A competitive enzyme-linked immunosorbent assay (ELISA) was developed for the quantitative detection of fenpropathrin \([\text{RS}] - \text{R} - \text{cyano-3-phenoxybenzyl-2,2,3,3-tetramethylcyclopropanecarboxylate}\). Polyclonal antisera were isolated from rabbits immunized with two different fenpropathrin hapten conjugates. One hapten contained an amino function; the other contained a carboxyl group for conjugation to carrier proteins. Mollusk hemocyanins, thyroglobulin, and fetuin were used as carrier proteins. The antisera varied greatly in their affinities for fenpropathrin. A homologous assay system using the coating antigen format was the most sensitive. The IC\(_{50}\) for fenpropathrin was 20 \(\mu\)g/L, and the lower detection limit was 2.5 \(\mu\)g/L. Pyrethroids, such as phenothrin, permethrin, resmethrin, fenvalerate, deltamethrin, cyfluthrin, and cypermethrin, and the pyrethroid metabolites, 3-phenoxybenzoic acid and fenpropathrin acid, did not cross-react significantly in this assay. Ten percent acetone or methanol and a pH of 4 were determined to be optimum assay conditions. Various cationic, anionic, and nonionic detergents had no significant effect on the assay.

**Keywords:** Fenpropathrin; pyrethroid; ELISA; pesticide; enzyme immunoassay; cross-reactivity

**INTRODUCTION**

Fenpropathrin (Figure 1), commercial name Danitol or Meothrin, is a synthetic pyrethroid insecticide, which is more photostable (Leahey, 1985) than the naturally occurring pyrethrum from which the synthetic pyrethroids were designed. Fenpropathrin is predominantly used to control various insects and mites that infest fruit plants, vegetables, and other crops. In the United States, as much as 28 800 kg of active ingredient is applied annually for crop protection (Giannessi and Anderson, 1995). Many pyrethroids are highly potent insecticides with relatively low toxicity to mammals (oral LD\(_{50}\) to female mice is 58 mg/kg; Fujita, 1981) and most nontarget organisms (Elliott, 1977). However, some nontarget organisms tested, such as fish, showed significant toxicity (LC\(_{50}\) for bluegill sunfish of 1.95 \(\mu\)g/L, 48 h; Tomlin, 1994). Thus, there is an interest in monitoring levels of pyrethroids in aquatic ecosystems.

Multistep sample cleanup procedures are used in conventional pyrethroid residue analysis followed by gas chromatography (GC) (Blass, 1990; Takimoto et al., 1984; Baker and Bottomley, 1982) or high-pressure liquid chromatography (HPLC) (Sakaue et al., 1982). Immunoassays are highly sensitive and selective analytical tools for detecting trace amounts of chemicals such as pesticides (Hammock et al., 1990; Meulenberg et al., 1995). They also offer the advantage of decreased sample preparation, in some cases resulting in increased sample throughput.

There are a variety of applications in which immunoassays may be advantageous. For example, one use for immunoassays would be to monitor the exposure of applicators and farm workers to pyrethroids, such as fenpropathrin, to reduce occupational exposure (Chen et al., 1991). Another possible application of immunoassays for pyrethroid residue analysis is the monitoring of food (Stanker et al., 1989; Skerritt et al., 1992; Hill et al., 1993) and environmental samples (Bonwick et al., 1994). The expected residues in food are generally low because of low application rates and relatively rapid degradation in the environment. Monitoring the aquatic environment and associated sediments for fenpropathrin content is equally important because of its long half-life (11–8520 days at 25 °C) in natural waters (Takahashi et al., 1985) and its relative toxicity to nontarget aquatic organisms.

One of the first immunoassays developed for a pyrethroid, S-bioallethrin (Wing et al., 1978), utilized antibodies that were stereoselective and could detect S-bioallethrin in the picomole range. Recently, monoclonal antibodies for the detection of allethrin have been developed and used in assays by Pullen and Hock (1995). Assays have also been reported for permethrin (Bonwick et al., 1994), bioresmethrin (Hill et al., 1993), and phenothrin (Skerritt et al., 1992).

Challenges encountered in developing immunoassays for pyrethroids lie in their lipophilicity and the synthesis of appropriate haptons (Skerritt and Lee, 1996). Fenpropathrin is very lipophilic (K\(_{OW}\) of 100 000, 20 °C; Tomlin, 1994), similar to other pyrethroids having a phenoxybenzyl group. The very low water solubility of fenpropathrin (14.1 \(\mu\)g/L at 25 °C) may cause yields from
the primarily aqueous conjugations to be low. The lipophilicity may also affect the ELISA format development, due to nonspecific binding of the lipophilic molecule to the surface of working materials such as microtiter plates or glass vials.

To our knowledge no work has been published on the development of immunoassays to fenpropathrin. Strategies for hapten synthesis for assays of other pyrethroids containing an ester of an aryl cyanohydrin have been comprehensively reviewed (Skerritt and Lee, 1996). Since antibodies bind to the portion of the molecule distal to the attachment site to the carrier protein, pyrethroid haptens have been developed that link the hapten to the protein (1) through the 3-phenoxybenzyl end of the molecule, (2) through the α-cyano moiety of the molecule, and (3) through fragments of the molecule such as metabolites. More selective assays would be developed from haptens made from the whole molecule. Thus, we opted to couple the hapten through the 3-phenoxybenzyl end of the fenpropathrin molecule. One of the haptens used was the same as reported by Demoute and Touer (1987; an alkyl side chain terminated by a carboxylic acid; hapten 6, Scheme 1) but synthesized by a different route. The other was a unique hapten containing an amine group on the 3-phenoxybenzyl moiety (hapten 8, Scheme 1). This paper describes the development of the immunoassay resulting from the above haptens.

MATERIALS AND METHODS

Chemicals and Immunoassay Reagents. The pyrethroid standards of fenvalerate, deltamethrin, phenothrin, permethrin, cypermethrin, cyfluthrin, and resmethrin were obtained from Riedel de Haen (Seelze, Germany). Racemic fenpropathrin was synthesized as described below with a purity of ≥99% based on analytical data. Other organic starting materials for hapten synthesis were obtained from Aldrich Chemical Co. (Milwaukee, WI). For thin-layer chromatography (TLC), 0.2 mm precoated silica gel 60 F 254 on glass plates from E. Merck (Darmstadt, Germany) was used. Spots were visualized under ultraviolet light or after staining with iodine vapor. Flash chromatographic separations were carried out on 40 μM average particle size Baker silica gel, packed in glass columns of such diameter to give a column height/diameter ratio of ≈7. Compounds were eluted using the indicated solvents, where the -- symbol denotes a stepwise concentration gradient.

