Evaluation of ELISA for the Multianalyte Analysis of s-Triazines in Pesticide Waste and Rinsate

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Immunoassay has been adapted for the analysis of pesticide waste and rinsate. Five s-triazine-specific monoclonal antibodies and three different enzyme-linked immunosorbent assay formats were evaluated for reactivity and sensitivity toward atrazine, simazine, and cyanazine. Three of the antibodies showed similar reactivity patterns toward the analytes. The two other antibodies had distinctively different patterns. Three antibodies that possessed different reactivity patterns were chosen for further assay format optimization and were evaluated for the ability to quantitate individual and total s-triazine analytes in a buffer mixture by solving simultaneous equations, each representative of individual antibody assays. This method was applied to pesticide waste and rinsate analysis. The quantities of individual and total s-trazines in actual pesticide waste samples containing atrazine, simazine, and cyanazine measured by immunoassay were compared to the quantities measured by high-pressure liquid chromatography. Correlation coefficients for the analysis of atrazine, simazine, cyanazine, and total s-triazine were 0.94, 0.90, 0.92, and 0.85, respectively.

INTRODUCTION

Large volumes of excess aqueous pesticide-containing materials are generated during normal agricultural operations. This waste is composed of excess pesticide product, leftover tank mixtures, and equipment rinsates and contains a combination of pesticide active ingredients, formulating agents, fertilizers, adjuvants, and wash-off debris. Pesticide concentrations can range from 10 000 to 1.0 ppm (Seiber, 1987), and improper disposal has been identified as a source of environmental contamination (Aharonson et al., 1987). On-site management of these materials includes reuse, recycling as subsequent make-up water, or, if necessary, disposal (Dwinell, 1992). If reuse or recycling is considered, pesticide content should be determined in the excess material to ensure product labeling restrictions are not exceeded in implemention. This requires the reliable detection of pesticide at or below its effective threshold level. Determination of pesticide content in the material is also needed for monitoring disposal processes. In each case, a simple analytical method for measuring pesticide content prior to reuse or during and following disposal would be useful.

Enzyme-linked immunosorbent assays (ELISAs) have been shown to be sensitive analytical methods for pesticide residue analysis (Van Emon et al., 1989) and are field adaptable (Bushway et al., 1988). These features make ELISAs particularly attractive for use in pesticide management applications. Existing immunoassay methods have been used as effective residue screening methods (Thurman et al., 1990), but because of differences in within-class reactivities (e.g., s-triazines; Bushway et al., 1988; Karu et al., 1991), their use for quantitating individual or total composition in a complex mixture has been limited. The cross-reactivity of immunoassays has been perceived by many analytical chemists as a disadvantage. However, with the increased availability of monoclonal antibodies with defined cross-reactivities and chemometric methods for data analysis, this feature can be utilized to design assays for predicting the amounts and types of cross-reactant analytes in a mixture.

The s-triazine class of herbicides is widely used in agriculture in a variety of applications. Recently, some s-triazines (atrazine, simazine, cyanazine) have been detected in a small percentage of groundwater samples at concentrations above health advisory levels (Parsons and Witt, 1988; Aharonson et al., 1987). Because of the importance of s-triazines in agriculture, the environmental concerns, and the availability of specific antibodies, this class of compounds was chosen for the current study.

An evaluation of ELISA for pesticide waste and rinsate analysis is reported here which considers sensitivity, specificity, and time and ease of analysis. An approach to quantifying individual and total s-triazine in a mixture is described. A series of monoclonal antibodies was screened for within-class specificity and sensitivity using competitive inhibition indirect ELISA, haptenated-enzyme ELISA, and modified haptenated-enzyme ELISA formats (Karu et al., 1991). Three antibodies with different within-class specificities were chosen and used with the modified ELISA format to quantitate individual and total analyte in a mixture by solving simultaneous equations derived from each of the antibodies. This technique was applied to the analysis of actual pesticide rinsates containing atrazine, simazine, and cyanazine. The results from ELISA and high-pressure liquid chromatography (HPLC) analyses were compared, and the correlation coefficients for atrazine, simazine, cyanazine, and total s-triazine found were 0.94, 0.90, 0.92 and 0.85, respectively.

MATERIALS AND METHODS

Immunochemicals. Mouse monoclonal s-triazine-specific antibodies (primary antibody) AM5C5.3, AM5D1.2, AM7B2.1, Am1B5.1, and SA5A1.1 were donated by Dr. A. E. Karu, Department of Plant Pathology, University of California, Berkeley, CA. The hybridoma cell culture supernatants were used unpurified. Alkaline phosphatase (AP), goat anti-mouse IgG (H+L) (GaMIgG; trapping antibody), goat anti-mouse IgG (H+L) conjugated to alkaline phosphatase (GaMIgG–AP), bovine serum albumin (BSA), and p-nitrophenylphosphate (enzyme substrate)

