

Development of an Immunoassay for the Pyrethroid Insecticide Esfenvalerate

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A competitive enzyme-linked immunosorbent assay was developed for the detection of the pyrethroid insecticide esfenvalerate. Two haptens containing amine or propanoic acid groups on the terminal aromatic ring of the fenvalerate molecule were synthesized and coupled to carrier proteins as immunogens. Five antisera were produced and screened against eight different coating antigens. The assay that had the least interference and was the most sensitive for esfenvalerate was optimized and characterized. The I_{50} for esfenvalerate was $30 \pm 6.2 \mu\text{g/L}$, and the lower detection limit (LDL) was $3.0 \pm 1.8 \mu\text{g/L}$. The assay was very selective. Other pyrethroid analogues and esfenvalerate metabolites tested did not cross-react significantly in this assay. To increase the sensitivity of the overall method, a C_{18} sorbent-based solid-phase extraction (SPE) was used for water matrix. With this SPE step, the LDL of the overall method for esfenvalerate was $0.1 \mu\text{g/L}$ in water samples.

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

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, >331500 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 (Boccelli et al., 1993; Wells et al., 1994) or electrolytic conductivity 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

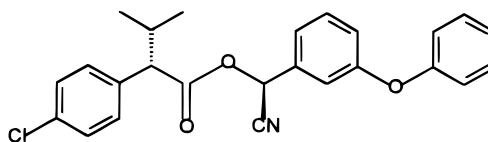


Figure 1. Structure of esfenvalerate.

trace levels (Hammock and Mumma, 1980; Gee et al., 1988; Hammock et al., 1990). A number of enzyme-linked immunosorbent assay (ELISA) methods have been reported for the detection of synthetic pyrethroids. They include allethrin (Pullen and Hock, 1995), *s*-bioallethrin (1*R*,3*R*,4'*S*-allethrin) (Wing et al., 1978), bioresmethrin (Hill et al., 1993), deltamethrin (Queffelec et al., 1998; Lee et al., 1998), fenpropathrin (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, fluvalinate, permethrin, phenothrin, and resmethrin standards (Figure 2) were obtained from Riedel de Haen (Seelze, Germany). ^{14}C -Labeled fenvalerate (14.9 mCi/mmol) was provided by Shell Development Co. (Modesto, CA). Racemic 4-chloro- α -(1-methylethyl)benzeneacetic 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 \rightarrow notation denotes a stepwise concentration gradient.

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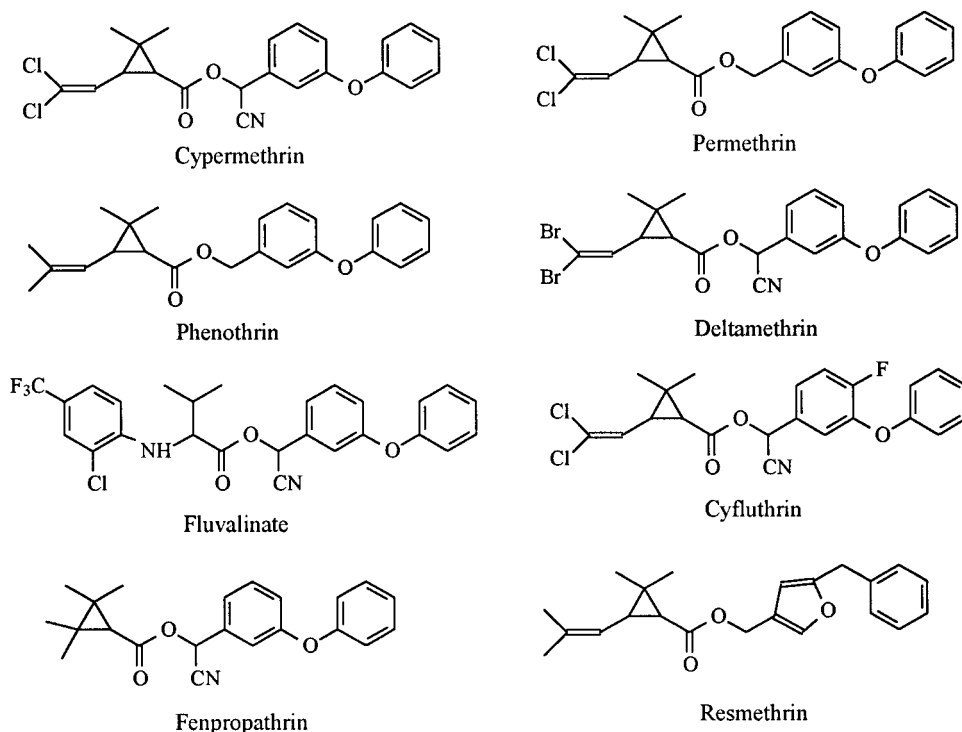


Figure 2. Structures of other pyrethroids used in this study.

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, Swedesborough, 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 μ m 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 high-resolution mass spectra (FAB-HRMS) were obtained on a ZAB-HS-2F mass spectrometer (VG Analytical, Wythenshawe, 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 ELISAs, and Dynatech microtiter plates (Dynatech Laboratories, Inc., Chantilly, VA) were used for preparing serial dilutions.

Hapten Synthesis and Verification. Syntheses of the haptens were carried out as outlined in Scheme 1. All reactions were straightforward, and yields were moderate to good. Analytical data supporting the structures are provided. Racemic 4-chloro- α -(1-methylethyl)benzeneacetic acid (fenvalerate acid) was resolved via the (*R*)- and (*S*)- α -methylbenzylamine salts as described.

