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ARTICLE

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Development of an Enzyme-Linked Immunosorbent Assay Based a Monoclonal Antibody for the Detection of Pyrethroids with Phenoxybenzene Multiresidue in River Water

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ABSTRACT: A sensitive and broad class selective direct competitive enzyme-linked immunosorbent assay (ELISA) using monoclonal antibody (McAb) has been described for the detection of pyrethroids with phenoxybenzene group. One monoclonal antibody, $2G_2E_7$, was obtained and characterized after fusion of myeloma cells with spleen cells isolated from BALB/c mice. The assay with the most selectivity for the family pyrethroids with phenoxybenzene group was optimized. The IC_{50} values of the optimized immunoassay were 1.8 $\mu g\ L^{-1}$ for deltamethrin, 1.5 $\mu g\ L^{-1}$ for cypermethrin, 2.0 $\mu g\ L^{-1}$ for fluvalinate and fenvalerate, 2.2 $\mu g\ L^{-1}$ for phenothrin, 2.4 $\mu g\ L^{-1}$ for flucythrinate, 3.0 $\mu g\ L^{-1}$ for fenpropathrin, and 5.0 $\mu g\ L^{-1}$ for permethrin. River water samples fortified with pyrethroids were analyzed with the ELISA to evaluate the accurancy of the assay. The recoveries of pyrethroids in spiked water samples ranged from 74 to 108%. The results indicate that the ELISA developed can accurately simultaneously determine pyrethroids with phenoxybenzene group in water samples.

KEYWORDS: pyrethroid, phenoxybenzene group, monoclonal antibody, ELISA, river water

INTRODUCTION

Recently, the pyrethroids such as deltamethrin, cypermethrin, and fluvalinate, et al. have been widely accepted for application in agriculture, forestry, horticulture, public health, and households all over the world.[1−4] Although pyrethroids are considered to be a group of considerably potent insecticides with relatively low toxicity to mammals and birds, there have been concerns arising from their potential effects on the immune system after long-term exposure.[5,6] Some of them may also cause lymph node and splenic damage as well as carcinogenesis.[7] It is reported that the pyrethroids have been determined as surface water contaminants, and their impacts on the aquatic ecosystems result in effects on ecosystem health.[8] Therefore, a rapid, selective and sensitive method is desirable for monitoring residue levels of pyrethroids.

Several methods to detect the presence of pyrethroids had been developed, including gas chromatography−mass spectrometry (GC−MS),[9,10] gas chromatography with an electron capture detector (GC−ECD),[11,12] liquid chromatography combined with postcolumn photochemically induced fluorometry derivatization and fluorescence detection (HPLC−FLD),[13,14] or high-performance liquid chromatography−mass spectrometry (HPLC−MS).[15] Although these methods have good sensitivity, they normally involve complicated sample cleanup procedures, and they are relatively time-consuming and expensive. Therefore, we need a more effective and cheaper method to detect pesticide residue. Over the past 20 years, the importance and application of immunoassays, especially ELISAs, have grown significantly. They have been widely used for the determination of both large and small analytes in the medical, biological, agricultural, and environmental area. Compared with the traditional instrumental methods, ELISAs have the advantages of high sensitivity and specificity, simple sample preparation, low cost, and high sample throughput.[16]

As shown in Table 1, some immunoassays based on polyclonal and monoclonal antibodies for the detection of pyrethroid pesticides have been developed. However, in most of those studies, only a single analyte could be analyzed. Although some of them could be applied to detect several pyrethroids simultaneously, the assay sensitivities were not perferable. As most of the pyrethroid pesticides contain a phenoxybenzene group, the aim of this study is to develop a more general and broader class selective ELISA based on monoclonal antibody for detecting pyrethroids with phenoxybenzene group simultaneously with higher sensitivity.

MATERIALS AND METHODS

Reagents and Chemicals. The pyrethroid standards of deltamethrin (99.0%), cypermethrin (94.0%), fluvalinate (93.8%), fenvalerate (99.0%), phenothrin (99.0%), flucythrinate (97.7%), fenpropathrin (99.0%), permethrin (98.0%), resmethrin (94.0%), and 3-phenoxybenzoic acid (98.0%), goat anti-mouse immunoglobulin G horseradish peroxidase conjugate (IgG-HRP), ovalbumin (OVA, MW 45,000), keyhole limpet hemocyanin (KLH), 3,3′,5,5′-tetramethylbenzidine (TMB), dimethyl sulfoxide (DMSO), Tween-20, *N*,*N*-dimethylformamide (DMF), and Freund's complete and incomplete adjuvants were from Sigma Chemical Co. (St. Louis, MO). Bovine serum albumin (BSA, MW 67,000) was obtained from Merck (Darmstadt, Germany). Hypoxanthine, aminopterin, and thymidine (HAT), hypoxanthine and thymidine (HT), Dulbecco's modified Eagle medium (DMEM) were all obtained from Sigma-Aldrich (USA). Female BALB/c mice were purchased from Vital River Laboratory Animal, Inc. (Beijing, China). Myeloma cells SP2/0 were provided by the China Center for Type

