JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Development of an Enzyme-Linked Immunosorbent Assay for the Pyrethroid Insecticide Cyhalothrin

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A competitive enzyme-linked immunosorbent assay (ELISA) was developed for detection of the pyrethroid insecticide cyhalothrin. Three haptens with an amine or propanoic acid terminus were synthesized and then conjugated with bovine serum albumin to give immunogens. Eight polyclonal antisera produced by rabbits were screened for titers and affinity using three different coating antigens. The antiserum CWB-C had the highest affinity with cyhalothrin and a low affinity with fenvalerate, fenpropathrin, deltamethrin, and fluvalinate. The half-maximum inhibition concentration for cyhalothrin was 37.2 μg/L, and the limit of detection was 4.7 μg/L. The recoveries of different concentrations of cyhalothrin (0.1−2500 μg/L) from fortified tap water, well water, and wastewater samples as determined with the ELISA were 81−114%.

KEYWORDS: ELISA; pyrethroid; cyhalothrin

INTRODUCTION

Cyhalothrin, registered by the U.S. Environmental Protection Agency in 1988 (*1*), is a synthetic pyrethroid and is widely used for the control of many common pests on crops and domestic animals (*2*, *3*). In China, cyhalothrin is widely used on cotton, teas, fruit trees, and vegetables. However, cyhalothrin is highly toxic when it is ingested. The acute oral LD_{50} values in rats and mice are 79 and 56 mg/kg, respectively (*4*). Cyhalothrin is especially highly toxic to fish (LC_{50}, 0.078−2.3 μg/L) and aquatic invertebrates (LC_{50}, 0.0023−3.3 μg/L). The half-life of cyhalothrin is 30, 40, and 23 days in soil, plants, and fat tissues, respectively (*4*−*6*). Laboratory studies indicated that cyhalothrin could be bioaccumulated in fish and that it had an adverse impact on human health (*4*, *7*). Thus, a sensitive, selective, and rapid method for monitoring residue levels of cyhalthrin in aquatic ecosystem is desirable.

The reported methods for detection of cyhalothrin include high-performance liquid chromatography (HPLC) (*8*), gas chromatography with electron capture detection (GC-ECD) (*9*), and GC-mass spectrometry (GC-MS) (*10*). These methods are expensive and labor intensive and require extensive analytical skills. Immunoassays have been proven to be a fast, sensitive, and selective method for detection of pesticides at a trace level (*11*). Several immunoassays have been well-developed for the detection of synthetic pyrethoids such as allethrin (*12*), (s)-bioallethrin (*13*, *14*), bioresmethrin (*15*), cypermethrin (*16*), deltamethrin (*16*, *17*), esfenvalerate (*11*), fenpropathrin (*18*), flucythrinate (*19*), permethrin (*20*, *21*), and phenothrin (*22*). An immunoassay was developed for cyhalothrin by Lee et al. (*16*). Because Lee et al. intended to develop a class-specific assay for type II pyrethroids, the cross-reaction of the deltamethrin immunoassay was 40−700% for cyhalothrin detection, and the assay was not applied for the analysis of environmental samples (*16*). A cyhalothrin-specific antibody will advance the study of cyhalothrin environmental fate and can be used in antibody microarray or immunoaffinity chromatography. The objective of this work was to develop a sensitive and selective immunoassay for the detection of cyhalothrin in tap water, well water, and wastewater.

MATERIALS AND METHODS

Reagents. Cyhalothrin, fenvalerate, fenpropathrin, deltamethrin, fluvalinate, and cypermethrin were obtained from China National Research Center for Certified Reference Materials (Beijing, China). All organic starting materials for hapten synthesis were purchased form Aldrich Chemical Co. (Milwaukee, WI). The coupling reagents goat-anti-rabbit (GAR) immunoglobin conjugated to horseradish peroxidase (HRP), bovine serum albumin (BSA), ovalbumin (OVA), and tetramethylbenzidine (TMB) were purchased from Sigma Chemical Co. (St. Louis, MO).

Apparatus. GC analysis was performed on a GC-9900 equipped with a flame ionization detector (Beijing Jiafen Analitical Instrument Factory, China). Separations were accomplished on a homemade capillary GC SE-54 column (40 m × 0.5 mm, 0.25 μm). The temperature of the injector port and the detector was 250 °C. The oven temperature was 230 °C. The carrier was nitrogen at a flow rate of 20 mL/min. Nuclear magnetic resonance (NMR) spectra was obtained with a General Electic QE-300 spectrometer. Chemical shift values were given in parts per million downfield from internal tetramethylsilane. Enzyme-linked immunosorbent assays (ELISAs) were carried out in

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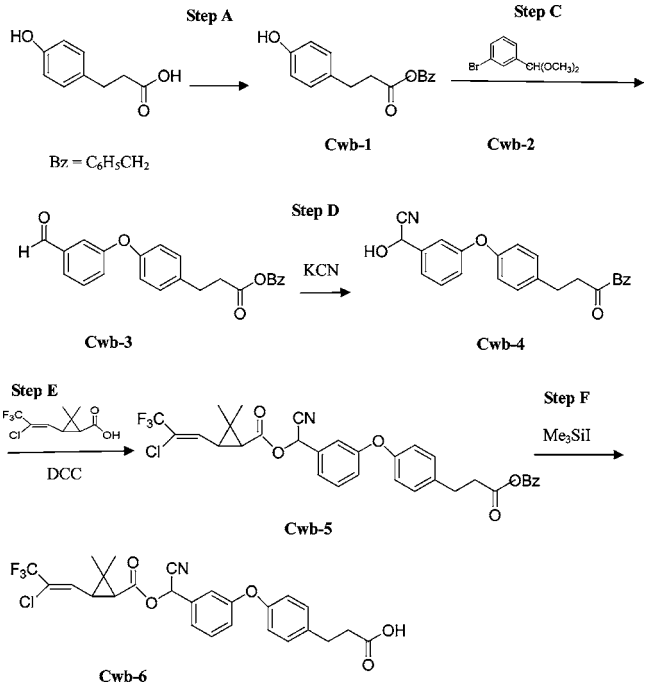
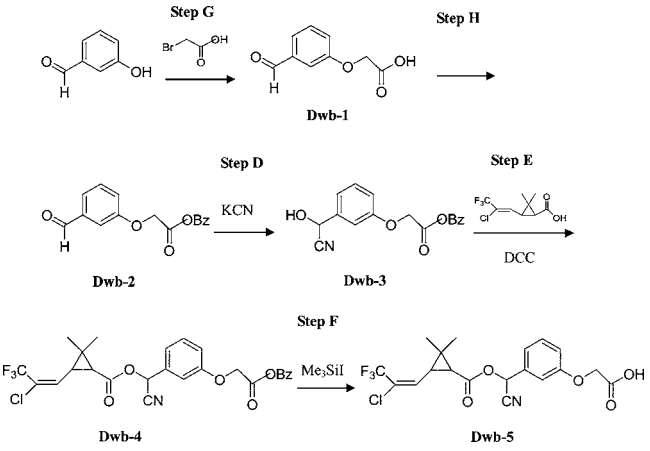
10.1021/jf0607009 CCC: $33.50
Published on Web 06/23/2006

