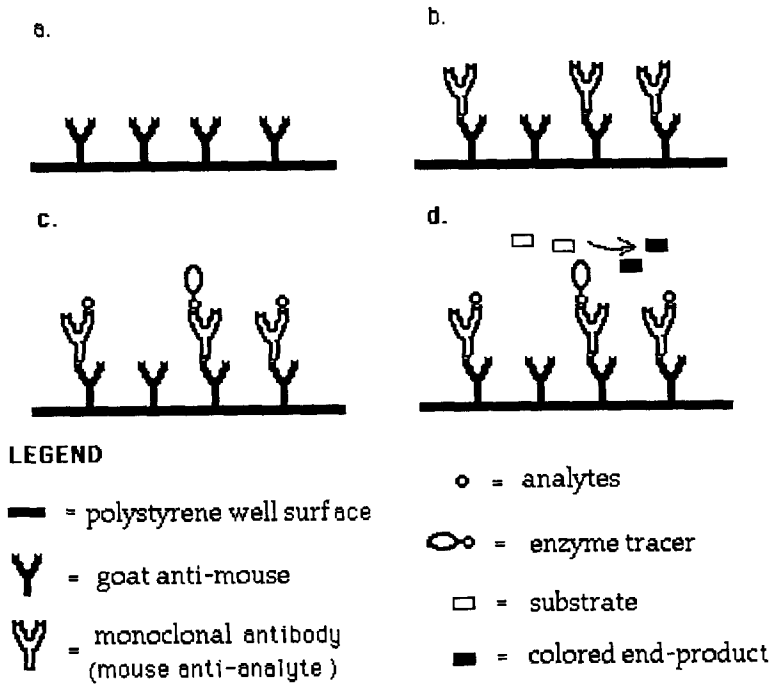

Figure 1. Double-antibody, competitive ELISA. a) Plate coating with goat anti-mouse antibody, b) trapping of monoclonal antibody AM7B2.1, c) competitive binding of analyte and hapten-enzyme conjugate for monoclonal antibody, and d) substrate conversion by enzyme tracer to blue color.

For GC analysis soil (25 g) was extracted using 50 mL of hexane:acetone (60:40) and 30 g of sodium sulfate by shaking for 2 h at 210 rpm on a G-10 Gyrotory shaker (New Brunswick Scientific; Edison, NJ). The extract was decanted through 20-g sodium sulfate on a #1 filter paper. Another 20 mL of hexane:acetone were added to the soil and extracted for 1-2 min, decanted and combined with the first extract. The filter funnel containing the sodium sulfate was washed with 10 mL of the solvents, and the final extract was brought to 75 mL. An aliquot (15 mL) of the extract was concentrated to 1 mL using a nitrogen evaporator (Myers Organomation Assoc., Inc.; South Berlin, MA) at 35°C. The sample was adjusted to 2 mL with hexane, and 2 g of sodium sulfate were added. The 2-mL sample was cleaned-up through a silica gel Sep-Pak® (Waters) which had been preconditioned with 4 mL of hexane. The hexane was discarded and the Sep-Pak® was centrifuged at 1100 rpm for 30 s to remove remaining hexane. The analyte was eluted from the Sep-

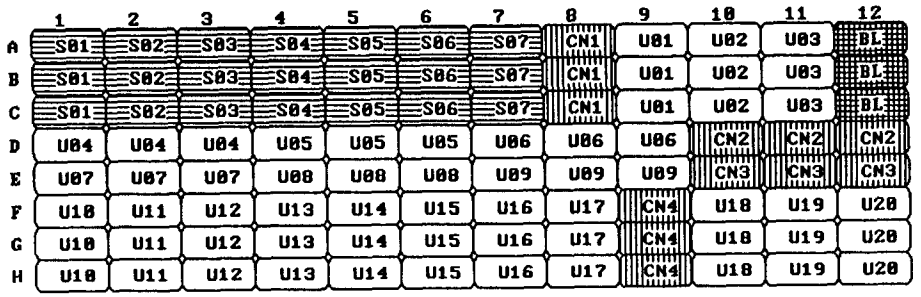

Figure 2. ELISA plate layout with wells designated for seven points calibration curve (three replications): S=Standard, S01=125 ppb, S02=31.3, S03=7.81, S04=1.95, S05=0.49, S06=0.12, S07=0.00; BL=blank; CN=control, CN1=10ppb, CN2=5, CN3=1.95, CN4=blank; U=unknown samples, U01-U20.

Pak® with 10 mL of methanol, and the eluant was concentrated to 3 mL using the nitrogen evaporator. The sample was analyzed using a Varian 3700 GC with a thermionic selective detector (Hewlett Packard) equipped with a FFAP 10M column, 30 m x 0.53 mm x 1.00-µm film, and helium as a carrier gas at 20 mL/min. The injector temperature was 230°C and detector was 250°C. The oven temperature was programmed at 10 deg C/min from 170 to 220°C with 1 min and 4 min hold times, respectively. The MDL (simazine or atrazine) was 10 ppb, and average recovery was determined to be 90%. ELISA and GC were compared using linear regression. Non-detect, false positive and false negative data were excluded from linear regression. Data were natural-log transformed as discussed in Goh et al. (1992a) using the Box-Cox family of power transformations (Draper and Smith 1981). All statistical analyses were performed using the SAS system (SAS Institute, Inc. 1987).

