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Enzyme-Linked Immunosorbent Assay for the Pyrethroid Deltamethrin

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A competitive enzyme-linked immunosorbent assay (ELISA) for the detection of deltamethrin was developed. Two haptens, cyano[3-(4-aminophenoxy)phenyl]methyl 1*R-cis*-3-(2,2−dibromoethenyl)-2,2-dimethylcyclopropanecarboxylate and 3-[(±)-cyano[1*R-cis*-3-(2,2−dibromoethenyl)-2,2-dimethylcyclopropan ecarbonyloxy]methyl]phenoxyacetic acid, were synthesized and conjugated with thyroglobulin as immunogens. Four antisera were generated and screened against six different coating antigens. The assay that was the most sensitive for deltamethrin was optimized and characterized. The I_{50} for deltamethrin was 17.5 ± 3.6 μg/L, and the lower detection limit was 1.1 ± 0.5 μg/L. This ELISA assay had relatively low cross-reactivities with other major pyrethroids, such as permethrin, phenothrin, bioresmethrin, cyfluthrin, and cypermethrin. Methanol was found to be the best organic cosolvent for this ELISA, with optimal sensitivity observed at a concentration of 40% (v/v). The assay parameters were unchanged at pH values between 5.0 and 8.0, whereas higher ionic strengths strongly suppressed the absorbances. To increase the sensitivity of the overall method, a C_{18} sorbent-based solid-phase extraction was used for river water samples. River water samples fortified with deltamethrin were analyzed according to this method. Good recoveries and correlation with spike levels were observed.

KEYWORDS: Deltamethrin; ELISA; environmental monitoring; pyrethroid

INTRODUCTION

Because pyrethroids are a group of highly potent insecticides with relatively low mammalian toxicity, they are being widely used in agriculture, forestry, horticulture, public health, and households throughout the world (*1*). These compounds have improved physical and chemical properties and biological activity compared to their natural analogues. Pyrethroids are the most potent lipophilic insecticides (*2*). They have been detected as surface water contaminants, and impacts to the environment leading to effects on ecosystem health have been reported. Many of the toxicological studies on pyrethroids focused on nontarget invertebrates and aquatic animals (*3*−*5*).

Deltamethrin (**Figure 1**) is a photostable pyrethroid with an insecticidal activity 10 times greater than that of permethrin on several major pests (*6*). Its greater stability to degradation than that of alternative pyrethroids has made it attractive for uses requiring longer residual activity (*7*). Thus, a sensitive, selective, and rapid method for monitoring residue levels of deltamethrin is needed, particularly in aquatic ecosystems. Most synthetic pyrethroids are marketed as mixtures of optical and geometrical isomers. In contrast, deltamethrin is 1*R-cis*- in the dibromochrysanthemate moiety and *S* at the α-cyano carbon.

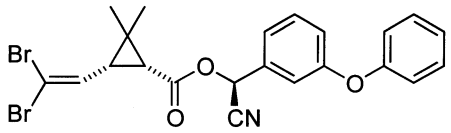

Figure 1. Structure of deltamethrin.

Current analytical methods for deltamethrin involve multistep sample cleanup procedures followed by gas chromatography (GC) and detection by electron capture (GC-EC) or high-performance liquid chromatography−mass spectrometry (HPLC-MS) (*2*, *6*, *8*, *9*). These methods are relatively intensive, time-consuming, and expensive, and not particularly suitable for large numbers of samples. An immunoassay would provide a sensitive, selective, and rapid method for the detection of this pyrethroid at trace levels (*10*−*16*).

To raise antibodies that would be selective for deltamethrin, the deltamethrin molecule must be modified to attach a spacer arm containing a functional group to facilitate coupling to a carrier protein. A monoclonal antibody-based assay was developed using a hapten in which a spacer that terminated in a carboxylic acid was added to the phenoxybenzyl moiety. This assay reported an I_{50} of ∼500 μg/L with a high degree of selectivity because there was no cross-reactivity with any pyrethroids tested (*17*). Another approach was to replace the α-cyano group with a spacer that terminated in a carboxylic

