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Development of a Monoclonal Antibody-Based Enzyme Immunoassay for the Pyrethroid Insecticide Deltamethrin

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With a screening hemisolid stem-cell culture, four positive hybridomas $2B_{12}$, $2C_1$, $2F_1$, and $3D_4$ were screened and used to prepare four correspondent monoclonal antibodies (McAbs) against the pyrethroid insecticide deltamethrin. These McAbs showed I_{50} values in a range of 17−94 ng mL^{-1}, from among which the antibody $2B_{12}$ with the lowest I_{50} value was selected to develop an optimized enzyme-linked immunosorbent assay (ELISA). In the developed ELISA, the I_{50} of deltamethrin was 17.0 ± 3.3 ng mL^{-1} and the limit of detection (LOD) was 1.2 ± 1.3 ng mL^{-1}. There seemed to be little or no cross-reactivity with other tested pyrethroids and their metabolites. For validation of the assay method, environmental water samples fortified with deltamethrin were analyzed with the ELISA and gas chromatography (GC) methods. The recoveries of the developed ELISA ranged from 82 to 117%, which were close to those of the GC method (94−103%). These results suggested that the developed ELISA based on the McAb $2B_{12}$ could be used for the rapid and sensitive determination of deltamethrin in environmental water.

KEYWORDS: Pyrethroid insecticide; deltamethrin; hapten; monoclonal antibody; enzyme immunoassay (EIA)

INTRODUCTION

Deltamethrin is a synthetic pyrethroid that is effective against a broad range of pests. Uses for deltamethrin range from agricultural uses (*1*−*4*) to home pest control (*5*, *6*). Relatively, deltamethrin is more stable to degradation than alternative pyrethroids, and it has longer residual activity (*7*). Deltamethrin residues have been seen in agricultural products (*8*, *9*) and food (*10*), and the limit of detection (LOD) for the insecticide has been lowered by many countries. Because deltamethrin is a neurotoxin, it is also highly toxic to aquatic life, particularly fish (*11*, *12*). Therefore, it is of great importance to develop a rapid, sensitive, and specific method for monitoring deltamethrin residues to meet requirements of the lower maximum residue limit of below 0.03 mg L^{-1} for surface water (*13*).

Current analytical methods for deltamethrin involve multistep sample cleanup procedures followed by multiple detection techniques, including gas chromatography with an electron capture detector (GC-EC) (*14*, *15*), gas chromatography−mass spectrometry (GC-MS) (*16*, *17*), liquid chromatography combined with postcolumn photochemically induced fluorometry derivatization and fluorescence detection (HPLC-FD) (*18*, *19*), or high-performance liquid chromatography−mass spectrometry (HPLC-MS) (*20*). These methods, on the one hand, can meet the required sensitivity and accuracy of determination. On the other hand, the above methods are relatively intensive, time-consuming, and expensive and not particularly suitable for the analysis of large numbers of samples.

Immunoassays have been considered as a valuable supplement to existing, and rapidly developing, chromatographic techniques, because they have attractive features including high sensitivity and selectivity, rapid detection, and the possibility of analysis of difficult matrices without extensive pretreatment (*21*).

In the past 10 years, some immunoassays based on polyclonal antibodies for pyrethroid insecticides were developed for the detection of pyrethroid insecticides at trace levels (*22*−*26*). These immunoassays showed high sensitivity and powerful use for pyrethroid chemical residues in spiked lake water samples. Compared with polyclonal antibodies, monoclonal antibodies (McAbs) have merits of constant properties and unlimited production possibility (*27*). Therefore, monoclonal antibodies can easily meet the demands of industry production. Here we aimed to prepare a specific McAb against the pyrethroid insecticide deltamethrin with high sensitivity.

MATERIALS AND METHODS

Chemicals. One of the coating conjugates, hapten I−THY (thyroglobulin), was prepared by Lee et al. previously (*22*). The pyrethroid standards of permethrin, bifenthrin, tetramethrin, 3-phenoxybenzoic acid (PBA), fenvalerate, cypermethrin, and deltamethrin were obtained from Jiangsu Pesticide Research Institute (Nanjing, China). Organic starting materials for hapten synthesis were obtained from Chemphy Chemical Co., Ltd. (Shanghai, China). Thin-layer chromatography (TLC) was

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carried out on 0.2 mm precoated silica gel F254 (100−200 mesh, particle size) on glass sheets, and detection was made by ultraviolet (UV) light. Goat anti-mouse immunoglobulin G horseradish peroxidase conjugate (IgG-HRP), ovalbumin (OVA, MW45,000), bovine serum albumin (BSA, MW 67000), culture media RPMI-1640, hypoxanthine, aminopterin, and thymidine (HAT), and hypoxanthine and thymidine (HT), *N*-hydroxysuccinimide (NHS), *N*,*N*′-dicyclohexylcarbodiimide (DCC), 3,3′,5,5′-tetramethylbenzidine (TMB), and complete and incomplete Freund's adjuvants were all obtained from Sigma-Aldrich (USA). L-Glutamine (L-Gla) was obtained from Invitrogen (USA). Hybridoma Fusion and Cloning Supplement (HFCS) was obtained from Roche (Switzerland). Female BALB/c mice were purchased from the Centers for Disease Control and Prevention of Hubei province and tested to be *Pasteurella* negative before use. SP2/0 myeloma cells were purchased from the China Center for Type Culture Collection (CCTCC). Cell culture plates (6, 24, and 96 wells) were from Shanghai Sunub Bio-Tech Development, Inc. Culture flasks were obtained from Iwaki, Japan. The ELISA was carried out in 96-well polystyrene microplates (Costar, USA).

