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Development of an Enzyme-Linked Immunosorbent Assay for the α-Cyano Pyrethroids Multiresidue in Tai Lake Water

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A general and broad class selective competitive enzyme-linked immunosorbent assay (ELISA) for the detection of the α-cyano pyrethroids was developed. One class specific hapten, the 5-(3-benzylphenyl)-5-cyanopentanoic acid (CPBA-BE), was conjugated with BSA as an immunogen. Polyclonal antibodies, PY-antibodies, were generated. The assay with the most selectivity for the family α-cyano pyrethroids such as cyphenothrin was further optimized and tested for tolerance to cosolvent, pH, and ionic strength changes. The IC_{50} values of the optimized immunoassay were 4.58 μg L^{-1} for cyphenothrin, 5.62 μg L^{-1} for fenpropathrin, 7.08 μg L^{-1} for deltamethrin, 10.72 μg L^{-1} for cypermethrin, 19.95 μg L^{-1} for flucythrinate, and 28.18 μg L^{-1} for esfenvalerate. The cross-reactivities of cyphenothrin, fenpropathrin, deltamethrin, cypermethrin, flucythrinate, and esfenvalerate were 100%, 81.49%, 64.68%, 42.72%, 22.96%, and 16.25%, respectively. The low detection limit (LDL) of this assay was 0.107 μg L^{-1} for cyphenothrin. This method was used for the detection of the α-cyano pyrethroids in Tai lake water.

KEYWORDS: α-Cyano pyrethroid; pesticide class; ELISA; tai lake water

INTRODUCTION

Pyrithroids are synthetic analogues of pyrethroids with relatively low mammalian toxicity and are used widely as insecticides in domestic, forestry, agriculture, horticulture, public health, and veterinary applications and in households all around the world (*1*−*4*). In recent years, the α-cyano pyrethroids such as cyphenothrin, fenpropathrin, deltamethrin, cypermethrin, flucythrinate, λ-cyhalothrin, and esfenvalerate (**Table 1**) are fastly becoming the dominant insecticide class. Although, α-cyano pyrethroids are thought to be safe for humans, most of them may cause lymph node and splenic damage as well as carcinogenesis (*5*), and more and more toxicological tests on pyrethroids showed that some pyrethroids belong to categories of endocrine disrupting compounds (*6*) and can impair the immune system (*7*).

Many methods for the detection of pyrethroids have been developed, such as high-performance liquid chromatography (HPLC) (*8*), gas chromatography with an electron capture detector (GC−ECD) (*8*−*11*), and with mass spectrometry (GC−MS) (*12, 13*). These methods are very sensitive, but in such methods, the procedure for sample preparation is complicated, relatively time-consuming, and expensive (*1*). In recent years, the water in Tai lake in Wuxi China has been badly polluted. Thus, a selective, rapid, and sensitive pesticide multiresidue analysis method for monitoring residue levels of pyrethroid in Tai lake water is required.

Class-selective immunoassay as a rapid screening method offers many potential advantages in pesticide multiresidue monitoring such as sensitivity, selectivity, speed of analysis, and cost and time effectiveness. Miyake used chrysanthemic acid (CAA) conjugated with ovalbumin (OVA) as the antigen to prepare polyclonal and monoclonal antibodies specific to non-α-cyano pyrethroids such as allethrin, bioallethrin, and tetramethrin (*14*). Lee Nanju et al. developed a class selective antibody against deltamethrin, cypermethrin, and λ-cyhalothrin (*15*). Takaho Watanabe generated polyclonal antibodies and developed a general enzyme-linked immunosorbent assay for the type I pyrethroid insecticides, such as permethrin, phenothrin, resmethrin, and bioresmethrin (*16*). Sally K. Mak et al. developed a class selective immunoassay for the type II pyrethroid insecticides cypermethrin, cyfluthrin, λ-cyhalothrin, deltamethrin, esfenvalerate, fenvalerate, and fluvalinate (*1*).

The aim of our work is to develop a more general and broader class selective competitive enzyme-linked immunosorbent assay (ELISA) for screening for the presence of compounds or their metabolites of the family α-cyano pyrethroids by using a new hapten design strategy (**Figure 1**).

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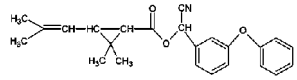
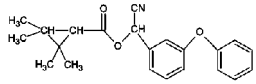
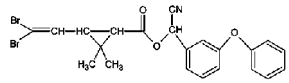
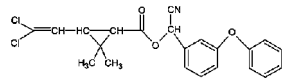
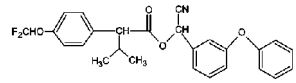
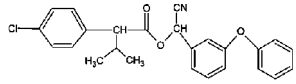
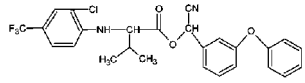
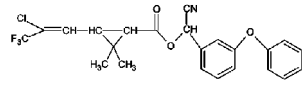
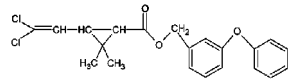
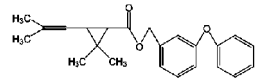
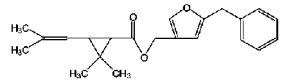
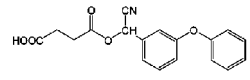
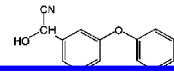
Table 1. Cross-Reactivities of Pyrethroids and Pyrethroid Intermediates[a]

Pyrethroids and hapten	Structures	$IC_{50}(\mu g\ L^{-1})$	CR(%)
cyphenothrin		4.58	100
fenpropathrin		5.62	81.49
deltamethrin		7.08	64.68
cypermethrin		10.72	42.72
flucythrinate		19.95	22.96
esfenvalerate		28.18	16.25
fluvalinate		>1000	<1
λ-cyhalothrin		>1000	<1
permethrin		Ni[b]	<0.01
phenothrin		Ni	<0.01
resmethrin		Ni	<0.01
CPBA-BE (hapten)		3.75	122
CPBA		5.19	88.24

[a] Cross-reactivity was calculated as (IC_{50} of cyphenothrin/IC_{50} of the structural related pyrethroids) × 100. [b] Ni means no inhibition.

