Evaluation and Validation of a Commercial ELISA for Diazinon in Surface Waters

Jonathan J. Sullivan[†] and Kean S. Goh*[,‡]

Department of Land, Air, and Water Resources, University of California, Davis, California 95616, and Environmental Monitoring and Pest Management Branch, Department of Pesticide Regulation, California Environmental Protection Agency, 830 K Street, Sacramento, California 95814

The performance of a commercially available microtiter plate ELISA kit for the determination of diazinon was evaluated for sensitivity, selectivity, intra-assay repeatability, accuracy, and matrix effects in fortified distilled water and filtered and unfiltered environmental surface water samples. Repeatability and reproducibility studies show that the kit satisfies current EPA criteria for the assessment of analytical methods. Mean recoveries from spiked samples averaged 80.3, 95.5, and 103.5% from distilled, unfiltered surface, and filtered surface waters, respectively. The experimentally determined method detection limit (MDL) for the commercial diazinon microtiter plate format (0.0159 μg L^{-1}) was comparable to the least detectable dose (LDD) established by the manufacturer (0.022 μg L^{-1}). Specificity studies indicate that the diazinon polyclonal antibody can readily distinguish the target compound from other structurally similar organophosphorus analogues, with the exception of diazoxon. Cross-reactivity with the oxon was approximately 29%, while reactivity with pirimiphos−methyl, pirimiphos−ethyl, and chlorpyrifos−ethyl was negligible. A slight matrix effect was discovered to be present in both filtered and unfiltered environmental water matrixes, but its effect on the immunoassays is insignificant within experimental error. For validation of the microtiter plate ELISA format, environmental surface and storm runoff water samples were collected, split, and analyzed directly by ELISA and by liquid−liquid extraction followed by GC (California State Department of Food and Agriculture method EM 46.0). Results of the two analytical methods were then compared statistically. A close correlation was found between methods for unspiked and untreated river water samples ($r = 0.969$) while a much less robust correlation was obtained for runoff waters ($r = 0.728$). Results from runoff waters exhibit a particularly high positive bias for the ELISA method relative to the GC method. Cross-reactivity of diazoxon and probably other unidentified cross-reacting components may be responsible for the exaggerated account of the target analyte in surface and runoff waters. While excellent for screening purposes, further study is required to elucidate and quantify the factors responsible for the consistent overestimation of ELISA results before the kit can be employed routinely for regulatory compliance monitoring.

Keywords: *ELISA; diazinon; microtiter plate kit; matrix effects; cross-reactivity; performance evaluation*

INTRODUCTION

Diazinon (*O*,*O*-diethyl-*O*-[2-isopropyl-4-methyl-6-pyrimidyl] phosphorothioate) is a nonselective organophosphorus insecticide used extensively on turf, alfalfa, lettuce, almonds, citrus, cotton, and other crops for dormant sprays in fruit and nut orchard crops, foundation and landscape applications, and urban pest control. In California, approximately 900 596 pounds of diazinon was applied in 1998 (California Department of Pesticide Regulation, 2000). As a result of its widespread agricultural and domestic use, diazinon residues have been found in homes, offices, soils, crops, commodities, urban stormwaters, and surface waters (Bailey et al., 1995; Currie et al., 1990; Tsuda et al., 1995). Its presence in surface waters is of particular concern since such waters supply approximately 50% of the drinking water in the United States and are vital aquatic ecosystems that provide important environmental and economic benefits (USGS, 1997).

In California, the Department of Pesticide Regulation's (DPR) surface water protection program monitors and protects the state's surface waters from contamination and assesses mitigation measures to prevent or reduce pollution associated with the use of pesticides. Determination of the presence and level of pesticide residues in surface waters is fundamental in such monitoring and regulatory programs. Current analytical methods for the determination of pesticides in water consist of gas chromatography (GC), high-pressure liquid chromatography (HPLC), and mass spectroscopy (MS). Over the past decade, immunoassays such as enzyme-linked immunosorbent assays (ELISA) have become an increasingly important alternative detection method for the determination of pesticides, particularly for the analysis of large numbers of samples and as a screening tool. Immunoassays are rapid, sensitive, and reliable and are generally cost-effective for large sample loads (Gee et al., 1996). For example, the cost of ELISA for routine testing or screening of pesticides is approximately $40-$60 per sample compared to $150-$250 per sample for GC/HPLC analyses (Goh et al., 1993;

* E-mail: kgoh@cdpr.ca.gov.
[†] Department of Land, Air, and Water Resources.
[‡] Environmental Monitoring and Pest Management Branch.

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Linde et al., 1996). The principles of ELISA for the analysis of pesticide residues have previously been described in detail by Hammock and Mumma (1980) and applied to the development of commercial ELISA kits for the trace-level analysis of numerous pesticides in environmental water samples (Fong et al., 1999). We have routinely used ELISAs developed in-house for monitoring herbicide residues in compliance monitoring (Goh et al., 1993; Linde et al., 1996) as well as research studies (Goh et al., 1992). This paper describes the evaluation of a commercially available microtiter-plate ELISA kit for diazinon in surface waters. The objectives of this study were (1) to evaluate the kit for sensitivity, precision, accuracy, matrix effects, and selectivity; (2) to compare the quality of ELISA results to those obtained by a liquid−liquid extraction and GC methodology; and (3) to appraise the overall cost and efficiency of the commercial kit.

