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Development of a class selective immunoassay for the type II pyrethroid insecticides

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Abstract

A general and broad class selective enzyme-linked immunosorbent assay was developed for the type II pyrethroid insecticides, such as cypermethrin, deltamethrin, cyhalothrin, cyfluthrin, fenvalerate, esfenvalerate and fluvalinate. Polyclonal antibodies were generated by immunizing with a type II pyrethroid immunogen ((*RS*)-α-cyano-3-phenoxybenzyl (*RS*)-*cis*,*trans*-2,2-dimethyl-3-carboxyl-cyclopropanecarboxylate) conjugated with thyroglobulin. Antisera were screened against nine different coating antigens. The antibody–antigen combination with the most selectivity for type II pyrethroids such as cypermethrin was further optimized and tested for tolerance to co-solvent, pH and ionic strength changes. The IC_{50}s of the optimized immunoassay were 78 $\mu g\,l^{-1}$ for cypermethrin, 205 $\mu g\,l^{-1}$ for cyfluthrin, 120 $\mu g\,l^{-1}$ for cyhalothrin, 13 $\mu g\,l^{-1}$ for deltamethrin, 6 $\mu g\,l^{-1}$ for esfenvalerate, 8 $\mu g\,l^{-1}$ for fenvalerate and 123 $\mu g\,l^{-1}$ for fluvalinate. No cross-reactivity was measured for the type I pyrethroids such as permethrin, bifenthrin, phenothrin, resmethrin and bioresmethrin. This assay can be used in monitoring studies to distinguish between type I and II pyrethroids.

Keywords: Pyrethroid; Insecticide; Pesticide class; Type II; Immunoassay; ELISA

1. Introduction

Synthetic pyrethroids are used widely in domestic, public health, agriculture, forestry, and veterinary applications [1]. The increasing use of synthetic pyrethroids, compared with that of other classes of insecticides, is attributed to their remarkably high insecticidal activities and low toxicity to mammals [2,3]. Although pyrethroids are thought to be safe for humans, reversible symptoms of poisoning and suppressive effects on the immune system have been reported after exposure [4–6]. Some pyrethroids may cause lymph node and splenic damage as well as carcinogenesis [7]. Because pyrethroids offer significant advantages to the agricultural ecosystem if used carefully, but have a potential for environmental damage, a sensitive, selective, and rapid method for monitoring residue levels of pyrethroids in aquatic ecosystems is desirable.

The synthetic pyrethroids and natural pyrethrins can be divided into two groups of compounds on the basis of chemical structure and mechanism of action at insect target sites. The type I compounds are simple cyclic alcohol esters of 2,2-dimethyl-3-(2-methyl-1-propenyl)cyclopropanecarboxylic acid. The type II compounds are esters of an arylcyanohydrin. The type I and II pyrethroids have different toxicological effects, and may have slightly different mechanisms of action at the insect neuron [1], thus the ability to monitor these two compound groups selectively would be an advantage.

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There are many methods for the detection of pyrethroids, such as high-performance liquid chromatography (HPLC) and gas chromatography with electron capture detector (GC-ECD) [8–10]. In spite of good sensitivity, the procedure for sample preparation in such methods is complicated, relatively time-consuming and expensive. In addition, these methods are not suitable for high throughput screening. Therefore, another effective and rapid method for analysis of several pyrethroids at residue levels is desirable. Hammock and Mumma [11] reported that immunoassay could be used for pesticide residue analysis and screening. Owing to the lower cost and higher sample throughput compared to instrumental analyses, enzyme linked immunosorbent assay (ELISA) is an alternative quantitative method. It also has the potential for use as a preliminary screen to reduce the number of samples that undergo subsequent instrumental analyses. ELISA can simplify the quantification of pesticide residues in complex matrices, such as sediments and soils or animal or plant tissues, and it may be possible to reduce the harmful organic solvents used for extraction [12].

Several immunoassays for pyrethroids have been reported. Stanker et al. [13] produced monoclonal antibodies against an immunogen containing the phenoxybenzyl moiety and a cyclopropane ring, and applied this method to detect permethrin in meat extracts. Skerritt et al. [14] described an ELISA format using the same antibodies to detect permethrin in grain and flour extracts. Class selective assays have been developed for those pyrethroids containing the chrysanthemic acid moiety [15]. Pullen and Hock [16] used the permethric acid moiety as a hapten to detect both permethrin (type I) and cypermethrin (type II). An assay that was highly selective for type I over type II pyrethroids has also been developed [17]. For this assay a permethrin-like hapten with a rigid double bond in a long linker attached to the permethric acid portion of the molecule was used. Class selective and compound selective immunoassays for the type II pyrethroid insecticides have also been developed [18,19]. For these assays the sensitivity is in the hundreds of ng ml^{-1}. However, if the analyte is isomerized the sensitivity increases to the tens of ng ml^{-1}. Selectivity was also greater with the isomerized analyte. The assay reported here has similar sensitivity to the isomerized assays, but does not require the isomerization step, and is more selective for type II pyrethroids than the previously reported assay.

In some situations, such as the analysis of storm water runoff, the ability to determine which pyrethroid is present is important. Thus, having a series of assays ranging in selectivity from broad to specific is useful. A class-selective immunoassay that can distinguish between types I and II pyrethroids is desirable. A class selective immunoassay for the type I pyrethroid insecticides has been already developed in this laboratory [17]. Using a similar hapten design strategy, a general and broad selective immunoassay for the type II pyrethroids is described here.

