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Development of a class-specific immunoassay for the type I pyrethroid insecticides

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Abstract

A general enzyme-linked immunosorbent assay was developed for the type I pyrethroid insecticides, such as permethrin, phenothrin, resmethrin and bioresmethrin. Polyclonal antibodies were generated by immunizing with a permethrin derivative, 2,2-dimethyl-3-(5′-carboxy-pent-1′-en-yl)cyclopropanecarboxylic acid-(3-phenoxybenzyl)ester conjugated with thyroglobulin, bovine serum albumin or ovalbumin. Antisera were screened against eight different coating antigens. The antibody–antigen combination with the lowest background, and highest sensitivity for permethrin was further optimized and tested for solvent and detergent tolerance. The I_{50}'s of the optimized immunoassay were 30 μg/l for permethrin and 20 μg/l for phenothrin, respectively. No cross-reactivities were measured to the type II pyrethroids, such as esfenvalerate, cyfluthrin, cypermethrin, deltamethrin, fenvalerate and fluvalinate. This assay can be used in monitoring studies to distinguish between types I and II pyrethroids.

Keywords: Permethrin; Pyrethroid; ELISA; Class-specific immunoassay

1. Introduction

The synthetic pyrethroids, such as permethrin, phenothrin, bioresmethrin, fenvalerate and deltamethrin differ in their toxicity, photostability and chemical structure [1]. They have been widely used to control insect pests in agriculture. Among type I pyrethroids, permethrin has been widely used for the control of insect disease vectors and as a moth-proofing agent by the textile industry [2,3] because it is less toxic to fish than the type II pyrethroids. The use of pyrethroid compounds has recently increased due to the low mammalian toxicity. Consequently, contamination of ecosystem components and foodstuffs derived from agriculture may occur. Therefore, a sensitive, selective and rapid method for monitoring residue pyrethroids is required.

There are many methods for the detection of pyrethroids, such as high-performance liquid chromatography (HPLC) [4], gas chromatography with electron capture detector (GC–ECD) [4] and with mass spectrometry (GC–MS) [5,6]. Though capable of good sensitivity, the procedure for sample preparation in such methods is very complicated, and highly skilled operations are required. Therefore, we need another effective and rapid method for analysis of many pesticides at residue levels. Hammock and Mumma [7] reported that immunoassay could be used

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for pesticide residue analysis and screening. Due to the lower cost and higher sample throughput achievable compared to instrumental analyses, ELISA can be an alternative or has the potential for use as a first screen to reduce the numbers of samples requiring subsequent instrumental analyses. It is easy to quantify 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 [8]. With this background, Stanker et al. [9] reported the production of monoclonal antibodies against an immunogen containing the phenoxybenzyl moiety and a cyclopropane ring, and applied this to detect permethrin in meat extracts. Skerritt et al. [10] described the ELISA format using the same antibodies to detect permethrin in grain and flour extracts. To study pyrethroid fate in the aquatic ecosystem, methods for analysis of large numbers of samples is necessary. In addition, in situation such as analysis of stormwater runoff, the ability to determine which pyrethroid is present is important. Thus, a series of assays beginning with broad, then narrowing specificities would be useful tools. Consequently, a class-specific immunoassay that can distinguish between types I and II pyrethroid is desirable. This goal necessitates careful hapten design. Therefore, we produced a polyclonal antibody against a permethrin analogue with a spacer of three carbons as an immunogen, and describe here the development of a class-specific immunoassay for the type I pyrethroids.

2. Experimental

2.1. Reagents

Permethrin (3-phenoxybenzyl-(*RS*)-*cis*,*trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate) (*cis*:*trans* = 25:75), *cis*- and *trans*-permethrin were obtained from Reidel de Haen (Seelze, Germany). The immunogen hapten I (2,2-dimethyl-3-(5′-carboxy-pent-1′-en-yl)cyclopropanecarboxylic acid-(3-phenoxybenzyl)ester) (Fig. 1) was provided by Bayer AG (Leverkusen, Germany). 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)-D-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-furylmethyl-(*R*)-*trans*-2,2-dimethyl-3-(2-methylprop-1-enyl)cyclopropanecarboxylate) were purchased from Reidel de Haen (Seelze, Germany) (Fig. 2). The eight coating antigen haptens used are shown in Table 1. Methanol was of GC Resolve grade and obtained from Fisher Scientific (Pittsburgh, PA), and dimethylsulfoxide (99.8%) was from Aldrich (St. Louis, MO). 1-[3-(Dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDC) (Aldrich), *N*,*N*-dimethylformamide (Aldrich) and *N*-hydroxysulfosuccinimide sodium salt (Fluka, Buchs, Switzerland) were used for the production of conjugate to protein. Thyroglobulin, bovine serum albumin and ovalbumin were obtained from Sigma (St. Louis, MO) as carrier proteins, and goat anti-rabbit immunoglobulin conjugated to horseradish-peroxidase (HRP) and 3,3′,5,5′-tetramethylbenzidine (TMB) were also purchased from Sigma. Water used was purified by a NANOpure II system (Barnstead, Newton, MA).