MATERIALS AND METHODS

Comparative ELISA-GC Analysis. *(a) Sampling.* Environmental water samples used in this study were collected as part of an ongoing DPR monitoring project assessing surface water quality of agricultural watersheds in California. Organophosphorus-free surface waters were obtained from the American River, CA (samples were screened using a multiresidue GC method for 14 currently used organophosphates) and were utilized to determine various kit evaluation parameters, e.g., reproducibility, interferences, and fortified sample recoveries. A total of thirty surface water and runoff field samples were collected for comparative ELISA-GC analysis (18 surface water samples from two sites on the Sacramento River and 12 runoff samples from nine Orange County, California, sites). All samples were untreated, split, packed in ice, and transported to the California Department of Food and Agriculture (CDFA) Center for Analytical Chemistry in Sacramento, California, for GC analysis and to the University of California in Davis, California, for ELISA analysis.

(b) Sample Preparation and GC Analysis and Apparatus. Sample preparation and analytical conditions for GC analyses were as follows: water samples were removed from the refrigerator and allowed to come to room temperature. Samples were extracted by shaking with 100 mL of methylene chloride for 2 min, after which the organic layer was drained through 20 g of anhydrous sodium sulfate into a clean boiling flask. The water layer was extracted two more times using 80 mL of methylene chloride, following the same procedure as above. After the final extraction, the sodium sulfate was rinsed with 25 mL of methylene chloride. The sample extract was evaporated to dryness on a rotary evaporator (Büchi/Brinkman) in a 35 °C water bath and at a vacuum of approximately 20 in. Hg. Acetone was added (5 mL) to the residue, and the contents were swirled to dissolve the solid extract. The extract solution was transferred to a clean, calibrated 15 mL graduated test tube. The flask was rinsed two more times with 2 mL of acetone, and the contents of each wash were combined. By use of a gentle stream of nitrogen, the acetone was evaporated to a volume slightly less than 1 mL, and the final volume was brought to 1 mL with the dropwise addition of acetone. The GC analysis was performed on a Hewlett-Packard 5890 series II gas chromatograph (Palo Alto, CA) equipped with a flame photometric detector (FPD) and using a 10 m × 0.53 mm × 2.65 μm HP-1 methyl silicone gum column with helium as a carrier gas at a flow rate of 20 mL/min. The injector and detector temperatures were 220 and 250 °C, respectively. Column temperature was held at 150 °C for 1 min, programmed to 200 °C at 10 °C min^{-1}, held for 2 min, programmed to 250 °C at 20 °C min^{-1}, and held for 5 min. The injection volume was 3 μL. The GC method described above has a method detection limit (MDL) of 0.0009 μg L^{-1}.

(c) ELISA Analysis. A diazinon EnviroGard kit (Strategic Diagnostic, Inc., Newark, N. J.) was employed for the ELISA analyses performed in this study. The diazinon kit is a 96-well microtiter plate design and has a detection range of 0.03−0.50 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 include eight strips (12-wells each) containing diazinon antibodies (rabbit polyclonal antidiazinon) immobilized on the walls of the test wells, diazinon horseradish peroxidase (HRP)-labeled enzyme conjugate, color solution, stopping solution, washing solution, and diazinon stock solution (100 μg L^{-1} in methanol). Standard solutions (0.030, 0.100, and 0.500 μg L^{-1}) were prepared from the provided stock solution in deionized (DI) water, which was also used as the negative control (reagent blank). Absorbances were measured with a Vmax microplate reader (Molecular Devices, Menlo Park, CA) in dual-wavelength mode (450−650 nm). Dynatech microtiter plates (Dynatech Laboratories, Inc., Chantilly, VA) were used for preparing serial dilutions. An Eppendorf series 2000 adjustable-volume (100−1000 μL) reference sampling pipet (Eppendorf, Hamburg, Germany) and an Eppendorf Titermate 12-channel adjustable-volume (100−300 μL) sampling pipet were used to dispense liquids.

(d) Microtiter Plate Kit Procedure. One hundred microliter reagent blank and each standard solution, and 100 μL of the samples to be analyzed were added to their respective wells. In the same order of addition, 100 μL of diazinonenzyme conjugate was added to each well, and the contents of the wells were mixed by gently moving the plate in a circular motion on the benchtop for 1 min. The wells were covered with tape to minimize evaporation and allowed to incubate at ambient temperature for 1 h. After incubation, the tape was removed, and the contents were shaken out of the wells into a sink. Wells were washed six times with DI water and tapped dry. Color substrate (100 μL) was added to each well, and the contents mixed, covered with tape, and allowed to incubate for 30 min at room temperature. After the incubation period was complete, 100 μL of stopping solution was added to each well. Quantitation was based on the optical density of the wells at 450−650 nm using a Vmax microplate reader.