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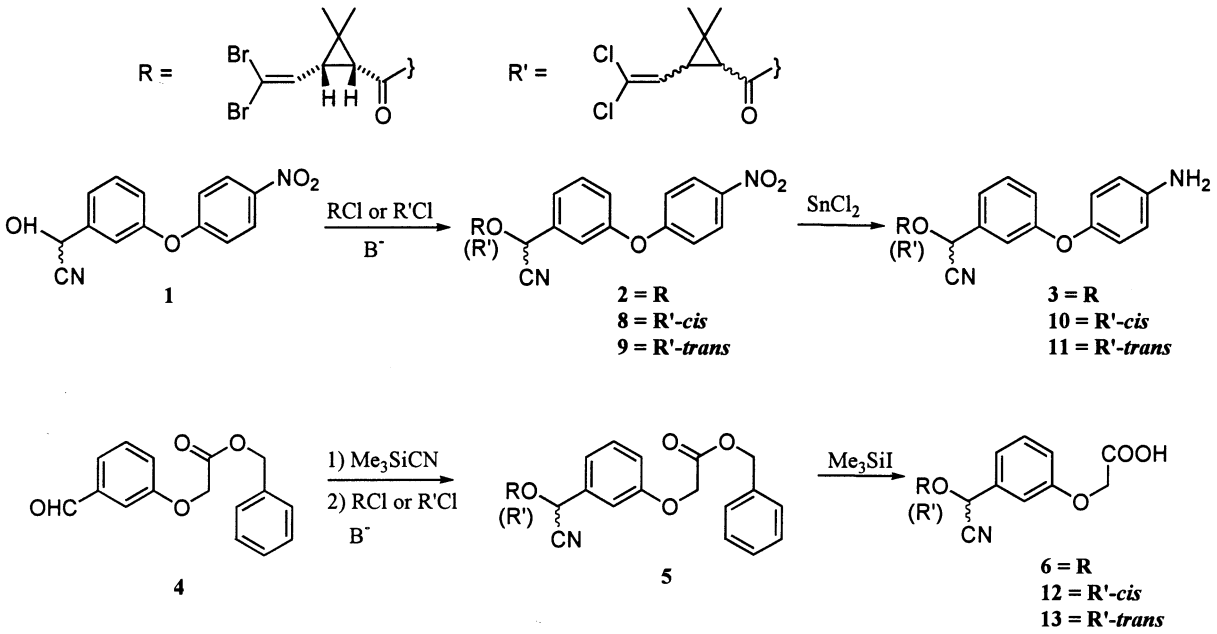

Figure 2. Scheme for hapten synthesis.

acid. Antibodies to this hapten resulted in an assay with an I_{50} of 130 μg/L, although treatment of sample with alkali to isomerize the deltamethrin increased the sensitivity of the assay to 4 μg/L (*18*, *19*). The antibodies reported here were developed from a novel deltamethrin hapten and resulted in an assay that is ~10 times more sensitive than previously reported for unisomerized deltamethrin.

MATERIALS AND METHODS

Chemicals and Immunoreagents. 1*R-cis*-3-(2,2-Dibromoethenyl)-2,2-dimethylcyclopropanecarboxylic acid was kindly supplied by The Institute of Arable Crops Research (Rothamsted Experimental Station, Long Ashton, U.K.). A number of synthetic routes to this acid are described in the literature (*20*−*22*). Haptens **12** and **13** were made in this laboratory previously (*14*). Other organic starting materials for hapten synthesis were purchased from Aldrich Chemical Co. (Milwaukee, WI) and Fisher Scientific (Pittsburgh, PA). Thin-layer chromatography (TLC) was performed using 0.2 mm precoated silica gel 60 F254 on glass plates from E. Merck (Darmstadt, Germany), and detection was made by ultraviolet (UV) light or iodine vapor stain. Flash chromatographic separations were carried out on Baker silica gel (40μm average particle size) using the indicated solvents, where the → notation denotes a stepwise concentration gradient.

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

Instruments. NMR spectra were obtained using a General Electric QE-300 spectrometer (Bruker NMR, Billerica, MA). Chemical shift values are given in parts per million (ppm) downfield from internal tetramethylsilane. 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-2SE mass spectrometer (VG Analytical, Wythenshawe, U.K.), using high-energy cesium ions at a density flux of 1−2 mA and 35−38 kV to generate secondary $[MH]^+$ ions. The liquid matrix was a 1:1 mixture of glycerol or 3-nitrobenzyl alcohol, and cesium iodide was used for mass calibration at a dynamic resolution of 5000:1. ELISA experiments were performed in 96-well microplates (Nunc, Roskilde, Denmark), and the absorbances were measured with a Vmax microplate reader (Molecular Devices, Menlo Park, CA) in dual-wavelength mode (450−650 nm).

Hapten Synthesis and Verification. Syntheses of the haptens were carried out as outlined in **Figure 2**. All reactions were straightforward and followed procedures employed in preceding publications (*12*−*14*, *23*). NMR spectral data supported all structures, and mass spectra further confirmed the structures of the final haptens.

