Enzyme-Linked Immunosorbent Assay for the Pyrethroid Permethrin

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Permethrin is a predominant pyrethroid widely used in agriculture and public health. A competitive enzyme-linked immunosorbent assay (ELISA) for the detection of permethrin was developed. Two haptens, the *trans*- and *cis*-isomers of 3-(4-aminophenoxy)benzyl-3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropanecarboxylate, were synthesized and conjugated with thyroglobulin as immunogens. Four antisera were generated and screened against six different coating antigens. The resulting ELISA has an I_{50} value of 2.50 μg/L and relatively low cross-reactivities with other major pyrethroids, such as esfenvalerate, cypermethrin, deltamethrin, and cyfluthrin. Methanol was found to be the best solvent for this ELISA, with optimal sensitivity observed at a concentration of 40% (v/v). The assay parameters are unchanged at pH values between 5.0 and 8.0, whereas higher ionic strengths (>0.2 M PBS) strongly suppress the absorbances. River water samples fortified with permethrin were analyzed according to this method and validated by GC-MS. Good recoveries and correlation with spike levels were observed, suggesting this immunoassay is valuable for environmental monitoring and toxicological studies at parts per trillion levels of permethrin.

Keywords: *Permethrin; ELISA; environmental monitoring; toxicological studies; pyrethroid*

INTRODUCTION

Pyrethroid insecticides are likely to become more widely used, particularly because organophosphate insecticides, which currently dominate the market, are being phased out in the United States. In 1997, >600000 lb of pyrethroid active ingredient were applied to various crops throughout California. Among those pyrethroids used in California, permethrin contributes >53% of the total amount (Pesticide Use Report, 1997). Permethrin is highly toxic to some nontarget invertebrates and fish (Tomlin, 1997) and has high bioconcentration factors in some aquatic animals (Schimmel et al., 1983). This compound has exhibited detrimental effects to aquatic species at low parts per billion levels of exposure (Kidd and James, 1995; Tomlin, 1997). In addition to ecosystem health, humans consume contaminated water and aquatic food, leading to potential exposure to pyrethroids. 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 (He et al., 1988, 1989; Repetto, 1996). Some pyrethroids may cause lymph node and splenic damage as well as carcinogenesis (Hallenbeck and Cunningham-Burns, 1985). 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.

Current analytical methods for the detection of permethrin involve multistep sample cleanup procedures followed by gas chromatography and detection by electron capture (GC-EC) or high-performance liquid chromatography (HPLC) (Wells et al., 1994; Boccelli et al., 1993; Hengel et al., 1997). Such methods are relatively time-consuming and expensive and, particularly, are not suitable for large sample screening. An immunoassay would provide a sensitive, selective, and rapid method for the detection of permethrin at trace levels (Hammock and Mumma, 1980; Hammock et al., 1990). Several immunoassays have been developed for the detection of permethrin (Stanker et al., 1989; Skerritt et al., 1992; Bonwick et al., 1994); however, these do not have the desired sensitivity for trace level analysis in environmental samples without significant sample cleanup and concentration. In this paper, we describe the development of a sensitive immunoassay for permethrin, the evaluation of the assay's performance in water matrix, and method validation by GC-MS.

MATERIALS AND METHODS

Chemicals and Immunoreagents. All pyrethroid standards were obtained from Riedel de Haen (Seelze, Germany). Organic starting materials for hapten synthesis were purchased from Aldrich Chemical Co. (Milwaukee, WI) and Fisher Scientific (Pittsburgh, PA). Thin-layer chromatography (TLC) was performed using 0.2 mm precoated silica gel 60 F254 on glass plates from E. Merck (Darmstadt, Germany), and detection was made by ultraviolet light or iodine vapor stain. Flash chromatographic separations were carried out on Baker silica gel (40 μm average particle size) using the indicated solvents where the → notation denotes a stepwise concentration gradient.

The coupling reagents were purchased from Aldrich. Goat anti-rabbit (GAR) immunoglobulin conjugated to horseradish

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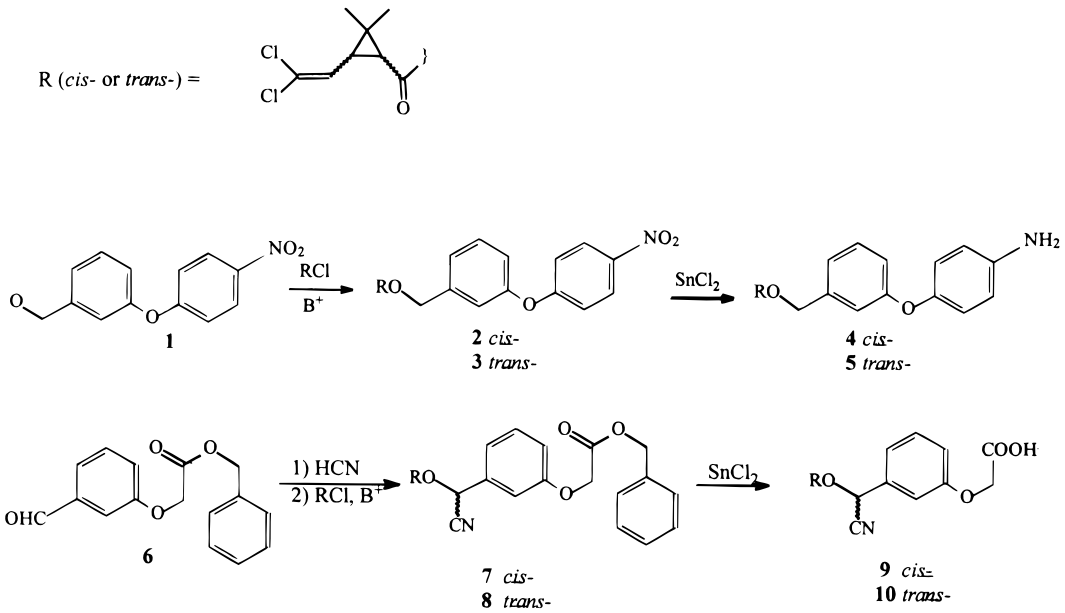


Figure 1. Scheme for hapten synthesis.

peroxidase (HRP), bovine serum albumin (BSA), thyroglobulin, Tween 20, and 3,3′,5,5′-tetramethylbenzidine (TMB) were purchased from Sigma Chemical Co. (St. Louis, MO).

Instruments. 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. Gas−liquid chromatograms were determined on an HP 5890 (Hewlett-Packard Corp., Avondale, PA) with a 15 m, 0.32 mm i.d. capillary column filled with a 0.25 μm film of dimethylpolysiloxane containing 5% of methyls substituted by phenyls (J&W Scientific, Folsom, CA). Radial chromatographic separations were carried out on a Chromatotron apparatus (Harrison Research, Inc., Palo Alto, CA), using 2-mm silica gel plates. Fast atom bombardment mass spectra (FAB-MS) were obtained on a ZAB-2SE mass spectrometer (VG Analytical, Wythenshawe, U.K.), using high-energy cesium ions at a density flux of 1−2 mA and 35−38 kV to generate secondary $[MH]^+$ ions. The liquid matrix was a 1:1 mixture of glycerol and 3-nitrobenzyl alcohol, and cesium iodide was used for mass calibration at a dynamic resolution of 5000:1. ELISA experiments were performed in 96-well microplates (Nunc, Roskilde, Denmark), and the absorbances were measured with a Vmax microplate reader (Molecular Devices, Menlo Park, CA) in dual-wavelength mode (450−650 nm).