Instruments. ^{1}H NMR (600 MHz) spectra were recorded on a Varian Unity INOVA 600 spectrometer in CD_3COCD_3 using tetramethylsilane (TMS) as internal standard. Chemical shift values are given in parts per million (ppm) downfield from internal TMS. UV−visible spectra were recorded on a ZF1-II spectrophotometer (Jiapeng, China). As ELISA equipment, a Wellwash 4 Mk 2 microplate strip washer (Thermo Electron Corp.), a microplate reader (Wallac 1420 Victor 3, Perkin-Elmer Inc.), and a 12-channel pipet (50−300 mL, Thermo Labsystems Co. Ltd., Shanghai, China) were used. For validation of the developed assay, a standard method of gas chromatography (Agilent 6890, Agilent Technologies Inc.) was used to detect the spiked samples.

Synthesis of Haptens. Three haptens shown in **Figure 1** were obtained by introducing amido and amino acid spacers with different C-chain lengths in the H-phenyl site of the deltamethrin molecular structure. Compound **6** (hapten II) was prepared according to the method described by Lee (*22*). Syntheses of the compound **7** (hapten III) were carried out as outlined in **Figure 1**. All reactions were straightforward using well-known procedures. NMR spectral data supported all structures of target molecules.

Cyano[*3*-(*4-aminophenoxy*)*phenyl*]*methyl 1R-cis-3*-(*2,2-dibromoethenyl*)-*2,2-dimethycyclopropanecarboxylate* (*6*). Compound **6**, named hapten II here, was synthesized with the same laboratory steps as in ref *22*. In this procedure, the structure of deltamethrin with a nitro group (compound **5**, shown in **Figure 1**) was synthesized in three steps using compounds **1** and **3** (**Figure 1**) as materials. Then compound **5** was deoxidized by stannous chloride to synthesize the target compound **6**. Finally, 340 mg of compound **6** purified with Lee's method (*22*) was obtained as a pale yellow oil: ^{1}H NMR (600 MHz, CD_3COCD_3, TMS) δ 1.23 (s, 6H, CH_3), 3.16 (d, J = 7.8 Hz, 1H, CHC═C), 3.53 (d, J = 6.6 Hz, 1H, CHCO), 6.28 (s, 1H, CHCN), 6.35 (s, 1H, C═CH), 6.72−7.26 (m, 8H, Ar−H).

4-(*4*-(*3*-(*Cyano*(*3*-(*2,2-dibromovinyl*)-*2,2-dimethylcyclopropanecarbonyloxy*)*methyl*)*phenoxy*)*phenylamino*)-*4-oxobutanoic Acid* (*7*). Compound **7**, named hapten III here, was synthesized as follows: A solution of **6** (73 mg, 0.14 mmol) in dichloromethane (5 mL) was stirred in a flask, and then succinic anhydride (22 mg, 0.22 mmol) was added. The mixture was stirred at room temperature (10 °C) overnight. The combined organics were extracted twice with dichloromethane. The organic phase was dried ($MgSO_4$) and stripped to a yellow oil. This oil was purified on silica gel (10 g) (dichloromethane/ethyl acetate = 1:1). Stripping fractions containing pure product gave 80 mg (92%) of **7** as a pale yellow oil: ^{1}H NMR (600 MHz, CD_3COCD_3, TMS) δ 1.23 (s, 6H, CH_3), 3.16 (d, J = 7.8 Hz, 1H, CHC═C), 3.53 (d, J = 6.6 Hz, 1H, CHCO), 6.28 (s, 1H, CHCN), 6.35 (s, 1H, C═CH), 6.72−7.26 (m, 8H, Ar−H), 2.586−2.682 (m, 2H, CH_2-NHCO), 2.896−3.067 (m, 2H, CH_2COOH).

Preparation of Hapten−Protein Conjugates. Conjugates were synthesized using a water-soluble carbodiimide method for acids and a diazotization method for anilines (*28*, *29*). To obtain the immunogens, haptens II and III were conjugated to BSA. Coating antigens were made by coupling haptens II and III to OVA.

Both immunogens (hapten−BSA conjugates) and coating antigens (hapten−OVA conjugates) were prepared by using the diazotization method. Hapten II was dissolved in 0.2 mL of ethanol and treated with 0.6 mL of 1 N HCl. The resulting solution was stirred in an ice bath as

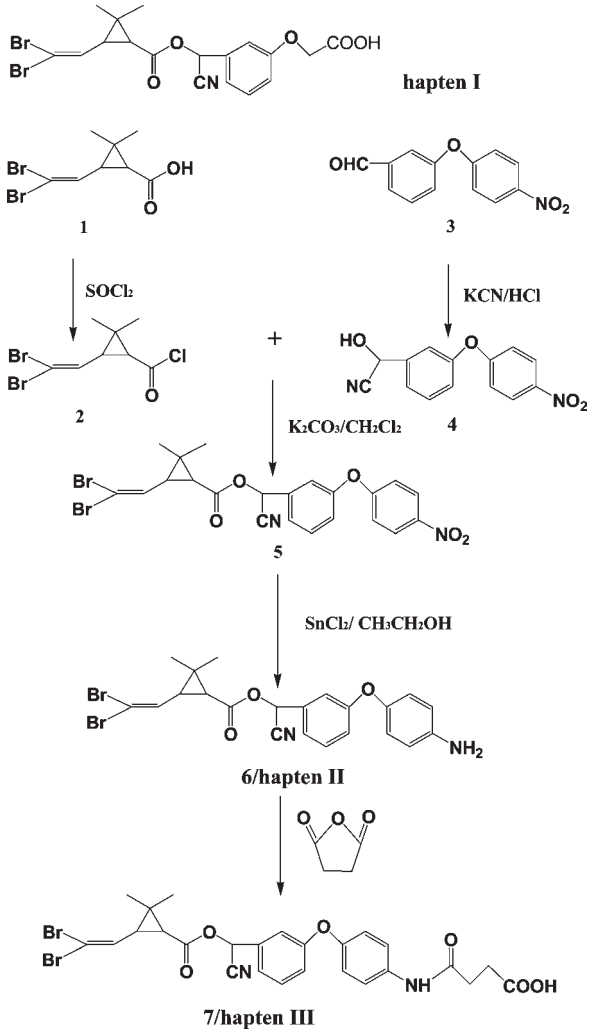


Figure 1. Reaction schemes of synthesis for haptens of deltamethrin.

