



MONTEREY COUNTY

WATER RESOURCES AGENCY

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CURTIS V. WEEKS
GENERAL MANAGER

809

October 18, 2006

STREET ADDRESS
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Song Her
Clerk to the Board
State Water Resources Control Board
1001 I Street
Sacramento, CA 95814

Dear Ms. Her:

Subject: **Comment Letter – 2006 Federal CWA Section 303(d) List**



This letter serves to provide comments to the proposed 2006 303(d) List, ITEM 10, being presented to the State Water Resources Control Board on October 25, 2006.

The draft document represents many hours of work by SWRCB and RB3 Staff.

GENERAL COMMENTS

In addition to referencing the table "Proposed 2006 CWA Section 303(d) List of Water Quality Limited Segments, CCRWQCB", a reference map on the web page would have been helpful in reviewing the proposed 303d list, along with the Hydrologic Area numbers on the Fact Sheets for each water body.

Missing Fact Sheets: There are missing fact sheets for the following two water bodies and associated constituents:

Salinas Reclamation Canal 309.11010

- Fecal Coliform
- Low Dissolved Oxygen
- Pesticides
- Priority Organics

Old Salinas River Estuary 309.11010

- Fecal Coliform
- Low Dissolved Oxygen
- Nutrients
- Pesticides

Tembladero Slough 309.11010

- Ammonia (added 2006)
- Fecal Coliform
- Nutrients
- Pesticides

Listed Water Bodies as Municipal and Domestic Supply Beneficial Use

The following water bodies are listed with a Municipal and Domestic Supply (MUN) designation in the Central Coast RWQCB Basin Plan. However, under Beneficial Use Definitions for "MUN", **these water bodies may not meet the requirements for MUN Beneficial Use: *The source is not sufficient to supply an average sustained yield of 200 gallons per day.***

- Gabilan Creek 309.19000
- Quail Creek 309.19000
- Santa Rita Creek 309.19000
- Natividad Creek 309.11010
- Alisal Creek 309.70093

Natividad Creek 309.11010 is listed in the incorrect category of Original Fact Sheets. It should reside under **New/Revised Fact Sheets**. This water body is not listed on the Central Coast Regional Board, Basin Plan, Chapter 2, Table 2-1, Identified Uses of Inland Surface and Inland Surface Waters, Sept. 8, 1994. Based on this, it is a newly proposed water body to the 303d list.

Tembladero Slough 309.1101 is not listed in the New or Revised Fact Sheets. Ammonia was added in 2006 as a POLLUTANT/STRESSOR. (See above under missing Fact Sheets)

Insufficient Current Data

Much of the data referred to on the fact sheets for the listed water bodies is not current, and may not reflect present conditions in the watershed. In the last few years, there has been considerable focus given to implementing improved management practices that reduce pollutant impact to surface waters of California. The Salinas Hydrologic Unit is primarily agriculture with some tributaries that flow through urbanized areas. Because of the recent efforts performed by growers related to the State-mandated Agricultural Discharge Waiver in Monterey County, along with increased implementation of irrigation management practices to reduce agricultural run off, it is felt that the limited surface water quality data from the early 2000's does not reflect current water quality for some constituents in the listed water bodies.

- Gabilan Creek 309.19000 Data 1999-2000 Nitrate
- Quail Creek 309.19000 Data 1999-2000 Nitrate
- Santa Rita Creek 309.19000 Data 1999-2000 Nitrate
- Natividad Creek 309.11010 2000 Nitrate
- Moro Cojo Slough 309.13011 Data 1999-2000 Unionized Ammonia

Question

Two water bodies are listed for Beneficial Uses: Cold Fresh Water (CO) and Warm Fresh Water (WA).

Can one water body be both cold and warm freshwater at the same time?

- Moro Cojo Slough 309.13011

Area Change

Salinas Reclamation Canal 309.11010

Proposed Area Change – There is no indication in the Fact Sheet as to the existing or to the proposed size change for this water body. The Monterey County Water Resources Agency, who oversees watershed management in Monterey County, requests to review the proposed change with your staff. Please contact Manuel Quezada, Associate Water Resources Engineer, at 831.755.4860 to discuss.

Santa Maria River 312.10030

It is well documented that certain testing methods for **Diazinon and Chlorpyrifos**, depending on the matrix and associated chemicals present, organic and inorganic, may produce a result that has a positive bias. The method in question is the **Enzyme-Linked Immunosorbent Assay (ELISA)** method. It is felt that, if these tests on this water body were performed with the ELISA method, a false positive may occur, which could compromise the ability of the SWRCB to regulate the Santa Maria River for water quality. It is our recommendation that an absolute, unbiased testing for Chlorpyrifos be performed, utilizing **Gas Chromatograph/Mass Spectrophotometry (GC/MS)** protocol.

Enclosed are four documents that reference the above statement. Another option would be for one of your staff to call Dr. Frank Spurlock, CA Dept. of Pesticide Regulation, at 916.324.4124, for further discussion.

Document A:

DPR Agreement 0-168-130-0, Kozlowski et al, **Monitoring Chlorpyrifos and Diazinon in Impaired Surface Waters of the Lower Salinas Region**, March 31, 2004 (cover and two pages)

Document B:

Final Report Agreement No. 00-0183S **“Evaluation of Potential Interferences for a Diazinon ELISA Test Kit”** Hammock and Gee for Spurlock Dept. of Pesticide Regulation (DPR), Environmental Monitoring and Pest Management, October 2002 (entire document)

Ms Song Her
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Document C:

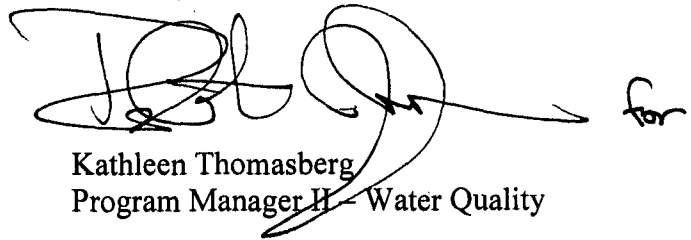
DPR Memorandum, January 13, 2002 **Study Summary: Evaluation of Interferences in Enzyme-Linked Immunosorbent Assay (ELISA) for Diazinon** (entire document)

Document D:

Evaluation and Validation of a Commercial ELISA for Diazinon in Surface Waters, Sullivan and Goh, *J. Agric. Food Chem.* 2000, 48, 4071-4078 (entire document)

Please feel free to follow up with me at 831.755.4860, if you have any questions regarding these comments.

Sincerely,

A handwritten signature in black ink, appearing to be 'K. Thomasberg', with a long horizontal flourish extending to the right. To the right of the signature, the word 'for' is written in a small, cursive script.

Kathleen Thomasberg
Program Manager II - Water Quality

Enclosures: (4)



Central
Coast
Watershed
Studies
CCoWS

Funded by:

California Dept. of Pesticide Regulation
Agreement 01-0183C

Central Coast Regional Water Quality
Control Board
Agreement 9-168-130-0

Report No. WI-2004-03
31 March 2004

The Watershed Institute

Earth Systems Science and Policy
California State University
Monterey Bay
<http://watershed.csumb.edu>

100 Campus Center, Seaside, CA,
93955-8001
831 582 4452 / 4431.

**Monitoring Chlorpyrifos and
Diazinon in Impaired Surface
Waters of the Lower Salinas Region**

*ELISA has
positive bias for
both Diazinon and
Chlorpyrifos.
See p. 142 and p. 162.*

Don Kozlowski¹
Dr Fred Watson^{1,2}
Mark Angelo³
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³Central Coast Regional Water Quality Control Board

Diazinon analysis of replicates tended to be more variable than chlorpyrifos. Chlorpyrifos replicates analyzed (n=74) averaged CV=15% (SD=23%). Diazinon replicates analyzed averaged CV= 29% (SD=32 %).

The variation between like environmental samples was less than the variation in test methodology. The average RPD for all (n=53) duplicates analyzed by ELISA was 30% (SD=36%); the average CV=21% (SD=26%). The CV for all duplicates (21%) is lower than the CV for all replicates (24%). This suggests that the variation that has been determined between like environmental samples (duplicates) is likely due to the analytical method used.

8.2.5 Inter-laboratory/inter-analysis method comparisons

Qualitative and quantitative comparisons of ELISA to GC analysis indicate that ELISA may be positively biased relative to GC analysis when reporting environmental values. Results obtained from APPL for duplicate samples are summarized in Appendix 2, Table 8.2. Full laboratory reports from APPL are presented in Appendix 2.

Many of the samples did not have directly quantifiable comparisons, but most of those had qualitatively consistent comparisons. Thirty-four sample values analyzed by APPL were below the PQL's for the test. Duplicate samples analyzed by ELISA had 27 values below or only slightly greater than the PQLs of the GC method. One sample had an ELISA value nearly 6 times greater than the PQL of GC suggesting the possibility of contamination of a duplicate sometime after sampling.

Twenty-four sample values had quantifiable results above the PQL of the test. ELISA analysis for chlorpyrifos (n=9) averaged a relative percent difference (RPD) of 32% higher than the GC value. ELISA analysis for diazinon (n=14) averaged a difference of 57% higher than the GC value. The Log of the determined concentrations by both labs are compared in Figure 8.1.

See also p. 162

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Table 8.2 Inter-Laboratory/Inter-Method Comparison Data.

(x, CCoWS value consistent with APPL; #, CCoWS value near EDL; nd, not determinable; *, see notes; C, chlorpyrifos; D, diazinon; w, water; b, benthic; RPD, relative percent difference)

run	site	Lab	C, water	RPD	C, benthic	RPD	D, water	RPD	D, benthic	RPD
DPRun1_Jul2002	sal-dav	CCoWS	102	x	37,548	-51	45	x	24,157	x
		APPL	<500		63,000		<500		<50,000	
DPRun2_Aug2002	sal-mon	CCoWS	50	#	20,735	x	37	x	3,947	x
		APPL	<50		<50,000		<50		<50,000	
DPRun3_Sep2002a	bla-coo	CCoWS	55	#	294,992	*	444	53	9,109	x
		APPL	<50		<50,000		290		<50,000	
DPRun4_Sep2002b	bla-pum	CCoWS	54	#	2,811	x	372	16	2,432	x
		APPL	<50		<50,000		320		<50,000	
DPRun5_Oct2002	rec-jon	CCoWS	111	94	147,715	39	309	23	103,097	53
		APPL	40		100,000		250		60,000	
DPRun6_Storm1	sal-mon-w	CCoWS	58	#	52,610	nd	<25	x	51,718	nd
	sal-dav-b	APPL	<50		<50,000		<50		<50,000	
DPRun7_Storm2	bla-coo-w	CCoWS	65	nd	19,114	x	1160	-35	10,095	x
	bla-pum-b	APPL	<50		<50,000		750		<50,000	
DPRun8_Storm3	rec-jon-w	CCoWS	75	61	17,701	x	247	66	4,577	x
	ols-pot-b	APPL	40		<50,000		410		<50,000	
DPRun9_Apr2003	ep1-rog	CCoWS	353	46	13,659	-114	2952	59	1,979,127	186
		APPL	220		50,000		1,600		70,000	
DPRun10_May2003	mos-san	CCoWS	84	nd	3,859	nd	80	0	1,699	nd
		APPL	<50		n/a		80		n/a	
DPRun11_Jun2003	ep1-rog	CCoWS	109	58	189,883	nd	308	25	2,329,568	179
		APPL	60		<50,000		240		130,000	
DPRun12_Jul2003	sal-mon	CCoWS	60	nd	23,631	x	23	x	non-detect	x
		APPL	<50		<50,000		<50		<50,000	
DPRun13_Aug2003	sal-dav	CCoWS	69	nd	23,309	x	25	-46	8,129	x
		APPL	<50		<50,000		40		<50,000	
DPRun14_Sep2003	ep1-rog	CCoWS	563	52	1,358,554	99	574	-15	20,367,689	130
		APPL	330		460,000		670		4,300,000	
DPRun15_Oct2003	ep1-ep1	CCoWS	51	#	nd	x	88	98	1,349	x
		APPL	<50		<50,000		30		<50,000	
Average RPD				62		-7	24		137	
					32				57	

Notes: 1) APPL labs used a higher detection limit for water samples on the first run (500, not 50)
 2) *, duplicate sample #202 value = 34,770, consistent with APPL labs. Duplicate sample #214 replicates averaged approximately 425,000. 3) Averages based on quantifiable values only.

