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Development of an Enzyme-Linked Immunosorbent Assay for the Pyrethroid Cypermethrin

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A competitive enzyme-linked immunosorbent assay (ELISA) for the detection of cypermethrin was developed. Two haptens, the *trans*- and *cis*-isomers of 3-[(±)-cyano-3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropanecarbonyloxy]methyl]phenoxyacetic acid, were conjugated with thyroglobulin as immunogens. Four antisera were generated and screened against six different coating antigens. The assay that was the most sensitive for cypermethrin was optimized and characterized. The IC_{50} for cypermethrin was 13.5 ± 4.3 μg/L, and the lower detection limit (LDL) was 1.3 ± 0.5 μg/L. This ELISA had relatively low cross-reactivities with other major pyrethroids, such as deltamethrin, phenothrin, resmethrin, fluvalinate, and permethrin. Methanol was found to be the best organic cosolvent for this ELISA, with an optimal sensitivity observed at a concentration of 40% (v/v). The assay parameters were unchanged at pH values between 5.0 and 8.0, whereas higher ionic strengths strongly suppressed the absorbances. To increase the sensitivity of the overall method, a C_{18} sorbent-based solid-phase extraction was applied to various domestic and environmental water samples. The water samples, fortified with cypermethrin, were analyzed according to this method. Good recoveries and correlation with spike levels were observed.

KEYWORDS: Cypermethrin; ELISA; environmental monitoring; pyrethroid

INTRODUCTION

Synthetic pyrethroids are widely used as insecticides in agriculture, forestry, domestic, and veterinary applications because of their general high bioefficacy, enhanced stability, and low toxicity to birds and mammals (*1*, *2*). However, the pyrethroids have been detected as surface water contaminants, and impacts on the aquatic environment leading to effects on ecosystem health have been reported (*3*). According to the toxicological studies on pyrethroids, nontarget invertebrates and aquatic organisms are extremely sensitive to the neurotoxic effects of these insecticides (*4*−*6*). In 2001, >610000 lb of pyrethroid active ingredient was applied to various crops throughout California (*7*). Among those pyrethroids used in California, cypermethrin contributed >26% of the total amount (*7*).

Cypermethrin (**Figure 1**) is light stable and has a moderate persistence in soil. Its greater stability to hydrolysis and photodegradation than that of alternative pyrethroids has made it attractive for uses requiring longer residual activity (*8*, *9*). Thus, a sensitive, selective, and rapid method for monitoring residue levels of cypermethrin is needed, particularly in aquatic environments. Most synthetic pyrethroids are a complex mixture of isomers rather than one single pure compound with the exception of deltamethrin and esfenvalerate (*10*). Cypermethrin possesses three chiral centers and thus consists of eight possible isomers. This compound is formulated as four different insecticides (α-, β-, θ-, and ζ-cypermethrin) depending upon the ratio of the different isomers (*11*).

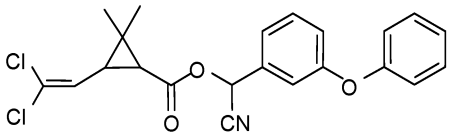

Figure 1. Structure of the pyrethroid insecticide cypermethrin.

Current analytical methods for the detection of cypermethrin involve multistep sample cleanup procedures followed by gas chromatography (GC) and detection by electron capture (GC-EC) or high-performance liquid chromatography−mass spectrometry (HPLC-MS) (*12*−*15*). These methods are relatively time-consuming and expensive and not particularly suitable for large numbers of samples. An immunoassay would provide a sensitive, selective, and rapid method for the detection of this pyrethroid at trace levels (*16*−*22*). For the purpose of raising antibodies that have selectivity for cypermethrin, the cypermethrin molecule must be modified to attach a spacer arm containing a functional group to facilitate coupling to a carrier

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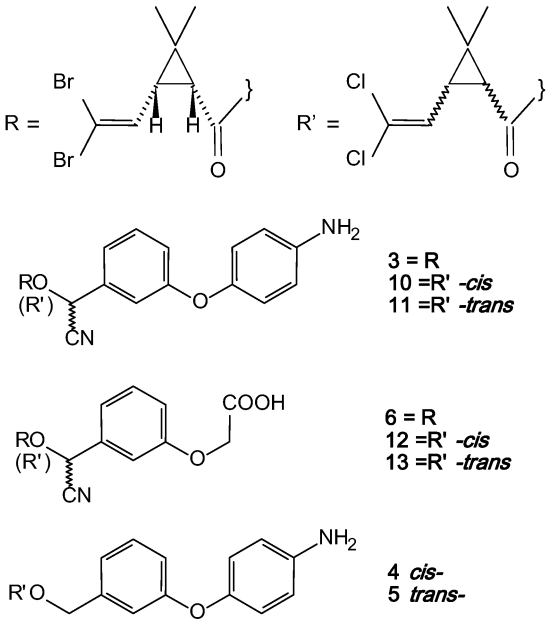