Scheme 1. Synthesis of Cwb-1, Cwb-2, Cwb-3, Cwb-4, Cwb-5, and Cwb-6



Scheme 2. Synthesis of Dwb-1, Dwb-2, Dwb-3, Dwb-4, and Dwb-5



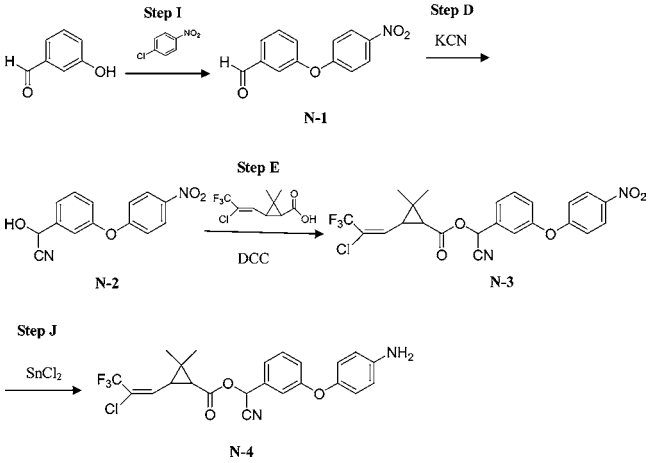
96 well microtiter plates, and the absorbance was read with a Vmax microplate reader (Molecular Devices, Menlo Park, CA).

Hapten Synthesis and Verification. Haptens were synthesized as outlined in **Schemes 1**−**3**.

Step A. Benzyl 4-hydroxybenzenepropanoate (Cwb-1) was synthesized according to the procedures of Wengatz et al. (*18*). A mixture of 4-hydroxybenzenepropanoic acid (14.4 g, 86.6 mmol), benzyl alcohol (27 mL), toluene (15 mL), and 4 drops of 85% phosphoric acid was heated to reflux under a dean-stark trap. Toluene was removed up to a kettle temperature of 145−150 °C. Heating was continued for 10 h to collect 1.50 mL of water. Excess benzyl alcohol was removed under vacuum to a kettle temperature of 150 °C. The residue was dissolved in ether, washed, and dried with anhydrous $MgSO_4$. The stripped residue was distilled through a short path heated from an oil bath at 230−240 °C to collect 16.1 g (73%) of product at a heat temperature of 185−194 °C. ^{1}H NMR ($CDCl_3$): δ 2.60−2.67 (2H, t, $COCH_2$), 2.85−2.89 (2H, t, $phCH_2CH_2$), 5.09 (2H, s, $phCH_2O$), 6.23 (1HS, b, phOH), 6.69−7.35 (9H, m, ArH).

Step B. Bromo-3-(dimethoxymethyl)benzene (Cwb-2) was obtained according to the procedure of Wengatz et al. (*18*). Two drops of concentrate sulfuric acid were added to a solution of 3-bromobenzaldehyed (18.5 g, 0.1 mol) and trimethylorthoformate (12.2 g, 0.1 mol) in 15 mL of methanol. After an immediate mild exotherm and 3 h at ambient temperature, the mixture was diluted with ether, washed with a sodium carbonate solution followed by a water wash, and dried with anhydrous $MgSO_4$. Solvents were evaporated, and the product was distilled under vacuum to yield 13.5 g, 57.9%, of Cwb-2 as a colorless oil. ^{1}H NMR ($CDCl_3$): 3.31, [6H, s, $(CH_3O)_2$], 5.36, (1H, s, phCH), 7.20−7.63 (4H, m, ArH).

Scheme 3. N-1, N-2, N-3, and N-4



Step C. Benzyl 4-(3-formylphenoxy)benzenepropanoate (Cwb-3) was formed by following the procedures of Wengatz et al. (*18*). Potassium tertbutoxide (65 mL, 1 M in tertbutyl alcohol) was added under N_2 to a stirred solution of Cwb-1 (16.6 g, 64.8 mmol) in 180 mL of xylene. The mixture was heated to remove 100 mL of distillate. An additional 40 mL of xylene was added and distilled to a kettle temperature of 136 °C. The resulting salt suspension was cooled and treated with pyridine (16 mL), cuprous chloride (0.6 g), copper powder (0.3 g), 18-crown-6 (100 mg), and 15.0 g (65 mmol) of Cwb-2. This mixture was refluxed under N_2 for 17 h, cooled, washed with water, and filtered through silica gel (25 g). The pad of silica gel was washed with CH_2Cl_2 (40 mL). The combined filtrate and washing were evaporated to a volume of 30 mL, diluted with wet CH_2Cl_2 (40 mL), and treated with acidified silica gel (30 g), previously treated in ether with 9 drops of 98% H_2SO_4 and stripped to a dry powder on a rotary evaporator. After water (1 mL) was added, this mixture was stirred for 2 h. After filtration and evaporation, the mixture was flash-chromatographed on a silica gel column (200 g), eluting with hexane/CH_2Cl_2 (80:20 → 0:100). Stripping at 30 °C and 0.1 Torr yielded 13.1 g (56%) of Cwb-3 as a pale yellow oil. ^{1}H NMR ($CDCl_3$): δ 2.66−2.71 (2H, t, $COCH_2$), 2.94−2.99 (2H, t, $phCH_2CH_2$), 5.12 (3H, s, $phCH_2O$), 6.92−7.59 (8H, m, ArH), 9.93 (1H, s, CHO).