Evaluation of Kit Performance and Specificity. *(a) Chemicals.* Certified analytical standards of diazinon (*O*,*O*-diethyl-*O*-[2-isopropyl-4-methyl-6-pyrimidyl] phosphorothioate), chlorpyrifos−ethyl (*O*,*O*-diethyl-*O*-[3,5,6-trichloro-2-pyridyl] phosphorothioate), diazoxon (*O*,*O*-diethyl-*O*-[2-isopropyl-4-methyl-6-pyrimidyl] phosphate), pirimiphos−ethyl (*O*,*O*-diethyl-*O*-[2-(diethylamino)-6-methyl-4-pyrimidynl] phosphorothioate), and pirimiphos−methyl (*O*,*O*-dimethyl-*O*-[2-(diethylamino)-6-methyl-4-pyrimidynl] phosphorothioate), were obtained from the standards repository at the CDFA Center for Analytical Chemistry. All analytical standards were prepared in HPLC-grade acetone (Fisher Scientific, Fair Lawn, NJ).

(b) Standard and Spike Preparation for Performance Evaluations. For the evaluation of the kits for accuracy, precision, reproducibility, and matrix effects, spiked samples were prepared with organic-free, Nanopure (Barnstead/Thermolyne, Dubuque, IA) distilled water and with filtered (45 μm) and unfiltered OP-free surface water. Standards provided with the kit, which are prepared in methanol, were not used for this portion of the study in order to minimize potential contrariety between standard and spiked solutions due to solvent or other effects. Five diazinon standards (0.016, 0.031, 0.125, 0.250, and 0.500 μg L^{-1}) were prepared with DI water for the evaluation of the microtiter plate kit. Spiked samples having concentrations ranging from 0.016 to 0.450 μg L^{-1} were prepared with deionized water and with filtered (45 μm) and unfiltered surface waters. All standards and spikes were made from 100 μg L^{-1} working stock solutions prepared from certified diazinon (0.9979 mg/mL) analytical standards provided by CDFA.

(c) Spike Preparation for Cross-Reactivity Studies. Certified CDFA analytical standard solutions of diazinon (0.9991 mg/mL), chlorpyrifos−ethyl (0.10009 mg/mL), diazoxon (0.9991 mg/mL), pirimiphos−ethyl (1.0016 mg/mL), and pirimiphos−methyl (1.0018 mg/mL) were used to prepare spikes for the analysis of kit selectivity. All spiked samples were made from 100 μg L^{-1} working stock solutions made in Nanopure organic-

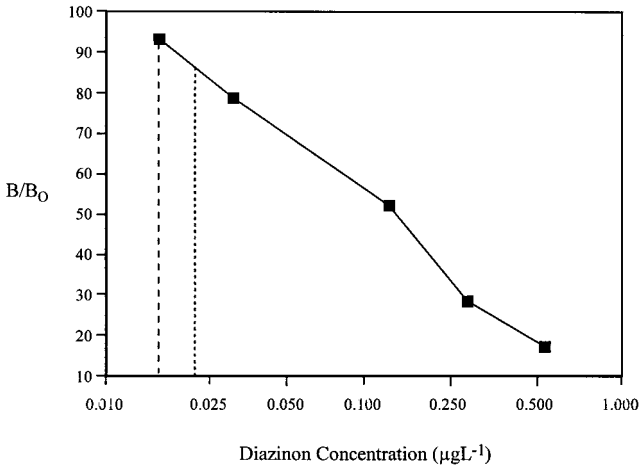


Figure 1. Standard curve (semilog scale) for diazinon used for the calibration of the EnviroGard ELISA kit. The dotted line represents the least detectable dose (LDD) for the kit (0.022 μg L^{-1}) determined by the manufacturer. The dashed line approximates both the experimentally determined LDD and the method detection limit (MDL) for the kit (0.016 μg^{-1}).

free, distilled water. Solutions having concentrations extending 8 orders of magnitude were prepared and run in duplicate. Spiked concentrations of 0.0006, 0.002, 0.005, 0.046, 0.14, 0.41, 1.24, 3.70, 11.1, 33.3, 100, and 1000 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

$$\%CR = (IC_{50}\ \text{target analyte}/IC_{50}\ \text{tested cross-reacting compound})(100) \quad (1)$$

where IC_{50} is the effective concentration of analyte that results in 50% enzyme conjugate inhibition. IC_{50} values for each cross-reactant were generated from a 4-parameter fit of experimentally determined absorbances versus spike concentration data. The equation for the 4-parameter fit (Rodbard, 1981) is

$$y = (A/D)/[1 + (x/C)^B] + D \quad (2)$$

where y is the absorbance, x is the concentration of the analyte, A and D are the upper and lower asymptotes, respectively, B is the slope and C is the central point of the linear portion of the curve, i.e., the IC_{50} (Gee et al., 1996). Standard curves resulting from a 4-parameter data reduction scheme are sigmoidal in shape. Both the upper and lower asymptotes must be well defined in sigmoidal dose−response relationships in order to ensure accurate IC_{50} values (Johnson et al., 1998).

