Development of an Immunoassay for the Pyrethroid Insecticide Esfenvalerate

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INTRODUCTION

Esfenvalerate (Figure 1) is a synthetic pyrethroid widely used for the control of many common pests on agricultural crops, such as apples, peaches, cotton, and almonds (Meister, 1996). Due to its excellent insecticidal properties and low mammalian toxicity (Ecobichon, 1996), the use of this compound has increased rapidly during the past 15 years. In 1994, > 33 1500 lb of the active ingredient of esfenvalerate was applied on various crops in the United States (Gianessi and Anderson, 1995). However, esfenvalerate is extremely toxic to many aquatic animals (Tomlin, 1997), such as fish, and amphibious and aquatic invertebrates, which may be exposed to field runoff or drift from aerial and ground-based spraying. Numerous studies have shown that esfenvalerate has detrimental effects on aquatic species by reduction or elimination of many crustaceans, chironomids, juvenile bluegills, and larval cyprinids at exposure levels of 1 ppb (Lozano et al., 1992; Tanner and Knuth, 1996). Thus, a sensitive, selective, and rapid method for monitoring residue levels of esfenvalerate in aquatic ecosystems is desirable.

Current analytical methods for esfenvalerate rely upon multistep sample cleanup procedures in conjunction with high-performance liquid chromatography (HPLC) (Wells et al., 1994) or gas–liquid chromatography (GLC) with either electron capture detection (Hengel et al., 1997). Such methods are relatively expensive and skill intensive. An immunoassay would provide a fast, sensitive, and selective method for the detection of this pesticide at trace levels (Hammock and Mumma, 1980; Gee et al., 1988; Hammock et al., 1990). A number of enzyme-linked immnosorbent assay (ELISA) methods have been reported for the detection of synthetic pyrethroids. They include allethrin (Pullen and Hock, 1995), S-bioallethrin (R,3R,4′S-allethrin) (Wing et al., 1978), bioresmethrin (Hill et al., 1993), deltamethrin (Queffelec et al., 1998; Lee et al., 1998), fenpropatrin (Wengatz et al., 1998), and permethrin (Stanker et al., 1989; Skerritt et al., 1992; Bonwick et al., 1994). To our knowledge no study has been reported on the development of immunoassays for esfenvalerate. In this paper, the development of an ELISA for esfenvalerate and the evaluation of the assay’s performance in water matrices are described.

MATERIALS AND METHODS

Reagents. Cyfluthrin, cypermethrin, deltamethrin, fenvalerate, fluvainlate, permethrin, phenothrin, and resmethrin standards (Figure 2) were obtained from Riedel de Haen (Seelze, Germany). 14C-Labeled fenvalerate (14.9 mCi/mmol) was provided by Shell Development Co. (Modesto, CA). Racemic 4-chloro-α-(1-methylethyl)benzenecetic acid was generously supplied by E. I. duPont de Nemours & Co. (Wilmington, DE). Organic starting materials for hapten synthesis were obtained from Aldrich Chemical Co. (Milwaukee, WI) and Fisher Scientific (Pittsburgh, PA). Thin-layer chromatography (TLC) utilized 0.2 mm precoated silica gel 60 F254 on glass plates from E. Merck (Darmstadt, Germany), and detection was made by ultraviolet (UV) light or iodine vapor stain. Flash chromatographic separations were carried out on 40 μm average particle size Baker silica gel using the indicated solvents, where the → notation denotes a stepwise concentration gradient.

Keywords: Immunoassay; esfenvalerate; pyrethroids; solid-phase extraction; residue analysis

Figure 1. Structure of esfenvalerate.

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The coupling reagents were purchased from Aldrich. Goat anti-rabbit (GAR) immunoglobulin conjugated to horseradish peroxidase (HRP), bovine serum albumin (BSA), ovalbumin (OVA), hemocyanin from Limulus polyphemus (LPH), Tween 20, and 3,3',5,5'-tetramethylbenzidine (TMB) were purchased from Sigma Chemical Co. (St. Louis, MO).

**Apparatus.** NMR spectra were obtained using a General Electric QE-300 spectrometer (Bruker NMR, Billerica, MA). Chemical shift values are given in parts per million downfield from internal tetramethylsilane. Melting points were determined on a Uni-Melt apparatus (Thomas Scientific, Swedesboro, NJ) and are uncorrected. 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). Fast atom bombardment high-resolution mass spectra (FAB-HRMS) were obtained on a Chromatotron apparatus (Harrison Research, Inc., Palo Alto, 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 high-resolution mass spectra (FAB-HRMS) were obtained on a ZAB-HS-2F mass spectrometer (VG Analytical, Wythenshaw, U.K.), using xenon (8 keV, 1 mA) for ionization and a ZAB-HS-2F mass spectrometer (VG Analytical, Wythenshaw, U.K.), using xenon (8 keV, 1 mA) for ionization and 3-nitrobenzyl alcohol or glycerol as the matrix. Poly(ethylene glycol) was added to the matrix as a mass calibrant. 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). Two brands of microplates were used: Nunc-Immuno plates were used for preparing serial dilutions. 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). Two brands of microplates were used: Nunc-Immuno plates were used for preparing serial dilutions.