Step D. Cwb-3 (1.3 g, 3.4 mmol) in 16 mL of tetrahydrofuran (THF) was added to a stirred solution of 1.5 g of KCN in 4 mL of H_2O precooled with ice followed by slow addition of 4 mL of H_2SO_4 (40%). After 30 min and on ice, the mixture was extracted with ether three times and dried with anhydrous $MgSO_4$. After the solvent was removed, 1.1 g of benzyl 3-[3-(cyano-hydroxy-methyl)phenoxy]benzenepropanoate (Cwb-4) was obtained in 77% yield, which was a pale solid. ^{1}H NMR ($CDCl_3$): δ 2.63−2.68 (2H, t, $COCH_2$), 2.88−2.94 (2H, t, $phCH_2CH_2$), 4.26 (1H, s, b, CNCHOH), 5.07 (2H, s, $phCH_2O$), 5.40 (1H, s, CNCH), 6.87−7.34 (13H, m, ArH). Following the above procedure for Cwb-4, the reaction of Dwb-2 (4.9 g, 18.2 mmol) instead of Cwb-3 gave 5.2 g of [3-(cyano-hydroxy-methyl)phenoxy]acetic acid benzyl ester (Dwb-3) as a white solid; yield, 96%. ^{1}H NMR ($CDCl_3$): δ 2.90 (1H, s, b, CNCHOH), 4.69 (2H, s, CH_2CO), 5.24 (2H, s, $phCH_2$), 5.46 (1H, s, CNCH), 6.92−7.37 (9H, m, ArH). The reaction of N-1 (1.68 g, 6.9 mmol) gave 2.34 g of 2-hyroxy-2-[3-(4-nitrophenoxy)-phenyl]acetonitrile (N-2) as a brown sticky liquid; yield, 89%. ^{1}H NMR ($CDCl_3$): δ 5.83−5.85 (1H, d, CNCH), 7.15−8.31 (9H, ArH, CHOH).

Step E. Dicyclohexylcarbodiimide (DCC) (1.3 g, 6.1 mmol) in 15 mL of CH_2Cl_2 was dropped into a stirred solution of Cwb-4 (2.4 g, 6.4 mmol) and (±)-(*cis*)-3-[(*Z*)-3-chloro-4,4,4-trifluorobut-1-enyl-2,2-dimethyl]cyclopropane carboxylic acid (1.3 g, 5.5 mmol) in 15 mL of CH_2Cl_2 with ice cooling. After DCC in 15 mL of CH_2Cl_2 was added for

about 2 h, the mixture was concentrated. Chromatographic purification on silica (R_f = 0.5, ethyl acetate:petroleum ether, 1:5) gave 2.5 g of benzyl-3-[(±)cyano[(±)-(*cis*)-3-[(*Z*)-3-chloro-4,4,4-trifluorobut-1-enyl-2,2-dimethyl]cyclopropanecarbonyloxy]phenoxy]benzenepropanoate (Cwb-5) as a straw yellow sticky liquid; yield, 75%. ^{1}H NMR ($CDCl_3$): δ 1.28−1.33 [6H, t, $(CH_3)_2C$], 2.01−2.05 (1H, d, CHCO), 2.49−2.31 (1H, t, =CHCH), 2.67−2.72 (2H, t, $COCH_2$), 2.95−2.99 (2H, t, $phCH_2CH_2$), 5.12 (2H, s, $phCH_2O$), 6.31, 6.37 (1H, s, CNCH), 6.80−6.81 (1H, d, =CH), 6.92−7.39 (13H, m, ArH). Following the above procedure for Cwb-5, the reaction of DCC (1.7 g, 8.2 mmol), Dwb-3 (2.2 g, 7.4 mmol), and (±)-(*cis*)-3-[(*Z*)-3-chloro-4,4,4-trifluorobut-1-enyl-2,2-dimethyl]cyclopropane carboxylic acid (2.41 g, 9.9 mmol) gave 2.57 g of 3-[(±)cyano[(±)-(*cis*)-3-[(*Z*)-3-chloro-4,4,4-trifluorobut-1-enyl-2,2-dimethyl]cyclopropanecarbonyloxy]phenoxy]-acetic acid benzyl ester (Dwb-4), yield 62%, which was a pea green sticky liquid. ^{1}H NMR ($CDCl_3$): δ 1.23−1.34 [6H, t, $(CH_3)_2C$], 2.00−2.04 (1H, dd, CHCO), 2.21−2.28 (1H, q, =CHCH), 4.69 (2H, s, CH_2-CO), 5.25 (2H, s, $phCH_2$), 6.30−6.36 (1H, d, CNCH), 6.81−6.85 (1H, d, =CH), 6.94−7.38 (9H, m, ArH). The reaction of N-2 (1.6 g, 5.9 mmol), (±)-(*cis*)-3-[(*Z*)-3-chloro-4,4,4-trifluorobut-1-enyl-2,2-dimethyl]-cyclopropane carboxylic acid (1.41 g, 5.3 mmol) and DCC (1.22 g, 5.9 mmol) gave 0.61 g of (±)cyano[3-(4-nitrophenoxy)phenyl]methyl-(±)-*cis*-3-(*Z*-2-chloro-3,3,3-trifluoroprop-1-enyl)-2,2-dimethylcyclopropane carboxylate (N-3), yield 21%, which was a brown sticky liquid. ^{1}H NMR ($CDCl_3$): δ 1.24−1.35 [6H, m, $(CH_3)_2C$], 2.04−2.07 (1H, d, CHCO), 2.24−2.31 (1H, q, =CHCH), 6.38−6.43 (1H, d, CNCH), 6.79−6.83, (1H, d, =CH), 7.03−8.26 (8H, m, ArH).

Step F. 3-[(±)Cyano[(±)-(*cis*)-3-[(*Z*)-3-chloro-4,4,4-trifluorobut-1-enyl-2,2-dimethyl]cyclopropane carbonyloxy]phenoxy]propanoic acid (Cwb-6) and 3-[(±)cyano[(±)-(*cis*)-3-[(*Z*)-3-chloro-4,4,4-trifluorobut-1-enyl-2,2-dimethyl]cyclopropane carbonyloxy]phenoxy]acetic acid (Dwb-5) were synthesized according to the procedure of Wengatz et al. (*18*). The ester Cwb-5 (1.4 g, 2.3 mmol) in 1.5 mL of dry CH_2Cl_2 was treated with 20 μL of bis(trimethylsilyl)trifluoroacetamide (BSTFA) followed by iodotrimethylsilane (TMSI, 0.18 mL). After 1.5 h, 5 drops of water and 1 mL of CH_2Cl_2 were added and the mixture was stirred for 5 min. The organic phase was immediately flash-chromatographed on 10 g of silica gel G450 (CH_2Cl_2 → ethyl acetate). The product was stripped from a thin-layer chromatography (TLC) plate (<1 mm) to yield 0.9 g of Cwb-6, yield 76%, which was a pale yellow gum. ^{1}H NMR ($CDCl_3$): δ 1.31 [6H, s, $(CH_3)_2C$], 2.01−2.06 (1H, d, CHCO), 2.22−2.28 (1H, t, =CH), 2.66−2.71 (2H, t, CH_2CO), 2.93−2.98 (2H, t, $phCH_2$), 6.31 (1H, s, CNCH), 6.82−6.85 (1H, d, =CH), 6.93−7.40 (8H, m, ArH), 10.50 (1H, S, b, COOH). Following the above procedure for Cwb-6, the reaction of Dwb-4 (2.6 g, 4.8 mmol) gave 0.8 g of Dwb-5, yield 35%, which was a yellow liquid. ^{1}H NMR ($CDCl_3$): δ 1.19−1.29 [6H, t, $(CH_3)_2C$], 1.97−2.01 (1H, d, CHCO), 2.18−2.24 (1H, t, =CHCH), 4.38 (2H, s, CH_2), 6.19−6.24 (1H, d, CNCH), 6.79−6.82 (1H, d, =CH), 6.82−7.19 (4H, m, ArH).

