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Chlorpyrifos in Catfish *(Ictalarus punctatus)* Tissue

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Chlorpyrifos [*o, o,* -diethyl-o- (3,5,6-trichloro-2-pyridyl) -phosphorothioate] is a broad spectrum organophosphorus insecticide commonly known as Dursban®. It is the active component of a number of formulations used in crop and turf applications (Racke 1993), as well as, control of household pests (Tomlin 1994). Chlorpyrifos is a potential replacement for organochlorine insecticides (e.g., aldrin and dieldrin) which are now either discouraged or banned for control of termites (Wright et al., 1988).

A recent report indicated that 10.5% of farm-raised channel catfish *(Ictalurus punctatus)* sampled fillets contained detectable chlorpyrifos residues (Santerre et al. 1999). Data from the National Contaminate Biomonitoring Program (EPA 1992) showed that chlorpyrifos was found in nine out of sixteen wild channel catfish samples with concentrations above the detection limit (i.e., 2.5 ppb). Since there is no established tolerance for chlorpyrifos in fish, contaminated fish entering interstate commerce would be deemed adulterated by the FDA.

Traditionally, residue analysis (FDA, Mills Method) relied upon classical analytical methods incorporating gas chromatography or mass spectrometry. Such methods require trained individuals to operate sophisticated instruments and interpret chromatograms or spectral results. Most traditional residue methods are accurate, but they are also time consuming, costly, and generally not adaptable to use in the field. The aquaculture industry needs a low cost and rapid method for measuring chlorpyrifos in fish.

An alternative analytical approach is to utilize immunochemical methods for residue analysis (Kaufman and Clower 1995). These methods are capable of achieving low detection levels, are adaptable to a variety of matrices, and require little in the way of instrumentation (Meulenberg 1995). However, researchers have had difficulty applying ELISA to the measurement of lipophilic compounds

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in high fat matrices (Zajicek 1996). This difficulty is due to problems encountered when removing interferences during cleanup to prevent false positive results. The Strategic Diagnostic Chlorpyrifos RaPID Assay® is an ELISA that employs paramagnetic particles coated with anti-chlorpyrifos antibodies. Kits have been successfully used for detection of chlorpyrifos in water; however, their application in high fat biological samples e.g. channel catfish *(Ictalarus punctatus)* tissue has been limited. The objective of this research was to develop a procedure for extraction of chlorpyrifos from fish tissue prior to analysis using a commercially-marketed ELISA kit.

MATERIALS AND METHODS

Catfish fillets, obtained from a local grocery store, were ground and spiked with chlorpyrifos dissolved in methanol (AccuStandard Inc., New Haven, CT). Extraction of fat and chlorpyrifos from fish tissue was performed according to a modified Mills Method (PAM 304 C1 ; FDA 1994). Five grams fish tissue was weighed into a cellulose thimble (33mm x 80mm, Whatman, Fisher Scientific, Fair Lawn, NJ), mixed with 40 g anhydrous sodium sulfate (Certified A.C.S., J.T.Baker, Philipsburg, NJ), 10 g washed sand (Fisher Scientific, Fair Lawn, NJ) and placed in a desiccator at ambient temperature overnight. Mixtures were extracted with a Soxhlet extractor for seven hours with 150 ml petroleum ether at a turnover rate of 8 min. The extract was evaporated under nitrogen (99.995%) at 52°C and transferred to a 150 ml separatory funnel. Lipid extracts were partitioned four times with 30 ml acetonitrile (HPLC, GC and Pesticide Grade, Burdick & Jackson, Muskegon, MI) saturated with petroleum ether (GC and Pesticide Grade, Burdick & Jackson) and shaken vigorously for 2 min each time. The separated acetonitrile layers were transferred into a 1 L separatory funnel containing 650 ml deionized water, 40 ml saturated NaCl (Ultrapure Grade, J.T.Baker) solution, and 100 ml petroleum ether. The aqueous layer was transferred to a second 1 L separator-y funnel and again extracted with 100 ml petroleum ether. The petroleum ether extracts were combined and washed with two 100 ml portions of water containing 5 ml saturated NaCl. After washing, solvent was evaporated to near dryness under nitrogen using Turbovap II (Zymark, Co. Hopkinton, MA) and N-Evap (Model 112, Organomation, South Berlin, MA) evaporators. Extract was diluted in 10 ml methanol. Since the assay can only detect total chlorpyrifos up to 3 ppb, a series of serial dilutions can be used. To analyze a sample with an unknown amount of chlorpyrifos, serial dilutions can also be applied. If sample concentration exceeds the upper limit for the standard curve with the lowest concentration of analyte, then further dilutions can be applied. A 20-fold dilution was made by adding 0.5 ml extract to 9.5 ml diluent (Strategic Diagnostic Inc., Newark, DE). To make a 200-fold dilution, we