2. Experimental

2.1. Materials

2.1.1. Reagents

Permethrin (3-phenoxybenzyl (*RS*)-*cis,trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate) (*cis:trans* = 25:75), esfenvalerate ((*S*)-α-cyano-3-phenoxybenzyl (*S*)-2-(4-chlorophenyl)-3-methylbutyrate), fenvalerate ((*RS*)-α-cyano-3-phenoxybenzyl (*RS*)-2-(4-chlorophenyl)-3-methylbutyrate), cypermethrin ((*RS*)-α-cyano-3-phenoxybenzyl (*RS*)-*cis,trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate), cyfluthrin ((*RS*)-α-cyano-4-fluoro-3-phenoxybenzyl (*RS*)-*cis,trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate), deltamethrin ((*S*)-α-cyano-3-phenoxybenzyl (*R*)-*cis*-3-(2,2-dibromovinyl)-2,2-dimethylcyclopropanecarboxylate), fluvalinate ((*RS*)-α-cyano-3-phenoxybenzyl *N*-(2-chloro-α,α,α-trifluoro-*p*-tolyl)valinate), phenothrin (3-phenoxybenzyl (*RS*)-*cis,trans*-2,2-dimethyl-3-(2-methylprop-1-enyl)cyclopropanecarboxylate), resmethrin (5-benzyl-3-furylmethyl (*RS*)-*cis,trans*-2,2-dimethyl-3-(2-methylprop-1-enyl) cyclopropanecarboxylate) and bioresmethrin ((5-benzyl-3-furyl)methyl (*R*)-*trans*-2,2-dimethyl-3-(2-methylprop-1-enyl)cyclopropanecarboxylate) were purchased from Reidel de Haen (Seelze, Germany) (Fig. 1). Bifenthrin (2-methylbiphenyl-3-ylmethyl 3-(2-chloro-3,3,3, trifluoro-propenyl)-2,2-dimethyl-cyclopropanecarboxylate and cyhalothrin (cyano-(3-phenoxy-phenyl)-methyl 3-(2-chloro-3,3,3-trifluoro-propenyl)-2,2-dimethylcyclopropanecarboxylate were purchased from Chem Service Inc. (West Chester, PA) (Fig. 1). Hapten **V** (3-phenoxybenzoic acid; Table 1), and 3-phenoxybenzyl alcohol were purchased from Aldrich (St. Louis, MO) (Fig. 1). Coating antigen haptens **IV**, **VI**, **VII**, and **VIII** (Table 1) were prepared in the laboratory [20]. Methanol (GC Resolve grade) was obtained from Fisher Scientific (Pittsburgh, PA), and dimethyl sulfoxide (DMSO; 99.8%) was purchased from Aldrich (St. Louis, MO). 1-[3-(Dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDC) (Aldrich), *N*,*N*-dimethylformamide (DMF; Aldrich) and *N*-hydroxysulfosuccinimide sodium salt (Fluka, Buchs, Switzerland) were used for the production of conjugate with protein. Thyroglobulin (THY), keyhole limpet hemocyanin (KLH), bovine serum albumin (BSA) and ovalbumin (OVA) were obtained from Sigma (St. Louis, MO) as carrier proteins, and goat anti-rabbit immunoglobulin conjugated to horseradish-peroxidase (HRP), Tween 20 and 3,3′,5,5′-tetramethylbenzidine (TMB) were also purchased from Sigma (St. Louis, MO). Water used was purified by a NANOpure II system (Barnstead, Newton, MA). Thin layer chromatography (TLC) was performed on 0.2 μm silica gel 60-F254 glass plates from E. Merck (Darmstadt, Germany). Flash chromatographic separations were carried out on 40 μm average particle size Baker silica gel, packed in glass columns of such diameter to give a column height:diameter ra-

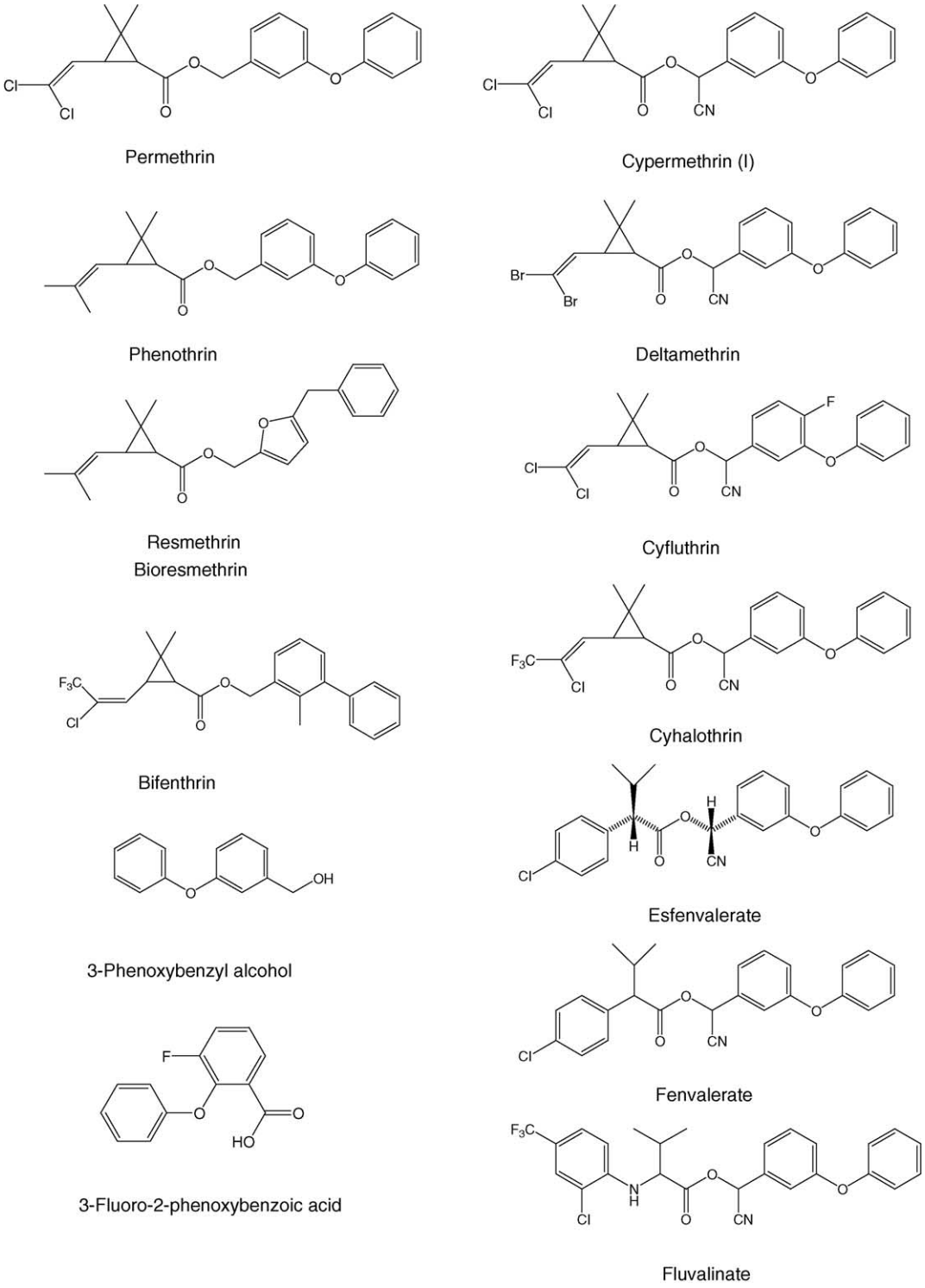


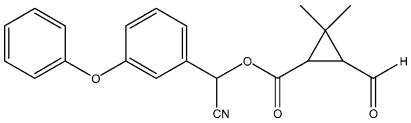
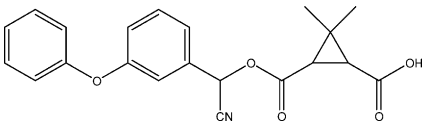
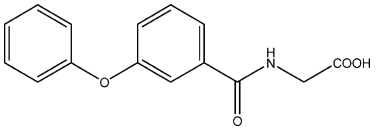
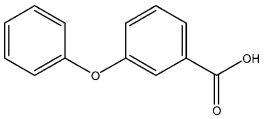
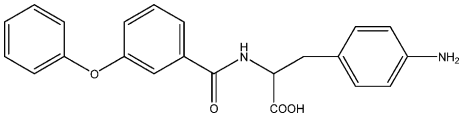
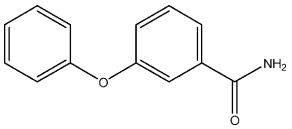
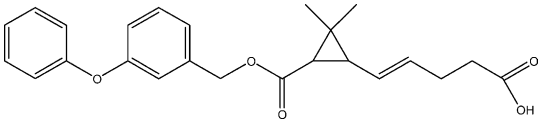
Fig. 1. Structures of the pyrethroids and pyrethroid metabolites.

tio of ∼7. The → notation denotes a stepwise solvent gradient.