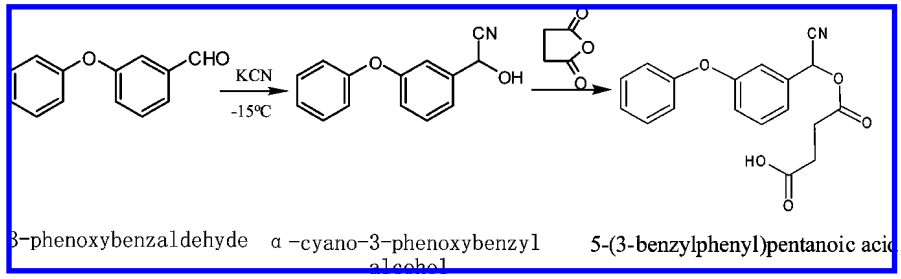


Figure 1. Synthetic route for hapten.

MATERIALS AND METHODS

Reagents. Cyphenothrin ((*RS*)-α-cyano-3-phenoxybenzyl (*RS*)-*cis*, *trans*-2,2-dimethyl-3-(2-methylprop-1-enyl) cyclopropanecarboxylate) were purchased from the Laboratories of Dr. Ehrenstorfer, Germany. Fenpropathrin ((*RS*)-α-cyano-3-phenoxybenzyl-2,2,3,3-tetramethylcyclopropanecarboxylate), deltamethrin ((*S*)-α-cyano-3-phenoxybenzyl(*R*)-*cis*-3-(2,2-dibromovinyl)-2,2-dimethylcyclopropanecarboxylate), cypermethrin ((*RS*)-α-cyano-3-phenoxybenzyl (*RS*)-*cis*,*trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate), flucythrinate ((*RS*)-α-cyano-3-phenoxybenzyl-(*S*)-2-(4-difluoromethoxyphenyl)-3-methylbutyrate), esfenvalerate ((*S*)-α-cyano-3-phenoxybenzyl(*S*)-2-(4-chlorophenyl)-3-methylbutyrate), fluvalinate ((*RS*)-α-cyano-3-phenoxybenzyl *N*-(2-chloro-α,α,α-trifluoro-*p*-tolyl)-DL-valinate), λ-cyhalothrin ((*RS*)-α-cyano-3-phenoxybenzyl(1*RS*)-*cis*-3-[(*Z*)-2-chloro-3,3,3-trifluoropropenyl]-2,2-dimethylcyclopropanecarboxylate), permethrin (3-phenoxybenzyl(*RS*)-*cis*,*trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate), phenothrin ((3-phenoxybenzyl(*RS*)-*cis*,*trans*-2,2-dimethyl-3-(2-methylprop-1-enyl)cyclopropanecarboxylate), and resmethrin (5-benzyl-3-furylmethyl(*RS*)-*cis*-

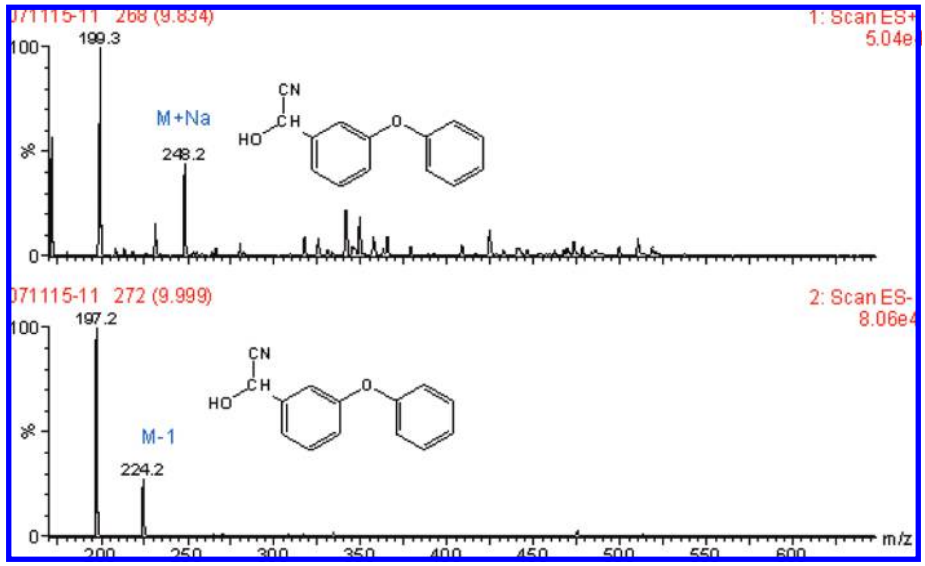


Figure 2. Identification of α-cyano-3-phenoxybenzyl alcohol analyzed by LC/MS.