Figure 2. Structures of haptens used in this study. Hapten synthesis was previously reported (*20*, *25*).

protein. An immunoassay for cypermethrin was developed in which the α-cyano group was replaced with a spacer that terminated in a carboxylic acid. Antibodies to this hapten resulted in an assay with an IC_{50} of 80 μg/L, although treatment of sample with alkali to isomerize the cypermethrin increased the sensitivity of the assay to 10 μg/L (*23*, *24*). In the present paper, the antibodies were developed from a novel cypermethrin hapten and resulted in an assay that is ~6 times more sensitive than previously reported for unisomerized cypermethrin and is as sensitive as the assay with isomerized cypermethrin but requires no alkali treatment.

MATERIALS AND METHODS

Chemicals and Immunoreagents. The haptens used in this study, **3**−**6** and **10**−**13**, were reported previously **(Figure 2)** (*20*, *25*). Numbers used to identify haptens conform to the numbering found in Lee et al. (*20*) and Shan et al. (*25*).

The coupling reagents were purchased from Aldrich (Milwaukee, WI). Goat anti-rabbit (GAR) immunoglobulin conjugated to horseradish peroxidase (HRP), bovine serum albumin (BSA), thyroglobulin (Thyr), Tween 20, and 3,3′,5,5′-tetramethylbenzidine (TMB) was purchased from Sigma Chemical Co. (St. Louis, MO).

Instruments. ELISA experiments were performed in 96-well microplates (Nunc, Roskilde, Denmark), and the absorbances were measured with a Vmax microplate reader (Molecular Devices, Menlo Park, CA) in dual-wavelength mode (450−650 nm).

Hapten Conjugation. Conjugates were synthesized using water-soluble carbodiimide or diazotization methods (*26*−*28*). To obtain immunogens, haptens **12** and **13** were conjugated to thyroglobulin. Coating antigens were made by coupling haptens **3**−**6** and **10**−**13** to BSA according to previous publications (*25*, *27*, *28*).

Immunization and Antiserum Preparation. Cypermethrin antisera were obtained following the protocol reported earlier for other pyrethroids (*20*, *25*). Briefly, two New Zealand white rabbits were immunized for each immunogen (rabbits 733 and 734 for **12**−Thyr and rabbits 735 and 736 for **13**−Thyr). The antigen solutions (100 μg in PBS) were emulsified with Freund's complete adjuvant (1:1, v/v) and injected subcutaneously. After 1 month, the animals were boosted with an additional 100 μg of immunogen that was emulsified with Freund's incomplete adjuvant (1:1, v/v). Booster injections were given at 3-week intervals. The rabbits were bled about 10 days after each boost. The serum was isolated by centrifugation at 800*g* for 10 min at 4 °C. The results of antibody characterization shown here were obtained from sera of terminal bleeds.

Enzyme Immunoassay. The method was identical to that reported by Shan et al. (*18*) with the following exception. The wash buffer was 0.05% Tween 20 in distilled water.

Antibody Characterization and Assay Optimization. Optimal concentrations for coating antigen and antisera were determined by screening in a two-dimensional titration. After comparison of the competitive inhibition curves for four antiserum and eight coating antigen combinations, the assay with the lowest IC_{50} was chosen for further assay development.

To investigate the effect of solvents, cypermethrin standards were dissolved in PBS buffer containing 0, 20, 40, 60, or 80% (v/v) methanol or 0, 10, 20, or 40% (v/v) DMSO.

The effects of pH (5, 6, 7, and 8) and ion strength (1×, 2×, 4×, and 8×) for PBS buffer were also tested in the same protocol as previously reported (*20*, *25*).

Cross-Reactivity (CR). Data were obtained from standard inhibition curves of cypermethrin and other structurally related pyrethroids by using the optimized ELISA system. The CR was calculated as (IC_{50} of cypermethrin/IC_{50} of analyte) × 100.