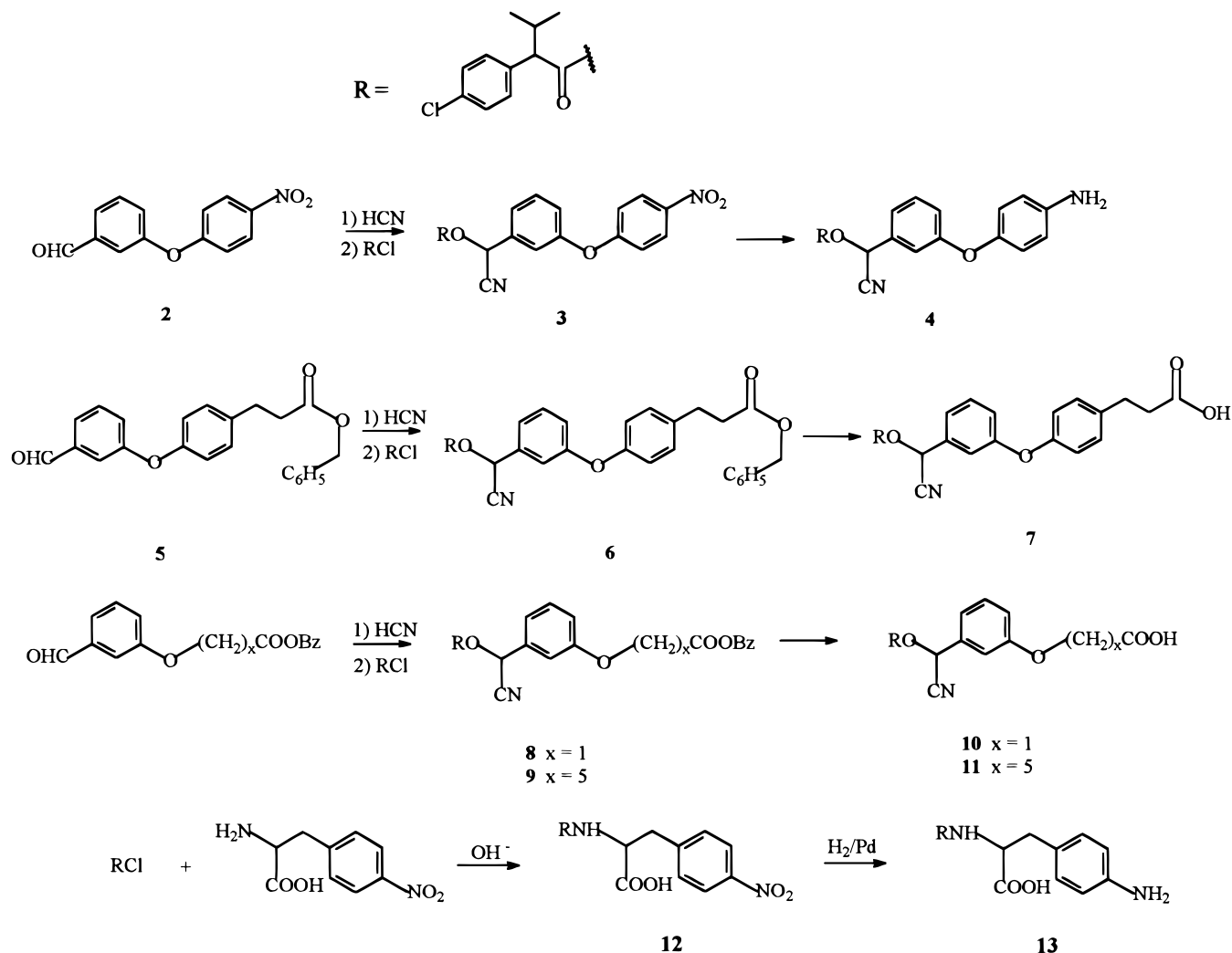
Resolution of 4-Chloro- α -(1-methylethyl)benzeneacetic Acid (Fenvalerate Acid). The racemic acid (128 g, 0.6 mol) in toluene (218 mL) and methanol (320 mL) was stirred and heated to reflux, and, with heating removed; (*S*)-(-)- α -

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, and the free acid was extracted into diethyl ether. 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 $^{\circ}$ C, $[\alpha]_D^{24}$ 48.1 (*c* 1.61, CHCl_3). The literature (Miyakado et al., 1975) gives mp 104–5 $^{\circ}$ C, $[\alpha]_D^{24}$ 46.8 (*c* 1–2, CHCl_3). 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 $^{\circ}$ C, $[\alpha]_D^{24}$ -47.5 (*c* 1.49, CHCl_3).

The optical purity of each acid was determined by treatment of a 100 mg (0.47 mmol) sample in 0.3 mL of CHCl_3 plus 0.5 μ L of *N,N*-dimethylformamide (DMF) with SOCl_2 (0.068 mL, 0.93 mmol) at 60 $^{\circ}$ C for 45 min. Vacuum stripping of the resulting acid chloride followed by addition of CH_2Cl_2 (0.5 mL) to an ice-chilled solution of (*S*)-(-)- α -methylbenzylamine (0.13 mL, 1.0 mmol) (Aldrich, 99.6% ee) in CH_2Cl_2 (0.5 mL) gave the diastereoisomeric amides. GLC analysis (50 \rightarrow 300 $^{\circ}$ C at 20 $^{\circ}$ C/min) gave complete separation of enantiomer pairs; the (*SS* + *RR*)/(*SR* + *RS*) ratios for the (*S*)- and (*R*)-acids were 98.3:1.7 and 0.8:99.2, respectively. Conversion of the acids to their respective amides using 1,3-dicyclohexylcarbodiimide (DCC) gave similar results: 97.7:2.3 and 0.6:99.4, respectively. The minimum optical purities of the two acids are therefore approximately 96 and 98% ee, respectively.

Fenvalerate Isomer Standards. 3-Phenoxybenzaldehyde (2.38 g, 12 mmol) and potassium cyanide (1.17 g, 18 mmol) in 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 CH_2Cl_2 and water, acidified with 3

Scheme 1. Synthesis of Haptens 4, 7, 10, 11, and 13



N HCl (caution, HCN released), washed three times with water, dried briefly over anhydrous MgSO_4 , and stripped to a colorless oil. Meanwhile, (*S*)-fenvalerate acid (2.55 g, 12 mmol) in 6 mL of CHCl_3 was treated with SOCl_2 (1.31 mL, 1.8 mmol) and DMF (0.5 μL) 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 CH_2Cl_2 was stirred with ice cooling, and the above cyanohydrin in 4.5 mL of CH_2Cl_2 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 MgSO_4 , and stripped to an oil, which was flash chromatographed on 100 g of silica gel (10 \rightarrow 60% CH_2Cl_2 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 reprocessed 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,S* isomer gave 620 mg of oil, which was crystallized from \sim 2 mL of MeOH at 0 °C to give 495 mg of white solid: mp 60–61.5 °C, $[\alpha]_{\text{D}}^{24} -11.1$ (*c* 1.5, CHCl_3); $^1\text{H NMR}$ (CDCl_3) δ 0.70 (d, *J* = 6.7 Hz, 3 H, CH_3), 0.95 (d, *J* = 6.5 Hz, 3 H, CH_3), 2.30 (m, *J* = 6.6, 10.5 Hz, 1 H, Me_2CH), 3.22 (d, *J* = 10.5 Hz, 1 H, $\text{CHC}(\text{O})$), 6.34 (s, 1 H, CHCN), 6.98–7.40 (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: $[\alpha]_{\text{D}}^{24} -11.2$ (*c* 0.985, CHCl_3); $^1\text{H NMR}$ (CDCl_3) δ 0.73 (d, *J* = 6.7 Hz, 3 H, CH_3), 1.06 (d, *J* = 6.5, 3 H, CH_3), 2.33 (m, *J* = 6.6, 10.4 Hz, 1 H, Me_2CH), 3.23 [d, *J* = 10.7 Hz, 1 H, $\text{CHC}(\text{O})$], 6.29 (s, 1 H, CHCN), 6.96–7.38 (m, 13 H, Ar).