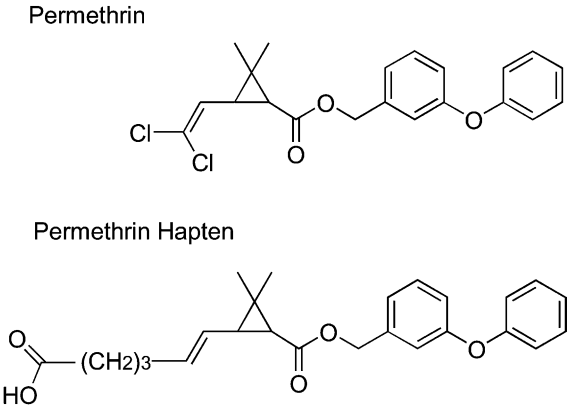


Fig. 1. Structure of permethrin and permethrin hapten.

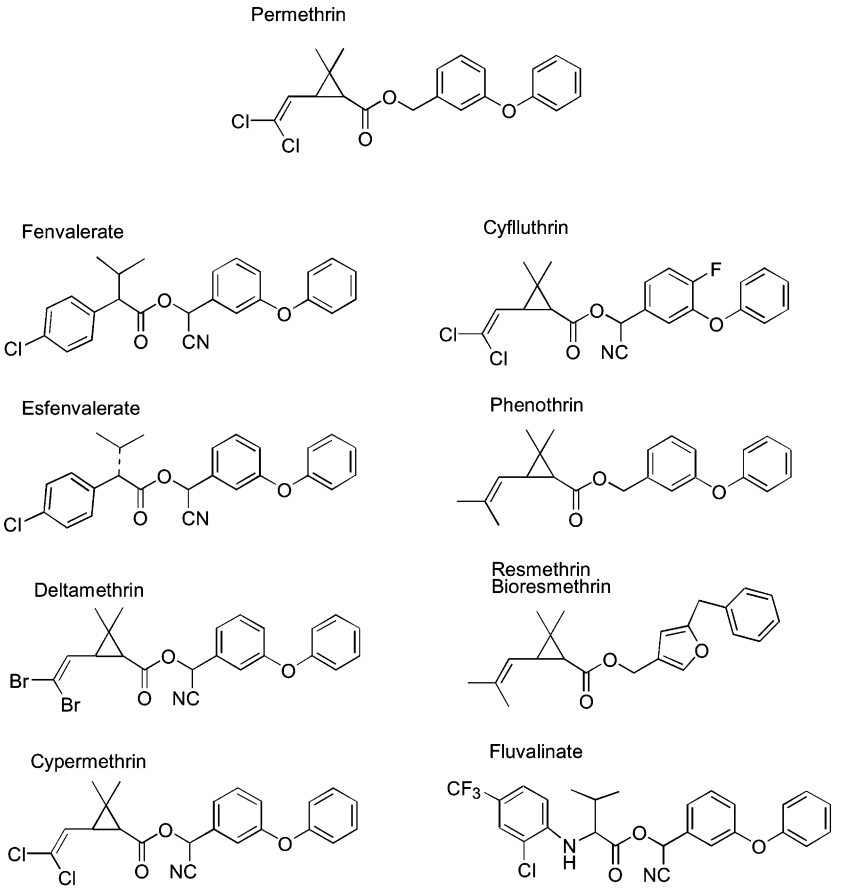


Fig. 2. Structures of some pyrethroids.