Hapten Synthesis and Verification. Syntheses of the haptens were carried out as outlined in Figure 1. All reactions were straightforward and followed procedures employed in preceding publications (Wengatz et al., 1998; Shan et al., 1999a). NMR spectral data supported all structures, and mass spectra further confirmed the structures of the final haptens.

3-(4-Nitrophenoxy)benzyl Alcohol (1). 3-(4-Nitrophenoxy)benzaldehyde (Loewe and Urbanietz, 1967) (3.0 g, 13.2 mmol) was added in portions over 1 h to a stirred solution of sodium borohydride (280 mg., 7.4 mmol) in ethyl alcohol (15 mL). After 3 h, the mixture was diluted with water, and the resulting solid was filtered, water washed, and dried. This was dissolved in butyl chloride/methylene chloride and filtered through silica gel (3 g) followed by methylene chloride to remove polar impurities. The stripped residue was recrystallized from butyl chloride by dilution with hexane to yield in two crops 2.86 g (95%) of a white solid: mp 73−75.5 °C; ^{1}H NMR ($CDCl_3$) δ 1.80 (bs, 1 H, OH), 4.73 (s, 2 H, CH_2), 7.02 (d, J = 9.2 Hz, 2 H, NO_2Ar), 7.03−8.02 (m, 4 H, Ar), 8.20 (d, J = 9.2 Hz, NO_2Ar).

3-(4-Nitrophenoxy)benzyl (±)-*cis*-3-(2,2-Dichloroethenyl)-2,2-dimethylcyclopropanecarboxylate [(±)-*cis*-4-Nitropermethrin] (2). (±)-*cis*-2-(2,2-Dichloroethenyl)-3,3-dimethylcyclopropanecarboxylic acid (0.75 g, 3.59 mmol) in chloroform (1 mL) containing 1 μL of dimethylformamide (DMF) was treated with thionyl chloride (0.52 mL, 7.12 mmol) and stirred under N_2 in an oil bath at 65 °C for 2 h. The mixture was stripped briefly of solvents, hexane (2 mL) was added, and the mixture was restripped. The residue was dissolved in chloroform (3 mL) and added all at once to an ice-cooled solution of **1** (0.88 g, 3.59 mmol) in chloroform (3 mL) and pyridine (0.348 mL), and the mixture was allowed to stand at ambient temperature for 3 h. The reaction mixture was washed in order with water, diluted HCl solution, saturated $NaHCO_3$ solution, and water and dried over Na_2SO_4. Stripping of solvent gave a pale yellow oil, which was flash chromatographed on silica gel (20 g) (5% → 100% CH_2Cl_2 in hexane) to recover after vacuum stripping 1.36 g (87%) of **2**: TLC R_f 0.27 (hexane/CH_2Cl_2 = 1:1); ^{1}H NMR ($CDCl_3$) δ 1.25 (s, 6 H, CH_3), 1.90 (d, J = 8.5 Hz, 1 H, $CHCO_2$), 2.06 (dd, J = 8.7, 8.7 Hz, 1 H, CH), 5.11 (s, 2 H, CH_2Ar), 6.24 (d, J = 8.9 Hz, 1 H, C=CH), 7.03 (d, J = 9.2 Hz, 2 H, NO_2Ar), 7.03−7.46 (m, 4 H, Ar), 8.21 (d, J = 9.2 Hz, 2 H, NO_2Ar).

3-(4-Nitrophenoxy)benzyl (±)-*trans*-3-(2,2-Dichloroethenyl)-2,2-dimethylcyclopropanecarboxylate [(±)-*trans*-4-Nitropermethrin] (3). Using (±)-*trans*-2-(2,2-dichloroethenyl)-3,3-dimethylcyclopropanecarboxylic acid in the same reaction procedure as described for **2** above, the product was obtained in 93% yield as a colorless oil: TLC R_f 0.30 (hexane/CH_2Cl_2 = 1:1); ^{1}H NMR ($CDCl_3$) δ 1.19 (s, 3 H, CH_3), 1.28 (s, 3 H, CH_3), 1.66 (d, J = 5.3 Hz, 1 H, $CHCO_2$), 2.27 (dd, J = 5.3, 8.3 Hz, 1 H, C=C−CH), 5.12 (d, J = 12.8 Hz, 1 H, OHCH), 5.18 (d, J = 12.8 Hz, 1 H, OHCH), 5.61 (d, J = 8.3 Hz, 1 H, C=CH), 7.00−8.23 (m, 8 H, Ar).

3-(4-Aminophenoxy)benzyl (±)-*cis*-3-(2,2-Dichloroethenyl)-2,2-dimethylcyclopropanecarboxylate [(±)-*cis*-4-Aminopermethrin] (4). A solution of **2** (218 mg, 0.5 mmol) in ethanol (2 mL) was treated with stannous chloride dihydrate (565 mg, 2.5 mmol), and the mixture was stirred in an oil bath at 70 °C for 30 min, then cooled, diluted with ether and water, and treated with Celite (0.45 g) and $NaHCO_3$ (0.42 g, 5 mmol) in portions (foaming). The mixture was filtered, and solids were washed with ether. Stripping the organic phase gave a heavy oil. TLC showed one primary product spot, which darkened, on exposure to light. This was flash chromatographed on silica gel (5 g) (50% CH_2Cl_2/hexane → 2−10% ether in hexane) to recover 25 mg of **2** and 144 mg (80%) of **4** as a yellow oil: TLC R_f 0.26 (CH_2Cl_2); ^{1}H NMR ($CDCl_3$) δ 1.23 (s, 6 H, CH_3), 1.87 (d, J = 8.5 Hz, 1 H, $CHCO_2$), 2.03 (dd, J = 8.66, 8.66 Hz, 1 H, C=C−CH), 3.5 (bs, 2 H, NH_2), 5.03 (s, 2 H, CH_2Ar), 6.26 (d, J = 8.86 Hz, 1 H, C=CH), 6.67−7.29 (m, 8 H,

Ar); addition of D_2O removed the NH_2 peak; FAB-MS *m/z* calcd for $[M]^+ = C_{21}H_{21}Cl_2NO_3$ 405, obsd 405.