Final Report
Agreement No. 00-0183S

“Evaluation of Potential Interferences for a Diazinon ELISA Test Kit”

Bruce Hammock and Shirley Gee
Department of Entomology
University of California
Davis, CA 95616

for
Frank Spurlock
Department of Pesticide Regulation
Environmental Monitoring and Pest Management
1001 I Street
P.O. Box 4015
Sacramento, CA 95812-4015

October 2002

Scope of Work

- Phase I. Evaluate specific and non-specific interferences on diazinon ELISA of storm water runoff using surrogate or actual water samples, potentially including factors such as diazinon degradates, co-occurring pesticides in surface water runoff, humic materials, salinity, pH, etc.
- Phase II. Evaluate methods to mitigate the presence of potential interferences to improve ELISA selectivity, such as clean up or extraction procedure modifications.
- Phase III. Conduct analysis of DPR-provided storm water runoff samples for diazinon using ELISA to compare results with gas chromatographic analysis conducted at the CDFA Center for Analytical Chemistry to test the modifications developed in steps 1 and 2 of the study discussed above.

Background

CDFA has evaluated the Diazinon test kit from Strategic Diagnostics (Sullivan and Goh, 2000) and found it to have a positive bias compared to results obtained by gas chromatography. The authors of this study postulated that the bias could be caused by the presence of a cross-reacting species, diazoxon. An alternative explanation offered was that the bias was a result of undetermined matrix effects.

In another comparative study conducted in the winter of 2000, a similar high bias was found compared to a GC method run by California Fish and Game (Appendix IID). Although the data correlate well ($r = 0.974$), the slope indicated a bias by the ELISA method. Examination of this data revealed that the magnitude of the bias was not related to site of collection, date of collection or limited to a particular concentration range. GC data was routinely lower than the ELISA data, ranging from 7 to 82% lower. Spikes and blind spikes were routinely run during the ELISA analyses. Spiking levels were 0.1, 0.2 or 0.5 ppb. With few exceptions, the recoveries were all greater than 100%. The upper control limit was set at 150%. This level was exceeded in three out of 14 analyses and in two other analyses the level was 140 and 145%, and these high recovery levels were associated with the high diazinon spike level of 0.5 ppb. The remainder of the recoveries was between 110 and 130%.

CDFA would like to use the diazinon ELISA kit for routine monitoring of storm water runoff. However, the high bias is a cause for concern. The goal of this project is to identify, if possible, the source of the difference in the ELISA and GC results and to develop or suggest ways to ameliorate the difference.

Materials and Methods

Immunoassay analyses: Diazinon test kits were obtained from Strategic Diagnostics (Newark, NJ). The test kits had been purchased by CDFA and were transferred to UC Davis. Eighteen test kits were obtained in this manner. UC Davis obtained additional test kits directly from Strategic Diagnostics as needed. All kits were stored at 4 °C according to the

manufacturer's instruction. The samples were analyzed by the test kit according to the test kit insert (Appendix IIA) with modifications as indicated in the CDFA Center for Analytical Chemistry method #EM18.0 (Appendix IIB).

GC analyses: GC analyses were conducted in the CDFA Center for Analytical Chemistry using a method entitled "Diazinon in Surface Water" dated 3/30/92 (Appendix IIC).

Solid phase extraction: Samples were treated to solid phase extraction after the method of Villarosa et al. (1994). The columns were Varian-Bond ELUT cartridges, 500 mg (catalog number 12113027). They were preconditioned with 3 mL diethyl ether, 3 mL methanol, 3 mL methanol:water (60:40) and 3 mL nanopure water. Samples (10 mL) were passed through the pretreated cartridges by vacuum at a rate of approximately 1 mL/min. The cartridges were dried under vacuum for about 20 min. The adsorbed diazinon was then eluted with 2 x 1 mL of diethyl ether. The eluate was collected and evaporated under a gentle stream of nitrogen until a few drops remained. The sample was then brought to a volume of 10 mL with nanopure water and analyzed by immunoassay.

Results and Discussion

I. Cross reactivity

The table below lists the cross reactivity for structurally related compounds. If a complete inhibition curve was obtained the IC_{50} was calculated and a % cross reactivity determined. In other cases there was a trend toward inhibition and the % inhibition at the highest concentration tested (i.e. 5000 ng/mL) is reported. All other compounds showed no inhibition at 5000 ng/mL.

A. Structurally related compounds. The oxon form of diazinon cross reacts to a small extent (2%), but the 2-isopropyl-6-methyl-4-pyrimidinolpyrimidone leaving group cross-reaction is extremely small, inhibiting the assay only 36% at 5000 ng/mL. Both of these findings are in agreement with the paper by Beasley et al., that describes the production of the antibody and initial assay development. Although the USEPA SAP states that the 2-isopropyl-4-methyl-6-hydroxypyrimidine is more mobile and more persistent than diazinon, it is not likely the cause of the bias in ELISA results since it does not cross-react. The diazoxon is known to cross react, however recent monitoring studies by the CDFA (Domagalski, 1996) indicate that the diazoxon accounts for only 1-3% of the total amount of the diazinon load, thus also would not account for the total bias.

Table 1. Cross reactivity of structurally related compounds.

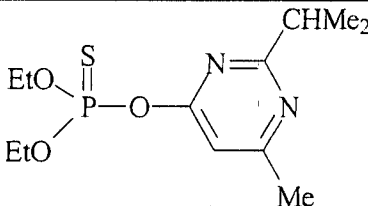
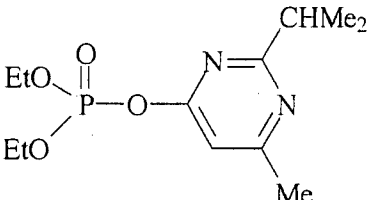
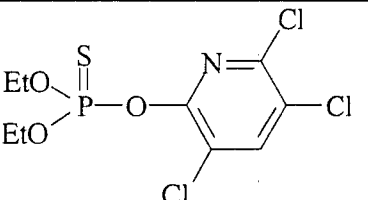
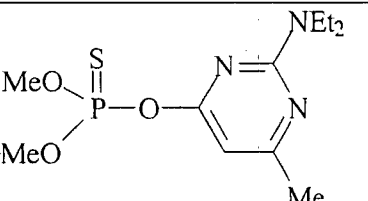
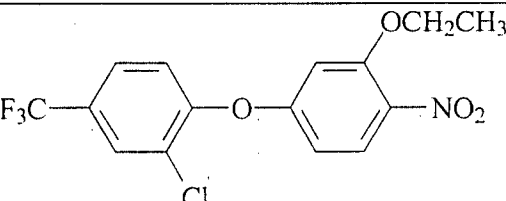
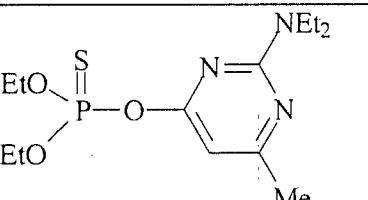
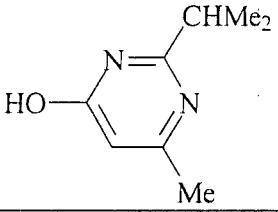
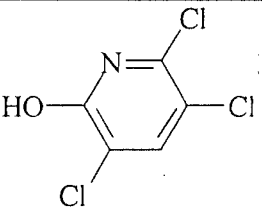
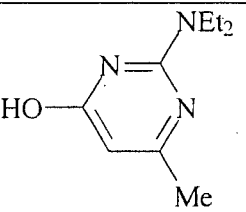
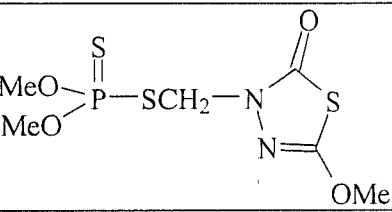
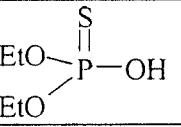
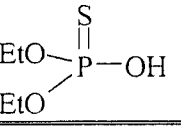
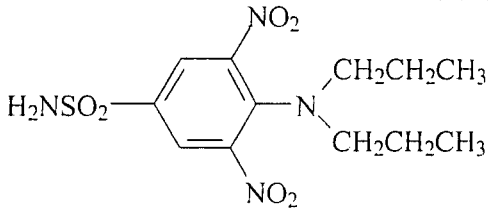
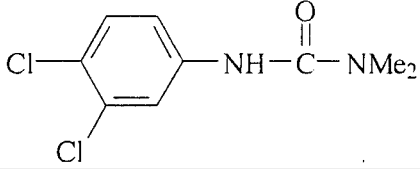
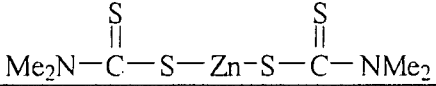
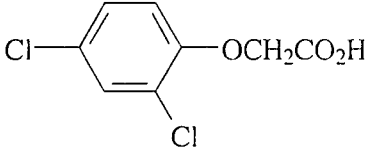
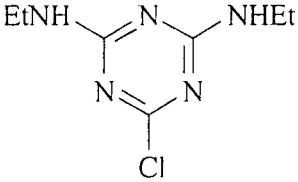
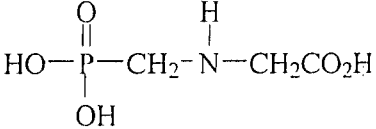
Name	Structure	IC ₅₀ (ppb)	% Cross Reactivity	% Inhibition at 5000 ng/mL
Diazinon		0.2	100%	
Diazoxon		9	2	
Chlorpyrifos		610	0.03	
Pyrimifos methyl				74
Oxyfluorfen				57
Pyrimiphos ethyl				60

Table 1. Cross reactivity of structurally related compounds (con't)

Name	Structure	IC ₅₀ (ppb)	% Cross Reactivity	% Inhibition at 5000 ng/mL
2-Isopropyl-6-methyl-4-pyrimidinol				36
3,5,6-Trichloropyridinol				37
2-Diethylamino-6-methyl-4-pyrimidinol				0
Methidathion				0
Diethyl phosphate				0
Diethylthiophosphate				0

B. High use compounds. The following table contains compounds used abundantly or during the dormant spray season. Diazinon, methidathion and oxyfluorfen are among the top ten, and are listed in the table above. Methyl bromide and 1,3-dichloropropene are also among the top ten, and were not tested. Due to their volatility, they are unlikely to be present in samples. Simazine has been found in runoff (Domalgalski, 1996).