A similar reaction and workup sequence starting with (*R*)-fenvalerate acid resulted in 441 mg of the *R,R* isomer [mp 59–60.5 °C, $[\alpha]_{\text{D}}^{24} 12.0$ (*c* 1.15, CHCl_3)] and 575 mg of the *R,S* isomer as an oil [$[\alpha]_{\text{D}}^{24} 12.7$ (*c* 1.72, CHCl_3)]. GLC analysis (15 m capillary DB-5 column, 50 \rightarrow 300 °C at 20 °C/min) of the four chiral fenvalerate isomers gave the following results: *S,S* isomer, >98% under one peak, 1.4% corresponding to *S,R/R,S* isomers; *S,R* isomer, >99% under one peak; *R,R* isomer, >98% under one peak, 1.4% corresponding to *R,S/S,R* isomers; *R,S* isomer, >99% under one peak.

Cyano[3-(4-nitrophenoxy)phenyl]methyl (S)-4-Chloro- α -(1-methylethyl)benzeneacetate (4-Nitrofenvalerate) (3). The cyanohydrin of 3-(4-nitrophenoxy)benzaldehyde (**2**) (Loewe and Urbanietz, 1967) (3 g, 12.3 mmol) 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 CH_2Cl_2 was stirred with ice cooling as the acid chloride in 10 mL of CH_2Cl_2 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 *R_f* 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 passes: 100 mg of the higher *R_f* *S,R* isomer [$[\alpha]_D^{21}$ -14.1 (c 1.13, CHCl₃); ¹H NMR δ 0.73 (d, *J* = 6.7 Hz, 3 H, CH₃), 1.03 (d, *J* = 6.5 Hz, 3 H, CH₃), 2.34 (m, *J* = 6.6, 10.5 Hz, 1 H, Me₂-CH), 3.24 [d, *J* = 10.5 Hz, 1 H, CHC(O)], 6.37 (s, 1 H, CHCN), 7.0–8.3 (m, 12 H, Ar)] and 89 mg of the lower *R_f* *S,S* isomer [$[\alpha]_D^{21}$ -7.3 (c 1.16, CHCl₃); ¹H NMR (CDCl₃) δ 0.72 (d, *J* = 6.7 Hz, 3 H, CH₃), 0.96 (d, *J* = 6.5 Hz, 3 H, CH₃), 2.30 (m, *J* = 6.6, 10.5 Hz, 1 H, Me₂CH), 3.24 [d, *J* = 10.5 Hz, 1 H, CHC(O)], 6.40 (s, 1 H, CHCN), 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 0.7 (d, *J* = 6.5 Hz, 3 H, CH₃), 0.95 (d, *J* = 6.5 Hz, 3 H, CH₃), 2.30 (m, 1 H, Me₂CH), 3.21 [d, *J* = 10.4 Hz, 1 H, CHC(O)], 3.64 (b, 2 H, NH₂), 6.30 (s, 1 H, CHCN), 6.68–7.36 (m, 12 H, Ar). This was dissolved in 0.3 mL of H₂O plus 0.22 mL of 3 N HCl plus 0.2 mL of EtOH, chilled in ice, and treated with 1 equiv of NaNO₂ solution followed by 0.15 mL of 50% H₃PO₂. Workup and purification by TLC gave an oil, which crystallized immediately on seeding with, and had an identical TLC to, (*S,S*)-fenvalerate [five solvent passes (5% THF in hexane) gave complete separation of the two diastereoisomers].

Cyano[3-(4-aminophenoxy)phenyl]methyl (S)-4-Chloro-α-(1-methylethyl)benzeneacetate (4-Aminoefenvalerate) (4). Stannous chloride hydrate (226 mg, 1 mmol) was added to a stirred solution of (*S,R,S*)-nitrofenvalerate **3** (93 mg, 0.2 mmol) in 0.75 mL of EtOH, and the mixture was heated under N₂ at 70–75 °C for 30 min. The cooled mixture was treated with 0.4 g of filter aid and diluted with water and ether. NaHCO₃ (168 mg, 2 mmol) was added in portions and stirred until CO₂ evolution ceased. The mixture was filtered, and solids were extracted with ether. Combined ether extracts were stripped, and the residue was chromatographed on 5 g of silica gel (50% CH₂Cl₂/hexane → CH₂Cl₂) to recover 5 mg of starting material and 53.9 mg (62%) of the aniline as a colorless viscous gum: TLC *R_f* 0.3 (CH₂Cl₂). The TLC spot rapidly darkened on exposure to light. A similar reduction of the *S,S* isomer (186 mg, 0.4 mmol) gave, after chromatography, 123 mg (70%) as a viscous colorless oil, which had a TLC *R_f* identical to that of the *S,R/S* isomer mix obtained above: ¹H NMR (CDCl₃) δ 0.70 (d, *J* = 6.7 Hz, 3 H, CH₃), 0.95 (d, *J* = 6.5 Hz, 3 H, CH₃), 2.30 (m, 1 H, Me₂CH), 3.21 [d, *J* = 10.4 Hz, 1 H, CHC(O)], 3.64 (b, 2 H, NH₂), 6.30 (s, 1 H, CHCN), 6.68–7.36 (m, 12 H, Ar); ¹³C NMR (CDCl₃) δ 19.9, 21.0, 31.9, 58.6, 62.7, 115.5, 115.9, 116.2, 118.7, 120.8, 121.2, 128.7, 129.7, 130.3, 133.0, 133.5, 135.1, 143.3, 147.4, 159.6, 171.3; FAB-HRMS *m/z* calcd for [M⁺] = C₂₅H₂₃ClN₂O₃ 434.1396, obsd 434.1384.

