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Performance Assessment and Validation of a Paramagnetic Particle-Based Enzyme-Linked Immunosorbent Assay for Chlorpyrifos in Agricultural Runoff Waters

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A commercial magnetic particle-based enzyme-linked immunosorbent assay (ELISA) kit for the insecticide chlorpyrifos [*O*,*O*-diethyl-*O*-(3,5,6-trichloro-2-pyridyl) phosphorothioate] was evaluated for its specificity, precision, and accuracy, its susceptibility to matrix interferences in agricultural and environmental surface waters, and its comparability to a gas chromatographic/flame photometric (GC/FPD) method for the determination of organophosphorus pesticides in natural waters. Repeatability, reproducibility, and accuracy studies show that the kit satisfies current U.S. Environmental Protection Agency criteria for the assessment of analytical methods. Observable matrix effects were found to be present in all of the environmental test waters, with the slopes of calibration curves generated in each of the test matrices deviating from that of the control matrix by as much as 16%. Specificity studies indicate that the chlorpyrifos polyclonal antibody adequately differentiates the target compound from other structurally similar organophosphorus pesticides, with the exception of its methyl analogue. Cross-reactivity with chlorpyrifos-methyl was approximately 37%, while reactivity with diazinon, pyridaphenthion, diclofenthion, bromiphos-ethyl, bromiphos-methyl, pirimiphos-ethyl, and chlorpyrifos oxon ranged from 1.6 to 10.7%. Cross-reactivity with pirimiphos-methyl, 3,5,6-trichloro-2-pyridinol, diethyl phosphate, and diethyl thiophosphate was negligible (<1%). Validation of the paramagnetic particle ELISA format was accomplished using water samples from two monitoring studies that were collected, split, and analyzed directly by ELISA and by GC/FPD. Results of the two analytical methods were then compared using standard *t* tests, regression analysis, and differences against mean measurement (bias) plots. While the agreement between the two methods was determined to be satisfactory, ELISA exhibits consistent positive bias in environmental matrices. Several preanalysis mitigation steps were suggested that may help moderate bias, but additional study is recommended to explicate the exact factors responsible for its consistent overestimation of results.

KEYWORDS: ELISA; chlorpyrifos; paramagnetic particles; performance evaluation; matrix effects; comparative validation

INTRODUCTION

Chlorpyrifos [*O*,*O*-diethyl-*O*-(3,5,6-trichloro-2-pyridyl) phosphorothioate] is a nonselective organophosphorus insecticide widely used in California agricultural production with 920545 kg of active ingredient applied in 2005 (*1*). Because of the concern of exposure to children, certain uses of chlorpyrifos such as for urban pest control are being phased out under the U.S. Food Quality Protection Act (*2*). In California, widespread detection of chlorpyrifos in surface water and associated toxicity to aquatic macroinvertebrates such as *Ceriodaphnia dubia* has caused the listing and regulation of ca. 25 water bodies under the U.S. Clean Water Act's [Section 303(d)] Total Maximum Daily Load and has caused them to be placed under re-evaluation by the California Department of Pesticide Regulation (CDPR) to mitigate the problems (*3*). In addition, the California State Water Resources Control Board has imposed the implementation of best management practices in mitigating agriculture pesticides that could potentially discharge to surface water from these nonpoint sources (*4*). These activities require intensive monitoring, which generates large sample loads.

Conventional analytical methods for the determination of pesticides in environmental water samples, for example, gas chromatography (GC), high-pressure liquid chromatography (HPLC), and mass spectroscopy (MS), have become increasingly costly and time-intensive. Over the past decade, enzyme-linked immunosorbent assays (ELISA) have become an important alternative detection method for pesticides, particularly for the analysis of large numbers of samples and as a screening tool.

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Immunoassays are characteristically rapid, sensitive, and reliable and are generally cost-effective for large sample loads (*5*). CDPR has routinely used ELISAs developed in-house for monitoring herbicide residues in compliance monitoring as well as research studies (*6*−*9*). This paper describes the evaluation of a commercially available magnetic particle-based ELISA kit for the determination of chlorpyrifos in surface waters. The objectives of this study were to (i) evaluate the kit for sensitivity, precision, accuracy, matrix effects, and specificity and (ii) compare the quality of ELISA results to those obtained by a typical liquid−liquid extraction and GC methodology.

MATERIALS AND METHODS

Matrix Effects and Comparative ELISA/GC Analysis. *Sampling.* Environmental water samples used in this study were collected as part of ongoing CDPR monitoring projects assessing surface water quality of agricultural watersheds in California. Organophosphorus-free surface water samples (previously evaluated using a California Department of Food and Agriculture organophosphate/organonitrogen multiresidue screen for 14 currently used organophosphates) from four surface water/agricultural runoff field sites in Placer County, CA (North Fork American River), Stanislaus County, CA (Pomelo drain, Del Puerto Creek), and San Joaquin County, CA (Walthall slough), were collected for matrix interference analysis. Water samples utilized for comparative ELISA/GC analysis were acquired from two recent monitoring studies conducted by CDPR to assess new proposed chlorpyrifos mitigation practices. CDPR Study 235 (http://www.cdpr.ca.gov/docs/sw/caps/swprot235.pdf) tested the use of constructed vegetated ditches to reduce chlorpyrifos runoff load from irrigated crops. CDPR Study 231 (http://www.cdpr.ca.gov/docs/empm/pubs/protocol/sw231protocol.pdf) evaluated the efficacy of polyacrylamide (PAM) in reducing chlorpyrifos loadings in sediment and surface water runoff from furrow-irrigated fields. Treated runoff and irrigation waters from these studies were collected as either duplicate (Study 231) or split (Study 235) samples. In both cases, half of the samples were transported from the field to the California Fish and Game Fish and Wildlife Water Pollution Control Laboratory (Rancho Cordova, CA) for GC analysis and half were transported to the laboratory and storage facilities at the CDPR West Sacramento Field Office (West Sacramento, CA) for ELISA analysis.

Sample Preparation and GC Analysis and Apparatus. GC analysis was performed by the California Fish and Game Fish and Wildlife Water Pollution Control Laboratory using a modified version of EPA Method 8141A, Organophosphorus Compounds By Gas Chromatography: Capillary Column Technique. Sample preparation and analytical conditions for GC analyses were as follows: Water samples were stored in amber glass bottles and refrigerated at 4 °C from the time of collection until extraction. Water samples were removed from the refrigerator, and the contents were transferred to a clean 2 L separatory funnel. A measured volume of sample (1000 mL) was extracted with methylene chloride in the separatory funnel. The extract was dried with sodium sulfate, evaporated using a Kuderna−Danish (K-D) apparatus, and solvent exchanged into petroleum ether. The extract was concentrated with a Micro Snyder (micro K-D) distilling column to approximately 1 mL and adjusted to 2.0 mL with isooctane. The extracts were analyzed by GC using conditions permitting the separation and measurement of the target analyte in the extracts by flame photometric detection (FPD). Florisil column cleanup or gel permeation chromatography (GPC) procedures were followed to eliminate or reduce interferences in very dirty samples whenever such samples were encountered. GC analysis was performed on a Agilent 6890 GC (Santa Clara, CA) equipped with a FPD in phosphorus mode and using 30 m × 0.32 mm i.d. × 25 μm film thickness DB5 and 30 m × 0.32 mm i.d. × 25 μm film thickness DB17 columns with helium as a carrier gas at a flow rate of 1 mL/min. The injector and detector temperatures were 200 and 225 °C, respectively. The column temperature was held at 90 °C for 1 min, programmed to 220 °C at 8 °C min^{-1} and held for 5 min, and programmed to 250 °C at 20 °C min^{-1} and held for 13 min. The injection volume was 3 μL. The GC method described above has a method detection limit (MDL) of 0.020 μg L^{-1} for chlorpyrifos.