Step G. Bromoacetic acid (1.1 g, 7.8 mmol) was added to a stirring solution of 0.6 g of NaOH in 4.8 mL of H_2O in a 50 mL vessel cooled with an ice bath. After 3-hydroxybenzaldehyde (0.9 g, 7.4 mmol) was added, the solution was refluxed for 2 h. The mixture was cooled with an ice bath, and the pH was adjusted with HCl to pH 1. Filtration under vacuum and then recrystallization in water gave 0.9 g of (3-formyl-phenoxy)acetic acid (Dwb-1); yield, 68%. ^{1}H NMR ($CDCl_3$): δ 4.79 (2H, s, CH_2), 7.25−7.56 (4H, m, ArH), 9.98 (1H, s, CHO), 13.10 (1H, s, b, COOH).

Step H. 1-(Chloromethyl)benzene (3.8 g, 30 mmol) and triethylamine (1.3 g, 12.5 mmol) were added to a stirred solution of Dwb-1 (4.5 g, 25 mmol) and NaH (1.2 g, 50%) in 30 mL of dried *N*,*N*-dimethylformamide (DMF). After the solution refluxed at 150 °C for 5 h, the solution was cooled, diluted with 100 mL of ethyl acetate, washed with water five times, and then dried by adding anhydrous $MgSO_4$ into the collected organic phase overnight. Chromatographic purification on silica (G450, ethyl acetate:petroleum ether = 1:6) and solvent removal gave 3.4 g of 3-formyl-phenoxy acetic acid benzyl ester (Dwb-2); yield, 51%. ^{1}H NMR ($CDCl_3$): δ 4.74 (2H, s, CH_2CO), 5.25 (2H, s, $phCH_2O$), 7.20−7.53 (9H, m, ArH), 9.94 (1H, s, CHO).

Step I. 3-Hydroxy-benzaldehyde (1.6 g, 14.4 mmol) and 1-choro-4-nitrobenzene (1.2 g, 7.7 mmol) were added to a solution of 1.4 g of K_2CO_3 in 11.5 mL of DMF. The mixture was heated to reflux at 150 °C for 10 h and then poured into 200 mL of ice water. A brown solid was formed. The brown solid was dissolved completely in the solution (ethyl acetate:petroleum ether = 1:1) when heated. The solution was kept in 4 °C overnight. After filtration, 2.1 g of 3-(4-nitrophenoxy)-benzaldehyde (N-1) as a yellow solid was obtained; yield, 87%. ^{1}H NMR ($CDCl_3$): δ 7.05−8.26 (8H, m ArH), 10.02 (1H, s, CHO).

Step J. (±)Cyano 3-(4-aminophenoxy)phenyl-(±)-*cis*-3-(*Z*-2-chloro-3,3,3-trifluoroprop-1-enyl)-2,2-dimethylcycloproanecarboxylate (N-4) was obtained according to the procedures of Shan et al. (*11*). Stannous chloride hydrate (1.4 g, 6.2 mmol) was added to a stirred solution of N-3 (0.6 g, 1.2 mmol) in 0.75 mL of ethanol (EtOH), and the mixture was heated under N_2 at 70−75 °C for 30 min. The mixture was cooled and poured into a slurry of water, ether, and Celiet (0.4 g). $NaHCO_3$ (1.4 g, 1.2 mmol) was added in portions and stirred until CO_2 evolution ceased. The mixture was filtered, and solids were extracted with ether. Combined ether extracts were stripped, and the residue was chromatographed on a 5 g silica gel column (50% CH_2Cl_2/hexane → CH_2Cl_2) to give 0.4 g of N-4, yield 70%, as a colorless viscous gum. TLC R_f 0.3 (CH_2Cl_2). The TLC spot rapidly darkened on exposure to sunlight. ^{1}H NMR ($CDCl_3$): δ 1.22−1.39 [6H, t, $(CH_3)_2C$], 2.01−2.05 (1H, d, COCH), 2.21−2.27 (1H, t, =CHCH), 3.60 (2H, s, b, NH_2), 6.28−6.35 (1H, d, CNCH), 6.68−6.70 (1H, d, =CH), 6.70−7.37 (8H, m, ArH).

Hapten Conjugation. Conjugates were synthesized according to water-soluble carbodiimide and diazotization methods (*11*). Immunogens were conjugates of haptens N-4, Cwb-6, or Dwb-5 with BSA. Coating antigens were conjugates of haptens Cwb-6, N-4, or Dwb-5 with OVA.

Polyclonal Antibody Production. Female New Zealand white rabbits were injected subcutaneously five times with 1 mg of BSA−hapten conjugate at 2 week intervals. The first injection consisted of 1 mL of the conjugate mixed with 1 mL of complete Freund's adjuvant. Incomplete adjuvant was used instead of complete in the subsequent boost injections. The rabbits were bled from the ear vein 10 days after each injection except for the initial injection.

Enzyme Immunoassay. The method was identical to that reported by Shan et al. (*11*) with the following modifications. The blocking solution was 0.5% OVA in phosphate-buffered saline (PBS). The incubation step with goat anti-rabbit-IgG-HRP conjugate or analytes was 30 min at 37 °C. The reaction of color was at 37 °C for 5−10 min. Standard curves were obtained by plotting absorbance against the logarithm of analyte concentration. The curves were fitted to a four-parameter logistic equation: $y = \{(A - D)/[1 + (x/C)^B]\} + D$. *A* is the maximum absorbance at no analyte present. *B* is the curve slop at the inflection point. *C* is the concentration of analyte giving 50% inhibition. *D* is the minimum absorbance at infinite concentration of the analyte. The I_{50} value and the limit of detection (LOD) (I_{15}) were found through the logistic equation.