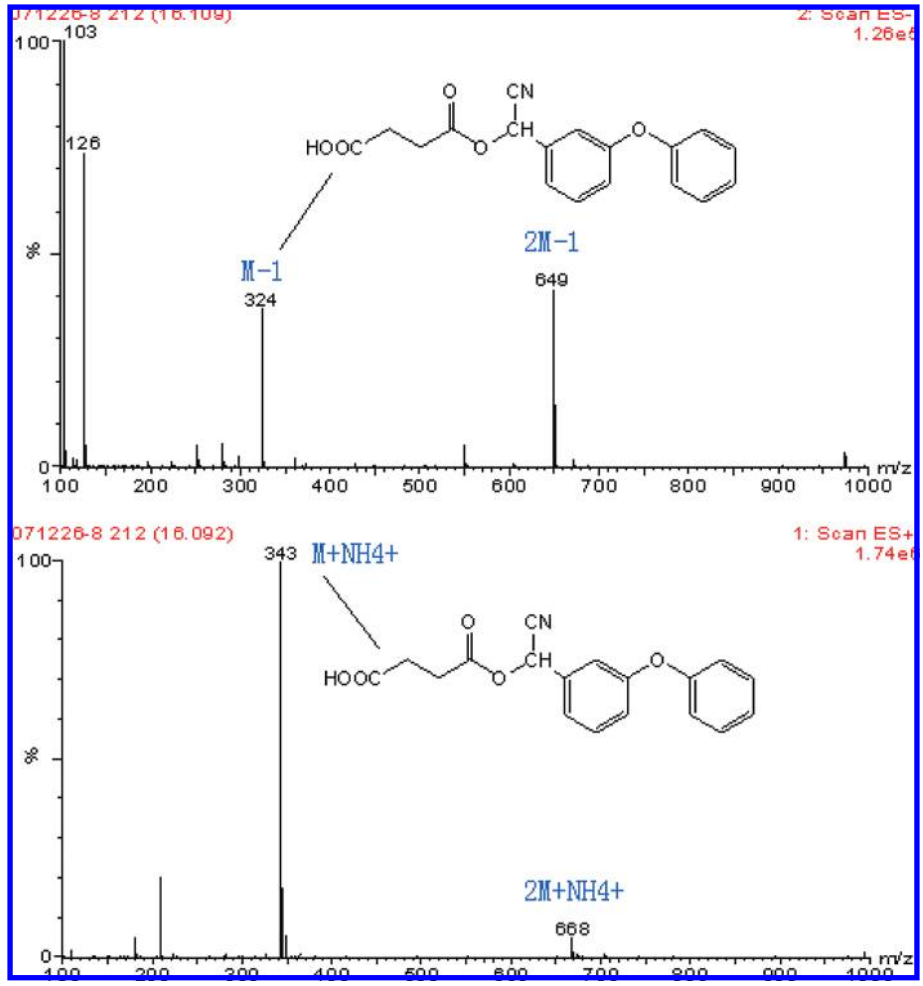


Figure 3. Identification of hapten CPBA-BE analyzed by LC/MS.

,*trans*-2,2-dimethyl-3-(2-methylprop-1-enyl)cyclopropanecarboxylate) were purchased from Sigma Chemical Company (**Table 1**).

3-Phenoxybenzaldehyde, methanol, KCN, and succinic anhydride were used for the production of hapten obtained from Sinopharm Medicine Holding Co., Ltd., Shanghai, China. Bovine serum albumin (BSA) and ovalbumin (OVA) were obtained from Sigma as carrier proteins, and goat antirabbit immunoglobulin conjugated to horseradish-peroxidase (HRP), Tween 20, 3,3′,5,5′-tetramethylbenzidine (TMB), 1-[3-(dimethylamino)-propyl]-3-ethylcarbodiimide hydrochloride (EDC), *N*,*N*-dimethylformamide (DMF), and gelatin (ELISA grade) were also purchased from Sigma. The water used was purified by a NANO pure II system.

Solutions and Buffers. The PBS buffer was 0.01 M at pH 7.4. PBST was the PBS buffer containing 0.05% (v/v) Tween-20. Each PY (0.1m g) was dissolved in 30 mL of methanol and then diluted with a PBS buffer (0.05 M at pH 6.5) to provide ten concentrations (0, 0. 01, 0.1, 1, 10, 100, 1000, and 10000 μg L^{-1}). These were used as standard solutions.

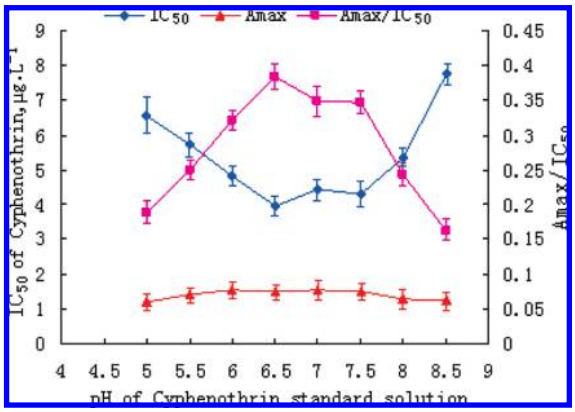


Figure 4. Effects of pH on the IC_{50} of cyphenothrin.

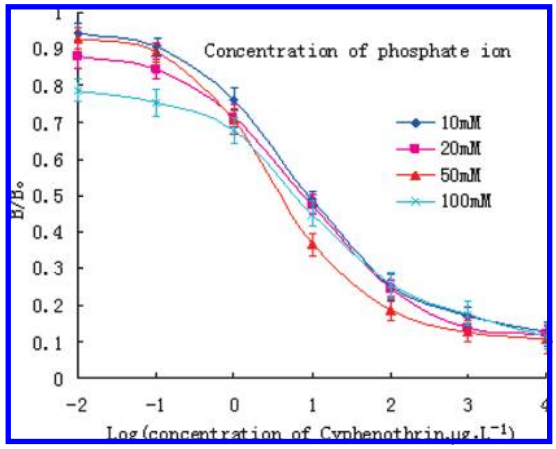


Figure 5. Effects of different ionic strength on cyphenothrin determination with competitive ELISA.

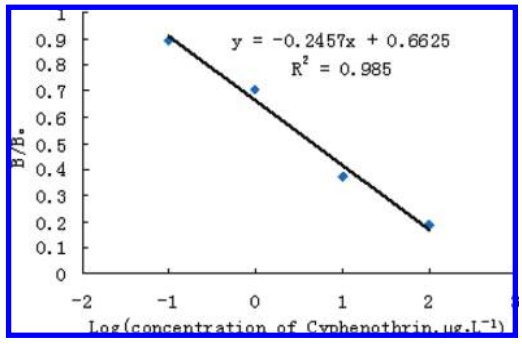


Figure 6. Standard curve for cyphenothrin.