2.1.2. Instruments

Proton NMR spectra were obtained using a General Electric QE-300 spectrometer (Bruker NMR, Billerica, MA). Chemical shift values are given in parts per million (ppm) downfield from internal standard tetramethylsilane. ELISA experiments were performed in 96-well microplates (Nunc, Roskilde, Denmark) and the absorbances were read with a Vmax microplate reader (Molecular Devices, Menlo Park, CA) in dual-wavelength mode (450–650 nm). The charac-

Table 1
Structure of coating antigen haptens[a]

Haptens	Structure
II	
III	
IV	
V	
VI	
VII	
VIII	

[a] Haptens were conjugated with BSA or OVA as described in Section 2.

terization of hapten with protein conjugate was done with a Shimadzu UV-2101PC UV–vis scanning spectrophotometer (Shimadzu, Kyoto, Japan).

2.2. Methods

2.2.1. Hapten synthesis and verification

Syntheses of the haptens **II** and **III** were carried out as outlined in Fig. 2. All reactions were straightforward using well-known procedures, and yields were good. NMR spectral data supported all structures, and mass spectra further supported the structure of target molecules.

*2.2.1.1. (RS)-α-Cyano-3-phenoxybenzyl (RS)-cis,trans-2,2-dimethyl-3-(2-methylprop-1-enyl)cyclopropanecarboxylate (**I**) [21].* (Note: the acid used in this preparation was a sample labeled as ±-*cis*-2,2-dimethyl-3-(2-methylprop-1-enyl)cyclopropanecarboxylic acid; however, NMR examination of the product, as noted below, indicated it to be about 13% of the *trans* isomer.)

A sample of ZnI_2 (0.5 mg) was added under N_2 with stirring and ice cooling to a solution of 3-phenoxybenzaldehyde (1.98 g, 0.01 M) in chloroform, and about one-half of a sample of cyanotrimethylsilane (1.47 ml, 0.011 M). The temperature rose slowly to ∼30 °C. The mixture was cooled to ∼10 °C and the remainder of the cyanotrimethylsilane was injected giving a second mild exotherm. After warming the mixture briefly to 40 °C, and allowing to stand at ambient temperature for 45 min, the mixture was poured into a mixture of glyme (15 ml) and 3N HCl (3.5 ml). There was a mild immediate exotherm in the two phase mixture which was then stirred for 30 min, diluted with water, phase separated, and the aqueous phase 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.

Meanwhile, a sample of the acid (1.68 g, 0.01 M) in chloroform (4 ml) plus dimethylformamide (0.5 ml) was treated with thionyl chloride (1.46 ml, 0.02 M) and stirred and heated in an oil bath at 60–65 °C under a short spiral bantam ware distillation column. Gas evolution stopped after ∼15 min. After 45 min, the oil bath temperature was increased to ∼110 °C to distill out most of the chloroform. Cyclohexane (5 ml) was added and distilled out to a pot temperature of 135 °C. A second 5 ml sample of cyclohexane was added and distilled out in the same way. The residual acid chloride, as a colorless oil, was dissolved in chloroform (4 ml), then added to a stirred and ice cooled solution of the above cyanohydrin in chloroform (2.5 ml). After 1 min, pyridine (1.01 ml, 0.0125 M) was injected over ∼2 min. The mixture was stirred for 30 min, then washed with dilute HCl solution (acidic wash), twice with water, dried ($MgSO_4$) and stripped. Flash chromatography on silica gel (25 g) eluting with 50 ml each of 25, 50, and 75% methylene chloride in hexane cleanly separated a small amount of unreacted cyanohydrin from product. Stripping product fractions gave 2.54 g of (**I**) as a mixture of isomers.

A 200 mg sample of **I** was separated by radial chromatography to give 161 mg of the higher R_f isomer mixture and 23 mg of the lower R_f isomer. The NMR spectra of these two were both consistent with the desired structure.

*2.2.1.2. (RS)-α-Cyano-3-phenoxybenzyl (RS)-cis,trans-2,2-dimethyl-3-formyl cyclopropanecarboxylate (**II**) and (RS)-α-cyano-3-phenoxybenzyl (RS)-cis,trans-2,2-dimethyl-3-carboxylcyclopropanecarboxylate (**III**).* The target compounds were prepared using ozonolysis of compound **I**. Compound **I** (0.305 g) was dissolved in 25 ml anhydrous methanol in a tubular reactor equipped with a magnetic stirrer, the reactor flask was placed in a liquid nitrogen bath. A stream of ozone-rich oxygen (from an ozone generator) was passed into the stirred, cold solution with a flow rate of 0.0248 l min^{-1}. The reaction solution was monitored by TLC (hexane:ethyl acetate = 4:1). After 60 min, the reactant was nearly gone and the ozonation was stopped. Acetic acid (1.2 ml) was added as the temperature was warmed to 30 °C by water bath. Then zinc powder (2.0 g) was added a small portion at a time, and the mixture was stirred overnight