3-(4-Aminophenoxy)benzyl (±)-*trans*-3-(2,2-Dichloroethenyl)-2,2-dimethylcyclopropanecarboxylate [(±)-*trans*-4-Aminopermethrin] (5). A sample of **3** (218 mg, 0.5 mmol) was reduced with stannous chloride dihydrate (620 mg, 3.0 mmol) according to the procedure described for **4** above to give 161 mg (80%) as a yellow oil: TLC R_f 0.2 (CH_2Cl_2); ^{1}H NMR ($CDCl_3$) δ 1.18 (s, 3 H, CH_3), 1.27 (s, 3 H, CH_3), 1.65 (d, J = 5.4 Hz, 1 H, $CHCO_2$), 2.25 (dd, J = 5.3, 8.4 Hz, 1 H, C=C−CH), 3.2 (bs, 2 H, NH_2), 5.06 (s, 2 H, CH_2), 5.60 (d, J = 8.4 Hz, 1 H, C=CH), 6.67−7.29 (m, 8 H, Ar); FAB-MS *m/z* calcd for $[M]^+ = C_{21}H_{21}Cl_2NO_3$ 405, obsd 405.

Benzyl 3-[(±)-Cyano[(±)-*cis*-3(2,2-dichloroethenyl)-2,2-dimethylcyclopropanecarbonyloxy]methyl]phenoxyacetate (7). Benzyl 2-(3-formylphenoxy)acetate (**6**) (0.970 g, 3.59 mmol) was converted to the cyanohydrin as described in a previous publication (Wengatz et al., 1998). Meanwhile, (±)-*cis*-3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropanecarboxylic acid (0.75 g, 3.59 mmol) was converted to the acid chloride as described above for **2**. The acid chloride was esterified with the cyanohydrin according to the procedure described above. The reaction mixture was washed with 1 N HCl solution (5 mL), saturated Na_2CO_3 solution, and water, dried ($MgSO_4$), and then filtered and stripped to give the crude product as a yellow-orange oil. This was flash chromatographed on silica gel (25 g) (10 → 100% CH_2Cl_2 in hexane) to recover 0.92 g (52%) of pure **7** showing one spot on TLC: R_f 0.40 (CH_2Cl_2).

A 76 mg sample of **7** was separated into its two diastereoisomer pairs by radial chromatography. The sample was applied to a 2 mm silica gel plate in 25% chlorobutane in hexane and eluted with 5% 1,2-dimethoxyethane in hexane. Separation of the isomer pairs was complete to give 40.7 mg of the higher R_f isomer pair [^{1}H NMR ($CDCl_3$) δ 1.29 (s, 3 H, CH_3), 1.30 (s, 3 H, CH_3), 1.89 (d, J = 8.4 Hz, 1 H, $CHCO_2$), 2.12,(dd, J = 8.5, 8.7 Hz, 1 H, C=C−CH), 4.70 (s, 2 H, CH_2), 5.25 (s, 2 H, CH_2), 6.18 (d, J = 8.8 Hz, 1 H, C=CH), 6.31 (s, 1 H, CHCN), 6.95−7.39 (m, 9 H, Ar); ^{13}C NMR ($CDCl_3$) δ 14.8, 28.1, 28.9, 31.0, 33.4, 62.3, 65.4, 67.1, 114.2, 116.0, 116.4 (2 C), 121.0, 121.8, 123.8, 128.5 (2 C), 128.6, (2 C), 130.5, 133.1, 135.0, 158.2, 168.2, 168.5] and 27.6 mg of the lower R_f isomer pair [^{1}H NMR ($CDCl_3$) δ 1.21 (s, 3 H, CH_3), 1.24 (s, 3 H, CH_3), 1.89 (d, J = 8.4 Hz, 1 H, $CHCO_2$), 2.15 (dd, J = 8.6 Hz, 1 H, C=C−CH), 4.70, (s, 2 H, OCH_2CO_2), 5.25 (s, 2 H, $PhCH_2$), 6.19 (d, J = 8.7 Hz, 1 H, C=CH), 6.35 (s, 1 H, CHCN), 6.94−7.39 (m, 9 H, Ar); ^{13}C NMR ($CDCl_3$) δ 14.8, 28.1, 28.8, 31.0. 33.5, 62.3, 65.4, 67.2, 114.4, 115.9, 116.3 (2 C), 121.0, 122.0, 123.7, 128.5 (2 C), 128.6, (2 C), 130.5, 133.4, 134.9, 158.2, 168 2, 168.5].

Benzyl 3-[(±)-Cyano[(±)-*trans*-3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropanecarbonyloxy]methyl]phenoxyacetate (8). By substituting (±)-*trans*-3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropanecarboxylic acid in the same reaction procedure as described for **7** above, pure **8** was obtained in 55% yield: TLC R_f 0.35 (CH_2Cl_2).

A 76 mg sample of **8** was separated into its two diastereoisomer pairs by radial chromatography as described above to give 40.5 mg of the higher R_f isomer pair [^{1}H NMR ($CDCl_3$) δ 1.22 (s, 3 H, CH_3), 1.33 (s, 3 H, CH_3), 1.65 (d, J = 5.3 Hz, 1 H, $CHCO_2$), 2.29 (dd, J = 5.3, 8.2 Hz, 1 H, C=C−CH), 4.69 (s, 2 H, CH_2CO_2), 5.24, (s, 2 H, OCH_2Ph), 5.59 (d, J = 8.2 Hz, 1 H, C=CH), 6.35 (s, 1 H, CHCN), 6.94−7.4 (m, 9 H, Ar); ^{13}C NMR ($CDCl_3$) δ 19.9, 22.4, 30.4, 33.8 (2 H), 62.6, 65.3, 67.1, 114.3, 116.0, 116.4 (2 C), 121.0, 122.9, 126.0, 128.5 (2 C), 128.6 (2 C), 130.5, 133.2, 134.9, 158.2, 168 2, 169.2] and 25.5 mg of the lower R_f isomer pair [^{1}H NMR ($CDCl_3$) δ 1.18 (s, 3 H, CH_3), 1.26 (s, 3 H, CH_3), 1.66 (d, J = 5.3 Hz, 1 H, CH_2CO_2), 2.32 (dd, J = 5.3, 8.2 Hz, 1 H, C=C−CH), 4.69 (s, 2 H, OCH_2CO_2), 5.25 (s, 2 H, OCH_2Ph), 5.62 (d, J = 8.2 Hz, 1 H, C=CH), 6.37 (s, 1 H, CHCN), 6.94−7.41 (m 9 H, Ar);^{13}C NMR ($CDCl_3$) δ 20.0, 22.4, 30.2, 33.8, 33.9, 62.6, 65.4, 67.2, 114.3, 115.9, 116.3, 116.4, 121.0, 123.1, 126.0, 128.5 (2 C), 128.6 (2 C), 130.5, 133.4, 135.0, 158.2, 168.2, 169.3].