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Table 1. Study on Pyrethroid Pesticides Residues by ELISA

target analyte	antibody	IC_{50} ($\mu g\ L^{-1}$)	author
cypermethrin	PcAb[a]	13.5	Lee[17]
deltamethrin	PcAb	17.5	Lee[18]
	McAb[b]	500	Queffelec[19]
esfenvalerate	PcAb	30	Shan[20]
fenpropathrin	PcAb	20	Wengatz[21]
type I pyrethroids[c]	PcAb	permethrin: 30	Watanabe[22]
		phenothrin: 20	
type II pyrethroids[d]	PcAb	cypermethrin: 78	Mak[1]
		cyfluthrin: 205	
		cyhalothrin: 120	
		deltamethrin: 13	
		esfenvalerate: 6	
		fenvalerate: 8	
		fluvalinate: 123	
α-cyano pyrethroids[e]	PcAb	cyphenothrin: 4.58	Hao[23]
		fenpropathrin: 5.62	
		deltamethrin: 7.08	
		cypermethrin: 10.72	
		flucythrinate: 19.95	
		esfenvalerate: 28.18	
5 pyrethroids[f]	PcAb	deltamethrin: 20	Lu[24]
		cypermethrin: 16	
		fluvalinate: 11	
		fenvalerate: 15	
		fenpropathrin: 20	
deltamethrin	McAb	17.0	Kong[25]

[a] PcAb, polyclonal antibodies. [b] McAb, monoclonal antibodies. [c] Permethrin, phenothrin. [d] Cypermethrin, cyfluthrin, cyhalothrin, deltamethrin, esfenvalerate, fenvalerate, fluvalinate. [e] Cyphenothrin, fenpropathrin, deltamethrin, cypermethrin, flucythrinate, esfenvalerate. [f] Deltamethrin, cypermethrin, fluvalinate, fenvalerate, fenpropathrin.

Culture Collection (CCTCC). Fetal bovine serum was the product of Gibco BRL (Paisley, U.K.). Polyethylene glycol (PEG 4000) and a Mouse Monoclonal Antibody Isotyping Kit were products of Boehringer Mannheim (Mannheim, Germany).

Materials and Instruments. Microtiter plates were from NUNC (Roskilde, Denmark). Immunoassay absorbance was measured with a Multiskan Spectrum purchased from Thermo (Labsystems, Vantaa, Finland). Cell culture plates (24 and 96 wells) and culture flasks were from Costar Inc. (Cambridge, MA, USA). Water was purified by a Milli-Q purification system from Millipore (Billerica, MA, USA). Gas chromatography/electron capture detector (GC/ECD) was obtained from Shimadzu GC-2010 Tokyo, Japan.

Buffers and Solutions. The following buffers were used in the experiments. Coating buffer: 50 mM sodium carbonate buffer, pH 9.6. Assay buffer: 10 mM phosphate-buffered saline (PBS), pH 7.5. Washing buffer (PBST): PBS with 0.05% Tween-20. Blocking buffer: 1% BSA (m/v) in PBS. TMB substrate solution: prepared by adding 3.3 mg of TMB in 250 μL of DMSO to 25 mL of phosphatecitrate buffer (0.1 mol L^{-1} citric acid + 0.2 mol L^{-1} Na_2HPO_4; pH 4.3), containing 3.25 μL of a 30% H_2O_2 solution. Stopping solution: 1.25 mol L^{-1} H_2SO_4.

Hapten Synthesis. The chemical structure of the hapten PBA is shown in Figure 1. Synthesis of PBA was described in a previous report by Lu et al.[24] Hapten PBA with a 3-carbon spacer attachment to 3-phenoxybenzoic acid was prepared.

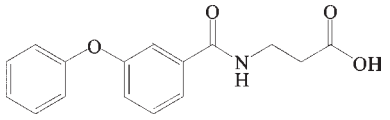


Figure 1. The structure of hapten PBA.