0.4 mL of 2 M sodium nitrite was added; dimethylformamide (DMF, 0.4 mL) was then added slowly to give a homogeneous solution. Forty milligrams of OVA or BSA was dissolved in a mixture of 5 mL of 0.2 M carbonic acid buffer (pH 9.6) and 1.2 mL of DMF. The activated hapten solution was added slowly to the two stirred protein solutions. The reaction mixtures were stirred in an ice bath overnight and then dialyzed against phosphate-buffered saline (PBS) over 72 h at 4 °C. The purified conjugates were suspended in water and stored in aliquots at −20 °C.

The conjugations of hapten III (compound **7**) and the estimations of hapten densities were carried out using the procedure described by Zhang (*30*). To generate the conjugate, hapten III was covalently attached through its carboxylic acid moiety to the lysine groups of BSA or OVA using the active ester method (DCC/NHS). The immunogens and coating antigens were purified by dialysis in phosphate buffer (PB: 0.02 mol L^{-1}, pH 6.8). The conjugates were stored at −20 °C until use. UV−vis spectral data were used to confirm the structures of the final conjugates following the previous method (*30*). Assuming that the molar absorptivity of haptens was the same for the free and conjugated forms, the hapten densities (the number of hapten molecules per molecule of protein) of the conjugates were estimated directly by the mole absorbance ε:

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

Finally, the hapten density of hapten II−BSA was determined as 11, that of hapten II−OVA as 8, that of hapten III−BSA as 12, and that of hapten III−OVA as 6.

Immunization. The antibodies for deltamethrin were prepared analogously to the method of Garrett et al. (*31*). BALB/c female mice (6−10 weeks old) were immunized subcutaneously with BSA−hapten II conjugate (150 μg) in physiological saline and complete Freund's adjuvant

1:1 (v/v) as an initial injection. The BSA−hapten III and THY−hapten I immunizations were the same as the BSA−hapten II conjugate. Three subsequent injections were given intradermally in incomplete Freund's adjuvant after the first immunization on days 28, 49, and 91, respectively. Ten days after the last injection, mice were tail bled to check for antibody titer by indirect ELISA and for analyte recognition properties by competitive indirect ELISA. Mice selected to be spleen donors for hybridoma production received a final soluble intraperitoneal injection with the same amounts of antigen in physiological saline (without adjuvant), 3 days prior to cell fusion.

Cell Fusion and Cloning. The spleen of a mouse of interest was removed aseptically, and splenocytes were fused with cells of the Sp2/0 myeloma cell line at a ratio of 1:10 in the presence of polyethylene glycol (PEG), molecular weight 1450 (Sigma-Aldrich, St. Louis, MO) according to the method described elsewhere (*32*). After fusion, cells were suspended in semisolid complete medium containing 20% fetal bovine serum (FBS) (Gibco catalog no. 263000-061), methylcellulose, HFCS, and hypoxanthine, aminopterin and thymidine medium (Gibco catalog no. 31062-011), and then the cell suspension was equally distributed into nine 6-well plates (*33*). After 14 days, culture supernatants from each well were assayed using indirect competitive ELISA (see below). Culture supernatants from the cells that gave an absorption value of >3 were transferred to 24-well microculture plates in complete RPMI-1640 medium containing 20% FBS, hypoxanthine, and thymidine. Supernatants from 24-well culture plates were rescreened by both indirect ELISAs and indirect competitive ELISAs (see below). Only those hybridomas that maintained absorption values, had no cross-reactivity with OVA, and had good reactivity with deltamethrin were chosen for further selection.

To ensure monoclonality, cells from each well of interest were subcloned by semisolid complete medium in 6-well culture plates. The plates were examined for the number of hybridomas. Those that contained some spots singularly distributing in each well were retained and were screened for neutralization titers. Stable antibody-producing clones were expanded and cryopreserved in freezing solution according to the freezing protocol: 30 min at 4 °C, 1 h at −20 °C, overnight in gas of liquid nitrogen jar, and then storage in liquid nitrogen. After titer testing with indirect ELISA of the mice ascites, the McAbs were separated according to the method of salting out (with caprylic acid−ammonium sulfate) as described by Zhu et al. (*34*).

Noncompetitive and Competitive Indirect ELISAs. The method was identical to that reported by Shan (*23*) with the following exceptions. The microtiter plates were coated overnight at 4 °C with 100 μL/well of the hapten−protein conjugate. The coated plates were washed with PBST (PBS plus Tween, 20.8 g L^{-1} NaCl, 1.15 g L^{-1} Na_2HPO_4, 0.2 g L^{-1} KH_2PO_4, 0.2 g L^{-1} KCl, and 0.05% Tween, v/v). The surface of the wells was blocked by adding a solution of OVA (1% in PBST, 200 μL/well) and incubating for 2 h at 37 °C. The 100 μL/well of antiserum diluted in PBST (for determination of antibody titer) or 50 μL/well of antiserum diluted in PBST and 50 μL/well of standard solution were dispensed into the wells incubated for 1 h at 37 °C, and the incubation step with goat anti-mouse−IgG−HRP conjugate was 100 μL/well incubated for 1 h at 37 °C; the substrate solution system was composed of 9.5 mL of pH 5.0 phosphate−citrate buffer, 0.5 mL of 2 mg mL^{-1} TMB (dissolved by ethanol), and 32 μL of 3% (w/v) urea hydrogen peroxide was pipeted into each well with 100 μL incubated at 37 °C for 15 min; the enzyme-catalyzed reaction was stopped after 15 min of incubation at 37 °C by the addition of 50 μL/well of stop solution. The absorbance was measured using a dual-wavelength mode at 450 nm.