TMB (60 mg) was dissolved in 100 mL of ethylene alcohol (solution A). The citrate buffer was a solution containing 0.1 M sodium citrate at pH 5.5. Solution B was the mixture of the citrate buffer, 0.2 M Na_2HPO_4, and water (24.3:25.7:50, v/v/v), with the addition of 0.05% H_2O_2. Enzymatic reactions were stopped by adding 2 M H_2SO_4.

Apparatus. Polystyrene microtiter plates were purchased from Costar. Pipettes and a Multiscan spectrophotometer were purchased from Thermo Labsystems Co., Ltd. The washing step was carried out on a shaker, which was acquired from Taicang Science and Education Equipment Company. Absorbances of the microplates were read on a spectrophotometric microplate reader. A TDL-4-C centrifuge was purchased from Shanghai Anting Science Apparatus Company. An Avortex shaker was purchased from Shanghai Huxi Analytical Apparatus Company, and a Nitrogen gas drier was obtained from Tianjin Hengao Instrument Company. Alliance 2695 liquid chromatograph equipped with a Quattro Ultima Pt (Micromass, UK) tandem mass spectrometer was from Waters Co., USA. A UV-2802PCS ultraviolet and visible range spectrophotometer was purchased from Unico (Shanghai) Instrument Co., Ltd. A Waters Platform ZMD 4000 LC-MS instrument (American Waters Co.) was used for comparative analyses.

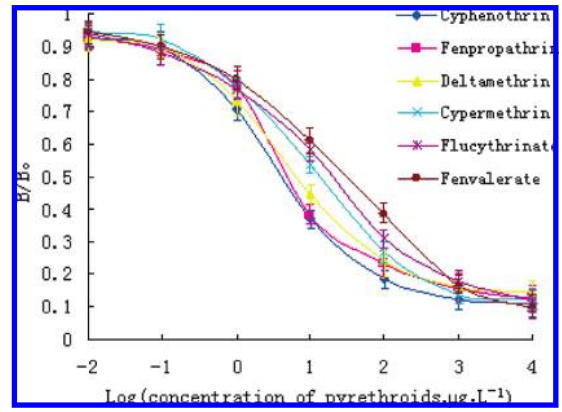


Figure 7. ELISA competitive curves of six pyrethroids.

Table 2. Effect of Organic Solvents on Assay Parameters[a,b]

organic solvent (%)	A_{max}	slope	A_{min}	IC_{50} (μg L^{-1})
		Methanol		
10	1.475	0.76	0.154	5.66
20	1.568	0.82	0.179	4.98
30	1.627	0.89	0.197	4.59
40	1.714	0.81	0.221	6.39
50	1.748	0.69	0.252	9.74
		Acetone		
10	1.324	0.64	0.219	8.33
20	1.416	0.68	0.198	7.45
30	1.479	0.71	0.227	9.97
40	1.533	0.69	0.231	11.25
50	1.601	0.75	0.254	15.88
		DMF		
10	1.265	0.81	0.099	9.96
20	1.189	0.62	0.178	8.31
30	0.971	0.56	0.155	13.03
40	0.836	0.54	0.124	19.29
50	0.695	0.51	0.106	28.71

[a] The assay solution contained PBS (10 mM), NaCl (137 mM), and KCl (2.7 mM) in addition to an organic solvent. [b] ELISA conditions: antiserum to CPBA-BE-BSA, diluted 1/4000 with 10 mM PBST with 0.1% gelatin; coating antigen, CPBA-BE-OVA, 100 μg L^{-1}; cyphenothrin standard solutions in various concentrations of methanol, acetone, or DMF; goat antirabbit IgG-HRP diluted 1/4000.

Preparation of α-Cyano-3-phenoxybenzyl Alcohol (CPBA) and Derivatives 5-(3-Benzylphenyl)-5-cyanopentanoic Acid Butyric Ester (CPBA-BE). A sample of KCN (0.3 g) was added carefully under N_2 with stirring and ice cooling to1 mL of water. The mixture was cooled to −15 °C. Then 3-phenoxybenzaldehyde (0.8 g) was added to the mixture. The temperature was kept at −15 °C. Then 40% sulfuric acid (1 mL) was injected in 10 min, stirred for another 15 min at −15 °C. The mixture was extracted twice with chloroform. The combined organic phase was washed twice with water, dried briefly over magnesium sulfate, and stripped to a tan oil (0.64 g) (*17*).

To a solution of the product of the above procedure (0.54 g, 2.4mmol) in pyridine (10 mL), was added succinic anhydride (0.27 g, 2.7mmol).Then the reactant was refluxed at 110° for 3 h. Then the reaction mixture was cooled to room temperature with cold water. Ten milliliters cold water was poured into it and the pH regulated to 2. The mixture was concentrated by stripping off the solvent under vacuum. The residue was separated by using C18 column chromatography with gradient elution consisting of methanol in water (v/v, 20−80%) and collecting the product that has a maximum absorbing wavelength of 269 nm as a maize syrup. TLC (ethyl acetate/petroleum ether, 2:8, R_f = 0.242).

Preparation of Immunogen and Coating Antigen. The hapten CPBA-BE was conjugated with BSA by the active ester method for

Table 3. Recovery Test of Cyphenothrin in Tai Lake Water

water sample	spiked concn[a] (mg L^{-1})	theor concn In ELISA[b] (μg L^{-1})	detected (μg L^{-1})[c]			mean ± SD (μg L^{-1})	mean recovery (%)
Tai lake water	0.5	5.0	5.63	5.58	5.39	5.53 ± 0.13	111.32 ± 2.6
	1.0	10.0	8.13	7.98	7.85	7.99 ± 0.14	80.20 ± 1.4
	5.0	50.0	4.8	46.1	45.7	45.5 ± 0.67	91.84 ± 1.4

[a] Water samples are from Tai lake in Wuxi, China. Different amounts of cyphenothrin (in methanol) were added to the samples to give final concentrations in water of 0.1, 0.2, and 1 μg L^{-1}. After thorough mixing and standing for at least 2 h, the samples were extracted by SPE prior to the immunoassay. Samples were analyzed blind. [b] Because of the matrix effect of lake water samples, it was diluted 100 times. [c] Three spiked samples were used in each study.