Antiserum Characterization and Assay Optimization. After an immune procedure, eight antisera were procured. However, the antiserum N-2 was hemolytic and not good to used in the immunoassay. Titers of other seven antisera were tested against three different coating antigens according to the procedure of Shan et al. (*11*). All seven antisera showed higher titers in a homologous system than those in the heterologous system, which was consistent with the findings of Shan et al. (*11*) and Wengatz et al. (*18*). The titers of different antisera varied with immnogens and coating antigens. The titer of CWB-C/Cwb-OVA (antiserum/coating antigen) was the highest titer among all of the tested combinations of the seven antisera with different coating antigens. The homologous systems, whose absorbance was higher than 0.75, were tested for cyhalothrin inhibition (**Table 1**). The I_{50} values ranged from 56 to 107 μg/L (**Table 2**), and the CWB-C/Cwb-OVA was the lowest I_{50}, which was chosen to be further optimized. Antisera dilution and coating antigens concentrations were further optimized in a two-dimensional titration (**Table 3**). The combination of antiserum dilution/coating antigen dilution (4000/1000) giving the highest value was selected for further assay development.

After the optimizing procedure, the conditions of the ELISA for cyhalothrin were found, and the inhibition curve was found (**Figure 1**). The I_{50} and LOD were 37.2 and 4.7 μg/L, respectively, and the calibration linear range was 5−500 μg/L.

Table 1. Titration Summary for Anti-cyhalothrin Antibody[a]

antiserum (1:6000)/ immunogen	coating antigen (1 μg/mL)		
	Cwb-OVA	Dwb-OVA	N-OVA
CWB-A/Cwb-BSA	+++	++	+
CWB-B/Cwb-BSA	+++	++	++
CWB-C/Cwb-BSA	++++	+++	+++
DWB-A/Dwb-BSA	++	++++	++
DWB-B/Dwb-BSA	+	+++	+
DWB-C/Dwb-BSA	++	+++	+
N-1/N-BSA	++	+	++++

[a] Absorbance: +, <0.25; ++, =0.25–0.50; +++, =0.50–0.75; ++++, >0.75. Goat anti-rabbit IgG-HRP dilution, 1/1000.

Table 2. Selected Competitive ELISA Results for Screening

antiserum (1:6000)	coating antigen (1 μg/mL)	A_{max}[a]	A_{min}[b]	I_{50} (μg/L)	R^2
CWB-C	Cwb-OVA	1.21	0.15	55.6	0.99
DWB-A	Dwb-OVA	0.97	0.26	106.8	0.99
N-1	N-OVA	1.08	0.16	87.3	1.0

[a] Maximum absorbance of no analyte control. [b] Minimum absorbance of 250 mg/L cyhalothrin. Goat anti-rabbit IgG-HRP dilution, 1/1000.

Table 3. Optimization of Coating Antigen Concentrations and Antiserum Dilutions

coating antigen (Cwb-OVA) concn (μg/L)	B_0-B[a]				
	1:500[b]	1:1000[b]	1:2000[b]	1:4000[b]	1:8000[b]
3.5	0.43[a]	0.58	0.85	0.91	0.65
1.4	0.52	0.70	1.12	1.21	0.82
0.7	0.61	0.85	1.43	1.65	0.92
0.35	0.49	0.68	1.02	1.17	0.77

[a] B_0 is the maximum absorbance at no analyte present. B is the inhibition absorbance at 250 mg/L cyhalothrin. [b] Antiserum (CWB-C) dilution. Goat anti-rabbit IgG-HRP dilution, 1/1000.

Effects of solvents, ionic strength, and pH were tested by running the assay in PBS, PBS at pH 3−9, PBS containing various percents of methanol (0, 10, 20, 50, 80, and 100%) or acetone (0, 10, 20, and 40%), and PBS containing different NaCl concentrations (0.2, 1, 2, 5, and 8%).

Cross-Reactivity. Cyhalothrin, fenvalerate, fenpropathrin, deltamethrin, fluvalinate, and cypermethrin were tested for cross-reactivity by preparing each compound in 50% methanol in PBS and determining the I_{50} in the ELISA. Cross-reactivity values were calculated as follows:

$$\text{CR\%} = (I_{50} \text{ of cyhalothrin}/I_{50} \text{ of tested compound}) \times 100$$

Fortification and Recovery of Water Samples. Well water, tap water, and wastewater were from China Agricultural University and XiaoQing River. Six pretreatments were designed for each sample. In pretreatment 1, each water sample was spiked with cyhalothrin at 100 μg/L and then diluted with an equal volume of methanol. The antiserum solution was 2-fold concentrated so the final concentration of PBS in the well was 1×. In pretreatment 2, each water sample was spiked with cyhalothrin at 500 μg/L and then diluted 10 times by PBS with 50% methanol. In pretreatment 3, each water sample was spiked with cyhalothrin at 2500 μg/L and then diluted 50 times by PBS with 50% methanol. Pretreatments 4−6 were the same as pretreatments 1−3 except that the antiserum PBS solution contained 0.2% bovine blood fibrinogen.

Solid Phase Extraction (SPE). A C18 SPE method was used for extraction of cyhalothrin from water samples, which was the same as the previously reported method for the extraction of esfenvalerate from water (*11*). This method did well in the extraction of pyrethroud insecticide from water, such as fenpropethrin (*18*), permethrin (*20*), deltamethrin (*23*), and esfenvalerate (*11*). In this study, the C18 SPE column (6 cm^3/500 mg, part 10188-1356AB) was obtained from Agilent Technologies Co. Ltd. (China). All of the three water samples (tap water, well water, and wastewater) were spiked with four different concentrations of cyhalothrin (0, 0.1, 0.5, and 5 μg/L) and then extracted by SPE. The final elute was evaporated under a gentle N_2 stream, and then, the residue was reconstituted in 1 mL of methanol and divided into two aliquots. To one aliquot was added an equal volume of PBS solution and analyzed by ELISA in a blind fashion; to the other was added an equal volume of methanol and analyzed by GC in a blind fashion.