Benzyl 4-[3-[Cyano[(S)-2-(4-chlorophenyl)-3-methyl-1-oxobutanoxy]methyl]phenoxy]benzenepropanoate (6). Benzyl 4-(3-formylphenoxy)benzenepropanoate (3.6 g, 10 mmol) was converted to the cyanohydrin as described in a previous paper (Wengatz et al., 1998). (*S*)-4-Chloro-α-(1-methylethyl)benzeneacetyl chloride was prepared from the corresponding (*S*)-acid (2.13 g, 10 mmol) as described above. The acid chloride in 7 mL of CH₂Cl₂ was stirred with ice cooling, and the cyanohydrin in 7 mL of CH₂Cl₂ was added all at once followed immediately by pyridine (1.01 mL, 12.5 mmol). The mixture was stirred at ambient temperature for 30 min and then washed with water, 3 N HCl, and water, dried (MgSO₄), and stripped to light brown oil. Flash chromatography on 80 g of silica gel (50% hexane/CH₂Cl₂ → ether) gave 4.96 g (85%) of product as a mixture of two diastereoisomers. ¹H NMR (CDCl₃) indicated a 1:1 mixture of 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* = 6.9 Hz, 3 H, CH₃), 1.05 (d, *J* = 6.5 Hz, 3 H,

CH₃), 2.3 (m, *J* = 6.5, 10.4 Hz, 1 H, Me₂CH), 2.7 (t, *J* = 7.6 Hz, 2 H, CH₂), 3.0 (t, *J* = 7.6 Hz, 2 H, CH₂), 3.23 [d, *J* = 10.4 Hz, 1 H, CHC(O)], 5.12 (s, 2 H, CH₂Ar), 6.28 (s, 1 H, CHCN), 6.86–7.4 (m, 17 H, Ar); *S,S* isomer δ 0.72 (d, *J* = 6.9 Hz, 3 H, CH₃), 0.94 (d, *J* = 6.5 Hz, 3 H, CH₃), 2.3 (m, *J* = 6.5, 10.4 Hz, 1 H, Me₂CH), 2.7 (t, *J* = 7.6 Hz, 2 H, CH₂), 3.0 (t, *J* = 7.6 Hz, 2 H, CH₂), 3.22 [d, *J* = 10.4 Hz, 1 H, CHC(O)], 5.12 (s, 2 H, CH₂Ar), 6.33 (s, 1 H, CHCN), 6.86–7.4 (m, 17 H, Ar).

4-[3-[Cyano[(S)-2-(4-chlorophenyl)-3-methyl-1-oxobutanoxy]methyl]phenoxy]benzenepropanoic Acid (7). The ester **6** as the diastereoisomer pair (0.97 g, 1.67 mmol) in 2 mL of CH₂Cl₂ was treated under N₂ with 25 μL of BSTFA followed by iodotrimethylsilane (0.249 mL, 1.75 mmol). After 3 h, the mixture was treated with 1 mL of water. The organic phase was then immediately flash 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: *R_f* 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, Me₂CH), 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, CHC(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, Me₂CH), 2.69 (t, *J* = 7.6 Hz, 2 H, CH₂), 2.96 (t, *J* = 7.7 Hz, 2 H, CH₂), 3.22 [d, *J* = 10.5 Hz, 1 H, CHC(O)], 6.34 (s, 1 H, CHCN), 6.9–7.39 (m, 12 H, Ar); FAB-HRMS *m/z* calcd for [M + H]⁺ = C₂₈H₂₇ClNO₅ 492.1552, obsd 492.1577.

Benzyl 3-[Cyano[(S)-2-(4-chlorophenyl)-3-methyl-1-oxobutanoxy]methyl]phenoxyacetate (8). Benzyl 2-(3-formylphenoxy)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-α-(1-methylethyl)benzeneacetyl chloride was prepared from the corresponding (*S*)-acid (1.57 g, 7.4 mmol) as described above. The cyanohydrin in 2.5 mL of CH₂Cl₂ was stirred with ice cooling, and the acid chloride in 3 mL of CH₂Cl₂ was added all at once followed 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) in ~200 mg batches to give nearly complete separation of isomers. For final purification, the higher *R_f* isomer was repassed with the same chromatographic condition to recover 613 mg of colorless viscous oil after high-vacuum stripping: $[\alpha]_D^{26}$ -5.42 (c 1.62, CHCl₃); ¹H NMR (CDCl₃) δ 0.73 (d, *J* = 6.6 Hz, 3 H, CH₃), 1.06 (d, *J* = 6.3 Hz, 3 H, CH₃), 2.33 (m, *J* = 6.7, 10.3 Hz, 1 H, Me₂CH), 3.23 [d, *J* = 10.5 Hz, 1 H, CHC(O)], 4.6 [s, 2 H, OCH₂C(O)], 5.24 (s, 2 H, CH₂Ph), 6.28 (s, 1 H, CHCN), 6.83–7.42 (m, 13 H, Ar). The lower *R_f* isomer was further purified in the same way to recover 586 mg of colorless viscous oil: $[\alpha]_D^{26}$ -8.26 (c 1.84, CHCl₃); ¹H NMR (CDCl₃) δ 0.70 (d, *J* = 6.7 Hz, 3 H, CH₃), 0.95 (d, *J* = 6.5 Hz, 3 H, CH₃), 2.30 (m, *J* = 6.4, 10.4 Hz, 1 H, Me₂CH), 3.22 [d, *J* = 10.5 Hz, 1 H, CHC(O)], 4.66 [s, 2 H, OCH₂C(O)], 5.24 (s, 2 H, CH₂Ph), 6.32 (s, 1 H, CHCN), 6.94–7.39 (m, 13 H, Ar). Comparison with the very consistent NMR shifts observed with the fenvalerate isomers and the nitro- and aminofenvalerate above allowed tentative assignment of the *S,R* and *S,S* configurations to the higher and lower *R_f* isomers, respectively.

Benzyl 6-[3-[Cyano[(S)-2-(4-chlorophenyl)-3-methyl-1-oxobutanoxy]methyl]phenoxy]hexanoate (9). Benzyl 6-(3-formylphenoxy)hexanoate (1.75 g, 5.36 mmol) was converted to the cyanohydrin as described in a previous paper (Wengatz et al., 1998). (*S*)-4-Chloro-α-(1-methylethyl)benzeneacetyl chloride was prepared from the corresponding (*S*)-acid (1.14 g, 5.36 mmol) as described above. These two materials were reacted

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 diastereoisomers, 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 reprocessed in two portions for further purification. Separation was followed by TLC (10 cm plates) with which five or more solvent passes (5% THF in hexane) were required to show complete separation of the two isomers. High-vacuum stripping of the higher *R_f* *S,S* isomer gave 637 mg of colorless oil: $[\alpha]_D^{24} -7.76$ (*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, Me₂CH), 2.4 (t, *J* = 7.4 Hz, 2 H, CH₂), 3.23 [d, *J* = 10.4 Hz, 1 H, CHC(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_f* *S,S* isomer gave 623 mg: $[\alpha]_D^{24} -13.8$ (*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, Me₂CH), 2.41 (t, *J* = 7.4 Hz, 2 H, CH₂), 3.22 [d, 1 H, CHC(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-[Cyano[(*S*)-2-(4-chlorophenyl)-3-methyl-1-oxobutan-oxymethyl]phenoxyacetic Acid (10). The ester **8** as the diastereoisomer pair (1.78 g, 3.6 mmol) in 3.5 mL of CH₂Cl₂ was treated with 30 μL 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, *R_f* 0.4 (CH₂Cl₂), were stripped under high vacuum to give 1.45 g (100%) of **10** as the diastereoisomer pair. A sample of the *S,S* isomer of **10** (450 mg, 0.915 mmol) was similarly cleaved and purified to give **10** (300 mg) as the pure *S,S* isomer: 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, Me₂CH), 3.23 [d, *J* = 10.5 Hz, 1 H, CHC(O)], 4.68 [s, 2 H, OCH₂C(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]⁺ = C₂₁H₂₁ClNO₅ 402.1, obsd 402.