ELISA Analysis. A chlorpyrifos RapidAssay kit (Strategic Diagnostic, Inc., Newark, NJ) was employed for the ELISA analyses performed in this study. The chlorpyrifos kit is a 100 tube magnetic particle design and has a detection range of 0.22−3.50 μg L^{-1}. For the comparative evaluation of ELISA and GC methodologies for surface water samples, immunochemical analysis was conducted according to instructions included with the kit using provided reagents. These reagents included chlorpyrifos antibody (mouse monoclonal antichlorpyrifos) covalently bound to paramagnetic particles in saline solution, chlorpyrifos-horseradish peroxidase (HRP) labeled enzyme conjugate, a color solution of hydrogen peroxide and 3,3′,5,5′- tetramethylbenzidine in an organic base, stopping solution of sulfuric acid (0.5%), washing solution, chlorpyrifos standard solutions (0.22, 1.0, and 3.0 μg L^{-1}), and a chlorpyrifos control solution (approximately 1.8 μg L^{-1} in saline solution). Spectrophotometric measurements for the chlorpyrifos RapidAssay tube kit samples were determined at 450 nm with a multiple wavelength, benchtop RPA-I RapidAnalyzer spectrophotometer (Strategic Diagnostic, Inc.). A two-piece magnetic separation rack (Strategic Diagnostic, Inc.) consisting of a 60-position tube rack that fits over a paramagnetic base is necessary for holding the magnetic particles in the tubes after incubation with enzyme conjugate and allowing unbound reagents to be decanted. Eppendorf deep well microtiter plates (Eppendorf Scientific, Inc., Hamburg, Germany) and an Eppendorf Titermate multichannel pipet (Brinkman Instruments, Inc., Westbury, NY) were used for performing serial dilutions in the specificity study. An Eppendorf Series 2000 adjustable volume (100−1000 μL) reference sampling pipet (Brinkman Instruments, Inc.), an Eppendorf Research Pro adjustable volume (20−300 μL) pipet (Brinkman Instruments, Inc.), and an Eppendorf Repeater 4780 repeating pipet (Brinkman Instruments, Inc.) were used to dispense liquids.

Chlorpyrifos Magnetic Particle Tube Kit Procedure. Disposable test tubes were labeled and placed in the tube rack. A 250 μL amount of the provided negative control and calibrators and 250 μL of unknowns to be analyzed were deposited in their respective tubes, after which 250 μL of HRP-labeled enzyme conjugate and 500 μL of chlorpyrifos antibody coupled paramagnetic particles were added. Each tube was vortexed for 1−2 s, and the mixture was allowed to incubate for 15 min at room temperature. At the end of the incubation period, the tube rack containing standards and samples and the paramagnetic base were joined and the magnetic particles in solution were allowed to separate for 2 min. Holding the base and the rack together, the contents of the tubes were decanted and the tubes were gently blotted to remove excess solution. One milliliter of washing solution was added and allowed to stand for 2 min. Tubes were then decanted and blotted, and the wash step was repeated two more times. After the tubes were thoroughly washed, the tube rack was removed from the paramagnetic base and 500 μL of color solution was added to all tubes. Tubes were vortexed for 1−2 s and incubated at room temperature for 20 min. After incubation, 500 μL of stopping solution was added to each tube, and results were read at 450 nm on the RPA-I photospectrometer. Because the HRP-labeled chlorpyrifos analogue was in competition with unlabeled chlorpyrifos in the samples for antibody sites on the paramagnetic particles, the color development was inversely proportional to the concentration of chlorpyrifos in the samples.

Evaluation of Kit Performance and Specificity. *Chemicals.* Certified analytical standards of chlorpyrifos-ethyl [99% *O*,*O*-diethyl-*O*-(3,5,6-trichloro-2-pyridyl) phosphorothioate], chlorpyrifos-methyl [99.5% *O*,*O*-dimethyl-*O*-(3,5,6-trichloro-2-pyridyl) phosphorothioate], pyridaphenthion [98.7% *O*-(1,6-dihydro-6-oxo-1-phenylpyridazin-3-yl) *O*,*O*-diethyl phosphorothioate], diazinon [98.1% *O*,*O*-diethyl-*O*-(2-isopropyl-4-methyl-6-pyrimidyl) phosphorothioate], diclofenthion (98% *O*-2,4-dichlorophenyl *O*,*O*-diethyl phosphorothioate), pirimiphos-methyl (99.5% *O*-2-diethylamino-6-methylpyrimidin-4-yl *O*,*O*-dimethyl phosphorothioate), bromiphos-ethyl (97% *O*-4-bromo-2,5-dichlorophenyl *O*,*O*-diethyl phosphorothioate), bromiphos-methyl (98% *O*-4-bromo-2,5-dichlorophenyl *O*,*O*-dimethyl phosphorothioate), pirimiphos-ethyl (97.5% *O*-2-diethylamino-6-methylpyrimidin-4-yl *O*,*O*-diethyl phosphorothioate), 3,5,6-trichloro-2-pyridinol (99%), diethyl phosphate (99.3%), diethyl thiophosphate (98%), and chlorpyrifos-oxon [*O*,*O*-diethyl-*O*-(3,5,6-trichloro-2-pyridyl) phosphate] were obtained from Chem Service, Inc. (West Chester, PA). All analytical standard stock

solutions were prepared in HPLC grade methanol (Fisher Scientific, Fair Lawn, NJ).

Standard and Spike Preparation for Performance Evaluations. For the evaluation of the kits for accuracy, precision, and reproducibility, nine fortified samples were prepared from stock solutions with organic-free, Nanopure (Barnstead, Newton, MA) distilled water at chlorpyrifos concentrations of 0.15, 0.25, 0.50, 1.0, 1.5, 2.0, 2.5, 3.0, and 3.5 μg L^{-1}. To establish an experimental limit of detection, the lowest concentration investigated (0.15 μg L^{-1}) was slightly below the reported lower linear range of the kit (0.22 μg L^{-1}). Similarly, the upper limit was evaluated by making the highest standard (3.5 μg L^{-1}) somewhat above the reported maximum range of the kit (3.0 μg L^{-1}). For the evaluation of the kits for potential matrix effects, six fortified samples (0.25, 0.50, 1.0, 2.0, 2.5, and 3.0 μg L^{-1}) were prepared with organic-free, Nanopure distilled water and with several OP-free agricultural and natural surface waters from various sources in Sacramento, Stanislaus, and San Joaquin Counties, California. All environmental test waters were gravity filtered using Whatman #2 filter paper (Whatman, Inc., Florham Park, NJ). Standards and spikes were made from 100 μg L^{-1} working stock solutions prepared from certified chlorpyrifos analytical standards provided by Chem Service, Inc.

