Department of Pesticide Regulation
Environmental Monitoring Branch
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STUDY 222: Evaluation and Validation of a Commercial ELISA for Chlorpyrifos in Surface Waters

I. INTRODUCTION

The Department of Pesticide Regulation Environmental Monitoring Branch has successfully used ELISA for analyses of pesticides in soil and water samples for over a decade using both in-house assays (Goh 1992a, 1992b, 1993, 1996) and commercially available kits (Goh 1990, 1991). We recently evaluated a commercial diazinon kit for its sensitivity, selectivity, accuracy and precision, etc. for surface water sample analyses (Sullivan and Goh, 2000). This study is a continuing effort in evaluating new ELISA kits for its reliability and usefulness as rapid and cost effective analytical tools for samples generated in our environmental monitoring project, regulatory compliance or controlled field experiment.

II. OBJECTIVES

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

III. PERSONNEL

Staff from the Registration and Environmental Monitoring Branch, Surface Water Protection Program, under the general direction of Kean S. Goh, PhD., Agricultural Program Supervisor IV, will conduct this study.

Key personnel are listed below:

Project Leader:	Jonathan Sullivan, Ph.D.
Senior Scientist:	Frank Spurlock, Ph.D.
Lab Liaison:	Carissa Ganapathy

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IV. STUDY PLAN

The performance of a commercial paramagnetic tube ELISA kit for the determination of chlorpyrifos will be 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 will show whether the kit satisfies current EPA criteria for the assessment of analytical methods. For validation of the paramagnetic tube ELISA format, environmental surface water samples will be collected, split, and analyzed directly by ELISA and by liquid-liquid extraction followed by GC. Results of the two analytical methods will then be compared statistically.

The chlorpyrifos RapidAssay kit (Strategic Diagnostics, Inc., Newark, N.J.), a 100-tube magnetic particle-based ELISA with an affirmed detection range of 0.22-3.0 μgL^{-1}, will be employed for all ELISA analyses performed in this study. For the comparative evaluation of ELISA and GC methodologies for surface water samples, all ELISA analyses will be conducted according to instructions included with the kits and using provided reagents. These reagents include chlorpyrifos antibody (mouse monoclonal antichlorpyrifos) coupled paramagnetic particles, chlorpyrifos horseradish peroxidase (HRP) labeled enzyme conjugate, color solution (hydrogen peroxide and 3,3',5,5'-tetra-methylbenzidine), stopping solution (0.5% sulfuric acid), washing solution (deionized water), standards (0, 0.22, 1.0, and 3.0 μgL^{-1}) and control (1.8 μgL^{-1}). Spectrophotometric measurements for the chlorpyrifos ELISA tube kit samples will be determined at 450 nm with a single wavelength, benchtop RPA-I photometric analyzer (Strategic Diagnostic, Inc). A two-piece magnetic separation rack consisting of a 60-position tube rack that fits over a paramagnetic base is employed for holding the magnetic particles in the tubes after incubation with enzyme conjugate and for allowing unbound reagents to be decanted. An Eppendorf Microman Positive Displacement Repeating Pipet (Eppendorf, Hamburg, Germany) and an Eppendorf 12-channel adjustable-volume sampling pipette will be used to dispense liquids.

The literature and practical experience has shown that the analysis of environmental samples by commercial ELISA kits are generally much less costly and labor-intensive than is the corresponding analysis performed by gas or liquid chromatography. This is particularly true for the analysis of environmental waters, samples of which can often be analyzed directly or after simple filtration without the need for lengthy extraction and cleanup steps. It cannot be presumed, however, that ELISA will yield analytical results of a quality comparable to that of GC with respect to method sensitivity, accuracy, and precision. These parameters must be established experimentally, and is accomplished by statistically comparing ELISA and GC data acquired from the analysis of split field water samples. Regression analysis of these data will allow the two methods to be evaluated in terms of such standard statistical parameters as the correlation coefficient, slope, F-test, and t-test.

These statistics make it possible to quantitatively assess the performance of the ELISA kit. GC analysis will be performed at a state laboratory .