**Hapten Synthesis and Verification.** Syntheses of the haptenes were straightforward, and yields were moderate to good. Analytical data supporting the structures are provided. Racemic acid (128 g, 0.6 mol) in toluene (218 mL) and methanol (320 mL) was stirred and heated to reflux, and, with heating removed; (S)−(−)−α- methylbenzylamine (45.8 g, 0.3 mmol) was added over 15 min. The mixture was allowed to cool over 1.25 h, then filtered, and washed with solvent mix. The solid was slurried in methanol (400 mL), refluxed for 1 h, cooled, and filtered to give 85 g of a white solid, which was immediately recrystallized from hot methanol (2.5 L) to give 55 and 22 g in two crops. The latter was again recrystallized from methanol to give 20 g of product. The two crops were combined and treated with 3 N HCl to recover the free acid. The ether extract was washed with dilute acid and water and stripped. Recrystallization of the residual solid from hexane (100 mL) provided 46.7 g of (S)-acid, mp 103.5–105.2 °C, [α]D 84.1 (c 1.61, CHCl3). The literature (Miyakado et al., 1975) gives mp 104–5 °C, [α]D 84.8 (c 1–2, CHCl3). The remaining amine salt concentrated in the (R)-acid fraction was treated with HCl to recover the free acid. Treatment in the same way with (R)-(+)-α-methylbenzylamine and workup as above led to 46 g of the (R)-acid as a white solid: mp 101–105.5 °C, [α]D 84.7 (c 1.49, CHCl3).

The optical purity of each acid was determined by treatment of a 100 mg (0.47 mmol) sample in 0.3 mL of CHCl3 plus 0.5 µL of N,N-dimethylformamide (DMF) with SOCl2 (0.068 mL, 0.93 mmol) at 60 °C for 45 min. Vacuum stripping of the resulting acid chloride followed by addition of CH2Cl2 (0.5 mL) to a mixture of 7 mL of tetrahydrofuran (THF) and 0.5 mL of water was stirred with ice cooling, and concentrated HCl (1.3 mL, 16 mmol) was added all at once. After a short time, the mixture became basic and a mild exotherm accompanied the formation of the cyanohydrin. The mixture was allowed to warm to room temperature with stirring over 30 min. The mixture was diluted with CH2Cl2 and water, acidified with 3

![Figure 2. Structures of other pyrethroids used in this study.](Image 126x471 to 486x744)
N HCl (caution, HCN released), washed three times with water, dried briefly over anhydrous MgSO₄, and stripped to a colorless oil. Meanwhile, (S)-fenvalerate acid (2.55 g, 12 mmol) in 6 mL of CHCl₃ was treated with SOCl₂ (1.31 mL, 1.8 mmol) and DMF (0.5 mL) and stirred while heating in an oil bath at 60 °C for 1 h. The mixture was stripped, and 5 mL of hexane was added and stripped to give the acid chloride as a colorless oil. This acid chloride in 4 mL of CH₂Cl₂ was stirred with ice cooling, and the above cyanohydrin in 4.5 mL of CH₂Cl₂ was added all at once followed immediately with 1.2 mL of pyridine. After an immediate mild exotherm, the mixture was stirred for 30 min, diluted with water, and acidified with 3 N HCl. The organic phase was washed with dilute HCl and water, dried over MgSO₄, and stripped to an oil, which was flash chromatographed on 100 g of silica gel (10% 60% CH₂Cl₂ in hexane). Stripping product fractions briefly to 60 °C under high vacuum gave 4.68 g (95%) of a mixture of the S,S, and S,R isomers of fenvalerate insecticide. A 1.2 g sample of this mixture was separated by radial chromatography (2 mm silica gel, 0.5% THF in hexane) in six 200 mg portions to give nearly complete separation of diastereoisomers. Each of the isomers was repassed in three portions for further purification. Separation was followed by TLC (10 cm plates), for which five or more solvent passes (5% THF in hexane) were required to show complete separation of the two isomers. Stripping the lower R,S isomer gave 620 mg of oil, which was crystallized from ~2 mL of MeOH at 0 °C to give 495 mg of white solid: mp 59–61 °C. Stripping the higher R,S isomer fractions in the same way gave 645 mg of a colorless viscous liquid: [α]D²⁴ +11.2 (c 0.985, CHCl₃); ¹H NMR (CDCl₃) δ 0.73 (d, J = 6.7 Hz, 3 H, CH₃), 1.06 (d, J = 6.5, 3 H, CH₃), 2.33 (m, J = 6.6, 10.4 Hz, 1 H, Me₂CH), 3.23 (d, J = 10.7 Hz, 1 H, CH₂C(O)), 6.29 (s, 1 H, CH-CN), 6.96–7.38 (m, 13 H, Ar).

A second crop was recrystallized to give 19 mg of additional product, mp 59–61 °C. Stripping the higher R,R isomer fractions in the same way gave 645 mg of a colorless viscous liquid: [α]D²⁴ +11.2 (c 0.985, CHCl₃); ¹H NMR (CDCl₃) δ 0.73 (d, J = 6.7 Hz, 3 H, CH₃), 1.06 (d, J = 6.5, 3 H, CH₃), 2.33 (m, J = 6.6, 10.4 Hz, 1 H, Me₂CH), 3.23 (d, J = 10.7 Hz, 1 H, CH₂C(O)), 6.29 (s, 1 H, CH-CN), 6.96–7.38 (m, 13 H, Ar).

Cyano[3-(4-nitrophenoxyl)phenyl]methyl (S)-4-Chloro-(1-methylethyl)benzeneacetate (4-Nitrofenvalerate) (3).