Spike Preparation for Cross-Reactivity Studies. Analytical standard solutions of chlorpyrifos-ethyl (1.002 mg/mL), chlorpyrifos-methyl (1.013 mg/mL), pyridaphenthion (0.981 mg/mL), diazinon (1.041 mg/mL), diclofenthion (0.971 mg/mL), pirimiphos-methyl (1.070 mg/mL), bromiphos-ethyl (0.338 mg/mL), bromiphos-methyl (0.514 mg/mL), pirimiphos-ethyl (0.980 mg/mL), 3,5,6-trichloro-2-pyridinol (1.035 mg/mL), diethylphosphate (1.002 mg/mL), diethylthiophosphate (1.002 mg/mL), and chlorpyrifos-oxon (1.002 mg/mL) were used to prepare spikes for the analysis of kit selectivity. All fortified samples were made from 100 μg L^{-1} working stock solutions prepared in Nanopure organic-free, distilled water. Solutions having concentrations extending eight orders of magnitude were prepared serially and run in triplicate. Spiked concentrations of 0.0256, 0.128, 0.64, 3.2, 16, 80, 400, 2000, and 10000 μg L^{-1} were used for obtaining standard curves. Assays were performed according to the procedures described earlier, and percent cross-reactivities (% CR) were determined from the formula

$$\% \text{ CR} = (\text{IC}_{50} \text{ chlorpyrifos}/\text{IC}_{50} \text{ structural analogue}) \times 100$$

where IC_{50} is the amount of substance that displaces 50% of the labeled antigen (kit enzyme conjugate) as compared to the IC_{50} of the antigen (chlorpyrifos). The IC_{50} is thus the effective concentration of analyte that results in 50% enzyme conjugate inhibition. IC_{50} values for each cross-reactant were generated from a four-parameter logistic fit of experimentally determined absorbances vs spike concentration data. Logistic dose−response (with variable slope) analysis was performed using OriginPro version 7.5885 for Windows (OriginLab Corp., Northampton, MA). The equation for the four-parameter dose−response model used for the analysis of ELISA data is

$$y = A_1 + \frac{A_2 - A_1}{1 + 10^{(\log X_0 - X)p}}$$

where y is the absorbance, X is the logarithm of analyte concentration, A_2 is the upper asymptote (zero dose), A_1 is the lower asymptote (infinite dose), p is the slope (hill slope), and log X_0 is the central point of the linear portion of the curve, that is, the IC_{50}. This equation is identical to the four-parameter logistic equation described by Rodbard (*10*). Standard dose−response curves resulting from a four-parameter data reduction scheme are sigmoidal in shape. Both the upper and the lower asymptotes must be well-defined in sigmoidal dose−response relationships to ensure accurate IC_{50} values (*11*).

RESULTS AND DISCUSSION

Performance Evaluations. *Standard Curve and Kit Sensitivity.* The standard curve used to investigate the sensitivity range of the RapidAssay chlorpyrifos magnetic particle kit is shown in **Figure 1**. The standard curve was based on triplicate samples in DI water and was linearly transformed using a log−linear curve fit as instructed by the kit manufacturer. Although the manufacturer recommends a lower limit for the ELISA linear range of 0.22 μg L^{-1}, a linear response was observed for the standards that ranged down to the reported least detectable dose (LDD, 0.10 μg L^{-1}). Precision was also excellent for the low concentration DI spike samples. For example, the mean absorbance standard error of the 0.15 μg L^{-1} spike was 0.976 ± 0.0419 (n = 6). To determine kit sensitivity, an experimental LDD was calculated as three times the mass equivalent of the standard deviation of four replicate sample blanks (negative controls) from its mean absorbance (*12*). The resulting value (0.107 μg L^{-1}) indicates that the sensitivity of the kit is equivalent to that reported by the manufacturer (i.e., 0.10 μg L^{-1}).

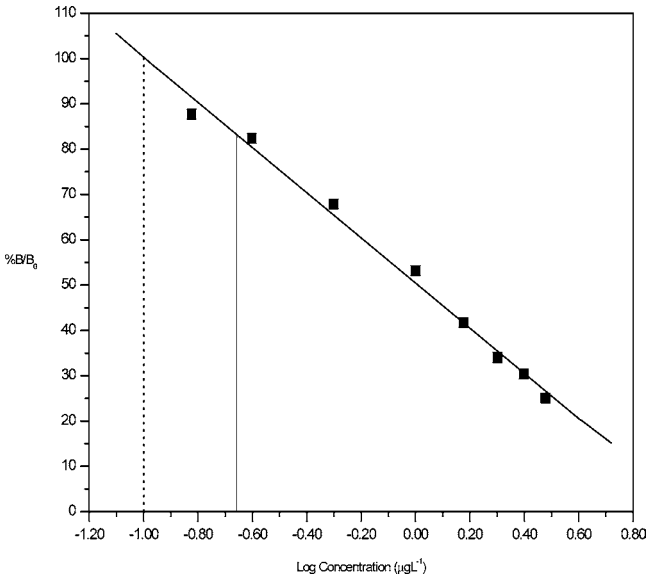


Figure 1. Standard curve (semilog scale) for chlorpyrifos used for the calibration of the RapidAssay ELISA kit. The standard curve was based on triplicate samples in DI water and was linearly transformed using a log–linear curve fit as instructed by the kit manufacturer. The dotted and solid lines correspond to the LDD (0.10 μg L^{-1}) and the lower detection limit (LDL, 0.22 μg L^{-1}) of the kit, respectively, as determined by the manufacturer. The dotted line also approximates the experimentally determined MDL of the kit (0.104 μg L^{-1}). The experimentally determined LDD (0.107 μg L^{-1}) is approximately equal to that established by the manufacturer. The equation of the line is $y = -49.82x + 50.45$ (n = 8, r^2 = 0.992, and $p < 0.0001$).

Kit Repeatability and Reproducibility. Intra-assay precision was tested by conducting six replicate analyses at nine concentrations ranging from 0.15 to 3.50 μg L^{-1} on a single rack in DI water. The mean absorbances and standard deviations were determined, and the percent coefficients of variation (% CV) were used to establish the precision of antibody response at each concentration. Quality control estimations for the RapidAssay kit demonstrate that the kit performs within a satisfactory range of variability (**Table 1**). The mean % CVs for intra-assay precision measurements (repeatability) and recoveries (accuracy) at all spike levels were 8.4 and 101.26%, respectively. Interassay precision (reproducibility) from three independent analyses on three different days had a CV of 15.1% (**Table 2**).