A. ELISA ANALYSIS

1) Materials And Equipment
   a) Chlorpyrifos RapidAssay ELISA kit (Strategic Diagnostics, Inc., Newark, N.J.)
      i) Chlorpyrifos horseradish peroxidase (HRP) labeled enzyme conjugate.
      ii) Color solution (hydrogen peroxide and 3,3',5,5'-tetra-methylbenzidine).
      iii) Stopping solution (0.5% sulfuric acid).
      iv) Washing solution (deionized water).
      v) Standards (0, 0.22, 1.0, and 3.0 μgL^{-1})
      vi) Control (1.8 μgL^{-1}).
      vii) 100 polystyrene tubes
   b) Single wavelength, benchtop RPA-I photometric analyzer (Strategic Diagnostics, Inc., Newark, N.J.).
   c) Two-piece 60-position magnetic separation rack (Strategic Diagnostics, Inc., Newark, N.J.)
   d) Vortexer (Vortex Genie)
   e) Digital balance & Timer
   f) Eppendorf adjustable-volume (100-1000μL) Reference sampling pipette (Eppendorf, Hamburg, Germany)
   g) Eppendorf Microman Positive Displacement Repeating Pipet (100-300μL).
   h) Origin Pro 7.5 Statistical Software

   2) Procedures
      a) Label disposable test tubes (calibrators, controls, matrix blanks, and samples) and place in the tube rack.
      b) Add 250 μL of the negative control and calibrators to the appropriate tubes.
      c) Add 250 μL of unknowns to be analyzed to their respective tubes.
      d) Add 250 μL of HRP-labeled enzyme conjugate to each tube.
      e) Add 500 μL of chlorpyrifos antibody coupled paramagnetic particles to each tube.
      f) Vortex each tube for 1-2 seconds.
      g) Allow the mixture to incubate for 15 minutes at room temperature.
      h) At the end of the incubation period, join the tube rack containing standards and samples and the paramagnetic base and allow the magnetic particles in solution to separate for two minutes.
      i) Holding the base and the rack together, slowly invert the rack and decant the contents of the tubes into a sink.
      j) While still inverted, gently blot the tubes on an absorbent pad to remove excess solution.
      k) Add 1 ml of washing solution and allow to stand for 2 minutes.
      l) Decant and tap the tubes dry on an absorbent pad.
      m) Repeat the wash step two more times.

n) After tubes were thoroughly washed, remove the tube rack from the paramagnetic base.
o) Add 500 μL of color solution to all tubes.
p) Vortex tubes for 1-2 seconds.
q) Incubate at room temperature for 20 minutes.
r) After incubation, add 500 μL of stopping solution to each tube.
s) Read results at 450 nm on the RPA-I photospectrometer.

B. GC ANALYSIS

1) Extraction Procedures (Based on CDFA Method EM 46.0)
   a) Remove water samples from the refrigerator and allowed to come to room temperature.
   b) Extract samples by shaking with 100 ml of methylene chloride for two minutes. Drain the organic layer through 20 g of anhydrous sodium sulfate into a clean boiling flask.
   c) Extract the water layer two more times using 80 ml of methylene chloride, following the same procedure as above.
   d) After the final extraction, rinse the sodium sulfate with 25 ml of methylene chloride.
   e) Evaporate the sample extract to dryness on a rotary evaporator (Büchi/Brinkman) in a 35 °C water bath and at a vacuum of approximately 20 inches Hg.
   f) Add 5 mL of acetone to the residue and swirl the contents to dissolve the solid extract.
   g) Transfer the extract solution to a clean, calibrated 15 ml graduated test tube.
   h) Rinse the flask two times with 2 ml of acetone and combine the contents of each wash.
   i) Using a gentle stream of nitrogen, evaporate the acetone to a volume slightly less than 1 ml and bring the final volume to 1 ml with the drop-wise addition of acetone.

2) Field runoff samples from a controlled experiment on PAM-calcium (protocol pending) will be analyzed by ELISA, and about 30 random split-samples will be analyzed by GC.

3) Analytical Procedures
   a) Exact analytical instrumentation and parameters will be determined. GC analysis will be performed at a state laboratory, the name and location of which will be determined at the time of need based on the availability of facilities and personnel.