6-[3-[Cyano[(*S*)-2-(4-chlorophenyl)-3-methyl-1-oxobutanoxymethyl]phenoxy]hexanoic Acid (11). The ester **9** as the *S,S* isomer (400 mg, 0.73 mmol) in 0.75 mL of CH₂Cl₂ was treated under N₂ with BSTFA (30 μL) and iodotrimethylsilane (0.109 mL, 7.65 mmol). After 12 h, the mixture was treated with 0.5 mL of methanol, washed three times with water, and stripped. Flash chromatography on 7 g of silica gel (CH₂Cl₂ → 3% HOAc in EtOAc) recovered 12 mg of the starting ester and 261 mg (78%) of the acid **11** as viscous oil: ¹H NMR (CDCl₃) δ 0.71 (d, *J* = 6.7 Hz, 3 H, CH₃), 0.97 (d, *J* = 6.5 Hz, 3 H, CH₃), 1.54 (m, 2 H, CH₂), 1.73 (m, 2 H, CH₂), 1.82 (m, 2 H, CH₂), 2.31 (m, *J* = 6.5, 10.5 Hz, 1 H, Me₂CH), 2.41 (t, *J* = 7.4 Hz, 2 H, CH₂), 3.25 [d, *J* = 10.5 Hz, 1 H, CHC(O)], 3.93 (t, *J* = 6.3 Hz, 2 H, CH₂), 6.35 (s, 1 H, CHCN), 6.93–7.34 (m, 8 H, Ar); ¹³C NMR (CDCl₃) δ 19.9 (CH₃), 21.0 (CH₃), 24.3, 25.4, 28.7, 31.9, 33.8, 58.7, 62.9, 67.7, 113.5, 115.6, 116.4, 119.6, 128.7 (2C), 129.7 (2C), 130.2, 132.8, 133.5, 135.2, 159.4, 171.4, 179.7; FAB-HRMS *m/z* calcd for [M + H]⁺ = C₂₅H₂₉ClNO₅ 458.1734, obsd 458.175.

N-[(*S*)-4-Chloro-α-(1-methylethyl)benzeneacetyl]-4-nitro-L-phenylalanine (12). (*S*)-4-Chloro-α-(1-methylethyl)-benzeneacetyl chloride was prepared from the corresponding (*S*)-acid (1.71 g, 8 mmol) as described above. This was added in portions over 15 min to a vigorously stirred ice-cooled solution of 4-nitro-L-phenylalanine hydrate (1.75 g, 7.67 mmol) and 4-(dimethylamino)pyridine (4 mg) in 3.84 mL of 2 N KOH and 3 mL of water. An additional 3.8 mL of 2 N KOH was added dropwise over 45 min to maintain a slightly basic pH. After an additional 2 h, the odor of acid chloride was no longer

detectable. The mixture was diluted with CH₂Cl₂ and acidified (3 N HCl) to precipitate a white solid. After filtration, the organic phase precipitated additional solid material. Trituration of the filtrate oil with ether gave additional solid. All three crops (~2.2 g) were a mixture of two components by TLC: *R_f* = 0.26 and 0.45 (3% HOAc in EtOAc); *R_f* = 0.53 and 0.65 (3% HOAc in acetone). These were separated with some difficulty by column chromatography on 215 g of silica gel [(3% HOAc) CH₂Cl₂ → EtOAc]. Recrystallization of the higher *R_f* product (THF diluted with *n*-butyl chloride) recovered 1.1 g (35%) of **12**: mp 187.5–188 °C; $[\alpha]_D^{24} -52.4$ (*c* 1.06, EtOH); ¹H NMR (DMSO-*d*₆) δ 0.51 (d, *J* = 6.6 Hz, 3 H, CH₃), 0.57 (d, *J* = 6.4 Hz, 3 H, CH₃), 2.05 (dq, *J* = 6.5, 10.8 Hz, 1 H, Me₂CH), 2.96 (d, *J* = 10.7 Hz, 1 H, Me₂CHCH), 3.0 (dd, *J* = 10.7, 13.7 Hz, 1 H, HCHAr), 3.22 (dd, *J* = 4.6, 13.6 Hz, 1 H, HCHAr), 4.50 [m, 1 H, NCHC(O)], 7.26 (d, *J* = 8.6 Hz, 2 H, ArCl), 7.33 (d, *J* = 8.6 Hz, 2 H, ArCl), 7.42 (d, *J* = 8.7 Hz, 2 H, ArNO₂), 8.07 (d, *J* = 8.7 Hz, 2 H, ArNO₂), 8.43 (d, *J* = 8.4 Hz, 1 H, NH), 12.7 (bs, 1 H, COOH); FAB-MS *m/z* calcd for [M + H]⁺ = C₂₀H₂₂ClN₂O₅ 405.12, obsd 405. Recrystallization of the lower *R_f* product from the same solvent mix gave 0.4 g of white solid: mp 244–247 °C; $[\alpha]_D^{21} -20.1$ (*c* 1.03, EtOH), which was indicated by NMR and MS to be the acyl-4-nitrophenylalanyl-4-nitrophenylalanine; FAB-MS *m/z* calcd for [M + H]⁺ = C₂₉H₃₀ClN₄O₈ 597.195, obsd 597.