The cyanohydrin of 3-(4-nitrophenoxy)benzaldehyde (2) was prepared as described in a previous publication (Wengatz et al., 1998). Meanwhile, the acid chloride of (S)-fenvalerate acid (2.62 g, 12.3 mmol) was prepared as described above. The above cyanohydrin in 10 mL of CH₂Cl₂ was stirred with ice cooling as the acid chloride in 10 mL of CH₂Cl₂ was added followed immediately with pyridine (1.25 mL, 15.4 mmol). After an initial mild exotherm, the mixture was stirred at room temperature for 20 min. The resulting mixture was washed with 3 N HCl solution and water, and the organic phase was
dried (MgSO₄) and stripped to a yellow gum. Flash chromatography on 85 g of silica gel (hexane → CH₂Cl₂) and high-vacuum stripping gave the pure ester as a mixture of the two diastereoisomers, 5.08 g (89%). TLC R₉ 0.55 (CH₂Cl₂). Separation of a ~200 mg sample by radial chromatography (2 mm silica gel, 5% THF in hexane) gave complete separation in two steps: 100 mg of the higher R₉ S iso (Rf 0.70; J 0.113, CH₃Cl), 1H NMR δ 0.73 (d, J = 6.7 Hz, 3 H, CH₃), 1.03 (d, J = 0.55 Hz, 3 H, CH₃), 2.34 (m, J = 6.5, 10.5 Hz, 1 H, Me-CH₂), 3.24 (d, J = 6.5 Hz, 1 H, CH(O)), 6.31 (2 H, CH₂), 7.0–8.3 (m, 12 H, Ar)] and 89 mg of the lower R₉ S iso (Rf 0.95; J 0.65, CH₃Cl), 2.30 (m, J = 6.6, 10.5 Hz, 1 H, Me-CH₂), 3.21 (d, J = 10.4 Hz, 1 H, CH(O)), 6.34 (2 H, CH₂), 7.0–8.3 (m, 12 H, Ar)]. The absolute configuration of the latter was determined as follows: A 15 mg sample was reduced in 0.25 mL of EtOH with 44 mg of SnCl₂·2H₂O (30 min/70 °C) to give 10.5 mg of the corresponding aniline after chromatography: 1H NMR (CDCl₃) δ 7.73 (d, J = 6.5 Hz, 3 H, CH₃), 6.37 (s, 1 H, CHCN), 6.94 (d, J = 7.4 Hz, 2 H, CH₂), 7.4 (m, 17 H, Ar). Treatment of this tryptone with 25 μL of BSTFA followed by dithioethersilane (0.249 mL, 1.75 mmol). After 3 h, the mixture was treated with 1 mL of water. The organic phase was then immediately and directly chromatographed on 20 g of silica gel (CH₂Cl₂ → EtOAc) to give 0.81 g (99%) of the acid as a pale yellow gum after high-vacuum stripping, which was one spot by TLC: R₉ 0.35 (EtOAc). 1H NMR (CDCl₃) showed a 1:1 mixture of two diastereoisomers. Comparison to spectra of fenvalerate and nitrofenvalerate isomers allowed tentative assignment of the two spectra as follows: S,R isomer δ 0.70 (d, J = 6.86 Hz, 3 H, CH₃), 1.05 (d, J = 6.5 Hz, 3 H, CH₃), 2.3 (m, J = 6.5, 10.2 Hz, 1 H, Me-CH₂), 2.69 (t, J = 7.6 Hz, 2 H, CH₂), 2.96 (t, J = 7.7 Hz, 2 H, CH₂), 3.23 (d, J = 10.5 Hz, 1 H, CH(O)), 6.29 (s, 1 H, CHCN), 6.9–7.39 (m, 12 H, Ar); S,S isomer δ 0.72 (d, J = 6.86 Hz, 3 H, CH₃), 0.95 (d, J = 6.5 Hz, 3 H, CH₃), 2.3 (m, J = 6.5, 10.2 Hz, 1 H, Me-CH₂), 2.69 (t, J = 7.6 Hz, 2 H, CH₂), 2.96 (t, J = 7.7 Hz, 2 H, CH₂), 3.23 (d, J = 10.5 Hz, 1 H, CH(O)), 6.29 (s, 1 H, CHCN), 6.9–7.39 (m, 12 H, Ar); FAB-HRMS m/z calcd for [M⁺ + H⁺]⁺ = C₂₂H₂₆Cl₂NO₃ 492.1552, obsd 492.1577.

Benzyl 3-[Cyano(S)-2-(4-chlorophenyl)-3-methyl-1-oxobutanoyloxy]methyl]phenylbenzenepropanoate (8). Benzyl 3-[formylphenyloxy]acetate (2.0 g, 7.4 mmol) was converted to the cyanohydrin as described in a previous paper (Wengatz et al., 1998). (S)-4-Chloro-a-(1-methylethyl)benzeneacetyl chloride was prepared from the corresponding (S)-acid (1.57 g, 7.4 mmol) as described above. The cyanohydryl in 2.5 mL of CH₂Cl₂ was stirred with ice cooling, and the acid chloride in 3 mL of CH₂Cl₂ was added at once after 5 min by pyridine (0.75 mL, 9.2 mmol). The mixture was stirred at room temperature for 30 min, then washed with water and a saturated NaCl solution, dried (MgSO₄), and stripped to a pale yellow oil. Flash chromatography on 70 g of silica gel (5 → 90%) CH₂Cl₂ in hexane and high-vacuum stripping of product fractions gave 3.25 g (90%) as a mixture of diastereoisomers. A sample of this mixture (1.23 g) was separated by radial chromatography (2 mm silica gel, 5% THF in hexane) and high-vacuum stripping to give 10.5 mg of the correspond-
using the same procedure as described for the ester 8 above. The crude stripped product was flash chromatographed on 60 g of silica gel (10% CH2Cl2/hexane — CH2Cl2) to give 7% of the starting aldehyde and 2.66 g (90%) of the ester 9, a mixture of two diasteroisomers, as a colorless viscous oil. A 1.3 g sample of this mixture was separated by radial chromatography (2 mm silica gel, 5% THF in hexane) in ~ 200 mg portions with recycle of overlap fractions into the following portion. Each of the isomers was recovered in two portions.