Cyano[3-(4-nitrophenoxy)phenyl]methyl 1R-cis-3-(2,2-dibromoethenyl)-2,2-dimethylcyclopropanecarboxylate (2). A solution of 1*R-cis*-3-(2,2-dibromoethenyl)-2,2-dimethylcyclopropanecarboxylic acid (300 mg, 1 mmol) in chloroform (0.3 mL) was treated with thionyl chloride (145 μL, ~2 mmol) and dimethyl formamide (0.2 μL) and heated in an oil bath at 60−65 °C for 1 h. Solvent and excess thionyl chloride were briefly stripped, then 0.5 mL of hexane was added, and the mixture was restripped to leave the acid chloride as a yellow oil. Meanwhile, zinc iodide (6 mg) was added to a solution of 3-(4-nitrophenoxy)-benzaldehyde (*24*) (245 mg, 1 mmol) in chloroform (0.6 mL) under nitrogen in a septum-capped vial. The mixture was chilled in ice, the bath was removed, and half of a sample of cyanotrimethylsilane (148 μL, 1.1 mmol) was injected. After a mild exotherm, the mixture was again cooled, and the remaining cyanotrimethylsilane was injected. After 2.5 h at ambient temperature, the reaction mixture was added to a mixture of glyme (3 mL) and 3 N HCl (0.75 mL). The mixture was stirred vigorously for 5 min, diluted with water (3 mL), and extracted twice with butyl chloride. The organic phase was dried ($MgSO_4$) and stripped to give the cyanohydrin as an oil.

A solution of the cyanohydrin in chloroform (1 mL) was stirred in an ice bath, and the acid chloride in chloroform (0.5 mL) was added, followed by a solution of pyridine (100 μL, 1.25 mmol) in chloroform (0.3 mL) injected over 5 min. After 30 min at ambient temperature, the mixture was washed with water, 1 N HCl, and sodium bicarbonate solution, dried ($MgSO_4$), and stripped to the heavy oil. This was flash chromatographed on silica gel (13 g) (10 → 100% methylene chloride in hexane), and fractions containing product were combined and stripped to yield 535 mg (95%) of **2** as a viscous almost colorless oil. Multiple development of a TLC with 5% glyme in hexane separated the two diastereoisomers. A 52 mg sample of **2** was separated by radial chromatography. The sample was injected in 50% butyl chloride in hexane and then eluted with 5% glyme in hexane to recover 26 mg of the higher R_f isomer [^{1}H NMR ($CDCl_3$) δ 1.29 (s, 3 H, CH_3), 1.32 (s, 3 H, CH_3), 1.92 [d, J = 8.4 Hz, 1 H, CHC(O)], 2.06 (dd, J = 8.4, 8.4 Hz, 1 H, C=CCH), 6.39 (s, 1 H, CHCN), 6.67 (d, J = 8.4 Hz, 1 H, C=CH), 7.04−8.26 (m, 8 H, Ar)] and 26 mg of the lower R_f isomer [^{1}H NMR ($CDCl_3$) δ 1.21 (s, 3 H, CH_3), 1.26 (s, 3 H, CH_3), 1.92 [d, J = 8.4 Hz, 1 H, CHC(O)], 2.10 (dd, J = 8.3, 8.3 Hz, 1 H, C=CCH), 6.42 (s, 1 H, CHCN), 6.68 (d, J = 8.2 Hz, 1 H, C=CH), 7.03−8.26 (m, 8H, Ar).

*Cyano[3-(4-aminophenoxy)phenyl]methyl 1R-cis-3-(2,2-dibromoethenyl)-2,2-dimethylcyclopropanecarboxylate (**3**).* Stannous chloride (345 mg, 1.5 mmol) was added to a solution of **2** (168 mg, 0.305 mmol) in ethanol (1.5 mL). The mixture was stirred under nitrogen and heated at 70−75 °C for 35 min. The mixture was cooled and diluted with water, and ether and Celite (0.4 g) were added, followed by solid $NaHCO_3$ (260 mg) in portions. The resulting mixture was filtered, and solids were washed with ethyl acetate. The combined organics were water washed and stripped to a dark oil. A TLC indicated complete reaction to one major product spot that rapidly darkened on exposure to light. This oil was flash chromatographed on silica gel (6 g) (25 → 100% CH_2Cl_2 in hexane followed by 2 → 10% EtOAc in CH_2Cl_2). Stripping fractions containing pure product gave 101 mg (64%) of **3** as a pale yellow oil: ^{1}H NMR ($CDCl_3$) δ 1.21 (s, 3 H, CH_3), 1.25 (s, 3 H, CH_3), *1.30* (s, 3 H, CH_3), *1.31* (s, 3 H, CH_3), 1.90 [d, J = 8.4 Hz, 1 H, CHC(O)], 1.91 [d, J = 8.4 Hz, 1 H, CHC(O)], *2.03* (t, J = 8.4 Hz, 1 H, C═CCH), 2.07 (t, J = 8.4 Hz, 1 H, C═CCH), 3.66 (bs, 2 × 2 H, NH_2), *6.28* (s, 1 H, CHCN), 6.34 (s, 1 H, CHCN), 6.67−7.38 (m, 2 × 8 H, Ar). The C═CH doublets are shifted downfield into the 6.65−6.67 ppm region and are overlapped by the NH_2 doublets. The italicized values are tentatively assigned to the diastereoisomer corresponding to the benzyl ester precursor having the higher R_f by TLC. FAB-HRMS m/z calcd for $[M]^+ = C_{22}H_{20}Br_2N_2O_3$ was 517.9841 (obsd 518.9844).