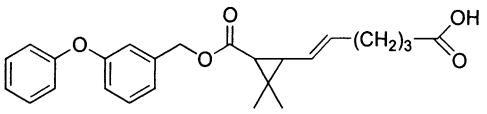
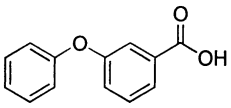
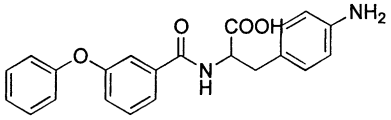
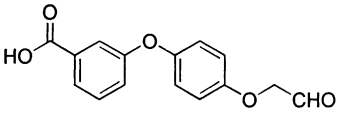
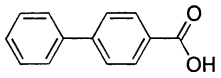
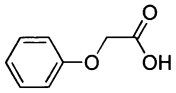
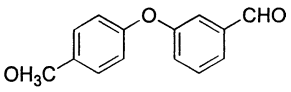
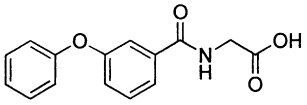
2.2. Instrument

ELISA experiments were performed in 96-well microplates (Nunc, Roskilde, Denmark) and the absorbances were read with a V_{max} microplate reader (Molecular Devices, Menlo Park, CA) in dual-wavelength mode (450–650 nm). The characterization of hapten to protein conjugate was done with a Shimadzu UV-2101PC UV–VIS scanning spectrophotometer (Shimadzu, Kyoto, Japan).

2.3. Production of hapten–protein conjugate

Because small molecular weight compounds are not immunogenic alone, haptens were conjugated with carrier proteins. The activated ester [11] was obtained from the reaction of the carboxyl group in the hapten and *N*-hydroxysuccinimide. The ester was then reacted with the amino group in the carrier protein in a separate step. This reaction was also used to conjugate hapten with enzyme. Briefly, a mixture of 40 μM permethrin hapten I, 50 μM EDC and 60 μM hydroxysulfosuccinimide in 3 ml of anhydrous DMF was stirred at room temperature overnight. BSA, OVA and THY (25 mg each) were dissolved in 6 ml of phosphate buffered saline (PBS) at 4°C. Aliquots (1 ml) of the activated hapten solution were added dropwise to the three stirred protein solutions. The reaction mixture was stirred at 4°C for 30 min, and stirring continued at room temperature overnight. The resulting product was dialyzed against PBS to eliminate the unreacted small molecular weight substances.

Table 1
Structures of coating antigen haptens[a]

Hapten	Structure
I	
II	
III	
IV	
V	
VI	
VII	
VIII	

[a] Haptens were coupled to BSA or OVA, as described in Section 2.

2.4. Immunization and analysis of the titer

Two New Zealand white rabbits were immunized intradermally with each immunogen (hapten I-BSA (rabbits #1603 and #1604), I-OVA (rabbits #1601 and #1602) and I-THY (rabbits #298 and #299)). One month after an initial immunization with 500 μg of the immunogen protein emulsified with Freund's complete adjuvant (1:1, v/v), further injections of 500 μg of the immunogen emulsified with Freund's incomplete adjuvant were given (1:1, v/v). Booster injections were given at 3-week intervals. The rabbits were bled 10 days after each boost. The serum was isolated by centrifugation for 10 min at 4°C.

The titer of the sera from each animal was screened by measuring the binding of serial dilutions to microtiter plates coated with several concentrations of I-BSA, I-OVA, I-THY, II-BSA, II-OVA, III-BSA, III-OVA, IV-BSA, V-BSA, VI-BSA, VII-BSA, VIII-BSA, and VIII-OVA. Optimal concentrations for coating antigen and antisera dilution were determined by performing two-dimensional titration experiments.

2.5. Enzyme-linked immunosorbent assay (ELISA)

The method was performed according to previous report by Shan et al. [12]. 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 washing with PBST (PBS with Tween 20: 8 g/l NaCl, 1.15 g/l Na_2HPO_4, 0.2 g/l KH_2PO_4, 0.2 g/l KCl, and 0.05% Tween, v/v), the surface of the wells were blocked with 200 μl of a 0.5% BSA in PBS for 30 min at room temperature. After another washing step, 50 μl per well of antiserum diluted in PBS with 0.2% BSA and 50 μl per well of analyte solution were added, and incubated for 1 h. Following a washing step, goat anti-rabbit HRP (1:3000 in PBST, 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 TMB 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 20–30 min with 2 M H_2SO_4 (50 μl per well). The absorbance was measured at 450 min and 650 nm. All experiments were conducted using three or four well replicates. Standard curves were obtained fitting data to a four-parameter logistic equation as follows: $y = \{(A - D)/[1 + (x/C)^B]\} + D$, 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 (I_{50}), and D is the minimum absorbance at infinite concentration.