In the competitive indirect ELISA procedure, just the specific antibody−antigen reaction step was different from noncompetitive ELISA, because analytes were added in the reaction solution.

Affinity, Sensitivity, and Cross-Reactivity. Titers of all antibody/antigen combinations were determined by measuring the binding of serial dilutions of the antibodies to the corresponding coating antigen with a noncompetitive ELISA. In the competitive indirect ELISA procedure with different concentrations of standard deltamethrin, I_{50} values (the concentration at which the binding of the antibody to the coating antigen is inhibited by 50%) of the analyte were determined. The cross-reactivity was studied by using the standard solution of the analytes and other structurally related compounds, such as permethrin, bifenthrin, tetramethrin, PBA, cypermethrin, and fenvalerate. Each compound was prepared in 20% methanol in PBS and tested over the range of 5−50000 ng mL^{-1}. The cross-reactivity (CR) was calculated as a ratio of the I_{50} of deltamethrin (assigned as 100%) to that of the test compound.

Optimization of a CI-ELISA. The screening of the variables to set up competitive ELISA procedures was performed in conjugate- and antibody-coated formats. Assay optimization was performed using deltamethrin as the competitor analyte. A set of experimental parameters (organic solvent concentration, blocking agents, and buffer) was studied sequentially to improve the sensitivity of the immunoassay. The main criteria used to evaluate immunoassay performance were I_{50}.

With the optimized working concentration of antibody and antigen, the influence of blocking reagent (1% OVA and 5% skimmed milk powder) was investigated, the effect of organic sovent (10, 20, 40, 60, and 80% of methanol, v/v) on immunoassay performance was studied, and the effect of reaction buffer (PBS and PBST) was determined.

According to the results, the optimized ELISA conditions were determined, and then under the best parameters, competitive indirect ELISA in serial concentrations of deltamethrin standard (0.001−100000 ng mL^{-1}) was repeated five times at different times to work out a quantitive standard curve for deltamethrin and evaluate the precision and sensitivity.

Analysis of Spiked Samples. To study the spike recovery, environmental water from Shahu Lake (Wuhan, Hubei) was used here. The environmental water was cleaned by filter paper, diluted five times with PBS containing 20% methanol, and spiked with deltamethrin standards (10, 100, and 1000 ng mL^{-1}). Then these spiked samples were detected by the developed ELISA. A GC method (*35*) was also performed to evaluate the accuracy of the ELISA, in which a solid phase extraction column (Florisil) was used for sample cleanup, and the analyte was separated by quartz capillary column at a programmed temperature (150 °C for 2 min, 6 °C/min, 270 °C for 23 min) and was determined by electron capture detector at 320 °C.

RESULTS AND DISCUSSION

Synthesis of Haptens and Their Conjugates. Deltamethrin, like most pesticides, is a small and simple organic molecule. It is nonimmunogenic by itself and lacks a functional group for coupling to proteins. Therefore, the primary goal of this study was to synthesize haptens to yield a specific immunoassay for deltamethrin. At the same time, a suitable hapten and its artificial antigen for immunization should preserve the structure of the target compound as much as possible. Because deltamethrin is highly lipophilic, a long side chain (with a 3- or 4-carbon spacer arm) may allow the lipophilic hapten to fold into the hydrophobic interior of the protein and decrease the affinity of the resulting antibodies. Accordingly, we obtained three immunizing haptens, namely, haptens I, II, and III, by the introduction of spacer arms of different lengths through the succinic anhydride group characteristic of this pesticide.

With regard to coating hapten strucutre, there are two kinds of ELISA systems: a homologous system (the coating hapten has the same structure as the immunizing hapten) and a heterologous system (the coating hapten has a structure different from the immunizing hapten). As is well-known, an excellent immunoassay may be developed using homologous haptens, especially with McAbs, but the synthesis of heterologous haptens is usually a very valuble approach to improve the sensitivity of immunoassays for pesticides. To synthesize this kind of hapten, the hapten II and hapten I synthesis method previously described by Lee (*22*) was used and here synthesized as a new compound **7**, which was named hapten III. From the data of ^{1}H NMR shown under Synthesis of Haptens, the products were confirmed.

Positive Monoclonal Hybridomas from the Screening Semisolid Medium. The screening semisolid medium was used for cell fusion because the conventional selection and cloning of hybridomas has a number of disadvantages in liquid medium (*36*, *37*): First, there is often a mixture of faster-growing cells and slower-growing cells

Table 1. Titer Values of Antisera (Absorbance at 450 nm)[a]

immunogen	antibody (1:200000)	antibody isotype	coating antigen (2.0 μg mL^{-1})		
			hapten I−THY	hapten II−OVA	hapten III−OVA
hapten II−BSA	$2B_{12}$	IgG_1	0.12 ± 0.01	1.22 ± 0.06	0.51 ± 0.04
	$2C_1$	IgG_{2b}	0.09 ± 0.01	0.94 ± 0.04	0.38 ± 0.04
	$2F_1$	IgG_1	0.07 ± 0.00	0.67 ± 0.03	0.20 ± 0.01
	$3D_4$	IgG_1	0.11 ± 0.01	0.90 ± 0.04	0.28 ± 0.02

[a] Absorbances were measured by a checkerboard pattern, with several coating conjugate concentrations and several antibody dilutions, and measured after a 15 min incubation with TMB at 37 °C. For convenience, only data from a coating antigen concentration of 2.0 μg per well and an antibody dilution of 1:200000 are shown. Titer values are the means of four replicates.

