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Study 268: Evaluation and Validation of a Commercial ELISA Kit for Permethrin in Surface Water

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I. Introduction

ELISA, enzyme-linked immunosorbent assay, is a biochemical technique used for detecting specific compound(s) in environmental and biological samples. The technique has been successfully applied for analyses of pesticides in soil and water samples by the Department of Pesticide Regulation, Environmental Monitoring Branch using in-house assays (Goh 1992a, 1992b, 1993, 1996) and commercially available kits (Goh 1990, 1991). Recently, the Environmental Monitoring Branch (Sullivan et al. 2000, 2007) also evaluated commercial diazinon and chlorpyrifos ELISA kits for their reliability and usefulness for surface water samples collected in environmental monitoring projects and controlled field experiments. Compared to conventional analytical methods such as gas chromatography (GC), high pressure liquid chromatography (HPLC) and mass spectroscopy, ELISA is considered a more rapid, cost-effective, and less labor-intensive alternative for analysis of pesticides in large numbers of environmental samples (Study 222 Protocol; Sullivan et al. 2000, 2007). However, ELISA is also prone to matrix effects – either due to the presence of cross-reactants or nonspecific interferences (Hammock and Gee, 2002).

II. Objectives

The objectives of this study are to evaluate a commercial permethrin ELISA kit for (1) sensitivity, precision, accuracy, matrix effects, and selectivity, and (2) comparability to a liquid-liquid GC method.

III. Personnel

Staff from the Surface Water Program, Environmental Monitoring Branch under the general direction of Sheryl Gill, Sr. Environmental Scientist, will conduct the study.

Key personnel are listed below:

Project Leader:	Xin Deng, Ph.D.
Senior Scientist:	Frank Spurlock, Ph.D.
Lab Liaison:	Sue Peoples

Questions concerning this study please contact Xin Deng, Environmental Scientist, at (916) 445-2506.

IV. Study Plan

The study will evaluate the performance of a commercial paramagnetic tube ELISA kit for the determination of total permethrin including 4 permethrin isomers for sensitivity, selectivity, intra-assay accuracy and matrix effects in fortified distilled water and environmental surface water samples. The repeatability and reproducibility of the ELISA kit will also be examined according to the current EPA criteria for the assessment of analytical methods (Lesnik 1994). Comparability between the ELISA kit and liquid-liquid GC method will be performed in environmental surface water samples upon the availability of sample pre-concentration method that will be developed by Abraxis.

The permethrin ELISA kit in a magnetic particle-based tube format (Abraxis LLC, Warminster, PA) will be employed in the study according to manufacturer's instructions. The kit provides a detection range of 1-15 ppb for permethrin. The detailed procedures for the analysis are described below in section 4.1. Briefly, the assay applies the principles of enzyme linked immunosorbent assay by mixing test samples with paramagnetic particles attached with antibodies specific to permethrin in glass tubes, followed by adding a pyrethroid enzyme conjugate. The permethrin in the sample and enzyme conjugate compete for the antibody binding sites on the magnetic particles. After an incubation period, a magnetic field created by a two-piece magnetic separation tube rack is applied to hold the paramagnetic particles with unknown pyrethroids and labeled permethrin analog bound to the antibodies on the particles, and allows the unbound reagents to be decanted. The particles in the tube are then washed and a color solution (a mixture of hydrogen peroxide and 3,3',5,5'-tetramethylbenzidine) is added. The chemicals in the color solution react with the enzyme-labeled permethrin analog bound to the antibodies and converts them to a color product. Absorbance of the color can then be measured on a spectrophotometer at 450 nm wavelength. Since the labeled permethrin conjugate is in competition with the unlabeled pyrethroids in the samples for the antibody sites, the color developed is inversely proportional to the concentration of permethrin in the sample (Pyrethoids ELISA Precedure, Abraxis LLC).