Table 4. Recovery Test of the α-Cyano Pyrethroids

compound	spiked concn[a] (mg L^{-1})	theor concn in ELISA[b] (μg L^{-1})	cyphenothrin equivalent (μg L^{-1})[c]	relative recovery (%)	mean ± S.D. (%)	actual recovery (%)
fenpropathrin	0.5	5.0	4.43	88.6	92.3 ± 4.0	113.3 ± 4.9
			4.59	91.7		
			4.83	96.6		
	1.0	10	6.64	66.4	72.7 ± 5.2	89.2 ± 6.4
			7.47	74.7		
			7.61	76.1		
deltamethrin	0.5	5.0	3.15	62.9	67.2 ± 4.5	103.9 ± 7.0
			3.41	68.2		
			3.60	71.9		
	1.0	10	6.38	63.8	63.5 ± 3.4	98.2 ± 5.3
			6.72	67.2		
			7.06	70.6		
cypermethrin	1.0	10	4.42	44.2	46.6 ± 2.3	109.1 ± 5.4
			4.68	46.8		
			4.88	48.8		
	5.0	50	20.9	41.7	42.6 ± 1.2	99.8 ± 2.8
			21.9	43.9		
			21.1	42.2		
flucythrinate	1.0	10	1.94	19.4	21.3 ± 1.7	92.6 ± 7.4
			2.27	22.7		
			2.18	21.8		
	5.0	50	8.75	17.5	19.2 ± 1.5	83.8 ± 6.5
			9.95	19.9		
			10.1	20.2		
esfenvalerate	1.0	10	1.52	15.2	16.4 ± 1.0	101.2 ± 6.2
			1.71	17.1		
			1.69	16.9		
	5.0	50	6.95	13.9	14.9 ± 0.9	91.8 ± 5.5
			7.55	15.1		
			7.85	15.7		
fluvalinate	10	100		no obvious relativity		
λ-cyhalothrin	10	100		no obvious relativity		
permethrin	100	1000		no relativity		
phenothrin	100	1000		no relativity		
resmethrin	100	1000		no relativity		

[a] Tai lake water was spiked with an appropriate concentration of cyphenothrin. [b] One hundred times dilution with 30% methanolic PBS (pH 6.5).

the immunogen CPBA-BE-BSA and was conjugated with OVA by the mixed anhydride method for the coating antigen CPBA-BE-OVA.

The hapten CPBA-BE (32.5 mg, 0.1mmol) was dissolved in 1 mL of dry *N*,*N*-dimethylformamide (DMF) with equimolar NHS and a 10% molar excess of DCC. After the mixture was stirred at room temperature overnight, the precipitate was removed by centrifugation, and about 1 mL of the active ester was added drop by drop to a solution of BSA (67 mg BSA was dissolved in 6 mL of BSA (0.2 M, pH 7.4)) with vigorous stirring at 4 °C. The reaction mixture was stirred gently at 4 °C for 3 h to complete the conjugation and then dialyzed in water that was changed with fresh water twice a day for 3 days at 4 °C (*18*). Finally, the conjugates were dispensed in 5 mL cryogenic vials and stored at −20 °C.

The hapten CPBA-BE (32.5 mg, 0.1 mmol) was dissolved in 1 mL of dry *N*,*N*-dimethylformamide (DMF), and tributylamine (40 μL), and isobutyl chloroformate (25 μL) were added to it. The reaction mixture was stirred at 4 °C for 3 h and then was added slowly to the OVA solution (45 mg of OVA was dissolved in 6 mL of borate buffer (0.2 M, pH 8.7)) with vigorous stirring at 4 °C (*19*). The following steps were the same as above.

UV−vis spectral data were used to confirm the structures of the final conjugates. The hapten densities (the number of hapten molecules per molecule of protein) of the conjugates were estimated directly by the mole absorbance ε:

$$\text{hapten density} = (\varepsilon_{\text{conjugation}} - \varepsilon_{\text{protein}})/\varepsilon_{\text{hapten}}$$

Preparation of Antibody. Immunogen CPBA-BSA was used to immunize two female and two male New Zealand white rabbits weighing 1.5−2 kg for 6 weeks to yield antibody Py-antibody, as described by Shen and Zhou (*20*). Routinely, 1 mg of immunogen for each animal dissolved in 0.5 mL of physiological saline was demulsified with Freund's complete adjuvant (1:1, v/v) and injected subcutaneously at multiple sites (10−15 sites) on the back. Further injections of the immunogen (1 mg for each animal) demulsified with Freund's incomplete adjuvant were given intramuscularly (1:1, v/v) after 4 weeks. Booster injections were given at 2-week intervals. On the seventh day after the third boost, about 10 mL of blood sample was taken from an ear vein to check the titer of the polyclonal antibodies. The blood sample was allowed to coagulate for about 2 h at room temperature and then stored overnight in a refrigerator. Serum was isolated by centrifugation (4,000 g, 10 min), and sodium azide was added as a preservative at a final concentration of 0.02% (v/v). Serum was then put up in several small plastic tubes and stored at −20 °C. Boosts were given six times. The protocol of the experiment was approved by the Committee of Ethics of Animal Experiments of Jiangsu Province Laboratory Animal Management Committee and conducted in accordance with the Guidelines of the Animal Screening of Antiserum.