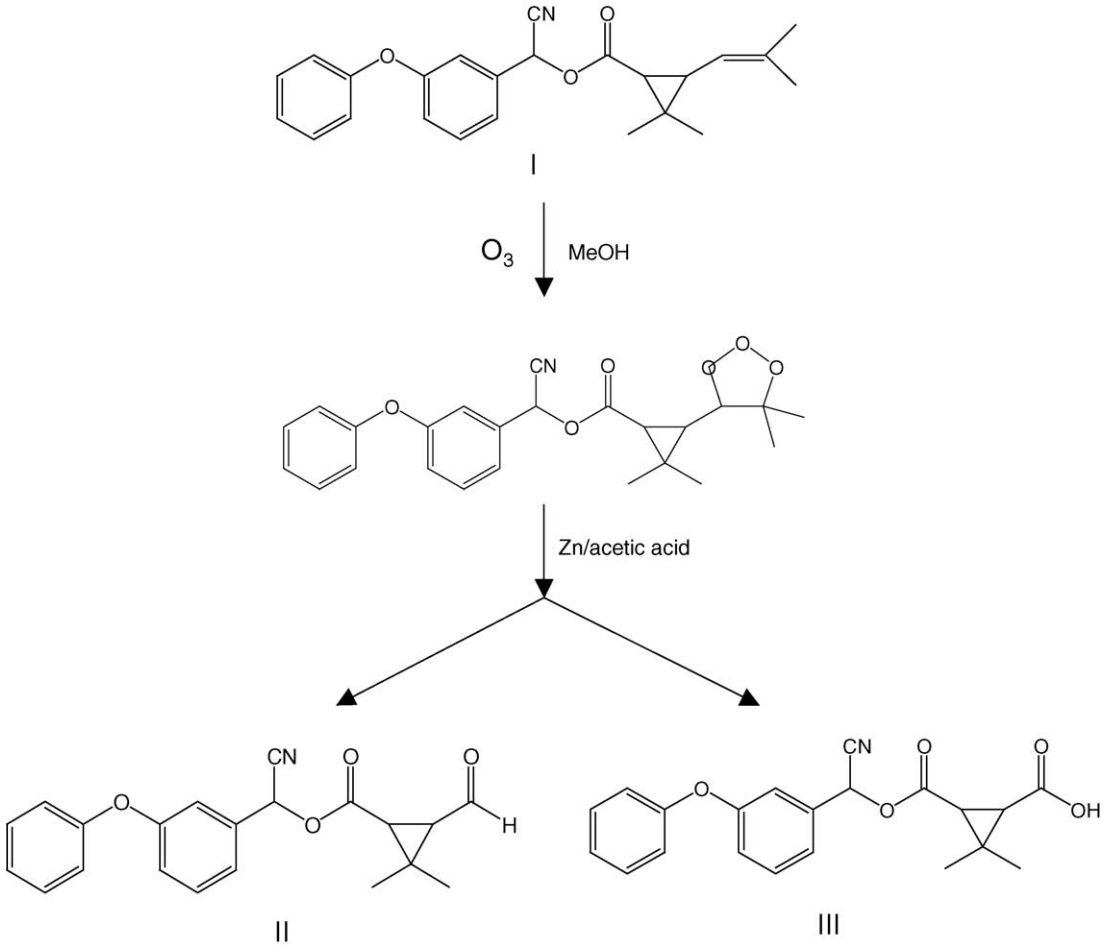


Fig. 2. Synthetic scheme for the preparation of haptens **II** and **III**.

at room temperature. The mixture was filtered to remove the precipitate. The solution was condensed to about 5 ml under vacuum. Flash chromatography on 40 g of silica gel (hexane → CH_2Cl_2) and high vacuum stripping gave the pure gummy aldehyde (**II**) 97 mg (35%) TLC $R_f = 0.65$, $M^+ = 349$. ^{1}H NMR ($CDCl_3$): δ 1.15 (d, 3H, CH_3), 1.25 (d, $J = 6.5$ Hz, 3H, CH_3), 1.64 (m, 1H, CH), 2.10 (d, 1H, CHC(O)O), 6.40 (s, 1H, CHCN), 7.0–7.8 (m, 10H, Ar), 9.92 (s, CHO), and further oxidized acid product (**III**), 40 mg (14%) TLC $R_f = 0.45$ CH_2Cl_2, $M^+ = 366$. ^{1}H NMR ($CDCl_3$): δ 1.10 (s, 3H, CH_3), 1.22 (s, 3H, CH_3), 1.62 (m, 1H, CH), 2.08 (d, 1H, CH), 6.35 (s, 1H, CHCN), 6.9–7.5 (m, 10H, Ar).

2.2.2. Production of hapten–protein conjugates

Hapten **II**-THY/BSA/KLH was synthesized using the Schiff base formation and reductive amination method [22]. Sixty milligrams of BSA (or 50 mg THY or 24 mg KLH) was dissolved in 5 ml of PBS (pH 7.4). Then compound **II** (0.02 mmol) in 200 μl DMSO was added with gentle stirring and followed by addition of 50 μL of 5 M sodium cyanoborohydride in 1N NaOH. The reaction mixture was stirred for 3 h at room temperature. The unreacted aldehyde sites were blocked by adding 100 μl of 3 M ethanolamine solution and reacted for 15 min at room temperature. The solution was then dialyzed against PBS over 48 h at 4 °C and stored at −20 °C. The **II**-THY and **II**-KLH conjugates were used for immunization and the **II**-BSA was used as a coating antigen.

Hapten **III**-THY/BSA was synthesized by conjugating hapten **III** with carrier proteins using an activated ester method [23]. Hapten **III** (45 μM) was dissolved in 1.0 ml of anhydrous DMF with equimolar (0.04 mmol) concentration of *N*-hydroxysuccinimide and a 10% molar excess of dicyclohexylcarbodiimide. After 3 h of stirring at room temperature, the precipitated dicyclohexylurea was removed by centrifugation, and the DMF supernatant was divided into two aliquots and added to protein solutions as below.

Fifty milligrams of BSA or THY was dissolved in 5 ml of borate buffer (pH 8.7) in a 10 ml glass vial with a Teflon stir bar. This solution was allowed to stir vigorously, and 1.05 ml of DMF was added very slowly to bring the DMF to 20%. Activated hapten then was added to the protein solution a few microliters each time at room temperature over about 20 min to finish this step. Then the mixture was stirred at 4 °C for 6 h. The solution was dialyzed against PBS for 72 h at 4 °C and stored at −20 °C. Hapten **III**-THY was used as

an immunogen and hapten **III**-BSA was used as a coating antigen.

2.2.3. Immunization and antiserum preparation

Cypermethrin antisera was obtained following the protocol described previously [24]. New Zealand white rabbits were immunized intradermally with each immunogen hapten **II**-KLH (rabbits #3540, and #3541), **II**-THY (rabbits #3543 and #3545) and **III**-THY (rabbits #3546 and #3547). One month after an initial immunization with 100 μg of the immunogen protein emulsified with Freund's complete adjuvant (1:1, v:v), further injections of 100 μg of the immunogen emulsified with Freund's incomplete adjuvant were given (1:1, v:v). Booster injections were given at 4-week intervals. The rabbits were bled 10 days after each boost. The serum was isolated by centrifugation for 10 min at 4 °C and stored at −20 °C. The results of antibody characterization were obtained from sera of terminal bleeds after four boosters.