N-[(*S*)-4-Chloro-2-(1-methylethyl)benzeneacetyl]-4-amino-L-phenylalanine (13). A 0.75 g (1.85 mmol) sample of **12** and 25 mg of 10% Pd on carbon in 20 mL of ethanol was stirred under an atmosphere of hydrogen for 2.5 h. A TLC indicated conversion to a single major product, *R_f* 0.46 (3% HOAc in EtOAc), which darkened on exposure to light. A slightly more polar impurity was assumed to be the dechlorinated analogue. This was removed by flash chromatography on silica gel (3% HOAc in CH₂Cl₂ → EtOAc) to recover a colorless gum. This product was crystallized from diethyl ether to give 0.664 g (72%) of a pale tan solid: mp 105–9 °C (gas); $[\alpha]_D^{24} 5.6$ (*c* 1.04, EtOH), which was shown by NMR to be a 1:1 molecular compound with ether; stripping the NMR sample and reanalysis removed the ether spectrum; ¹H NMR (DMSO-*d*₆) δ 0.54 (d, *J* = 6.6 Hz, 3 H, CH₃), 0.70 (d, *J* = 6.4 Hz, 3 H, CH₃), 2.07 (dq, *J* = 6.6, 10.7 Hz, 1 H, Me₂CH), 2.69 (dd, *J* = 9.6, 13.8 Hz, 1 H, CH₂), 2.87 (dd, *J* = 4.7, 13.8 Hz, 1 H, CH₂), 3.08 (d, *J* = 10.6 Hz, 1 H, Me₂CHCH), 4.30 (dq, *J* = 4.6, 8.5 Hz, 1 H, NCHCOO), 6.51 (d, *J* = 8.2 Hz, 2 H, Ar), 6.86 (d, *J* = 8.2, 2 H, Ar), 7.28–7.35 (m, 4 H, Ar), 8.29 (d, *J* = 8.2 Hz, 1 H, NHCO); FAB-MS *m/z* calcd for [M + H]⁺ = C₂₀H₂₄ClN₂O₃ 375.04, obsd 375.

Hapten Conjugation. Conjugates were synthesized using three different methods: water soluble carbodiimide, diazotization, and activated ester method (Tijssen, 1985; Erlanger, 1973). To obtain immunogens, haptens **4** and **7** were conjugated to LPH. Coating antigens were made by coupling haptens **4**, **7**, **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 as 0.5 mL of a 0.201 M solution of sodium nitrite was injected. Some product appeared as a gel. DMF (0.4 mL) was added dropwise to give a homogeneous solution, which was divided into two equal aliquots (each aliquot was used for one protein). Ninety-eight milligrams of LPH or 101 mg of BSA or 100 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 and purified by acetone precipitation as described in Wengatz et al. (1998). The purified conjugates were suspended in water and stored in aliquots at –80, –20, and 4 °C.

Conjugates of 7-BSA/LPH. Morpho-CDI (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). 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 bleeds.

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: $y = \{A - D/[1 + (x/C)^B]\} + D$, 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 (I_{50}), 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 I_{50} in the ELISA. Cross-reactivity values were calculated as follows:

$$\text{CR}\% = (I_{50} \text{ of esfenvalerate}/I_{50} \text{ of tested compound}) \times 100$$

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.

Solid-Phase Extraction (SPE). An SPE method was developed for isolating esfenvalerate from water samples. Esfenvalerate was extracted from water using a C₁₈ SPE column (10 cm³/500 mg; part 1211-3027, Varian Sample Preparation Products, Harbor City, CA). The column was preconditioned with methanol (3.5 mL) and deionized water (3.5 mL) before sample application. Water samples (200 mL) were loaded on the column and eluted with 3–5 mL/min flows. The containers (glass flask) were washed with deionized water (5–8 × 5 mL), and all washes were applied onto the column as well. After the water had passed through, the column was dried under vacuum for 15 min and then eluted with 100% methanol (3.5 mL). After one drop of propylglycol was added, this methanol extract was evaporated to dryness under a gentle stream of nitrogen and the residue was redissolved in 1 mL of methanol. An aliquot was analyzed by ELISA diluted with 50% methanol in PBS (1:4).

Recovery of SPE. Radiolabeled fenvalerate (¹⁴C) was used for the investigation of esfenvalerate SPE recovery and elution behavior in both C₈ and C₁₈ columns. Extraction procedures similar to those described above were used. Each blank water sample (200 mL) was spiked with 1.35 nmol of ¹⁴C-fenvalerate (45000 dpm). After preconditioning, extraction, washing, and column drying steps, fenvalerate was eluted with 100% methanol (five fractions × 3.5 mL). Each fraction was collected in a different tube. To monitor fenvalerate losses after extraction, the filtrate (postcolumn water solution) was collected and extracted with ethyl acetate (3 × 10 mL). This combined ethyl acetate extract was assigned as solution A. The residue fenvalerate on the surface of glass flask (container) was extracted by rinsing the container with ethyl acetate, which was assigned as solution B. Each of final eluate fractions and surface washing and postcolumn extraction solutions were evaporated to dryness (one drop of propylglycol was added to each tube before evaporation) under nitrogen, and then residues were dissolved into Scintiverse BD cocktail (3 mL) prior to analysis by a 1409 liquid scintillation counter (Wallac, Gaithersburg, MD).

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-methylethyl)benzeneacetic 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 (Friend, 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 *R_f* isomer to the aminofenvalerate, which was diazo-

Table 1. Titration Summary for Anti-esfenvalerate Antisera

antibody/immunogen	coating antigen							
	4-BSA	7-BSA	10-BSA	10-OVA	11-BSA	11-OVA	13-BSA	13-OVA
AS7584/4-LPH	+++ ^{a,b}	++	++	+	-	-	+	+
AS7585/4-LPH	++ ^b	+	++	+	+	-	-	-
AS7586/4-LPH	+++ ^b	++	++	+	+	+	-	-
AS7588/7-LPH	+++	+++ ^b	+++	+++	++	++	++	++
AS7589/7-LPH	+	++ ^b	+	+	+	-	+	+

^a The data shown were determined at a coating antigen concentration of 1.0 $\mu\text{g/mL}$ and an antibody dilution of 16000. (-) Absorbance < 0.25; (+) absorbance = 0.25–0.50; (++) absorbance = 0.50–0.75; (+++) absorbance > 0.75. ^b Homologous format.

Table 2. Selected Competitive ELISA Results for Screening

combination ^a Ab/cAg	coating antigen ($\mu\text{g/mL}$)	antiserum (dilution)	A_{max} (A)	slope (B)	I_{50} ($\mu\text{g/L}$) (C)	A_{min} (D)	R^2
7586/4-BSA	0.5	1:5000	0.59	0.98	285	0.03	0.99
7586/10-BSA	0.5	1:5000	0.68	0.80	296	0.04	1.00
7586/7-BSA	0.5	1:5000	0.24	0.83	74	0.01	1.00
7588/4-BSA	0.5	1:6000	0.47	0.81	158	0.03	0.99
7588/10-BSA	0.5	1:8000	0.63	0.61	102	0.04	1.00
7588/7-BSA	0.5	1:8000	0.66	0.81	29	0.06	1.00

^a Other combinations with $I_{50} > 500 \mu\text{g/L}$ are not listed in this table. Analytes were prepared in 50% methanolic PBS solution.