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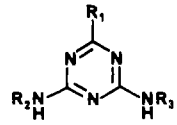
Table I. Structure and Nomenclature of s-Triazine Herbicides and Haptens



name	Cook system[a]	R_1	R_2	R_3
atrazine	CIET	Cl	$CH(CH_3)_2$	CH_2CH_3
simazine	CEET	Cl	CH_2CH_3	CH_2CH_3
cyanazine	CENT	Cl	CH_2CH_3	$C(CH_3)_2CN$
propazine	CIIT	Cl	$CH(CH_3)_2$	$CH(CH_3)_2$
	CEPrT	Cl	CH_2CH_3	$(CH_2)_2COOH$
	CIPrT	Cl	$CH(CH_3)_2$	$(CH_2)_2COOH$
	CIHeT	Cl	$CH(CH_3)_2$	$(CH_2)_5COOH$
	SPrIET	$S(CH_2)_2COOH$	$CH(CH_3)_2$	CH_2CH_3

[a] T, s-triazine ring; C, chlorine substituent; I, isopropylamino substituent; E, ethylamino substituent; N, cyanoisopropylamino substituent; Pr, aminopropanoic acid substituent; He, aminohexanoic acid substituent; SPr, thiopropanoic acid substituent. Adapted from Cook et al. (1981).

were purchased from Sigma Chemical Co. (St. Louis, MO). s-Triazine haptens and BSA protein conjugates were synthesized according to the procedures of Goodrow et al. (1990). Hapten–alkaline phosphatase enzyme conjugates CIPrT–AP and CEPrT–AP (Table I) were synthesized by active ester activation of the hapten followed by carbodiimide coupling to the enzyme (Langone and Van Vunakis, 1975).

Buffers. Phosphate-buffered saline (pH 7.5) containing Tween 20 and sodium azide (PBSTA) was used for dilution of immunoreagents and samples prior to immunoassay and for microtiter plate washing. Sodium carbonate buffer (pH 9.6) was used in coating microtiter plates with antibodies and hapten–BSA conjugates. Enzyme substrate buffer was diethanolamine, pH 9.8. The compositions of the various buffers used have been described in detail elsewhere (Lucas et al., 1991).

Equipment. The microtiter plates used were Nunc Immunoplate II Maxisorp (Nunc, No. 442964). Optical density (OD) measurements and calculations were made using a ThermoMax microplate reader with associated SOFTmax software (Molecular Devices Corp., Menlo Park, CA) on an IBM PC.

HPLC measurements were made using a Waters 712 WISP automatic sample injector, two Waters Model 510 HPLC pumps, a Waters Model 490 UV detector (210, 225, and 260 nm monitored), and NEC APC-IV controller with Maxima 820 software. The column was a Waters Nova-Pak 4 μm C-18 in a 8 mm × 10 cm radial compression module. The solvent system was 0–75% acetonitrile/phosphoric acid buffer (pH 2), 15-min gradient (Waters curve 6) at a flow rate of 2.0 mL/min. The final condition was maintained for 5 min.

ELISA Formats. For each of the assay formats, the amounts of the various immunochemical reagents used were determined by checkerboard titration. Coating of microtiter plates with antibodies and hapten–BSA conjugates was performed at 4 °C for 18 h. All other incubations were carried out at ambient room temperature. ELISAs 2 and 3 were adaptations from Karu et al. (1991).

Indirect Competitive Inhibition ELISA (ELISA 1). Twenty microliters of diluted primary antibody was mixed with 300 μL of sample in a separate microwell and incubated for 60 min. Fifty microliters of each mixture was applied to triplicate wells of an antigen-coated plate blocked with BSA and incubated 60 min. After a PBSTA wash, 100 μL of diluted GaMIgG–AP was added and incubated for 60 min. The plate was washed, and enzyme substrate (1 mg/mL in substrate buffer) was added. OD measurements (405–650 nm) were made 60 min following the addition of substrate. The relative sensitivities and within-class reactivities of the five antibodies were determined as described below. Coating antigen CIHeT–BSA was evaluated with all of the antibodies. Coating antigen SPrIET–BSA was evaluated using primary antibodies AM7B2.1, AM1B5.1, and SA5A1.1.

Standard Haptenated-Enzyme Competitive Inhibition ELISA (ELISA 2). One hundred microliters of diluted haptenated-enzyme and 100 μL of diluted primary antibody were added to 40 μL of sample and allowed to react for 60 min. Fifty microliters of each mixture was applied to triplicate wells of a trapping antibody-coated plate blocked with BSA. Primary antibody trapping proceeded for 60 min and was followed by a PBSTA wash and the addition of enzyme substrate. OD measurement was made 60 min following the addition of enzyme substrate. The relative sensitivities and within-class reactivities of the five antibodies were determined as described below. Haptenated-enzyme conjugates CEPrT–AP and CIPrT–AP and all of the primary antibodies were evaluated using this assay.