3-[(±)-Cyano[(±)-*cis*-3(2,2-dichloroethenyl)-2,2-dimethylcyclopropanecarbonyloxy]methyl]phenoxyacetic Acid (9). A solution of **7** (200 mg, 0.409 mmol) in CH_2Cl_2 (0.5 mL) was stirred under nitrogen, and iodotrimethylsilane (77 μL, 0.54 mmol) was added. After 3 h at ambient temperature and 1 h at 35 °C, the mixture was added to methanol (0.5 mL). After 5 min, it was diluted with water and extracted with CH_2Cl_2, and the extracts were washed with water and stripped. The resulting gum was immediately flash chromatographed on silica gel (5 g) eluting with CH_2Cl_2, then 40% EtOAc in hexane followed by 20 → 40% EtOAc in CH_2Cl_2 containing 1.5% acetic acid. The stripped product slowly crystallized on standing to 128 mg (78%) of a white solid. The ^{1}H NMR ($CDCl_3$) spectrum clearly indicated a mixture of two diastereoisomer pairs in a ratio of about 3:2 as follows: δ 1.22 (s, 3H, CH_3), 1.26 (s, 3H, CH_3), 1.30 (s, 3H, CH_3), 1.32 (s, 3H, CH_3), 1.92 (d, J = 8.4 Hz, 2 × 1 H, $CHCO_2$), 2.15 (dd, J = 8.6, 11.2 Hz, 1 H, C=C−CH), 2.17 (dd, J = 8.6, 11.2 Hz, 1 H, C=C−CH), 4.74 (s, 2 × 2 H, CH_2), 6.19 (d, J = 8.8 Hz, 1 H, C=CH), 6.20 (d, J = 8.7 Hz, 1 H, C=CH), 6.36 (s, 1 H, CHCN), 6.39 (s, 1 H, CHCN), 7.00−7.44 (m, 2 × 4 H, Ar) (the underlined values are the more intense of the pair): FAB-MS *m/z* calcd for $[M + H]^+ = C_{18}H_{18}Cl_2NO_5$ 398, obsd 398.

3-[(±)-Cyano[(±)-*trans*-3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropanecarbonyloxy]methyl]phenoxyacetic Acid (10). A solution of **8** (200 mg, 0.409 mmol) in CH_2Cl_2 (0.5 mL) was cleaved with iodotrimethylsilane, and the reaction mixture was worked up as described above for the preparation of **9** to give a slightly gummy white solid. This was triturated with a small amount of 50% butyl chloride/hexane and filtered to yield 97 mg (60%) of **10** as a white solid. The ^{1}H NMR ($CDCl_3$) spectrum clearly indicated a mixture of two diastereoisomer pairs in a ratio of about 3:2: ^{1}H NMR ($CDCl_3$) δ 1.19 (s, 3 H, CH_3), 1.23 (s, 3 H, CH_3), 1.26 (s, 3 H, CH_3), 1.34 (s, 3 H, CH_3), 1.67 (d, J = 5.3 Hz, 1 H, $CHCO_2$), 1.69 (d, J = 5.3 Hz, 1 H, $CHCO_2$), 2.29 (dd, J = 5.3, 8.2 Hz, C=C−CH), 2.33 (dd, J = 5.4, 8.3 Hz, 1 H, C=C−CH), 4.73 (s, 2 × 2 H, CH_2), 5.60 (d, J = 8.2 Hz, 1 H, C=CH), 5.63 (d, J = 8.1 Hz, 1 H, C=CH), 6.40 (s, 1 H, CHCN), 6.41 (s, 1 H, CHCN), 6.98−7.43 (m, 2 × 4 H, Ar) (the underlined values are the more intense of the pair); FAB-MS *m/z* calcd for $[M + H]^+ = C_{18}H_{18}Cl_2NO_5$ 398, obsd 398.

Hapten Conjugation. Conjugates were synthesized using a water-soluble carbodiimide method for acids and a diazotization method for anilines (Tijssen, 1985; Erlanger, 1973). To obtain the immunogens, haptens **4** and **5** were conjugated to thyroglobulin. Coating antigens were made by coupling haptens **4**, **5**, **9**, and **10**, and *trans*-and *cis*-permethrin acids (PA) to BSA.

Conjugates of Haptens 4 and 5 with Thyroglobulin and BSA. Hapten **4** or **5** (0.10 mmol) was dissolved in 4 drops of ethanol and treated with 1 mL of 1 N HCl. The resulting solution was stirred in an ice bath as 0.5 mL of 0.20 M sodium nitrite was added. DMF (0.4 mL) was then added dropwise to give a homogeneous solution, which was divided into two equal aliquots (each aliquot was used for one protein). Fifty milligrams of thyroglobulin or 45 mg of BSA was dissolved in 5 mL of 0.2 M borate buffer (pH 9.6) and 1.5 mL of DMF. Aliquots (~0.9 mL each) of the activated hapten solution were added dropwise to the two stirred protein solutions. The reaction mixture was stirred in an ice bath for 45 min and then dialyzed against PBS over 72 h at 4 °C. The purified conjugates were suspended in water and stored in aliquots at −80, −20, and 4 °C.

Conjugates of Haptens 9, 10, (±)-*trans*-PA, and (±)-*cis*-PA with BSA. Hapten (0.025 mmol) was dissolved in 2 mL of dry DMF, and then 6 mg (0.05 mmol) of *N*-hydroxysuccinimide (NHS) and 5.8 mg (0.03 mmol) of 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide hydrochloride (EDC) were added. The reaction mixture was stirred overnight at room temperature. Forty-five milligrams of BSA was dissolved in 8 mL of PBS and 2 mL of carbonate buffer (pH 9). The activated hapten was added dropwise to the protein solution. The mixture was stirred for 30 min at room temperature and for 6 h at 4 °C.

The solution was then dialyzed against PBS over 72 h at 4 °C and stored at −20 °C.

Immunization and Antiserum Preparation. Permethrin antisera were obtained following the protocol reported earlier (Shan et al., 1999b). Briefly, for each immunogen (**4**−Thyr, **5**−Thyr), two New Zealand white rabbits were immunized (rabbits 546 and 548 for **4**−Thyr and rabbits 549 and 550 for **5**−Thyr). The antigen solutions (100 μg in PBS) were emulsified with Freund's complete adjuvant (1:1, v/v) and injected intradermally. After 1 month, the animals were boosted with an additional 100 μg of immunogen that was emulsified with Freund's incomplete adjuvant (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. The results of antibody characterization were obtained from sera of terminal bleeds after four boosters.

Enzyme Immunoassay. The competitive inhibition ELISA format in this study was based on methods described by Voller et al. (1976). Microplates were coated overnight at 4 °C with 100 μL/well of the appropriate coating antigen concentration in 0.1 M carbonate−bicarbonate buffer (pH 9.6). After the plates had been washed with washing solution (0.05% Tween in distilled water), the surface of the wells was blocked with 200 μL of a 0.5% BSA−PBS solution by incubation for 30 min at room temperature to minimize nonspecific binding in the plate. After another washing step, 100 μL of antiserum diluted in PBS (PBS: 8 g/L NaCl, 1.15 g/L Na_2HPO_4, 0.2 g/L KCl) per well (for titration experiment) or 50 μL/well of antiserum diluted in PBS with 0.2% BSA (PBSB) and 50 μL/well of standard analyte or sample solution were added and incubated for 1 h. The standard analyte concentrations ranged from 0.05 μg/L to 5 mg/L. Following a washing step, GAR−HRP (diluted 1:3000 in PBS with 0.05% Tween 20, 100 μL/well) was added and incubated for 1 h at room temperature. The plates were washed again, and 100 μL/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 15 min with 50 μL/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, B is the curve slope at the inflection point, C is the concentration of analyte giving 50% inhibition (I_{50}), and D is the minimum absorbance at infinite concentration.