first prepared a lo-fold dilution by adding 0.5 ml extract in 4.5 ml diluent, then diluted each extract 20 fold. The 400-fold dilution was achieved in a similar manner. First 0.5 ml extract was diluted in 9.5 ml diluent to get a 20-fold dilution then 0.5 ml of the prior 20-fold dilution was added to 9.5 ml diluent. Then, 250 µl diluted extract was added, along with an enzyme conjugate (250 µl), to a disposable test tube and combined with 500 µl paramagnetic particles that had antibodies specific to chlorpyrifos attached. Both the chlorpyrifos, in the sample and the horseradish peroxidase-conjugated-chlorpyrifos, competed for antibody (mouse monoclonal anti-chlorpyrifos) binding sites on the magnetic particles. After a 15 min incubation period, a magnetic field was applied to hold the pammagnetic particles with antibodies in the tube and the tubes were decanted. After decanting, the particles were washed with deionized water. The presence of chlorpyrifos in the samples was detected by adding 500 µl color solution containing enzyme substrate, hydrogen peroxide, and the chromogen, 3,3', 5,5'-tetramethylbenzidine. The enzyme conjugated chlorpyrifos analog bound to the antibody catalyzed the conversion of the substrate to a colored product. After 20 min incubation, the reaction was stopped and stabilized by the addition of 500 µl 0.5% sulfuric acid. Since the labeled chlorpyrifos (conjugate) competes with the unlabeled chlorpyrifos (sample) for antibody binding sites, the color developed is inversely proportional to the concentration of chlorpyrifos in the sample. Absorption values (λ=450 nm) were measured and the concentration of chlorpyrifos in the samples was calculated. Standard curves were constructed by plotting the Logit(B/B_0) vs. chlorpyrifos concentration as follows:

$$Logit\left(\frac{B}{B_0}\right) = k * \ln(X) + b$$

Where:

k = slope
b= y- intercept
B = fortified sample absorption
B_o= blank absorption
X= sample chlorpyrifos concentration

Three standard curves were made with three dilution factors 40, 200, 800 with linear ranges of 0.01-0.09 ppm, 0.09-0.46 ppm, 0.47-1.91 ppm, respectively. Lower limit of detection (LOD) was calculated as described by Manclús and Montoya (1995).

To determine recovery, catfish samples that had been previously shown by the FDA Mills Method to contain no chlorpyrifos residue, were spiked with chlorpyrifos solution. Chlorpyrifos fortifications were added to the fish tissues which were then homogenized with a tissue tearor (Model 985-370, Biospec

Products Inc. Bartlesville, OK). Six replicates with 0.09 ppm and 0.46 ppm chlorpyrifos were analyzed. Recoveries were then calculated.

The effects of methanol on ELISA performance were examined by spiking fish tissue with chlorpyrifos methanol solution (50, 100, 200 ppb). Extracts were obtained by adding 2 g fish tissue to 10 ml methanol in centrifuge tubes and centrifuged at a speed of 2200 G for 15 min. Supernatants were filtered using 5 ml plastic syringes fitted with 0.45 µm membrane syringe filters (Gelman, Fisher Scientific, Fair Lawn, NJ). Filtrates were diluted and a 250 µl aliquot was assayed with ELISA. Methanol concentration ranged from 1% to **20%.**

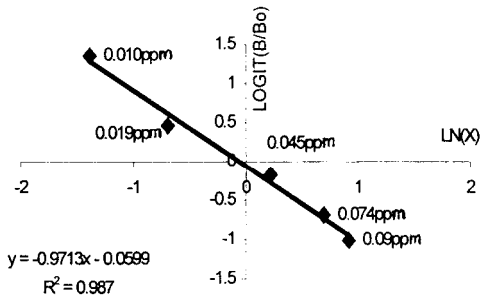

Figure1. Chlorpyrifos standard curve (0.01-0.09 ppm)