Table 2. Cross reactivity of some "high use" compounds.

Name	Structure	IC ₅₀ (ppb)	% Cross Reactivity	% Inhibition at 5000 ng/mL
Oryzalin				0
Diuron				0
Ziram				22
2,4-D				0
Simazine				40
Glyphosate				0

C. Other pesticides tested are in the table below. Molinate and its degradates have also been found in runoff, as has carbofuran which was not tested. (Domagalski, 1996).

Table 3. Cross reactivity of some other pesticides.

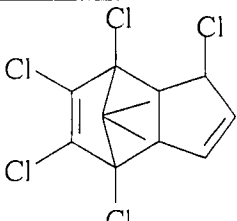
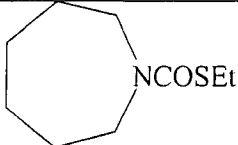
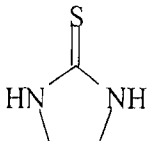
Name	Structure	IC ₅₀ (ppb)	% Cross Reactivity	% Inhibition at 5000 ng/mL
Atrazine	$\text{EtNH} \begin{array}{c} \diagup \text{N} \diagdown \\ \diagdown \text{N} \diagup \\ \text{N} \\ \text{Cl} \end{array} \text{NHPr}$			57
Thiobencarb	$\text{Et}_2\text{NC(=O)SCH}_2\text{-C}_6\text{H}_4\text{-Cl}$			42
Carbaryl	$\text{MeNHCO-C}_6\text{H}_4\text{-C}_6\text{H}_5$			0
Propoxur	$\text{MeNHCO-C}_6\text{H}_3(\text{OCHMe}_2)\text{-C}_6\text{H}_5$			0
Aldicarb	$\text{MeNHCO-N=CH-C}(\text{Me})_2\text{-SMe}$			0
Trichlopyr	$\text{Cl-C}_6\text{H}_2(\text{Cl})_2(\text{N=OCH}_2\text{COOH})$			0
Paraquat dichloride	$\text{Me-N}^+\text{C}_5\text{H}_4\text{-C}_6\text{H}_4\text{-N}^+\text{C}_5\text{H}_4\text{-Me} \cdot 2\text{Cl}^-$			0
Heptachlor				0

Table 3. Cross reactivity of some other pesticides (con't)

Name	Structure	IC ₅₀ (ppb)	% Cross Reactivity	% Inhibition at 5000 ng/mL
Molinate				0
Ethylenethiourea				0

D. Other potential interferents. An examination of the Pesticide Use Report shows that many inorganics are used during the dormant spray season in amounts much larger than the pesticides. Among them are copper sulfate, sulfur and mineral oil. We tested solutions of these and found the following: A 1 mM copper sulfate solution corresponded to 49 ppt of diazinon. Nanopure water saturated with sulfur corresponded to 151 ppt of diazinon. Nanopure water saturated with mineral oil corresponded to 22 ppt of diazinon.

There appear to be several organic and inorganic chemicals that can affect the assay either selectively (a cross-reactant) or non-selectively (interferent). No single compound can account for the high bias seen in the 2000 samples discussed above. However, it is possible that a combination of chemicals could be responsible for some of the bias.

II. Methods Evaluation

A. Old vs new test kits. Some of the test kits received from CDFA were near the expiration date. Tests were conducted with these "nearly" expired and new kits to determine the usefulness of the old kits for this study.

Table 4. Evaluation of old vs new test kits

Sample #	ELISA	ELISA	GC
	Old Test Kit	New Test Kit	
	Diazinon Found, ppt	Diazinon Found, ppt	Diazinon Found, ppt
48	35	38	50
66	13	29	50
103	28	73	85
138	16	59	67
153	11	34	42

Samples were from study 199 and were measured without pretreatment.
Samples run in three well replicates on the same day.

The data in Table 4 show that the old test kits gave diazinon concentrations for these samples that were lower than might be expected compared to GC values and different from the values for the new kits. The new kits, however, gave values closer to the GC values. Most of the test kit parameters for the old kit were within the quality control values reported in the test kit insert. New kits were used where quantitative data was needed. The old kits were used in range-finding and methods development studies and in the determination of cross reactivity.

B. Recovery studies. Background water was collected from Wadsworth (Butte County) and Karnak (Sutter Bypass) collection sites. The Karnak sample contained some suspended sediment. An aliquot of each of the water types was spiked with the indicated amount of diazinon and analyzed without pretreatment. Three well replicates of each sample were run on the same day.

Table 5. Spike recovery.

Sampling Site	Nominal Spike Level ppt	Diazinon Found ppt	% Recovery
Wadsworth	30	38.9	129.7
	60	54.8	91.3
	100	136.4	136.4
	200	200.2	100.1
	500	478	95.6
	1000	841.7***	84.2
Karnak	30	31.2	104.0
	60	43.2	72.0
	100	64.2	64.2
	500	485.9	97.2
	1000	831.4***	83.1

***Indicates that absorbances were outside the range of the highest standard, 500 ppt.

The data in Table 5 indicate that recovery of diazinon from these water samples was variable with no particular relationship to spike concentration. In addition, some of the spikes were outside the normally acceptable range of 80-120% recoveries. Thus, some matrix effect is presumed. To test this idea, samples were subjected to solid phase extraction.

C. Recovery studies following SPE. Background water was collected from Wadsworth (Butte County) and Karnak (Sutter Bypass) collection sites. The Karnak sample contained some suspended sediment. Aliquots (10 mL) of each of the water types was spiked with the indicated amount of diazinon in duplicate and analyzed by SPE. Three well replicates of each sample were run on the same day.

Table 6. Spike recovery following SPE

	Nominal Spike Level ppt	Mean* Diazinon Found ppt	Mean % Recovery
Wadsworth			
	60	105.0	175.0
	100	116.8	116.8
	500	485.8	97.2
Karnak			
	60	77.2	128.7
	100	105.5	105.5
	500	428.2	85.6

*n=2.

The data in Table 6 indicate that the solid phase extraction method used, results in good recovery of diazinon. From the limited concentrations tested, the SPE does seem to eliminate some interference. However, the recoveries for the 60 ppt samples are outside the acceptable range. This was not the case when the samples were analyzed directly, which implies that something may be co-eluting with diazinon that is interfering. Further work on optimizing a solid phase extraction method is needed.

D. Day-to-day variation. Data were compiled from those experiments in which the same samples were run on more than one day to examine day-to-day variation. On each day, three independent replicates of each sample were run. One set of samples was run without pretreatment. A second set of data is for samples that had been analyzed following solid phase extraction. With this limited data set, there were no differences in values obtained from day to day.

Table 7. Day-to-day variation.

Sample #	03/08/01 ELISA	03/09/01 ELISA
418	406 ± 17	408 ± 75
441	59 ± 20	56 ± 12
466	578 ± 49	493 ± 37
491	69 ± 13	81 ± 25
376	209 ± 19	222 ± 24
389	370 ± 11	217 ± 31
	02/22/01 SPE ELISA	02/23/01 SPE ELISA
376A	147 ± 21	158 ± 25
376B	129 ± 16	105 ± 7
389A	243 ± 9	253 ± 13
389B	232 ± 4	221 ± 38

Table 7 shows that samples analyzed on two consecutive days give values that are identical or overlapping within the standard deviation of the determinations.

E. Evaluation of calibration curve. The absorbances for each calibrator are used to back calculate each concentration as another estimate of the fit of the calibration curve. Table 8 is a compilation of this data collected over several months.

Table 8. "Recovered" diazinon using absorbances from calibration standards.

Nominal Diazinon Conc ppt	Mean Found Conc ppt	SD	Mean %Difference	SD
30	29.91	2.37	6.13	4.69
60	55.94	6.20	10.50	6.15
100	107.35	12.91	11.78	8.71
200	202.73	21.74	8.14	7.01
500	501.43	49.06	7.57	5.91

The curve fit data (Table 8) show that the calibrators selected fit the semi-log regression well (mean % differences between found and nominal were between 6 and 12%) and accurate quantitation should be expected within the range of calibrators used. The %CVs on the found concentrations were between 8 and 12%.

III. Sample Analysis

A. Study #199. A series of samples were collected from Wadsworth Canal over a three-month period. These samples were analyzed by GC and by immunoassay. For immunoassay the samples were analyzed without pretreatment and in addition a portion were analyzed following solid phase extraction. The MDL for the immunoassay method was set, conservatively, at 30 ppt as that was the lowest concentration tested in recovery studies. The LLD for the kit assay was 22 ppt according to the manufacturer.

Table 9. Immunoassay and GC results for samples collected for Study #199.

Sample #	ELISA Result (ppt) - direct				ELISA Result (ppt) - SPE				GC Result (ppt)
	Std Dev	%CV	n	Std Dev	%CV	n			
47	NT*				NT				59
48	39.8				NT				
65	NT				NT				
66	ND				NT				53
103	73.2				NT				85
138	59.6				NT				67
153	35.3				NT				42
182	ND				69.1	0.8	1.2	2	40
201	248.6	38.5	15.5	3	178.7	9.3	5.2	2	185
230	53.9				73.1	5.6	7.7	2	69
249	43.9				51.6	1.8	3.5	2	49
278	ND				42.3	2.7	6.4	2	ND
297	43.5	7.3	16.8	2	51.2	0.8	1.6	2	ND
328	609.2	63.5	10.4	4	495.5	54.9	11.1	8	536
341	218.4	30.8	14.1	4	147.6	23.5	15.9	4	164
376	200.4	8	4.0	3	132.3	17.3	13.1	6	119
389	296.3	40.5	13.7	3	215	15.9	7.4	6	154
418	362	39.8	11.0	4	292.3	73.5	25.2	3	289
441	58.5	1.3	2.2	2	50.8	2.8	5.5	2	49
466	452.6	87.8	19.4	4	282.7	51.6	18.3	6	393
491	74.7	7.8	10.4	2	50.3	12.9	25.7	2	64
555	ND			2	ND			4	ND
590	53.9				63.4	23.8	37.5	2	ND

*NT = not tested; ND = not detectable. Immunoassay MDL = 30 ppt; GC MDL = 40 ppt.