Antibody Characterization and Assay Optimization. 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, and the one with the lowest I_{50} was selected for further assay development.

The effects of solvents were tested by dissolving the analyte in PBS buffers containing various proportions of solvent (0, 10, 20, 40, 60, and 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 and added to a coated plate. pH values of 5, 6, 7, and 8 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.

Cross-Reactivity (CR). The optimized assays were applied to cross-reactivity studies by using the standard solution of the analyte and other structurally related compounds (listed in Table 3). The CR was determined by dividing the I_{50} of the chemical assigned to be 100% by the I_{50} of another compound and multiplying by 100 to obtain a percent figure.

SPE and GC-MS. The SPE method used in this study was the same as a previously reported method for the extraction of esfenvalerate from water using a C18 column (Shan et al., 1999a). The final eluent in methanol was used directly for immunoassay. An eluent aliquot was evaporated to dryness and then redissolved in toluene for GC-MS measurement. Assay validation was conducted with an HP 5890 GC equipped with an HP 5973 mass selective detector and a 30 m × 0.25 mm i.d. (d_f = 0.25 μm) HP-5MS 5% phenyl methyl siloxane column. The injector was operated at 280 °C. An HP model 6890 series autoinjector was used to inject 1 μL of sample. The oven temperature was programmed from 200 to 280 °C at 5 °C/min and held for 5 min. Helium was used as a carrier gas (1 mL/min). The detection limit of quantitative analysis was 10 pg for permethrin.

RESULTS AND DISCUSSION

Hapten Synthesis. Permethrin insecticide is a mixture of four chiral isomers, a consequence of the two chiral centers in the cyclopropane ring of the molecule. The insecticidal activities of the *cis*- and *trans*-isomers differ depending on the target insect (Elliott et al., 1978); however, *trans*-permethrin is metabolized much more rapidly than the *cis*-isomer in mammals (Gaughan et al., 1979; Casida and Ruzo, 1980). Consequently, the *trans*-isomers are made to predominate (60−75%) in the commercially produced product. The haptens were thus designed to contain both the *cis*- and *trans*-isomers. The primary goal of this study was to develop a permethrin-specific antibody. Because the alcohol portion of permethrin, the phenoxybenzyl group, is common to many of the synthetic pyrethroids, the strategy for designing the immunizing hapten was to link the protein through the aromatic end of the permethrin molecule and leave the relatively unique acid portion distal from the protein to improve antibody specificity (Goodrow et al., 1995). In addition, due to the lipophilicity of pyrethroids, a long side chain (handle) could allow the lipophilic hapten to fold back into the hydrophobic interior of the protein and decrease the affinity of the resulting antibodies. In our experience, a rigid or shorter side chain in an immunizing hapten is important for the generation of sensitive and selective antibodies (Sanborn et al., 1998). Thus, the hapten we have chosen for immunization contains the whole permethrin molecule and has no side chain (Figure 1). This approach differed from that considered in previous papers (Stanker et al., 1989; Skerritt et al., 1992; Bonwick et al., 1994), in which either a long side chain or a modification of the acid moiety of permethrin was chosen for coupling.

Thyroglobulin conjugates of haptens **4** and **5** were used for antibody production, and their BSA conjugates and conjugates of other haptens served as coating antigens. To facilitate the synthesis, isolation, and purification of the haptens, the *cis*- and *trans*-isomer mixtures were prepared separately. The permethrin acids were prepared according to literature procedures (Nakada et al., 1979; Kleschick 1986). The *cis*- and *trans*-acids were separated by fractional vacuum distillation of the ethyl esters through an annular still. Subsequent hydrolysis and crystallization gave the racemic *cis*- and *trans*-permethrin acids. GLC of the methyl esters prepared via trimethylsilyldiazomethane gave isomeric purity of >99% for both acids.

The synthesis routes for the haptens are summarized in Figure 1. The intermediate nitrophenoxybenzyl alcohol **1** was prepared by sodium borohydride reduction of the corresponding aldehyde (Loewe and Urbanietz, 1967). Esterification with the acid chlorides of the racemic *cis*- and *trans*-permethrin acids gave the corresponding nitropermethrins **2** and **3**. Subsequent re-

Table 1. Summary of Optical Density of Titration Tests[a]

Ab/immunogen	**4**−BSA	**5**−BSA	**9**−BSA	**10**−BSA	*trans*-PA−BSA	*cis*-PA−BSA
546/**4**-Thyr	++	+	−	+	−	−
548/**4**-Thyr	+++	+++	++	+++	−	−
549/**5**-Thyr	+++	++++	+++	++++	−	−
550/**5**-Thyr	+++	++++	+++	++++	−	−

[a] The data shown are at an antibody dilution of 1:16000 and a coating antigen concentration of 1 mg/L. Symbols refer to optical density ranges after 15 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.

duction with stannous chloride (Bellamy and Ou, 1984) followed by flash chromatography gave the pure haptens **4** and **5**. Both gave TLC spots that rapidly darkened on exposure to light as is typical of anilines in general.

The coating haptens **9** and **10** were prepared by a similar acylation of the cyanohydrin of the aldehyde **6** as described previously (Wengatz et al., 1998). This introduces an additional chiral center alpha to the nitrile group that results in two diastereoisomer pairs for each of the intermediate esters **7** and **8**. A small sample of each was separated by radial chromatography to obtain definitive confirming NMR spectra; however, the respective mixtures were carried through to the final haptens. Cleavage of the benzyl esters with iodotrimethylsilane gave the haptens **9** and **10** as mixtures of two diastereoisomer pairs. Flash chromatography gave pure mixtures that crystallized to white solids. NMR spectra indicated these pairs to be in a ratio of ~3:2 in both cases, allowing tentative separation of the spectra. No attempt was made to separate isomer pairs or to determine relative chiral assignments.

Screening and Selection of Antisera. The antisera of four rabbits were screened against six 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 1. In all combinations, the titer of Ab546 and Ab548 was much lower than those of Ab549 and Ab550. The homologous assay, in which the same hapten was used in the coating antigen and immunogen, had a higher titer than the heterologous assay. For Ab549, which was generated from the *trans*-permethrin hapten immunogen, coating antigen **5**−BSA had the highest affinity, followed by **10**−BSA, then **4**−BSA and **9**−BSA. No affinity was measured for the *trans*- and *cis*-PA−BSA coating antigens with all four antibodies. Combinations of coating antigen and antiserum that resulted in high optical densities (OD > 0.75) were selected for further development.