RESULTS AND DISCUSSION

Performance Evaluations. *Standard Curves and Kit Sensitivity.* The standard curve for the diazinon standards is shown in Figure 1. The standard curve was based on duplicate samples in DI water and was linearly transformed using a log-linear curve fit as instructed by the kit manufacturer. Lowest standard concentrations were prepared below the normal linear range (0.030−0.500 μg L^{-1}) and below the least detectable dose (LDD) determined by the manufacturer (0.022 μg L^{-1}) in order to examine the linearity of responses in this region. The kit displayed a high degree of linearity below the kit manufacturer's established sensitivity, and the accuracy of absorbance values for the lowest standards was good (e.g., mean optical density value for the 0.016 μg L^{-1} spike was 1.0165 ± 0.0161). The LDD of the EnviroGard diazinon microtiter plate kit was calculated by the manufacturer as the amount of diazinon required to achieve 85% B/B_O, where B/B_O is the mean absorbance of a given sample divided by the mean absorbance of the negative control (Midgley et al., 1996). Absorbances for six replicate sample blanks were used to establish an experimentally based LDD to compare against that obtained by the manufacturer. Experimental LDDs were calculated as 3 times the mass equivalent of the standard deviation of the negative control from its mean absorbance (ACS, 1980). LDDs calculated by this method (0.015 μg L^{-1}) suggest that sensitivities are somewhat higher (approximately 32%) than those determined by the manufacturer using the B/B_O method (i.e., 0.022 μg L^{-1}). The correlation coefficient (r) for the diazinon standard curve was 0.9964.

Table 1. Intra-assay Reproducibility of ELISA Kit Spiked with Diazinon in DI Water at Eight Concentrations and Assayed Seven Times[a]

spike level (μg/L)	mean OD	SD	%CV	mean concn (μg/L)	SD	%CV	% recov.
0.035	0.743	0.0378	5.08	0.0329	0.0050	15.20	93.99
0.050	0.632	0.0373	5.90	0.0546	0.0089	16.43	109.21
0.075	0.565	0.0382	6.76	0.0743	0.0121	16.29	99.11
0.100	0.519	0.0354	6.82	0.0917	0.0157	17.12	91.78
0.150	0.365	0.0201	5.50	0.1851	0.0171	9.24	123.39
0.250	0.312	0.0170	5.44	0.2354	0.0185	7.86	94.17
0.350	0.215	0.0193	8.97	0.3674	0.0326	8.87	104.98
0.450	0.187	0.0162	8.65	0.4182	0.0308	7.36	92.94

[a] The acronyms OD, SD, and CV represent optical density, standard deviation, and coefficient of variation, respectively.

Intra-Assay Reproducibility. Results of reproducibility studies, in which DI samples spiked with diazinon at eight concentrations falling within the linear range of the kit were each assayed seven times, is shown in Table 1. The mean percent coefficient of variation (%CV) for the kit was 6.4% for optical density and 12.6% for concentrations. The microtiter plate test produced some %CV values in excess of 10%, all at concentration levels ≤0.100 μg L^{-1}. Such variability may be due in part to operator error, but it is more likely that observed variabilities are due to the lack of uniformity in the antibody coating on the walls of the plate wells or to leaching of the coating material. Variability of wells within microtiter plates has been shown to be the largest contributor to total assay imprecision (Mouvet et al., 1997).

The lowest spiked concentrations (0.035 μg L^{-1}) used for precision determinations were also utilized to calculate the MDL for the diazinon kit. The 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 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 (US EPA, 1996):

$$\text{MDL} = \sigma t_{(n-1,1-\alpha=0.99)} \quad (3)$$

By use of standard statistical tables and standard deviations obtained from Table 1, the MDL for the diazinon kit was calculated to be 0.0158 μg L^{-1}, which is comparable to the experimentally determined LDD (0.015 μg L^{-1}) presented earlier.

Table 2. Agreement between Known and Measured Concentrations of Diazinon in Distilled Water, Unfiltered Stream Water, and Filtered Stream Water.

spike level (μg L^{-1})	mean[a] (μg L^{-1})	SD	%CV	% recovery
		DI Water		
0.450	0.4552	0.0156	3.43	101.16
0.225	0.2112	0.0031	1.47	93.87
0.113	0.0816	0.0068	8.33	72.21
0.056	0.0396	0.0031	7.83	70.71
0.028	0.0179	0.0012	6.70	63.93
			5.55[b]	80.38[c]
		Unfiltered Streamwater		
0.450	0.4986	0.0086	1.72	110.8
0.225	0.2647	0.0104	3.93	117.64
0.113	0.0993	0.0153	15.41	87.88
0.056	0.0445	0.0042	9.44	79.46
0.028	0.0229	0.0005	2.18	81.79
			6.54[b]	95.51[c]
		Filtered Streamwater		
0.450	0.5194	0.0030	0.58	115.42
0.225	0.2780	0.0256	9.21	123.56
0.113	0.1121	0.0181	16.15	99.20
0.056	0.0481	0.0008	1.66	85.89
0.028	0.0261	0.0003	1.15	93.21
			5.75[b]	103.46[c]

[a] Mean of three (diazinon) replicated measurements. [b] Mean CV (%). [c] Mean recovery (%).