The lowest spiked concentration (0.15 μg L^{-1}) was used to calculate the MDL for the chlorpyrifos kit, where MDL is defined as the minimum concentration of a substance that can be measured and reported with 99% confidence that the analyte concentration is greater than zero. The MDL is determined by multiplying the appropriate one-tailed 99% t-statistic by the

Table 1. Intra-assay Reproducibility and Accuracy of ELISA Kit Spiked with Chlorpyrifos in DI Water at Nine Concentrations and Assayed Six Times[a]

spike level (μg L^{-1})	mean ABS	SD	% CV	mean concn (μg L^{-1})	SD	% CV	% recovery
3.50	0.268	0.0339	12.660	3.542	0.2818	7.96	101.21
3.00	0.315	0.0474	15.079	2.939	0.5862	19.95	97.96
2.50	0.339	0.0373	11.023	2.650	0.3480	13.13	106.01
2.00	0.379	0.0375	9.910	2.230	0.2645	11.86	111.48
1.50	0.464	0.0213	4.590	1.543	0.0601	3.90	102.83
1.00	0.592	0.0304	5.137	0.898	0.0570	6.35	89.84
0.50	0.755	0.0445	5.894	0.455	0.0810	17.80	90.96
0.25	0.916	0.0676	7.381	0.231	0.0416	18.06	92.25
0.15	0.976	0.0419	4.290	0.178	0.0309	17.36	118.76

[a] The acronyms ABS, SD, and CV represent absorbance, standard deviation, and coefficient of variation, respectively.

Table 2. Interassay Reproducibility of Chlorpyrifos ELISA Kit in Nanopure Water at Nine Concentrations and Assayed 20 Times over Three Analyses Performed on Three Separate Days

spike level	mean absorbance	standard deviation	% CV
3.50	0.381	0.0803	21.05
3.00	0.406	0.0679	16.71
2.50	0.455	0.0849	18.65
2.00	0.479	0.0786	16.39
1.50	0.535	0.0682	12.75
1.00	0.755	0.1182	15.64
0.50	0.913	0.1201	13.16
0.25	1.052	0.1286	12.22
0.15	1.095	0.0991	9.05

standard deviation (σ) obtained from a minimum of three replicates (seven recommended) of a matrix spike subsample containing the analyte of interest at a concentration 1−5 times the estimated MDL (*13*). The MDL for the kit was calculated from nine replicates to be 0.104 μg L^{-1}, which is comparable to the manufacturer's estimated LDD (0.10 μg L^{-1}).

Kit Accuracy. The accuracy of the RapidAssay kit was determined by calculating the mean percent recovery of six subsamples at nine fortified concentrations in DI water (**Table 1**) and by comparing known and measured concentrations of chlorpyrifos derived from assays that were performed in DI water and in several agricultural runoff waters to test for matrix effects (**Table 3**). **Figure 2** shows the statistical relationship between known and measured concentrations taken from values in **Table 1**. Each point on the curve represents the mean concentration derived from six regression models, and the error bars represent the variation between known and estimated values. A strong correlation ($R^2 = 0.998$) indicates that the kit is able to adequately estimate known concentrations from measured absorbances. Recoveries in the DI control matrix ranged from 89.8 to 118.8%, and the mean recovery for all spike levels was 101.26%. Note that while measured values at the lower (0.15 μg L^{-1}) and upper (3.50 μg L^{-1}) ends of the curve are outside the recommended linear range of the kit, the concentrations at these levels were satisfactorily reproduced.

Accuracy was further evaluated by comparing known fortified concentrations with measured values obtained from field samples used for the matrix effects studies. Recoveries for these relationships were expressed as relative percent differences (RPD), where

$$\mathrm{RPD} = [(X_1 - X_2) \times 100]/[(X_1 + X_2)/2]$$

Table 3. Agreement between Known and Measured Concentrations of Chlorpyrifos in DI Water and Several Environmental Water Matrices

	spike level	mean absorbance[a]	standard deviation	% CV	concn[b]	RPD
DI water	3.00	0.330	0.011	3.265	2.798	6.97
	2.50	0.363	0.010	2.645	2.518	0.72
	2.00	0.429	0.009	2.168	2.044	2.18
	1.00	0.641	0.006	0.940	1.037	3.63
	0.50	0.843	0.032	3.738	0.546	8.80
	0.25	1.113	0.012	1.037	0.230	8.33
				2.299[c]		5.10[d]
Del Puerto Creek	3.00	0.354	0.011	3.216	2.723	9.68
	2.50	0.376	0.026	6.799	2.503	0.12
	2.00	0.426	0.009	1.995	2.062	3.05
	1.00	0.598	0.020	3.298	1.068	6.58
	0.50	0.765	0.022	2.879	0.563	11.85
	0.25	1.009	0.001	0.099	0.222	11.86
				3.048[c]		7.19[d]
Pomelo drain	3.00	0.360	0.040	11.240	2.441	20.55
	2.50	0.314	0.012	3.861	2.899	14.78
	2.00	0.383	0.003	0.797	2.234	11.05
	1.00	0.625	0.034	5.471	0.899	10.64
	0.50	0.748	0.013	1.720	0.568	12.73
	0.25	0.986	0.009	0.943	0.232	7.47
				4.005[c]		12.87[d]
American River	3.00	0.341	0.005	1.503	2.812	6.47
	2.50	0.378	0.007	1.948	2.500	0.00
	2.00	0.431	0.009	2.157	1.982	0.90
	1.00	0.626	0.024	3.837	1.011	1.09
	0.50	0.764	0.021	2.749	0.635	23.79
	0.25	0.896	0.008	0.867	0.210	17.39
				2.177[c]		8.27[d]
Walthall slough	3.00	0.328	0.013	4.033	2.775	7.79
	2.50	0.401	0.016	3.949	2.998	18.12
	2.00	0.449	0.013	2.805	1.979	1.06
	1.00	0.649	0.049	7.559	0.771	25.86
	0.50	0.786	0.007	0.847	0.627	22.54
	0.25	0.873	0.013	1.496	0.235	6.19
				3.448[c]		13.59[d]

[a] Mean of three (chlorpyrifos) replicated measurements. [b] Estimated concentrations from mean absorbance. [c] Mean % CV. [d] Mean RPD.

and X_1 is the larger and X_2 is the smaller measured or estimated concentration at each spike level. The results of these analyses are summarized in **Table 3**. Recoveries, variability, and RPDs in all water types satisfy current EPA criteria for the assessment of analytical methods, which maintain that mean recoveries must lie in the range of 70−120%, and RPDs/CVs must lie within the range of ±20% (*14*).

Matrix Effects. The RapidAssay system is a competitive assay format; that is, the enzyme-labeled conjugate and the antigen compete for antibody binding sites on the paramagnetic particles and are consequently prone to interferences due to nonspecific binding between antibodies and nontarget analytes that may be present in a particular matrix. In addition, the tracer enzyme of the detection system is under matrix influence. Because the labeled conjugate is in competition with the unlabeled antigen for antibody sites, color development is inversely proportional to antigen concentration in the sample. Matrix effects in competitive assays, if present, are generally manifested by a decrease in color development. Such effects are evaluated by comparing the standard curve produced in the control matrix with analogous curves generated in the matrices of interest. For this study, chlorpyrifos standard curves were prepared in DI water, three agricultural runoff waters, and a river water matrix, each fortified at six concentrations (0.25, 0.50, 1.00, 2.00, 3.00, and 3.50 μg L^{-1}) and analyzed in triplicate. Curves were derived by regressing normalized absorbances (%B/B_0) vs concentration in each matrix. The resulting statistical relationships are shown in **Figure 3**.