Preparation of Protein and Enzyme Conjugates. The hapten PBA was conjugated with KLH to be used as immunogen, with OVA as the coating antigen for antisera screening, and with HRP as enzyme tracer respectively, by the active ester method, which was described by Wang et al.[26] Resultant ester (1 mg) was dissolved in 200 μL of dry DMF. Then the mixture was added drop by drop to a solution of protein (10 mg of KLH, OVA, HRP was dissolved in 2 mL of 50 mM, pH 9.3 dipotassium phosphate buffer) with vigorous stirring at 4 °C. The reaction mixture was stirred overnight on the mixing wheel at 4 °C and then dialyzed against PBS for three days at 4 °C. The resultant conjugates were stored at −20 °C.

Immunization. Five BALB/c female mice (7 weeks old) were immunized intraperitoneally with 1:1 mixture (v/v, 200 μL) of PBA−KLH conjugates (100 μg) in a saline solution (0.9% NaCl) and Freund's complete adjuvant. Booster injections were given intraperitoneally at 2-week intervals with the same amount of immunogen emulsified with incomplete Freund's adjuvant. One week after each booster injection, mice were tail-bled and antisera titer was tested by an indirect ELISA. Two weeks after the last booster injection, mice selected to be spleen donors for hybridoma production received a final soluble intraperitoneal injection with the same amount of conjugate in a saline solution (0.9% NaCl). The mice selected were prepared for cell fusion 3 days after the final injection.

Cell Fusion and Hybridoma Selection. SP2/0 murine myeloma cells were cultured in low-glucose DMEM media including 20% fetal bovine serum and 1% penicillin−streptomycin. Cell fusion schedules were performed essentially the same as those described by Galfre and Milstein.[27] The splenocytes from the immunized mice were mixed with the myeloma cells at a 5:1 ratio in the presence of PEG 4000. Then the fused cells were centrifuged and diluted with fresh DMEM including 20% fetal bovine serum (referred to as s-DMEM) at an approximate density of 4×10^3 cells μL^{-1} before they were distributed in a dose of 50 μL/well in 96-well culture plates. After culturing overnight, 50 μL of HAT selection medium was added to each well. The HAT medium consisted of s-DMEM supplemented with 100 μM hypoxanthine, 0.4 μM aminopterine and 16 μM thymidine. Half of the medium in the wells was replaced by fresh HAT medium every third day. When most of the nonfused cells disappeared, HAT was substituted by HT medium with no aminopterin. The cultures in the plates were incubated at 37 °C with 5% CO_2. After about 10 days, hybridoma culture supernatants were screened for the presence of antibodies that recognized deltamethrin by an indirect ELISA. Hybridoma supernatants from the cells which gave an absorbency of over 3 were transferred to 24-well microculture plates in HT medium. Supernatants from 24-well culture plates were tested, and only those that exhibited inhibition by deltamethrin at 1 $\mu g\ mL^{-1}$ were transferred to cell culture bottle. Supernatants from cell culture bottle were tested again, and only those that exhibited high inhibition by eight pyrethroids at 1 $\mu g\ mL^{-1}$ were chosen for further selection. Selected positive hybridoma cell lines were subsequently subcloned by limited dilution technique, and stable antibody-producing clones were expanded. Selected clones were cryopreserved in liquid nitrogen.

Production, Purification and Isotyping of Monoclonal Antibodies. Ascites fluid was produced in female BALB/c mice which were preinjected with 0.5 mL of pristane 1 week ago by intraperitoneal injection of 1×10^6 hybridoma cells. Two weeks after the injection, the ascites fluid was harvested and subjected to purification by salting out (with caprylic acid−ammonium sulfate) as described by Zhu et al.[28] The

Table 2. Sensitivity (IC_{50}) of Indirect Competive ELISA for Five McAbs[a]

McAb	ascites dilution	IC_{50} ($\mu g\ L^{-1}$) DEL[b]	CYP[c]	FLU1[d]	FEN1[e]	PHE[f]	FLU2[g]	FEN2[h]	PER[i]
$2G_2E_7$	1:600000	3.4	5.2	4.5	2.8	4.8	3.6	5.8	8.7
$14B_8G_4$	1:400000	4.5	9.4	7.2	5.4	12.4	8.7	15.3	16.4
$17G_7C_3$	1:300000	15.5	10.0	6.4	15.7	18.5	4.8	19.6	21.6
$17G_7F_3$	1:400000	7.3	10.3	14.9	5.2	15.5	18.3	20.4	18.2
$27C_8E_{11}$	1:600000	13.7	14.4	16.8	20.3	18.7	8.9	22.5	30.5