tized, then reduced with hypophosphorous acid to give (*S,S*)-fenvalerate identical to an authentic sample. A similar stannous chloride reduction of **3** as the diastereoisomeric pair gave hapten **4** also as a mixture of isomers. This latter mixture was used for protein coupling because some isomerization of esfenvalerate and similar pyrethroids is known to occur under field conditions. Thus, detection of both is desirable. Cleavage of the benzyl ester **6** with iodotrimethylsilane gave the hapten **7** having a terminal propanoic acid for protein coupling. The deltamethrin hapten corresponding to hapten **7** was recently reported (Queffelec et al., 1998) synthesized according to a slightly different route. Haptens **10** and **11** lack the terminal phenyl group in fenvalerate and contain, respectively, acetate and hexanoate chains linked to the *m*-phenoxy group. Hapten **10** was conjugated as the *S,R/S* diastereoisomeric pair, whereas hapten **11** was conjugated as the pure *S,S* isomer. Compound **13** was prepared as a hapten for the glycine conjugate of fenvalerate acid, which is proposed as a major metabolite of fenvalerate in humans (Miyamoto et al., 1981). This was prepared by reaction of the acid chlorides of (*S*)-4-chloro- α -(1-methylethyl)benzeneacetic acid with 4-nitro-L-phenylalanine through a modified Schotten-Baumann reaction to give the N-acylated nitro intermediate **12**. Subsequent catalytic reduction of the nitro group to the amine over palladium catalyst gave the corresponding amino hapten **13**. This reduction resulted in some dechlorination; however, the product was easily purified by flash chromatography and crystallization from ether to give **13** as a 1:1 etherate.

Because the phenoxybenzyl (PB) group is common to many of the synthetic pyrethroids, the strategy for choosing the immunogen hapten was to link the protein through the aromatic end of the esfenvalerate molecule to minimize the contribution of PB to antibody specificity. Therefore, LPH conjugates of haptens **4** and **7** were used for antibody production, and their BSA conjugates and conjugates of other haptens 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 hapten (but 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 fenprothrin 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 antiserum and coating antigen with OD absorbance of >0.50 in Table 1 were screened for competition by esfenvalerate. The I_{50} values ranged from 30 to 300 $\mu\text{g/L}$ in the homologous and heterologous systems tested (Table 2). The homologous system for Ab7588 (with cAg 7-BSA) showed the lowest I_{50} (29 $\mu\text{g/L}$), which was at least 4 times better than the heterologous format. However, a heterologous system worked best for Ab7586 (with cAg 7-BSA, $I_{50} = 74 \mu\text{g/L}$), 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 esfenvalerate 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

Table 3. Effects of Methanol Concentration^a

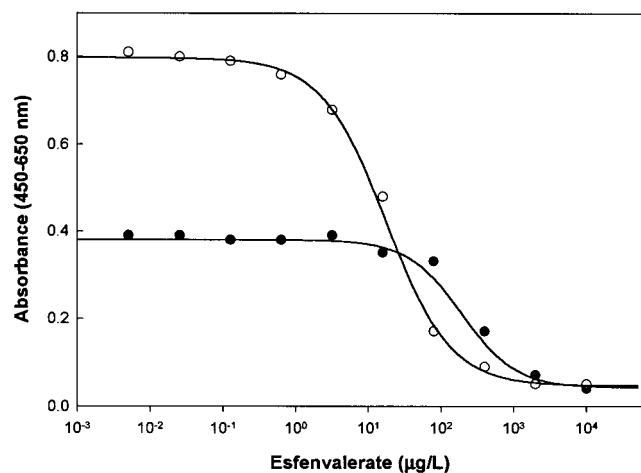
MeOH ^b (%)	A _{max} (A)	slope (B)	I ₅₀ (μg/L) (C)	A _{min} (D)	A/D	R ²
0	0.61 ± 0.02 ^c	0.89	247 ± 24.2 ^c	0.09	6.3	0.99
10	0.65 ± 0.03	0.78	155 ± 9.2	0.10	6.2	1.00
25	0.71 ± 0.03	0.74	68 ± 10.0	0.08	8.8	0.99
50	0.84 ± 0.02	0.83	32 ± 5.5	0.09	9.3	1.00
75	0.90 ± 0.04	1.08	41 ± 6.6	0.18	5.0	1.00
100	0.95 ± 0.04	1.07	117 ± 17.0	0.31	3.5	0.99

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

Table 4. Effects of DMSO Concentration^a

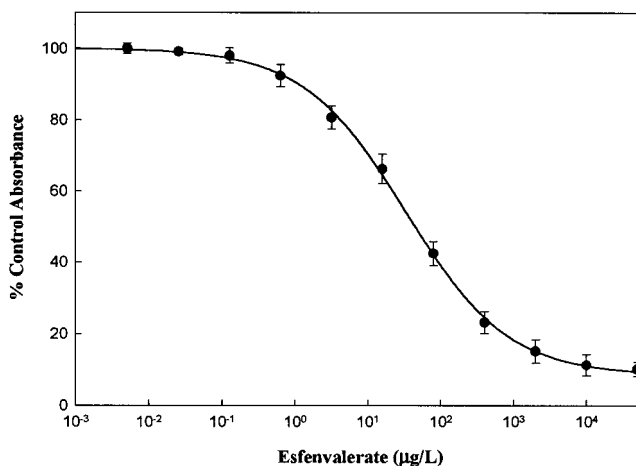
DMSO ^b (%)	A _{max} (A)	slope (B)	I ₅₀ (ppb) (C)	A _{min} (D)	A/D	R ²
0	0.61 ± 0.02 ^c	0.89	247 ± 24.2 ^c	0.09	6.3	0.99
10	0.45 ± 0.04	0.70	207 ± 32.0	0.06	7.0	1.00
25	0.37 ± 0.03	0.76	219 ± 18.5	0.06	6.2	0.99

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

**Figure 3.** Effect of detergent (Tween 20): (○) 0%; (●) 0.05%.

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 μg/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