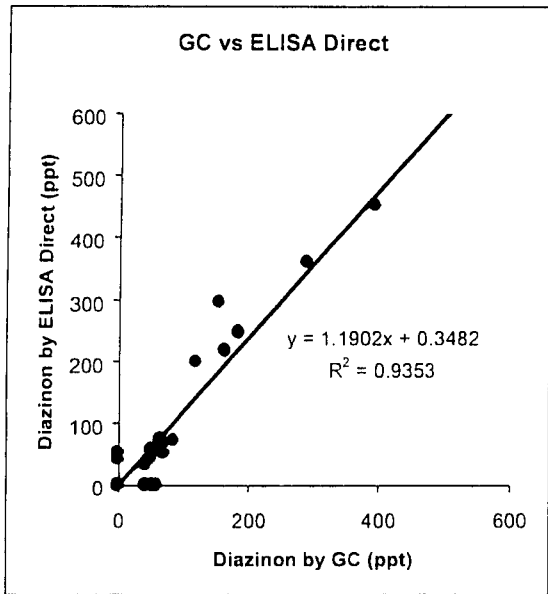


Figure 1. Comparison of analysis of samples by GC and by immunoassay without sample pretreatment.

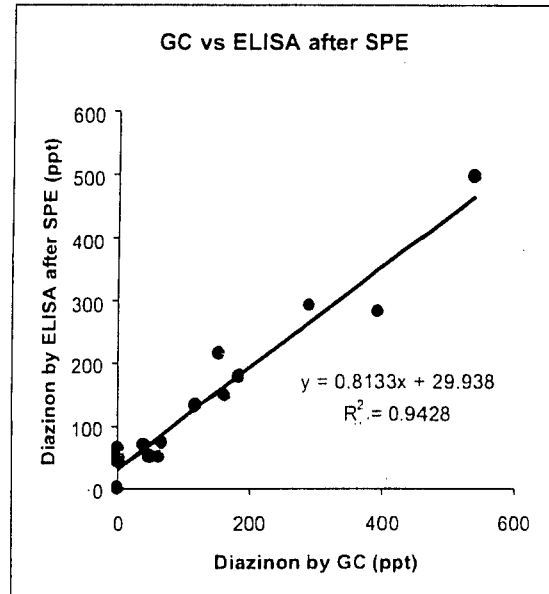


Figure 2. Comparison of analysis of samples by GC and by immunoassay following SPE.

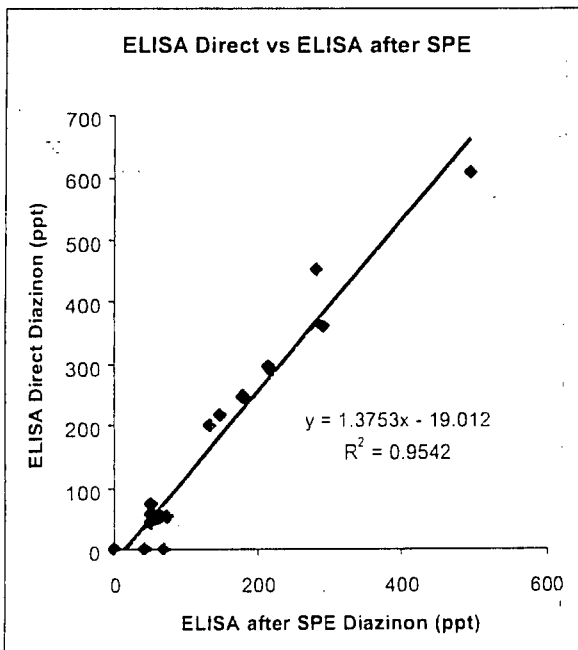


Figure 3. Comparison of analysis of samples by immunoassay without pretreatment (direct) and by immunoassay following SPE.

Figure 1 shows that the analysis of samples directly by ELISA agrees well with the GC data, with a slight high bias for the immunoassay. After solid phase extraction, values between immunoassay and GC still agree well, but the bias for the immunoassay is low. An examination of Table 9 shows that the high bias for samples analyzed by immunoassay and run directly is primarily due to samples containing high levels of diazinon (>200 ppt by immunoassay). Following cleanup, the data by immunoassay for these high level samples agrees better with GC data, implying that if a matrix effect was present, it was eliminated. However, because the values are low, there may be a recovery problem. This supports the idea that further work is necessary on the solid phase extraction method.

B. Study #201. A series of samples were collected during rain events in February 2001. The samples were analyzed by both GC and ELISA and the results shown in Table 10.

Table 10. Diazinon concentrations in water samples by ELISA and GC, organized by sample number.

Site	Date/Time	Sample #	ELISA Result (ppt)	GC Result (ppt)
Sacramento Outfall	2/10/01 10:55	10	ND*	125
Alamar	2/10/01 10:50	12	ND	ND
Butte Slough @ Lower Pass Road	2/9/01 12:00	60	ND	30
Mud Creek	2/9/01 16:00	87	ND	48
Lindo Channel	2/9/01 16:55	89	83	129
Big Chico Creek @ River Road	2/9/01 17:55	91	95	62
Big Chico Creek @ Rose Avenue	2/9/01 19:10	94	ND	ND
Big Chico Creek @ River Road	2/10/01 8:50	96	ND	ND
Big Chico Creek @ River Road	2/10/01 10:20	121	ND	ND
Stony Creek	2/10/01 11:20	122	ND	ND
Sacramento River @ Hamilton City	2/10/01 12:10	126	ND	ND
Mud Creek	2/10/01 13:10	130	ND	20
Lindo Channel	2/10/01 13:40	132	155	185
Sacramento River @ Hamilton City	2/11/01 11:50	133	ND	ND
Sacramento Outfall	2/11/01 11:30	158	73	141
Obanion South	2/11/01 12:03	160	87	143
Obanion North	2/11/01 12:17	162	ND	38
Sacramento Outfall	2/12/01 12:00	164	112	107
		168	ND	61
Sacramento River @ Hamilton City	2/14/01 11:10	182	ND	ND
Little Chico Creek	2/10/01 14:50	218	ND	20
Big Chico Creek @ Rose Avenue	2/10/01 15:25	221	ND	ND
Big Chico Creek @ River Road	2/10/01 16:00	223	ND	22
Big Chico Creek @ Rose Avenue	2/11/01 8:55	225	ND	20
Big Chico Creek @ River Road	2/22/01 9:45	227	ND	ND
Big Chico Creek @ River Road	2/9/01 9:20	242	ND	ND
Stony Creek	2/9/01 10:55	244	ND	21
Big Chico Creek @ Rose Avenue	2/9/01 12:10	247	ND	24

Table 10. Diazinon concentrations in water samples by ELISA and GC, organized by sample number (con't).

Site	Date/Time	Sample #	ELISA Result (ppt)	GC Result (ppt)
Little Chico Creek	2/9/01 13:35		249	62 133
Sacramento River @ Hamilton City	2/9/01 15:25		251	ND ND
Sacramento River @ Colusa	2/14/01 10:00		351	ND ND
Butte Slough @ Lower Pass Road	2/14/01 11:15		353	ND 25
Feather River @ Yuba City	2/14/01 11:50		356	ND 20
Jack Slough @ Doc Adams Road	2/14/01 12:20		358	ND 53
Sacramento Outfall	2/9/01 12:10		384	57 111
Sacramento River @ Colusa	2/10/01 10:55		434	ND 38
Butte Slough @ Lower Pass Road	2/11/01 11:25		436	ND 38
Feather River @ Yuba City	2/11/01 12:10		438	ND ND
Jack Slough @ Doc Adams Road	2/11/01 12:45		440	ND 102
Sacramento River @ Colusa	2/12/01 10:45		442	ND 20
Butte Slough @ Lower Pass Road	2/12/01 11:15		444	ND 27
Wadsworth	2/11/01 13:00		446	322 770
Butte	2/11/01 14:05		448	71 24
Main Canal	2/11/01 14:30		450	253 175
Main Canal	2/11/01 18:00		453	55 20
Main Canal	2/11/01 18:15		455	74 134
Main Canal	2/9/01 18:30		457	50 23
Wadsworth	2/9/01 19:20		459	76 61
Butte Canal	2/9/01 22:17		461	ND 28
Main Canal	2/9/01 22:40		463	128 101
Wadsworth	2/9/01 23:20		465	192 358
Butte Creek	2/10/01 2:20		467	76 26
Butte Creek	2/10/01 10:30		470	51 24
Main Canal	2/10/01 10:55		472	77 51
Wadsworth	2/10/01 14:10		474	323 513
Butte Creek	2/10/01 15:25		476	53 23
Main Canal	2/10/01 15:40		478	61 47
Butte Creek	2/11/01 2:10		480	69 25
Feather River @ Star Bend	2/14/01 10:40		486	34 ND
Feather River @ Star Bend	2/10/01 13:00		495	42 26
Bear River @ Berry Road	2/11/01 10:30		497	ND ND
Feather River @ Star Bend	2/11/01 13:00		500	ND ND
Bear River @ Berry Road	2/12/01 11:00		502	27 43
Feather River @ Star Bend	2/12/01 13:00		504	ND ND
Jack Slough @ Doc Adams Road	2/9/01 13:15		506	57 96
Sacramento River @ Colusa	2/10/01 10:20		508	ND ND
Butte Slough @ Lower Pass Road	2/10/01 11:20		510	ND 36
Feather River @ Yuba City	2/10/01 12:10		513	ND ND
Jack Slough @ Doc Adams Road	2/10/01 12:45		515	54 86

Table 10. Diazinon concentrations in water samples by ELISA and GC, organized by sample number (con't).

Site	Date/Time	Sample #	ELISA	GC
			Result (ppt)	Result (ppt)
Obanion South	2/10/01 12:22		558	65 58
Obanion North	2/10/01 12:39		561	ND 25
Bear River @ Berry Road	2/9/01 10:50		584	ND 50
Bear River @ Berry Road	2/10/01 10:30		586	ND 42
Feather River @ Yuba City	2/12/01 12:30		615	ND ND
Jack Slough @ Doc Adams Road	2/12/01 13:00		617	ND 66
Butte Creek	2/9/01 12:25		626	ND 32
Main Canal	2/9/01 12:50		630	ND 20
Wadsworth	2/9/01 16:05		632	ND 38
Butte Creek	2/9/01 18:15		635	ND 28
Main Canal	2/10/01 2:38		637	68 83
Wadsworth	2/10/01 3:20		639	160 207
Butte Creek	2/10/01 6:15		642	37 28
Wadsworth	2/10/01 7:35		648	108 1380
Butte Creek	2/11/01 6:10		661	ND 20
Main Canal	2/11/01 7:00		665	56 91
Wadsworth	2/11/01 8:25		668	294 418
Butte Creek	2/11/10 10:15		670	ND 23
Main Canal	2/11/01 10:30		672	72 95
Butte Canal	2/12/01 18:15		696	ND ND
Obanion South	2/12/01 12:50		699	ND 31
Obanion South	2/9/01 12:50		701	ND ND
Obanion North	2/9/01 13:10		705	ND 24
Butte	2/13/01 10:31		710	ND 21
Main Canal	2/13/01 10:45		713	ND 47
Main Canal	2/11/01 2:20		722	37 92
Wadsworth	2/11/01 3:00		724	257 453
Wadsworth	2/11/01 19:00		725	159 829
Butte Creek	2/9/01 18:55		746	ND 22
Main Canal	2/10/01 19:10		748	ND 44
Wadsworth	2/10/01 20:00		750	178 872
Butte Creek	2/10/01 22:05		752	ND 22
Main Canal	2/10/01 22:20		754	33 54
Wadsworth	2/10/01 23:00		756	212 630

* ND = not detected, below the reporting limit. The reporting limit for GC was 20 ppt. The reporting limit for the ELISA was 30 ppt.