2.2.4. Enzyme-linked immunosorbent assay (ELISA)

The method was performed as previously described by Shan et al. [25]. Microplates were coated overnight at 4 °C with 100 μl per well of the appropriate coating antigen concentration in 0.1 M carbonate-bicarbonate buffer (pH 9.6). After the plate had been washed with washing solution (0.05% Tween 20 in distilled water), the surface of the wells were blocked with 200 μl of 0.5% BSA in 0.1 M PBS (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) by incubation for 30 min at room temperature to minimize the non-specific binding in the plate. After another washing step, 100 μl of antiserum per well diluted in PBS (for titration experiments) or 50 μl per well of antiserum diluted in PBS with 0.2% BSA (PBSB) and 50 μl per well of analyte solution were added and incubated for 1 h at room temperature. The standard analyte concentrations ranged from 0.05 to 5000 μg l^{-1}. Following a washing step, goat anti-rabbit IgG-HRP conjugate (diluted in 1:3000 in PBS with 0.05% Tween 20, 100 μl per well) was added and incubated for 1 h at room temperature. The plates were washed again, and 100 μl per well of substrate solution (3.3 μl of 30% H_2O_2, 400 μl of 0.6% TMB in DMSO per 25 ml of acetate buffer, pH 5.5) was added. The color development was stopped after 20 min with 50 μl per well of 2 M H_2SO_4. The absorbance was measured using a dual wavelength mode at 450 nm minus 650 nm. Standard curves were obtained by plotting absorbance against the logarithm of analyte concentration, which were fitted to a four-parameter logistic equation: $y = \{(A - D)/[1 + (x/C)^B]\} + D$, where A is the maximum absorbance at no analyte present, B the curve slope at the inflection point, C the concentration of analyte giving 50% inhibition (IC_{50}), and D the minimum absorbance at infinite concentration.

2.2.5. Antibody characterization and assay optimisation

Antibodies and antigens were screened in a two-dimensional titration for the best dilution of coating antigen and antiserum. Then the competitive inhibition curves were measured for different antibody and antigen combinations. In this study, the combination of antibody and coating antigen with the lowest IC_{50} and more selectivity to cypermethrin (a type II pyrethroid over permethrin which is a type I analog) was selected for further assay development.

The effects of the solvents were tested by dissolving the analyte in PBS buffers containing various proportions of solvent (0, 10, 20, 40, 60, 80% solvent) and incubating these with antibody in PBSB on the coated plate. Methanol and DMSO were tested in this way.

In the experiment to evaluate pH effects, both the analyte and antiserum were dissolved in PBS buffer at the specified pH values of 5, 6, 7, and 8 and were tested in this incubation step with all other parameters of the assay fixed. Ionic strength effects were determined in the same manner as previously mentioned except that, instead of pH, PBS concentration was varied. PBS concentrations of 0.1, 0.2, 0.3, and 0.5 M were tested.

2.2.6. Cross-reactivity

The optimized assays were applied to cross-reactivity studies by using the standard solution of the analyte and other structurally related compounds. The cross-reactivity (CR) was determined by dividing the IC_{50} of the chemical, cypermethrin, assigned to be 100% by the IC_{50} of another compound and multiplying by 100 to obtain a percent figure.

2.2.7. Recovery

Industrial waters diluted 100 times with 20% methanolic PBS were spiked with cypermethrin standards (0.5, 1.0, 2.5, 5.0, and 10 μg l^{-1}) to estimate the recoveries from water samples.

3. Results and discussion

3.1. Hapten synthesis

The primary goal of this study was to develop a class specific immunoassay for type II pyrethroids. To design a hapten that has a common portion of type II pyrethroids and differs from type I pyrethroids is critical for such a purpose. Structurally, most pyrethroids (type I and II) are esters containing a phenoxybenzyl group and a dimethylcyclopropane. Type II pyrethroids can be distinguished from type I pyrethroids by having an α-cyano group in its alcohol moiety. The haptens were thus designed to contain the α-cyano phenoxybenzyl and dimethylcyclopropane. The carrier protein was to link through the dimethylcyclopropane end of the cypermethrin molecule. Since pyrethroids are highly lipophilic, a long side chain (handle) may allow the lipophilic hapten to fold into the hydrophobic interior of the protein and decrease the affinity of the resulting antibodies. Studied with dioxins, Sanborn et al. [26] demonstrated that a rigid or shorter side chain in an immunizing hapten is important for generation of sensitive and selective antibodies for a lipophilic target. A longer

Table 2
Summary of optical density of titration tests[a]

Ab/immunogen	**II**-BSA	**IV**-BSA	**IV**-OVA	**V**-BSA	**V**-OVA	**VI**-BSA	**VII**-BSA	**VIII**-BSA	**VIII**-OVA
3540/**II**-KLH	++++	++++	++++	++++	++++	++++	+	–	–
3541/**II**-KLH	–	+++	++++	+++	–	+++	–	–	–
3543/**II**-THY	–	–	–	–	–	+++	–	–	–
3545/**II**-THY	++++	++++	++++	++++	++++	++++	+	–	–
3546/**III**-THY	+++	–	++++	+++	+++	+	–	–	–
3547/**III**-THY	–	–	–	–	+++	+	–	–	–

Symbols refer to optical density ranges after 20 min of color development. (–) absorbance < 0.25; (+) absorbance = 0.25–0.50; (++) absorbance = 0.50–0.75; (+++) absorbance = 0.75–1.00; (++++) absorbance > 1.00.

[a] The data shown are at an antibody dilution of 1:16,000 and coating antigen concentration of 1 $\mu g\,ml^{-1}$.

linker that contained a double bond for rigidity was used for the development of a type I pyrethroid assay [17]. The hapten chosen for this study for immunization contains most of the cypermethrin molecule and has no side chain (Fig. 2). This approach differed from previous reports in which a long side chain was placed off the cyclopropane ring or attached at the α-cyano portion of pyrethroid [18]. In addition, the two haptens differed in the functional group used for coupling to carrier protein. This offered options for slightly different conditions for coupling, while not altering the actual linker.

3.2. Screening and selection of antisera

The antisera of six rabbits were screened against nine different coating antigens using a two-dimensional titration method with the coated antigen format. The objective was to find the coating antigen that has the highest affinity with tested antibody but could still be replaced by target analyte. The results of the titration experiments using the final bleeds are shown in Table 2. Animal to animal variability likely accounts for the highly different responses between rabbits 3543 and 3545 and 3546 and 3547 even though each pair was immunized with the same antigen. In all combinations, the titer of 3546 and 3547, which was generated from the immunogen **III**-THY, was much lower than those of 3540, 3541 and 3545, which were produced from the immunogens **II**-KLH and **II**-THY. Some of the difference in titer may be due to the difference in the coupling method used. The different methods used slightly different conditions that may have favored/hindered the optimal loading of the protein. Thus immunogens from hapten **III** may have had lower hapten loads than the immunogens from hapten **II** resulting in the lower titers for rabbit immunized with hapten **III**. The homologous assay, in which the same hapten was used in the coating antigen and immunogen, had a similar titer with the heterologous assay.

In general, good affinity was obtained with coating antigen haptens **II**, **IV**, **V** and **VI**. These haptens have in common, the exposed phenoxyphenyl moiety. On the other hand, little affinity was measured for coating antigen hapten **VII** and none measured for hapten **VIII** with all six antibodies. The antibodies appear to have a high degree of selectivity for the phenoxyphenyl ring and the presence of a methoxy group in hapten **VII** is likely the reason for the low binding to this antigen. Surprisingly hapten **VIII**, which is structurally similar to the immunizing haptens, is not bound by any of the antibodies. Pyrethroids are lipophilic molecules and the long linker may allow the highly recognized phenoxyphenyl moiety to fold back into the protein making it unavailable for binding to the antibody.