[a] All analytes were prepared in PBS, coating antigen (PBA−OVA) concentration, 1 μg/well; goat anti-mouse IgG-HRP dilution, 1/10000. All data are means of three replicates. [b] DEL, deltamethrin. [c] CYP, cypermethrin. [d] FLU1, fluvalinate. [e] FEN1, fenvalerate. [f] PHE, phenothrin. [g] FLU2, flucythrinate. [h] FEN2, fenpropathrin. [i] PER, permethrin.

isotype of the antibodies was determined by a Mouse Monoclonal Antibody Isotyping kit. Purified antibodies were stored at −20 °C in the presence of 50% glycerol.

Indirect Competitive ELISA. Culture supernatants were screened by indirect competitive ELISA: Microtiter plates were coated with 100 μL/well of a hapten−OVA conjugate in carbonate−bicarbonate buffer by overnight incubation at 4 °C. Plates were washed three times and then blocked by incubation with 1% BSA in PBS (200 μL/well) for 1 h at 37 °C. After another washing step, serial dilutions of the analyte in PBS (50 μL/well) were added, followed by 50 μL/well of previously diluted hybridoma supernatants in PBS. After incubation for 1 h at room temperature, 100 μL/well of a diluted (1/10000) goat anti-mouse IgG-HRP was added. The mixture was allowed to incubate for 0.5 h, and 100 μL/well of TMB/peroxide-based substrate solution was added. The reaction was stopped after 20 min by addition of 50 μL of 1.25 M H_2SO_4, and absorbance was read using a microplate reader in dual wavelength mode (450 nm as test and 650 nm as reference).

Direct Competitive ELISA. Direct competitive ELISA was developed; it was performed as follows. The microtiter plates were coated at 37 °C with antibody (100 μL/well) previously diluted (10 $\mu g\ mL^{-1}$) in 50 mM sodium carbonate buffer for 3 h. After washing with PBST for three times, the surface of the wells was blocked with 200 μL/well of 1% BSA in PBS for 1 h at 37 °C. After another washing step, 50 μL/well of diluted pyrethroid standard or sample, and then 50 μL/well of enzyme conjugate (diluted in PBS) was added, incubated for 1 h at room temperature. The plates were washed, 100 μL/well of a TMB solution was added, and the reaction was stopped by addition of 50 μL/well 1.25 M H_2SO_4 after incubating for 30 min. The absorbance was measured using a microplate reader. Competition curves were obtained by plotting absorbance against the logarithm of analyte concentration. Sigmoidal curves were fitted to a four parameter logistic equation, from which IC_{50} values were determined.[29]

Optimization of Direct Competitive ELISA. Assay optimization was carried out using deltamethrin as the competitor analyte. The effects of the ionic strength and the pH value were studied to improve the performances of the selected immunoassay format. The experiments were carried out using the direct procedure described above. In order to examine the influence of the ionic strength on the assay system, a series of PBS solutions (2.5%, 4.5%, 6.5%, and 8.5% NaCl) were prepared to dilute the pyrethroids. Competitive curves were also performed using assay buffers at different pH values (4.0, 5.0, 6.0, 7.0, 8.0 and 9.0). The criteria of immunoassay performance were evaluated using the value of IC_{50}.

Determination of Cross-Reactivities. To assess the specificity of the McAb, tests were made by using nine pyrethroids, including the

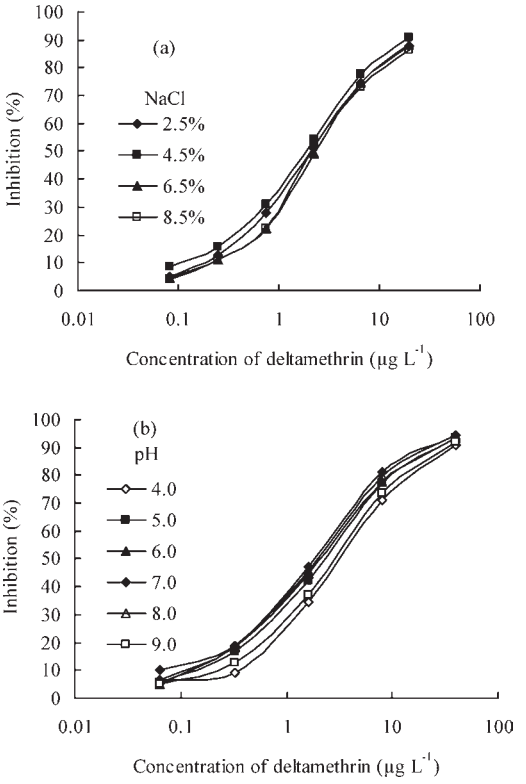


Figure 2. Effects of different ionic strength, pH on direct competitive ELISA of deltamethrin: (a) direct competitive ELISA curves of deltamethrin in buffers with various ionic strengths; (b) direct competitive ELISA curves of deltamethrin in buffers at various pH. All values are means of three replicates.