Solid-Phase Extraction (SPE). To analyze industrial water samples by using the optimized assay, the method of the extraction of cypermethrin from water using a C_{18} column was the same as that of a method previously reported by Shan et al. (*18*). Briefly C_{18} SPE columns (Varian Sample Preparation Products, Harbor City, CA) were preconditioned with 3.5 mL each of methanol and deionized water. Water samples (200 mL) and five to eight 5 mL water washes of the sample container were loaded, and then the column was dried under vacuum for 15 min. The column was eluted with 3.5 mL of methanol. A drop of propylene glycol was added to the methanol, and then the methanol was evaporated under a stream of nitrogen. The residue was resuspended in 1 mL of methanol, an aliquot of which was diluted with PBS to 40% methanol for ELISA analysis.

RESULTS AND DISCUSSION

Design of the immunogen hapten is very important for development of a sensitive and specific cypermethrin immunoassay. It is desirable to immunize with a hapten that exposes the most unique portions of the target analyte for antibody development. Because of the lipophilicity of the pyrethroids, a handle that is more rigid which would prevent the hapten from folding into the core of the carrier protein is also desirable. Therefore, two haptens containing the partial cypermethrin molecule with a short handle on aromatic rings (compounds **12** and **13**) were chosen for immunization. In compounds **12** and **13**, the distal phenyl group of cypermethrin was eliminated and a carboxylic acid was directly linked to first aromatic ring. This exposed the dichlorovinylcyclopropane portion of the molecule, which is relatively unique to cypermethrin among the type II pyrethroids.

Screening and Selection of Antisera. The antisera of terminal bleeds from four rabbits were screened against six different coating antigens using a two-dimensional titration method with the coated antigen format. Among the heterologous coating antigens, Ab735 had the highest affinity for the coating antigen **4**−BSA, followed by **5**−BSA, and then **3**−BSA, **13**−BSA, **10**−BSA, and **6**−BSA. Combinations of coating antigen and antiserum that resulted in high optical densities (OD > 0.75) were selected for further development.

To find systems that yielded the highest sensitivity for cypermethrin, competitive inhibition experiments were conducted in parallel to optimization of antiserum and coating antigen concentrations. The reagent concentrations with optical densities of ~0.8 and lowest IC_{50} values were chosen as the optimal combinations. The IC_{50} values for each combination ranged from 26.5 to 1.27 × 10^3 μg/L (**Table 1**). The heterolog-

Table 1. Selected Competitive ELISA Screening Data against Cypermethrin[a]

immunogen	antiserum	coating antigen	IC_{50} (μg/L)
12–THY	733	**3**–BSA	706
		10–BSA	650
		11–BSA	186
		6–BSA	1230
		12–BSA	362
	734	**3**–BSA	1030
		10–BSA	714
		11–BSA	178
		6–BSA	1270
		12–BSA	140
		4–BSA	183
		5–BSA	117
13–THY	735	**3**–BSA	166
		10–BSA	234
		6–BSA	504
		13–BSA	170
		4–BSA	26.5
		5–BSA	41
	736	**3**–BSA	294
		10–BSA	163
		6–BSA	567
		13–BSA	142

[a] Optimized assay conditions were used. The cypermethrin analyte standards were prepared in a 40% methanol/PBS solution.

ous system, in which the hapten used in the coating antigen was different from that used in the immunogen, for Ab735 (with cAg **4**−BSA) showed the lowest IC_{50} (26.5 μg/L in 40% methanol/PBS). In this study, only the heterologous system of Ab735 (1/7000) and cAg **4**−BSA (0.16 μg/mL) was used for further assay development and optimization.

Optimization. Because the pyrethroids are lipophilic, a water-miscible organic cosolvent is needed to ensure the solubility. MeOH and DMSO were tested. As observed in our previous studies for esfenvalerate, permethrin, and deltamethrin immunoassays (*18*, *20*, *25*), MeOH and DMSO significantly influenced the cypermethrin assay sensitivity and absorbance. The IC_{50} value of the assay varied depending upon the different concentrations of the cosolvents MeOH or DMSO. The lowest IC_{50} was found at 40% MeOH (8.16 μg/L), which is ~4 times lower than that at 20% MeOH (34.6 μg/L) and 5 times lower than that at 40% DMSO (41.9 μg/L). As the concentration of MeOH increased the maximum absorbance and background tended to increase, but the improvement in IC_{50} at 40% methanol is likely due to improved solubility of the analyte. With DMSO, as the concentration increased, the overall absorbance decreased. On the basis of the IC_{50} values and the ratios of maximum and minimum absorbances for the cypermethrin standard inhibition curve, a MeOH concentration of 40% was selected for subsequent experiments.