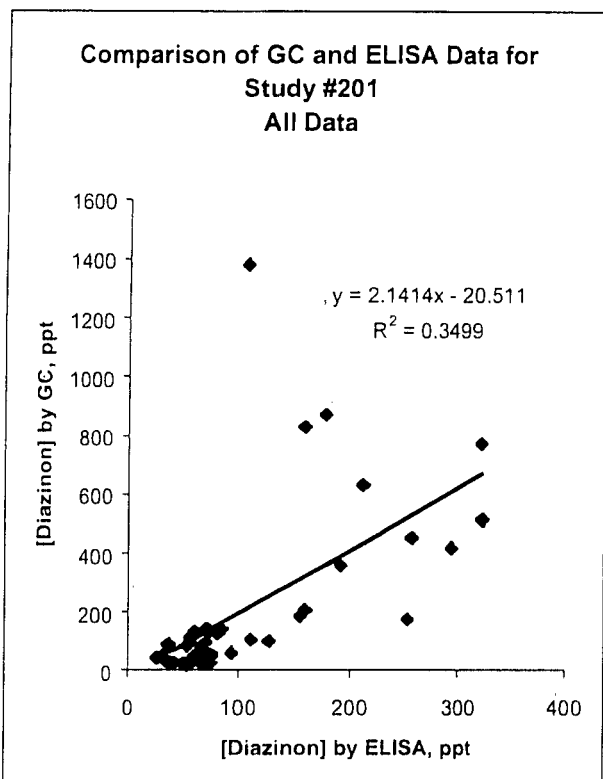


Figure 4. Comparison of GC and ELISA data for samples from study #201. All data are shown on this graph except for non-detects.

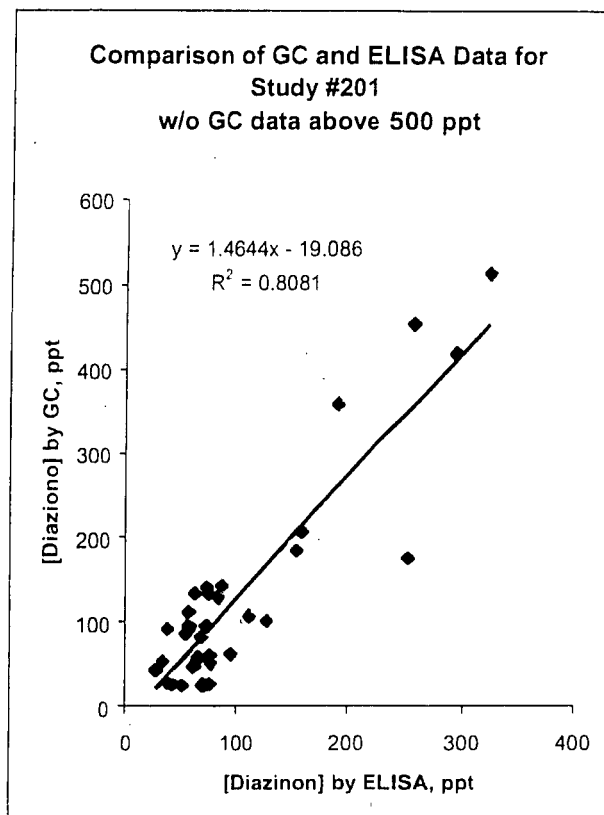


Figure 5. Comparison of GC and ELISA data for samples from Study #201. All points that were non-detects or correlated to GC values above 500 ppt were omitted.

The correlation in Figure 4 for all data points is poor. GC values were much higher than ELISA values in this study. Examination of data in Table 10 show that discrepancies were greatest at concentrations of 500 ppt and greater as measured by GC. When these data are omitted, (Figure 5), the correlation greatly improves. Nevertheless, there is still a strong high bias for GC values. This is in contrast to results of Study #199 where the high bias was in favor of the ELISA.

Conclusion

The Strategic Diagnostics test kits performed to the specifications given in the test kit insert. The curve fit data (Table 8) show that the calibrators selected fit the semi-log regression well (mean % differences between found and nominal were between 6 and 12%) and accurate quantitation should be expected within the range of calibrators used. The %CVs on the found concentrations were between 8 and 12%. The kits should be used before their expiration date to assure quality data.

The high bias detected in earlier studies was not reproduced in the work conducted here. The test kit itself, does not seem to be a problem, thus the most likely explanation is interference from the matrix and that this interferent(s) was not present this season.

The solid phase extraction method used was apparently successful in removing interferences, but further work is needed to optimize and characterize the method.

A wide variety of compounds can interfere with the assay (as seen in the cross reactivity studies), but no one compound is likely responsible for the bias seen earlier. Significant interference might be seen if several of these compounds were present simultaneously.

The correlation among study #201 immunoassay and GC data was less strong than for study #199. This might be because study #199 samples were all collected at the same sight, although sorting data in study #201 by site does not show any particular correlation to variability.

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Department of Pesticide Regulation



Gray Davis
Governor

Winston H. Hickox
Secretary, California
Environmental
Protection Agency

Paul E. Helliker
Director

MEMORANDUM

TO: John Sanders, Ph.D.
Chief, Environmental Monitoring Branch
Department of Pesticide Regulation

FROM: Frank Spurlock, Ph.D.
Senior Environmental Research Scientist (specialist)
Environmental Monitoring Branch

DATE: January 13, 2002

SUBJECT: **STUDY SUMMARY: EVALUATION OF INTERFERENCES IN ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) FOR DIAZINON**

BACKGROUND

In January and February, 2000, a Sacramento Valley surface water monitoring study was jointly conducted by the U.S. Geological Survey (USGS) and the California Department of Pesticide Regulation (DPR) (Dileanis et al., 2002). The purpose of the study was to characterize the rainy season occurrence and sources of diazinon in the Sacramento and Feather Rivers. Water samples were collected from 17 monitoring sites and analyzed for the presence of diazinon and other selected pesticides. Diazinon analysis on most samples was conducted using enzyme-linked immunosorbent assay (ELISA, 412 samples), while replicate splits from approximately 30 percent of those samples were also analyzed using gas chromatography/thermionic specific detection (GC/TSD, 107 samples) for confirmation. A small number of samples were analyzed using gas chromatography/mass spectrometry (GC/MS, 31 samples), but only 10 samples were also analyzed by ELISA and/or GC/TSD. Additional details on sampling locations, sampling procedures and analytical methods are discussed by Dileanis et al. (2002).

There were 87 split samples in which diazinon was detected in both the ELISA and GC/TSD methods above their respective limits of detection (20 ng/L for GC/TSD, 30 ng/L for ELISA). The ELISA method yielded higher concentrations than GC/TSD in every sample (Figure 1), with percent differences between ELISA and GC/TSD ($=\frac{[ELISA-GC/TSD]}{[GC/TSD]} * 100$) ranging from 7.5 to 429 percent, with a median of 81 percent (Figure 2). The ELISA method demonstrated a similar positive bias relative to the GC/MS method in nine of 10 samples in which detections were reported for both methods. The percent difference data were analyzed to determine if larger differences between the two analytical methods were associated with specific sampling sites, types of sampling sites (river vs. tributary), or varied systematically with concentration (Figure 3). No significant differences between sites, types of sites or concentration were evident.

The quality assurance/quality control (QA/QC) plan of the USGS/DPR winter 2000 Sacramento Valley diazinon study included rinse blanks, field blanks, reagent blanks, blank spikes, and matrix spikes (Dileanis et al., 2002). Diazinon was not detected in any rinse blank or field blank



samples. ELISA matrix spike recoveries were elevated, with an average recovery of 130% and a range in spike recoveries of 111-161% (n=14). These QC data are limited, but suggest some bias in the ELISA diazinon results for the Sacramento Valley samples due to matrix effects. The GC/TSD matrix spikes yielded a mean recovery of 87% (n=4), while GC/TSD analysis of American River water sample spikes demonstrated a mean recovery of 85% (n=11). Any possible matrix effect on GC/TSD is apparently smaller in magnitude than that observed for ELISA, and reduces instead of enhances GC/TSD analytical results.

Traditional GC-based methods for determination of diazinon in water have a demonstrated history of quantitative recoveries and reproducibility and so are usually considered to be the "gold standard" relative to newer methods such as ELISA. In addition, ELISA is also prone to matrix effects – either due to the presence of cross-reactants or nonspecific interferences. Sullivan and Goh (2000) reported that ELISA yielded elevated diazinon concentrations in storm runoff water samples relative to a gas chromatography/flame photometric detection method (GC/FPD). These researchers were unable to determine the specific cause of the apparently elevated ELISA results. Sullivan and Goh concluded "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."

Consequently DPR designed a study in conjunction with the University of California (UC) with the primary objective of identifying any specific or non-specific interferences in Sacramento Valley dormant season runoff water that may be responsible for the high biased winter 2000 ELISA concentration data.

The study was performed under contract with Dr. B. Hammock and Shirley Gee of UC Davis; and detailed study data for this project are provided in the final report (Hammock and Gee, 2002). This memo is a summary of the main study conclusions and provides general recommendations for use of ELISA in future studies.

SUMMARY OF STUDY RESULTS

1. Cross-reactivity

Thirty different chemicals were tested for cross-reactivity in the laboratory using the brand of diazinon ELISA kit used to analyze the winter 2000 dormant spray runoff samples of Dileanis et al. (2002). These chemicals included structurally similar pesticides and degradates, other dormant-season high use organic pesticides, a variety of other organic pesticides, and inorganic pesticides. In certain cases a small degree of cross-reactivity was observed, but at levels too small to explain the consistent high bias in the winter 2000 dormant spray ELISA analytical results.

2. Recovery studies of spiked environmental water samples

Water samples were collected from two sampling sites in the Sacramento Valley in early December 2000, immediately prior to the 2001 dormant spray season diazinon applications. These samples were spiked with known amounts of diazinon and analyzed using ELISA; spike recoveries were variable, and there was no consistent bias in analytical recoveries relative to the known spike levels. The apparent bias that was observed in the previous year's sampling was not evident in these matrix spikes.

3. Comparison of ELISA to gas chromatography/flame photometric detection (GC/FPD) analysis of 2001 dormant season water sample splits

Water samples were collected during the 2001 dormant spray season and analyzed by GC/TSD and ELISA. Many of the 2001 sampling locations were either identical to or very close to those used during the 2000 dormant spray runoff sampling of Dileanis et al. (2002). There were 50 of the 2001 dormant season samples in which diazinon was detected by both the ELISA and GC/TSD methods. Among these data the median percent difference of the two methods was not significantly different than zero (Wilcoxon 1-sample test, $p=0.98$). No high bias in ELISA results relative to GC/TSD was evident. However, the percent differences between the two methods were highly variable, ranging from approximately -90% to 200% (Figure 4).