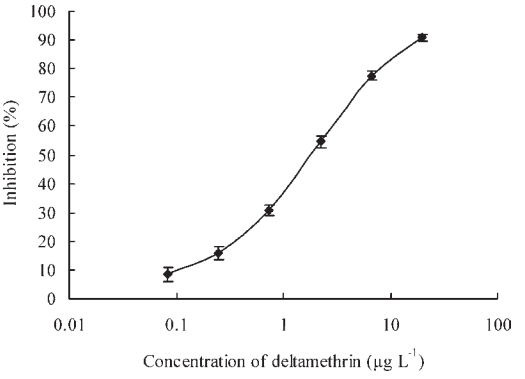


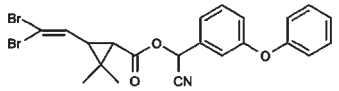
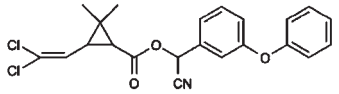
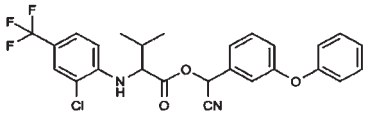
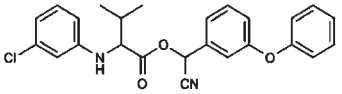
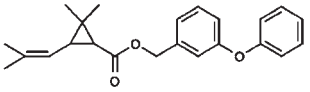
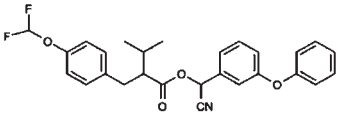
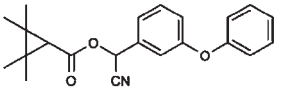
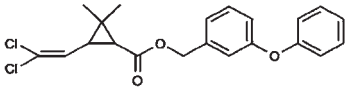
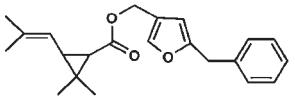
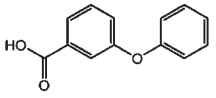
Figure 3. Standard inhibition curves of deltamethrin dissolved in PBS. The values are means of three replicates.

pyrethroids with the phenoxybenzene group, such as deltamethrin, cypermethrin, fluvalinate, fenvalerate, phenothrin, flucythrinate, fenpropathrin, and permethrin; the pyrethroids without phenoxybenzene group, such as resmethrin; and the metabolite analogue of deltamethrin, 3-phenoxybenzoic acid. The cross-reactivity (CR) was determined by the IC_{50} of deltamethrin assigned to be 100% comparing with the IC_{50} of other compounds. The CR values were calculated according to the following formula:

$$\text{cross-reactivity rate (\%)} = (IC_{50} \text{ of deltamethrin})/(IC_{50} \text{ of other compounds}) \times 100$$

GC/ECD Analysis of Pyrethroids. To evaluate the accuracy of the ELISA, a GC method was also performed. A Shimadzu GC-2010

Table 3. Cross-Reactivities of the Monoclonal Antibody with Various Pyrethroid Insecticides[a]

Analyte	Structure	IC_{50} (μg L^{-1})	CR (%)
Deltamethrin		1.8	100
Cypermethrin		1.5	120
Fluvalinate		2.0	90
Fenvalerate		2.0	90
Phenothrin		2.2	82
Flucythrinate		2.4	75
Fenpropathrin		3.0	60
Permethrin		5.0	36
Resmethrin		>1000	<1
3-phenoxybenzoic acid		0.8	225

[a] Cross-reactivity values were calculated as percentages of the IC_{50} of the standard deltamethrin to the IC_{50} of other compounds. All data are means of three replicates.