Table 2. ELISA Sensitivity (I_{50}) for Different Combinations of Antibodies and Coating Antigens (Nanograms per Milliliter)[a]

antibody	ascites dilution	coating conjugate	
		hapten II−OVA	hpaten III−OVA
$2B_{12}$	1:250000	17.0	N[b]
$2B_{12}$	1:80000	N	60.1
$2C_1$	1:200000	45.6	N
$2F_1$	1:100000	94.2	N
$3D_4$	1:200000	89.6	N

[a] The average coefficient of variation (CV) was 6.4%, and all were below 10%. [b] N, no detection due to very low titer.

together in a single culture, and the culture eventually becomes dominated by the faster-growing cells; second, nonproducing hybridoma cells often grow faster than antibody-producing clones, and this can result in a failure to obtain the desired hybridomas as the nonproducing hybridomas constitute the majority of clones screened. The semisolid medium contains growth factors and medium supplements optimized to support the selection and growth of hybridoma clones, which includes HAT for the selection of hybridomas. As described in Davis (*38*), growing clones in semisolid media eliminates the potential for overgrowth of antibody-producing clones by nonproducers and allows the clone progeny of a single cell to stay together. The clones are therefore monoclonal from the start, eliminating the need to perform cloning by limiting dilution. Under this method, clone cells reduce the overall time necessary to produce McAbs by up to 26 days and decrease the technical work hours required by ~30%.

Through noncompetitive indirect ELISA and competitive indirect ELISA screening procedures, finally, four monoclonal hybridomas (**Table 1**) producing antibodies against deltamethrin were found and selected to prepare ascites antibody for further study.

Affinity, Sensitivity, and Cross-Reactivity. To develop a sensitive immunoassay, sufficient titer value is necessary for coating hapten and antibody. Here titers of 12 combinations between 4 monoclonal antibodies and 3 coating conjugates were determined and are shown in **Table 1**. The antibodies against hapten I could almost be seen to have no reaction with hapten I−THY. The combinations with high affinity were selected for determination of sensitivity.

Values of I_{50} in the ELISA system were used to evaluate the sensitivity of the obtained antibodies, and the results are shown in **Table 2**. The homologous system of $2B_{12}$/II−OVA showed the lowest I_{50} (17.0 ng mL^{-1}), which was at least ~3 times better than that of the heterologous system of $2B_{12}$/7−OVA (60.1 ng mL^{-1}). As in previous studies on homology and heterology of the ELISA system (*37*, *39*), some papers stated that a heterologous ELISA system would improve the sensitivity of McAbs (*22*, *24*, *39*), but it did not happen every time (*23*, *40*). The result here perhaps resulted from no moderate difference between the structure of the coating hapten (hapten III) and that of the immunizing hapten (hapten II). Therefore, the combination of $2B_{12}$/II−OVA was selected for ELISA optimization, and the cross-reactivities were determined.

Table 3 demonstrates the cross-reactivity of the optimized ELISA system with different pyrethroids. This assay system had less cross-reactivity with type II pyrethroids such as cypermethrin (25.95%), followed by fenvalerate (16.95%). No cross-reactivity was measured for type I pyrethroids such as permethrin, bifenthrin, and tetramethrin or the metabolite PBA in this system. These results suggests that binding to this antibody is inhibited by the non-cyano group, so that no cross-reactivity was measured with type I pyrethroids without a cyano group.

Establishment of an Optimized CI-ELISA. Considering the highest sensitivity of the combination of $2B_{12}$/II−OVA, the CI-ELISA of $2B_{12}$/II−OVA was optimized to develop an assay.

The results of **Figure 2** show that the I_{50} of deltamethrin when using OVA as blocking reagent was 47.1 ng mL^{-1}, less than one-third that of skimmed milk powder (147.8 ng mL^{-1}). For solvent optimization, we tested different concentrations of methanol because it is usually used as cosolvent in immunoassays to improve analyte solubility. As observed in **Figure 3**, the lowest I_{50} was found at 20% methanol (33.5 ng mL^{-1}). As for the effect of reaction buffer, PBS and PBST were tested (**Figure 4**). Finally, PBS in the lower I_{50} (17.0 ng mL^{-1}) was selected.

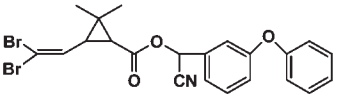
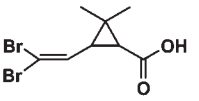
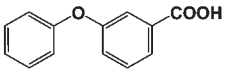
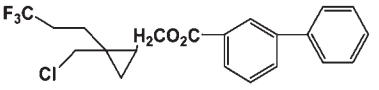
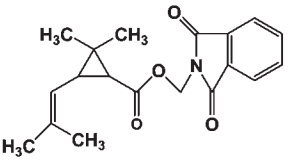
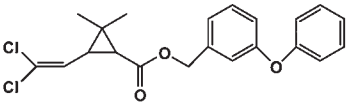
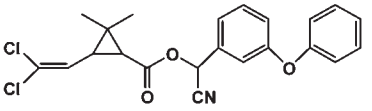
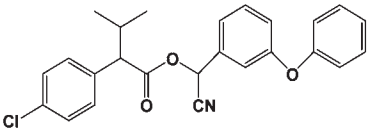
With the above optimized conditions, a typical standard curve of the deltamethrin immunoassay is given in **Figure 5**. The average I_{50} for deltamethrin was 17.0 ± 3.3 ng mL^{-1}, and the LOD was 1.2 ± 1.3 ng mL^{-1}. The sensitivity here is higher than that reported with a McAb-based assay (I_{50} for deltamethrin was 500 ng mL^{-1}) (*41*), close to the pAb-based assay (I_{50} for deltamethrin was 17.5 ± 3.6 ng mL^{-1}) by Lee et al. (*22*), and a little lower than Lee's (I_{50} for deltamethrin was 2 ng mL^{-1}) (*25*).