Competitive Indirect ELISA. Competitive indirect ELISA wa developed. The procedure of the competitive assay was described similarly by Shan et al. (*21*). The microtiter plates were coated at 4 °C for 12 h with 100 μL/well of coating antigen (CPBA-BE-OVA) in carbonate−bicarbonate buffer (pH 9.6, Na_2CO_3 1.59 g, $NaHCO_3$ 2.93 g in 1 L H_2O). After washing with 200 μL/well PBST (0.05% Tween 20 in 0.01 M PBS, pH 7.4), the plates were incubated with 200 μL/well of 0.1% gelatin in carbonate−bicarbonate buffer for another 2 h at 37 °C. Then the solution was discarded in wells and the plates dried by patting on bibulous paper. The analyte standard was prepared with concentration ranging from 0.01 μg L^{-1} to 10000 μg L^{-1} in PBS buffer. Fifty microliters/well of standard solution was added, and then 50 μL/well of antiserum diluted in PBS (0.01M, pH 7.4) was added, and the plates were incubated at 37 °C for 30 min. After washing three times, 100 μL/well goat antirabbit IgG labeled with horseradish peroxidase (1:4000 in PBST with 0.1% gelatin) was added and incubated at 37 °C for 30 min. The plates were washed, and 100 μL /well substrate solution A and B (1:5,v/v) were added. After incubating at 37 °C for 15 min, the reaction was stopped using 2 M sulfuric acid. The absorbance was measured at 450 nm and recorded. Standard curves were obtained by plotting absorbance against the logarithm of analyte concentration, which were fitted to a four-parameter logistic equation.

Optimization of the ELISA Method. A set of experimental parameters (pH, ionic strength, and organic solvents of standard solution) were studied sequentially to improve the immunoassay sensitivity and to study the immunoassay performance under several conditions. These experiments were carried out using the indirect protocol described above. The PY-antibody diluted to 1/20000 in PBST with 0.1% gelatin and the coating antigen with a quantity of 100 μg L^{-1} of the CPBA-BE-OVA conjugate in carbonate−bicarbonate buffer were the immunoreactives chosen. The criteria used to evaluate immunoassay performance were IC_{50}, maximum absorbance (A_{max}), IC_{50}/A_{max}, and the slope of the ELISA competitive curves (*22*).

Effect of Organic Solvent. The effects of organic solvents on the ELISA system were evaluated when the concentration varies.

Effect of the Assay Buffer pH. To determine the effect of pH on the assay, the phosphate buffer was used to dilute cyphenothrin in the range of pH 5−8.5.

Effect of the Ionic Strength. As ionic strength is always an important parameter, a series of PBS solutions (10, 20, 50, and 100 mM) containing 30% methanol were used to dilute the pyrethroid cyphenothrin.

Cross-Reactivity. Standard solutions of 11 pyrethroids were prepared in the PBS buffer (pH 6.5, 50 mM) containing 30% methanol covering the concentration range 0.01−10000 μg L^{-1}. Standard curves were obtained using the optimized ELISA. The CR values were calculated according to the following formula:

$$\text{cross-reactivity rate (\%)} = (IC_{50} \text{ of cyphenothrin})/(IC_{50} \text{ of the structural related pyrethroids}) \times 100$$

Recovery Test. Tai lake water diluted 100 times with 30% methanolic PBS was spiked with cyphenothrin standards (5, 10, and 50 μg L^{-1}) to estimate the recoveries from water samples.

Sample Extraction. The samples were extracted by SPE prior to the immunoassay.

To analyze Tai lake 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 etal (*21*, *25*). Briefly, C_{18} SPE columns (Waters Sample Preparation Products, Shanghai, CA) were preconditioned with 4 mL each of methanol and ultrapure 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 20 min. The column was eluted with 4 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 30% methanol for ELISA analysis.

RESULTS AND DISCUSSION

Verification of Derivatives of CPBA. Identification of the LC-MS, the purity of the product can be used as a raw material to obtain the hapten α-cyano-3-phenoxybenzyl alcohol butyric ester without other purification. **Figure 2** shows the LC/MS chromatography of the CPBA product. In the LC chromatography, there is one main peak in TIC under ES^+ mode, and there is one smaller peak in TIC under ES^- mode. The molecular weight of CPBA was 225, and from the MS chromatography, it can be concluded that there is CPBA in the sample because of the existence of two fragments with molecular weights of 224.2 and 248.2. In general, the LC/MS chromatography validated the successful synthesis of CPBA.

Verification of Derivatives of CPBA-BE. Figure 3 shows the LC/MS chromatography of the CPBA-BE product. In the LC chromatography, there are two main peaks in TIC under ES^+ mode, and there is one bigger peak and one smaller peak in TIC under ES^-mode. The molecular weight of CPBA-BE was 325, and from the MS chromatography, it can be concluded that there is CPBA-BE in the sample because of the existence of two fragments with molecular weights of 324 and 343, and the existence of diploid moleculars of two fragments with molecular weights of 649 and 668. In general, the LC/MS chromatography validated the successful synthesis of the α-cyano-3-phenoxybenzyl alcohol butyric ester. Because of the identification of the LC-MS, the purity of the product could be used as hapten to conjugate with carrier proteins.

Characterization of Immunogen. In the UV−vis spectra obtained from continuous wavelength scanning, there are obvious differences between the spectra of the conjugate and that of the corresponding carrier protein, especially at around 260 nm. The conjugate of uptake peak shape is the result of lapping between the pristine carrier protein and hapten that have been coupled (*23*). The molar ratio of hapten to protein (as assessed by this spectrophotometric method) was 17.9:1 and 13.2:1 for CPBA-BE-BSA and CPBA-BE-OVA, respectively.

Optimization of the Method. ELISA conditions such as organic solvent, buffer pH, and ionic strength were optimized, and the main effective parameters were as follows.