*Benzyl 3-[(±)-Cyano[1R-cis-3-(2,2-dibromoethenyl)-2,2-dimethylcyclopropanecarbony loxy]methyl]phenoxyacetate (**5**).* A sample of benzyl 2-(3-formylphenoxy)acetate (**4**) (*20*) (273 mg, 1.0 mmol) was converted to the cyanohydrin as a colorless oil as described for **2** above. This was reacted as described above with 3-(2,2-dibromoethenyl)-2,2-dimethylcyclopropanecarbonyl chloride prepared from the acid (300 mg, 1.0 mmol) as described above. Flash chromatography of the resulting product recovered 525 mg (90%) of **5** as a viscous oil. A 51 mg sample was separated by radial chromatography as described above to recover 25 mg of the higher R_f isomer [^{1}H NMR ($CDCl_3$) δ 1.29 (s, 3 H, CH_3), 1.31 (s, 3 H, CH_3), 1.90 [d, J = 8.4 Hz, 1 H, CHC(O)], 2.04 (dd, J = 8.4, 8.4 Hz, 1 H, C═CCH), 4.70 [s, 2 H, $CH_2C(O)$], 5.25 (s, 2H, CH_2Ph), 6.31 (s, 1 H, CHCN), 6.70 (d, J = 8.4 Hz, 1 H, C═CH), 6.95−7.40 (m, 9 H, Ar)] and 25 mg of the lower R_f isomer [^{1}H NMR ($CDCl_3$) δ 1.21 (s, 3 H, CH_3), 1.24 (s, 3 H, CH_3), 1.90 [d, J = 8.4 Hz, 1 H, CHC(O)], 2.07 (dd, J = 8.4, 8.4 Hz, 1 H, C═CCH), 4.70 [s, 2 H, $CH_2C(O)$], 5.25 (s, 2 H, CH_2Ph), 6.36 (s, 1 H, CHCN), 6.71 (d, J = 8.3 Hz, 1 H, C═CH), 6.94−7.41 (m, 9 H, Ar)].

*3-[(±)-Cyano[1R-cis-3-(2,2-dibromoethenyl)-2,2-dimethylcyclopropanecarbon yloxy]methyl]phenoxyacetic Acid (**6**).* Iodotrimethylsilane (43 μL, 0.3 mmol) was injected into a solution of **5** (144 mg, 0.25 mmol) in methylene chloride (0.3 mL) contained in a septum-capped nitrogen-filled test tube. The mixture was warmed in an oil bath at 35 °C for 2 h and then diluted with methanol (1 mL) and methylene chloride (2 mL). The resulting mixture was water washed and stripped to a brown oil. This was immediately flash chromatographed on silica gel (5 g) eluting with 50%CH_2Cl_2 in hexane (10 mL), 2% EtOAc in CH_2Cl_2 (10 mL), and then 20 → 100% EtOAc in CH_2Cl_2 containing 1.5% v/v HOAc. Fractions containing pure product were stripped, and EtOAc (5 mL) was added and restripped to remove traces of acetic acid to yield 94 mg (75%) of **6** as a colorless gum: ^{1}H NMR ($CDCl_3$) δ 1.22 (s, 3 H, CH_3), 1.26 (s, 3 H, CH_3), *1.30* (s, 3 H, CH_3), *1.32* (s, 3 H, CH_3), 1.92 (×2) [d, J = 8.4 Hz, 2 × 1 H, CHC(O)], *2.05* (dd, J = 8.4, 8.4 Hz, 1 H, C═CCH), 2.09 (dd, J = 8.4, 8.4 Hz, 1 H, C═CCH), 4.74 (×2) [s, 2 × 2 H, $CH_2C(O)$], *6.35* (s, 1 H, CHCN), 6.39 (s, 1 H, CHCN), *6.70* (d, J = 8.4 Hz, C═CH), 6.71 (d, J = 8.4 Hz, C═CH), 6.98−7.44 (m, 2 × 4 H, Ar). The italicized values are tentatively assigned to the diastereoisomer corresponding to the benzyl ester precursor having the higher R_f by TLC. FAB-HRMS m/z calcd for $[M + H]^+ = C_{18}H_{18}Br_2NO_5$ was 485.9552 (obsd 485.9547).