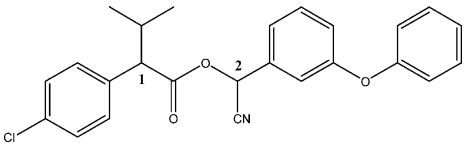
Interestingly, antibody 3546 shows a high titer for **IV**-OVA, but a low titer for **IV**-BSA. This same trend is true with antibody 3547 and coating antigen **V**-OVA and **V**-BSA. On the other hand, antibody 3541 shows a high titer for coating

Table 3
Selected competitive ELISA screening data against cypermethrin[a]

Immunogen	Ab/cAg	Cypermethrin				Permethrin			
		A_{max} (*A*)	Slope (*B*)	A_{min} (*D*)	IC_{50} ($\mu g\,l^{-1}$) (*C*)	A_{max} (*A*)	Slope (*B*)	A_{min} (*D*)	IC_{50} ($\mu g\,l^{-1}$) (*C*)
II-KLH	3540/**II**-BSA				>5000	0.63	1.21	0.21	589
II-THY	3545/**II**-BSA	0.36	1.05	0.12	165	0.32	1.00	0.10	137
II-KLH	3541/**IV**-BSA	0.48	0.40	0.04	56.8	0.48	0.67	0.11	87.5
II-KLH	3541/**IV**-OVA	0.72	1.31	0.12	598	0.82	1.29	0.07	562
II-THY	3545/**IV**-OVA	1.20	0.81	0.05	914	1.16	1.40	0.19	342
II-KLH	3541/**V**-BSA	0.69	1.13	0.13	278	0.75	0.81	0.06	282
II-THY	3545/**V**-BSA	1.64	0.89	0.20	596	1.57	0.95	0.15	515
III-THY	3546/**IV**-BSA	0.57	1.10	0.08	346	0.55	1.03	0.07	319
III-THY	3546/**IV**-OVA	0.42	0.69	0.06	792	0.43	1.25	0.06	459
III-THY	3546/**V**-BSA	1.13	1.25	0.42	467				>5000
III-THY	3546/**VI**-BSA	0.65	1.03	0.05	193				>5000
III-THY	3546/**VII**-BSA	0.53	0.702	0.07	958	0.55	0.84	0.01	603

[a] The cypermethrin analyte standards were prepared in a 30% methanol–PBS solution.

Table 4
Cross-reactivities of pyrethroids and their metabolites

Analyte	A_{max} (A)	Slope (B)	A_{min} (D)	IC_{50} ($\mu g\,l^{-1}$)[c] (C)	R^2	Cross-reactivity (%)[a]
Cypermethrin	0.53	0.97	0.03	78.4 ± 11	0.99	100
Type I pyrethroid insecticide						
Permethrin						N.I.
cis-Permethrin						N.I.
trans-Permethrin						N.I.
Bifenthrin						N.I.
Phenothrin						N.I.
Bioresmethrin						N.I.
Resmethrin						N.I.
Type II pyrethroid insecticide 						
Fenvalerate isomers						
1*S*,2*S* (esfenvalerate)	0.57	0.97	0.03	1086 ± 109	0.99	1386.2 ± 140
1*R*,2*S*	0.55	0.92	0.05	945 ± 77	0.99	1206 ± 98
1*S*,2*R*	0.52	0.74	0.03	56 ± 9	0.99	72.1 ± 11
1*R*,2*R*	0.52	0.68	0.04	16 ± 2	0.99	20.8 ± 3
1*R*,2*R*/*S*	0.58	0.94	0.06	1011 ± 86	0.99	1289.2 ± 110
1*S*,2*R*/*S*	0.50	0.70	0.07	50 ± 14	0.99	63.6 ± 18
1*R*/*S*,2*R*/*S* (fenvalerate)	0.56	0.90	0.06	772 ± 60	0.99	984.2 ± 76
Cyfluthrin	0.53	0.70	0.05	30 ± 4	0.99	38.2 ± 5
Cyhalothrin	0.50	0.74	0.04	51 ± 9	0.99	65.4 ± 12
Deltamethrin	0.55	0.89	0.07	486 ± 37	0.99	619.6 ± 47
Fluvalinate	0.51	0.83	0.06	50 ± 7	0.99	63.5 ± 9
Metabolites						
Phenoxybenzyl alcohol						N.I.
Phenoxybenzoic acid						N.I.
Fluorophenoxybenzoic acid						N.I.

[a] ELISA system 3540/**VI**-BSA was used in the cross-reactivity studies. Cypermethrin was assigned as 100%. Values are the means and standard deviations of three experiments.

antigen **V**-BSA, but not **V**-OVA. With these three examples, two have higher titers on OVA and one on BSA, thus it is not likely that one carrier protein is better than another. With other antibodies, no difference in titer is noted between BSA and OVA antigens ruling out likely differences due to hapten load. Because other antibodies do bind to these antigens, failed coupling is also ruled out. Thus this observation is most probably due to animal variability.

Competitive inhibition experiments were performed to optimize antiserum and coating antigen concentrations for high sensitivity. In addition, to distinguish the selectivity of the combinations of antisera and coating antigens to the type **II** pyrethroids, the competitive inhibition experiments to cypermethrin and permethrin were conducted simultaneously (Table 3). The IC_{50} of the homologous assay (3540 or 3545/**II**-BSA) showed that this combination was not selective for cypermethrin. Even though some combinations of antibody and coating antigen had lower IC_{50}s, they did not show good selectivity to cypermethrin. After preliminary screening, the combination of antiserum 3546 with coating antigen **VI**-BSA gave the lowest IC_{50} values for cypermethrin and no inhibition for permethrin despite the lower titer. This system was selected for cross-reactivity studies and was chosen for further assay development and optimization.

3.3. Cross-reactivity

Table 4 demonstrates the cross-reactivity of the ELISA system 3546/**VI**-BSA with different pyrethroids and pyrethroid metabolites. This system had the highest cross-reactivity with the type II pyrethroids such as esfenvalerate (1386%), followed by fenvalerate (984%), deltamethrin (620%), cyhalothrin (65%), fluvalinate (64%) and cyfluthrin (38%). Less or no cross-reactivity was measured for the type I pyrethroids such as bifenthrin, bioresmethrin, permethrin, phenothrin, resmethrin and permethrin metabolites in this system. Since our primary goal was to develop a class-selective immunoassay with no or low cross-reactivity to the type I pyrethroids, 3546/**VI**-BSA was selected for further optimization.