4. An additional observation

Shortly after the present study was initiated an additional possible cause for high bias in ELISA concentrations was discovered: use of expired ELISA kits. During analysis of diazinon samples from an unrelated DPR Environmental Monitoring study, the analyst discovered a strong high bias for the "expired" ELISA results (> 1 month past expiration) relative to GC/FPD (Figure 5, Appendix 1). It is possible that if expired or compromised ELISA kits were inadvertently used to analyze the winter 2000 dormant season samples, this would explain some or all of the apparent bias in those ELISA data. At this time there is no way to determine the status of the ELISA kits that were used to analyze the winter 2000 dormant spray data of Dileanis et al. (2002).

CONCLUSION

This study failed to identify a definitive cause for the (apparently) high-biased diazinon ELISA concentrations in Sacramento Valley water samples reported by Dileanis et al. (2002). It appears unlikely that a particular constituent was the cause of high biased ELISA concentrations in the winter 2000 monitoring study of Delineas et al. (2002) because (a) the high bias was apparent for ELISA-determined diazinon concentrations in all samples from every location in 2000, (b) 2001 ELISA samples displayed no such consistent bias, and (c) several pesticides with high use in the Sacramento Valley were shown to have no or little effect on the SDI immunoassay.

During the course of this study it was discovered that expired or compromised ELISA kits may yield data that are too high. While this is one possible explanation for the consistent bias observed between ELISA and GC/TSD in the 2000 data, there is no way to determine the status of the kits that were used to obtain those data.

The percentage differences between GC and ELISA results obtained on sample splits were highly variable in both 2000 and 2001: in 2000, the percentage differences ranged from 8 to 430 percent, whereas the range in 2001 was -92 to 196 percent. The inter-quartile range (25th to 75th centiles) was greater than 50 percentage points in both years: 54% - 107% in 2000, and -39% to 33% in 2001. Finally, the standard deviation of percent difference between GC and ELISA was 41 and 70% in 2000 and 2001, respectively. These and similar data (e.g., Holmes et al., 1998) illustrate the variability among analytical methods, and emphasize the need to thoroughly vet newer methods such as ELISA.

It is obvious that a robust QA/QC plan is imperative for all studies, and particularly the use of matrix spikes and control limits to confirm the veracity of data from each analytical set. If control limits are exceeded, analysis should always stop and diagnostic procedures should be used to identify problems in the analytical procedure. Finally, in those instances that the Environmental Monitoring Branch utilizes ELISA for diazinon analysis, we should continue to analyze splits of a substantial portion of ELISA samples using standard chromatographic methods for confirmatory purposes.

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**1. diazinon concentrations -
Elisa vs GC/TSD results - winter 2000 samples**

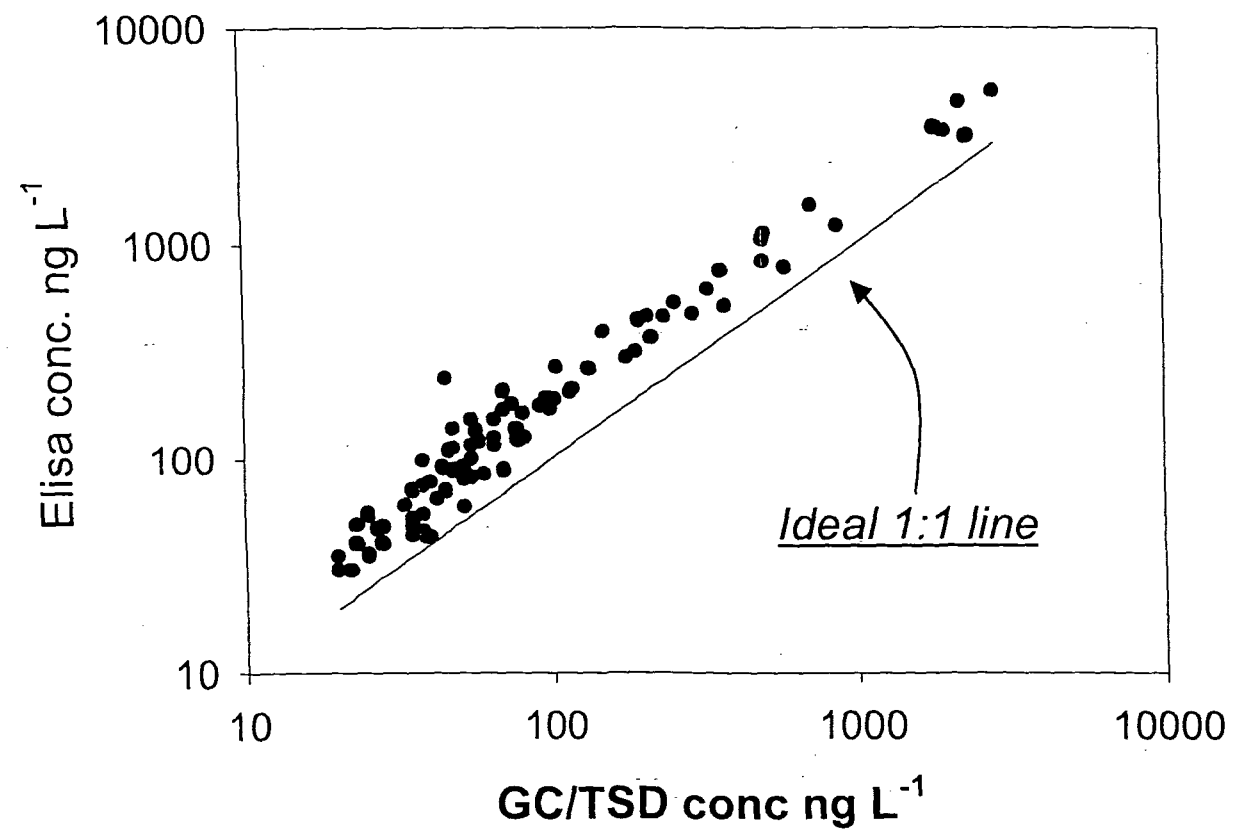


Figure 2. distribution of percent difference for winter 2000 dormant season runoff data (Dileanis et al. ,2002) (n=87)

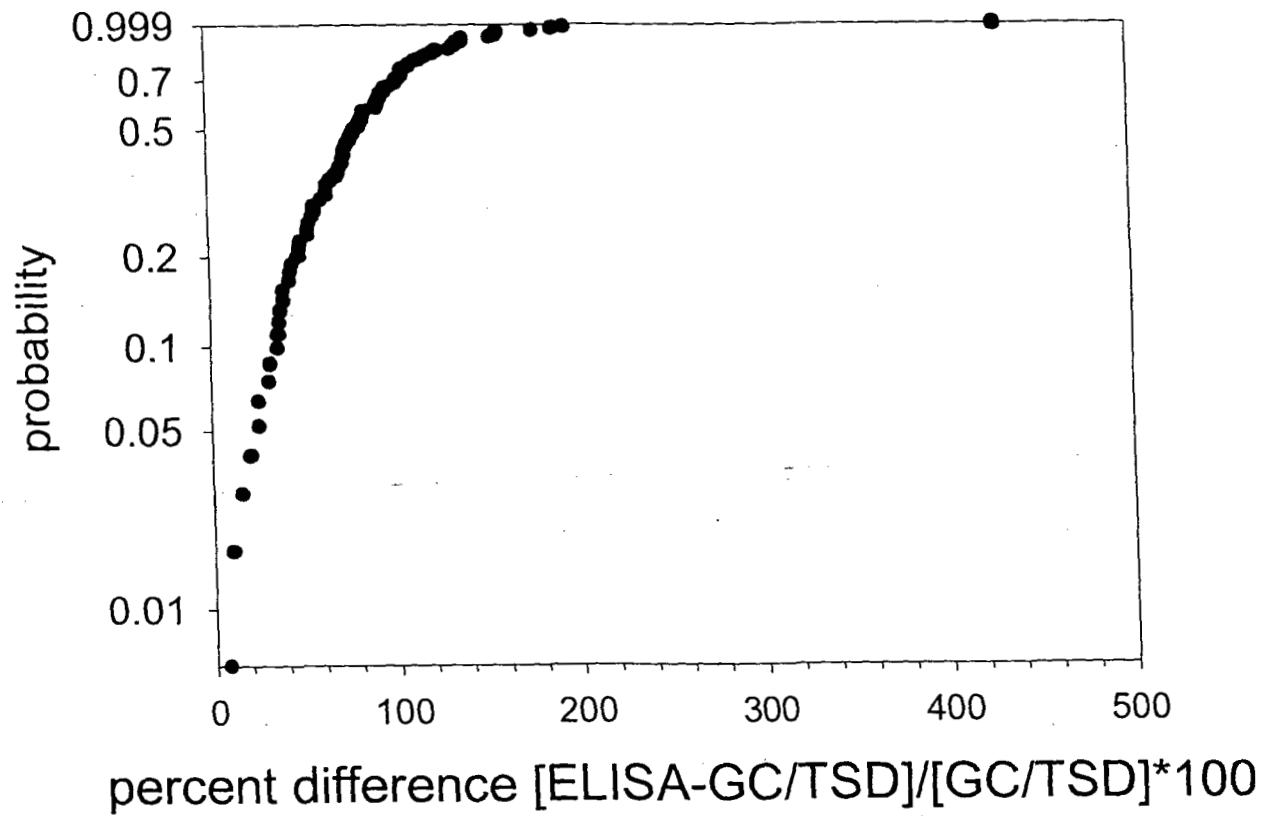


Figure 3. percent difference vs GC/TSD diazinon conc. (ng/L) for winter 2000 runoff (Dileanis et al., 2002)