To evaluate potential interferences that may be encountered, for example, in environmental water samples, the effects of pH and ionic strength on the ELISA system were tested in this study. In system **4**/735, there was no significant effect upon the IC_{50} in PBS buffer at various pH values, indicating that the assay could effectively detect cypermethrin at pH values ranging from 5.0 to 8.0. On the other hand, ionic strength strongly influenced the ELISA system because a higher salt concentration of PBS buffer resulted in lower absorbances. The absorbance values at salt concentrations of 4× and 8× PBS decreased by 38 and 72%, respectively, from the absorbance value at a salt concentration of 1× PBS. The lowest IC_{50} (20.7 μg/L) was found for 1× PBS, followed by 2× PBS (26.4 μg/L), 4× PBS (70.6 μg/L), and 8× PBS (121 μg/L), indicating that high salt concentrations are a source of interference.

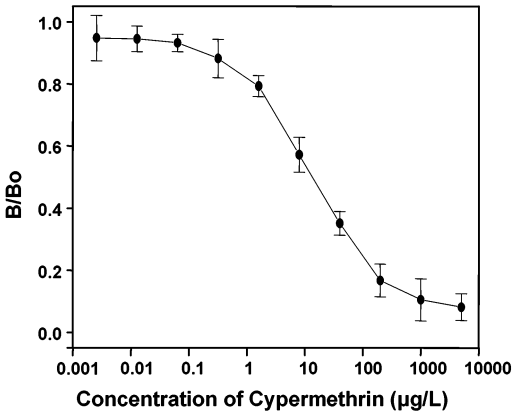

Figure 3. ELISA inhibition curves for cypermethrin. Reagent concentration: antiserum 735, 1/7000 (final dilution in wells); coating antigen **4**–BSA, 0.16 μg/mL. Calibration standards were prepared in 40% MeOH/PBS. Standard curves represent the average of 13 curves (±SD) run over a period of 3 months.

Table 2. Cross-Reactivities of Pyrethroids

analyte	cross-reactivity[a] (%)	analyte	cross-reactivity[a] (%)
cypermethrin	100	cyfluthrin	7.2
permethrin	4.3	deltamethrin	0.004
phenothrin	0.029	esfenvalerate	7.0
resmethrin	0.007	fenvalerate	7.7
bioresmethrin	3.5	fluvalinate	3.1
λ-cyhalothrin	0.97		

[a] Cross-reactivity was calculated as (IC_{50} of cypermethrin/IC_{50} of analyte) × 100.

The optimized cypermethrin ELISA used 0.16 μg/mL of coating antigen **4**−BSA, antibody 735 at a dilution of 1/7000, cypermethrin in 40% methanol/PBS buffer, pH 7, and PBS concentration of 1×. The IC_{50} value of this assay was 13.5 ± 4.3 μg/L with a limit of quantitation (LOQ) of 1.3 ± 0.5 μg/L in buffer (**Figure 3**). The LOQ was estimated as the concentration that corresponded to the absorbance of the control (zero concentration of analyte minus 3 times the standard deviation of the control) (*29*).

Cross-Reactivities. The results of the cross-reactivity of various pyrethroids are shown in **Table 2**. The optimized ELISA system (Ab735/**4**−BSA) had low CR for fenvalerate (7.7%), cyfluthrin (7.2%), esfenvalerate (7.0%), permethrin (4.3%), and bioresmethrin (3.5%). The antibody recognized substitutions on the ethenyl as Cl > Br > CH_3 as demonstrated by decreasing CR for cypermethrin versus deltamethrin and permethrin versus phenothrin showing the predicted high selectivity. Because fenvalerate, cyfluthrin, esfenvalerate, fluvalinate, and permethrin cross-react relatively higher than other pyrethroids, and because deltamethrin, the bromo analogue, does not cross-react, the chloride group is an important determinant for binding. In addition, the antibody is less selective in the alcohol portion of the molecule as bioresmethrin can also cross-react. Although the CR of bioresmethrin (1*R-trans*) is low, it is interesting that the *trans*-bioresmethrin isomer shows stronger CR than the *cis*-/*trans*-resmethrin mixture. This shows that the antibody binding is directed more toward the 1*R-trans* configuration as might be expected because this antibody was generated from a 1*R-trans* hapten. In addition, the commercial deltamethrin and λ-cyha-