The coupling reagents 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide-metho-p-toluenesulfonate (morpho-CDI), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (DAPEC), N-hydroxysuccinimide (NHS), and N-hydroxysulfosuccinimide (sulfo-NHS) were purchased from Aldrich. Presto desalting plastic columns (5 mL volume) were obtained from Pierce (Rockford, IL).

Anti-rabbit immunoglobulin, raised in goats and conjugated to horseradish peroxidase (GAR/HRP), 3,3′,5,5′-tetramethylbenzidine (TMB), bovine serum albumin (BSA), ovalbumin (OVA), hemocyanin from Limulus polyphemus (LPH), hemocyanin from keyhole limpet (KLH), thyroglobulin, and fetuin were purchased from Sigma Chemical Co. (St. Louis, MO). Microtiter plates 4-42404 were purchased from Nunc (Roskilde, Denmark).

Instruments. NMR spectra were obtained using a General Electric QE-300 spectrometer (Bruker NMR, Billerica, MA). Chemical shift values are given in parts per million 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 GLC (Hewlett-Packard Corp., Avondale, PA) fitted with a 15 m, 0.32 mm i.d., capillary column with a 0.25 μm film of dimethylpolysiloxane containing 5% of the methyl groups substituted by phenyl groups (J&W Scientific, Folsom, CA). 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. Polye(ethylene glycol) was added to the matrix as a mass calibrant. ELISAs were carried out using 96-well microtiter plates and the absorbances read with the Vmax reader from Molecular Devices (Menlo Park, CA).

Hapten Synthesis and Verification. Syntheses of the haptens were carried out as outlined in Schemes 1 and 2. Analytical data verifying the structures are provided. Racemic fenpropathrin standard was prepared from 2,2,3,3-tetrameth-
Scheme 2

CHCl₃. The mixture was stirred and heated in an oil bath at 60–65 °C for 95 min. The mixture was stripped, hexane was added to the residue, and the solution was evaporated to yield the acid chloride, which was a colorless oil. 3-Phenoxybenzaldehyde (4.18 g, 21.1 mmol) and potassium cyanide (2.1 g, 31.1 mmol) of 1-bromo-3-(dimethoxymethyl) benzene (2). This mixture was refluxed under nitrogen for 17 h, cooled, washed with water, and filtered through silica gel (25 g). The pad of silica gel was washed with wet CH₂Cl₂ (40 mL), and the combined filtrate was evaporated to a volume of 30 mL, diluted with wet CH₂Cl₂ (40 mL), and treated with acidified silica gel (30 g, previously treated in ether with 9 drops of 98% H₂SO₄ and stripped to a dry powder on a rotary evaporator). Water (1 mL) was added, and this mixture was stirred for 2 h. After filtration (30 mL) and evaporation, the mixture was flash chromatographed on silica gel (200 g), eluting with hexane/CH₂Cl₂ (40:20 → 0:100). Stripping at 30 °C and 1 Torr yielded 13.1 g (56%) of 4, which was a pale yellow oil showing only one spot by TLC (CH₂Cl₂): 1R 0.36; ¹H NMR (CDCl₃) δ 7.63 (m, 4 H, Ar). GLC analysis indicated a purity of > 99% according to TLC, NMR, and GLC analyses. All intermediates and haptons were synthesized as racemic mixtures.

Fenpropathrin (1). Thionyl chloride (2.1 mL, 29 mmol) was added to a mixture of 2,2,3,3-tetramethylcyclopropanecarboxylic acid and 3-phenoxybenzaldehyde cyanohydrin and assigned a purity of ≥ 99% according to TLC, NMR, and GLC analyses. All intermediates and haptons were synthesized as racemic mixtures.

Development of a Fenpropathrin ELISA


was continued for 10 h to collect 1.48 mL of water. Excess benzyl alcohol was removed under vacuum (1 Torr) to a kettle temperature of 150 °C. The residue was dissolved in ether, washed with sodium bicarbonate solution, followed by a water wash, and dried (MgSO₄). The stripped residue was distilled through a short-path head from an oil bath at 230–240 °C to collect 20 g (91%) of product at a head temperature of 185–194 °C (0.08 Torr), which displayed only one spot by TLC (ether/CH₂Cl₂, 1:9): R² 0.47; ¹H NMR (CDCl₃) δ 2.64 (t, J = 7.8 Hz, 2 H, CH₂), 2.89 (t, 2 H, J = 7.7 Hz, CH₂), 5.1 (2 s, 2 H, CH₂), 5.5 (s, 1 H, OH), 6.73 (d, J = 8.4 Hz, 2 H), 7.03 (d, J = 8.4 Hz, 2 H), 7.27–7.33 (m, 5 H, Ar).

Benzyl 4-(3-Formylphenoxy)benzenepropanoate (4). Potassium tert-butoxide (65 mL, 1 M in tert-butyl alcohol) was added under N₂ to a stirred solution of benzyl 4-hydroxybenzenepropanoate (3) (16.6 g, 64.8 mmol), in 180 mL of xylene, and the mixture was heated to remove 100 mL of distillate. An additional 40 mL of xylene was added and distilled to a kettle temperature of 136 °C. The resulting salt suspension was cooled, treated with pyridine (16 mL), cuprous chloride (0.6 g, copper powder (0.3 g), 18-crown-6 (100 mg), and 15.0 g (65 mmol) of 1-bromo-3-(dimethylmethyl) benzene (2). This mixture was refluxed under N₂ for 17 h, cooled, washed with water, and filtered through silica gel (25 g). The pad of silica gel was washed with CH₂Cl₂ (40 mL), and the combined filtrate was evaporated to a volume of 30 mL, diluted with wet CH₂Cl₂ (40 mL), and treated with acidified silica gel (30 g, previously treated in ether with 9 drops of 98% H₂SO₄ and stripped to a dry powder on a rotary evaporator). Water (1 mL) was added, and this mixture was stirred for 2 h. After filtration (30 mL) and evaporation, the mixture was flash chromatographed on silica gel (200 g), eluting with hexane/CH₂Cl₂ (40:20 → 0:100). Stripping at 30 °C and 1 Torr yielded 13.1 g (56%) of 4, which was a pale yellow oil showing only one spot by TLC (CH₂Cl₂): 1R 0.36; ¹H NMR (CDCl₃) δ 2.64 (t, J = 8.0 Hz, 2 H, CH₂), 2.97 (t, J = 7.8 Hz, 2 H, CH₂), 5.12 (2 s, 2 H, CH₂Ar), 6.92–7.57 (m, 13 H, Ar), 9.94 (s, 1 H, CHO).¹³C NMR (CDCl₃) δ 130.0 (CH₃), 35.7 (CH₂CO), 66.1 (CH₂Ar), 117.7, 119.4 (2 C), 124.1, 124.3, 128.07 (3 C), 128.4 (2 C), 129.7 (2 C), 130.2, 135.8, 136.2, 137.9, 154.3, 158.4, 172.3 (COOR), 191.3 (CHO).