Modified Haptenated-Enzyme Competitive Inhibition ELISA (ELISA 3). Primary antibody dilutions were made in PBSTA containing 0.5 mg/mL BSA. One hundred microliters of this solution was applied to wells of a trapping antibody-coated plate, incubated for 60 min, and frozen with liquid remaining in the wells. When needed, the plates were thawed and washed with PBSTA. For competitive inhibition assays, 200 μL of diluted haptenated-enzyme was mixed with 40 μL of sample. Fifty microliters of each mixture was immediately added to triplicate wells of a trapping antibody/primary antibody-coated plate and reacted for 30 min. The plate was washed with PBSTA and then enzyme substrate added. The OD mesurement was made 30 min after the addition of enzyme substrate. The relative sensitivities and within-class reactivities of the five antibodies were determined as described below. Haptenated-enzyme conjugate CEPrT–AP and primary antibodies AM7B2.1, AM1B5.1, and SA5A.1. were evaluated using this assay.

Determination of Relative Sensitivities and Within-Class Reactivities. The relative sensitivities of the five primary antibodies were determined by assaying a dilution series of standard atrazine, simazine, cyanazine, and propazine in PBSTA and comparing the IC_{50}s (concentration of analyte which produces a 50% decrease in the maximum normalized response) generated from the four-parameter logistic curve fitting function in SOFTmax (parameter C). Within-class reactivities were interpreted relative to atrazine (=100%) according to the formula

$$\% \text{ reactivity} = (IC_{50} \text{ atrazine}/IC_{50} \text{ class analog}) \times 100 \quad (1)$$

Rationale for Discrimination of Individual Components in a Mixture. The experimental approach described here is based on the premise that the observed response of antibody binding to ligands present in a sample as measured by immunoassay (e.g., ELISA) is a "summed response" to all of the reactive ligands. This summed response is modified by each reactive ligand's "reactivity coefficient" toward the antibody. Therefore, the observed ELISA response would follow the equation

$$\text{ELISA response} = A(X_A) + B(X_B) + C(X_C) + ... + Z(X_Z) \quad (2)$$

where A, B, C, and Z are concentrations of the different analytes and X_A, X_B, X_C, and X_Z, are the reactivity coefficients of the analytes A, B, C, and Z, respectively. The ELISA response is expressed in the units used for the standard curve for one analyte (e.g., atrazine, reactivity coefficient = 1.00); therefore, reactivity coefficients for the other components would be relative to the analyte used in the standard curve (see previous section). By using one antibody (one equation) for each cross-reactive analyte in the mixture, it is possible to solve simultaneous equations to derive quantities of each analyte. The results from the analysis of a sample containing three cross-reactive analytes using three different antibody ELISAs were written in equation form as

$$\text{ELISA response Ab 1} = A(X_{A1}) + B(X_{B1}) + C(X_{C1}) \quad (3)$$

$$\text{ELISA response Ab 2} = A(X_{A2}) + B(X_{B2}) + C(X_{C2}) \quad (4)$$

$$\text{ELISA response Ab 3} = A(X_{A3}) + B(X_{B3}) + C(X_{C3}) \quad (5)$$

where, in eq 3, ELISA response Ab 1 is the amount determined by ELISA using Ab 1, expressed in units of the standard curve (i.e., micromolar atrazine), A, B, and C are the unknown concentrations of the analytes A, B, and C, and X_{A1}, X_{B1}, and X_{C1} are the known reactivity coefficients for antibody 1 for the analytes A, B, and C. Respective designations are also given to

Table II. Antibody and ELISA Format Characterization

antibody	ELISA format	ELISA antigen	IC_{50} atrazine, μM	% reactivity			
				atrazine	simazine	cyanazine	propazine
AM5C5.3	1	ClHeT-BSA	0.153	100	25	87	92
	2	CEPrT-AP	1.120	100	39	72	88
	2	ClPrT-AP	0.877	100	31	68	83
AM5D1.2	1	ClHeT-BSA	0.007	100	24	118	175
	2	CEPrT-AP	0.054	100	23	70	116
	2	ClPrT-AP	0.047	100	32	86	127
AM7B2.1	1	ClHeT-BSA	0.002	100	33	100	250
	1	SPrIET-BSA[a]	0.047	100	46	168	294
	2	CEPrT-AP	0.029	100	31	78	223
	2	ClPrT-AP	0.030	100	35	76	215
	3	CEPrT-AP	0.040	100	25	92	207
AM1B5.1	1	ClHeT-BSA	0.029	100	6	0	967
	1	SPrIET-BSA[a]	0.022	100	4	12	723
	2	CEPrT-AP	0.051	100	6	0	204
	2	ClPrT-AP	0.084	100	6	1	336
	3	CEPrT-AP	0.078	100	6	6	419
SA5A1.1	1	ClHeT-BSA[b]	4.780	100	141	0	122
	1	SPrIET-BSA	0.011	100	70	13	62
	2	CEPrT-AP	0.715	100	91	7	136
	2	ClPrT-AP	0.843	100	96	19	180
	3	CEPrT-AP	1.010	100	80	5	131

[a] Homologous antigen; hapten is same as that used in immunization. [b] Semihomologous antigen; hapten structure is very similar to that used in immunization.

eqs 4 and 5. The three equations were solved simultaneously for the unknown concentrations of analytes A–C by matrix inversion.