High-vacuum stripping of the higher R, S, R isomer gave 637 mg of colorless oil: [α]D 28.8° − 7.76 (c 1.12, CHCl3); 1H NMR (CDCl3) δ 0.72 (d, J = 6.6 Hz, 3 H, CH3), 1.07 (d, J = 6.4 Hz, 3 H, CH3), 1.49 (m, 2 H, CH2), 1.75 (m, 4 H, 2 CH2), 2.35 (m, 1 H, MeCH), 2.4 (t, J = 7.4 Hz, 2 H, CH2), 3.23 [d, J = 10.4 Hz, 1 H, CH(O)], 3.84 (t, J = 6.18 Hz, 2 H, CH2), 5.12 (s, 2 H, C6H5CH2), 6.3 (s, 1 H, CHCN), 6.78–7.35 (m, 13 H, Ar). Similar stripping of the lower R, S, R isomer gave 623 mg: [α]D 26.7° − 1.38 (c 1.31, CHCl3); 1H NMR (CDCl3) δ 0.70 (d, J = 6.7 Hz, 3 H, CH3), 0.97 (d, J = 6.5 Hz, 3 H, CH3), 1.51 (m, 2 H, CH2), 1.77 (m, 4 H, 2 CH2), 2.31 (m, 1 H, MeCH2), 2.41 (t, J = 7.4 Hz, 2 H, CH2), 3.22 [d, J = 10.8 Hz, 1 CH(O)], 3.91 (t, J = 6.3 Hz, 2 H, CH2), 5.12 (s, 2 H, C6H5CH2), 6.34 (s, 1 H, CHCN), 6.91–7.36 (m, 13 H, Ar).

3-[Cyanos(L)-2-(4-chlorophenyl)-3-methyl-1-oxobutanoyloxy|methyl]phenoxycetic Acid (10). The ester 8 as the diastereoisomer pair (1.78 g, 3.6 mmol) in 3.5 mL of CH2Cl2 was treated with 0.5 mL of BSTFA and iodotrimethylsilane (0.523 mL, 3.68 mmol). After 20 h at ambient temperature, 1.5 mL of methanol was added, and the mixture was washed with water and saturated NaCl solution, dried (MgSO4), and stripped. The resulting gum was immediately flash chromatographed on 25 g of silica gel (CH2Cl2 → EtOAc → 3% HOAc in EtOAc). Fractions containing only product by TLC, Rf 0.4 (CH2Cl2), were treated with 0.5 mL of methanol, washed three times with water and stripped. Flash chromatography on 7 g of silica gel (CH2Cl2 → EtOAc → 3% HOAc in EtOAc) recovered 1.1 g (35%) of the starting ester and 261 mg (78%) of the acid 11 as viscous oil: [α]D 26.4° + 1.67 (c 1.16, CHCl3); 1H NMR (CDCl3) δ 0.71 (d, J = 6.7 Hz, 3 H, CH3), 0.96 (d, J = 6.5 Hz, 3 H, CH3), 2.31 (m, J = 6.6, 10.4 Hz, 1 H, MeCH2), 3.23 [d, J = 10.5 Hz, 1 CH(O)], 4.68 [s, 2 H, OCH2CH(O)], 6.35 (s, 1 H, CHCN), 6.75 (b, 1 H, COOH), 6.97–7.39 (m, 8 H, Ar); FAB-MS m/z calcd for [M + H]+ = C25H29ClNO5 402.1, obsd 401.2, obsd 402.

Hapten Conjugation. Conjugates were synthesized using three different methods: water soluble carbodiimide diazotization, and activated ester method (Tijssen, 1985; Erlanger, 1973). To obtain immunogens, hapten 4 and 7 were conjugated to LPH. Coating antigens were made by coupling hapten 4, 10, 11, and 13 to BSA and/or OVA.

Conjugates of 4-BSA/LPH and 13-BSA/OVA. Hapten 4 or 13 (0.104 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 at 0.5 mL of a 0.201 M solution of sodium nitrite was injected. Some product appeared as a gel. DMF (0.1 mL) was added dropwise to remove the gel, which was divided into two equal aliquots (each aliquot was used for one protein). Ninety-eight milligrams of LPH and 101 mg of OVA was dissolved in 30 mL of 0.2 M borate buffer (pH 8.8) 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 (hapten 4 for LPH and BSA; hapten 13 for BSA and OVA). The reaction mixture was stirred in an ice bath for 45 min to give a homoged mixture, then diluted with water and stored in aliquots at −80, −20, and 4 °C.