RESULTS AND DISCUSSION

Of the 90 split-samples, triazine was not detected in 35 samples at a MDL of 0.015 ppm for ELISA and 0.010 ppm for GC. Agreement of detections between methods was obtained for 39 samples. ELISA gave 15 false positives and one false negative when compared to GC results. For the 39 detections, triazine concentrations determined by ELISA and GC were correlated ($\ln y=1.01+0.82 \ln x$; $R^2=88.7\%$) (Fig. 3). Because the data violated the assumption of homogeneity of variance, natural log transformation was performed. The regression coeffient for the transformed data was significantly less than 1 (slope=0.82, t=-3.71, df=35, $p<0.00$) and the intercept was

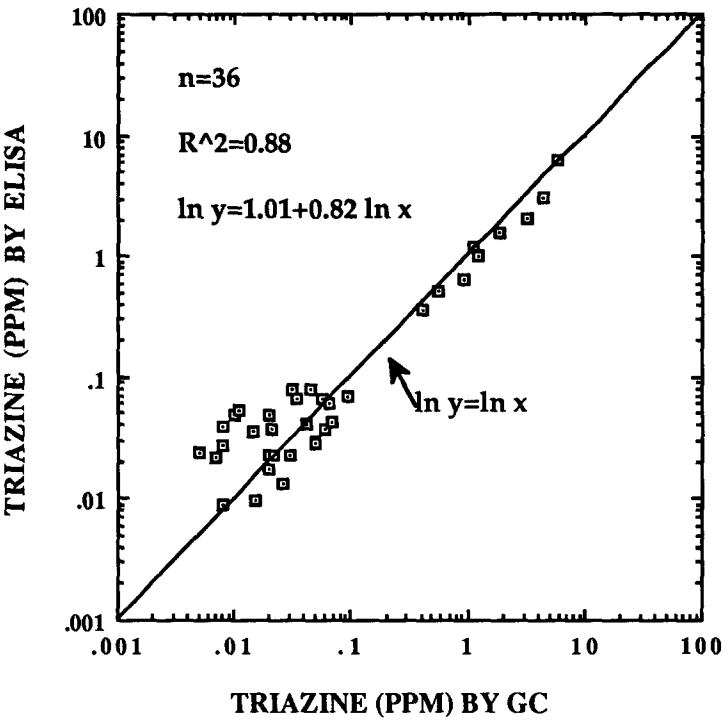

Figure 3. Regression of triazines concentration in soils as estimated by ELISA versus GC analysis.

significantly greater than 0 (intercept=1.01, t=4.37, df=35, p<0.00). These indicated that below 235 ppb, values generated by ELISA tended to be higher than GC values, but above 235 ppb ELISA tended to give lower values than GC. This general trend is in agreement with our earlier field soil studies (Goh et al. 1992b). The 15 ELISA false positives ranged from 0.01 to 0.07 ppm from four sampling sites. These are probably detections of prometon (3% cross reactive), trace amounts of triazine breakdown products (dealkylated or hydroxy triazines) or from interferences of soil constituents. The only false negative was a detection of 0.03 ppm of simazine by GC. These false positives and false negative are inconsequential because they are at least 1/60 the compliance guidance levels. The compliance guidance levels were estimated to be from 4.3 to 9.0 ppm based on the herbicide-label application rates that range from 5.4 kg ai/ha for deciduous and vine crops to 11.2 kg ai/ha for industrial and rights-of-way weed control, respectively. Estimates were also based on the assumption that herbicide distributed in the top 10 cm of soil and concentrations were at maximum as measured at time of application.

Two samples (ELISA=6.2, GC=5.7ppm; ELISA=3.1, GC=4.4 ppm; Fig. 3) approached or exceeded the compliance guidance levels of 4.3 or 9 ppm. These samples indicated a high probability that recent applications of atrazine/simazine had been made, violating prohibition of their use in the Pesticide Management Zone. This study demonstrated that the ELISA is a valid and rapid analytical tool for the initial screening of simazine and atrazine for some California soils for regulatory compliance monitoring. Only violative samples need be definitively confirmed by a second method using GC as well as mass spectrometry. By using this ELISA single plate versus previous double plate format (Goh et al. 1992b), incubation time was shortened from 4 h to 40 min for each run. Furthermore, for each sample, the ELISA extraction method generated only 20 mL of methanol waste compared to 86 mL of more hazardous hexane and acetone, and 10 mL of methanol waste for GC extraction. In addition, ELISA offered a cost savings of about $113 per sample (GC $150, ELISA $37) for a potential savings of $19,000 for each annual compliance monitoring study at the current sampling rate of 170 samples/year.

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