Competitive inhibition experiments were performed to optimize antiserum and coating antigen concentrations for a high sensitivity. The reagent concentrations having optical densities of ~0.8 and lowest I_{50} values were chosen as the optimal conditions. The I_{50} values for each combination ranged from 2.60 to 500 μg/L (Table 2). The stereoselectivity is significant in assay sensitivity. The I_{50} of the homologous assay (*trans*-hapten **5** and **10**) was ~100 times higher than that of the heterologous (*cis*-hapten **4** and **9**) assay. The stereoselectivity of antibodies has been reported in bioallethrin and esfenvalerate immunoassay studies (Wing and Hammock, 1979; Shan et al., 1999a). These results further suggested that antibody stereoselectivity could play an important role in the assay development by the design of coating antigen and immunogen haptens. After screening, the combination of antiserum 549 with coating antigen **4**−BSA (hereafter designated **4**/549) and coating antigen **9**−BSA (hereafter designated **9**/549) gave the lowest I_{50} values (2.6 and 4.6 μg/L, respectively) when using permethrin as the inhibitor. These systems were selected for CR studies, and one of them (**4**/549) was chosen for further assay development and optimization.

Table 2. Selected Competitive ELISA Screening Data against Permethrin[a]

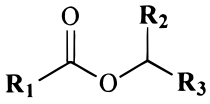
immunogen	antiserum	coating antigen	I_{50} (μg/L)
4-Thyr	Ab549	**4**−BSA	2.60
		5−BSA	289
		9−BSA	4.60
		10−BSA	470
	Ab550	**4**−BSA	4.00
		5−BSA	420
		9−BSA	6.84
		10−BSA	486
5-Thyr	Ab548	**4**−BSA	400
		5−BSA	12.9
		10−BSA	526

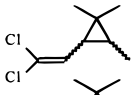
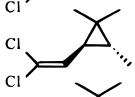
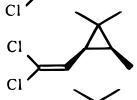
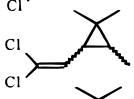
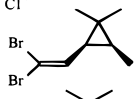
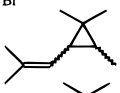
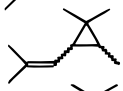
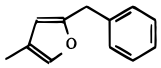
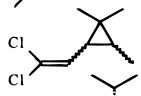
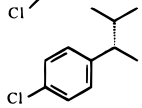
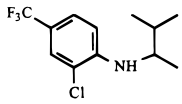
[a] The permethrin analyte standards were prepared in a 40% methanol/PBS solution.

CR. The ELISA systems **4**/549 and **9**/549 expressed different selectivity patterns to pyrethroid analogues (Table 3). As expected, because the *trans*-isomer hapten was used for immunization, *trans*-permethrin was more strongly recognized by both systems than the other compounds tested. System **4**/549 had the highest CR with phenothrin (72%) followed by cypermethin (28%), cyfluthrin (11%), and resmethrin (4%), whereas system **9**/549 strongly cross-reacted with cypermethrin (56%), phenothrin (42%), and cyfluthrin (24%). Little or no CR was measured for deltamethrin, esfenvalerate, fluvalinate, or permethrin metabolites in either system. Among the pyrethroids displaying CR, cypermethrin and cyfluthrin are actively used in the agricultural field; however, the use of phenothrin is negligible in the United States (Pesticide Use Report, 1997). Therefore, cypermethrin and cyfluthrin may be important sources of interference when these assays are used for real world sample measurement. Because our primary goal was to develop a compound-specific immunoassay, low CR to common pyrethroids was an important factor in assay development, and only assay **4**/549 was selected for further optimization.

Solvent, pH, and Salt Effects. Finding a proper cosolvent for an immunoassay is very important for the analysis of hydrophobic chemicals such as permethrin. The solvent (methanol or DMSO) effects on the ELISA system (**4**/549) were evaluated by preparing permethrin in PBS containing various amounts of solvent. DMSO strongly interferes with the assay sensitivity and maximum absorbance (Table 4). Using methanol as a cosolvent, absorbance was enhanced with higher MeOH concentration (Figure 2): a similar effect was noticed during our previous esfenvalerate immunoassay development (Shan et al., 1999a). The I_{50} value of the assay varied depending upon the different concentrations of the cosolvent MeOH. The lowest I_{50} was found at 40%

Table 3. Cross-Reactivites of Pyrethroids and Their Metabolites



Analyte	R_1	R_2	R_3	4-BSA/Ab549 I_{50} (μg/L)	4-BSA/Ab549 CR%*	9-BSA/Ab549 I_{50} (μg/L)	9-BSA/Ab549 CR%
Permethrin		H	3-PhOPh	2.50	100	4.20	100
trans-Permethrin		H	3-PhOPh	2.10	120	3.20	130
cis-Permethrin		H	3-PhOPh	15.2	16.4	32.3	13
Cypermethrin		CN	3-PhOPh	8.80	28	7.50	65
Deltamethrin		CN	3-PhOPh	645	0.4	1585	0.3
Phenothrin		H	3-PhOPh	3.50	72	10.2	42
Resmethrin		H		44	5	117	3.6
Cyfluthrin		CN	3-PhO(4-F)Ph	22.7	11	17.5	24
Esfenvalerate		CN	3-PhOPh	>10,000	--	>10,000	--
Fluvalinate		CN	3-PhOPh	>10,000	--	>10,000	--
Fenpropathrin		CN	3-PhOPh	>10,000	--	>10,000	--
trans-Permethrin acid				2500	0.1	6500	0.06
cis-Permethrin acid				>10,000	--	>10,000	--
4-Phenoxybenzyl alcohol				>10,000	--	>10,000	--

*Permethrin is assigned as 100%

Table 4. Effects of DMSO Concentration on Assay Parameters[a]

DMSO[b] (%)	ABS_{max} (*A*)	slope (*B*)	I_{50} (ppb) (*C*)	ABS_{min} (*D*)	*A*/*D*	R^2
0	0.86 ± 0.10[c]	0.89	18.4 ± 2[c]	0.09	9.5	0.99
10	0.52 ± 0.06	0.63	110 ± 12.0	0.06	8.6	1.00
20	0.45 ± 0.03	0.61	190 ± 18.5	0.06	7.5	0.99
40	0.35 ± 0.03	0.72	400 ± 25	0.07	5.0	0.99

[a] ELISA conditions: coating antigen **4**−BSA (0.4 μg/mL); antiserum 549 (1/5000); goat anti-rabbit IgG−HRP (1/3000). [b] Concentration of DMSO in permethrin standard solution (PBS−DMSO). [c] Mean value ± SD. Each set of data represents the average of three experiments.

MeOH (2.40 ppb), which is ~15 times lower than that at 20% MeOH (32 ppb) and 3 times lower than that at 60% MeOH (8.60 ppb). At concentrations up to 80% MeOH (I_{50} = 13.6 ppb), the assay might still be useful for permethrin quantitationn; however, the high background makes it impractical (*A*/*D* = 3.6). These data suggest that the cosolvent could be an important factor in assay performance, especially for hydrophobic compounds. A high concentration of cosolvent will help the solubility of hydrophobic permethrin in reaction solution, but it could also affect the interaction of antibody and antigen, resulting in a high background. On the basis of the I_{50} values and the ratios of maximum and minimum absorbances for the permethrin standard curves, a MeOH concentration of 40% was selected for subsequent experiments.