Conjugates of 7-BSA/LPH. Morpho-CD1 (100 mg, 0.236 mmol) was added to a solution of 55 mg (0.112 mmol) of hapten 7 in 1 mL of DMF. The resulting mixture was diluted with additional DMF (2 mL) and then diluted dropwise with water.
almost to the point of oil-out. The solution was divided into two aliquots. Ninety-eight milligrams of LPH or 102 mg of BSA was dissolved in 16 mL of cold distilled H2O and the pH was adjusted to 6.5 with 0.2 M HCl. DMF (0.75 mL) was then added to each protein solution. Aliquots (2.25 mL) of the activated hapten solution were added slowly with stirring. The reaction mixture was stirred for 2 h at room temperature and purified and stored as described above.

**Conjugates of 10 (and 11)–BSA/OVA.** Hapten (0.05 mmol) was dissolved in 2 mL of dry DMF, and then NHS (18 mg, 0.08 mmol) and DAPEC (13.6 mg, 0.06 mmol) were added. The reaction mixture was stirred overnight at room temperature and divided into two aliquots. Each aliquot was used for one protein. Twenty milligrams of BSA or OVA was dissolved in 3 mL of PBS (pH 7.6). All aliquots of the activated hapten solutions were added dropwise to the two protein solutions. The mixture was stirred for 1.5 h at 4 °C and for 7 h at room temperature and then purified using dextran desalting columns as described in Wengatz et al. (1998) and stored as described above.

**Immunization and Antiserum Preparation.** Esfenvalerate antisera were obtained following the protocol reported earlier (Wengatz et al., 1998). Briefly, for each immunogen (4–LPH, 7–LPH), three New Zealand white rabbits were immunized. 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 3 week intervals. The rabbits were bled 10 days after each boost. One rabbit (no. 7587) died prior to the final bleed. The serum was isolated by centrifugation for 10 min at 4 °C. The results of antibody characterization were obtained from sera of terminal bleed.

**ELISA.** The method was similar to that previously described by Wengatz et al. (1998). 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 coated plates were washed with PBST (PBS plus Tween 20: 8 g/L NaCl, 1.15 g/L Na2HPO4, 0.2 g/L KH2PO4, 0.2 g/L KCl, and 0.05% Tween, v/v), 200 μL of blocking solution (0.5% BSA in PBS) was added and incubated for 30 min at room temperature. After another washing step, 50 μL/well antiserum diluted in PBS with 0.2% BSA and 50 μL/well of inhibitor solution were added. The plate was incubated for 1 h and then washed for 8–10 times. GAR–HRP (diluted 1:3000 in PBST, 100 μL/well) was added and incubated for 1 h at room temperature. Following another washing step (8–10 times), tetramethylbenzidine (TMB) substrate solution (100 μL/well; 3.3 μL of 30% H2O2, 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 15–20 min with 2 M H2SO4 (50 μL/well), and absorbances were measured at 450–650 nm. All experiments were conducted in triplicate or quadruplicate. Standard curves were obtained by plotting absorbance against the logarithm of analyte concentration. The curves were fitted to a four-parameter logistic equation: \( \text{y} = \left( \frac{I_A - D}{(1 + (x/C)^n)} \right) + D \), where \( A \) is the maximum absorbance at no analyte present, \( B \) is the curve slope at the inflection point, \( C \) is the concentration of analyte giving 50% inhibition (I50), and \( D \) is the minimum absorbance at infinite concentration.

**Cross-Reactivity.** The compounds listed in Tables 5 and 6 were tested for cross-reactivity by preparing each compound in 50% methanol in PBS and determining the I50 in the ELISA. Cross-reactivity values were calculated as follows:

\[ \text{CR}\% = \left( \frac{I_{50} \text{ of esfenvalerate}}{I_{50} \text{ of tested compound}} \right) \times 100 \]

**Assay Optimization.** Esfenvalerate standard curves were prepared in PBS buffer containing 0, 10, 25, 50, 75, and 100% (v/v) methanol or DMSO to determine the effects of solvent. Curves also were prepared in PBS buffer containing 0 and 0.05% of Tween 20 to determine the effect of detergent.

**RESULTS AND DISCUSSION**

**Hapten Synthesis.** Fenvalerate insecticide contains four chiral isomers, a result of its two chiral centers. The commercially produced esfenvalerate (such as Asana) is primarily the active S,S isomer, where the first and second assignments represent the absolute chirality of the acid and cyanohydrin centers, respectively. For this reason, and to facilitate synthesis and purification, the (S)-4-chloro-α-(1-methyleryl)benzenacetic acid was used for the synthesis of the haptens. This acid was resolved using the R and S isomers of α-methylbenzylamine according to a modified literature procedure (Freund, 1985). The optical purities were determined by conversion to the amide of (S)-α-methylbenzylamine having an assigned purity of 99.6% ee (Aldrich). Analysis by GLC gave baseline separation of enantiomer pairs. Conversion via the acid chloride and via DCC coupling gave nearly identical results and indicated optical purities for the S and R isomers to be a minimum of 96 and 98% ee, respectively.

The synthesis routes for the haptens are summarized in Scheme 1. Haptens 4 and 7 were synthesized via acylation of the cyanohydrins of the aldehydes 2 (Loewe and Urbanietz, 1967) and 5 (Wengatz et al., 1998) to give the intermediates 3 and 6 as mixture of the diastereoisomeric pairs. A sample of the mixture, 3, was separated into the S,S and S,R isomers by radial chromatography. The absolute configurations were determined via a stannous chloride reduction of the lower R4 isomer to the aminofenvalerate, which was diazo-
Acetic acid with 4-nitro-L-phenylalanine through a 11 where hapten was conjugated as the anioate chains linked to the moto et al., 1981). This was prepared by reaction of the glycine conjugate of fenvalerate acid, which is proposed to minimize the contribution of PB to antibody specificity. Therefore, LPH conjugates of haptenes 4 and 7 were used for antibody production, and their BSA conjugates and conjugates of other haptenes served as coating antigens.