Cyanos-[3-4-[3-(oxo-3-benzyl oxyl)propyl phenoxyl]phenyl]-methyl 2,2,3,3-tetramethylcyclopropanecarboxylate (5). The aldehyde (4) (0.75 g, 2.1 mmol), in 1 mL of THF and 0.2 mL of H₂O, was cooled in ice and treated with powdered KCN (203 mg, 3.1 mmol) followed by 0.215 mL of 12.1 N HCl (Caution! Excess HCN generated!). The reaction was mildly exothermic. After stirring for 30 min, the mixture was acidified with 3 N HCl and extracted with ether, and the organic phase was washed with water, dried (MgSO₄), and stripped to give the cyanohydride, which was a brown oil. The cyanohydride, in 1 mL of CH₂Cl₂, was stirred and cooled in ice and treated with 2,2,3,3-tetramethylcyclopropanecarbonyl chloride (prepared as described from 311 mg of the acid above) in 1 mL of CH₂Cl₂, and pyridine (0.23 mL) was injected immediately. After 10 min of ice cooling, the mixture was stirred for 20 min at ambient temperature, washed twice with water, and stripped to yield a brown gum. Chromatography on silica gel (hexane → CH₂Cl₂) gave the pure ester, 0.71 g (88%), which was a colorless gum: ¹H NMR (CDCl₃) δ 1.17 (s, 3 H, CH₃), 1.21 (s, 3 H, CH₃), 1.22 (s, 3 H, CH₃), 1.26 (s, 1 H, CHCO), 1.27 (s, 3 H, CH₃), 6.35 (s, 1H, CHCN), 7.01–7.42 (m, 9 H, Ar).¹³C NMR (CDCl₃) δ 164.2 (CH₂), 23.3 (2 CH₃), 31.9, 32.1, 35.0, 61.5 (CCN), 116.4 (CN), 117.5, 119.2 (2 CH), 119.8, 121.9, 123.9, 129.9 (2 CH), 130.4, 134.1, 156.2, 158.0, 169.7 (C=O). GC analysis indicated a purity of ≥ 99%.

1-Bromo-3-(dimethylmethyl) benzene (2). This product was prepared using a modification of literature procedures (Young et al., 1980; Creary and Mollen 1991). Two drops of concentrated sulfuric acid was added to a solution of 3-bromobenzaldehyde (18.5 g, 0.1 mol) and trimethyl orthoformaldehyde (12.2 g, 0.115 mol) in 15 mL of methanol. After an immediate mild exotherm and 3 h at ambient temperature, the mixture was diluted with ether, washed with a sodium carbonate solution, followed by a water wash, dried (MgSO₄), stripped, and vacuum distilled to yield 22 g, 95% of 2, which was a colorless oil: bp 60–63 °C (0.05 Torr); ¹H NMR (CDCl₃) δ 3.32 (s, 6 H, CH₃), 5.36 (s, 1 H), 7.21–7.63 (m, 11 H, Ar).

Benzyl 4-Hydroxybenzenepropanoate (3). A mixture of 4-hydroxybenzenepropanoic acid (14.4 g, 86.6 mmol), benzyl alcohol (27 mL), toluene (15 mL), and 4 drops of 85% phosphoric acid was heated to reflux under a Dean–Stark trap. Toluene was removed up to a kettle temperature of 145–150 °C.
The product was stripped (<1 mm) to yield 0.47 g of 6, which was a pale yellow gum: \( ^1H \) NMR (CDCl3) \( \delta 1.18 \) (s, 3 H, CH3), 1.21 (s, 3 H, CH3), 1.22 (s, 3 H, CH3), 1.26 (s, 1 H, CHCOO), 1.27 (s, 3 H, CH3), 2.69 (t, \( J = 7.7 \) Hz, 2 H, CH2), 2.96 (t, \( J = 7.7 \) Hz, 2 H, CH2), 3.64 (s, 1 H, CHCNC), 6.94–7.42 (m, 8 H, Ar); \( ^13C \) NMR (CDCl3) \( \delta 16.4 \) (2 CH3), 23.3 (2 CH2), 29.8 (CH3), 31.9 and 32.1 (cyclopropane), 35.0 (CH0), 35.6, 61.6, 116.4 (CN), 117.4, 119.1 (CO), 126.5, 127.7, 130.4, 134.0, 135.8, 154.7, 158.1, 169.8 (COO), 178.9 (COO); MS: m/z 364.2 (M+).

Benzyl 2-(3-Formylphenoxy)acetate (10). Potassium tert-butoxide (10.1 g, 90 mmol) was added with cooling under \( \text{N}_2 \) to a stirred solution of 3-hydroxybenzaldehyde (11.0 g, 90 mmol) in 70 mL of dimethyl sulfoxide (DMSO). Benzyl bromoacetate (19.6 g, 86 mmol) was added over ~5 min. After 2.5 h at ambient temperature, the mixture was diluted with water and (300 mL) and extracted with diethyl ether. The hexane solution was washed with water and stripped of solvent to yield a yellow oil. Flash chromatography on 200 g of silica gel (n-\( \text{BuCl} \rightarrow \text{CHCl}_3 \)) and vacuum stripping of fractions containing pure product yielded 18.2 g, (75%) of 10, which was a light yellow oil: \( ^1H \) NMR (CDCl3) \( \delta 4.74 \) (2 H, CH2COO), 5.25 (2 s, 2 H, CH2Ar), 7.2–7.53 (m, 9 H, Ar), 9.94 (s, 1 H, CHO).