The ELISA kit is the only commercial kit available for pyrethroids analysis on the market. It has the lowest detection limit of 0.75 ppb for permethrin and cross-reacts with several pyrethroids at higher concentrations (Table 1). The kit could be potentially used for screening permethrin in environmental samples upon validation. However, results from both urban and agricultural surface water monitoring projects have shown that pyrethroid concentrations including permethrin are typically at parts per trillion levels in both urban and agricultural settings, below the lowest detection limit of the ELISA kit. Therefore, pre-concentration steps in water samples are necessary in order to meet its detection range. The Abraxis laboratory is developing a pre-concentration method to lower the detection limits of the kit. Consequently, the study will focus on the objective 1 by validating sensitivity, precision, accuracy and selectivity in clean spiked matrices. Evaluation for the pre-concentration method and comparability study between ELISA and GC analyses for environmental samples will be conducted in the future while the pre-concentration and extraction methods are available.

Table 1 Cross-reactivity pattern of the Abraxis pyrethroid ELISA kit expressed as the least detectable concentration (LDC)

Compound	LDC (ppb)
Permethrin	0.75
Cypermethrin	4.75
Lamda Cyhalothrin	9.2
Bifenthrin	13.5
Resmethrin	200
Cyfluthrin	200
Tetramethrin	>1000
3-phenoxybenzoic acid	170

V. ELISA Analysis

A. Materials and Equipment

i) Permethrin ELISA kit produced by Abraxis LLC, Warminster, PA
Permethrin Antibody Coupled Paramagnetic Particles
Permethrin Enzyme Conjugate, horseradish peroxidase (HRP) labeled
Permethrin Standards: Five concentrations (0.75, 2.5, 5.0, 15.0 ppb)
Positive Control: A concentration 3.0 ppb
Diluent/Zero Standard
Color Solution: A solution of hydrogen peroxide and 3,3',5,5'-tetramethylbenzidine in an organic base.
Stopping Solution: diluted sulfuric acid (0.5%).
Washing Solution: preserved deionized water
Test Tubes: 100 glass tubes per kit

ii) Single wavelength benchtop RPA-1 photometric analyzer (Strategic Diagnostics, Inc., Newark, N.J.)

iii) Two-piece 60-position magnetic separation rack (Strategic Diagnostics, Inc., Newark, N.J.)

iv) Vortex Mixer (Vortex Genie)

v) Digital balance and timer

vi) Eppendorf adjustable-volume (100-1000 µL) reference sampling pipette (Eppendorf, Hamburg, Germany)

vii) Eppendorf microman positive displacement repeating pipette (100-300 µL)

B. Procedures

i) Label glass test tubes for standards, control, and samples.

ii) Add 250 µL of the appropriate standard, control, or sample to the test tube. Preparation for environmental samples is described under Section VI

iii) Mix the Pyrethroid Antibody Coupled Paramagnetic Particles thoroughly and add 500 µL to each tube.

iv) Vortex for 1 to 2 seconds minimizing foaming.
v) Incubate for 20 minutes at room temperature.
vi) Add 250 µL of Pyrethroid Enzyme Conjugate to each tube.
vii) Vortex for 1 to 2 seconds minimizing foaming.
viii) Incubate for 30 minutes at room temperature.
ix) Separate in the Magnetic Separation Rack for two (2) minutes.
x) Decant and gently blot all tubes briefly in a consistent manner.
xi) Add 1 mL of Washing Solution to each tube and vortex tubes for 1-2 seconds. Return tubes and allow to remain in the magnetic separation unit for two (2) minutes.
xii) Decant and gently blot all tubes briefly in a consistent manner.
xiii) Repeat Steps 12 and 13 an additional time.
xiv) Remove the rack from the separator and add 500 uL of Color Solution to each tube.
xv) Vortex for 1 to 2 seconds minimizing foaming.
xvi) Incubate for 30 minutes at room temperature.
xvii) Add 500 µL of Stopping Solution to each tube.
xviii) Add 1 mL Washing Solution to a clean test tube. Use as blank in Step 20.
xix) Read results at 450 nm within 15 minutes after adding the Stopping Solution.