V. DATA ANALYSIS

1) CALCULATIONS
   a) Spectrophotometric Measurement and Analysis

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

      Calculate B/B_0 for each of the standards/samples:

      B/B_0 = (mean absorbance of standards or samples)/(mean absorbance of negative control)

      Construct a standard curve by plotting the B/B_0 for each standard on a vertical logit (Y) axis versus the corresponding chlorpyrifos concentration on a horizontal logarithmic (X) axis:

      $$\text{logit}\ (B/B_0) = m(\ln C) + b$$

      where

      $$\text{logit}\ (B/B_0) = \ln\ [(B/B_0)/(1- (B/B_0))]$$

      The calibration curve should have a correlation coefficient $r > 0.99$ ($R^2 > 0.98$).

      %B/Bo for controls and samples will then yield levels in ppb of chlorpyrifos by direct calculation solving the resulting equation of the line for ln C:

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

      The manufacturer (Strategic Diagnostics, Inc., Newark, N.J.) defines the limit of detection (LOD) as 90% B/B_0. For the determination of the LOD on an assay to assay basis, subtract three times the standard deviation (SD) of the negative control from the its mean absorbance (MABS)

      $$\text{LOD}\ (\%B/B_0) = \text{MABS}_{\text{negative control}} - 3*\text{SD}_{\text{negative control}}$$

      which gives the LOD in terms of % B/B_0. Substitute LOD ($\%B/B_0$) 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). Determine the MDL as follows:

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

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
2) 3	3) 6.96
4) 4	5) 4.54
6) 5	7) 3.75
8) 6	9) 3.36
10) 7	11) 3.14
12) 8	13) 3.00
14) 9	15) 2.90
16) 10	17) 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 [ppb]) * 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 [ppb]) * 100

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

iii) Reproducibility.

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

$$\text{reproducibility} = (C_{observed}/C_{expected}) * 100$$

Percent recovery for each sample should fall within the 80% to 120% range (Krotzky 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 (Manclús and Montoya, 1995). Cross-reactivity for individual compounds are calculated as the ratio of target analyte concentration to the concentration of the cross-reacting compound at 50% of the maximum signal (IC_{50}). If specificity studies are done, IC_{50} values for each potential cross-reactant are generated from a 4-parameter fit of experimentally-determined absorbances versus spike concentration data, and percent cross-reactivities are 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 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} (Rodbarb, 1981). Once IC_{50} values for each cross-reacting compound is determined, percent cross-reactivities are calculated from the formula

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

d) Matrix Effects

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 ensuing relative slope of a standard calibration curve in a matrix containing interferences is less steep than with the control system Thus,

$$m_{\text{matrix system}} < m_{\text{control system}}$$

for those matrices having interfering components (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,

$$I_m =[ABS_{\text{Blank A}} - ABS_{\text{Blank B}}] / ABS_{\text{Blank A}}$$

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 I_m is known as the *index of matrix interference*. Upon calculating I_m for a particular matrix, it is then used to derive a correction factor, N

$$N = [(100\text{-}I_m)/100]$$

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

$$C_X = NC_{measured}$$

where C_X is the actual 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 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

$$Bias = (x_S - x_u) - K$$

where

x_S = measured value of spiked sample
x_u = measured value of unspiked sample
K = known value of spike in the sample

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

$$\%R = [(x_S - x_u)/K]*100$$

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 (0.22 ppb) are reported as "< 0.22 ppb". If samples yield concentrations greater than the linear range of the kit (3.0 ppb), they are reported as "> 3.0 ppb".

VI. TIMETABLE

Lab setup: April 2004
Chemical Analysis: May 2004

Preliminary Memorandum: November 2004
Final Report: January 2005

VII. BUDGET

Personnel & Benefits (7 days) ……	$1,820
Cross-reactivity: 2 kits (100 tubes each kit) ……. ……	1,100
Specificity: 2 kits (100 tubes each kit) ……	1,100
Matrix effects/Accuracy/Precision: 1 kit (100 tubes) ……	550
Intra-Assay Reproducibility: 1 kit (100 tubes)……..	550
Field Samples (ELISA)……..	550
Field samples (GC, 30 samples) …… ……	5,000
TOTAL ……	**$10,650**

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