Benzyl 6-(3-Formylphenoxy)hexanoate (11). A solution of 3-hydroxybenzaldehyde (4.8 g, 37 mmol) in 3 mL of ethanol. The mixture was heated at 70 °C for 35 min and poured into water (10 mL) containing 0.7 g of Celite and 0.72 g of KHCNO. Filtration, followed by extraction of solids with ethyl acetate and stripping of the solvent, yielded a red gum. Flash chromatography on silica gel (hexane → CH2Cl2 → ether) yielded 12 mg of starting ester and 190 mg (69%) of 8, which was a pale yellow gum: TLC (CH2Cl2), Rf 0.24; \( ^1H \) NMR (CDCl3) \( \delta 1.19 \) (s, 3 H, CH3), 1.22 (s, 3 H, CH3), 1.24 (s, 3 H, CH3), 1.27 (s, 1 H, CHCOO), 1.28 (s, 3 H, CH3), 1.68 (s, 1 H, CHCNC), 5.70 and 8.24 (m, J = 9.2 Hz, 4 H, NOAr), 7.0–7.6 (m, 4 H, Ar).

The ester was dissolved in 2 mL of chlorinated CH2Cl2 and added all at once to a stirred ice-cooled solution of 2,2,3,3-tetramethylcyclopropanecarbonyl chloride (2.0 mmol of CH2Cl2) as prepared as described above. Pyridine (0.18 mL, 2.2 mmol) was immediately added with stirring. After an immediate mod exotherm and stirring at ambient temperature for 2 h, the mixture was acidified with 3 N HCl, washed twice with ether, filtered through 3 g of silica gel, followed by 25 mL of CH2Cl2, and stripped to yield a yellow oil. Flash chromatography on 20 g of silica gel (20–80% CH2Cl2 in hexane) yielded 0.60 g (47%) of 7, which was a pure colorless gum, plus an additional 0.45 g (34%) of compound 6b, which made the purification by flash chromatography difficult. Only multiple development of the TLC plate using 2% acetic acid in isopropyl alcohol/ethyl acetate, 1:2, contaminated with 1% acetic acid in hexane/n-ethylacetate, 1:9, which made the purification by flash chromatography of the residue on silica gel (BuClCH2Cl2) and vacuum stripping of fractions containing pure product yielded 18.2 g, (75%) of 10, which was a light yellow oil: \( ^1H \) NMR (CDCl3) \( \delta 4.74 \) (2 H, CH2COO), 5.25 (2 s, 2 H, CH2Ar), 7.2–7.53 (m, 9 H, Ar), 9.94 (s, 1 H, CHO).

Benzyl 3-(Cyanomethyl)-2,2,3,3-tetramethylcyclopropanecarboxylate (11). A mixture of 6-bromohexanoic acid (12.6 g, 65 mmol), benzyl alcohol (10 mL), 85% phosphoric acid (10 drops), and 30 mL of benzene was heated under a Dean–Stark trap with removal of solvent to a kettle temperature of 135 °C. Boiling was continued for ~3.5 h to remove 1.38 mL of water. The reaction mixture was diluted with hexane, washed with sodium bicarbonate solution followed by water, and distilled through a short-path head to yield 14.4 g (78%) of a colorless liquid: bp 129–145 °C (0.08 Torr); \( ^1H \) NMR (CDCl3) \( \delta 1.46 \) (m, \( J = 7.4 \) Hz, 2 H, CH2), 1.67 (quin, \( J = 7.4 \) Hz, 2 H, CH2), 1.84 (quin, \( J = 7.1 \) Hz, 2 H, CH2), 2.38 (t, \( J = 7.4 \) Hz, 2 H, CH2), 3.39 (t, \( J = 6.8 \) Hz, 2 H, CH2), 5.12 (s, 2 H, CH2Ar), 7.32–7.36 (m, 5 H, Ar).

Benzyl 2-(3-Formylphenoxy)acetate (10). Potassium tert-butoxide (10.1 g, 90 mmol) was added with cooling under \( \text{N}_2 \) to a stirred solution of 3-hydroxybenzaldehyde (11.0 g, 90 mmol) in 70 mL of dimethyl sulfoxide (DMSO). Benzyl bromoacetate (19.6 g, 86 mmol) was added over ~5 min. After 2.5 h at ambient temperature, the mixture was diluted with water (300 mL) and extracted with diethyl ether. The hexane solution was washed with water and stripped of solvent to yield a yellow oil. Flash chromatography on 200 g of silica gel (n-\( \text{BuCl} \rightarrow \text{CHCl}_3 \)) and vacuum stripping of fractions containing pure product yielded 18.2 g, (75%) of 10, which was a light yellow oil: \( ^1H \) NMR (CDCl3) \( \delta 4.74 \) (2 H, CH2COO), 5.25 (2 s, 2 H, CH2Ar), 7.2–7.53 (m, 9 H, Ar), 9.94 (s, 1 H, CHO).
two crops (n-BuCl/hexane) to yield 0.51 g of 15, which was a white solid: mp 80–82.5 °C (84% based on recovered starting material); H NMR (CDCl3) δ 1.18 (s, 3 H, CH3), 1.23 (s, 6 H, 2 CH3), 1.26 (s, 1 H, CHCO2), 1.28 (s, 3 H, CH3), 1.54 (m, 2 H, CH2), 1.73 (quin, J = 7.5 Hz, 2 H, CH2), 1.83 (quin, J = 7.1 Hz, 2 H, CH2), 2.41 (t, J = 7.4 Hz, 2 H, CH2CO2), 3.98 (t, J = 6.3 Hz, 2 H, CH2O), 6.35 (s, 1 H, CHCN), 6.93–7.36 (m, 4 H, Ar).

**Hapten Conjugation.** Four different conjugation methods were utilized—the water soluble carbodiimide, diazotization, periodate, and activated ester methods (Tijssen, 1985; Ehringer, 1973). To obtain immunogens, hapten 6 was conjugated to KLH, and hapten 8 was conjugated to the carrier proteins LPH, thyroglobulin, and fetuin. Coating antigens were made by coupling haptons 6, 8, 13, and 15 to BSA and OVA (Table 1).