Determination of *s*-Triazines in a Mixture. Mixtures of 0, 0.5, and 1.0 μM atrazine, simazine, and cyanazine were made in PBSTA, resulting in 27 combinations of *s*-triazine composition. Three dilutions of each sample were assayed in triplicate wells using each of the antibodies AM7B2.1, AM1B5.1, and SA5A1.1 with ELISA 3. Concentrations of *s*-triazine were initially calculated as micromolar atrazine equivalents (based on a standard curve for atrazine for each assay) using the lowest sample dilution that gave an OD value within the working range of the assay, defined as 70–20% of the maximum normalized response (parameter A of the four-parameter logistic equation determined by SOFTmax). The analyte reactivity coefficients for each antibody were determined by fitting the data set to linear regression. The model used antibody response (micromolar, atrazine equivalents) as the dependent variable and the individual analyte concentration as the independent variable. The slope of the line is the reactivity coefficient. Individual single antibody ELISAs were evaluated by linear regression of a plot of the amount found by ELISA vs the expected response to total *s*-triazine added, defined as the sum of each individual analytes' reactivity coefficient multiplied by the amount of individual analyte added. Quantitation of individual *s*-triazine in each sample was made using the analyte reactivity coefficients for each antibody and solving three simultaneous equations (one per antibody) with three unknowns (one per analyte) by matrix inversion. Total *s*-triazine was calculated as the sum of the individual analytes. Estimation of individual and total *s*-triazines in the samples was evaluated by linear regression of a plot of added vs found.

Sampling and HPLC Analysis of Pesticide Waste and Rinsate (PWR). PWR samples were obtained from various collection facilities at the Beltsville Agricultural Research Center (BARC), ARS, USDA, Beltsville, MD, during the spring 1991 growing season. Samples were diluted 1:2 in acetonitrile and were analyzed by direct injection reversed-phase HPLC (Somich et al., 1990). Pesticide composition was determined using authentic analytical standards and utilizing farm records of spraying operations. Subsamples of each of the seven PWR samples were spiked to either 100 or 200 μM atrazine above the original concentration. In addition, three of the samples were spiked to either 100 or 200 μM simazine or cyanazine above the original concentration. All 39 samples were diluted 1:2 in acetonitrile and analyzed by HPLC.

PWR ELISA Analysis. The acetonitrile-diluted samples were further diluted in PBSTA and analyzed by ELISA 3 using antibodies AM7B2.1, AM1B5.1, and SA5A1.1. Each microplate (one for each individual antibody analysis) consisted of an atrazine standard curve (seven concentrations), six dilutions each of three samples (which ensured that at least one dilution produced an OD measurement within the working range of the assay) and three dilutions each of 1 μM simazine and 1 μM cyanazine to obtain reactivity coefficients for these analytes. Each sample dilution was assayed in triplicate wells.

Concentrations of *s*-triazine were initially calculated as micromolar atrazine equivalents (based on the standard curve for atrazine) using the two lowest sample dilutions which gave an OD value within the working range of the assay (defined above). For simplicity, the analyte reactivity coefficients for simazine and cyanazine were calculated directly as atrazine equivalents from the analysis of the 1 μM standards of simazine and cyanazine (atrazine = 1.00). Quantitation of individual *s*-triazine in each sample was made using the analyte reactivity coefficients for each antibody and solving three simultaneous equations (one per antibody) with three unknowns (one per analyte) by matrix inversion. Sample interferences were analyzed by testing the parallelism of sample dilutions with the standard curve for atrazine by linear regression. Individual single antibody ELISAs were evaluated by geometric mean regression (Sokal and Rohlf, 1981) of the amount found by ELISA on the expected response to total *s*-triazine determined by HPLC utilizing the individual antibody/analyte reactivity coefficients. Estimation of individual and total *s*-triazines in the samples was evaluated by geometric mean regression of the amount found by ELISA after simultaneous equations were solved on the amount determined by HPLC.

RESULTS AND DISCUSSION

Assay Format Characterization. The pooled results of screening the various antibodies using various assay formats for sensitivity and within-class reactivity is shown in Table II. Some useful trends were observed; however, meaningful statistical comparisons could not be made due to low replication. In general, the highest sensitivity was found when a heterologous hapten (different from the hapten used in animal immunization) was used as the ELISA hapten. These results could not be accounted for by a difference in hapten density on the BSA proteins (data not shown). Since an antibody usually has a lower affinity toward a heterologous hapten than toward a homologous one, less free analyte is required to inhibit