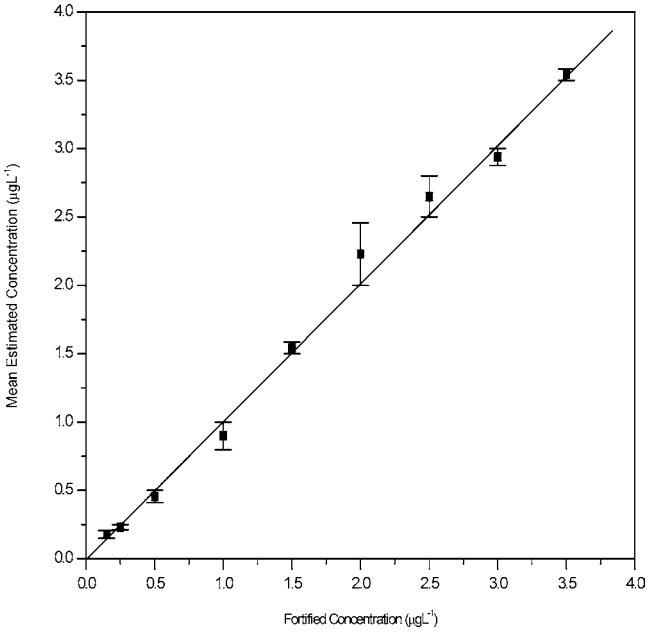


Figure 2. Accuracy of the RapidAssay ELISA kit. Plot of known vs measured values at nine concentrations falling within the linear range of the kit. The error bars represent the variation between measured and theoretical values. The equation of the line is $y = 1.0098x - 0.0092$ ($n = 9$, $r^2 = 0.998$, and $p < 0.0001$).

The presence of material nonspecifically interfering with an ELISA may alter, even at very low concentrations, two principle assay parameters: the shape of the standard calibration curve and test accuracy (inconsistent recoveries). The slope of a standard curve in a matrix containing interferences is always less than that of the control system (*15*). As shown in **Figure 3**, the slopes of all test matrix regression lines are less steep than that derived for DI water, suggesting that the chlorpyrifos antibody is inhibited to some extent in each matrix. To quantitate the inhibitory effects of a matrix on antibody binding capacity relative to the control matrix, a useful indicator may be expressed as follows:

$$\text{matrix inhibition index} = M_i = 100 \times ([\text{slope}_{\text{control}} - \text{slope}_{\text{matrix}}]/\text{slope}_{\text{control}})$$

Values of M_i represent the comparative steepness of slopes derived from standard curves generated in each matrix and correspond to the percent of matrix interference on assay performance relative to that of the control matrix, which is always zero. Accordingly, the larger the value of M_i is, then the greater the deviation from the control system and the more prominent the effect. In the present study, M_i values of 16.6, 15.1, 15.3, and 4.3 have been calculated for the Del Puerto Creek, Pomelo drain, Walthal slough, and American River waters, respectively; that is, there is roughly a 4−17% increase in antibody inhibition in the environmental systems as compared to the control system due to the presence of matrix interferences. Calculated values of M_i thus permit quantitative comparisons of matrix influences to be made among the various test matrices and provide a simple gauge to rapidly assess the inhibitory character of real-world test systems. Observed digression from the control system in this study may have been induced by differences in pH, ionic strength (electrical conductivity, EC), or other water quality parameters, although moderate variations in pH, ionic strength, and dissolved organic carbon (DOC) have been shown to not adversely affect analyte−antibody affinity in most other competitive ELISA kits and formats (*16*, *17*). Results of fortified sample recovery studies in filtered and unfiltered river waters in previous published studies (*18*, *19*) have shown that matrix effects appear to be minimal in the presence of the representative chemical constituents of natural waters (e.g., salts, metals, particulates, and humics) and with variations in pH. Other studies, however, suggest that small variations in ionic strength and organic matter may sometimes affect kit sensitivity (*20*). In the present study, basic water quality measurements for Del Puerto Creek (pH = 7.77, EC = 536.2 μS/cm, and DOC = 7.34 ppm), Pomelo drain (pH = 8.09, EC = 1287.7 μS/cm, and DOC = 7.42 ppm), Walthal slough (pH = 6.8, EC = 616.2 μS/cm, and DOC = 6.89 ppm), and North Fork American River (pH = 7.01, EC = 56.5 μS/cm, and DOC = 11.01 ppm) do not show obvious patterns sufficient to explain observed variations in M_i (i.e., slopes), although those matrices most affected tend to have lower DOCs and higher ionic strengths.

Mechanistically, inhibitory effects in ELISA may be induced by interference with antibody binding due to physical masking of the antibody (due to turbidity), interference with the detection systems caused by enzyme inhibitors (e.g., polyphenols, Fe) and catalysts (e.g., metals such as Cu), alternation of antigen concentration due to adsorption to solids (e.g., in turbid water), or alteration of antigen conformation. Mean absorbance data tabulated in **Table 3** indicate that there was usually inhibition of the antibody−antigen reaction (decrease in assay absorbance values relative to that of the control) rather than a decrease in assay sensitivity (highly variable absorbance values) in the test matrices relative to DI water. Because sensitivity is more an issue of affinity than inhibition, these data suggest that inhibitory effects are the prevailing source of diminished function in the present matrix effects study. The regression lines in **Figure 3** show that even a slight performance loss may lead to significant overestimation of chlorpyrifos residues in samples analyzed in test waters, particularly at low analyte concentrations. In addition, recoveries for the test waters (**Table 3**) in most cases exhibit greater variability than those obtained for the control matrix. Such characteristics also support the notion that the kit may be prone to miscalculation at low concentrations in affected waters (*21*).

Cross-Reactivity. The IC_{50} values, determined from the semilog calibration curves of chlorpyrifos and its structural analogues that were generated in DI water from the four-parameter dose−response model described earlier, are expressed as the concentrations required to displace 50% of the labeled antigen as compared to the IC_{50} of the antigen. In pesticide analysis, this single parameter is only a guide to the relative sensitivity of the kit to various pesticides. IC_{50} values and percent cross-reactivities derived for chlorpyrifos and 12 structurally similar analogues are shown in **Table 4**. The standard curves for chlorpyrifos and the most significant cross-reactants are shown in **Figure 4**. The kit's antibody has been shown to be highly selective toward chlorpyrifos, and only chlorpyrifos-methyl has been determined to exhibit significant cross-reactivity. Results for this study are consistent with these earlier findings (*22*). The high affinity of the chlorpyrifos antibody for the methyl analogue may lead to overestimations of the target antigen. This, however, should not be problematic since chlorpyrifos-methyl is no longer registered for use in California, and its uses have been largely phased out by the U.S. EPA.