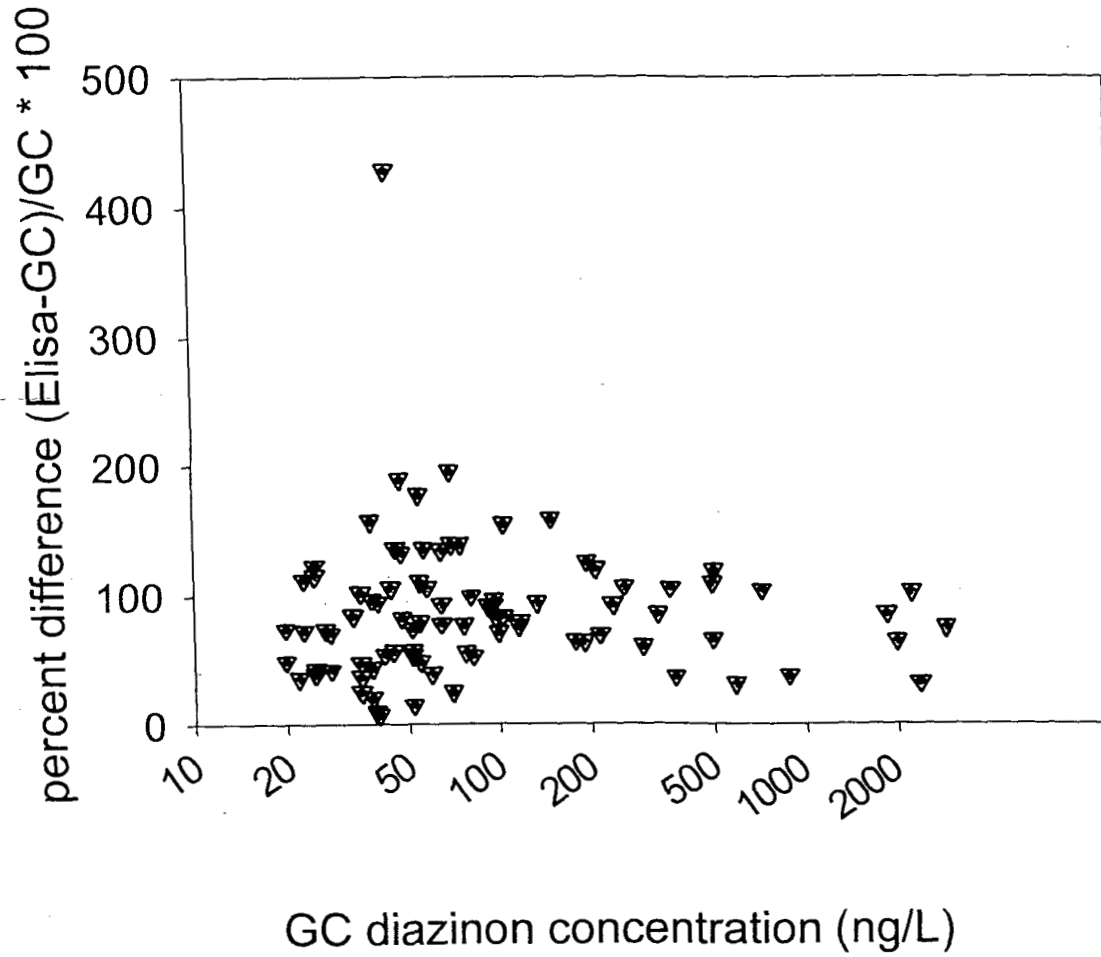


Figure 4. Distribution of percent difference for winter 2001 Sacramento Valley dormant season samples (n=56)

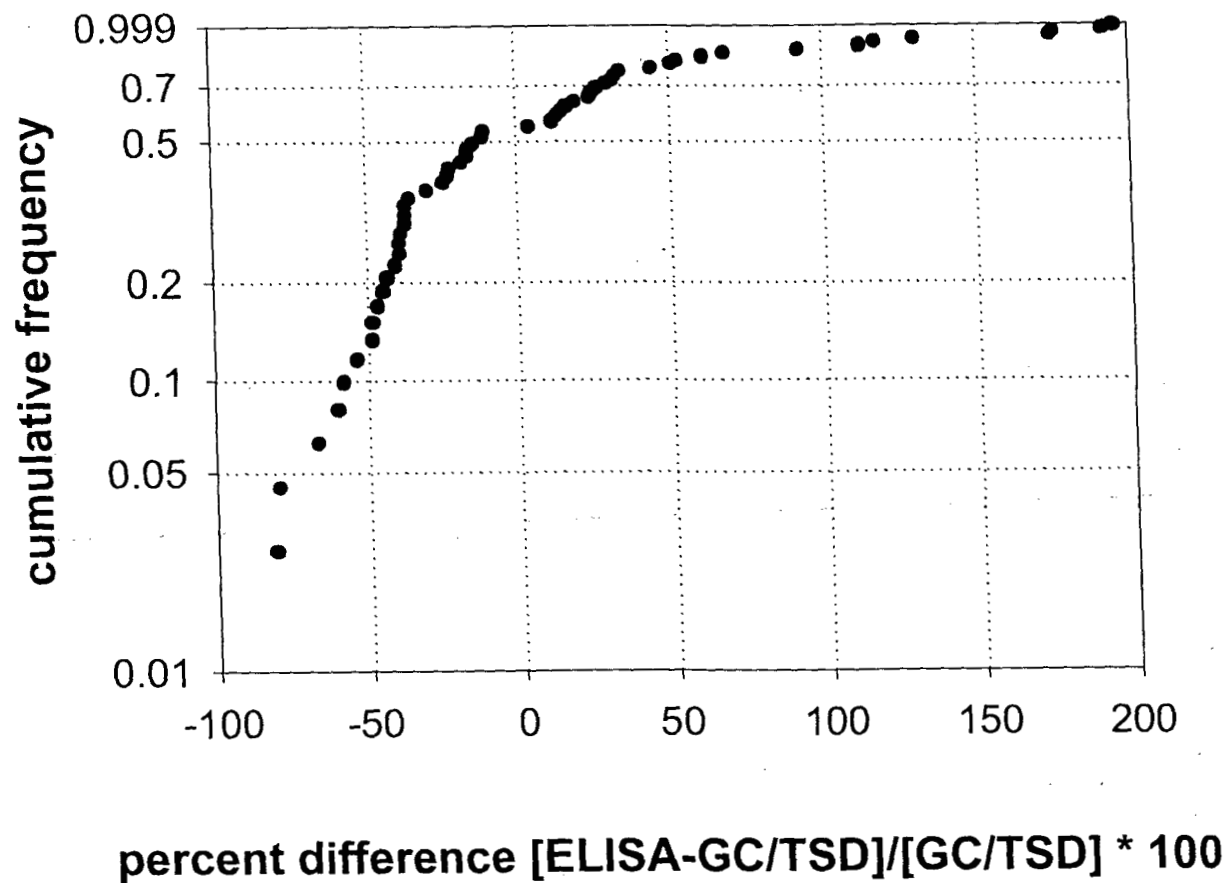
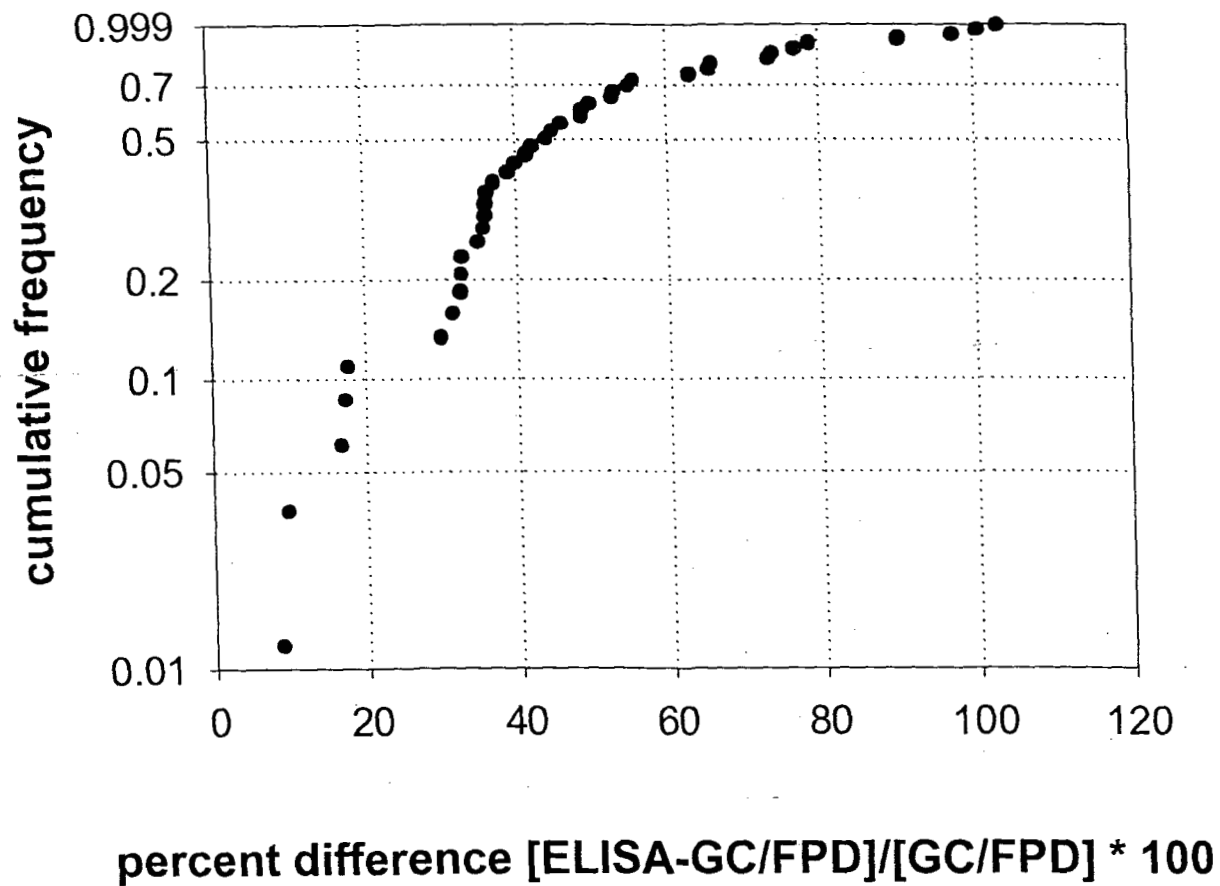
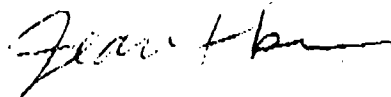


Figure 5. Percent difference between "expired" ELISA kit data and GC/FPD (n=41)



Appendix 1

Date: December 22, 2000
To: Catherine Cooper
From: Jean Hsu 
Subject: The Results of **Diazinon** Analysis in Water by **ELISA**

The Strategic Diagnostics Inc. (SDI) **ELISA** plate kit was used for the determination of **Diazinon** in this study. The kits **expired** in 3 1/2000. Since I observed that one of the reagent (substrate) had color change from colorless to light blue, I became **concerned** the **accuracy** of the test results.

For samples (194-61 to 194- 1 0 1), the **results** by **ELISA** were **much higher** than the results by GC method. See attached result **table**.

Since all the samples **have** been diluted 1: 10,000 times before analysis, there **should** not be any background interferences. Even after I tried to use a fresh substrate prepared in-house to substitute the **reagent** of the kit, the results were still unacceptable. The **color** turned out to be **too pale** to generate a good standard curve.

In order to have **reliable results** by **ELISA**, **expired kits** should never be used. In addition, we should not substitute **any components** of the kits with in-house reagent.