**Table 1. Summary of Homologous Assay Components**

<table>
<thead>
<tr>
<th>Immunogen structure and name</th>
<th>anti sera no.</th>
<th>Coating antigen structure and name</th>
<th>IC50</th>
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<tr>
<td>LPH (131 mg) were dissolved in 20 mL of ice-cold borate buffer (0.2 M, pH 8.9). The reaction mixtures were cooled in an ice bath and stirred continuously for 30 min. The pH of the yellow solutions was adjusted to 7.0 with 1 N NaOH. Each mixture was purified by acetone precipitation and stored as described above.</td>
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<tr>
<td>BSA and</td>
<td>8-BSA</td>
<td>with AS 56 or 58:</td>
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<td>12 mg (0.002 mmol in 2 mL of PBS) was added dropwise aqueous NaOH (200 μL of a 100 mM solution). Stirring was continued for 20 min at room temperature. The solution was dialyzed overnight at 4 °C against 1 mM sodium acetate buffer (pH 4.4). This dialysate was transferred into a vial, and 450 μL of a 200 mM carbonate buffer (pH 9.5) was added, followed by 6.2 mg (0.015 mmol) of hapten 8 in 2 mL of THF. This mixture was stirred for 4.5 h at room temperature. Finally, 100 μL of freshly prepared NaBH4 solution (4 mg/mL water) was added, and the solution was incubated for 2 h at room temperature. The reaction mixture was purified by using a dextran desalting column and PBS (pH 7.5) solution as the eluant. Column fractions containing purified conjugates were orange. These were stored as described above.</td>
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<tr>
<td>BSA (Approach A)</td>
<td></td>
<td>IC50 20 μg/L</td>
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<tr>
<td>12 mg (0.002 mmol in 2 mL of PBS) was added dropwise aqueous NaOH (200 μL of a 100 mM solution). Stirring was continued for 20 min at room temperature. The solution was dialyzed overnight at 4 °C against 1 mM sodium acetate buffer (pH 4.4). This dialysate was transferred into a vial, and 450 μL of a 200 mM carbonate buffer (pH 9.5) was added, followed by 6.2 mg (0.015 mmol) of hapten 8 in 2 mL of THF. This mixture was stirred for 4.5 h at room temperature. Finally, 100 μL of freshly prepared NaBH4 solution (4 mg/mL water) was added, and the solution was incubated for 2 h at room temperature. The reaction mixture was purified by using a dextran desalting column and PBS (pH 7.5) solution as the eluant.</td>
<td>with AS 55: no competition</td>
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<tr>
<td>BSA (Approach B)</td>
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<td>IC50 2.5 μg/L</td>
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<td>Hapten Conjugates: 8-Thyroglobulin and 8-BSA (Approach B). Three vials, each containing a solution of hapten 8 (9 mg, 0.025 mmol) in 1.0 mL of 37.5 mM H2SO4 in DMSO, were cooled to 16 °C. Butyl nitrite (100 μL of 0.375 M butyl nitrite in DMSO) was added dropwise with stirring. Stirring was continued for 10 min. The contents of each vial was added to a solution of BSA, the other to a solution of thyroglobulin, and the third to a solution of LPH. Each protein (20 mg) was dissolved in 5 mL of borate buffer (0.1 M, pH 9.4). The stirring was continued overnight at 4 °C. Each yellow reaction mixture was purified by size exclusion chromatography, using a 5 mL dextran desalting column and phosphate-buffered saline (PBS; pH 7.5) solution as the eluant. Column fractions containing purified conjugates were orange. These were stored as described above.</td>
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<tr>
<td>Hapten Conjugates: 8-Fetuin. To a stirred solution of fetuin (12 mg, 0.002 mmol in 2 mL of PBS) was added dropwise aqueous NaOH (200 μL of a 100 mM solution). Stirring was continued for 20 min at room temperature. The solution was dialyzed overnight at 4 °C against 1 mM sodium acetate buffer (pH 4.4). This dialysate was transferred into a vial, and 450 μL of a 200 mM carbonate buffer (pH 9.5) was added, followed by 6.2 mg (0.015 mmol) of hapten 8 in 2 mL of THF. This mixture was stirred for 4.5 h at room temperature. Finally, 100 μL of freshly prepared NaBH4 solution (4 mg/mL water) was added, and the solution was incubated for 2 h at room temperature. The reaction mixture was purified by using a dextran desalting column and PBS (pH 7.5) solution as the eluant. The conjugate was stored as described above.</td>
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</tr>
<tr>
<td>Hapten Conjugates: 13-BSA and 13-OVA. To 19.8 mg (0.05 mmol) of hapten 13 in 2 mL of dry DMF were added 12.0 mg (0.10 mmol) of NHS and 13.6 mg (0.060 mmol) of DAPEC. The mixture was stirred overnight at ambient temperature and divided into two equal aliquots. One aliquot was added to a solution of BSA, the other one to a solution of OVA. Each protein solution (10 mg) was dissolved in 7 mL of PBS. The reaction mixtures were stirred for 1 h at ambient temperature followed by 5 h at 4 °C, purified using dextran desalting columns, and stored as described above.</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
| Hapten Conjugates: 15-BSA and 15-OVA. To 8.3 mg (0.025 mmol) of hapten 15 in 2 mL of dry DMF were added 9.0 mg (0.040 mmol) of sulfo-NHS and 5.8 mg (0.030 mmol) of DAPEC (Bekheit et al., 1993). The mixture was stirred overnight at ambient temperature and then divided into two equal aliquots. One aliquot was added to a solution of BSA, the other to a
solution of OVA. Each protein (20 mg) was dissolved in 3 mL of PBS. Both reaction mixtures were stirred for 1.5 h at 4 °C, followed by 7 h at ambient temperature, purified using dextran desalting columns, and stored as described above.

**Immunization and Antiserum Preparation.** Each female New Zealand white rabbit was immunized intradermally (Gee et al., 1988) with one of the immunogens listed in Tables 1 and 2. One month after an initial immunization with 100 μg of the immunogen protein dissolved in PBS and emulsified with Freund's complete adjuvant (1:1 v/v), further injections of 100 μg of the immunogen that was emulsified with Freund's incomplete adjuvant were given. Booster injections were given at 3 week intervals. The rabbits were bled 10 days after each boost. After coagulation of the blood, the serum was isolated by centrifugation for 10 min at 4 °C. Preimmune sera were collected prior to immunization to provide control sera having no related humoral immune response.

**Enzyme-Linked Immunosorbent Assay (ELISA).** The coating antigen format was used in which BSA or OVA conjugates were used as coating antigens. Each coating antigen was diluted with coating buffer (100 mM carbonate-bicarbonate buffer, pH 9.6). Microtiter plates were coated with antigenwas diluted with coating buffer (100 mM carbonate-bicarbonate buffer, pH 9.6) and incubated for 30 min at room temperature. After another washing step, 200 μL/well of antiserum diluted in PBS (for determination of antibody titer) or 100 μL/well of antiserum diluted in PBS and 100 μL/well of standard solution were dispensed into the wells and incubated for 1–2 h at room temperature. Following another washing step (four times), 200 μL/well of substrate solution (3.3 μL of 30% H2O2, 200 μL of 1.2% TMB in DMSO per 25 mL of acetate buffer, pH 5.5) was pipetted into each well. Ten to fifteen minutes later the color development was stopped by adding 50 μL/well of 2 M H2SO4. The plates were read in a dual-wavelength mode, subtracting the absorbance at 650 nm from the absorbance at 450 nm. All experiments were conducted using two or three well replicates.