The hapten density (the number of hapten molecules per molecule of protein) of conjugates was estimated indirectly by competitive ELISA (Wengatz et al., 1998). The hapten densities for 4–BSA and 7–BSA are 28 and 22, respectively. Because the antibodies used in this study were raised against LPH conjugates, their hapten densities could not be measured by using this method. The LPH protein molecule is ~5 times bigger than BSA, and it might have more free amine or carboxylic acid residues on the protein surface. By using the same coupling methodology, the immunogens 4–LPH and 7–LPH would have a higher hapten density than their BSA conjugates.

**Screening of Antisera.** Titers of all five antisera were tested against eight different coating antigens using a checkerboard titration system (Gee et al., 1994). The results of the titration experiments with the terminal bleeds are shown in Table 1. All raw antisera showed higher titers in a homologous system, in which the same antigen (a different carrier protein) was used for both immunogen and coating antigen (cAg), than in heterologous systems. These results are consistent with a previous study on the pyrethroid fenpropathrin by Wengatz et al. (1998). The titers were also varied among different immunogens and rabbits. Among the five antisera tested, antibody 7588, which was generated against antigen 7–LPH, exhibited the highest titer with all coating antigens. Only those combinations of antiseros and coating antigen with OD absorbance of >0.50 in Table 1 were screened for competition by fenvalerate. The 1/50 values ranged from 30 to 300 μg/L in the homologous and heterologous systems tested (Table 2). The homologous system for Ab7588 (with cAg 7–BSA) showed the lowest 1/50 (29 μg/L), which was at least 4 times better than the heterologous format. However, a heterologous system worked best for Ab7586 (with cAg 7–BSA, 1/50 = 74 μg/L), which was ~4 times more sensitive than the homologous one. In this study, only the homologous system of Ab7588 and cAg 7–BSA was used for further assay development.

**Optimization.** The effects of the solvents (methanol or DMSO) and detergent on the ELISA system (Ab7588/7–BSA) were evaluated by preparing fenvalerate in a buffer containing various amounts of solvent or Tween 20. The results (Tables 3 and 4) showed that these solvents significantly influence assay sensitivity and absorbance. The maximum absorbance (without analyte) was enhanced with increasing MeOH concentration. However, it was significantly decreased in the presence of DMSO (Table 4), indicating that high concentration of DMSO suppresses antibody–hapten
binding. Consequently, only MeOH was chosen for further optimization. The optimal MeOH concentration was selected on the basis of \(I_{50}\) values and the ratios of maximum and minimum absorbances for esfenvalerate standard curves (A/D). Because esfenvalerate is highly lipophilic and will adhere to glass and plastic surfaces (Sharom and Solomon, 1981), higher concentrations of MeOH might reduce such losses by solution of adsorbed material. The lowest \(I_{50}\) values were measured at 50 and 75% MeOH (32 and 41 \(\mu\)g/L, respectively). Because of a low A/D ratio for 75% MeOH, a MeOH concentration of 50% was selected for subsequent experiments.

Tween 20, a nonionic detergent, is commonly used in many immunoassays to reduce nonspecific binding and improve sensitivity (Vanderlaan et al., 1988; Chiu et al., 1995). In this study, Tween 20 significantly affected the binding between antibody and hapten (Figure 3). At the concentration tested (0.05% of Tween 20), the \(I_{50}\) with Tween 20 was ~20 times higher than that without the detergent. The maximum absorbance was significantly suppressed in addition. This is probably caused by nonspecific hydrophobic interaction between detergent and lipophilic esfenvalerate molecules in an aqueous system (Manclus and Montoya, 1996; Sugawara et al., 1998). Therefore, no Tween 20 was used in either the analyte solution or the antiserum solution in this assay.

The optimized esfenvalerate ELISA used 0.4 \(\mu\)g/mL of coating antigen 7–BSA, antibody 7588 at a dilution 1:12000, final dilution in well, and coating antigen 7–BSA (0.4 \(\mu\)g/mL). This standard curve represents the average of 25 curves.

Table 3. Effects of Methanol Concentration

<table>
<thead>
<tr>
<th>MeOH (%)</th>
<th>(A_{\text{max}}) (A)</th>
<th>slope (B)</th>
<th>(I_{50}) ((\mu)g/L) (C)</th>
<th>(A_{\text{min}}) (D)</th>
<th>A/D</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.61 ± 0.02</td>
<td>0.89</td>
<td>247 ± 24.2</td>
<td>0.09</td>
<td>6.3</td>
<td>0.99</td>
</tr>
<tr>
<td>10</td>
<td>0.65 ± 0.03</td>
<td>0.78</td>
<td>155 ± 9.2</td>
<td>0.10</td>
<td>6.2</td>
<td>1.00</td>
</tr>
<tr>
<td>25</td>
<td>0.71 ± 0.03</td>
<td>0.74</td>
<td>68 ± 10.0</td>
<td>0.08</td>
<td>8.8</td>
<td>0.99</td>
</tr>
<tr>
<td>50</td>
<td>0.84 ± 0.02</td>
<td>0.83</td>
<td>32 ± 5.5</td>
<td>0.09</td>
<td>9.3</td>
<td>1.00</td>
</tr>
<tr>
<td>75</td>
<td>0.90 ± 0.04</td>
<td>1.08</td>
<td>41 ± 6.6</td>
<td>0.18</td>
<td>5.0</td>
<td>1.00</td>
</tr>
<tr>
<td>100</td>
<td>0.95 ± 0.04</td>
<td>1.07</td>
<td>117 ± 17.0</td>
<td>0.31</td>
<td>3.5</td>
<td>0.99</td>
</tr>
</tbody>
</table>