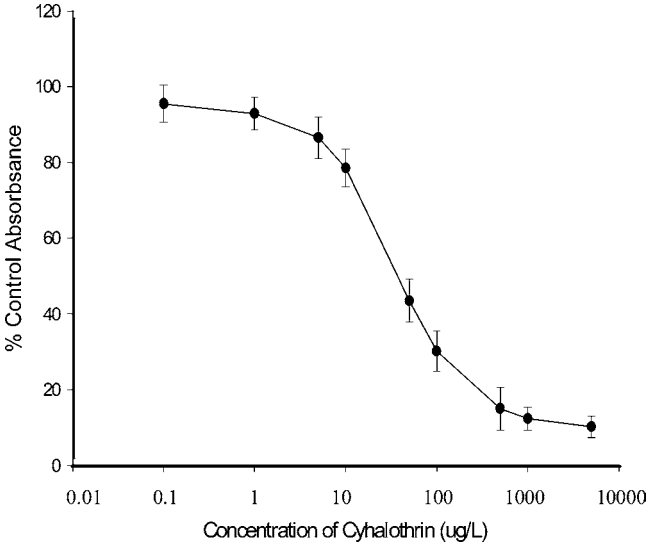


Figure 1. Inhibition curve of the optimized ELISA for cyhalothrin. Coating antigen (Cwb-OVA) concentration, 1/1000 (0.7 μg/L); antiserum (CWB-C) dilution, 1/4000; and goat anti-rabbit IgG-HRP dilution, 1/1000. The values are the means ± SD of three replicates. The cyhalothrin analyte standards were prepared in a 50% methanol/PBS solution. The pH value was 7.0.

RESULTS AND DISCUSSION

Hapten Synthesis. There are 16 theoretical cyhalothrin isomers. Commercial cyhalothrin insecticide products typically contain four isomers that are (±)cyano-3-(4-phenoxyphenyl)-(±)-*cis*-3-(*Z*-2-chloro-3,3,3-trifluoroprop-1-enyl)-2,2-dimethylcycloproane carboxylate [*Z*-(1*R*,3*R*)-*cis*,*S*; *Z*-(1*S*,3*S*)-*cis*,*R*; *Z*-(1*R*,3*R*)-*cis*,*R*; *Z*-(1*S*,3*S*)-*cis*,*S*]. Cyhalothrin haptens were designed to mimic the four cyhalothrin isomers. The (±)-(*cis*)-3-[(*Z*)-3-chloro-4,4,4-trifluorobut-1-enyl-2,2-dimethyl]cyclopropanecarboxylic acid moiety was kept as a major part of the haptens. To keep the (±)-(*cis*)-3-[(*Z*)-3-chloro-4,4,4-trifluorobut-1-enyl-2,2-dimethyl]cyclopropanecarboxylic acid moiety in haptens from isomerizing during synthesis, a two-step approach was used. Cwb-3, Dwb-2, and N-1 were converted to Cwb-4, Dwb-3, and N-2, respectively, followed by conversion to corresponding Cwb-5, Dwb-4, and N-3 using DCC.

Pyrethroid insecticides differ from their cyclopropane end of the structures. Therefore, haptens were designed to have a functional group at the aromatic moiety of the cyhalothrin molecule for coupling with protein. The cyclopropane end of the hapten molecules is distal to the conjugate target to elicit cyhalothrin-specific antibodies. Haptens Cwb-6, Dwb-5, and N-4 were conjugated with BSA to give the immunogens Cwb-BSA, Dwb-BSA, and N-BSA, respectively, and conjugated with OVA to give the corresponding coating antigens Cwb-OVA, Dwb-OVA, and N-OVA.

Optimization. Effects of methanol or acetone on the ELISA using the antiserum CWB-C and the coating antigen Cwb-OVA

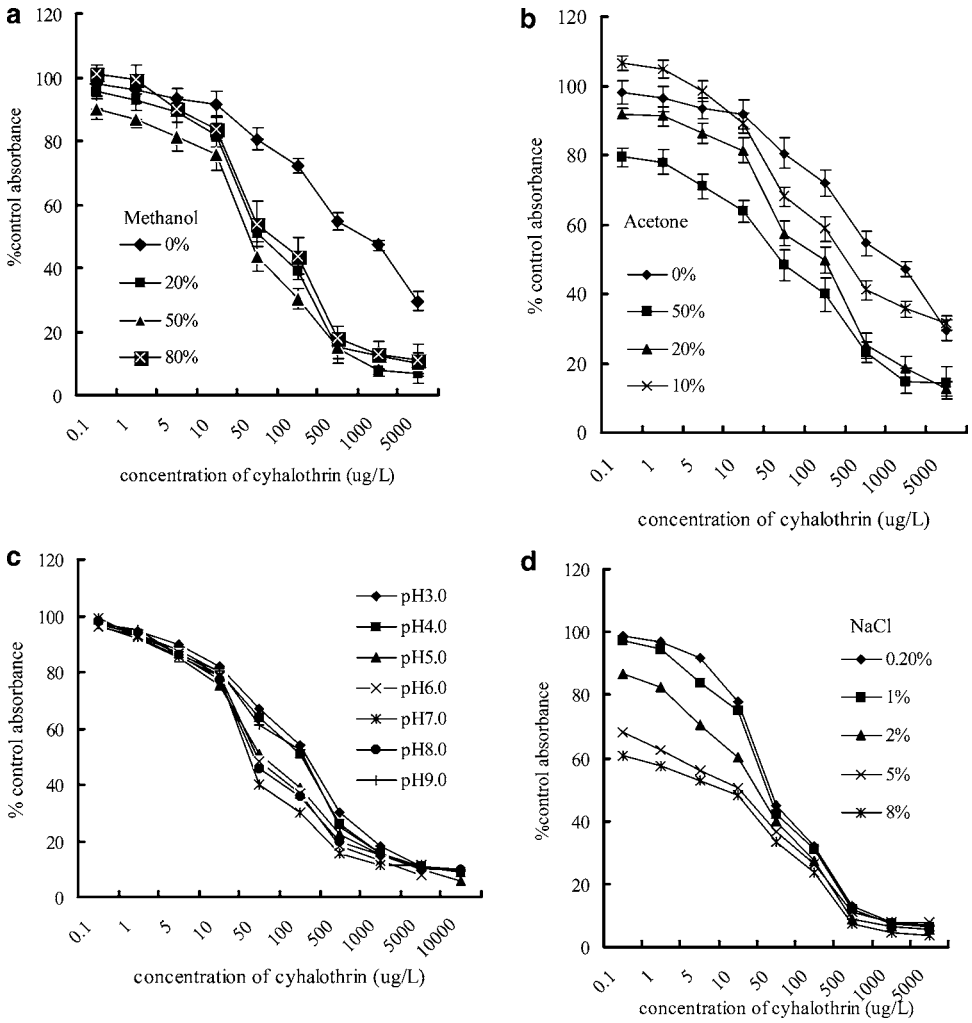


Figure 2. Effects of solvents, pH and anionic strength on the ELISA for cyhalothrin. Coating antigen (Cwb-OVA) concentration, 1/1000 (0.7 μg/L); antiserum (CWB-C) dilution, 1/4000; and goat anti-rabbit IgG-HRP dilution, 1/1000. (**a**) ELISA competition curves for cyhalothrin in PBS buffer containing various concentrations of methanol. The concentrations of methanol shown in this figure are the methanol concentrations of the inhibitor solution before it was added into the well of a 96 well plate. (**b**) ELISA competition curves for cyhalothrin in PBS buffer containing various concentrations of acetone. The concentrations of acetone shown in this figure are the acetone concentrations of the inhibitor solution before it was added into the well of a 96 well plate. (**c**) ELISA competition curves for cyhalothrin in solutions at various pH values. The average of SD = 3.1. (**d**) ELISA competition curves for cyhalothrin in buffers with various ionic strengths. The average of SD = 2.7.