Comparative Validation Study. *Study Parameters.* Test waters used for comparing ELISA and GC analytical method-

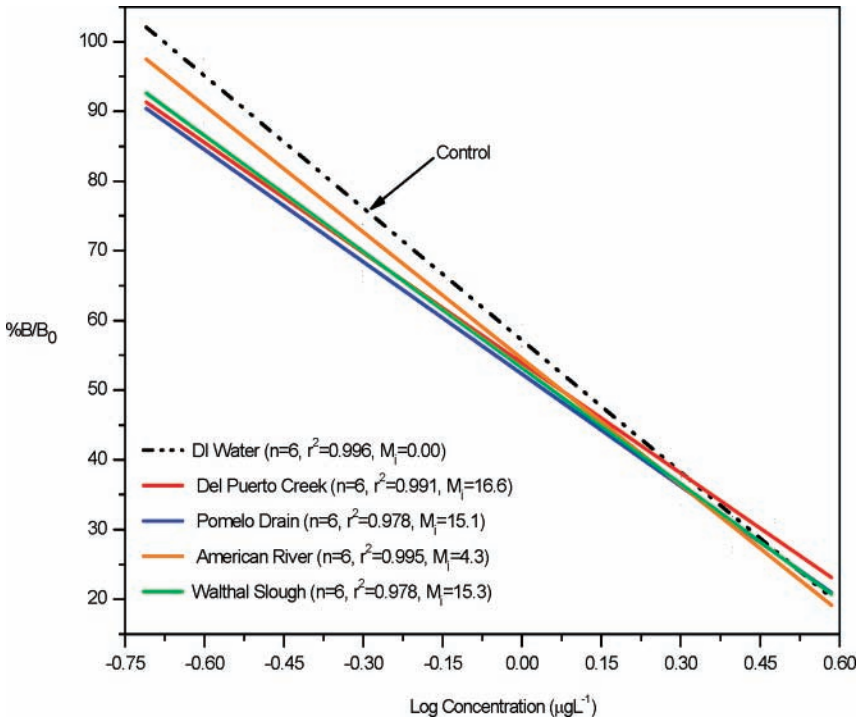


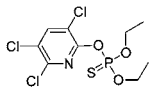
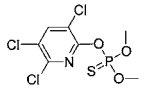
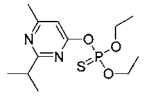
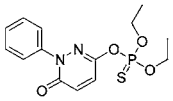
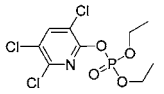
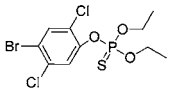
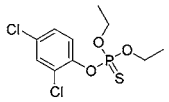
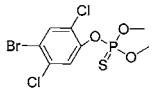
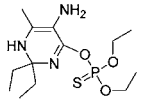
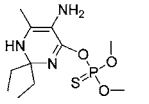
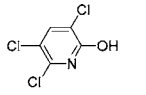
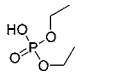
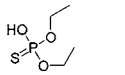
Figure 3. Graphical representation of matrix effects from standard curves (semilog scale) for the chlorpyrifos RapidAssay ELISA kit in DI (control) and several environmental surface waters. The values M_I are indicative of the comparative steepness of derived slopes relative to that of the control matrix. The larger that M_I is, the more prominent the influence of the matrix and the larger the divergence from the control. All curves were generated from six calibrators ranging in concentration from 0.25 to 3.0 ppb.

ologies were collected at the respective CDPR PAM (Study 231) and vegetative ditch (Study 231) field sites in Stanislaus County, CA, during the month of July, 2006. The PAM study was designed to measure the effect of polyacrylamide in reducing chlorpyrifos movement offsite in irrigation tail water. In this study, pairs of side-by-side irrigation furrows in a fallow field were flooded with water from an irrigation canal 24 h after a broadcast application (per label directions and at the maximum label rate) of a liquid chlorpyrifos formulation was made. PAM was injected at the head of each PAM-treated row at the onset of irrigation, while the adjacent control row was untreated. Irrigation runoff waters were collected at the base of each row, and samples were split and transported in ice to the respective labs for ELISA and GC analyses. Both the control and the PAM-treated samples were analyzed. Because samples collected for the PAM study had chlorpyrifos concentrations far exceeding the linear range of the RapidAssay kit, all samples required a 5:1 dilution in DDI water. The vegetative ditch study was designed to evaluate the use of constructed vegetated ditches to reduce chlorpyrifos loading in return water from irrigated crops as a means of mitigating its movement to surface water. Half of the water used to irrigate a 75 acre alfalfa field treated with chlorpyrifos was diverted into an interception ditch and through a weir into a 200 m long constructed ditch containing a variety of California native grasses. Chlorpyrifos flux was measured before and after the irrigation water flowed through the ditch. Samples were collected during the first irrigation events following two separate chlorpyrifos applications. Duplicate samples were collected each time and transported under ice to the respective labs for ELISA and GC analyses. All collected field samples were analyzed in duplicate by the protocols described in the Materials and Methods. For each study, ELISA and GC results were compiled and organized according to samples numbers, and the two methods were compared using several statistical techniques.

Standard t Tests. Paired, two-tailed *t* tests were performed on both the PAM and the vegetative ditch study data sets, and estimated *t* values were compared to those obtained from a standard *t* distribution table. For the PAM study ($n = 204$), the calculated value of *t* (10.775) was greater than the table value at the 95% confidence level and yielded a *P* value of <0.0001. For the vegetative ditch study ($n = 88$), the calculated value of *t* (12.763) was also larger than the table value and had a *P* value of <0.0001. These tests indicate that there is no significant statistical difference between the ELISA and the GC methods for either data set.

Comparative Regression Analysis. Regression analyses were conducted on the PAM and vegetative ditch data sets using GraphPad Prism version 4.03 for Windows (GraphPad Software, San Diego, CA, www.graphpad.com), and the resulting data plots are shown in **Figures 5** and **6**. To help in visually gauging the degree of agreement between the two methods, each plot includes the line of equality ($y = x$) on which all points would lie if the two methods gave exactly the same measurement at each concentration within the range of concentrations considered. ELISA concentrations in both the vegetative ditch study ($n = 88$, $r = 0.850$, and $p < 0.0001$, **Figure 5**) and the PAM study ($n = 205$, $r = 0.824$, and $p < 0.0001$, **Figure 6**) analyses were found significantly correlated with the respective GC values. The vegetative ditch ELISA results were generally higher than the corresponding GC results, with an average RPD of 23.4%. Similarly, the PAM ELISA concentrations were usually higher than the corresponding GC concentrations, with an average RPD of 45.3%. The regression lines derived for each test system have different intercepts but slopes that are nearly identical (parallel) to the respective lines of equality ($y = x$). In the vegetative ditch plot, nearly all of the points lie to the left of the line of equality, and there is a clear bias for ELISA. The ELISA measurements tend to exceed the GC measurements by the value of the intercept, at least for those points on or near

Table 4. Specificity of the RapidAssay Chlorpyrifos Antibody toward Other Structurally Similar Organophosphorus Analogues

Analogues	Structure	IC_{50} ($\mu g\ L^{-1}$)	% Cross Reactivity
Chlorpyrifos-ethyl		6.74	100
Chlorpyrifos-methyl		18.24	36.9
Diazinon		63.09	10.7
Pyridaphenthion		93.74	7.2
Chlorpyrifos-Oxon		179.44	3.8
Bromiphos-ethyl		220.62	3.1
Diclofenthion		281.92	2.4
Bromiphos-methyl		339.31	2.0
Pirimiphos-ethyl		426.29	1.6
Pirimiphos-methyl		869.19	<1.00
3,5,6-trichloro-2-pyridinol		>1000	<1.00
Diethyl Phosphate		>1000	<1.00
Diethyl Thiophosphate		>1000	<1.00