VI. GC Analysis (Based on WPCL Method #53, DPR Method #299)

Samples are collected in one liter amber glass bottles and iced or refrigerated at 4 °C from time of collection until extraction. GC analysis will follow the DPR method for synthetic pyrethroids in water samples (Method #299). Briefly, permethrin will be analyzed by solvent extraction of the "whole" water sample (water + sediment) with methylene chloride (DCM), followed by a subsequent rinse of the sample bottle twice with DCM and combining extracts. Extracts will be dried over anhydrous Na_2SO_4, reconstituted in isooctane after solvent exchange and analyzed by Agilent 6890 GC/ECD. Paired standard t-test will be performed to compare the results between GC and ELISA methods at a significance level of 0.05.

VII. Data Analysis

Calculations

A. Spectrophotometric Measurements and Analysis

i) From measured absorbencies, calculate the mean absorbance value (B) for the standards and samples.

Calculate B/Bo for each of the standards/samples:

B/Bo = (mean absorbance of standards or samples) / (mean absorbance of negative control)

Construct a standard curve by plotting the B/Bo for each standard on a vertical logit (Y) axis versus the corresponding permetherin concentration on a horizontal logarithmic (X) axis:

$$\text{Logit } (B/Bo) = m(\ln C) + b$$

Where

$$\text{Logit } (B/Bo) = \ln [(B/Bo)/(1-(B/Bo))]$$

The calibration curve should have a correlation coefficient $r > 0.99$ ($R2 > 0.98$).

The manufacturer (Abraxis, Inc) defines the limit of detection (LOD) as 90% B/Bo. For the determination of the LOD on an assay to assay basis, subtract three times the standard deviation (SD) of the negative control from its mean absorbance (MABS)

$$\text{LOD } (\%B/Bo) = \text{MABS negative control} - 3*\text{SD negative control}$$

Which gives the LOD in terms of % B/Bo. Substitute LOD into the equation of the line to convert to units of concentration. Alternatively, the minimum concentration of substance that can be measured may be expressed in terms of the method detection limit (MDL). The MDL should be determined at the 99% confidence level by multiplying the appropriate one-tailed 99% t-statistic by the standard deviation obtained from a minimum of three analyses of a matrix spike containing the analyte of interest at a concentration three to five times the estimated MDL (SW-846, US EPA, 1992).

$$\text{MDL} = t(n-1, \alpha = 0.99) \text{ (standard deviation)}$$

The estimated MDL can be determined by locating the concentration value that corresponds to an instrument signal/noise ratio within the range of 2.5 to 5.0. The t-statistic is obtained from standard reference tables or from the table below:

Number of Samples	t-statistic
3	6.96
4	4.54
5	3.75
6	3.36
7	3.14
8	3.00
9	2.90
10	2.82

B. Quality Control

i) Accuracy

Measure sample replicates in each matrix and express as the percent coefficient of variation (% CV):

%CV = (standard deviation of sample/mean concentration of sample [pbb])*100

ii) Precision

Both intra-assay and inter-assay precision, i.e., the agreement among a set of replicate measurements without assumption of knowledge of the true value, is determined from the repeated measurement of the same control sample. Precision may be expressed as the relative standard deviation (RSD) or the percent coefficient of variation (%CV):

RSD = %CV = (standard deviation of control/mean concentration of control [pbb])*100

Values of %CV should not exceed ± 20% (Krotzke and Seeh 1995).

iii) Reproducibility

Measure sample concentrations (C) in each matrix and then compare observed values to expected values:

Reproducibility = (C observed / C expected) *100

Percent recovery for each sample should fall within the 80% to 120% range (Krotzke and Seeh 1995).