Both homologous and heterologous assays were assessed (Tables 2 and 3). In the homologous assays the hapten conjugation chemistry was the same for the immunogen and the coating antigen. In the heterologous assays coating antigens having coupling chemistry different from that used for the immunogen were used. For standard curves, stock solutions of pyrethroids in methanol or acetonitrile (10 mg/2.5 mL) were diluted from 1 mg/L to 1 μg/L in 1:2 dilution steps using 10% methanol in PBS as the diluent. The final concentration of methanol in the well was 5%.

**Assay Optimization.** The following assay parameters were investigated.

- **pH Effect.** To vary the pH, the antibody was prepared in phosphate buffer with pH values from 4.0 to 10.0. Analyte was prepared as described above in 10% methanol in PBS. All other assay conditions were as described above. The standard curve was run in three well replicates.

- **Ionic Strength.** The effect of PBS buffer with increasing content of NaCl (0.1–1.0 M) was studied. As above, the antibody was diluted in buffers of various ionic strengths, and the assays were conducted as above.

- **Solvent Effect.** The tolerance to various water-miscible solvents used to dissolve pyrethroids (methanol, ethanol, acetone, acetonitrile, and DMF) was tested. The assays were dissolved in solvents of various concentration levels (1, 2.5, 5, 7.5, 10, 20, and 40% in PBS). In this case, a fixed amount of analyte (fenpropatrin) at the approximate IC50 level (20 μg/L) was used in the ELISA. The control contained antibody but no analyte.

- **Effect of Gelatin and Detergents as Additives.** In one assay, gelatin was added to the antiserum solution at six concentration levels (0.1–1.0%). In a second experiment, the antiserum solution was prepared in several detergents. Neutral detergents, Tween 20 (0.02%), PEG 3400 (0.02%), PEG 5000 (0.02%), and Triton X-100 (0.05%), were tested as well as anionic detergents such as cholic acid (0.02%), deoxycholic acid

---

**Table 2. Summary of Heterologous Assay Components**

<table>
<thead>
<tr>
<th>Immunogen structure and name</th>
<th>Antiserum no.</th>
<th>Coating antigen structure and name</th>
<th>IC50</th>
</tr>
</thead>
<tbody>
<tr>
<td>8-LPH</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15-BSA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13-BSA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15-OVA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13-OVA</td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

*Structures of the immunogens used to elicit antisera (AS) and the coating antigens are shown. The IC50 is listed for each assay utilizing the respective coating antigen and antiserum combination."

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**Table 3. Cross-Reactivities of Pyrethroids and Other Structurally Related Compounds**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Cross-reactivity&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Compound</th>
<th>Cross-reactivity&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>fenpropathrin (1)</td>
<td>100.0</td>
<td>deltamethrin (21)</td>
<td>18.8</td>
</tr>
<tr>
<td>fenvalerate (16)</td>
<td>2.3</td>
<td>DDT (23)</td>
<td>ni&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>phenathrin (17)</td>
<td>ni</td>
<td>fenoxycarb (24)</td>
<td>ni</td>
</tr>
<tr>
<td>permethrin (18)</td>
<td>ni</td>
<td>chlorpropham (26)</td>
<td>16.3</td>
</tr>
<tr>
<td>cypermethrin (20)</td>
<td>ni</td>
<td>3-phenoxybenzoic acid (22)</td>
<td>ni</td>
</tr>
<tr>
<td>resmethrin (19)</td>
<td>ni</td>
<td>fenpropatrin acid (25)</td>
<td>ni</td>
</tr>
</tbody>
</table>

<sup>a</sup> The assay consisted of coating antigen 8-BSA (1/8000) and AS 58 (1/40000 dilution). All assay conditions were as described under Materials and Methods. Cross-reactivity was calculated as follows: (IC50 of fenpropathrin/IC50 of compound tested) × 100. The IC50 of fenpropathrin was 16 μg/L. No inhibition at the highest concentration tested (1000 μg/L).

for the immunogen were used. For standard curves, stock solutions of pyrethroids in methanol or acetonitrile (10 mg/2.5 mL) were diluted from 1 mg/L to 1 μg/L in 1:2 dilution steps using 10% methanol in PBS as the diluent. The final concentration of methanol in the well was 5%.
Development of a Fenpropathrin ELISA

(0.02%), SDS (sodium dodecyl sulfate 0.05%), glyceroxyeholic acid (0.02%), diocysulfosuccinate (0.025% and 0.01%), and 3-(3-cholamidopropyl)dimethylamino-1-propanesulfate (0.02%) and the cationic detergents dodecytrimethylammonium bromide (0.015%), dodecylethylammonium bromide (0.015%), and Aliquat 336 (0.03%). These concentrations were chosen because they were clearly below the critical micellar concentrations of the detergents used.

Cross-Reactivities. Data were obtained from standard curves of fenpropathrin, the pyrethroids phenothrin, permethrin, resmethrin, fenvalerate, deltamethrin, cyanonmethrin, and the metabolites 3-phenoxynbenzonic acid and fenpropathrin acid. In addition, other pesticides such as fenoxycarb, chlorpropham, and DDT were also tested for cross-reactivity. Each compound was prepared in 10% methanol in PBS and tested at the concentration range 0.1–1000 µg/L. The cross-reactivities (CR) were calculated as a ratio of the ICSO of fenpropathrin to that of the tested compound. CR was set at 100% for fenpropathrin.