are shown in **Figure 2a**,**b**, respectively. The results show that the solvents significantly reduce the ELISA sensitivities. Methanol or acetone enhanced the binding of antibody-hapten and kept cyhalothrin soluble in the solution, which was consistent with the results of fenpropathrin (*18*). However, excess high concentrations of solvents would affect the identification and binding of the antibody-hapten and, furthermore, denature protein reagents. The I_{50} of 50% methanol was the lowest I_{50}, which was two times lower than that of 80% methanol and 50% acetone.

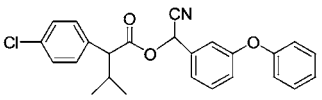
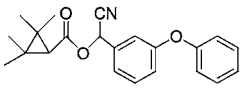
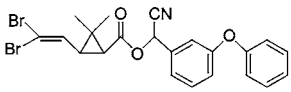
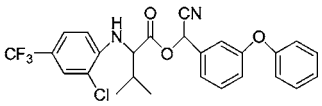
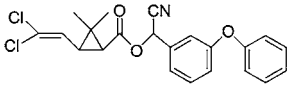
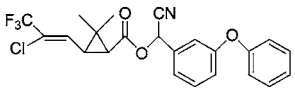
To estimate potential interferences resulting from pH and salt concentration variations in environmental water samples, the effects of pH and ionic strength on the ELISA performance were evaluated in this study. The pH effects on the ELISA (CWB-C/Cwb-OVA) are shown in **Figure 2c**. These results showed that this ELISA was stable in the range of pH 5.0−8.0 and higher or lower pH values significantly increased the I_{50} values, which was similar to the trends of pH effects on deltamethrin (*23*) and flucythrinate (*19*) ELISAs. The assay buffer, pH 7.0, was stable and most sensitive.

The effects of ionic strength on the ELISA (CWB-C/Cwb-OVA) are shown in **Figure 2d**. Increasing salinity decreased significantly not only the I_{50} of cyhalothrin but also the control absorbance. The optimum range of NaCl concentration was 0.2 → 2.0%.

Cross-Reactivity. The ELISA using the antiserum CWB-C and the coating antigen Cwb-OVA was selective for cyhalothrin. Most of the tested compounds showed no significant cross-reactivity in a range of 0.1−10000 μg/L except fenpropathrin (CR, <0.05%) and cypermethrin (CR, 2.5%) (**Table 4**). In this study, the haptens derived from the aromatic moiety of the cyhalothrin molecule, which was different from the haptens in the research of Lee et al. (*16*), used the hapten deriving from the cyano of cyhalothrin molecule as the immunogen. Using the haptens similar to this study, the ELISA selective for esfevalerate (*11*), the ELISA selective for deltamethrin (*16*, *17*), and the ELISA selective for permethrin were found.

Recovery of Water Samples. A rapid and efficient SPE procedure was used to extract and concentrate cyhalothrin in water samples for its detection at sub-part per billion levels. When 200 mL of water sample was extracted with SPE and analyzed by ELISA and GC, no matrix effect was measured. All recoveries from the water samples were >80% of the spiked values (**Table 5**). Because pyrethroids were hydrophobic

Table 4. Assay of Cross-Reactivity among Different Pyrhthroids[a]

Analyte	*Structure*	*Cross-reactivity (%) (n=4)*
Fenvelerate		ni
Fenpropethrin		< 0.05
Deltamethrin		ni
Fluvalinate		ni
Cypermethrin		2.5
Cyhalothrin		100

[a] Cross-reactivity was calculated as (I_{50} of cyhalothrin/I_{50} of analyte) × 100%; ni, <10% inhibition was measured at the highest concentration (10 mg/L). All analytes were prepared in PBS with 50% methanol. Coating antigen (Cwb-OVA) concentration, 1/1000 (0.7 μg/L); antiserum (CWB-C) dilution, 1/4000; and goat anti-rabbit IgG-HRP dilution, 1/1000.

Table 5. Comparison of SPE Cyhalothrin Recoveries Determined by ELISA with Those by GC[a]

water type	spiked concn (μg/L)	average recovery (%) ($n = 4$) ELISA	GC
tap water	0.1	100 ± 9	104 ± 2.5
	0.5	96 ± 6	96 ± 1
	5	98 ± 5	96 ± 1
well water	0.1	89 ± 10	88 ± 2
	0.5	88 ± 7	95 ± 3
	5	104 ± 4	100 ± 3
wastewater	0.1	81 ± 3	85 ± 1.5
	0.5	91 ± 9	84 ± 2.5
	5	85 ± 8	84 ± 2

[a] Coating antigen (Cwb-OVA) concentration, 1/1000 (0.7 μg/L); antiserum (CWB-C) dilution, 1/4000; and goat anti-rabbit IgG-HRP dilution, 1/1000.

compounds and trapped by C18 column, the matrix effects of water samples were largely eliminated by prior SPE. However, considering a simple pretreatment without cleaning up, it is necessary to test the matrix effect from a real water sample. In real water sample analysis, interference in the assay has been caused by sample characteristics, such as ionic strength, pH, and organic impurity (*24*). To adjust the ionic composition and pH value of the samples, the samples were diluted in 50% methanol−PBS. The ionic concentration of tap water was low, and the pH value was close to 7. There were little organic chemicals in the tap water. There were not many differences between the recoveries of the tap water pretreatments 2, 3, 5, and 6, but they were higher than the pretreatments 1 and 4

Table 6. Recovery of Cyhalothrin from the Fortified Water Samples

pretreatment	spiked concn (μg/L)	average recovery (%) ± SE ($n = 4$) tap water	well water	wastewater
1	100	90.3 ± 0.7	84.9 ± 1.2	*a*
2	500	96.7 ± 1.2	92.8 ± 1.0	125 ± 3.8
3	2500	96.4 ± 0.5	95.3 ± 0.8	120 ± 2.7
4	100	91.7 ± 1.3	84.5 ± 1.1	*a*
5	500	92.4 ± 1.1	96.7 ± 1.2	124 ± 5.6
6	2500	96.7 ± 2.0	96.5 ± 1.8	111 ± 4.3

[a] Because of lots of precipitate after adding methanol, the treatment could not be analyzed. Coating antigen (Cwb-OVA) concentration, 1/1000 (0.7 μg/L); antiserum (CWB-C) dilution, 1/4000; and goat anti-rabbit IgG-HRP dilution, 1/1000.