\(\ast\) ELISA conditions: coating antigen 7–BSA (0.4 \(\mu\)g/mL); antiserum 7588 (1:6000); goat anti-rabbit IgG-HRP (1:3000). \(\ast\) Concentration of methanol in esfenvalerate standard solution (PBS/MeOH). \(\ast\) Mean value ± SD. Each set of data represents the average of three experiments.

Table 4. Effects of DMSO Concentration

<table>
<thead>
<tr>
<th>DMSO (%)</th>
<th>(A_{\text{max}}) (A)</th>
<th>slope (B)</th>
<th>(I_{50}) (xppb) (C)</th>
<th>(A_{\text{min}}) (D)</th>
<th>A/D</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.61 ± 0.02</td>
<td>0.89</td>
<td>247 ± 24.2</td>
<td>0.09</td>
<td>6.3</td>
<td>0.99</td>
</tr>
<tr>
<td>10</td>
<td>0.45 ± 0.04</td>
<td>0.70</td>
<td>207 ± 32.0</td>
<td>0.06</td>
<td>7.0</td>
<td>1.00</td>
</tr>
<tr>
<td>25</td>
<td>0.37 ± 0.03</td>
<td>0.76</td>
<td>219 ± 18.5</td>
<td>0.06</td>
<td>6.2</td>
<td>0.99</td>
</tr>
</tbody>
</table>

\(\ast\) ELISA conditions: coating antigen 7–BSA (0.4 \(\mu\)g/mL); antiserum 7588 (1:6000); goat anti-rabbit IgG-HRP (1:3000). \(\ast\) Concentration of DMSO in esfenvalerate standard solution (PBS/DMSO). \(\ast\) Mean value ± SD. Each set of data represents the average of three experiments.

Figure 3. Effect of detergent (Tween 20): (○) 0% (●) 0.05%.
pyrethroids such as permethrin, cypermethrin, and deltamethrin do not interfere with this assay, which makes this technique very useful for the selective detection of this compound. Compared to (S,S)-fenvalerate as a reference, Ab7588 fully recognized the S,R enantiomer of fenvalerate, and the S,R/S pair of diastereoisomers, which have the same absolute configuration as immunogen hapten. However, isomers that have the R configuration at the acid portion of the fenvalerate molecule have a very low cross-reactivity (<14%). This is consistent with the study by Wing and Hammock (1979) in which their immunoassay showed the highest selectivity for the optical center most distal to the site of coupling. The data indicate that in this study the stereostructure of the acid moiety of fenvalerate is more critical than that of alcohol portion in determining antibody stereospecificity. These further supported the hypothesis that the portion of hapten farthest from the protein is the most important in causing antibody specificity (Parker, 1976; Wing and Hammock, 1979). It must be pointed out that the (S)-fenvalerate acid used in the synthesis of the haptons contained ~1% of R isomer (see synthesis above). If this carried through the synthetic steps to the final haptons, it would generate some antibodies to the R,R/S isomers and thus contribute to the cross-reactivity.

Finally, cross-reactivity experiments were conducted for haptons, which were used for preparing antigens (Table 5). A high inhibition, ~25 times better than with esfenvalerate, was detected for immunogen hapten 7. Similar phenomena have been reported (Wing et al., 1978; Stanker et al., 1987) for (S)-bioallethrin and dioxin immunoassays, respectively, in which hapten handle recognition was assumed to be a contributing factor. However, this assay was also extremely sensitive to haptons 4, 10, and 11, which have handles different from that of the immunogen hapten. Esfenvalerate is a very lipophilic compound, which may limit the interaction between antibody and anlyte. In addition, haptons 4, 7, 10, and 11 all contain −NH₂ or −COOH groups, which make these compounds more polar and more soluble in aqueous system. Therefore, the solubility of those haptons could be a contributing factor for their high cross-reactivity (Stanker et al., 1987). This hypothesis is also supported by the fact that the cross-reactivity was significantly lowered when those −COOH-containing haptons (4, 10, and 11) were esterified as benzyl esters (Table 5).