2.6. Cross-reactivity

Data were obtained from standard curves of permethrin, phenothrin, resmethrin, and bioresmethrin

Table 2
Titration results for anti-permethrin antisera

Rabbit no.	Coating antigen[a]												
	I-BSA	I-OVA	I-THY	II-BSA	II-OVA	III-BSA	III-OVA	IV-BSA	V-BSA	VI-BSA	VII-BSA	VIII-BSA	VIII-OVA
298	H	H	–	M	H	H	M	L	L	L	L	H	H
299	H	H	–	M	M	H	H	L	M	M	M	M	H
1601	M	–	H	L	–	L	–	L	L	L	L	M	–
1602	H	–	H	L	–	H	–	L	L	L	L	H	M
1603	–	M	M	–	M	–	M	–	–	–	–	–	M
1604	–	H	H	–	H	–	M	–	–	–	–	–	H

[a] H, M, and L indicate the serum dilution factor range that produced absorbances of 0.5 after 30 min. H: more than 1:16 000; M: between 1:1000 and 1:16 000; L: less than 1:1000. Antisera #298 and #299 were raised against I-THY and #1601 and #1602 against I-OVA and #1603 and #1604 against I-BSA. '–' indicates not tested.

representative of type I pyrethroids, and fenvalerate, esfenvalerate, cyfluthrin, cypermethrin, deltamethrin, and fluvalinate as type II pyrethroids. Each compound was prepared in 30% methanol in PBS and tested in the concentration range from 0.002 to 5000 μg/l. The cross-reactivities (CR, %) were calculated as follows:

$$\mathrm{CR} = \left(\frac{I_{50} \text{ of permethrin}}{I_{50} \text{ of tested compound}} \right) \times 100$$

2.7. Matrix effect studies

To investigate the effect of solvent, permethrin standards were dissolved in methanol of varying concentration levels (10, 20, 30, 40, 60, 80, and 100% in PBS). The effect of DMSO (10, 20, 30, and 40% in PBS) was also tested. Industrial water samples were serially diluted and spiked with permethrin in concentrations ranging from 0.02 to 5000 μg/l and were analyzed for recovery.

2.8. Recovery test

Industrial waters diluted 50 or 100 times with 30% methanolic PBS were spiked with permethrin standards (0.5, 1.0, 2.5, 5.0, and 10 mg/l) to estimate the recoveries from water samples.

3. Results and discussion

3.1. Screening of antisera

To screen the titer of six antisera, the eight coating antigens were produced as shown in Table 1. Table 2 shows the titration results for antisera with the terminal bleeds using a checkerboard titration system. It is well known that the titer of different rabbits will vary even when the same antigen is used for immunization. As shown in Table 2, coating antigens I, II, III, and VIII, which included the phenoxybenzyl

Table 3
Combination data for screening with competitive ELISA[a]

Immunogen	Combination Ab/cAg	A_{max} (A)	Slope (B)	A_{min} (D)	I_{50} (μg/l)
I-THY	#298/I-BSA	0.987	0.645	0.097	299
	#298/I-OVA	1.160	0.765	0.253	269
	#298/II-BSA	0.679	0.748	0.155	118
	#298/II-OVA	0.776	0.694	0.161	171
	#298/III-BSA	0.434	0.760	0.150	52.9
	#298/III-OVA	0.544	0.881	0.172	58.0
	#298/VIII-BSA	1.050	0.789	0.302	50.1
	#298/VIII-OVA	0.727	0.670	0.088	153
	#299/I-BSA	0.840	0.956	0.075	466
	#299/I-OVA	0.727	0.647	0.036	199
	#299/II-BSA	0.458	0.605	0.127	125
	#299/II-OVA	0.607	0.661	0.132	62.7
	#299/III-BSA	0.465	0.642	0.154	45.5
	#299/III-OVA	0.202	0.516	0.047	59.6
	#299/VIII-BSA	0.647	0.677	0.095	67.4
	#299/VIII-OVA	0.514	0.699	0.038	110
I-OVA	#1601/I-BSA	0.491	0.685	0.011	79.8
	#1601/I-THY	0.986	0.749	0.158	283
	#1601/VIII-BSA	0.258	1.090	0.076	19.6
	#1602/I-BSA	0.582	0.890	0.026	265
	#1602/I-THY	0.697	0.812	0.039	209
	#1602/III-BSA	0.140	1.310	0.082	21.1
	#1602/VIII-BSA	0.427	0.980	0.126	43.2
I-BSA	#1604/I-OVA	0.641	0.893	0.172	103
	#1604/I-THY	0.584	0.926	0.156	97.6

[a] ELISA assay conditions described in methods. A, B, D and I_{50} were derived from a four-parameter logistic equation.