Analysis of Spiked Samples. Recovery of deltamethrin in water was determined with a competitive ELISA method. To study the spike recovery, river water from Shahu Lake (Wuhan, HuBei) was spiked with three different concentrations of deltamethrin (10, 100, 1000 ng mL^{-1}). Because there are many metal ions and other potential interferences in environmental water (*39*), it is necessary to filter the samples and dilute those with PBS prior to analysis. **Table 4** shows the recovery of deltamethrin from environmental water. The recoveries of the developed ELISA ranged from 82 to 117% (89 ± 2.5% for 10 ng mL^{-1}, 93.9 ± 1.5% for 100 ng mL^{-1}, and 107.9 ± 6.3% for 1000 ng mL^{-1}). These recoveries are close to those of the GC method, which showed a range of 94−103%. The results demonstrate that the McAb-based assay developed here can be suitable for the detection of trace levels of deltamethrin in environmental water.

ABBREVIATIONS USED

BSA, bovine serum albumin; CI-ELISA, competitive inhibition enzyme-linked immunosorbent assay, ELISA, enzyme-linked

Table 3. Cross-Reactivity of the Monoclonal Antibody against Different Pyrethroid Insecticides[a]

Analyte	Structure	I_{50} ng mL^{-1}	Cross-reactivity %
Deltamethrin		17.0	100
Deltamethric acid		>1700	<1
PBA		>1700	<1
Bifenthrin		>1700	<1
Tetramethrin		>1700	<1
Permethrin		>1700	<1
Cypermethrin		65.5	25.95
Fenvalerate		100.3	16.95

[a] ELISA system 2B$_{12}$/II−OVA was used in the cross-reactivity studies. Deltamethrin was assigned as 100%. Percentage of cross-reactivity = (I_{50} of deltamethrin/I_{50} of other compound) × 100.

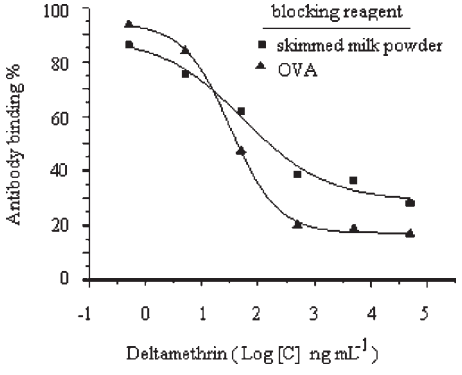


Figure 2. Effect of blocking reagent on ELISA curve. I_{50} values of OVA and skimmed milk powder are 47.1 and 147.8 ng mL^{-1}, respectively.

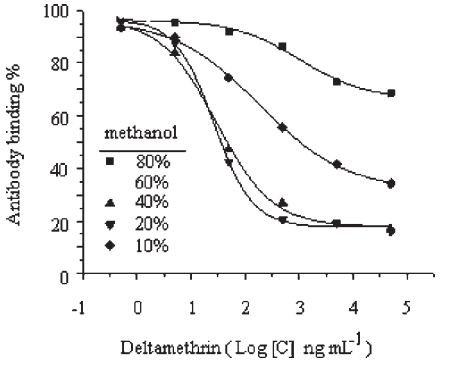


Figure 3. Effect of concentration of cosovlent methanol on ELISA curve. I_{50} values are worked out as the following: 5941.5 ng mL^{-1} (60%), 1085.1 ng mL^{-1} (10%), 33.5 ng mL^{-1} (20%), and 41.8 ng mL^{-1} (40%).

immunosorbent assay, EIA, enzyme immunoassay; CR, cross-reactivity; DCC, *N*,*N*′-dicyclohexylcarbodiimide; DMF, dimethylformamide; FBS, fetal bovine serum; GC, gas chromatography, GC-EC, GC with an electron capture detector, GC-MS, GC−mass spectrometry; HFCS, Hybridoma Fusion and Cloning Supplement; HPLC-FD, high-performance liquid chromatography−fluorescence detection, HPLC-MS, high-performance

Table 4. Recovery Test of Deltamethrin in Environmental Water for Validation

		ELISA			GC		
spiked[a] (ng mL^{-1})	theoretical[b] (ng mL^{-1})	measured (ng mL^{-1})	recovery[c] (%)	mean ± SD	measured (ng mL^{-1})	recovery[c] (%)	mean ± SD
50	10	8.20	82.0	89.0 ± 2.5	9.4	94.08	95.6 ± 1.6
		10.04	100.4		9.7	97.2	
		8.49	84.9		9.6	95.5	
500	100	92.72	92.72	93.9 ± 1.5	98	98.16	98.2 ± 3.2
		92.15	92.15		95	95.03	
		96.3	96.3		101	101.47	
5000	1000	1067.94	106.79	107.9 ± 6.3	1032	103.19	102.1 ± 1.9
		1173.42	117.34		1032	103.25	
		996.09	99.61		999.4	99.94	

[a] Environmental water was spiked with an appropriate concentration of deltamethrin. Three spiked samples were used in each study. [b] Five times dilution with 20% methanolic PBS. [c] Percent recovery was calculated as the measured spiked concentration of deltamethrin divided by the theoretical spiked concentration of deltamethrin × 100.

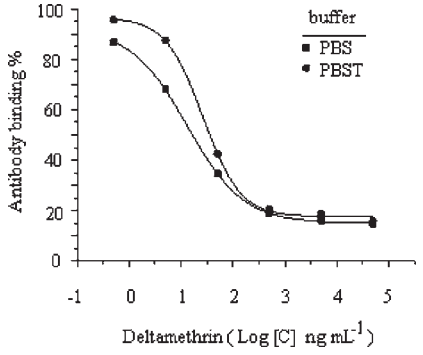


Figure 4. Effect of reaction buffer on ELISA curve. I_{50} values of PBS and PBST are 17.0 and 33.5 ng mL^{-1}, respectively.