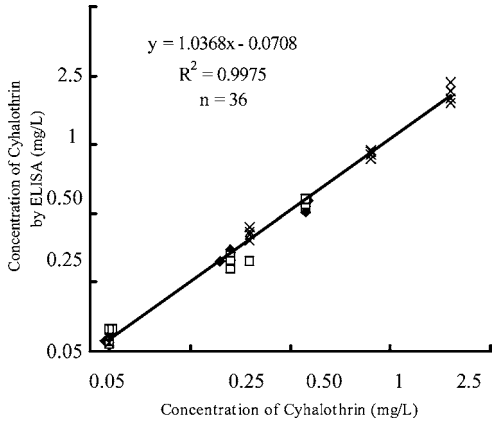


Figure 3. Relationship between the concentration of cyhalothrin fortified into water samples and those determined by ELISA. Tap water (♦), well water (□), and wastewater (×).

(**Table 6**). The results indicated that simple dilution could effectively minimize interferences as long as the concentrations were above the LOD. Dankwardt et al. (*25*) reported that the influence of humic substances in soil samples on ELISA was eliminated by addition of BSA solution to the assay buffer. Similar results were observed in wheat samples (*26*), in apple sample using rabbit serum albumin (RSA) (*19*), and in industrial water samples using BSA (*20*). In this study, bovine blood fibrinogen, differing from the immunogens (BSA) and the coating antigen (OVA) for no interference with the ELISA, was chosen to reduce the interference from the sample impurities with the ELISA. Without cleanup, well water had more ionic impurities and other impurities than tap water. Pretreatment 5 (10-fold dilution and addition of bovine blood fibrinogen) for well water had a better recovery (96.7%) than other pretreatments. Wastewater had lots of salts and chemical impurities. The permethrin immunoassay required 50 times dilution of industrial water in order to obtain good analysis results (*20*). The results of this study suggested that wastewater samples were required to be diluted 50-fold (pretreatment 6) or higher and bovine blood fibrinogen needed to be added for accurate analysis of cyhalothrin in wastewater (**Table 6**). **Figure 3** shows a good correlation between the fortification levels of cyhalothrin in the water samples and those determined recovery by the ELISA. The average recoveries of cyhalothrin ranged from 90 to 120% (**Table 7**). The results demonstrate that these pretreatments and assays are suitable for the detection of the trace levels of cyhalothrin in tap water, well water, and wastewater. Comparing SPE and pretreatment, the method of SPE was more time-consuming than the method of pretreatment but it made the ELISA detect cyhalothrin in water at sub-part per billion levels. The method of pretreatment was simple and timesaving, but it increased the detection lower limit of the ELISA for cyhalothrin

Table 7. Recovery of Cyhalothrin from the Fortified Water Samples by Pretreatment

analyte	spiked concn (mg/L)	average recovery (%) ± SE ($n = 4$)
tap water[a]	0.05	95 ± 6
	0.2	98 ± 8
	0.5	97 ± 4
well water[b]	0.05	100 ± 8
	0.2	98 ± 11
	0.5	97 ± 3
wastewater[c]	0.25	114 ± 5
	1	108 ± 5
	2.5	111 ± 7

[a] Ten-fold dilution in PBS with 50% methanol. [b] Ten-fold dilution in PBS with 50% methanol and added 0.2% bovine blood fibrinogen in the PBS solution for diluting antibody. [c] Fifty-fold dilution in PBS with 50% methanol and added 0.2% bovine blood fibrinogen in the PBS solution for diluting antibody. Coating antigen (Cwb-OVA) concentration, 1/1000 (0.7 μg/L); antiserum (CWB-C) dilution, 1/4000; and goat anti-rabbit IgG-HRP dilution, 1/1000.

in water. In this test, SPE and pretreatment supplemented each other. Using the method of SPE, the ELISA could detect cyhalothrin in water at sub-part per billion levels. Using the method of pretreatment, the ELISA could detect cyhalothrin in water at part per billion levels and part per million levels. The methods of SPE and pretreatment extended the range of the ELISA detection on cyhalothrin in water.

Conclusions. Antisera selective to cyhalothrin were produced in rabbits. An ELISA procedure was optimized for the analysis of cyhalothrin in various types of water samples. The I_{50} and LOD were 37.2 and 4.7 μg/L, respectively, and the calibration linear range was 5−500 μg/L. This assay showed recoveries averaging 97% for cyhalothrin spiked in tap water, well water, and wastewater samples. The matrix effect of different water samples on the ELISA was eliminated in the ELISA using a SPE procedure. The results of ELISA agreed very well with those of GC. Simple dilution can eliminate some matrix effects on the assay for the analysis of cyhalothrin in tap water, well water, and wastewater. The concentrations determined by ELISA after simple dilution correlate well with the concentrations of cyhalothrin fortified in water samples.

ABBREVIATION USED

BSA, bovine serum albumin; DAPEC, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide; DCC, 1,3-dicyclohexylcarbodiimide; DMF, *N*,*N*-dimethylformamide; ELISA, enzyme-linked immunosorbent assay; GAR-HRP, goat anti-rabbit immunoglobulin conjugated to horseradish peroxidase; GC, gas chromatography; HPLC, high-performance liquid chromatography; I_{50}, the concentration analyte giving 50% inhibition; LOD, limit of detection; NHS, N-hydroxysuccinimide; NMR, nuclear magnetic resonance; OVA, ovalbumin; PBS, phosphate-buffered saline; RSA, rabbit serum albumin; SPE, solid phase extraction; TLC, thin-layer chromatography; TMB, tetramethylbenzidine.

ACKNOWLEDGMENT

We thank Dr. Xinlin Yang and Dr. Shangzhong Liu of China Agricultural University for their assistance on hapten synthesis and Dr. Guomin Shan of Dow AgroSciences LLC for suggestions on the hapten design.

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Received for review March 12, 2006. Revised manuscript received May 17, 2006. Accepted May 18, 2006. This study was partially supported by a National Key Project of Science Research for the 10th Five-Year Plan of China.

JF0607009