Accuracy. The accuracy of the ELISA kit was investigated by performing recovery studies in which measured concentrations in DI water and in unfiltered and filtered river waters were determined and compared to expected values. The accuracy of the EnviroGard kit was determined by spiking each water matrix with diazinon (0.028, 0.056, 0.100, 0.113, 0.225, and 0.450 μg L^{-1}) and analyzing all samples in triplicate. The results of these analyses are summarized in Table 2. Matrix blanks indicated that no residual amounts of diazinon were present in any of the water matrixes. Mean percent recoveries for the diazinon microtiter plate were best in unfiltered (95.5) and filtered (103.5) surface waters, although results in unfiltered water also exhibited the highest percent variability (6.54). Overall recoveries in DI water were significantly lower than expected (80.4%) due to poor recoveries for spiked samples at the 0.028−0.100 μg L^{-1} range. Preliminary trials with the diazinon kit were performed in DI water, and these experiments consistently exhibited recoveries in excess of 90%. Consequently, operator error is suspected as the most likely explanation for the poor recoveries observed in the DI matrix in the final analyses. The recovery pattern for spikes in DI water shown in Table 2 suggest that a serial dilution error may have occurred. Overall, however, despite slightly greater variance in the distribution of estimated values for diazinon in surface waters, the accuracy of all results was found to be acceptable. The highest mean recovery of diazinon (103.5%) was observed for spiked samples in filtered surface water. The lowest mean variability (5.5%) was observed for spiked samples in DI water. The average percent recovery for diazinon for all water types and at all spike levels was 93.1, and the average variability was 5.95 (range 63.9 to 123.6 with %CV varying from 0.58 to 16.15). Mean variabilities and recoveries in all water types satisfy current EPA criteria for the assessment of analytical methods. EPA standards maintain that mean recoveries must lie in the range of 70−120% with a maximum coefficient of variation of ±20% (Hammock et al., 1990).

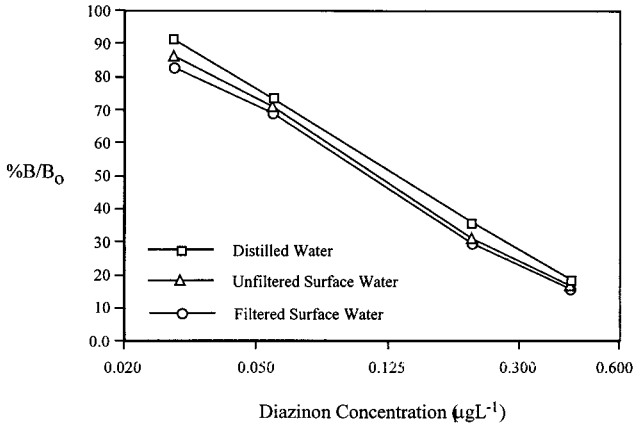


Figure 2. Graphical representation of matrix interference effects from standard curves (semilog scale) for the diazinon EnviroGard ELISA kit in distilled, unfiltered, and filtered surface water.

Matrix Effects. Immunoassays are rapid and convenient for environmental water analysis primarily because they usually do not require sample preconcentration and cleanup steps. ELISA methods, however, often have a high potential for nonspecific binding between nontarget analytes and antibodies and are consequently prone to matrix interferences, even in "clean" matrixes. There are several methods available for the quantitative evaluation of so-called matrix effects, two of which were employed in this study. Typically, interferences are quantified by comparing a standard curve produced in a control matrix such as distilled or buffered water with a calibration curve generated in the matrix of interest. The slope of a standard curve in a matrix containing interferences is less than that of the control system (Krotzky and Zeeh, 1995). For the current investigation, the ELISA kit was used to generate three diazinon standard curves, one in distilled water, one in unfiltered surface water, and one in filtered surface water. All curves were generated from four spiked samples having concentrations of 0.028, 0.056, 0.112, and 0.450 μg L^{-1}.

The resulting statistical relationships (Figure 2) indicate that a strong parallelism exists between each of the three curves. There was little difference (<5%) between the slopes of curves generated in unfiltered and filtered surface water relative to that of the control matrix. Estimated concentrations of diazinon tend to be slightly lower in surface waters due to a minor decrease in sensitivity compared to the control (Figure 2 and Table 2). It is possible that small variations in sensitivity between the natural and control systems are induced by differences in pH or ionic strength (electrical conductivity, EC). However, DI water (pH = 6.81, EC = 5.98 μS/m), unfiltered surface water (pH = 7.41, EC = 606 μS/m), and filtered surface water (pH = 7.39, EC = 666 μS/m) all had similar pH's near neutral and low conductivity. Moderate variations in pH and dissolved organic carbon (DOC) has been shown to not adversely affect analyte−antibody affinity in most other competitive ELISA kits and formats (Watts et al., 1997; Lawruk et al., 1993). Other studies, however, suggest that small variations in ionic strength and organic matter may sometimes affect kit sensitivity (Manclús and Montoya, 1995). In the present study, the removal of particulates and organic matter by filtering had little impact on inhibition, as evidenced by the similarity of the slopes of the standard curves in unfiltered and filtered waters.