Effect of Organic Solvent. Table 1 shows the effect of methanol, acetone, and DMF concentration on sensitivity. The results indicated that these solvents significantly influenced assay performance. The rate of color development (estimated from maximum absorbance, A_{max}) decreased rapidly with increasing concentration of acetone and DMF. Methanol concentration does not affect absorbance remarkably. IC_{50} values at 30% concentration of methanol, acetone, and DMF were 4.58, 9.97, and 13.03 μg L^{-1}, respectively. Since methanol provided higher sensitivity and faster enzyme reaction, we selected methanol as the most suitable cosolvent. The optimum concentration selected was 30%, where the IC_{50} value is the lowest, and A_{max} is the highest.

Effect of the Assay Buffer pH. As shown in **Figure 4**, the ELISA was more sensitive under neutral and slightly acidic conditions than under slightly alkaline ones. The acid matrix resulted in the rate of the color development being much slower at pH 5. However, the ratio of maximum absorbance to IC_{50} for cyphenothrin standard curve at pH 8.5 was lower than that of others. On the basis of the relatively high A_{max} value and sharpest slope of the calibration curve, pH 6.5 was selected as the best one.

Effect of the Ionic Strength. Figure 5 presents the effect of the phosphate ion concentration at the competition step on ELISA characteristics. The optimum concentration selected was 50 mM where the IC_{50} value is the lowest.

On the basis of these results, the optimal conditions for the cyphenothrin ELISA are summarized as follows: The PY-antiserum was diluted with PBST and competed with the target analyte dissolved in PBS (50 mM, 30% methanol, pH 6.5). **Figure 6** shows typical calibration curves obtained under these optimum conditions.

With optimized combination, a standard curve for cyphenothrin was obtained by B/B_0 versus concentration of cyphenothrin and by plotting inhibition rate versus log (concentration of cyphenothrin, μg L^{-1}): $y = -0.2457 \times +0.6625$, $r = 0.985$ (**Figure 6**) (working range was assigned to concentrations 0.28−76 μg L^{-1} for cyphenothrin). In the assay, the IC_{50} value was 4.58 μg L^{-1}, and the low detection limit (LDL) was 0.107 μg L^{-1}.

Cross-Reactivities. With the optimized combination, most of the pyrethriod compounds were tested, and six ELISA competitive curves for cyphenothrin, fenpropathrin, deltamethrin, cypermethrin, flucythrinate, and esfenvalerate were obtained by B/B_0 versus concentration of these target analytes and by plotting inhibition rate versus log(concentration of pyrethroids, μg L^{-1}) (**Figure 7**). Including six α-cyano pyrethroids and pyrethroid metabolite CPBA and hapten CPBA-BE showed good cross-reactivity, but the pyrethroids without α-cyano-, such as permethrin, phenothrin and resmethrin, show no significant cross-reactivity (**Table 1**).

Recovery Test. To estimate reliability, a recovery test was performed using Tai lake water samples. The samples were diluted with PBS to eliminate the matrix effect prior to analysis. The recoveries of cyphenothrin were during 80.20% to 111.32%, and the relative standard deviation of this method is within 2.6%. In addition, the recoveries of other α-cyano pyrethroids using a cyphenothrin standard curve were tested. Tai lake waters were

spiked with 0.5, 1.0, and 5.0 mg L^{-1} fenpropathrin, deltamethrin, cypermethrin, flucythrinate, and esfenvalerate, and were diluted in an appropriate dilution factor with 30% methanolic PBS (pH 6.5) (Table **4**) (*24*). The results indicate that this assay will be a useful screening test for the six α-cyano pyrethroids mentioned above.

Conclusions. In previous publications, immunoassays for fenpropathrin (*26*), deltamethrin (*27*), and cyphenothrin (*21*) reported that the LDLs in those studies could reach the need of detection. But in those studies, only one analyte could be analyzed by each method.

In this study, we designed and synthesized the hapten for the α-cyano pyrethroids, which contain the alcohol moiety with α-cyano. A general and broad selective immunoassay for the α-cyano pyrethroid insecticides has been developed. This ELISA method had a relatively lower IC_{50} and also demonstrated no inhibition to the pyrethroids without α-cyano. The IC_{50} values of the optimized immunoassay were 4.58 μg L^{-1} for cyphenothrin, 5.62 μg L^{-1} for fenpropathrin, 7.08 μg L^{-1} for deltamethrin, 10.72 μg L^{-1} for cypermethrin, 19.95 μg L^{-1} for flucythrinate, and 28.18 μg L^{-1} for esfenvalerate. The LDL of this assay was 0.107 μg L^{-1} for cyphenothrin. This assay can be used in monitoring the α-cyano pyrethroids in Tai lake water and also can be used to detect pyrethroid residues in other waters due to class-specific antibodies for these compounds.

LITERATURE CITED

(1) Mak, S. K.; Shan, G. M.; Lee, H. J.; Watanabe, T.; Stoutamire, D. W.; Gee, S. J.; Hammock, B. D. Development of a class selective immunoassay for the type II pyrethroid insecticides. *Anal. Chim. Acta* **2005**, *534*, 109–120.

(2) Shan, G. M.; Wengatz, I.; Stoutamire, D. W.; Gee, S. J.; Hammock, B. D. An enzyme-linked immunosorbent assay for the detection of esfenvalerate metabolites in human urine. *Chem. Res. Toxicol*. **1999**, *12*, 1033–1041.

(3) Leahey, J. P. Metabolism and Environmental Degradation. In *The Pyrethroid Insecticides*; Leahey, J. P., Ed.; Taylor and Francis: London, U.K., 1985; Chapter 5, pp 263−341.

(4) Miadokova, E.; Vickova, V.; Trebaticka, M.; Garajova, L.; Grolmus, J.; Podstavkova, S.; Vieck, D. Effects of supercypermethrin, a synthetic development pyrethroid on four biological test systems. *Mutat. Res*. **1992**, *280*, 161–168.