antibody binding to an immobilized heterologous ELISA antigen, hence a lower IC_{50}. For example, when AM7B2.1, in which the immunizing hapten was SPrIET (Karu et al., 1991), was used with ClHeT–BSA as coating antigen in ELISA 1, a much lower IC_{50} for atrazine was observed than when SPrIET–BSA was used. SA5A1.1 showed a similar trend; however, in this case SPrIET was the heterologous hapten. The structure of ClHeT is very similar to that of the immunizing hapten, possessing an isopropylamino group instead of an ethylamino group in the side chain. Use of heterologous haptens in s-triazine immunoassay development has been emphasized in previous studies (Goodrow et al., 1991; Harrison et al., 1991). The heterologous hapten ELISA 1 gave higher sensitivity than the haptenated-enzyme formats but could be accounted for by the different quantities of the immunoreagents used in the various formats. In ELISA 2, with antibodies AM1B5.1 and SA5A1.1, some improvement in sensitivity (lower IC_{50}) was observed when CEPrT–AP was used as the haptenated-enzyme instead of ClPrT–AP. Improved sensitivity was not observed with antibody AM7B2.1, and this is in contrast to results reported by Schneider and Hammock (1992). The latter result may be due to differences in the conditions used for hapten–enzyme conjugation that caused different hapten loading densities, which in turn could affect binding kinetics. It should be noted that antibody AM7B2.1, despite its complex reactivity pattern, was most sensitive for every individual s-triazine tested. This antibody is superior for use in a sensitive broad class screening assay. Recently, a very sensitive ELISA method for s-triazines in water and soil was developed which utilized a solid-phase extraction step and antibody AM7B2.1 (Lucas et al., 1991).

The antibody reactivity patterns did not change with assay format. In ELISAs 1 and 2, the primary antibody was reacted with free analyte and ELISA antigen while in solution, whereas in ELISA 3 the primary antibody is immobilized, frozen, and thawed prior to the competition step. This suggests that the hapten binding site of the primary antibody was not appreciably altered as result of the various immobilization chemistries involved in the ELISA formats. For the haptenated-enzyme formats, changing the alkylamino side chain of the s-triazine hapten from ethylamino (CEPrT) to isopropylamino (ClPrT) did not change the reactivity pattern of the antibodies toward the other analytes. This is to be expected when using a monoclonal antibody preparation. Overall, AM5C5.3, AM5D1.2, and AM7B2.1 showed very similar reactivity patterns toward atrazine, simazine, and cyanazine in each of the formats. AM1B5.1 and SA5A1.1 showed individually different patterns. The isopropylamino side chain of the substituted s-triazine appears to be the dominant binding epitope for the antibodies studied. The highest degree of binding was seen with the bis(isopropylamino)-substituted s-triazine propazine. For antibodies AM5C5.3, AM5D1.2, and AM7B2.1, substitution of the isopropylamino for cyanoisopropylamino resulted in only a slight decrease in binding, whereas an ethylamino substitution resulted in nearly a 70% decrease in binding. The same substitutions resulted in a near loss of recognition by antibody AM1B5.1. Antibody SA5A1.1 recognized the alkylamino side chains nearly equally; however, inclusion of a cyano moiety resulted in a loss of recognition. Because of their distinctive reactivity patterns, antibodies AM7B2.1, AM1B5.1, and SA5A1.1 were further evaluated using ELISA 3 with CEPrT–AP.

A savings of about 2 h in analysis time was achieved using ELISA 3 with a decrease in sensitivity compared to ELISA 2. The primary antibody was pretrapped on the ELISA plate and could be stored frozen for up to 60 days without loss of activity. This feature makes ELISA 3 more suitable for field use. However, when using three replicate wells per sample analysis, this format requires 3 times more antibody per sample compared to ELISA 2.

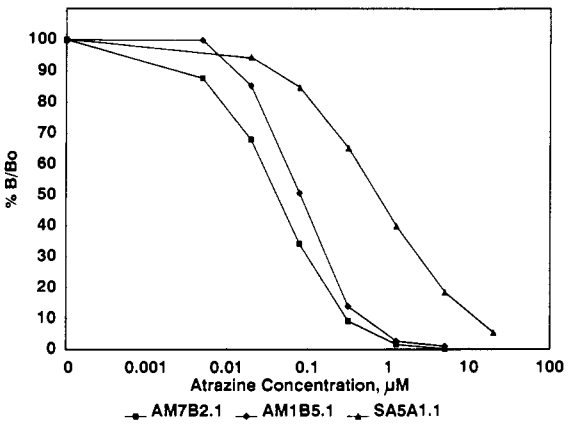


Figure 1. Atrazine competitive inhibition curves using ELISA 3, with antibodies AM7B2.1, AM1B5.1, and SA5A1.1 and haptenated-enzyme CEPrT–AP. Absorbance measurements were normalized by conversion to $\%B/B_0$ values according to the formula below. Differences in assay working ranges required different sample dilutions to be utilized in measurement.

$$\%B/B_0 = [(A_{sample} - A_{blank})/(A_{zero\ dose} - A_{blank})] \times 100$$

Table III. Working Parameters and Summed Response of Individual ELISAs in Analysis of s-Triazine Mixture in PBSTA

antibody	AM7B2.1	AM1B5.1	SA5A1.1
IC_{50} (% CV),[a] μM atrazine	0.04 (10.3)	0.08 (4.7)	0.68 (4.5)
working range, μM atrazine	0.13–0.02	0.25–0.04	3.52–0.23
reactivity coefficients[b] (SE)[c]			
atrazine	0.93 (0.03)	0.98 (0.03)	1.14 (0.04)
simazine	0.37 (0.03)	0.03 (0.03)	0.82 (0.04)
cyanazine	0.71 (0.03)	−0.04 (0.03)	0.06 (0.04)
regression (fnd/expctd)[d]			
slope (SE)	1.11 (0.04)	1.05 (0.03)	1.00 (0.03)
intercept	−0.07	0.00	0.00
R^2	0.97	0.99	0.97

[a] Percent coefficient of variation from four determinations. [b] Reactivity coefficients were determined for each antibody by linear regression within the data set of micromolar atrazine equivalents found on micromolar individual analyte added (three measurements). [c] Standard error. [d] Linear regression of the summed response to total triazine on the expected response to total triazine added (27 measurements) based on the reactivity coefficients.