Table 3. Slopes Obtained for Standard Curves in DI, Filtered Surface, and Unfiltered Surface Waters and Their Percent Differences Relative to the Control Matrix (DI) and Values of the Index of Matrix Interference, I_M, and the Correction Factor, N, Calculated for Unfiltered and Filtered Surface Waters

	rel. stand. curves		index of matrix interfer.	
matrix	slope	% diff.	I_m	N
distilled water:	1.030	0.00	0.00	0.00
unfiltered river water:	1.003	2.62	3.57	0.96
filtered river water:	0.998	3.11	4.89	0.95

An alternative methodology to that of comparative standard curve analysis for providing a general quantitative account of matrix effects has been proposed (Cairoli et al., 1996) and was used in this investigation to corroborate the more conventional statistical approach. In this technique, experimentally determined absorbance values for matrix blanks are normalized against those of the blank control matrix, which yields a unitless term called the index of matrix interference, I_m

$$I_m = [ABS_{blank\ A} - ABS_{blank\ B}]/ABS_{blank\ A} \quad (4)$$

where ABS is the mean absorbance determined from experiment, Blank A is the control matrix (DI water in the present study), and blank B is the unspiked environmental matrix. I_m for a particular matrix is then used to derive a correction factor, N

$$N = [(100 - I_m)/100] \quad (5)$$

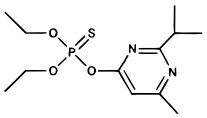
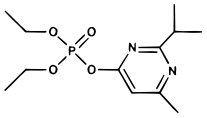
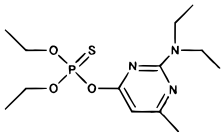
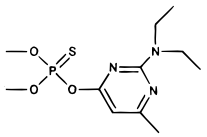
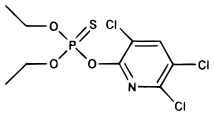
which is subsequently employed for the direct quantitation of a particular analyte of interest

$$C_x = NC_{measured} \quad (6)$$

where C_x is the matrix-corrected estimated analyte concentration and $C_{measured}$ is the analyte concentration determined from the calibration curve. With this approach, the calculated I_m values can be considered a "true" matrix interference, thus allowing the determination of the analyte in each matrix directly from the calibration curve in DI water using eq 4. Values of I_m and N were calculated for unfiltered and filtered surface waters using mean absorbance values for control and matrix blanks obtained from the EnviroGard kit (Table 3).

Evidence supporting this approach is shown in Table 3. The percent difference between the slope of the standard curve generated for the control matrix and that of each natural water matrix is shown to approximate closely their respective I_m values, i.e., $[slope_{control} - slope_{matrix}]/slope_{control} \approx I_m$. In the present study, the index of matrix interference appears to be comparable to the standard curve method for the quantification of general matrix effects. These results merit further study, since the ability to assess potential matrix effects through simple calculation rather than through additional experiment is clearly advantageous in terms of time and cost. Values of N derived for each surface water matrix were used to calculate corrected values for mean concentrations shown in Table 2. When corrected concentrations were used to recalculate recoveries, observable improvements were noted (not shown), although all such improvements fell within the range of experimental error (%CV). Thus, observed variations in sensitivity for natural waters occurring in the microtiter ELISA kit are probably not due to matrix interferences but may instead be the result of variability in well to well binding capacity or, in particular, temperature. With the 96-well microtiter plate format, the outer wells tend to reach optimum temperature sooner than the inner wells, which then has an effect on the equilibrium reactions which drive the binding process. Variations in final absorbances due to this phenomenon are generally manifested in what is called an "edge effect" (Gee et al., 1996).

Table 4. Specificity of Diazinon Antibody toward Other Structurally Similar Organophosphorus Analogues

Analogues	Structure	IC_{50} ($\mu g\ L^{-1}$)	%Cross Reactivity
Diazinon		0.289	100
Diazoxon		0.986	29.31
Pirimiphos-Et		>700	< 0.01
Pirimiphos-Me		>1000	< 0.001
Chlorpyrifos		>1000	< 0.001

Cross-Reactivity. Cross-reactivity between antibodies and compounds that are structurally similar to the target compound is an inherent problem with ELISA (Meulenberg et al., 1995). Cross-reactions can affect analytical results by either indicating that the target compound is present when it is not (false positive) or by elevating the predicted concentration of the target compound when both the target and one or more structurally similar compounds are present. Therefore the specificity of each kit toward the target compound and its most probable cross-reactants should be determined. The EnviroGard diazinon antibody has been shown to be highly selective toward diazinon (Beasely et al., 1997). Only diazoxon, the O-analogue of the target compound, has been found to exhibit significant cross-reactivity ($IC_{50} < 1.000\ \mu g\ L^{-1}$) (Fan and Bushway, 1997). The current results (Table 4, Figure 3) are consistent with these earlier findings. Cross-reaction of the diazinon antibody with the oxon form of diazinon was approximately 29%, while reaction with pirimiphos−methyl and pirimiphos−ethyl, which share the disubstituted pyrimidine ring structure, and chlorpyrifos−ethyl, was minor (<1%). The affinity of the diazinon