*(±)-Cyano[3-(4−nitrophenoxy)phenyl]methyl (±)-cis-3-(2,2-Dichloroethenyl)-2,2-dimethylcyclopropanecarboxylate (**8**).* Using the same procedures as those described above for the preparation of **3**, (±)-*cis*-3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropanecarbonyl chloride was prepared from the corresponding acid [(±)-*cis*-permethric acid] (0.75 g, 3.59 mmol), and the cyanohydrin was prepared from 3-(4-nitrophenoxy)benzaldehyde (0.875 g, 3.59 mmol). These were coupled as described above to yield the crude ester as an oil. Flash chromatography on silica gel (25 g) (5 → 80% CH_2Cl_2 in hexane) gave 1.06 g (64%) of the pure isomer mix, **8**, as a colorless gum. A 54 mg sample of **8** was separated by radial chromatography into the two diastereomer pairs as described for **2** above to return 33.9 mg of the higher R_f isomer pair [^{1}H NMR ($CDCl_3$) δ 1.29 (s, 3 H, CH_3), 1.31 (s, 3 H, CH_3), 1.91 (d, J = 8.4 Hz, 1 H, CHCOO), 2.14 (dd, J = 8.6, 8.6 Hz, 1 H, C═CCH), 6.15 (d, J = 8.8 Hz, 1 H, C═CH), 6.39 (s, 1 H, CHCN), 7.04−8.26 (m, 8 H, Ar); ^{13}C NMR ($CDCl_3$) δ 14.7, 28.1, 29.0, 31.0, 33.5, 62.0, 115.8, 117.5 (2 C), 119.6, 121.9, 122.0, 123.7, 124.3, 126.0 (2 C), 131.2, 134.2, 143.1, 155.5, 162.3, 168.4] and 19.8 mg of the lower R_f isomer pair [^{1}H NMR ($CDCl_3$) δ 1.21 (s, 3 H, CH_3), 1.26 (s, 3 H, CH_3), 1.92 (d, J = 8.4 Hz, 1 H, CHCOO), 2.18 (dd, J = 8.6, 8.6 Hz, 1 H, C═CCH), 6.16 (d, J = 8.5 Hz, 1 H, C═CH, 6.41 (s, 1 H, CHCN), 7.04−8.27 (m, 8 H, Ar); ^{13}C NMR ($CDCl_3$) δ 14.8, 28.1, 28.9, 30.9, 33.6, 62.0, 115.7, 117.6 (2 C), 119.6, 122.0, 122,2, 123.5, 124.4, 126.1 (2 C), 131.2, 134.4, 143.2, 155.5, 162.4, 168.4].

*(±)-Cyano[3-(4−nitrophenoxy)phenyl]methyl (±)-trans-3-(2,2-Dichloroethenyl)-2,2-dimethylcyclopropanecarboxylate (**9**).* This material was prepared on the same scale and by the same procedure as described above for **8** using (±)-*trans*-permethric acid to yield 1.06 g (64%) of the pure isomer mix, **9**, as a colorless gum. A 70 mg sample of **9** was separated by radial chromatography as described above for **2** to yield 45 mg of the higher R_f isomer pair [^{1}H NMR ($CDCl_3$) δ 1.24 (s, 3 H, CH_3), 1.34 (s, 1 H, CH_3), 1.67 (d, J = 5.3 Hz, 1 H, CHCOO), 2.29 (dd, J = 5.3, 8.1 Hz, 1 H, C═CCH), 5.61 (d, J = 8.1 Hz, 1 H, C═CH), 6.44 (s, 1 H, CHCN), 7.03−8.27 (m, 8 H, Ar); ^{13}C NMR ($CDCl_3$) δ 19.9, 22.3, 30.5, 33.7, 34.0, 62.3, 115.8, 117.5 (2 C), 119.6, 122.0, 123.1, 124.4, 125.9, 126.0 (2 C), 131.2, 134.2, 143.1, 155.5, 162.3, 169.2] and 25 mg of the lower R_f isomer pair [^{1}H NMR ($CDCl_3$) δ 1.20 (s, 3 H, CH_3), 1.25 (s, 3 H, CH_3), 1.69 (d, J = 5.3 Hz, 1 H, CHCOO), 2.33 (dd, J = 5.3, 8.1 Hz, 1 H, C═CCH), 5.63 (d, J = 8.1 Hz, 1 H, C═CH), 6.44 (s, 1 H, CHCN), 7.02−8.25 (m, 8 H, Ar); ^{13}C NMR ($CDCl_3$) δ 20.0, 22.4, 30.3, 33.7, 34.0, 62.3, 115.6, 117.6 (2 C), 119.5, 122.0, 123.3, 124.3, 125.8, 126.0 (2 C), 131.2, 134.4, 143.2, 155.5, 162.3, 169.2].