moiety in the structure, gave the higher titer. Generally it is easier to obtain higher titer antiserum when a rabbit is immunized with an immunogen conjugated to a large molecular weight protein such as THY rather than OVA. However, from these results, the antiserum raised against the OVA-conjugate, also gave high titers, possibly due to a higher hapten to protein ratio. The combinations of the antiserum and coating antigens were further tested with competitive inhibition experiments as shown in Table 3. Although the titer of the homologous assay was higher than that of the heterologous assay, the I_{50} was generally more than about 200 μg/l. On the other hand, with VIII-BSA as a coating antigen, a high maximum absorbance and a low I_{50} were obtained. Thus, the dilution factors for this coating antigen and the antiserum and the concentration of secondary antibody were further investigated. The standard curves plotted for each antiserum were also compared under the optimum conditions (Fig. 3). The I_{50}'s of the #298 antiserum generated by immunizing with the I-THY and the #1602 were 50 μg/l (cAg: VIII-BSA; 1:50 000, Ab: 1:8000) and 30 μg/l (cAg: VIII-BSA; 1:12 800, Ab: 1:4000), respectively. The #298 and the #1602 antiserum were used for further assay development.

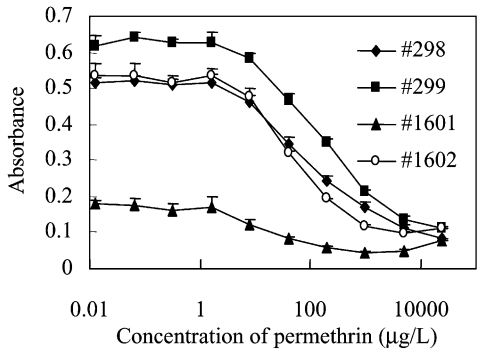


Fig. 3. Standard curves for different antisera as the competitor. Data represent mean ± S.D. of four determinations. ELISA assay conditions are as follows: #298 (1:8000), coating antigen, VIII-BSA (0.08 μg/ml), goat anti-rabbit IgG-HRP (1:3000); #299 (1:4000), coating antigen, VIII-BSA (0.15 μg/ml), goat anti-rabbit IgG-HRP (1:3000); #1601 (1:2000), coating antigen, VIII-BSA (0.3 μg/ml), goat anti-rabbit IgG-HRP (1:3000); #1602 (1:4000), coating antigen, VIII-BSA (0.3 μg/ml), goat anti-rabbit IgG-HRP (1:3000).

3.2. Assay optimization

Organic solvent or detergent is needed for this assay because pyrethroids are hydrophobic compounds. Organic solvents can influence the binding of the antibody to hapten. Table 4 shows the effect of methanol concentration on sensitivity. The results indicate that the methanol concentration significantly influenced assay sensitivity and absorbance. The maximum absorbance was decreased with an increase in methanol concentration, and absorbance and background were inversely increased in more than 80% methanol. In the case of #298 antiserum, the maximum absorbance was increased with an increase in methanol concentration (data not shown). Fig. 4 illustrates the effects of DMSO. The maximum absorbance was significantly decreased with an increase in DMSO concentration because of the inhibition of the binding of antibody to hapten. Consequently, methanol was used in this assay. The optimum methanol concentration

Table 4
Effect of methanol concentration[a]

MeOH[b] (%)	A_{max} (A)	Slope (B)	I_{50} (μg/l) (C)	A_{min} (D)	A/D	R
0	0.647	0.962	29.2	0.133	4.9	0.995
10	0.547	0.965	22.7	0.125	4.4	0.997
20	0.561	0.966	28.3	0.149	3.8	0.997
30	0.508	0.913	21.8	0.145	3.5	0.997
40	0.464	1.040	32.4	0.170	2.7	0.992
60	0.435	1.010	39.8	0.234	1.9	0.990
80	1.210	0.256	1.75×10^{6}	0.364	3.3	0.880
100	1.170	0.057	1.02×10^{-8}	1.640	0.7	−0.905

[a] ELISA assay conditions were as follows: coating antigen; VIII-BSA (0.3 μg/ml); antiserum; #1602 (1:4000); goat anti-rabbit IgG-HRP (1:3000).

[b] Concentration of methanol in permethrin standard solution (PBS/MeOH) prior to analysis.