Determination of s-Triazines in a Mixture. Atrazine competitive inhibition curves for each of the antibodies using ELISA 3 is shown in Figure 1. Since each antibody has different sensitivities toward the analytes (see IC_{50} s, Table III), it is necessary to use different sample dilutions for each of the assays. All of the assays showed acceptable reproducibility (IC_{50} %CVs ≤ 10.4). Upper and lower limits of the ELISA working ranges for atrazine are also given in Table III. The observed response of the various antibodies to total s-triazine added (expressed as atrazine equivalence) was compared to the expected response based on the analyte reactivity coefficients for that antibody. The independent analyte reactivity coefficients were generated by linear regression (three concentrations), and then individual antibody response was reanalyzed by linear regression over the entire data set which consisted of concentrations of the other analytes. These results are presented in Table III. The single antibody assays were highly accurate. Antibody reactivity toward a single

Table IV. Discrimination and Recovery of s-Triazines in PBSTA Mixture

analyte	slope[a] (SE)[b]	intercept	R^2
atrazine	1.06 (0.03)	0.00	0.98
simazine	0.88 (0.03)	0.00	0.81
cyanazine	1.07 (0.07)	0.01	0.92
total	1.04 (0.04)	−0.02	0.97

[a] Linear regression of amount found by ELISA (micromolar analyte) on amount added (micromolar analyte) (27 measurements). [b] Standard error.

analyte was relatively constant in the presence of cross-reacting analytes. This is important when a mixture of analytes, such as is encountered in waste samples, is analyzed. Table IV shows the recovery data for atrazine, simazine, cyanazine, and total s-triazines in the mixture after simultaneous equations were solved. Of the individual s-triazines, estimation of atrazine was most accurate and precise due to the high selectivity of AM1B5.1 for this analyte. Quantitation of simazine and cyanazine is less accurate as their estimates are dependent on the analyses with AM7B2.1, AM1B5.1, and SA5A1.1, resulting in a less precise measurement. Total s-triazine estimation was very accurate and precise. Although sensitivity toward any individual s-triazine is dependent on the most sensitive antibody (AM7B2.1), quantitation of s-triazines in a mixture is interdependent on the analyses using the three antibodies and is therefore determined by the least sensitive antibody (SA5A1.1). The detection limit for total s-triazine in the mixture was approximately 1 μM (216 ppb of atrazine). Although this level of sensitivity may not be suitable for residue analysis without a preconcentration step, it is more than sufficient for waste analysis where higher pesticide concentrations are normally encountered.

PWR Analysis. The results from HPLC analysis of the seven unspiked PWR samples are shown in Table V. Other pesticides reported to have been used in spraying operations but not detected by HPLC were metribuzin, permethrin, and trifluralin. In addition, propazine and the s-triazine degradates, hydroxyatrazine, deethylsimazine, deisopropylatrazine, and chlorodiamino-s-triazine, were not detected in the samples. The composition of PWR found may be typical of that found from a farm in corn, wheat, and soybean production.

Figure 2 depicts the summed response to s-triazine by ELISA using AM7B2.1. Geometric mean regression was used to compare ELISA to HPLC results to correct for the bias of the least-squares estimates caused by a random regressor. Geometric mean regression data are given in Table VI for this and each of the other antibodies based on the mean reactivity coefficients for simazine and cyanazine from 13 microplates per antibody (atrazine = 1.00). Measured response was highly correlated with s-triazine content with all antibodies. The variation in antibody atrazine IC_{50} determinations for the experiment was relatively high (% CVs ≤ 19.5) but could be attributed to day effects such as ambient temperature differences and pipetting errors. These effects were less important in the analyses of s-triazine mixtures in buffer (previous section) which were performed on the same day and resulted in less variability between assays. Variation between batches of trapping antibody/primary antibody-coated plates could have contributed to the differences observed in IC_{50} values reported in Tables II, III, and VI. These sources of variation were minimized because sample concentration estimates were based on an atrazine standard curve assayed on the same plate for each antibody ELISA. The upper and lower limits of the ELISA working ranges are given in Table VI.