*(±)-Cyano[3-(4-aminophenoxy)phenyl]methyl (±)-cis-3-(2,2-Dichloroethenyl)-2,2-dimethylcyclopropanecarboxylate (**10**).* A 5 mL reaction flask with a magnetic stir bar was charged with compound **8** (250 mg, 0.54 mmol), ethanol (2 mL), and stannous chloride dihydrate (612 mg, 2.7 mmol). The mixture was stirred and heated in an oil bath at 70 °C for 30 min. The mixture was cooled and poured into a slurry of water, ether, and Celite (0.45 g). $NaHCO_3$ (0.455 g, 5.4 mmol) was added in portions (foaming). The neutral mixture was filtered, and solids were washed with water and ether. The ether phase was separated, dried (Na_2SO_4), and stripped to a dark oil. Flash chromatography on 5.5 g of silica gel (50 → 100% CH_2Cl_2 in hexane) recovered 16 mg (6.4%) of **8** and 129 mg (59% based on recovered starting material) of isomer mix **10** as a stripped gum, R_f 0.15 (silica gel, CH_2Cl_2). FAB-HRMS m/z calcd for $[M]^+ = C_{22}H_{20}Cl_2N_2O_3$ was 430.0851 (obsd 430.0854). ^{1}H NMR ($CDCl_3$) confirmed a mixture of two isomer pairs in a ∼3:2 ratio: δ 1.20 (s, 3 H, CH_3), 1.25 (s, 3 H, CH_3), *1.29* (s, 3 H, CH_3), *1.30* (s, 3 H, CH_3), 1.89 (d, J = 8.4 Hz, 2 × 1 H, $CHCO_2$), 2.09−2.15 (m, 2 × 1 H, C═CCH), 3.6 (bs, 2 × 2 H, NH_2), *6.18* (d, J = 8.8 Hz, 1 H, C═CH), 6.19 (d, J = 8.8 Hz, 1 H, C═CH), *6.29* (s, 1 H, CHCN), 6.34 (s, 1 H, CHCN), 6.69−7.38 (m, 2 × 8 H, Ar). The italicized peak values are the more intense of the pair.

*(±)-Cyano[3-(4-aminophenoxy)phenyl]methyl (±)-trans-3-(2,2-Dichloroethenyl)-2,2-dimethylcyclopropanecarboxylate (**11**).* This material was prepared from **9** on the same scale and by the same procedures as for the preparation of **10** above to yield 128 mg (55%) of the isomer mix **11** as a gum, R_f 0.15 (silica gel, CH_2Cl_2). FAB-HRMS m/z calcd for $[M]^+ = C_{22}H_{20}Cl_2N_2O_3$ was 430.0851 (obsd 430.0852). ^{1}H NMR ($CDCl_3$) confirmed a mixture of two isomer pairs in a ∼3:2 ratio: δ 1.18 (s, 3 H, CH_3), *1.22* (s, 3 H, CH_3), 1.24 (s, 3 H, CH_3), *1.32* (s, 3 H, CH_3), *1.65* (d, J = 5.4 Hz, 1 H, $CHCO_2$), 1.67 (d, J = 5.3 Hz, 1 H, $CHCO_2$), 2.25−2.33 (m, 2 × 1 H, C═CCH), 3.64 (bs, 2 × 2 H, NH_2), *5.60* (d, J = 7.9 Hz, 1 H, C═CH), 5.62 (d, J = 7.9 HZ, 1 H, C═CH), *6.34* (s, 1 H, CHCN), 6.36 (s, 1 H, CHCN), 6.68−7.37 (m, 2 × 8 H, Ar). The italicized peak values are the more intense of the pair.

Hapten Conjugation. Conjugates were synthesized using a water-soluble carbodiimide or diazotization methods (*25*, *26*). To obtain

immunogens, haptens **3** and **6** were conjugated to thyroglobulin. Coating antigens were made by coupling haptens **3**, **6**, **10**, **11**, **12**, and **13** to BSA.

Hapten **3**, **10**, or **11** (0.05 mmol) was dissolved in 4 drops of ethanol and treated with 0.6 mL of 1 N HCl. The resulting solution was stirred in an ice bath as 0.4 mL of 0.20 M sodium nitrite was added. DMF (0.4 mL) was then added dropwise to give a homogeneous solution. Forty-five milligrams of thyroglobulin or BSA was dissolved in a mixture of 5 mL of 0.2 M borate buffer (pH 8.8) and 1.2 mL of DMF. The activated hapten solution was added dropwise to the two stirred protein solutions. The reaction mixtures were stirred in an ice bath for 45 min and then dialyzed against phosphate-buffered saline (PBS) over 72 h at 4 °C. The purified conjugates were suspended in water and stored in aliquots at −20 °C.

Haptens **6**, **12**, and **13** were conjugated according to ref *14*.

Immunization and Antiserum Preparation. Deltamethrin antisera were obtained following the protocol reported earlier for other pyrethroids (*14*).