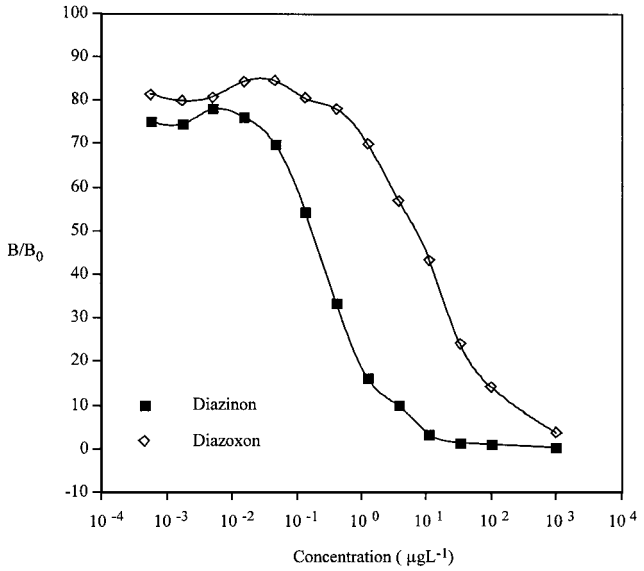


Figure 3. Standard curves showing specificity EnviroGard diazinon kit antibody for diazinon and diazinon O-analogue (diazoxon).

antibody for the oxon analogue may lead to difficulties for the quantitative determination of diazinon in water samples where its degradation products are present in significant amounts. In such cases, the antibody may not accurately differentiate between diazinon and its oxon, leading to exaggerated estimations of the target compound.

METHOD COMPARISON

Unfiltered environmental water samples were used for comparing ELISA and GC methodologies and were collected at the Sacramento River and Orange County field sites. All field samples were analyzed in duplicate by the appropriate protocols described in the Materials and Methods section and the results are shown graphically in Figures 4 and 5. For both the ELISA and GC datasets, paired, two-tailed *t*-tests were performed, and calculated *t*-values were compared to those obtained from a standard *t*-distribution table. For the Sacramento River dataset ($n = 18$), the calculated value of t was determined to be larger than the table value at the 95% confidence level and yielded a P value of 0.021. For the Orange County dataset ($n = 12$), the calculated value of t was found to be smaller than the table value and had a P value of 0.1188. These results infer that there is no significant statistical difference between the ELISA and GC methods for the analysis of river water samples, whereas a considerable difference exists between the two methods for the analysis of runoff waters. These characteristics can be seen graphically in Figures 4 and 5, which show the correlation between GC and ELISA results for the detection of diazinon in untreated Sacramento River and Orange County runoff water samples, respectively. Regression analysis of the Sacramento River samples (Figure 4) yielded a good linear relationship having a correlation coefficient of 0.969 and a slope of 1.178 between the two methods ($F = 243.5$, $s = 0.028$, $p < 0.0001$). A much poorer quality linear relationship was observed for the runoff samples (Figure 5). These samples had a correlation coefficient of 0.728 and a slope of 1.452 ($F = 11.25$, $s = 0.254$, $p < 0.0073$). The slopes from both figures are greater than 1.0 and indicate a high positive bias for the ELISA method

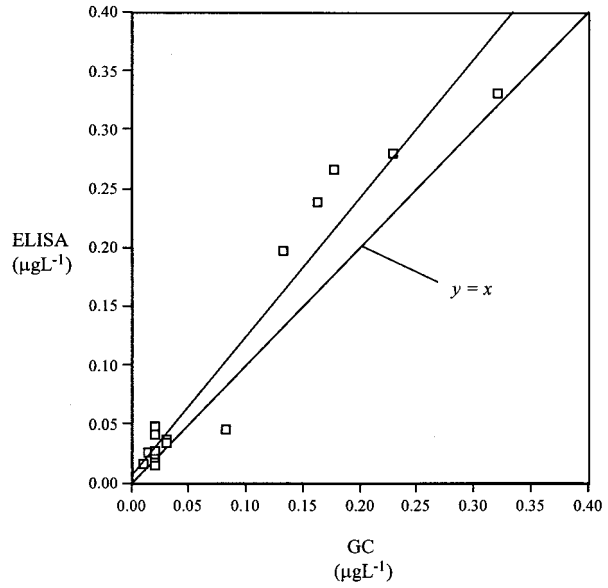


Figure 4. Correlation between GC and ELISA results for the analysis of field water samples obtained from two sampling sites on the Sacramento River, CA. The equation of the line is $y = 1.178x + 0.006$ ($n = 18$, $r = 0.969$, $F = 243.5$, $s = 0.028$, $p < 0.0001$).

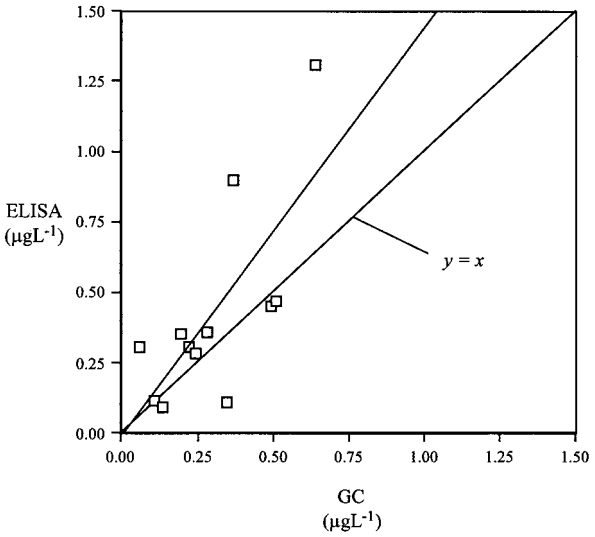


Figure 5. Correlation between GC and ELISA results for the analysis of field water samples obtained from nine sampling sites in Orange County, CA. The equation of the line is $y = 1.452x - 0.10$ ($n = 12$, $r = 0.728$, $F = 11.25$, $s = 0.254$, $p < 0.0073$).

relative to the GC method, particularly for runoff samples obtained from Orange County sites. Although positive bias can be beneficial to a screening method as it reduces the possibility of generating false negatives, the consistent overestimation of values is an undesirable trait for quantitative applications.