To address potential interferences from aqueous environmental samples, the effects of pH and ionic

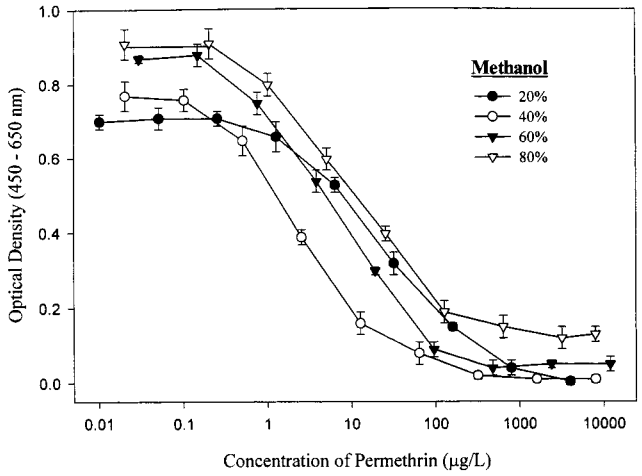


Figure 2. ELISA competition curves of permethrin prepared in PBS buffer containing various concentrations of methanol. Reagent concentrations: coating antigen (**4**−BSA), 1/8000; antiserum (Ab549), 1/6000 (final concentration in wells); goat anti-rabbit IgG−HRP, 1/3000. Error bars represent standard deviation of three replicates.

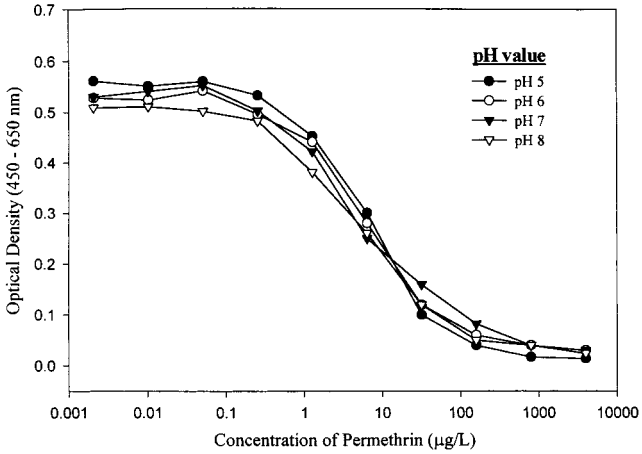


Figure 3. ELISA competition curves of permethrin prepared at various pH values. Reagent concentrations: coating antigen (**4**−BSA), 0.4 μg/mL; antiserum (Ab549), 1/6000 (final concentration in wells); goat anti-rabbit IgG−HRP, 1/3000. Each curve represents the average of three replicates.

strength on ELISA performance were evaluated in this study. In system **4**/549, when analyte was dissolved in buffer at various pH values, no significant effect upon the I_{50} was detected, indicating that the assay could effectively detect permethrin at pH values ranging from 5.0 to 8.0 (Figure 3). Ionic strength strongly influenced ELISA performance (Figure 4). A higher salt concentration in the assay system resulted in lower optical densities (OD) and higher I_{50} values. The OD values at salt concentrations of 0.3 and 0.5 M PBS decreased by approximately 20 and 65%, respectively, from the OD value at a salt concentration 0.1 M PBS. Thus, the maintenance of a minimal ionic strength appears to be important.

The optimized permethrin ELISA used coating antigen **4**−BSA at 0.4 μg/mL, antibody 549 at a dilution of 1/5000, and permethrin in 40% methanol−PBS buffer. The I_{50} value of this assay was 2.50 ± 0.6 μg/L (Figure 5) with a limit of quantitation (LOQ) of 0.30 ± 0.08 μg/L in buffer. The LOQ was estimated as the concentration that corresponded to the absorbance of the control (zero concentration of analyte) minus 3 times the standard deviation of the control (Grotjan and Keel, 1996).

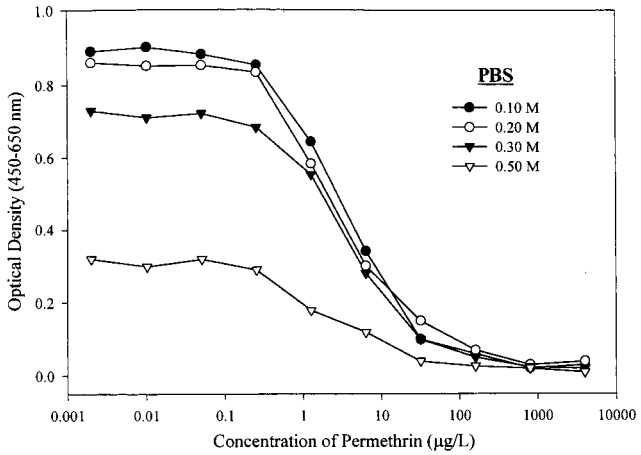


Figure 4. ELISA competition curves of permethrin prepared in buffer at various ionic strengths. Reagent concentrations: coating antigen (**4**−BSA), 0.4 μg/mL; antiserum (Ab549), 1/6000 (final concentration in wells); goat anti-rabbit IgG−HRP, 1/3000. Each curve represents the average of three replicates.

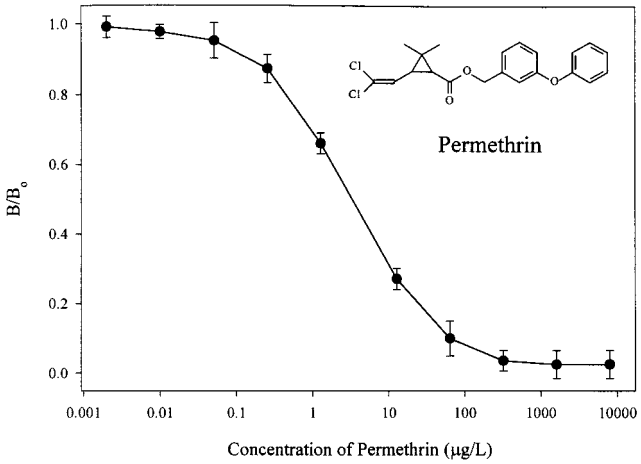


Figure 5. ELISA inhibition curves for permethrin. Reagent concentrations: antiserum 549, 1/6000 (final dilution in wells); coating antigen (**4**−BSA), 0.4 μg/mL. Standard curves represent the average of 16 curves.