Figure 3 illustrates the discrimination and recovery of atrazine in the PWR samples utilizing the response from the three antibodies and solving simultaneous equations. Geometric mean regression data of ELISA results on HPLC are given in Table VII. Quantitation of the individual analytes was highly correlated with HPLC results. The slopes obtained for atrazine and cyanazine were very close to 1.00, indicating a highly accurate measurement. Large errors occurred in the estimation of simazine, but these were probably due to the low amounts of this analyte relative to the others in the samples. Total s-triazine content estimated by ELISA (Figure 4) was highly correlated with HPLC results (Table VII) and resulted in a slope of 0.83. The intercepts of the geometric mean regression models were variable betwen analytes. Atrazine and cyanazine were present in relatively high concentrations in the unspiked samples (35–85 ppm, Table V) and gave large negative intercept values. Simazine was present in lower concentrations and resulted in an intercept value much closer to 0.00. Analysis of unspiked samples with lower concentrations of atrazine and cyanazine should also give intercept values closer to 0.00 for these analytes.

No sample interferences were detected by parallelism analysis of standard and sample dilution curves (data not shown). The high concentrations of analyte found in this sample type required a minimum 100-fold dilution (for SA5A1.1) to give OD measurements within the working range of the assay. This was probably effective in diluting out any potential sample matrix interferences if present. Examination of the slopes for regression of ELISA results on HPLC (Tables VI and VII) suggests that there may be an interaction effect present. Higher concentrations of pesticide are associated with higher concentrations of surfactants and adjuvants in waste materials. In addition, sample dilution may introduce an important source of assay error and may not be practical when low amounts of analyte are present. Further studies on the quantitative effect of specific waste components on ELISA are planned.

CONCLUSIONS

Immunoassay technology is particularly attractive for pesticide waste and rinsate analysis. To develop an ELISA for waste analysis in the field, sensitivity, specificity, and time and ease of analysis must be considered. Optimization of one parameter may have the effect of compromising another parameter. Therefore, when optimizing an ELISA format, one should continuously account for the important parameters in the process. The advantages of haptenated-enzyme ELISA formats (ELISAs 2 and 3) over an indirect ELISA format (ELISA 1) have been emphasized in other papers (Karu et al., 1991; Jung et al., 1988). Here, it was shown that the haptenated-enzyme format could be modified to further reduce analysis time without sacrificing specificity; however, some loss of sensitivity was observed. In addition, this is readily adaptable to a tube-based format (Bushway et al., 1988).

Waste materials usually contain a combination of pesticide active ingredients, in addition to other components which could potentially interfere with immunoassay results. Multianalyte analysis by immunoassay is limited by the specificity of the antibody used in the assay. Unless the antibody used can detect each analyte equally, the single assay cannot accurately estimate total composition. In this study, multiple antibodies with different within-class reactivities for each of the potential analytes were

Table V. Concentrations of Pesticides Detected in Unspiked Pesticide Waste and Rinsate (PWR) As Determined by High-Pressure Liquid Chromatography

sample	pesticide concentration, ppm						
	atrazine	simazine	cyanazine	dicamba	paraquat	metolachlor	2,4-D[b]
BW2B	45.4	ND[a]	63.9	ND	65.9	170.9	ND
BW2T	46.5	ND	55.2	ND	70.7	224.8	ND
BE1B	35.6	3.3	14.8	1.3	ND	13.5	ND
BE2M	39.0	5.4	50.0	16.4	58.6	64.2	ND
HF1M	43.8	ND	83.9	51.5	118.1	188.8	10.2
HF2M	42.1	ND	79.7	50.8	111.4	174.5	ND
HF3B	37.0	ND	71.8	16.6	67.9	179.6	ND

[a] Not detected at a detection limit of approximately 0.5 ppm. [b] 2,4-D free acid.

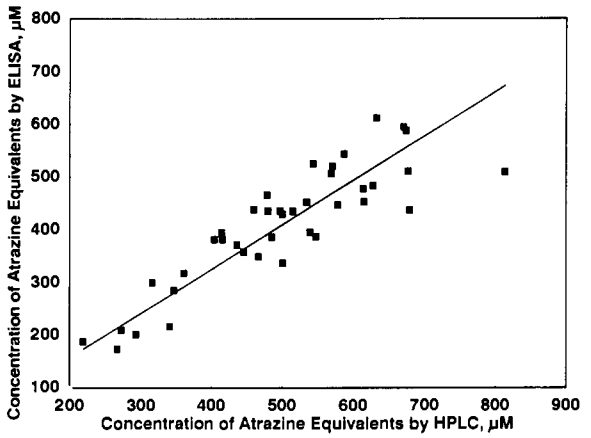


Figure 2. Summed response of AM7B2.1 in analysis of PWR. The expected response was calculated from HPLC results and reactivity coefficients generated for the antibody.