The hapten used in this study for generating antibodies contained a very short side chain that resulted in antibodies that were highly selective for type II pyrethroids. Short or rigid side chains are considered desirable for lipophilic hap-

Table 5
Effects of DMSO concentration on assay parameters[a]

DMSO (%)[b]	A_{max} (A)	Slope (B)	A_{min} (D)	IC_{50} ($\mu g\,l^{-1}$)[c] (C)	A/D	R^2
0	0.59 ± 0.03	0.96	0.06	113.0 ± 6.22	10.70	0.99
10	0.28 ± 0.04	0.63	0.04	167.4 ± 7.78	7.65	0.99
20	0.11 ± 0.01	0.43	0.03	280.0 ± 16.09	3.27	0.99
40	0.10 ± 0.01	0.24	0.04	1041.3 ± 29.14	2.39	0.99

[a] ELISA conditions: coating antigen **IV**-BSA (0.25 $\mu g\,ml^{-1}$); antiserum 3546 (1:5000) (final concentration in wells); goat anti-rabbit IgG HRP (1:3000).
[b] Concentration of DMSO in cypermethrin standard solution (PBS–DMSO).
[c] Mean value ± S.D. Each set of data represents the average of three experiments.

tens to prevent their folding back onto the protein surface. Such folding may result in less antibody selectivity and sensitivity. Although it is difficult to compare antibodies from one laboratory to another, the antibodies derived from longer chain, non-rigid haptens resulted in assays that were not as selective, but of similar sensitivity as the assay reported here [18].

3.4. Solvent, pH, and salt effects

Finding a proper co-solvent for an immunoassay is very important for the analysis of hydrophobic chemicals such as cypermethrin. The solvent (methanol or DMSO) effects on the ELISA system (3546/**VI**-BSA) were evaluated by preparing cypermethrin in PBS containing various amounts of solvent. DMSO strongly interferes with the assay sensitivity and maximum absorbance (Table 5). Using methanol as a cosolvent, absorbance was enhanced with higher methanol concentration (Fig. 3), which was also found by Shan et al. [25]. The IC_{50} value of the assay varied depending upon the different concentrations of the cosolvent methanol. The lowest IC_{50} was found at 20% methanol (70.2 $\mu g\,l^{-1}$), which is approximately two times and four times lower than that at 40% methanol (148 $\mu g\,l^{-1}$) and 60% methanol (300 $\mu g\,l^{-1}$) respectively. At concentrations up to 80% methanol, an incomplete inhibition curve was demonstrated. The co-solvent could be an important factor in assay performance, especially for hydrophobic compounds. A high concentration of co-solvent will help the solubility of hydrophobic cypermethrin in reaction solution, but it could also affect the interaction of antibody and antigen, especially the low affinity of antibody and antigen. On the basis of the IC_{50} values and the ratios of maximum and minimum absorbances for the cypermethrin standard curves, a methanol concentration of 20% was selected for subsequent experiments.

To address potential interferences from aqueous environmental samples, the effects of pH and ionic strength on ELISA performance were evaluated in this study. In system 3546/**VI**-BSA, when analyte was dissolved in buffer at various pH values, no significant effect upon the IC_{50} was detected, indicating that the assay could effectively detect cypermethrin at pH values ranging from 5.0 to 8.0 (Fig. 4). Ionic strength strongly influenced ELISA performance (Fig. 5). A higher salt concentration in the assay system resulted in lower absorbance and higher IC_{50} values. The absorbance values at salt concentrations of 0.3 and 0.5 M PBS decreased by approximately 53 and 68%, respectively, from the absorbance value at a salt concentration 0.1 M PBS. Therefore, the maintenance of a minimal ionic strength appears to be important.

The optimized cypermethrin ELISA used coating antigen **VI**-BSA at 0.25 $\mu g\,ml^{-1}$, antibody 3546 at a dilution of 1:5000, and cypermethrin in 20% methanol–PBS buffer. The IC_{50} value of this assay was 78.2 ± 5.0 $\mu g\,l^{-1}$ (Fig. 6).

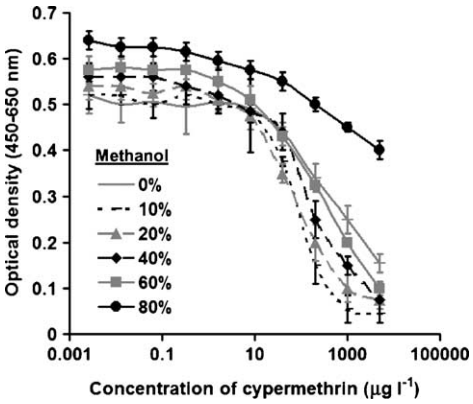


Fig. 3. ELISA competitive curves of cypermethrin prepared in PBS buffer containing various concentrations of methanol. Reagent concentrations: coating antigen **IV**-BSA (0.25 $\mu g\,ml^{-1}$); antiserum 3546 (1:5000) (final concentration in wells); goat anti-rabbit IgG HRP (1:3000). Error bars represent S.D. of three experiments.

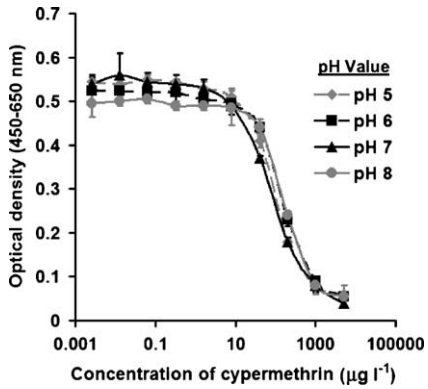


Fig. 4. ELISA competitive curves of cypermethrin prepared in PBS buffer containing various pH values. Reagent concentrations: coating antigen **IV**-BSA (0.25 $\mu g\,ml^{-1}$); antiserum 3546 (1:5000) (final concentration in wells); goat anti-rabbit IgG HRP (1:3000). Error bars represent S.D. of three experiments.