(5) Hallenbeck, W. H. ; Cunningham-Burns, K. M. *Pesticides and Human Health*, Springer-Verlag: New York, 1985; p 118.

(6) Vera, G.; Joan, G.; Mary, S. W.; Beattiz, G. T. Pogo estrogenic potential of certain pyrethroid compounds in the MCF-7 human breast carcinoma cell line. *Environ. Health Perspect*. **1999**, *107*, 173–177.

(7) Cox, C. "Inert" ingredient factsheet: Nonyl phenol and related chemicals. *J. Pestic. Reform* **1996**, *16*, 15–20.

(8) Bottomley, P.; Baker, P. G. Multi-residue determination of organochlorine, organophosphorus and synthetic pyrethroid pesticides in grain by gas-liqyuid and high performance liquid chromatography. *Analyst* **1984**, *109*, 85–90.

(9) Boccelli, R.; Capri, E.; Delre, A. A. M. Residues of esfenvalerate in irrigation water, stubble, husked and unusked rice samples of a paddy-field. *Toxicol. Environ. Chem*. **1993**, *37*, 185–189.

(10) Hengel, M. J.; Mourer, C. R.; Shibamoto, T. New method for analysis of pyrethroid insecticides: esfenvalerate, cis-permethrin, and trans-permethrin, in surface waters using solid-phase extraction and gas chromatography. *Bull. Environ. Contam. Toxicol*. **1997**, *59*, 171–178.

(11) Wells, M. J. M.; Riemer, D. D.; Wellsknecht, M. C. Development and optimization of a solid-phase extraction scheme for determination of the pesticides metribuzin atrazine metolachlor and esfenvalerate in agricultural runoff water. *J. Chromatogr., A* **1994**, *659*, 337–348.

(12) Roberts, T. R. Current trends in pesticide residue analysis. *Trends. Anal. Chem*. **1985**, *4*, 3–7.

(13) Fleet, I. A.; Tetler, L. W.; Monaghan, J. J. Study of the major fragmentation pathways of cypermethrin and related synthetic insecticides using tandem mass spectrometry. *Org. Mass Spectrom*. **1993**, *28*, 626–634.

(14) Shiro, M.; Rie, B.; Yuki, Y.; Hideo, K.; Hideo, O. Polyclonal and monoclonal antibodies specific to the chrysanthemic acid moiety of pyrethroid insecticides. *Pestic. Sci*. **1998**, *54*, 189–194.

(15) Lee, N. J.; McAdam, P. D.; Sherritt, H. J. Develoment of immunoassays for type II synthetic pyrethroids 1 hapten design and application to heterologous and homologous assays. *J. Agric. Food. Chem*. **1998**, *46*, 520–534.

(16) Watanabe, T.; Shan, G.; Stoutamire, D. W.; Gee, S. J.; Hammock, B. D. Development of a class-specific immunoassay forthe type I pyrethroid insecticides. *Anal. Chim. Acta* **2001**, *444*, 119–129.

(17) Kequan, G. *Pyrethroids [M]*; East China Normal University Press, 1984; p 187.

(18) Lee, J. K.; Ahn, K. C.; Stoutamire, D. W.; Gee, S. J.; Hammock, B. D. Development of an enzyme-linked immunosorbent assay for the detection of the organophosphorus insecticide acephate. *J. Agric. Food. Chem*. **2003**, *51*, 3695–3703.

(19) Ballesteros, B.; Barcelo, D.; Camps, F.; Marco, M. P. Preparation of antisera and development of a direct enzyme-linked immunosorbent assay for the determination of the antifouling agent Irgarol 1051. *Anal. Chim. Acta* **1997**, *347*, 139–147.

(20) Shen, G. X.; Zhou, R. L. *Modern Immuno-Experiment Technology*; Hubei Science and Technology Press: Hubei, China, 2002; p 30.

(21) Shan, G.; Stoutamire, D. W.; Wengatz, I.; Gee, S. J.; Hammock, B. D. Development of an immunoassay for the pyrethriod insecticide esfenvalerate. *J. Agric. Food Chem*. **1999**, *47*, 2145–2155.

(22) Oubin, A.; Ballesterosa, B.; Galve, R.; Barcelo, D.; Marco, M. P. Development and optimization of an indirect enzyme-linked immunosorbent assay for 4-nitrophenol. Application to the analysis of certified water samples. *Anal. Chim. Acta* **1999**, *387*, 255–266.

(23) Zhu, G. N.; Wu, Y. L.; Cheng, J. L. Synthesis and identification of the antigens for carbofuran. *J. Zhejiang Univ. (Agric. & LifeSci.)* **2002**, *28* (1), 47–53.

(24) Wortberg, M.; Jones, G.; Kreissig, S. B.; Rocke, D. M.; Gee, S. J.; Hammock, B. D. An approach to the construction of an immunoarray for differentiating and quantitating cross reacting analytes. *Anal. Chim. Acta* **1996**, *319*, 291–303.

(25) Lee, H. J.; Shan, G.; Ahn, K.; Park, E.; Watanabe, T.; GEE, S. J.; Hammock, B. D. Development of an enzyme-linked immunosorbent assay for the pyrethroid cypermethrin. *J. Agric. Food Chem*. **2004**, *52*, 1039–1043.

(26) Wengatz, I.; Stoutamire, D. W.; Gee, S. J.; Hammock, B. D. Development of an enzyme-linked immunosorbent assay for the detection of the pyrethroid insecticide fenpropathrin. *J. Agric. Food Chem*. **1998**, *42*, 2211–2221.

(27) Lee, H. J.; Shan, G; Watanabe, T; Stoutamire, D. W.; Gee, S. J.; Hammock, B. D. Enzyme-linked immunosorbent assay for the pyrethroid deltamethrin. *J. Agric. Food Chem*. **2002**, *50*, 5526–5532.

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