**SPE and Assay Validation.** To detect esfenvalerate in water samples at a sub parts per billion level, a rapid and efficient SPE procedure was developed. The C₁₈ column has been considered the primary choice for extracting nonpolar or semipolar pesticides including pyrethroids from natural water sources (Junk and Richard, 1988; Swineford and Belisle, 1989; Durhan et al., 1990). A recent study by Woin (1994) showed that C₈ sorbent is as effective as C₁₈ for pyrethroid extraction, showing very good recoveries (80–100%). In the present study, high recovery rates were achieved for both C₈ and C₁₈ columns (82 and 85%, respectively) when applied to tap and river water samples (Table 7). Although the C₈ column had higher eluting efficiency with methanol than the C₁₈ column (0.84 versus 1.6% of analyte in second through fifth eluate fractions), the C₁₈ column is much more effective for extracting fenvalerate from water than the C₈ column (~0.05 versus 2.2% of analyte lost in filtrate). To ensure a higher rate of recovery, a C₁₈ SPE column was used in this assay. Because fenvalerate can be easily adsorbed to glass and plastic materials (Schoor and McKenney, 1983; Day, 1991; Woin, 1994), sample handling is of great importance for the accurate detection of this compound in water. In this study, only 13 ± 2.7% of fenvalerate was found to adhere to the surface of the glass container, which is fairly low compared with previous studies (Schoor and McKenney, 1983; Day, 1991). In our experience, to maintain a consistently low level of adsorption, a thorough rinse of the glass containers with deionized water is required. This reduces adsorption by 6–8% (data not shown).

Adding a trap solvent such as propylglycol is also very helpful. Finally, sonication was proposed when the water samples were kept in a container for >12 h (reducing losses by 5–10%, data not shown).

Assay validation was conducted in a blind fashion by SPE plus ELISA for tap and Sacramento River water samples, which were spiked with esfenvalerate concentrations ranging from 0 to 50 ppb (Figure 5). Good correlation between spiked and ELISA-measured esfenvalerate was obtained from linear regression analysis (Y = 0.98X − 0.079, R² = 0.96, n = 16). All recoveries were >80% of spiked value. Recovery of esfenvalerate for five water samples spiked at 0.1 μg/L was 0.102 ± 0.018 μg/L (102 ± 18%). These results demonstrate that this ELISA is suitable for the detection of esfenvalerate in water contamination monitoring, which requires high sensitivity.

In conclusion, careful hapten design and preparation, extensive studies with various antibody–antigen combinations, and optimization of an ELISA resulted in a homologous immunoassay (Ab75887–BSA) that is highly selective and fairly sensitive for esfenvalerate. With a rapid and simple SPE step, this ELISA was successfully applied to the quantitative detection of sub parts per billion amounts of esfenvalerate in water. A high concentration of cosolvent (methanol) in this ELISA system (50% in antibody and standard solution) is extremely important for the accurate performance of the ELISA for the highly lipophilic esfenvalerate. Finally,

| Table 6. Cross-Reactivities of Pyrethroids and Esfenvalerate Metabolites |
|-----------------------------|-----------------------------|-----------------------------|
| analyte                      | % cross-reactivity<sup>a</sup> | analyte                      | % cross-reactivity<sup>a</sup> |
| esfenvalerate acid           | 100                         | phenothrin                  | ni                          |
| phenoxynbenzoic acid         | <0.01                       | cyfluthrin                  | ni                          |
| (S)-fenvalerate acid         | <0.01                       | fenpropothrin               | ni                          |
| permethrin                   | ni<sup>b</sup>              | resmethrin                  | ni                          |
| cypermethrin                 | ni                          | fluvalinate                 | ni                          |
| deltamethrin                 | ni                          |                             |                             |

<sup>a</sup> Cross-reactivity was calculated as (I₅₀ of esfenvalerate/I₅₀ of analyte) × 100%. <sup>b</sup> < 10% inhibition was measured at highest concentration (10000 μg/L).

<table>
<thead>
<tr>
<th>Table 7. SPE Method Development</th>
</tr>
</thead>
<tbody>
<tr>
<td>%&lt;sup&gt;14&lt;/sup&gt;C-esfenvalerate recovered&lt;sup&gt;a&lt;/sup&gt; (SD)</td>
</tr>
<tr>
<td>C₈ column (n = 3)</td>
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<tr>
<td>-------------------</td>
</tr>
<tr>
<td>eluate&lt;sup&gt;a&lt;/sup&gt;</td>
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<td></td>
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<tr>
<td></td>
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<tr>
<td></td>
</tr>
<tr>
<td>solution A&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>solution B&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Analyte was eluted with 100% methanol, 3.5 mL each fraction. <sup>b</sup> Solution washed from glass container. <sup>c</sup> Solution extracted from postcolumn solution (filtrate).
in the integration with traditional analytical methods, this relatively simple, sensitive, and highly selective immunochemical assay could play an important role for environmental contamination studies and monitoring.

ABBREVIATIONS USED

Ab, antibody; BSA, bovine serum albumin; BSTFA, bis(trimethylsilyl)trifluoroacetamide; cAg, coating antigen; DAPEC, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide; DCC, 1,3-dicyclohexylcarbodiimide; DMF, N,N-dimethylformamide; DMSO, dimethyl sulfoxide; ELISA, enzyme-linked immunosorbent assay; FAB-HRMS, fast atom bombardment high-resolution mass spectrometry; GAR-HP, goat anti-rabbit immunoglobulin conjugated to horseradish peroxidase; GLC, gas-liquid chromatography; HPLC, high-performance liquid chromatography; I$_{50}$, the concentration of analyte giving 50% inhibition; LDL, lower detection limit; LPH, hemocyanin from Limulus polyphemus; Morpho-CDI, 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide-metho-toluene-sulfonate; NHS, N-hydroxysuccinimide; NMR, nuclear magnetic resonance; OVA, ovalbumin; PB, phenoxycarbonyl; PBS, phosphate-buffered saline; PBT, phosphate-buffered saline with 0.05% of Tween 20; RT, room temperature; SPE, solid-phase extraction; THF, tetrahydrofuran; TLC, thin-layer chromatography; TMB, tetramethylbenzidine.

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