(Tokyo, Japan) gas chromatograph equipped with a GC/ECD detector was used for instrumental analysis of pyrethroids with external calibration. Chromatographic separation was analyzed using a low polarity fused silica capillary column DB-5 (30 m × 0.25 mm i.d. with 0.25 μm film thickness) obtained by Agilent and with the column temperature as follows: 75 °C which was maintained for 2 min, then programmed to 260 °C by the speed of 20 °C min^{-1}, held 40 min. The carrier gas was high-purity (over 99.999%) nitrogen at 1.2 mL min^{-1}.

Recovery of Pyrethroids in Spiked Water Sample. River water samples (Hai River, TianJin, China) used for recovery studies were previously verified as free of deltamethrin (cypermethrin, fluvalinate, fenvalerate, flucythrinate, and fenpropathrin) by GC/ECD.

For direct ELISA, the water samples were fortified with pyrethroids to give the final concentrations at 10, 20, and 30 μg L^{-1}. Spiked samples were filtered through a 0.22 μm nylon membrane filter to remove the particles. The samples were diluted with PBS and detected by the optimized ELISA.

For GC/ECD, the water samples were spiked with pyrethroids at three different concentrations (0.5, 1.0, and 1.5 mg L^{-1}). The spiked samples (50 mL) filtered through a 0.22 μm nylon membrane filter were extracted by 7.5 mL *n*-hexane, and then the extracts were filtered by a sodium sulfate anhydrous column. The filtrate was evaporated to 5 mL under a gentle N_2 stream, and then it was extracted by SPE (5 mm ×70 mm) which contained 500 mg of Florisil. SPE cartridges were first conditioned with 5 mL of *n*-hexane and 5 mL of acetone/*n*-hexane (1:9, V/V), and then extracts were applied, followed by eluting from the column with 5 mL acetone/*n*-hexane (1:9, V/V). The solutions were dried under a stream of nitrogen and then resuspended in 1 mL of methanol to analyze by GC/ECD.

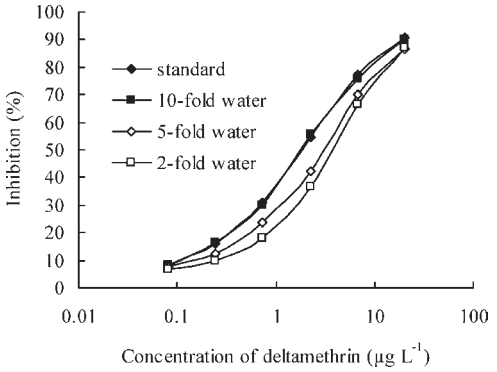


Figure 4. Effects of the dilution folds on the direct competitive ELISA of deltamethrin in water sample. Samples were diluted with PBS. All values are means of three replicates.

Table 4. Recoveries of Pyrethroids in Spiked Water Samples

	concn (μg L^{-1})			
analyte	spiked	theor[a]	mean recovery ± SD[b]	CV (%)
deltamethrin	10	1.0	87.7 ± 2.2	2.5
	20	2.0	84.7 ± 3.2	3.7
	30	3.0	78.0 ± 5.9	7.6
cypermethrin	10	1.0	90.0 ± 4.1	4.5
	20	2.0	81.6 ± 7.4	9.1
	30	3.0	107.8 ± 1.5	1.3
fluvalinate	10	1.0	84.2 ± 8.5	10.1
	20	2.0	89.1 ± 3.9	4.3
	30	3.0	102.8 ± 2.7	2.6
fenvalerate	10	1.0	79.0 ± 4.7	6.0
	20	2.0	90.8 ± 2.3	2.5
	30	3.0	84.8 ± 1.4	1.7
phenothrin	10	1.0	85.5 ± 1.8	2.0
	20	2.0	78.2 ± 2.5	3.2
	30	3.0	95.6 ± 2.8	2.9
flucythrinate	10	1.0	74.0 ± 4.6	6.2
	20	2.0	106.1 ± 3.7	3.5
	30	3.0	92.6 ± 5.0	5.4
fenpropathrin	10	1.0	87.2 ± 3.6	4.1
	20	2.0	101.1 ± 3.8	3.7
	30	3.0	96.7 ± 3.1	3.2
permethrin	10	1.0	80.3 ± 3.5	4.4
	20	2.0	84.7 ± 4.3	5.1
	30	3.0	92.1 ± 3.9	4.2

[a] Ten times dilution with PBS. [b] All data are means of three replicates.

RESULTS AND DISCUSSION

Production of Hybridomas and Cloning. Among the antisera collected from the mice previously injected, the one with the highest antibody titer was chosen for cell fusion. The supernatants from hybridoma cells were detected by indirect competitive ELISA for the presence of antibodies against pyrethroids. In all the wells tested, 29 wells gave an absorbency of over 3 and then were transferred to 24-well microculture plates in HT medium. Among the 29 wells, 20 wells exhibiting inhibition by deltamethrin at 1 μg mL^{-1} were transferred to cell culture bottle.