**Figure 4.** ELISA inhibition curve for esfenvalerate, using antiserum 7588 (diluted 1:12000, final dilution in well), and coating antigen 7-BSA (0.4 μg/L). This standard curve represents the average of 25 curves.**Table 5. Cross-Reactivities of Fenvalerate Isomers and Other Structurally Related Compounds**

analyte	R	% cross-reactivity ^a (n)
fenvalerate isomers		
1 <i>S</i> ,2 <i>S</i> (esfenvalerate)	Ph	100 (9) ^b
1 <i>S</i> ,2 <i>R</i>	Ph	93 ± 7 (3)
1 <i>R</i> ,2 <i>S</i>	Ph	14 ± 4 (3)
1 <i>R</i> ,2 <i>R</i>	Ph	1.8 ± 0.8 (3)
1 <i>S</i> ,2 <i>R</i> / <i>S</i>	Ph	97 ± 3 (3)
1 <i>R</i> ,2 <i>R</i> / <i>S</i>	Ph	14 ± 2 (3)
1 <i>R</i> / <i>S</i> ,2 <i>R</i> / <i>S</i> (fenvalerate)	Ph	60 ± 9 (3)
haptens		
4	4-PhNH ₂	750 ± 85 (3)
6	4-PhCH ₂ CH ₂ COOCH ₂ Ph	44 ± 9 (3)
7	4-PhCH ₂ CH ₂ COOH	2050 ± 220 (3)
8	CH ₂ COOCH ₂ Ph	143 ± 35 (3)
10	CH ₂ COOH	610 ± 110 (3)
9	CH ₂ (CH ₂) ₄ COOCH ₂ Ph	39 ± 20 (3)
11	CH ₂ (CH ₂) ₄ COOH	4000 ± 330 (3)

^a Cross-reactivity was calculated as (*I*₅₀ of esfenvalerate/*I*₅₀ of analyte) × 100%. ^b n, number of times cross-reactivity experiment was conducted.

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 μg/mL of coating antigen 7-BSA, antibody 7588 at a dilution of 1:6000, and esfenvalerate in MeOH/PBS buffer (1:1). The *I*₅₀ value of this homologous assay was 30 ± 6.2 μg/L (Figure 4) with a lower detection limit (LDL) of 3.0 ± 1.8 μg/L. The LDL 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).

Cross-Reactivity. The antibody from rabbit 7588 was highly selective for esfenvalerate, and its ability to bind with fenvalerate depended upon the isomer analyzed (Table 5). In an optimized homologous system (Ab7588/7-BSA), no significant cross-reactivity was measured for most compounds tested, including pyrethroids and fenvalerate metabolites (Table 6). It is of importance that several widely used, PB-containing

Table 6. Cross-Reactivities of Pyrethroids and Esfenvalerate Metabolites

analyte	% cross-reactivity ^a	analyte	% cross-reactivity ^a
esfenvalerate	100	phenothrin	ni
phenoxybenzoic acid	<0.01	cyfluthrin	ni
(<i>S,S</i>)-fenvalerate acid	<0.01	fenpropathrin	ni
permethrin	ni ^b	resmethrin	ni
cypermethrin	ni	fluvalinate	ni
deltamethrin	ni		

^a Cross-reactivity was calculated as (I_{50} of esfenvalerate/ I_{50} of analyte) \times 100%. ^b ni, <10% inhibition was measured at highest concentration (10000 μ g/L).

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 stereoselectivity. 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 haptens contained ~1% of *R* isomer (see synthesis above). If this carried through the synthetic steps to the final haptens, 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 haptens, 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 haptens 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 analyte. In addition, haptens 4, 7, 10, and 11 all contain $-\text{NH}_2$ or $-\text{COOH}$ groups, which make these compounds more polar and more soluble in aqueous system. Therefore, the solubility of those haptens 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 $-\text{COOH}$ -containing haptens (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 C_{18} column has been considered the primary choice for extracting nonpolar or semipolar pesticides including pyrethroids from natural water sources (Junk and

Table 7. SPE Method Development

		% ¹⁴ C-fenvalerate recovered ^a (SD)	
		C_8 column ($n = 3$)	C_{18} column ($n = 3$)
eluate ^a	fraction 1	82 \pm 3.1	85 \pm 3.9
	fraction 2	0.48 \pm 0.16	1.05 \pm 0.15
	fraction 3	0.25 \pm 0.08	0.31 \pm 0.08
	fraction 4	0.11 \pm 0.04	0.17 \pm 0.04
	fraction 5	<0.05	0.11 \pm 0.01
solution A ^b		13 \pm 2.2	13 \pm 2.7
solution B ^c		2.2 \pm 0.20	<0.05

^a Analyte was eluted with 100% methanol, 3.5 mL each fraction. ^b Solution washed from glass container. ^c Solution extracted from postcolumn solution (filtrate).

Richard, 1988; Swineford and Belisle, 1989; Durhan et al., 1990). A recent study by Woin (1994) showed that C_8 sorbent is as effective as C_{18} for pyrethroid extraction, showing very good recoveries (80–100%). In the present study, high recovery rates were achieved for both C_8 and C_{18} columns (82 and 85%, respectively) when applied to tap and river water samples (Table 7). Although the C_8 column had higher eluting efficiency with methanol than the C_{18} column (0.84 versus 1.6% of analyte in second through fifth eluate fractions), the C_{18} column is much more effective for extracting fenvalerate from water than the C_8 column (<0.05 versus 2.2% of analyte lost in filtrate). To ensure a higher rate of recovery, a C_{18} 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 \pm 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 ($Y = 0.98x - 0.079$, $R^2 = 0.96$, $n = 16$). All recoveries were >80% of spiked value. Recovery of esfenvalerate for five water samples spiked at 0.1 μ g/L was 0.102 \pm 0.018 μ g/L (102 \pm 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 (Ab7588/7–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 analyte and standard solution) is extremely important for the accurate performance of the ELISA for the highly lipophilic esfenvalerate. Finally,

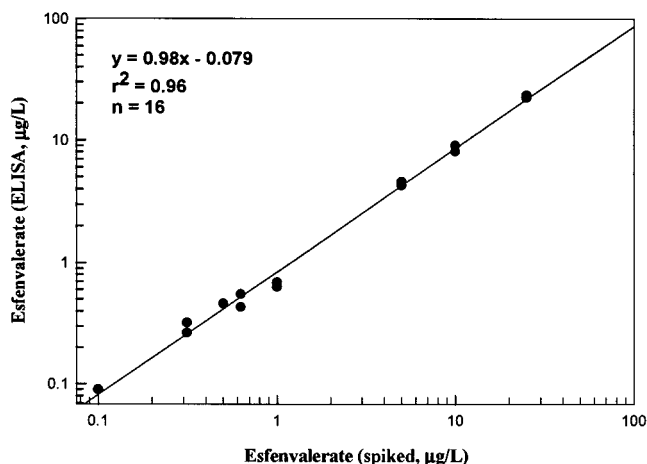


Figure 5. Relationship between esfenvalerate spiked into water and determined by ELISA. Water samples were from tap water (Davis, CA) and the Sacramento River.

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 spectrum; GAR-HRP, 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-*p*-toluenesulfonate; NHS, *N*-hydroxysuccinimide; NMR, nuclear magnetic resonance; OVA, ovalbumin; PB, phenoxybenzyl; PBS, phosphate-buffered saline; PBST, 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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