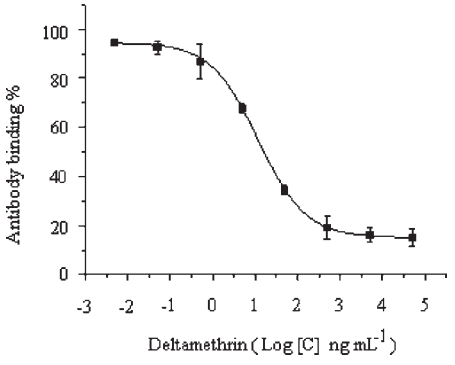


Figure 5. ELISA competition curves of deltamethrin. Hapten II−OVA was coated at 2 μg mL^{-1}, ascites $2B_{12}$ was diluted as 1:250000 (final concentration in wells), and goat anti-mouse IgG HRP was diluted as 1:20000. Standard curve represents the average of five repeats.

liquid chromatography−mass spectrometry; HT, hypoxanthine and thymidine, HAT, HA, and thymidine; I_{50}, concentration at which the binding of the antibody to the coating antigen is inhibited by 50%; IgG−HRP, goat anti-mouse immunoglobulin G horseradish peroxidase conjugate; L-Gla, L-glutamine; LOD, limit of detection; McAb, monoclonal antibody; pAb, polyclonal antibody; NHS, N-hydroxysuccinimide; OVA, ovalbumin; PB, phosphate buffer, PBS, phosphate-buffered saline, PBST, phosphate-buffered saline with Tween-20; PBA, phenoxyphenylmethyl acid; PEG, polyethylene glycol; THY, thyroglobulin; TLC, thin-layer chromatography; TMB, 3,3′,5,5′-tetramethylbenzidine; TMS, tetramethylsilane; UV, ultraviolet.

LITERATURE CITED

(1) Fabellar, L. T.; Heinrichs, E. A. Relative toxicity of insecticides to rice planthoppers and leafhoppers and their predators. *Crop Prot.* **1986**, *5*, 254–258.
(2) Li, Y.; Luo, W.; Zhao, S. Effects of cruciferous hosts on susceptibility of deltamethrin and cypermethrin in the diamondback moth, *Plutella xylostella* (L.) (lepidoptera: plutellidae). *J. Shandong Agric. Univ.* **1996**, *27*, 269–274.
(3) Chen, S.; Li, F.; He, J.; Chen, X.; Wang, D.; Wei, L.; Yang, H.; Guan, L. Experimental study on prevention of dog—sandfly contact by deltamethrin collar. *Endemic Dis. Bull.* **2001**, *16*, 17–19.
(4) Arthur, F. H. Evaluation of a new insecticide formulation (F2) as a protectant of stored wheat, maize, and rice. *J. Stored Prod. Res.* **2004**, *40*, 317–330.
(5) Shao, X.; Huang, Q.; Zhou, G. Study on the pest control with 2.5% control release micro-emulsion of deltamethrin. *Chin. J. Vector Biol. Control* **2001**, *12*, 53–55.
(6) Zhang, Y.; Meng, F.; Liu, Q.; Xu, F. The susceptibility of female adult house fly (*Musca domestica*) at different closion days to deltamethrin. *Chin. J. Vector. Biol. Control* **2005**, *16*, 95–97.
(7) Bengston, M.; Davises, R. A. H.; Desmarchelier, J. M.; Henning, R.; Murray, W.; Simpson, B. W.; Snelson, J. T.; Sticka, R.; Wallbank, B. E. Organophosphorothioates and synergised synthetic pyrethroids as grain protectants on bulk wheat. *Pestic. Sci.* **1983**, *14*, 373–384.
(8) Sharma, J.; Satya, S.; Kumar, V.; Tewary, D. K. Dissipation of pesticides during bread-making. *J. Chem. Health Saf.* **2005**, *12*, 17–22.
(9) Jimenez, J. J.; Bernal, J. L.; del Nozal, M. J.; Bernal, J.; Toribio, L. Persistence and degradation of metalaxyl, lindane, fenvalerate and deltamethrin during the wine making process. *Food Chem.* **2007**, *104*, 216–223.
(10) Bouwman, H.; Sereda, B.; Meinhardt, H. M. Simultaneous presence of DDT and pyrethroid residues in human breast milk from a malaria endemic area in South Africa. *Environ. Pollut.* **2006**, *144*, 902–917.
(11) Erstfeld, K. M. Environmental fate of synthetic pyrethroids during spray drift and field runoff treatments in aquatic microcosms. *Chemosphere* **1999**, *39*, 1737–1769.
(12) Velisek, J.; Jurcikova, J.; Dobsikova, R.; Svobodova, Z.; Piackova, V.; Machova, J.; Novotny, L. Effects of deltamethrin on rainbow trout (*Oncorhynchus mykiss*). *Environ. Toxicol. Pharmacol.* **2007**, *23*, 297–301.
(13) Zhang, Z.; Dai, X.; Zhou, D. The study on the method of establishment of hygienic standard of deltamethrine in surface water. *J. Environ. Health* **1998**, *15*, 49–54.
(14) Akhtar, M. H. Gas chromatographic determination of deltamethrin in biological samples. *J. Chromatogr., A* **1982**, *246*, 81–87.
(15) Ding, H.; Bao, X.; Zheng, Z. Determination of 7 pyrethroid residues in tea. *J. Instrum. Anal.* **2000**, *19*, 31–34.
(16) Li, Y.; Huang, Z.; Dai, H.; Zhang, Y. Determination of pyrethroids pesticides multi-residues in tea by gas chromatography/mass spectrometry. *Chin. J. Anal. Chem.* **2002**, *30*, 865–868.