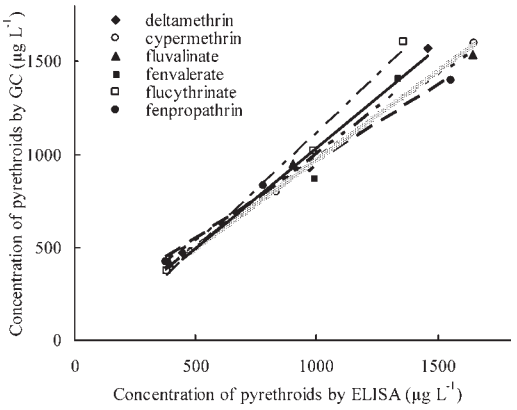


Figure 5. Correlation between ELISA and GC/ECD results for water samples spiked with pyrethroids. The values of each curve are means of three replicates .

Four positive wells which exhibited high inhibition in the presence of 1 μg mL^{-1} pyrethroids with phenoxybenzene group were chosen to be cloned by limiting dilution. Final clones were obtained from wells that had been seeded with 1 cell/well. Finally, after three times of cloning, we selected five stable hybridomas $2G_2E_7$, $14B_8G_4$, $17G_7C_3$, $17G_7F_3$ and $27C_8E_{11}$, which were used to produce ascites. IC_{50} values obtained by an indirect competitive ELISA were used to evaluate the sensitivity of the obtained antibodies, and the results are shown in Table 2. The hybridoma $2G_2E_7$ showed the lowest IC_{50} and had similar cross-reactivities among the eight pyrethroids. Therefore, the hybridoma $2G_2E_7$ was selected for ELISA optimization, and the cross-reactivities were determined. The McAb was of the IgG_1 isotype.

Optimization of dcELISA. The effects of pH and ionic strength on dcELISA performance were evaluated in this study. Figure 2a presents the effect of the salt concentration on dcELISA characteristics. A higher or lower salt concentration in the assay system resulted in higher IC_{50} values. The optimum concentration of NaCl was 4.5%. The pH effects on the dcELISA are shown in Figure 2b. These results showed that no significant effect upon the IC_{50} was detected when analyte was dissolved in buffer in the range of pH 5.0−8.0, but higher or lower pH values significantly increased the IC_{50} values, which was similar to the trends of pH effects on deltamethrin[18] and flucythrinate[30] ELISAs. The optimized ELISA used coating antibody at a dilution of 1:5000, enzyme tracer at a dilution of 1:9 × 10^5, pH 5.0−8.0, and PBS of 4.5% NaCl. The resulting IC_{50} values of the optimized immunoassay were 1.8 ± 0.30 μg L^{-1} for deltamethrin (Figure 3), 1.5 ± 0.42 μg L^{-1} for cypermethrin, 2.0 ± 0.58 μg L^{-1} for fluvalinate, 2.0 ± 0.35 μg L^{-1} fenvalerate, 2.2 ± 0.64 μg L^{-1} for phenothrin, 2.4 ± 0.40 μg L^{-1} for flucythrinate, 3.0 ± 0.45 μg L^{-1} for fenpropathrin, 5.0 ± 0.38 μg L^{-1} for permethrin, and 0.8 ± 0.32 μg L^{-1} for 3-phenoxybenzoic acid. The low limit of detection (LOD) of this assay was 0.24 ± 0.06 μg L^{-1} for deltamethrin, 0.10 ± 0.05 μg L^{-1} for cypermethrin, 0.21 ± 0.03 μg L^{-1} for fluvalinate, 0.20 ± 0.08 μg L^{-1} for fenvalerate, 0.20 ± 0.10 μg L^{-1} for phenothrin, 0.10 ± 0.04 μg L^{-1} for flucythrinate, 0.15 ± 0.04 μg L^{-1} for fenpropathrin, 0.52 ± 0.06 μg L^{-1} for permethrin, and 0.08 ± 0.06 μg L^{-1} for 3-phenoxybenzoic acid.