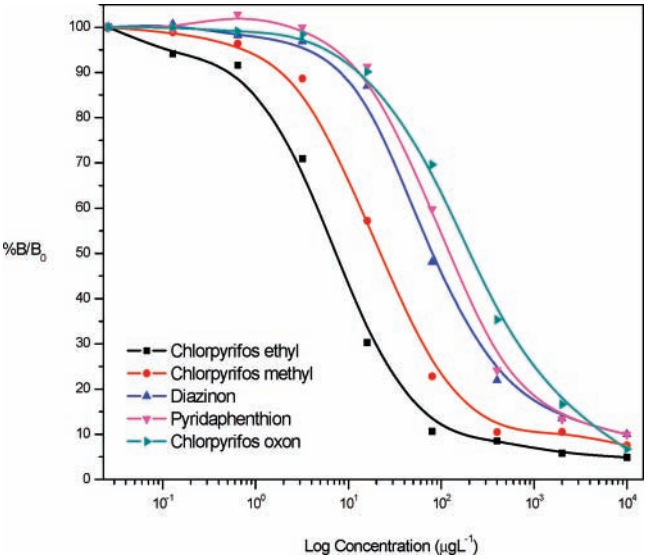


Figure 4. Standard curves showing the specificity of the RaPIDAssay chlorpyrifos kit antibody for chlorpyrifos and those structural analogues whose cross-reactivities were 3% or greater. Curves were normalized with respect to the lowest calibration standards, which had B/B_0 values ranging from 98 to 111%. All curves except the curve representing diazinon were generated from duplicate assays run simultaneously. The diazinon curve was derived from a single assay.

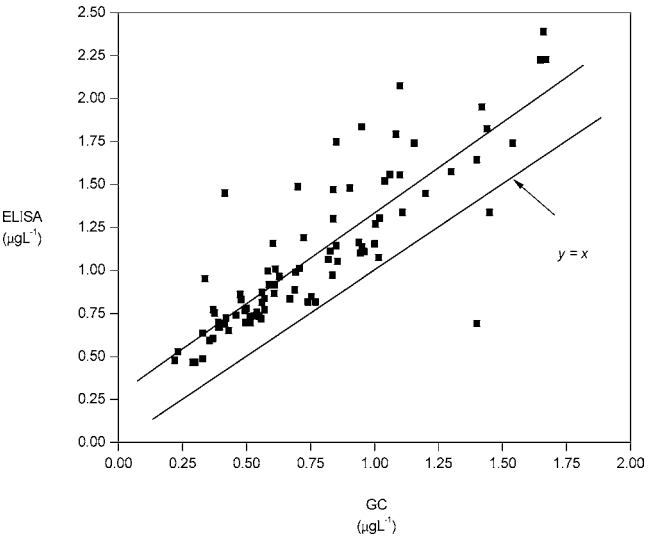


Figure 5. Correlation between GC and ELISA results for the analysis of vegetative ditch samples (DPR study no. 235) obtained from irrigation runoff waters in the Salinas Valley, CA. The equation of the line is $y = 1.054x + 0.2802$ ($n = 88$, $r = 0.850$, and $p < 0.0001$).

the regression line. The trend diverges somewhat in the PAM plot, where points tend to lie along the line of equality in the lower concentration range and gradually begin to drift away from it with increasing variability as concentrations increase. However, there again is clearly a positive bias for ELISA. To evaluate the possibility of inhibitory effects from PAM on analytical results, control and PAM-treated samples were regressed both independently and collectively. The plots for the independent analyses were scaled appropriately and superimposed, and the resulting composite plot (**Figure 6**) reveals the shape and variability of both subpopulations within the context of the collective distribution. The slopes derived for control samples (0.935) and PAM samples (0.977) differ little from the slope obtained for the entire population (0.962). In addition, the regression lines for each independent analysis are displaced to the left relative to the line of equality in a manner that is nearly analogous in scale and magnitude to that of the collective analysis. There is no apparent bias for either control or PAM samples in the composite scattergram.

Bias Analysis. Although correlation is routinely used to establish agreement between ELISA and other analytical methods of measurement, the correlation coefficient describes only the straight-line association (the linear relation) between two variables and is not an indicator of agreement; that is, it is possible to have a high correlation coefficient when agreement is poor (*23*). A bias plot (sometimes called a Bland−Altman plot) is more appropriate to show agreement between two methods of measurement (*24*). A bias plot is constructed by plotting the difference between two methods of measurement (the ordinate) against the mean of the two methods (the

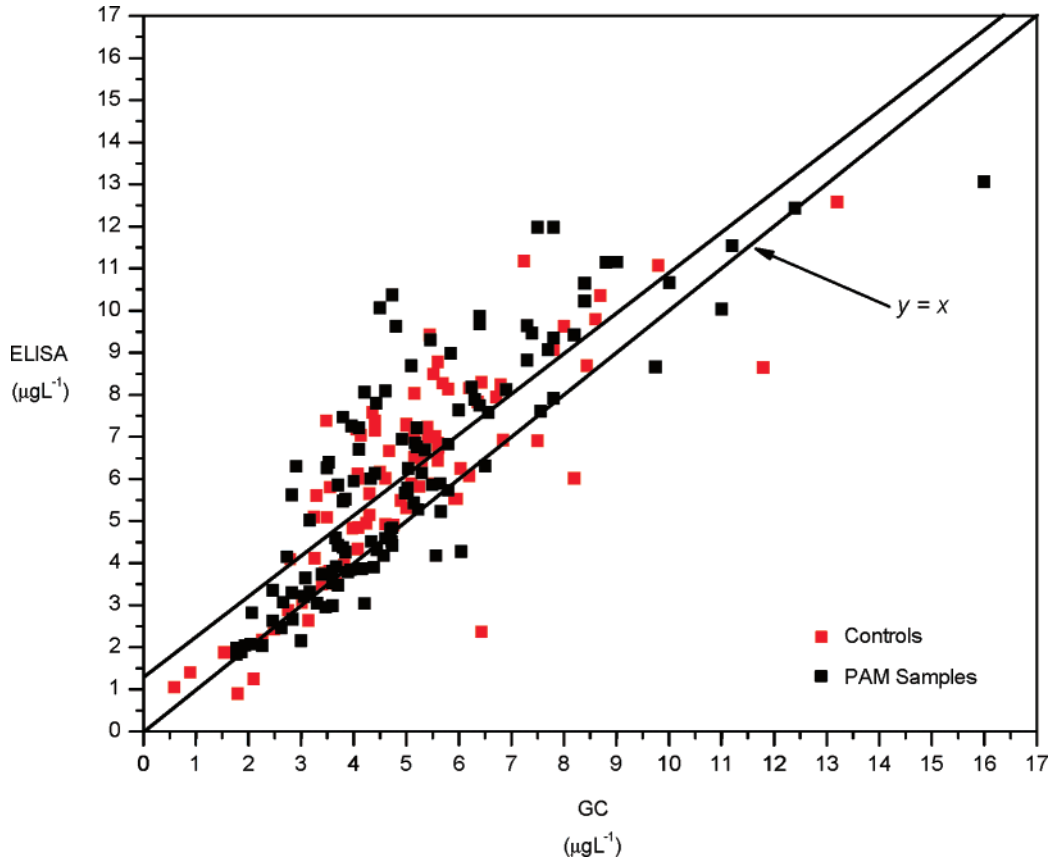


Figure 6. Correlation between GC and ELISA results for the analysis of PAM samples (DPR study no. 231) obtained from irrigation tailwaters in San Joaquin County, CA. Comparative ELISA/GC overlay plot shows the shape and variability of control and PAM samples within the context of the global distribution. The overall equation of the line is $y = 1.0063x + 1.0655$ ($n = 204$, $r = 0.823$, and $p < 0.0001$). Results obtained for both treated and control samples exhibit a positive bias toward ELISA. No evidence suggests that PAM-treated samples significantly affected the bias observed in the overall regression relationship.

abscissa). The 95% confidence limits are also calculated and displayed on the graph (mean difference ± 2 × standard deviation). If the two methods are measuring the same thing in an unbiased way, the resulting plot should show a random scatter of points between the upper and the lower confidence limits. If there is a pattern to the points, there may be bias associated with the measurements.