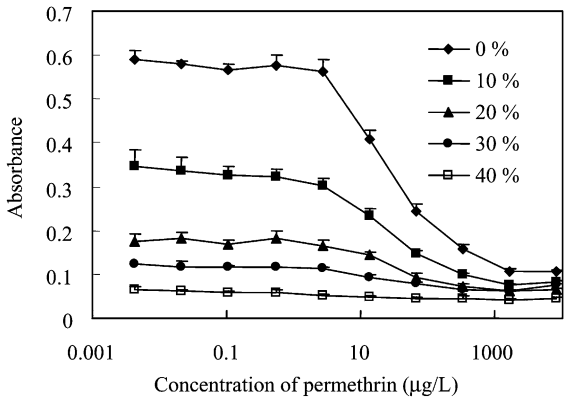


Fig. 4. Effect of DMSO concentration. Data represent mean ± S.D. of four determinations. ELISA assay conditions are as follows: coating antigen VIII-BSA (0.3 μg/ml); antiserum #1602 (1:4000); goat anti-rabbit IgG-HRP (1:3000). DMSO concentrations are expressed as v/v.

was chosen based on the ratio of maximum absorbance to minimum absorbance for permethrin standard curve (A/D) and I_{50} value obtained from standard curve. As mentioned above, permethrin is a non-polar compound, so that permethrin is easily adsorbed to glass or plastic vessels. Therefore, 30% methanol concentration was selected for subsequent experiments. Fig. 5 illustrates the typical standard curve for permethrin under the optimum conditions. The lower detection limit (0.3 μg/l) was estimated as the concentration that corresponded to the absorbance

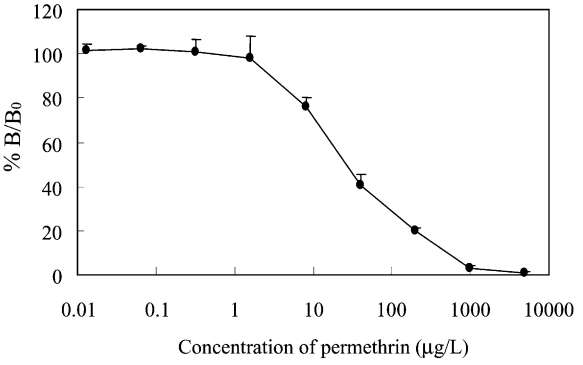


Fig. 5. Inhibition curve for permethrin obtained from a four-parameter curve fit. Data represent mean ± S.D. of four determinations. ELISA assay conditions are as follows: coating antigen VIII-BSA (0.3 μg/ml); antiserum #1602 (1:4000); goat anti-rabbit IgG-HRP (1:3000).

of the control (zero concentration of analyte) minus three times the standard deviation of the control [13].

3.3. Cross-reactivity

Table 5 shows the results of the cross-reactivities of various pyrethroids. The structures of pyrethroid insecticide analogs are as shown in Fig. 2. *Trans*-, *cis*-permethrin, phenothrin, resmethrin and bioresmethrin (type I pyrethroids), and cypermethrin, cyfluthrin, esfenvalerate, fenvalerate, deltamethrin and fluvalinate (type II pyrethroids) were tested. The cross-reactivities of phenothrin were 136% with antiserum from #1602 and 113% with #298, which are equivalent or greater than permethrin cross-reactivity. Since the difference in structure between permethrin and phenothrin is the terminal chlorines, the antibody recognized both similarly because the hapten used was conjugated to the carrier protein through the cyclopropane end of the molecule at the chlorine position (Fig. 1). The #1602 antiserum recognized resmethrin (10.9%) and bioresmethrin (25.3%), and no cross-reactivity was measured with the #298 antiserum. Slight cross-reactivity was measured to cypermethrin, which is a type II pyrethroid, no inhibition was observed for the other type II pyrethroids

Table 5
Cross-reactivities of pyrethroids

Analyte	Cross-reactivity (%)[a]	
	#1602[b]	#298[c]
Permethrin	100	100
Trans-permethrin	25.9	30.2
Cis-permethrin	142	120
Phenothrin	136	113
Resmethrin	10.9	0.005
Bioresmethrin	25.3	0.013
Cyfluthrin	NI	NI
Cypermethrin	2.4	0.008
Deltamethrin	NI	NI
Esfenvalerate	NI	NI
Fenvalerate	NI	0.006
Fluvalinate	NI	0.004

[a] Cross-reactivity was calculated as follows: (I_{50} of permethrin/I_{50} of analyte tested) × 100.

[b] The antiserum generated by immunization with I-OVA conjugate.

[c] The antiserum generated by immunization with I-THY conjugate.