Table VI. Working Parameters and Summed Response of Individual ELISAs in the Analysis of s-Triazines in Pesticide Waste and Rinsate

antibody	AM7B2.1	AM1B5.1	SA5A1.1
IC_{50} (% CV),[a] μM atrazine	0.04 (19.5)	0.07 (16.0)	0.83 (14.0)
working range, μM atrazine	0.14–0.02	0.23–0.04	4.37–0.25
reactivity coefficients[b] SE[c]			
atrazine	1.00	1.00	1.00
simazine	0.33 (0.06)	0.08 (0.01)	0.76 (0.09)
cyanazine	0.78 (0.12)	0.01 (0.01)	0.03 (0.02)
regression (fnd/expctd)[d]			
slope (SE)[e]	0.84 (0.07)	0.98 (0.06)	0.82 (0.07)
intercept	−10.19	−38.55	−13.90
R	0.88	0.94	0.86

[a] Percent coefficient of variation from 13 determinations. [b] Reactivity coefficients were determined for each antibody as atrazine equivalents by analysis of a 1 μM solution of either simazine or cyanazine in PBSTA. The analysis was performed on the same microtiter plate on which the sample was analyzed. The value presented here is the mean from 13 determinations. [c] Standard error. Standard errors are not presented for atrazine reactivity coefficients due to the use of atrazine in the standard curve for the ELISAs. [d] Geometric mean regression of the summed response to total triazine on the expected response to total triazine as determined by HPLC (39 measurements) based on the reactivity coefficients. [e] Standard error approximation from least-squares estimates (Sokal and Rohlf, 1981).

used to estimate individual components within a sample by solving simultaneous equations. Individual and total s-triazine contents were accurately estimated in pesticide waste samples using this approach. This simultaneous equation method does not account for the random error associated with the measurement of the antibody reactivity coefficients or the ELISA response. However, the mathematical operations involved can be performed on some pocket calculators and may offer an advantage over more sophisticated pattern recognition techniques (Kauvar et al., 1992). A systematic comparison of the various analyte discrimination techniques would be valuable.

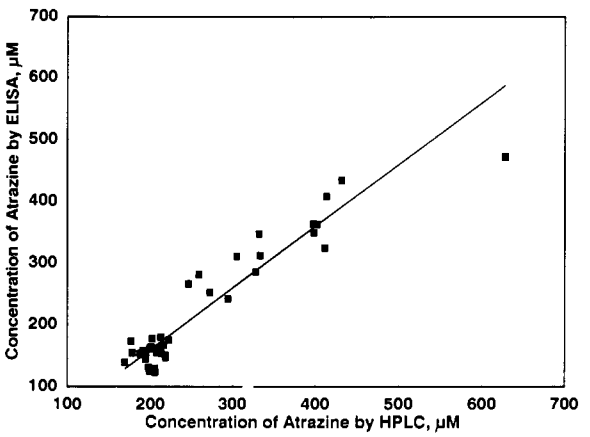


Figure 3. Atrazine discrimination and quantitation in PWR. Each analyte was discriminated and quantitated in the PWR samples by solving simultaneous equations derived from the ELISA response of three s-triazine-specific antibodies with different reactivity coefficients for the components in the sample.

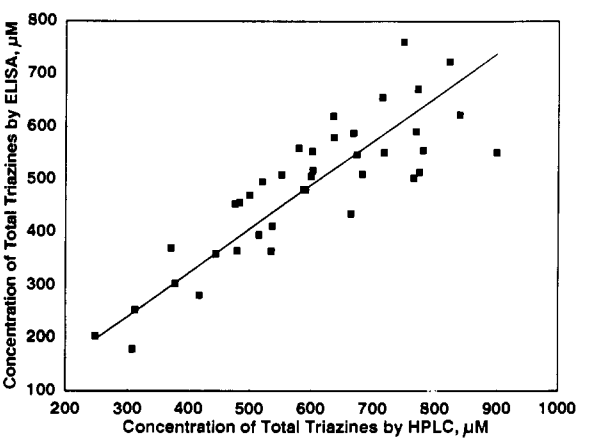


Figure 4. Total s-triazine quantitation in PWR. Total s-triazine quantitation was obtained by summation of the concentration of individual analytes.

Table VII. Discrimination and Recovery of s-Triazines in Pesticide Waste and Rinsate

analyte	slope[a] (SE)[b]	intercept	R
atrazine	1.00 (0.05)	−39.72	0.94
simazine	0.66 (0.05)	−5.00	0.90
cyanazine	1.19 (0.08)	−98.08	0.92
total	0.83 (0.07)	−7.80	0.85

[a] Geometric mean regression of amount found by ELISA (micromolar analyte) on amount determined by HPLC (micromolar analyte) (39 measurements). [b] Standard error approximation from least-squares estimates (Sokal and Rohlf, 1981).

In a pesticide waste disposal process monitoring situation, initial characterization of the waste materials would be performed using current chromatographic techniques such as HPLC. Once characterized, treatment could be

monitored using the current immunoassay. Total *s*-triazine composition would be estimated using a multiple-antibody assay and monitored with a more sensitive and simple single antibody assay, for instance, using antibody AM7B2.1, the latter assay calibrated by the first. In one pesticide waste treatment system which utilizes ozone to oxidize pesticides to more biodegradable products (Somich et al., 1990), loss of atrazine was slower than that of most other pesticides in the waste. By monitoring the loss of *s*-triazines, one could predict sufficient oxidation of the other pesticides, allowing for subsequent biodegradation. Therefore, it would not be necessary to monitor all of the various pesticidal components in the waste.

The use of multiple antibodies for multianalyte discrimination may have application in biosensor configuration, and the analytical response is conducive to more complex mathematical iterations. Future research will focus on integrating this analytical approach with disposal process monitoring.

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