Enzyme Immunoassay. The method was identical to that reported by Shan et al. (*12*) with the following exceptions. The wash buffer was 0.05% Tween 20 in distilled water. The incubation step with inhibitor was 30 min, and the incubation step with goat anti-rabbit-IgG-HRP conjugate was 45 min.

Antibody Characterization and Assay Optimization. Antibodies and antigens were screened in a two-dimensional titration for the best dilution of coating antigen and antiserum. Then the competitive inhibition curves were measured for different antibody and antigen combinations, and the one with the lowest I_{50} was selected for further assay development.

The effects of solvents were tested by dissolving the analyte in PBS buffers containing various proportions of solvent and incubating these with antibody in PBST on the coated plate. We tested methanol (0, 10, 20,40, 60, and 80%) and DMSO (0, 10, 20, and 40%) in this fashion.

In the experiment to evaluate pH effects, both the analyte and antiserum were dissolved in PBS buffer at the specified pH and added to a coated plate. pH values of 5, 6, 7, and 8 were tested in this incubation step with all other parameters of the assay fixed. Ionic strength effects were determined in the same manner as previously mentioned except that, instead of pH, PBS concentration was varied. PBS concentrations of normal strength (1×), 2×, 4×, and 8× were tested. Deltamethrin standards were prepared in PBS buffer containing 0, 10, 20, 40, 60, and 80% (v/v) methanol or 0, 10, 20, and 40% (v/v) DMSO to determine the effects of solvent.

Cross-Reactivity. The optimized assays were applied to cross-reactivity studies by using the standard solution of the analytes and other structurally related compounds. The crosw-reactivity (CR) was determined by dividing the I_{50} of the cross-reactant by the I_{50} of deltamethrin (assigned as 100%) and multiplying by 100 to obtain a percent figure.

Solid-Phase Extraction. An SPE method used in this study was the same as a previously reported method for the extraction of esfenvalerate from water using a C_{18} column (*12*). Deltamethrin was extracted from water using a C_{18} SPE column (10 cm^3/500 mg; part 1211-3027, Varian Samples Preparation Products, Harbor City, CA). The column was preconditioned with 3.5 mL of methanol and deionized water 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 a thorough drying with vacuum air, the containers were washed with methanol (3−4 × 1 mL) and all washes added to the final methanol extract. After the water had passed through, the column was dried under vacuum for 15 min and then eluted with 100% methanol (3.5 mL). The mixture of the methanol extract and washes was evaporated to dryness under a gentle stream of nitrogen and the residue was redissolved in 1 mL of methanol. An aliquot diluted with 40% methanol in PBS (1:4) was analyzed by ELISA.

RESULTS AND DISCUSSION

Hapten Synthesis. The haptens were synthesized according to **Figure 2**. The synthetic routes parallel those described for the synthesis of permethrin haptens in a previous publication (*14*). Deltamethrin is a potent pyrethroid insecticide containing three chiral centers. The commercial material is produced through a chiral synthesis scheme by which the acid moiety has the more active 1*R*-cis configuration and the cyanohydrin center the *S* configuration. Because racemization of the cyanohydrin center is known to occur under some field conditions (*27*), the haptens were synthesized as mixtures of the two diastereoisomers racemic at the cyanohydrin center to allow detection of both of these isomers. Although separation of the immediate precursors was possible as demonstrated under Materials and Methods, no attempt was made to prepare the pure chiral isomers or diastereoisomers of the final haptens in this study.

To develop a sensitive and specific deltamethrin immunoassay, immunogen hapten design is important. First, because the alcohol portion of deltamethrin, the phenoxybenzyl group, is common in many commercial pyrethroids and its acid portion is relatively unique, our strategy for designing the immunizing hapten was to link carrier protein through the aromatic end to improve antibody specificity. Second, a highly lipophilic pyrethroid conjugated on the protein surface may fold into the hydrophobic interior of the protein and, thus, result in a low-affinity antibody. Our previous studies on pyrethroid and dioxin immunoassays suggested that a short or rigid handle (side chain) could prevent or minimize such folding (*14*, *28*). Therefore, two haptens containing the whole or partial deltamethrin molecule with a short handle on aromatic rings (compounds **3** and **6**) were chosen for immunization. In compound **6**, the distal phenyl group of deltamethrin was eliminated and a carboxylic acid was directly linked to first aromatic ring.

The deltamethrin haptens **3** and **6** were prepared via acylation of the cyanohydrins of 3-(4-nitrophenoxy)benzaldehyde and benzyl 2-(3-formylphenoxy)acetate with the acid chloride of 1*R*-*cis*-deltamethric acid to give intermediates **2** and **5**. A small sample of each was separated by radial chromatography into the two isomers in order to obtain definitive confirming NMR spectra. Reduction of the nitro ester **2** with stannous chloride gave the amino-substituted hapten **3**, and cleavage of the benzyl ester with iodotrimethylsilane gave the hapten **6**, each as a mixture of isomers racemic at the cyanohydrin center.