RESULTS AND DISCUSSION

Hapten Synthesis and Conjugation. The main steps in the synthesis of haptenes are summarized in Schemes 1 and 2. The intermediate aldehyde 4 was synthesized by an Ullman coupling (Moroz and Shvartsberg, 1974) of the dimethyl acetal of 3-bromobenzaldehyde and benzyl 4-hydroxybenzenepropanoate 3 followed by acid-catalyzed deprotection in wet solvent. The synthesis of the analogous tert-butyl ester, prepared by a more circuitous route as an intermediate for a pyrethroid molecule, was described by Demouze and Touer (1987); however, no details of immunoassay development were given. The intermediate aldehydes 10 and 11, which lack the terminal phenyl group of fenpropathrin, were readily prepared by treatment of the potassium salt of 3-hydroxybenzenaldehyde with benzyl bromacetate and benzyl 6-bromohexanoate 9. Conversion of these aldehydes 4, 10, and 11 to their cyanohydrins followed by acylation with tetramethylcyclopropanecarbonyl chloride gave the hapten benzyl esters 5, 12, and 14, respectively. Cleavage of these benzyl esters with iodotrimethylsilane yielded haptenes 6, 13, and 15. Hapten 6 was found to be contaminated with ~10% of the tetramethylcyclopropanecarboxylate ester 6b of 4-hydroxybenzenepropanoic acid, resulting from the carry-through of unreacted phenol 3 because of its Rf value being identical to that of the intermediate aldehyde 4. Identical Rf values for subsequent products 5 and 6a, and 6 and 6b, made removal of these contaminants extremely difficult. The Rf values of 6 and 6b were identical in a variety of solvents, giving values ranging from 0.1 to 0.9. Nevertheless, our use of this preparation of compound 6 as the immunizing hapten gave excellent antibodies to fenpropathrin. Future preparation of this material should include a careful stepwise extraction of the intermediate acetal of compound 4 with aqueous base before proceeding to the subsequent step. Care should be taken in the extraction, since any base in excess over the contained phenol 3 can hydrolyze the benzyl ester function. Hapten 8, substituted by a terminal aromatic amino group, was synthesized from 3-(4-nitrophenoxo)benzaldehyde (Loewe and Urbanietz, 1967). Conversion to the cyanohydrin followed by acylation yielded the nitro-substituted fenpropathrin 7. Reduction of the nitro group with stannous chloride (Bellamy and Ou, 1984) yielded the amino-substituted fenpropathrin 8, which was a pale yellow gum. After TLC development, the product spot rapidly changed to dark orange-brown on exposure to light, as is typical of anilines.

In general, we chose to prepare haptenes that contain a functional group for coupling at the aromatic moiety of the pyrethroid molecule. The cyclopropane moiety of the molecule would be distal to the conjugation site, and therefore it would present a high target to elicit compound-specific antibodies that have an affinity for this part of the molecule. A set of immunogens was synthesized using different haptenes, containing amino or carboxyl functionalities, and different carrier proteins (Tables 1 and 2).

For example, hapten 6 has two methylene groups linking the carboxylic acid group to the aromatic portion of the molecule. The conjugates were made using a carbodiimide method (Tijssen, 1985). This method is advantageous in that a lipophilic hapten is first dissolved in organic solvent. This serves to help keep it in solution during further reaction with the protein in the mostly aqueous medium. It was anticipated that the short aliphatic spacer of the hapten would result in an immunogen that would generate antibodies that also bind to the aromatic moiety of the pyrethroid. In fact, the antibodies generated (AS 6982) were able to bind to a fenvalerate-based coating antigen (Table 1) that has only the aromatic moiety in common with fenpropathrin. In addition, the affinity of this antiserum for the fenvalerate moiety is very high, since free fenpropathrin was not easily able to displace the antibody from this coating antigen, as evidenced by the high IC50.

Hapten 8 features an amino group in the para position of the phenoxybenzyl group in contrast to the carboxylic acid group of hapten 6. The aromatic amino group was coupled to carrier proteins using a diazotization method (Tijssen, 1985). This method is advantageous in that the reaction product is brightly colored, making confirmation of the success of the reaction immediate. In addition, the reactive intermediate is water soluble, an advantage when working with lipophilic haptenes. To synthesize immunogen and coating antigens, diazo salts were made using NaNO2 under acidic conditions (approach A). The resulting immunogen, although highly colored, did not generate antibodies that could be used in a competitive assay format. In another approach (B) hapten 8 was coupled to the carrier proteins by a modified diazotization reaction using butyl nitrite in DMF as described under Materials and Methods. The antisera generated from this immunogen (AS 56 and AS 58) were useful in both homologous and heterologous assay formats (Tables 1 and 2).

Hapten 8 was also conjugated to bovine fetuin using a sodium periodate method (Tijssen, 1985). Fetuin has a molecular weight of ~42 000 (Carr et al., 1993) with an estimated carbohydrate content of 7% N-glycolylneuraminic acid (Noguchi et al., 1995) consisting largely of galactose, mannose, and glucose oligosaccharides (Rice et al., 1990). Because it has been shown that an increased carbohydrate content of the carrier increases the antigenicity of the immunogen (Noguchi et al., 1995), we used this glycoprotein as a carrier. Another potential advantage is that the hydrophilic carbohydrate moiety acts as a spacer between the lipophilic hapten and the carrier surface, allowing the immunogen to remain fairly hydrophilic despite attachment of a rather lipophilic hapten. One rabbit was immunized with this immunogen. The resulting antisera (AS 55) bound
Table 4. Determination of the Ratio of Hapten to Protein Molecules Following Conjugation a

<table>
<thead>
<tr>
<th>conjugate (MW of carrier protein)</th>
<th>molar ratio hapten/conjugate</th>
<th>hapten molecules per molecule of carrier protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immunogen</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8 - fetuin (42 000)</td>
<td>5.5 × 10⁻⁹/8.8 × 10⁻¹0</td>
<td>62.5</td>
</tr>
<tr>
<td>8 - thyroglobulin (660 000)</td>
<td>5.5 × 10⁻⁹/2.5 × 10⁻¹0</td>
<td>220</td>
</tr>
<tr>
<td>8 - LPH (335 000)</td>
<td>5.5 × 10⁻⁹/5.0 × 10⁻⁸</td>
<td>1b</td>
</tr>
<tr>
<td>Coating antigen</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8 - BSA (56 000)</td>
<td>5.5 × 10⁻⁹/1.0 × 10⁻⁸</td>
<td>55</td>
</tr>
</tbody>
</table>

The ratio of hapten to protein molecules was determined indirectly by the optimized ELISA described under Materials and Methods. A solution of conjugate with a known protein concentration was tested for its ability to inhibit the binding of antibodies in the ELISA. The percent inhibition was thus referenced to the calculated amount of hapten in the conjugate to the concentration of conjugate protein used to determine the ratios reported above. b The LPH conjugate was not completely soluble during analysis, which may contribute to the low ratio found.