(17) Esteve-Turrillas, F. A.; Pastor, A.; de la Guardia, M. Determination of pyrethroid insecticide residues in vegetable oils by using combined solid-phases extraction and tandem mass spectrometry detection. *Anal. Chim. Acta* **2005**, *553*, 50–57.
(18) Lopez-Lopez, T.; Gil-Garcia, M. D.; Martinez-Vidal, J. L.; Martinez-Galera, M. Determination of pyrethroids in vegetables by HPLC using continuous on-line post-elution photoirradiation with fluorescence detection. *Anal. Chim. Acta* **2001**, *447*, 101–111.
(19) Vazquez, P. P.; Mughari, A. R.; Martinez, G. M. Solid-phase microextraction (SPME) for the determination of pyrethroids in cucumber and watermelon using liquid chromatography combined with post-column photochemically induced fluorimetry derivatization and fluorescence detection. *Anal. Chim. Acta* **2008**, *607*, 74–82.
(20) Ferrer, C.; Gomez, M. J.; Garcia-Reyes, J. F.; Ferrer, I.; Thurman, E. M.; Fernandez-Alba, A. R. Determination of pesticide residues in olives and olive oil by matrix solid-phase dispersion followed by gas chromatography/mass spectrometry and liquid chromatography/tandem mass spectrometry. *J. Chromatogr., A* **2005**, *1069*, 183–194.
(21) Knopp, D. Immunoassay development for environmental analysis. *Anal. Bioanal. Chem.* **2006**, *385*, 425–427.
(22) Lee, H. J.; Shan, G. M.; 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.
(23) Shan, G. M.; Stoutamire, D. W.; Wengatz, I.; Gee, S. J.; Hammock, B. D. Development of an immunoassay for the pyrethroid insecticide esfenvalerate. *J. Agric. Food Chem.* **1999**, *47*, 2145–2155.
(24) 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.
(25) Lee, N.; McAdam, D. P.; Skerritt, J. H. Development 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.
(26) Wengatz, I.; Stoutamire, D. W.; Gee, S. J.; Hammock, B. D. Development of an enzyme-linked immunosorbent assay for the detection of pyrithroid insecticide fenpropathrin. *J. Agric. Food Chem.* **1998**, *46*, 2211–2221.
(27) Dankwardt, A. Determination of pesticides by enzyme immunoassay. *Encyclopedia of Analytical Chemistry*; Wiley: Chichester, U.K., 2006.
(28) Tijssen, P. *Practice and Theory of Enzyme Immunoassay*; Elsevier: Amsterdam, The Netherlands, 1985.
(29) Erlanger, B. F. Principles and methods for the preparation of drug protein conjugates for immunological studies. *Pharmacol. Rev.* **1973**, *25*, 271–280.
(30) Zhang, Q.; Wang, L.; Ahn, K. C.; Sun, Q.; Hu, B.; Wang, J.; Liu, F. Hapten heterology for a specific and sensitive indirect enzyme-linked immunosorbent assay for organophosphorus insecticide fenthion. *Anal. Chim. Acta* **2007**, *596*, 303–311.
(31) Garrett, S. D.; Appleford, D. J. A.; Wyatt, G. M.; Lee, H. A.; Morgan, M. R. A. Production of a recombinant anti-parathion antibody (ScFv): stability in methanolic food extracts and comparison to an anti-parathion monoclonal antibody. *J. Agric. Food Chem.* **1997**, *45*, 4183–4189.
(32) Yu, K. O. A.; Im, J. S.; Illarionov, P. A.; Ndonye, R. M.; Howell, A. R.; Besra, G. S.; Porcelli, S. A. Production and characterization of monoclonal antibodies against complexes of the NKT cell ligand α-galactosylceramide bound to mouse CD1d. *J. Immunol. Methods* **2007**, *323*, 11–23.
(33) Wang, J. S.; Abubaker, S.; He, X.; Sun, G.; Strickland, P. T.; Groopman, J. D. Development of aflatoxin B1−lysine adduct monoclonal antibody for human exposure studies. *Appl. Environ. Microbiol.* **2001**, *67*, 2712–2717.
(34) Zhu, G. N.; Wu, G.; Wu, H. M. Synthesis and identification of the antigens for the organophpsphorus insecticide chlorpyrifos. *Sci. Agric. Sin.* **2003**, *36*, 657–662.
(35) Liu, C.; Liu, X.; Liu, F.; Mai, G.; Li, P.; Zhao, M.; Zhen, M.; Wang, Y. Multiresidue analysis method for organophosphorus, organochlorine and pyrethroids pesticides in vegetables and fruits. *Agricultural Standards NY/T 761.2−2008: Part 2*; Agricultural Press: Beijing, China, 2008.
(36) Gee, S. J.; Hammock, B. D.; Van Emon, J. M. *A User's Guide to Environmental Immunochemical Analysis*; EPA/540/R-94/509; U.S EPAOffice of Research and Development, Environmental Monitoring Systems Laboratory: Las Vegas, NV, 1994.
(37) Pearson, T. W.; Pinder, M.; Roelants, G.; Kar, S.; Lundin, L.; Mayor-Whithey, K. S.; Hewett, R. S. Methods for derivation and detection of anti-parasite monoclonal antibodies. *J. Immunol. Methods* **1980**, *34*, 141–154.
(38) Davis, J. M.; Pennington, J. E.; Kubler, A. M.; Conscience, J. F. A simple, single-step technique for selecting and cloning hybridomas for the production of monoclonal antibodies. *J. Immunol. Methods* **1982**, *50*, 161–171.
(39) Gui, W.; Jin, R.; Chen, Z.; Cheng, J.; Zhu, G. Hapten synthesis for enzyme-linked immunoassay of the insecticide triazophos. *Anal. Biochem.* **2006**, *357*, 9–14.
(40) Abad, A.; Moreno, M. J.; Montoya, A. Hapten synthesis and production of monoclonal antibodies to the *N*-methylcarbamate pesticide methiocarb. *J. Agric. Food Chem.* **1998**, *46*, 2417–2426.
(41) Queffelec, A. L.; Nodet, P.; Haelters, J.-P.; Thouvenot, D.; Corbel, B. Hapten synthesis for a monoclonal antibody based ELISA for deltamethrin. *J. Agric. Food Chem.* **1998**, *46*, 1670–1676.

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