Cross-Reactivities. The cross-reactivity values with various pyrethroid pesticides and 3-phenoxybenzoic acid are summarized in Table 3. Most of the test pyrethroids and metabolite analogue

showed high cross-reactivities. With respect to chemical structure, the hapten PBA contained the characteristic molecule group of pyrethroids, phenoxybenzene group, and generated monoclonal antibodies could recognize this structure of pyrethroids well, but pyrethroids without phenoxybenzene group, such as resmethrin, showed no significant cross-reactivity. Therefore, the dcELISA could potentially be applied to simultaneously determine the pyrethroids and metabolite, which contained the characteristic molecule group, phenoxybenzene group.

Analysis of Spiked Water Samples. As there are many metal ions and other potential interferences in environmental water,[31] removing the matrix effects is important in the study of ELISA. The matrix effect can be evaluated by comparing a standard curve with a calibration curve generated in the sample matrix. If the two curves are superimposed, the standard curve can then be used to analyze the samples.[32] To remove the matrix effect, it is necessary to filter the samples and dilute those with PBS prior to analysis. The water sample was diluted 2-fold, 5-fold and 10-fold, and the results of Figure 4 indicate that the matrix effect could be eliminated with 10 times dilution with PBS .

Water samples were spiked with pyrethroid standards and analyzed by optimized ELISA. Three different concentrations of pyrethroids were spiked (10, 20, and 30 $\mu g\ L^{-1}$). Assay accuracy and precision were estimated by measuring three replicates. The results of precision and accuracy obtained from spiked water sample measured by ELISA were shown in Table 4. The recoveries of the developed ELISA ranged from 74 to 108%. Overall, the results proved that the assay was suitable for rapid screening and sensitive determination of pyrethroids with phenoxybenzene group simultaneously in water sample; moreover, we use a monoclonal antibody for which there are no similar reports. The detection limits of ELISA in water sample were 2.4 $\mu g\ L^{-1}$ for deltamethrin, 1.0 $\mu g\ L^{-1}$ for cypermethrin and flucythrinate, 2.1 $\mu g\ L^{-1}$ for fluvalinate, 2.0 $\mu g\ L^{-1}$ for fenvalerate and phenothrin, 1.5 $\mu g\ L^{-1}$ for fenpropathrin, and 5.2 $\mu g\ L^{-1}$ for permethrin.

Correlation between ELISA and GC/ECD Analysis. The river water samples spiked with pyrethroids (0.5, 1.0, and 1.5 mg L^{-1}) were analyzed by ELISA and GC/ECD. The linear regression equations between the ELISA and GC/ECD were $y = 1.1648x - 94.4210$, $R^2 = 0.9861$ for deltamethrin; $y = 0.9676x + 0.5219$, $R^2 = 0.9997$ for cypermethrin; $y = 0.8870x + 96.0900$, $R^2 = 0.9944$ for fluvalinate; $y = 0.9965x + 5.9638$, $R^2 = 0.9542$ for fenvalerate; $y = 1.2450x - 133.3700$, $R^2 = 0.9887$ for flucythrinate; $y = 0.8185x + 143.0400$, $R^2 = 0.9936$ for fenpropathrin. It showed that there was a good correlation between data obtained using the two methods (Figure 5), indicating the accuracy of this ELISA. Therefore, the method developed here was suitable for the detection of trace levels of pyrethroids with phenoxybenzene group residues in river water. This sensitive and broad class selective ELISA also can potentially be applied in monitoring pyrethroids with phenoxybenzene group residues in other environmental waters due to class-specific monoclonal antibodies for these pesticides.

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ABBREVIATIONS USED

BSA, bovine serum albumin; KLH, keyhole limpet hemocyanin; OVA, ovalbumin; ELISA, enzyme-linked immunosorbent assay; indirect ELISA, indirect competitive enzyme-linked immunosorbent assay; direct ELISA, direct competitive enzyme-linked immunosorbent assay; IgG-HRP, goat anti-mouse immunoglobulin G horseradish peroxidase conjugate; DMF, *N*,*N*-dimethylformamide; DMSO, dimethyl sufoxide; HAT, hypoxanthine, aminopterin, and thymidine; HT, hypoxanthine and thymidine; DMEM, dulbecco's modified eagle medium; PEG, polyethylene glycol; s-DMEM, DMEM including 20% fetal bovine serum; IC_{50}, concentration of analyte giving 50% inhibition of color development; IC_{15}, concentration of analyte giving 15% inhibition of color development; PBS, phosphate-buffered saline; PBST, phosphate-buffered saline with 0.05% Tween 20; TMB, 3, 3′, 5, 5′-tetramethylbenzidine; CR, cross-reactivity; SPE, solid phase extraction; GC/ECD, gas chromatography/electron capture detector

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