Table 5. Representative Titer Results a

<table>
<thead>
<tr>
<th>Antiserum (immunogen)</th>
<th>6 - BSA</th>
<th>8 - BSA (B)</th>
<th>13 - BSA</th>
<th>13 - OVA</th>
<th>15 - BSA</th>
<th>15 - OVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>6982 (6 - KLH)</td>
<td>+++</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>56 (8 - LPH)</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>58 (8 - THY)</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>55 (8 - fetuin)</td>
<td>–</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
</tbody>
</table>

Data were determined from a coating antigen concentration of 1.5 μg/mL and an antibody dilution of 1/80000. All other ELISA conditions were as described under Materials and Methods. Antiseras and coating antigen combinations showing a titer response > 0.3 were further screened for inhibition by fenpropathrin. nd, not determined; -, OD of < 0.3; +, OD of 0.3–0.6; ++, OD of 0.6–1.0; ++++, OD of 1.0–1.5.

Screening for Competition by Fenpropathrin. The results of the competition screening are shown in Tables 1 and 2. The IC₅₀ values ranged from 20 to 500 μg/L in the homologous and heterologous systems tested. Concurrently, we were developing immunoassays for fenvalerate; thus, coating antigens designed for this assay were tested for binding with fenpropathrin antibodies. Table 1 shows that antibody 6982 was able to bind to and compete with the fenvalerate antigen. However, the IC₅₀ for fenpropathrin indicated that detection limits would be higher than those of conventional methods. Some differences in IC₅₀ were observed when different coating proteins were used. When the coating antigen was an OVA conjugate, the IC₅₀ values were lower compared to those using the BSA conjugates. The most sensitive assay was a homologous assay that used antibody 58 and coating antigen 8 - BSA. This assay was used for further assay optimization.

Assay Optimization. Many parameters can influence the binding of the antibody to the hapten. These parameters include the pH of the assay buffer during the competition step and the ionic strength of the buffer. Organic solvents, gelatin, or detergents are often added to the assay buffer to improve the solubility of the analyte or to decrease nonspecific binding. The effects of these were also determined.

pH Effect. Figure 3 shows the influence of varying the pH of the assay buffer during the competition step in the presence of the antibody. Because the control absorbance varied at each pH, the data are represented as a percent of the control absorbance at each pH. The assay sensitivity increased at more acidic or basic pH values (< 5 or > 9). However, at basic pH values the control absorbances decreased. Because the assay was more sensitive and had higher control absorbances under more acidic conditions, a pH of 4 was used for subsequent assays.

Ionic Strength. Because the ionic strength of the buffer containing the antibody can affect antibody binding (Tijssen, 1985), the ionic strength of the assay buffer was varied by increasing the NaCl concentration from 0.05 to 1.0 M (Figure 4). Increasing the salinity of the assay buffer resulted in an increase in sensitivity (IC₅₀ decreased significantly) but also resulted in a decrease of the control absorbance. The optimum range of ionic strength was 0.2–0.4 M NaCl.

Solvent Effect. Water-miscible organic solvents were used to keep fenpropathrin in solution while minimizing denaturation of the antibody during incubation. Figures 5 shows the effect of methanol, ethanol, acetone, DMF,
and acetonitrile on the control absorbance. The absorbances of the controls containing only organic solvent increased with increasing concentration of organic solvent, especially at concentrations >10%. Thus, the binding of the antibodies to the coating antigen was not adversely affected and, in fact, was enhanced. An increase in the absorbance was also observed in the presence of fenpropathrin. With acetonitrile, DMF, and ethanol, the absorbance was similar in the presence or absence of fenpropathrin. Since little inhibition was observed in the presence of fenpropathrin, these solvents were unacceptable for assay purposes. In contrast, a concentration of up to 10% methanol or 10% acetone resulted in high absorbance values in the absence of fenpropathrin and a significant inhibition of the signal in the presence of the inhibitor. Thus, 10% methanol was used in subsequent experiments.
Effect of Additives. The addition of gelatin has been found to increase the sensitivity and stability of immunoassays (Forlani et al., 1992). Gelatin was tested at 0.1–1.0%. No changes in the assay parameters were seen except for an increase in the background signal.

A series of nonionic, cationic, and anionic detergents were added in the competition step to reduce nonspecific binding and improve solubility of the analyte. At the concentrations tested, no changes in the fenpropathrin concentration curve were observed.

The optimized fenpropathrin assay used a 1/8000 dilution of the coating antigen BSA preparation and antibody 58 at a dilution of 1/40000. This homologous assay showed an IC_{50} value of ~20 \mu g/L (Figure 6). The assay detects fenpropathrin within a linear range of 2.5–200 \mu g/L.

Cross-Reactivity. The antibodies from antisera 58 were relatively selective for fenpropathrin. Most of the compounds tested, including a group of pyrethroids, and pyrethroid metabolites showed no significant cross-reactivity in the concentration range of 0.1–1000 \mu g/L (Table 3), except deltamethrin (CR = 18.8%).

Conclusion. The synthesis of different haptens for fenpropathrin allowed us to test different immunogens for the antibody production. Comparison of homologous and heterologous assays revealed that the highest sensitivity was achieved with an homologous assay. Since heterology often results in more sensitive assays, the discrepancy may be due to differences in hapten load among the homologous and heterologous coating antigens. Using antisera 58 in the coating antigen format, the IC_{50} was 20 \mu g/L with a lower detection limit of 2.5 \mu g/L. This is similar to the detection limit of the GC method reported by Takimoto et al. (1984).

The assay optimization studies revealed that methanol or acetone at concentrations >10% and assay buffer at pH 4 resulted in the most sensitive assay. None of the added detergents or gelatin increased the sensitivity of the assay. The assay had little cross-reactivity to other pyrethroids or pyrethroid metabolites, making it useful for the selective detection of fenpropathrin.

ABBREVIATIONS USED

AS, antiserum; BSA, bovine serum albumin; BSTFA, bis(trimethylsilyl)trifluoroacetamide; t-BuOH, tert-butyl alcohol; BuCl, butyl chloride; DAPEC, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride; DCC, dicyclohexylcarbodiimide; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; DMAP, 4-dimethylaminopyridine; ELISA, enzyme-linked immunosorbent assay; GAR/HRP, goat-anti-rabbit immunoglobulin conjugated to horseradish peroxidase; KLH, hemocyanin of keyhole limpet; LPH, hemocyanin of Limulus polyphemus; NHS, N-hydroxysuccinimide; OVA, ovalbumin; PBS, phosphate-buffered saline; sulfo-NHS, N-hydroxysulfosuccinimide; THF, tetrahydrofuran; TMSI, iodo(trimethyl)silane; TMB, tetramethylbenzidine.

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