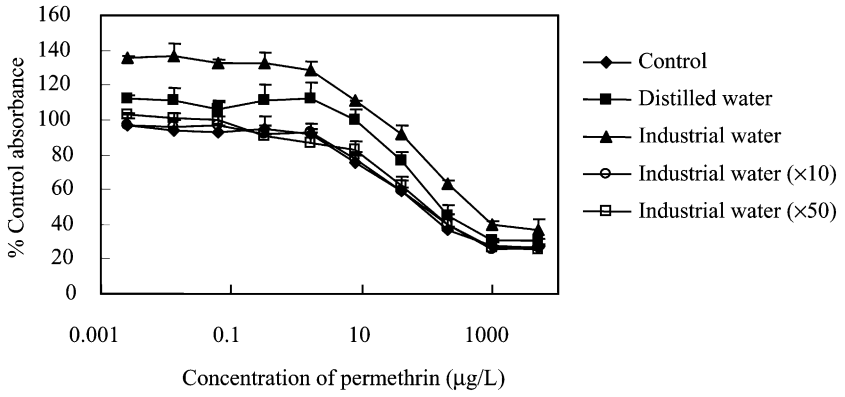


Fig. 6. Effect of the dilution of the matrix on the reliability of ELISA. Data represent mean ± S.D. of four determinations. ELISA assay conditions are as follows: coating antigen VIII-BSA (0.3 μg/ml); antiserum #1602 (1:4000); goat anti-rabbit IgG-HRP (1:3000). Samples were diluted with 30% methanolic PBS.

tested. From these results, it was found that the #1602 antiserum recognized the type I pyrethroids more than the #298 antiserum. It suggests that binding to this antibody is inhibited by the cyano group, so that no cross-reactivity was measured with type II pyrethroids with a cyano group. In comparison with the previous reports, this ELISA, as a general assay, more clearly distinguishes type I from type II pyrethroids.

Table 6
Recovery test of permethrin in industrial water

Spiked (ppm)	Theoretical (ppb)	Measure (ppb)	Recovery (%)	Mean ± S.D.
0.5[a]	10	10.6	106	
		10.1	101	
		13.8	138	119 ± 17.9
		12.9	129	
1.0[b]	10	10.0	100	
		10.6	106	
		12.8	128	108 ± 14
		9.7	97	
2.5[a]	50	55.2	110	
		58.2	116	
		62.8	127	119 ± 7.7
		62.2	124	
5.0[b]	50	55.3	110	
		56.9	114	111 ± 2.6
		54.3	109	
5.0[a]	100	99.1	99	
		125.9	126	114 ± 17.4
		98.4	98	
10.0[b]	100	129	129	
		112.4	112	112 ± 16.5
		96.2	96	

[a] Fifty times dilution with 30% methanolic PBS.
[b] Hundred times dilution with 30% methanolic PBS.

Table 7
Recovery test of type I pyrethroids

Analyte	Spiked[a] (ppm)	Theoretical[b] (ppm)	Measure (ppm)	Recovery (%)	Mean ± S.D.
Phenothrin	2.5	50	73.0	146	
			71.2	142	144 ± 2.1
			71.3	143	
	5.0	100	196	196	
			236	236	204 ± 37
			229	229	
Resmethrin	2.5	50	3.06	6.1	
			2.83	5.7	5.9 ± 0.2
			2.93	5.9	
	5.0	100	14.7	14.7	
			18.0	18.0	
			21.1	21.1	16.5 ± 3.9
			12.2	12.2	
Bioresmethrin	2.5	50	2.68	5.4	
			7.30	14.6	9.2 ± 4.8
			3.74	7.5	
	5.0	100	19.3	19.3	
			23.0	23.0	
			17.9	17.9	19.1 ± 2.9
			16.1	16.1	

[a] Industrial water were spiked with 2.5 and 5.0 ppm pyrethroid standard.
[b] Fifty times dilution with 30% methanolic PBS.

3.4. Recovery test

To estimate reliability, a recovery test was performed using an industrial water sample. When the standard curve was plotted with industrial water spiked with permethrin standards, the absorbance was significantly increased. Since there are many metal ions and other matrices in industrial water, dilution with PBS was needed. In the case of water samples, matrix effect largely are eliminated by prior solid phase extraction, because pyrethroids, such as permethrin are hydrophobic compounds and are trapped with a C18 column. However, considering a simple pretreatment without clean-up, it is necessary to test the matrix effects from a real sample. The effect of dilution factors on sensitivity was tested as shown in Fig. 6. The results indicate that the matrix effect was eliminated with more than 10 times dilution with 30% methanolic PBS. Table 6 shows the recovery of permethrin from industrial water. Industrial waters diluted 50 and 100 times with 30% methanolic PBS were tested in the linear range from 10 to 100 μg/l. The recoveries of permethrin (>100%) were satisfactory. In the case of the sample diluted 100 times, the standard deviation for recovery was smaller than for the samples diluted 50 times, and recovery was also somewhat lower, although not significantly different. This result suggests that the background in the inhibition curve was affected by ions in industrial water. However, recoveries indicate a reasonable result would be obtained even if a 50 times dilution was selected. Furthermore, we tested the recoveries of other type I pyrethroids using a permethrin standard curve. Industrial waters were spiked with 2.5 and 5.0 mg/l phenothrin, resmethrin, or bioresmethrin standard (dissolved in methanol), and were diluted 50 times with 30% methanolic PBS (Table 7). When recoveries of each sample were calculated, we can obtain the values that reflect the relative cross-reactivities as shown in Table 5. Thus, it is possible to calculate the amount of type I pyrethroid using this assay by correcting with a cross-reactivity factor.