Table 3. Recovery of Cypermethrin from Spiked Water Samples

water sample	spiked concn (μg/L)	theor concn in ELISA[a] (μg/L)	ELISA detected (μg/L)	ELISA mean recovery (%, $n = 3$)
tap water	0	0	<1	
	0.2	10	9.15	92 ± 5.1
	2.0	100	76.15	76 ± 8.4
	10	500	427.40	85 ± 15.8
lake water	0	0	<10	
	2	20	25.80	129 ± 13.2
	10	100	71.19	71 ± 5.9
	20	200	206.01	103 ± 1.6
runoff water	0	0	<10	
	2	20	14.01	71 ± 11.8
	10	100	69.06	69 ± 7.4
	20	200	135.20	68 ± 6.0

[a] Water samples are from an industrial tap, lake water, and runoff water, Davis, CA. Different amounts of cypermethrin (in methanol) were added to the samples to give final concentrations in water of 0, 0.2, 2, 10, and 20 μg/L. After thorough mixing and standing for at least 2 h, the samples were extracted by SPE prior to immunoassay. Samples were analyzed blind. Due to the matrix effect of different water samples, tap and lake and runoff water were diluted 50 and 10 times, respectively.

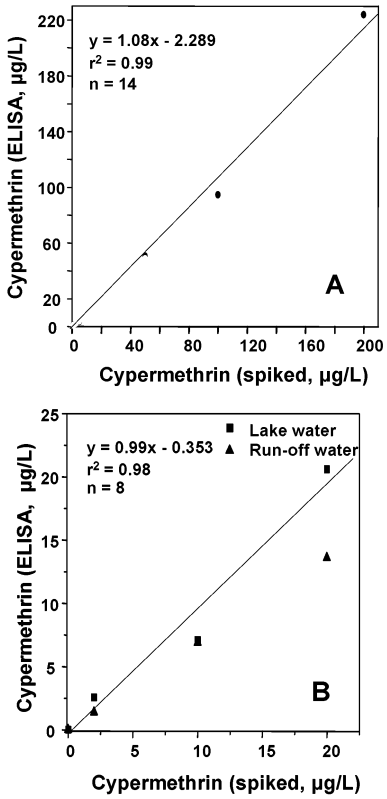

Figure 4. Relationship between nominal cypermethrin concentrations and concentrations determined by ELISA. Industrial water samples (**A**) and lake and runoff water samples (**B**) were analyzed following SPE as indicated under Materials and Methods.

lothrin are only *cis*-isomers, and their low CR indicates that the antibody generated against the *trans* configuration does not recognize the *cis* configuration of the cyclopropane ring as well.

Analysis of Spiked Industrial Water Sample. Water samples collected from an industrial tap, Putah Creek (Davis, CA, pH 9.4), and runoff from agricultural drainage (pH 6.72) were spiked with five different concentrations of cypermethrin (0, 0.2, 2, 10, and 20 μg/L) and analyzed in a blind fashion by the ELISA. Putah Creek is an oxbow lake with high levels of particulates, nitrogen salts, and algae. The runoff water, collected after a spring rain (May 2003), contained suspended soil particles and some dissolved organic matter. Before extraction, the water samples were filtered through a 0.22 μm nylon membrane filter to remove the particles.

The samples were extracted using SPE. Only a slight matrix effect was measured when 200 mL of water sample was extracted with SPE, eluted into 1 mL of methanol, and analyzed in the ELISA following 4 and 20× dilutions (concentration factors of 50 and 10). All recoveries in the ELISA were >68% of the spiked values (**Table 3**). High correlations between nominal and ELISA measurements of cypermethrin ($r^2 = 0.98-0.99$) were obtained for spiked industrial water samples from 2.5 to 200 μg/L (**Figure 4A**) and lake and runoff water samples from 2 to 20 μg/L (**Figure 4B**). The results demonstrate that these assays are suitable for the detection of cypermethrin in domestic or environmental water samples.

ABBREVIATIONS USED

Ab, antibody; BSA, bovine serum albumin; cAg, coating antigen; CR, cross-reactivity; DMSO, dimethyl sulfoxide; ELISA, enzyme-linked immunosorbent assay; GAR−HRP, goat anti-rabbit immunoglobulin conjugated to horseradish peroxidase; IC_{50}, concentration of analyte giving 50% inhibition; LOQ, limit of quantitation; MeOH, methanol; PBS, phosphate-buffered saline; SD, standard deviation; SPE, solid-phase extraction; TMB, tetramethylbenzidine; Thyr, thyroglobulin.

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