For the PAM and vegetative ditch data sets, the difference (ELISA minus GC) and the mean for each measured value were calculated, as well as the mean difference, d, and the standard deviation of the difference, σ. Plotting the difference between ELISA and GC against the mean for all measured values yields the statistical relationships shown in **Figures 7** and **8**. Assuming that the differences are normally distributed, 95% of the differences between the two methods (the bias) should lie between the limits given by $d \pm 2\sigma$, which are represented by the dashed lines in **Figures 7** and **8**. For the Study 231 data set, the mean difference and 95% limits are given by 1.077 + 1.878 and 1.077 − 4.033, and for the Study 235 data set, these values are 0.321 + 0.793 and 0.321 − 0.151. The standard errors of the limits are given by the square root of $3\sigma^2/N$, which were determined to be ±0.147 and ±0.090 for the PAM and vegetative ditch data sets, respectively. Provided it is agreed that differences within $d \pm 1.96\sigma$ are acceptable limits, if 95% of the values are distributed within these limits (i.e., between the dashed lines), then the two methods may be considered amendable (*25*). Inspection of **Figures 7** and **8** shows that, by this approach and given the imposed limits, the ELISA and GC methods are agreeable for both studies. The limits reveal that for the PAM study, in about 95% of the cases, the ELISA

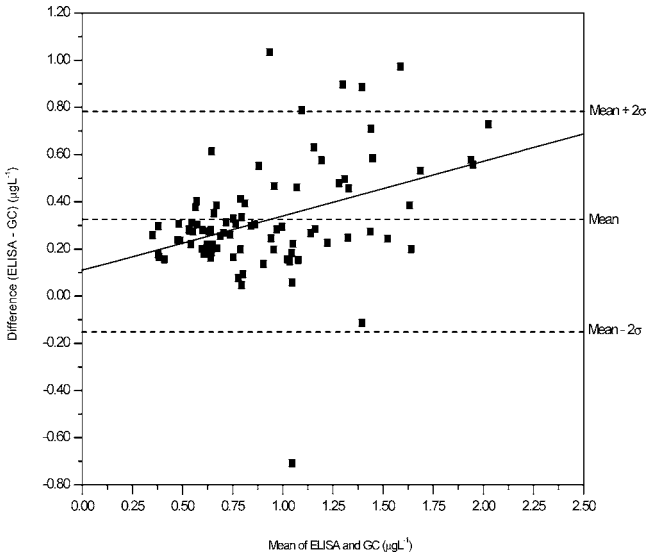


Figure 7. Difference against mean (bias) plot for ELISA/GC data from the vegetative ditch study.

measurements will be between −1.9 and four times the GC measurement. For the vegetative ditch study, in about 95% of the cases, the ELISA measurements will be between −0.15 and 0.79 times the GC measurement. The positive slopes of the regression lines and the steady increase in variability with concentration apparent in **Figures 7** and **8** indicate that in both studies the two methods do not agree uniformly throughout the range of concentrations considered and that the positive bias

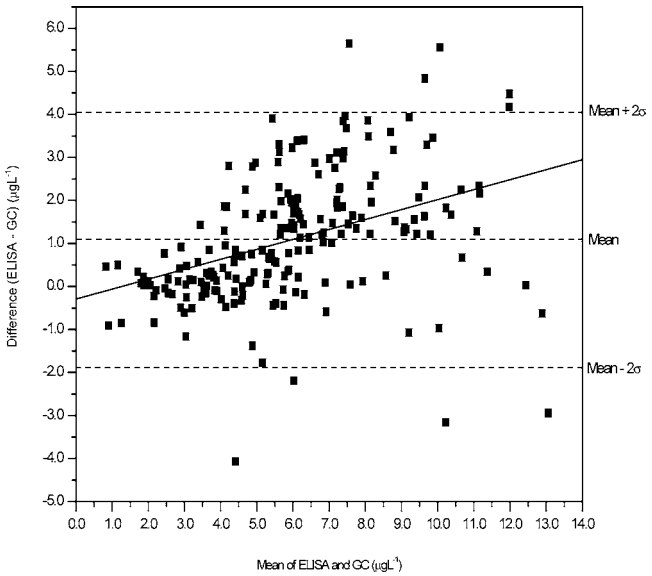


Figure 8. Difference against mean (bias) plot for ELISA/GC data from the PAM study.

for ELISA increases with increasing concentration. This trend is also evident in the regression plots generated for each study (**Figures 5** and **6**). The difference against mean analysis suggests that, while the majority of concentration values occurred within the range of the mean $\pm$ 2σ (i.e., the two methods are in general accord), ELISA may not yet be a dependable analytical alternative to GC for environmental analysis given that the high variability and bias observed between the two methods generally yields disproportionate results.

Conclusions. The routine use of commercial ELISA kits for pesticide screening and compliance monitoring has long been anticipated in the regulatory community because of its many practical benefits, for example, cost efficiency, consistency, portability, and ease of use. As demonstrated in the present work, ELISA performs exceptionally well under controlled conditions, exhibiting excellent accuracy and low variability in rigorous quality control evaluations. The chlorpyrifos ELISA kit was also shown to have high specificity and sensitivity to the target analyte, and its overall performance characteristics in controlled assays were found to fall within the current U.S. EPA criteria for the assessment of analytical methods. Unfortunately, ELISA is susceptible to matrix influences in real-world applications. These effects were shown to lead to the inhibition of antibody activity and to subsequent positive bias in measured values. Previous work by CDPR and others suggests that such bias is characteristic of ELISA in environmental applications, and this limitation has thus far impeded its use as a practical data-gathering alternative to conventional chromatographic methods of analysis. Because the observed bias for ELISA appears to be most clearly associated with matrix interactions, it is recommended that additional resources and effort be directed at mitigating such influences to help improve the analytical viability of ELISA. For example, the use of more aggressive preanalysis filtering techniques, centrifugation, or detergents or binding agents to reduce the amount of suspended solids and other interferences might be profitable and should be studied more thoroughly.

Despite its current analytical limitations, ELISA still has practical application in the implementation and fulfillment of regulatory policy. Effective environmental decision making depends upon credible and timely information about the state of the environment, and comprehensive monitoring capabilities are essential to provide such information to support the development of prudent management strategies. To this end, ELISA remains a dependable and cost-efficient complementary tool for screening prior to the employment of more expensive and laborious GC or HPLC methods.

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