The observed bias for the ELISA test kit appears to imply the presence of a significant matrix effect, particularly for samples taken from storm runoff. Matrix effects, however, are typically manifested by diminished rather than enhanced ELISA responses, since interfering components tend to inhibit selective interactions between the target analyte and antibody. Runoff waters used for this comparative method study were determined to have higher electrical conductivity (EC), alkalinity, and ammonia concentrations than corresponding river waters, while dissolved oxygen (DO) and

pH were similar for both matrixes (Ganapathy, 1999; Kim et al., 1999). Nevertheless, results of fortified sample recovery studies in filtered and unfiltered river waters in this study (Table 2) and in previous studies (Lawruk et al., 1993; Oubiña et al., 1996) 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, humics) and with variations in pH. It therefore seems unlikely that bias induced by antibody interactions with these components would be as extensive as those observed in the method comparison study.

Enhanced responses are most likely due to cross-reactivity with unidentified metabolites, degradation products, or other components present in the water matrixes. If the cross-reacting component or components are detected by ELISA but not by GC, the immunoassay will exhibit a positive bias. Since diazoxon has been found to be the most significant cross-reacting analogue for the EnviroGard plate kit, having a sensitivity (0.200 μg L^{-1}) only around 10 times less than that of diazinon (0.022 μg L^{-1}), it is a logical cross-reacting candidate. It is well-known that phosphorothionates are subject to oxidative desulfuration in the environment through either photochemical or in vivo processes, or by interaction with common chemical constituents of natural waters, such as dissolved oxygen, ozone, metals, and halides (Eto, 1979; Ohashi et al., 1994; Ku et al., 1998; Zhang and Pehkonen, 1999). However, we have no quantifiable evidence which suggests that diazoxon was present in the samples analyzed and that it is responsible for the observed bias for ELISA in the current study. Phosphate esters are, in fact, considerably less stable in the environment than their corresponding thiophosphate analogues due to the greater polarity of the P═O bond. Diazoxon, for instance, hydrolyzes about 6 times faster in water under neutral conditions than diazinon and about 14 times faster in water under basic conditions (Falah and Hammers, 1994). Consequently, diazoxon tends to degrade rapidly and is not generally found in significant quantities in the environment. Recent studies conducted by DPR to monitor the levels and estimate the changes in concentration of diazinon and diazoxon over time on surface soil and turfgrass (Rodriguez, 1995) and to assess the distribution and mass loading of insecticides in the San Joaquin River, California (Ross et al., 1999), found that diazoxon was seldom detectable in either soil or water. On the few occasions it was detected, it was consistently quantified at concentrations less than 2% of the parent thioester. In runoff waters, Domagalski (1996) determined that diazoxon only made up approximately 1−3% of the diazinon load in stormwater runoff in the Sacramento River Basin, California. Accordingly, it is evident that diazoxon is not likely to be present in tested samples at concentrations high enough to explain the observed overestimation of ELISA results. The positive bias for ELISA observed in this study is probably due to the combined inhibitory effects of several unknown interferences rather than to secondary antibody inhibition by diazoxon alone. Before the diazinon kit can be employed routinely for regulatory compliance monitoring, particularly for quantifying runoff water from a storm event, further study is required to elucidate and quantify the factors responsible for its consistent overestimation of ELISA results.

CONCLUSIONS

The results of recovery, reproducibility, and sample comparison studies indicate that the EnviroGard ELISA kit is a satisfactory and cost-effective method for the analysis of diazinon in surface water samples. Most of the mean variabilities and recoveries in all water types satisfy current U. S. EPA criteria for the assessment of analytical methods, i.e., recoveries in the 70−120% range with a maximum variation coefficient of ±20%. Few differences were observed at low levels between spiked filtered and nonfiltered environmental waters. These results suggest that the ELISA kit may be effectively employed for the direct analysis of diazinon in surface waters without the need for sample cleanup or filtration. The kit also exhibits good accuracy and precision, which helps ensure the consistent monitoring and screening of environmental waters. The specific antibody employed allows for the detection of diazinon in the presence of other structurally similar pesticides, with the possible exception of diazoxon, the O-analogue of the target compound, which displayed significant reactivity (approximately 29%) toward it. The commercial assay compares favorably with results from GC analysis of diazinon in environmental surface waters, but the kit exhibits substantial positive bias for ELISA in runoff waters. This may be attributed to the presence of higher concentrations of cross-reacting interferences in runoff waters than in surface waters. Despite these limitations, the relatively low cost ($42 per sample), low amount of sample required (200 μL), minimal sample preparation and solvent waste, rapid analysis time, and ease of use of the microtiter plate ELISA make it well suited for adaptation to screening low levels of diazinon in environmental surface waters. Before the diazinon kit can be employed routinely for regulatory compliance monitoring, however, further study is required to identify and quantify the factors responsible for its observed bias for ELISA.

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