①

Appendix 1

The Diazinon results for water by SDI ELISA				
Rec'd	12/14/2000			
Allquoted	12/14/2000			
Diluted:	12/18/2000	All samples have been diluted 1:10,000 and not been filtered.		
Analysis:	12/18-21/00			
Sample	CDF#	ELISA result ug/L	GC Result ug/L	% Different
194-61	2000-2620	8280	5707	9.24%
194-62	2000-2621	6510	3626	49.62%
194-63	2000-2622	6900	4215	33.32%
194-64	2000-2623	5180	3816	26.01%
194-65	2000-2624	4880	3584	26.62%
194-66	2000-2625	4550	3429	34.33%
194-67	2000-2626	3720	3158	16.09%
194-68	2000-2627	3970	2895	31.32%
1 9463	2000-2628	4080	3007	30.52%
1 w-70	2000-2629	3870	2187	55.57%
194-71	2000-2630	3960	2214	58.68%
194-72	2000-2631	3650	2359	42.97%
194-73	2000-2632	3550	2045	53.80%
194-74	2000-2633	3100	2357	27.23%
194-75	2000-2634	3450	2599	26.14%
194-76	200012635	2560	1882	30.53%
194-77	2000-2636	2320	1989	16.41%
194-78	2000-2637	2320	1889	21.63%
194-79	2000-2638	3480	1753	65.49%
194-80	2000-2639	2770	1779	43.67%
194-81	2000-2640	3100	2180	34.66%
194-82	2000-2641	7830	4715	49.69%
194-83	2000-2642	6030	3441	37.52%
194-84	2000-2643	4720	2713	54.00%
194-85	2000-2644	5080	2523	66.91%
194-86	2000-2645	3630	2438	39.37%
194-87	2000-2646	3710	2474	39.97%
194-88	2000-2647	3740	2442	41.98%
194-89	2000-2648	4000	2862	29.62%
194-90	2000-2649	4170	2180	62.26%
194-91	2000-2650	2810	2400	8.38%
194-92	2000-2651	2830	2179	25.99%
194-93	2000-2652	2580	2183	15.90%
194-94	2000-2653	3330	2454	30.29%
194 - e	2000-2654	3360	231%	38.66%
194 - a	2000-2655	3050	1897	42.21%
194-97	2000-2656	3170	2127	38.38%
194-98	2000-2657	3450	1898	58.04%
194-99	2000-2658	2700	1788	41.83%
194-100	2000-2659	2370	1784	28.21%
194-101	2000-2660	2810	1950	36.13%
BK				
Spike	1ppm	1.36	1.36%	
Spike	1ppm	1.36	1.36%	
Spike	1ppm	1.19	1.19%	
Spike	1ppm	0.981	95.1%	
Spike	1ppm	1.37	1.37%	
Spike	1ppm	1.18	1.18%	
Spike(PE)	175ppt	201	1.15%	
Spike(PE)	175ppt	209	1.19%	

% Different: (ELISA result - GC result)/Average of ELISA & GC result X 100

2

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

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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 ($0.0159 \mu\text{g L}^{-1}$) was comparable to the least detectable dose (LDD) established by the manufacturer ($0.022 \mu\text{g L}^{-1}$). 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;

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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 multi-residue 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 diazinon enzyme 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-pyrimidinyl] phosphorothioate), and pirimiphos-methyl (*O,O*-dimethyl-*O*-[2-(diethylamino)-6-methyl-4-pyrimidinyl] 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/ThermoLynce, 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-

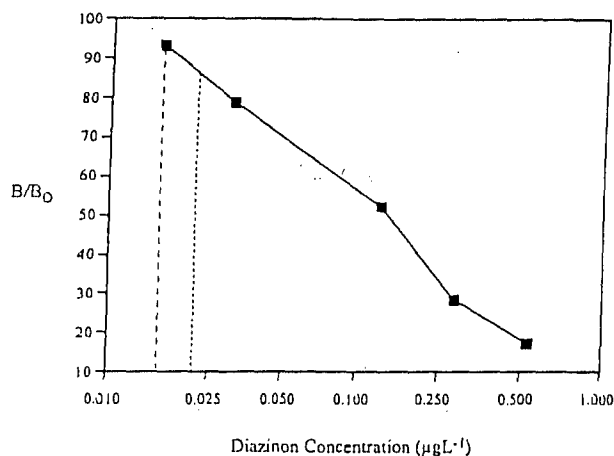


Figure 1. Standard curve (semilog scale) for diazinon used for the calibration of the EnviroGard ELISA kit. The dotted line represents the least detectable dose (LDD) for the kit ($0.022 \mu\text{g L}^{-1}$) determined by the manufacturer. The dashed line approximates both the experimentally determined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

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 $1000 \mu\text{g L}^{-1}$ 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

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

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 ($0.030\text{--}0.500 \mu\text{g L}^{-1}$) and below the least detectable dose (LDD) determined by the manufacturer ($0.022 \mu\text{g L}^{-1}$) 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 $0.016 \mu\text{g L}^{-1}$ spike was 1.0165 ± 0.0161). The LDD of the EnviroGard diazinon microtiter plate kit was calculated

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

spike level ($\mu\text{g/L}$)	mean OD	SD	%CV	mean concn ($\mu\text{g/L}$)	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

^aThe acronyms OD, SD, and CV represent optical density, standard deviation, and coefficient of variation, respectively.

by the manufacturer as the amount of diazinon required to achieve 85% B/B_0 , where B/B_0 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 ($0.015 \mu\text{g L}^{-1}$) suggest that sensitivities are somewhat higher (approximately 32%) than those determined by the manufacturer using the B/B_0 method (i.e., $0.022 \mu\text{g L}^{-1}$). The correlation coefficient (r) for the diazinon standard curve was 0.9964.

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 $\leq 0.100 \mu\text{g L}^{-1}$. 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 ($0.035 \mu\text{g L}^{-1}$) 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):

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

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

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

spike level ($\mu\text{g L}^{-1}$)	mean ^a ($\mu\text{g L}^{-1}$)	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 $\mu\text{g L}^{-1}$) 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 $\mu\text{g L}^{-1}$ 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 $\pm 20\%$ (Hammock et al., 1990).

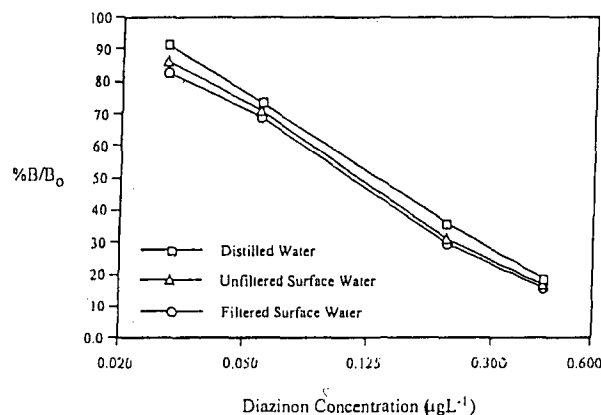


Figure 2. Graphical representation of matrix interference effects from standard curves (semilog scale) for the diazinon EnviroGard ELISA kit in distilled, unfiltered, and filtered surface water.

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 $\mu\text{g L}^{-1}$.

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 $\mu\text{S/m}$), unfiltered surface water (pH = 7.41, EC = 606 $\mu\text{S/m}$), and filtered surface water (pH = 7.39, EC = 666 $\mu\text{S/m}$) 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

matrix	rel. stand. curves		index of matrix interfer.	
	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

$$I_m = \frac{|\text{ABS}_{\text{blank A}} - \text{ABS}_{\text{blank B}}|}{\text{ABS}_{\text{blank A}}} \quad (4)$$

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

$$N = \frac{100 - I_m}{100} \quad (5)$$

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

$$C_x = N C_{\text{measured}} \quad (6)$$

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., $[\text{slope}_{\text{control}} - \text{slope}_{\text{matrix}}]/\text{slope}_{\text{control}} \approx I_m$. 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

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

Analogues	Structure	IC ₅₀ ($\mu\text{g L}^{-1}$)	%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

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).

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 (Beasley et al., 1997). Only diazoxon, the O-analogue of the target compound, has been found to exhibit significant cross-reactivity ($\text{IC}_{50} < 1,000 \mu\text{g L}^{-1}$) (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

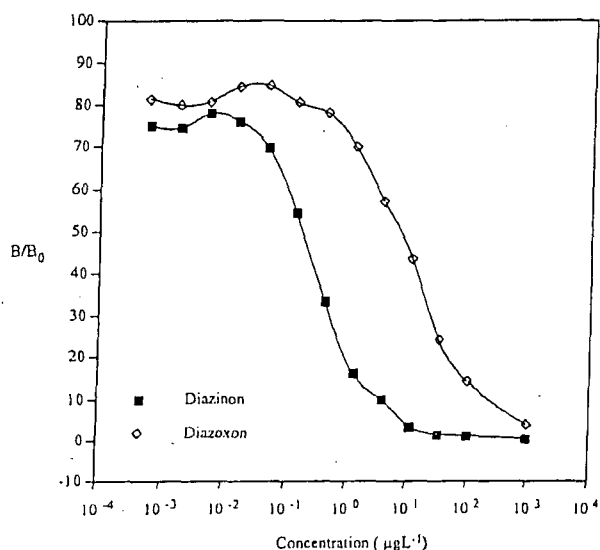


Figure 3. Standard curves showing specificity EnviroGard diazinon kit antibody for diazinon and diazinon O-analogue (diazoxon).

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

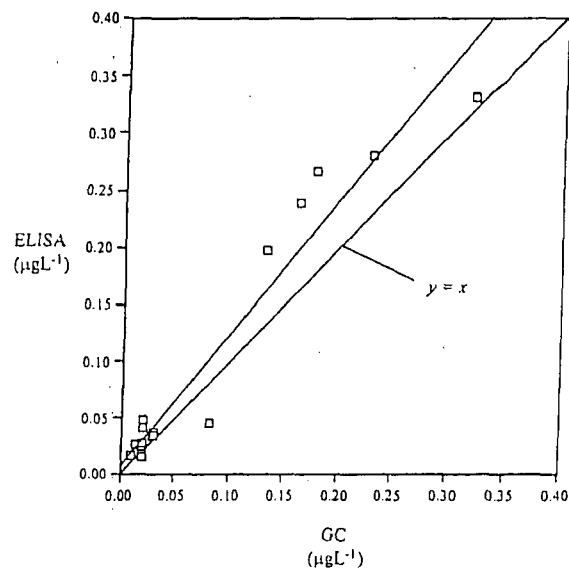


Figure 4. Correlation between GC and ELISA results for the analysis of field water samples obtained from two sampling sites on the Sacramento River, CA. The equation of the line is $y = 1.178x + 0.006$ ($n = 18$, $r = 0.969$, $F = 243.5$, $s = 0.028$, $p < 0.0001$).

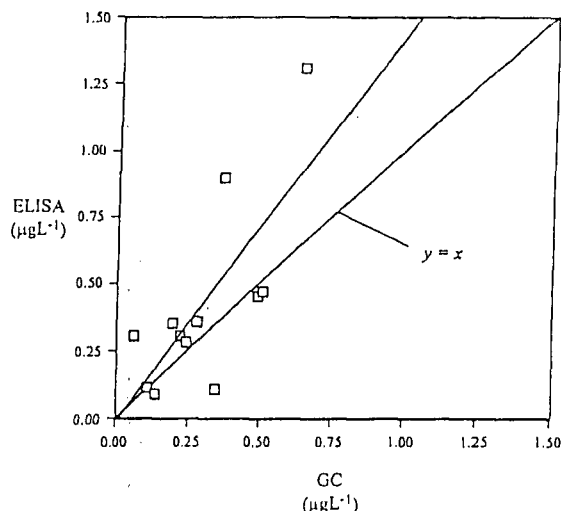


Figure 5. Correlation between GC and ELISA results for the analysis of field water samples obtained from nine sampling sites in Orange County, CA. The equation of the line is $y = 1.452x - 0.10$ ($n = 12$, $r = 0.728$, $F = 11.25$, $s = 0.254$, $p < 0.0073$).

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 ($0.200 \mu\text{g L}^{-1}$) only around 10 times less than that of diazinon ($0.022 \mu\text{g L}^{-1}$), 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 $\pm 20\%$. 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 ($200 \mu\text{L}$), 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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