Table 5. Retention Times and Detected Masses of Pyrethroids

pyrethroid	retention time (min)	detected masses, *m/z* (% base peak)
permethrin	13.50	163 (32), 183 (100)
cyfluthrin	14.80	163 (100), 226 (52)
cypermethrin	16.01	163 (100), 181 (51)
esfenvaleate	17.10	125 (100), 167 (81), 225 (60)
deltamethrin	19.20	181 (81), 253 (100)

GC-MS Analysis Assessment. A sensitive GC-MS method was developed for the assay validation. Under the GC conditions described, permethrin was completely separated from other pyrethroids such as esfenvalerate, cyfluthrin, and cypermethrin (Table 5). After GC separation, the substances were detected using MS in the selected ion monitoring (SIM) mode. The mass selective detector provided characteristic information about each of the separated substances, which resulted in high specificity regarding the determination of a single analyte. Due to the lower noise level, better sensitivity was achieved using SIM. In the case of permethrin, the most important peak of fragmentation (*m/z* 183) was used for quantitation (Figure 6). The detection limit was calculated from a signal-to-noise ratio of 3:1. The

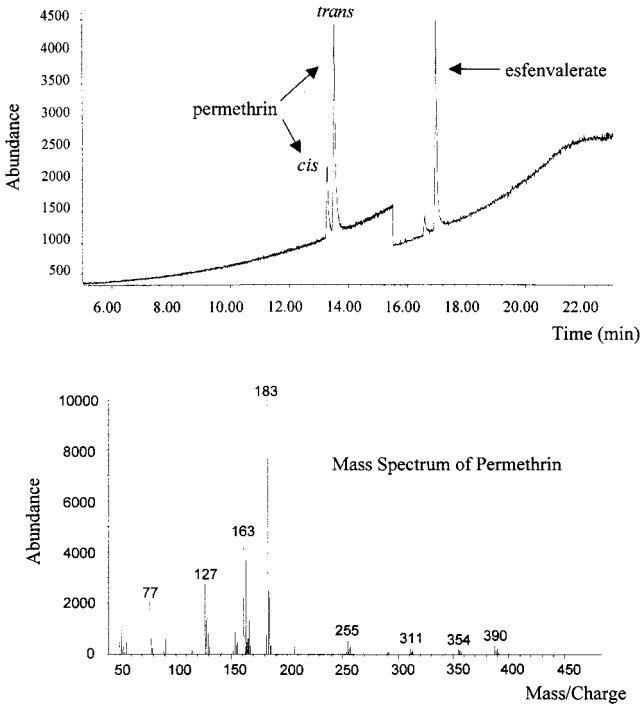


Figure 6. GC-MS chromatogram and the corresponding mass spectrum of permethrin.

resulting detection limit for permethrin in water ranged from 0.005 to 0.01 μg/L.

Analysis of Spiked Water Samples. To study the spike recovery, water samples from the Sacramento River (Sacramento, CA), Putah Creek (Davis, CA), Lake Berryessa (CA), and local tap water (Davis, CA) were spiked with permethrin (with concentrations ranging from 0 to 10 ppb) in a blind fashion. The samples were extracted using SPE and measured by ELISA and GC-MS. No matrix effects were measured when 200 mL of water sample was extracted with SPE and eluted with 3.5 mL of methanol. The volume of methanol was finalized at 1 mL and diluted by a factor 5 before ELISA analysis, resulting in a concentration factor of 40. Because permethrin is highly hydrophobic, it can be easily sorbed to glass or plastic surfaces. In one of our studies, after leaving the spiked sample in the bottle overnight, we found that >85% of the permethrin was trapped to the glass surface (data not shown). Therefore, a small amount of methanol was used for rinsing the container, and this methanol was combined with the SPE eluent. A good correlation between permethrin concentration measured by the GC-MS and ELISA (R^2 = 0.900, a = 1.21, and b = −0.068) was obtained from linear regression analysis (Figure 7). In a subsequent study, water samples from the Sacramento River were spiked with four different concentrations of permethrin (0, 0.01, 0.1, and 0.5 ppb) and analyzed (Table 6). Spike recoveries in both ELISA and GC-MS methods were compared. All recoveries were >72% of the spiked values, and again, a good agreement between these two detection methods was obtained. The results demonstrate that these assays are suitable for the quantitative detection of permethrin at trace levels in water samples.

Conclusions. A sensitive and selective immunoassay for permethrin has been developed by using **5** [(±)-*trans*-isomer] as the immunizing hapten and **4** [(±)-*cis*-isomer] as the coating antigen hapten. ELISA **4**/549 has a very low I_{50} value and also exhibits good performance characteristics at various pH values and high solvent (40% methanol) levels. Even though the assay is sensitive to high ionic strength, with the implementation of dilution or SPE, interferences can be minimized for field sample measurement. With a simple SPE step, this ELISA was successfully applied to quantitate low parts per trillion (ppt) of permethrin in water. A good correlation between ELISA and GC-MS results was achieved in the validation study, suggesting that this permethrin immunoassay is useful for environmental monitoring and toxicological studies.

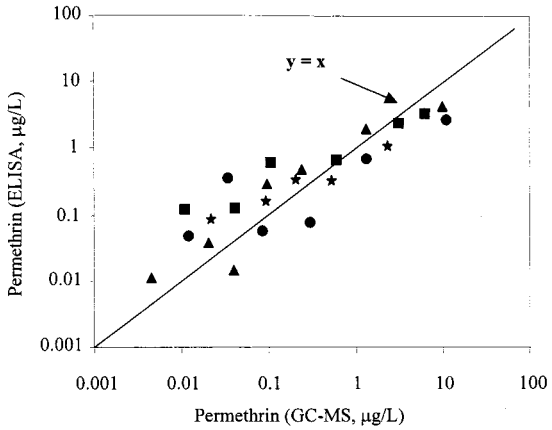


Figure 7. Relationship between permethrin levels as measured by GS-MS and ELISA. Water samples were from the Sacramento River (■), Putah Creek (Davis) (●), Lake Berryessa (▲), and tap water (Davis) (★). $Y = 1.211x - 0.068$, $R^2 = 0.900$, $n = 24$.

Table 6. Spike Recovery in Water Matrix[a]

spiked concn (μg/L)	ELISA		GC-MS	
	measured	% recovery	measured	% recovery
0	<0.002		<0.002	
0.01	0.011 ± 0.002	110 ± 20	0.008 ± 0.002	80 ± 20
0.05	0.038 ± 0.005	76 ± 10	0.036 ± 0.006	72 ± 12
0.5	0.41 ± 0.042	82 ± 8.4	0.38 ± 0.035	76 ± 7.0

[a] Water samples are from the Sacramento River, Sacramento, CA. Different amounts of permethrin (in methanol) were added to water samples to final concentrations in water of 0.01, 0.05, and 0.5 μg/L. After thorough mixing and sitting for at least 2 h, these samples were extracted by SPE before immunoassay and GC-MS measurement.

ABBREVIATIONS USED

Ab, antibody; BSA, bovine serum albumin; cAg, coating antigen; CR, cross-reactivity; DMF, *N*,*N*-dimethylformamide; DMSO, dimethyl sulfoxide; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; ELISA, enzyme-linked immunosorbent assay; FAB-MS, fast atom bombardment mass spectrum; GAR−HRP, goat anti-rabbit immunoglobulin conjugated to horseradish peroxidase; GC-MS, gas chromatography with mass spectral detection; I_{50}, concentration of analyte giving 50% inhibition; LOQ, limit of quantitation; NHS, *N*-hydroxysuccinimide; NMR, nuclear magnetic resonance; OD, optical density; PA, permethrin acid; PBS, phosphate-buffered saline; PBST, phosphate-buffered saline with 0.05% of Tween 20; SD, standard deviation; SIM, selected ion monitoring; SPE, solid-phase extraction; TLC, thin-layer chromatography; TMB, tetramethylbenzidine.

ACKNOWLEDGMENT

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