4. Conclusions

We developed an ELISA that is more selective for the detection of type I pyrethroid insecticides than type

II. Polyclonal antibody was generated by immunizing with a permethrin analog as a hapten conjugated to ovalbumin. The I_{50} of the optimized immunoassay was 30 μg/l for permethrin and 20 μg/l for phenothrin. Though the type I pyrethroids, such as permethrin, phenothrin, resmethrin, and bioresmethrin were recognized with #1602 antiserum, no cross-reactivities were measured for the type II pyrethroids, such as cypermethrin, cyfluthrin, deltamethrin, esfenvalerate, fenvalerate, and fluvalinate. This assay can be used in monitoring studies to distinguish between types I and II pyrethroids due to class-specific antibodies for type I pyrethroids. Class-specific assays can be used in a variety of ways in environmental chemistry. For example, such assays in conjunction with other general and specific assays can be used in an array format as an environmental taster. Such an antibody array can provide both quantitative and qualitative information on pyrethroid concentration in complex mixtures [14]. Class-specific assays can be used in a tiered system where the class assay is performed first. If it gives a positive response other immunochemical and/or chromatographic assays can be used to speciate the particular compounds present. This has the advantage of excluding samples which have no detectable residues and ranking the others for order of analysis. A class-specific assay also can be used as a highly sensitive and specific detector following chromatographic separation. For highly lipophilic compounds like pyrethroids one can concentrate them on a hydrophobic precolumn and then elute the separate compounds with a methanol gradient [15]. As shown in this study, methanol at even high levels can be tolerated by the assay.

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References

[1] F. Matsumura, Toxicology of Insecticides, 2nd Edition, Plenum Press, New York, 1985.
[2] T.F. Zabel, J. Seager, S.D. Oakley, Proposed environmental quality standards for list II substances in water: mothproofing agents, Water Research Centre Technical Report 201, WRC, Marlow, Buckinghamshire, UK.
[3] S.W. Lindsay, P.L. Alonso, J.R.M.A. Schhellenberg, J. Hemingway, J.H. Adiamah, F.C. Shenton, M. Jawara, B.M. Greenwood, Trans. R. Soc. Med. Hyg. 87 (1993) 45.
[4] P. Bottomley, P.G. Baker, Analyst 85 (1984) 90.
[5] T.R. Roberts, Trends Anal. Chem. 4 (1985) 3.
[6] I.A. Fleet, L.W. Tetler, J.J. Monaghan, Org. Mass Spectrom. 28 (1993) 626.
[7] B.D. Hammock, R.O. Mumma, Potential of immunochemical technology for pesticide analysis, in: J. Harvey, G. Zweig (Eds.), Pesticide Analytical Methodology, ACS Symposium Series 136, American Chemical Society, Washington, DC, 1980, p. 321.
[8] L. Dohrman, Am. Lab. 23 (1991) 29.
[9] L.H. Stanker, C. Bigbee, J. Van Emon, B. Watkins, R.H. Jensen, C. Morris, M. Vanderlaan, J. Agric. Food Chem. 37 (1989) 834.
[10] J.H. Skerritt, A.S. Hill, D.P. McAdam, L.H. Stanker, J. Agric. Food Chem. 40 (1992) 1287.
[11] P. Tijssen, Practice and Theory of Enzyme Immunoassay, Elsevier, Amsterdam, The Netherlands, 1985.
[12] G. Shan, D.W. Stoutamire, I. Wengatz, S.J. Gee, B.D. Hammock, J. Agric. Food Chem. 47 (1999) 2145.
[13] H.E. Grotjan, B.A. Keel, Data interpretation and quality control, in: E. Diamandis, T. Christopoulos (Eds.), Immunoassay, Academic Press, San Diego, CA, 1996.
[14] M. Wortberg, G. Jones, S.B. Kreissig, D.M. Rocke, S.J. Gee, B.D. Hammock, Anal. Chim. Acta 319 (1996) 291.
[15] P.M. Kramer, Lab. Robotics Automat. 9 (1997) 81.