C. Cross-Reactivity

i) Assay specificity is evaluated by using compounds that are structurally related to the target analyte as antibody competitors (Manclus and Montoya, 1995). Cross-reactivity for individual compounds is calculated as the ratio of target analyte concentration to the concentration of the cross-reacting compound at 50% of the maximum signal (IC50). If specificity studies are done, IC50 values for each potential cross-reactant are generated from a 4-parameter fit of experimentally-determined absorbance versus spike concentration data, and percent cross-reactivity is calculated from these data (Gee et al. 1996). The equation for the 4-parameter fit is given by:

$$Y = (A/D) / [1 + (x/C)B] + D$$

Where y is the absorbance, x is the analyte concentration, A and D are the upper and low asymptotes, respectively, B is the slope, and C is the central point of the linear portion of the curve, i.e., the IC50 (Rodbarb 1981). Once IC50 values for each cross-reacting compound is determined, percent cross- reactivity is calculated:
%CR = (IC50 target analyte / IC50 tested cross-reacting compound)*100

Pyrethroid analytes to be tested for cross-reactivity will include those that are commonly used in urban and agricultural settings, i.e. lamda-cyhalothrin, cyfluthrin, cypermethrin, bifenthrin, esfenvalerate, and resmethrin.

D. Matrix Effects

Typically, interferences are quantified by comparing a standard calibration curve produced in a control matrix such as distilled or buffered water with a calibration curve generated in the matrix containing interferences. The slope of a calibration curve with interferences is less steep than with the control system. (Midgley et al. 1996). An alternative method for quantitatively assessing matrix interferences is also available (Cairoli et al. 1996). Absorbance values for matrix blanks are first normalized with respect to the absorbance of the blank control matrix,

$$Im = (ABSBlank\ A - ABSBlank\ B) / ABSBlank\ A$$

Where ABS is the mean absorbance determined from experiment, A is the control matrix (e.g., DI or buffered water), and B is the unspiked environmental matrix. The term Im is known as the index of matrix interference. Upon calculating Im for a particular matrix, it is then used to derive a correction factor, N:

$$N = (100-Im) / 100$$

Which is subsequently employed for the direct quantitation of the analyte of interest

$$Cx = NCmeasured$$

Where Cx is the actual analyte concentration and Cmeasured is the analyte concentration determined from the calibration curve. With this approach, the calculated Im values can be considered a "true" matrix interference, thus allowing the determination of the analyte in each matrix directly from the calibration curve in the control matrix.

E. Bias

The situation often arises that a kit can react with far more substances that can be measured by full protocol methods, thus biasing the ELISA results on the high side (U.S. EPA 1996). To account for potential bias due to matrix effects, measured values are compared to an accepted reference value in a sample of known concentration or by determining the recovery of a known amount of contaminant spiked into a sample, i.e., a matrix spike. Bias due to matrix effects based on a matrix spike is calculated as

$$Bias = (Xs - Xu) - K$$

Where

Xs = measured value of spiked sample
Xu = measured value of unspiked sample
K = known value of spike in the sample

The percent recovery (%R) is then determined from the following equation:

$$\%R = [(Xs - Xu)/K] * 100$$

Samples yielding measured concentrations less than the LOD are reported as nd or "none detected". Samples which yield concentrations greater than the LOD but less than the linear range of the kit (0.75 ppb) are reported as "< 0.75 ppb". If samples yield concentrations greater than the linear range of the kit (15 ppb), they are reported as ">15 ppb".

VIII. Timetable

Lab set up: June 2010
Chemical Analysis: July-September 2010
Preliminary Memorandum: December 2010
Final Report: February 2011

IX. Budget

Personnel & Benefits (7 days)	1820
Cross-reactivity: 2 kits (100 tubes each kit)	1000
Specificity: 2 kits	1000
Matrix effects/Accuracy/Precision: 1 kit	500
Intra-assay reproducibility: 1 kit	500
Field samples: 1 kit	500
Field samples (GC, 30 samples)	5000
Total	$10,320

X. References

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