Table 6
Recovery test of cypermethrin in industrial water

Analyte	Spiked ($\mu g\,ml^{-1}$)[a]	Theoretical ($\mu g\,l^{-1}$)[b]	Measured ($\mu g\,l^{-1}$)	Recovery (%)[c]	Mean ± S.D.
Cypermethrin	1	20	18.4	92.0	92.7 ± 1.6
			18.9	92.5	
			18.3	91.5	
	4	80	87.0	108.8	107.0 ± 3.6
			87.5	109.4	
			82.3	102.9	
	10	200	201.2	100.6	101.5 ± 2.2
			198.2	99.0	
			210.0	105.0	

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

Table 7
Recovery test of the type II pyrethroids

Analyte	Spiked ($\mu g\,ml^{-1}$)[a]	Theoretical ($\mu g\,l^{-1}$)[b]	Cypermethrin equivalent ($\mu g\,l^{-1}$)	Relative recovery (%)	Mean ± S.D. (%)	Actual recovery (%)
Cyfluthrin	4	80	22.4	28.0	31.6 ± 4.3	82.7 ± 11.2
			29.1	36.4		
			24.3	30.4		
	10	200	70.4	35.2	37.6 ± 2.7	98.4 ± 7.2
			74.0	37.0		
			80.2	40.6		
Cyhalothrin	4	80	47.2	59.0	59.4 ± 6.0	90.9 ± 9.2
			42.9	53.6		
			52.5	65.6		
	10	200	128.6	64.3	67.6 ± 2.9	103.3 ± 4.4
			137.6	68.8		
			139.2	69.6		
Deltamethrin[c]	1	5	39.1	782.0	697.3 ± 2.9	112.5 ± 16.2
			29.3	586.0		
			36.2	724.0		
	4	20	113.1	565.5	546.2 ± 17.6	88.1 ± 2.8
			106.2	531.0		
			108.4	542.0		
Esfenvalerate[d]	1	2.5	40.2	1608.0	1440 ± 257.1	103.9 ± 18.5
			39.2	1568.0		
			28.6	1144.0		
	4	10	132.8	1328.0	1407.3 ± 134.0	101.5 ± 9.7
			156.2	1562.0		
			133.2	1332.0		
Fluvalinate	4	80	43.2	54.0	58.7 ± 5.0	92.4 ± 7.9
			51.6	64.0		
			46.5	58.1		
	10	200	107.2	53.6	54.8 ± 2.4	86.2 ± 3.8
			115.1	57.6		
			106.2	53.1		

[a] Industrial water was spiked with an appropriate concentration of cypermethrin.
[b] Fifty times dilution with 20% methanolic PBS.
[c] Two hundred times dilution with 20% methanolic PBS.
[d] Four hundred times dilution with 20% methanolic PBS.

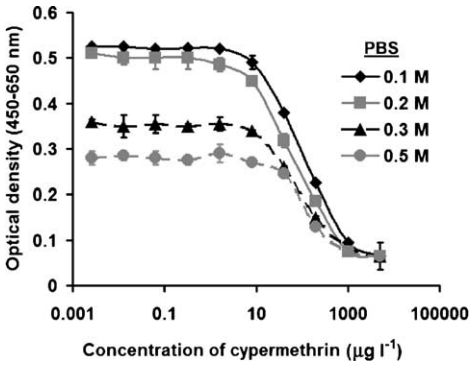


Fig. 5. ELISA competitive curves of cypermethrin prepared in PBS buffer containing various concentrations of ionic strength. Reagent concentrations: coating antigen **IV**-BSA (0.25 μg ml^{-1}); antiserum 3546 (1:5000) (final concentration in wells); goat anti-rabbit IgG HRP (1:3000). Error bars represent S.D. of three experiments.

3.5. Recovery

To estimate reliability, a recovery test was performed using an industrial water sample. When the standard curve was plotted with industrial water spiked with cypermethrin, the absorbance was significantly increased. There are many metal ions and other potential interferences in industrial water that could account for this enhanced response. It was necessary to dilute the samples with PBS prior to analysis. Although the matrix effect can be largely eliminated by solid phase extraction, a simple pretreatment without cleanup is important for real water analysis to enhance the speed of analysis. The effect of dilution factors on sensitivity was tested as shown in Fig. 7. The results indicate that the matrix effect was eliminated with more than 10-fold dilution with 20% methanolic PBS. Table 6 shows the recovery of cypermethrin from industrial water. Industrial waters, diluted 50-fold with 20% methanolic PBS, were tested in the linear range from 20 to 200 μg l^{-1}. The recoveries of cypermethrin (∼100 or >100%) were satisfactory. In addition, the recoveries of other type II

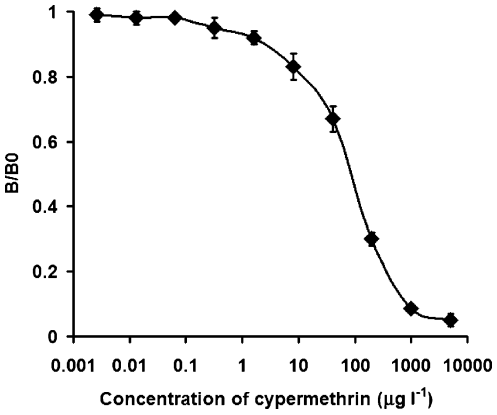


Fig. 6. ELISA inhibition curves of cypermethrin. Reagent concentrations: coating antigen **IV**-BSA (0.25 μg ml^{-1}); antiserum 3546 (1:5000) (final concentration in wells); goat anti-rabbit IgG HRP (1:3000). Standard curve represent the average of 16 curves.

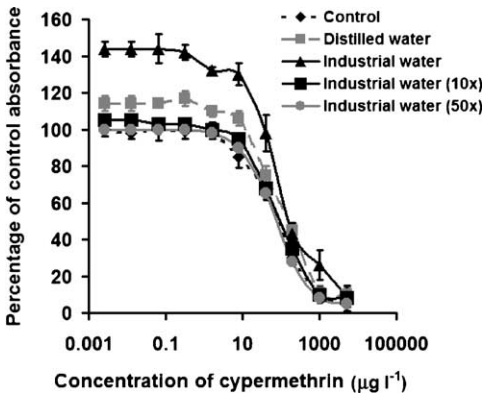


Fig. 7. Effects of the dilution of the matrix on the reliability of ELISA. Samples were diluted with 20% methanolic PBS.

pyrethroids using a cypermethrin standard curve were tested. Tap waters were spiked with 1, 4 and 10 μg l^{-1} cyfluthrin, cyhalothrin, deltamethrin, esfenvalerate, and fluvalinate (dissolved in methanol), and were diluted in an appropriate dilution factor with 20% methanolic PBS (Table 7). The results indicate that this assay will be a useful screening test for type II pyrethroids.

In order to estimate the recoveries of different kinds of type II pyrethroids, the relative cross-reactivity factor (shown in Table 4) could be used as reference values. Along with assays for parent compounds it may be possible to estimate the amount of different type II pyrethroids using mathematical approaches for multianalyte analysis similar to those developed by Wortberg et al. [27] for the triazine herbicides.

4. Conclusions

A general and broad selective immunoassay for the type II pyrethroid insecticides has been developed by using **III**-THY as the immunizing hapten and **VI**-BSA as the coating antigen. ELISA 3546/**VI**-BSA had a relatively lower IC_{50} and also demonstrated no or little inhibition to the type I pyrethroids. Also, this system exhibited good performance characteristics at various pH values and medium solvent (20% methanol) levels. Although the assay is sensitive to high ionic strength, with the implementation of dilution, interferences can be minimized for field sample measurement. This assay is useful for screening type II pyrethroids and the use of such a general assay has several advantages. One such advantage is to screen out samples containing non-detectable residues thus enhancing efficiency of further instrumental analysis such as GC–MS.

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