RESULTS AND DISCUSSION

Standard curves were linear from 0.01 to 1.91 ppm. (Figures l-3). The correlation coefficients for the standard curves are 0.987, 0.974, 0.919, respectively. Sample chlorpyrifos concentration can be determined by reading absorption values and caculating from the appropriate standard curve. Percent recovery for the fortified chlorpyrifos samples was between 82-113% (Table 1). A LOD of 0.004 ppm chlorpyrifos in fish tissue was calculated. Experimental results indicated the importance of removing solvents before extracts enter into ELISA analysis. In an ELISA analysis for PCB residues in fish, Zajicek (1994) found that the assay could tolerate up to 0.2% isooctane in extracts. Concentrations as low as 1% reduced recovery by as much as 96%. It's believed that components of the sample matrix may bind to the antibody in such a way that they interfere with the assay's response. (Moye 1999). The matrix effect was also demonstrated in our experiments, i.e., blank fish samples that have been previously shown by the FDA

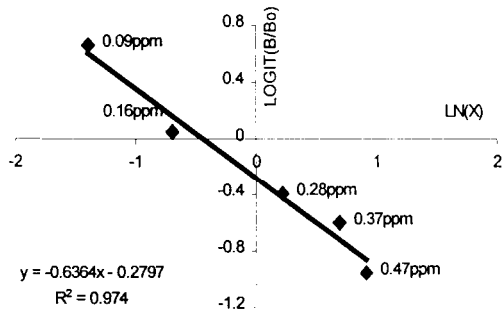

Figure. 2 Chlorpyrifos standard curve

Mills Method to not contain detectable chlorpyrifos residue, appeared to be positive in ELISA analysis when petroleum ether was present during assay. Certain Certain solvents (e.g., methanol) may also cause a false plosive effect possibly due to co-extraction of interfering compounds. The recovery of chlorpyrifos extracted with methanol ranges from 122 to 186% (Table 2). The elevated recovery, which could result in false positive, is likely due to polar substances that were extracted by the methanol and interfere with the immunoassay. Lipid extracted from fatty fish tissue may sometimes interfere with acetonitrile extraction of lipophilic organochlorine residues (Frank et al. 1996). To resolve fat interferences, a florisil column is often used during cleanup to remove lipid. Solid phase extraction may provide advantages by reducing solvent consumption, decreasing interferences and improving accuracy and precision (Odanaka et al. 1991). Combining SPE technique with ELISA for analysis of fish tissue for chlorpyrifos may further simplify the extraction and cleanup procedure.

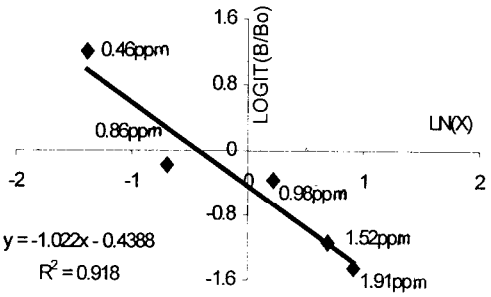

Figure 3. Chlorpyrifos standard curve (0.46-1.91 ppm)

Table 1. Recovery and σ of fish tissue with fortified with chlorpyrifos (n=6)

Added (ppm)	Recovery	standard deviation(σ)
0.09	81%	0.004
0.47	113%	0.009

Analysis of chlorpyrifos in fish using an ELISA can be performed quantitatively with a wide linear range from 0.01 to 1.91 ppm. This technique offers a more economical and time efficient procedure than traditional GC methods. Solvent waste for traditional FDA Mills method is about 4 liters per sample compared to 0.5 liter for this method. With this method, we are able to complete a sample analysis in approximately half the time required for the FDA method which normally requires three days.

Table 2. Recovery of chlorpyrifos from fortified fish tissue using methanol extraction (n=3)

Spiked sample	50 ppb	100 ppb	200 ppb
Recovery	122%	186%	134%

The method described here could be employed as part of a processor's HACCP program for monitoring residues in fish following harvest. Under field conditions, this method can screen fish for chlorpyrifos residues. If residues are confirmed, approaches that can eliminate chlorpyrifos from fish may be explored so that product loss due to contamination can be minimized. The reduced time requirement and relative ease of ELISA analysis makes this method suitable for laboratories that must screen and analyze large numbers of samples with the need to shorten analysis reporting time. Quick determination by ELISA of some pesticides under field conditions should improve agricultural management practices. Whereby, its application in fatty samples is still limited by solvent-solvent extraction procedure. Further research is necessary to shorten the extraction procedure and improve efficiency.

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