The coating haptens **10** and **11** were prepared via similar acylations of the cyanohydrin of 3-(4-nitrophenoxy)benzaldehyde as described above using the acid chlorides of (±)-*cis*- and (±)-*trans*-permethric acid. This introduces an additional chiral center resulting in two diastereoisomer pairs for each of the intermediate esters, **8** and **9**. A small sample of each ester was separated by radial chromatography into the two diastereoisomer pairs in order to obtain definitive confirming NMR spectra; however, the respective mixtures were carried through to the final haptens **10** and **11** by the same reduction reaction as described for hapten **3**. The NMR spectra of the intermediate esters indicated these pairs to be in a ratio of ∼3:2 in each case. This ratio allowed tentative spectral assignments to the isomer pairs of the final haptens. The preparation of haptens **12** and **13** is described in a previous publication (*14*).

Screening and Selection of Antisera. The antisera of terminal bleeds from four rabbits were screened against six different coating antigens using a two-dimensional titration method with the coated antigen format. The homologous assay, in which the same hapten was used in coating antigen and immunogen, had a higher titer than the heterologous assay. These results are consistent with a previous study on the pyrethroid esfenvalerate (*12*). Ab732 had the highest affinity

Table 1. Selected Competitive ELISA Screening Data against Deltamethrin[a]

immunogen	antiserum	coating antigen	I_{50} (μg/L)
3–THY	729	**3**–BSA	2.61×10^6
	730	**3**–BSA	2.99×10^3
6–THY	731	**12**–BSA	119
		13–BSA	122
		11–BSA	275
		10–BSA	148
		3–BSA	160
		6–BSA	173
	732	**12**–BSA	203
		13–BSA	22.8
		11–BSA	42.2
		10–BSA	166
		3–BSA	458
		6–BSA	129

[a] Optimized assay conditions were used. The deltamethrin analyte standards were prepared in a 40% methanol/PBS solution.

for coating antigen **6**−BSA, followed by **13**−BSA and then **3**−BSA, **10**−BSA, **11**−BSA, and **12**−BSA. Combinations of coating antigen and antiserum that resulted in high optical densities (OD > 0.75) were selected for further development.

To identify systems that yielded the highest sensitivity for deltamethrin, competitive inhibition experiments were conducted in parallel with optimization of antiserum and coating antigen concentrations. The reagent concentrations with optical densities of ~0.8 and lowest I_{50} values were chosen as the optimal combinations. The I_{50} values for each combination ranged from 22.8 to 2.61×10^6 μg/L (**Table 1**). The heterologous system for Ab732 (with cAg **13**−BSA) showed the lowest I_{50} (22.8 μg/L), which was ~6 times lower than the homologous assay with cAg **6**−BSA. In this study, only the heterologous system of Ab732 (1/22000) and cAg **13**−BSA (0.06 μg/mL) was used for further assay development and optimization.

It is also interesting to note that for one rabbit (731), the I_{50} values are all similar, indicating that the relationship between the affinity for the coating antigen and the affinity for deltamethrin is similar in all cases, regardless of the structure of the coating antigen. This antiserum may be useful in a more general pyrethroid screen.

Optimization. For solvent optimization, we tested methanol as it is commonly used as the eluant in SPE and DMSO because it is a common cosolvent used in immunoassay to improve analyte solubility. As observed in our previous studies for esfenvalerate and permethrin immunoassays (*12*, *14*), MeOH and DMSO significantly influence the deltamethrin assay sensitivity and absorbance. The I_{50} value of the assay varied depending upon the different concentration of the cosolvent MeOH or DMSO. The lowest I_{50} was found at 40% MeOH (22.3 μg/L), which is ~25 times lower than that at 10% MeOH (501 μg/L) and 2 times lower than that at 10% DMSO (42.3 μg/L). The maximum absorbance was enhanced with higher methanol concentration up to 40% MeOH but was suppressed in a dose-dependent fashion with DMSO as a cosolvent. A high concentration of cosolvent facilitates the distribution of hydrophobic deltamethrin in the reaction solution. However, very high levels of organic cosolvents will affect and retard the interaction of antibody and antigen and may denature protein reagents. Therefore, a proper concentration of cosolvent is important for the performance of antibody and assay sensitivity. On the basis of the I_{50} values and the ratios of maximum and minimum absorbances for the deltamethrin standard curve, an MeOH concentration of 40% was selected for subsequent experiments.

To identify potential interferences from aqueous environmental samples, the effects of pH and ionic strength on ELISA performance were evaluated in this study. In system **13**/732, when analyte was dissolved in buffer at different pH values, no significant effect upon the I_{50} was detected, indicating that the assay could effectively detect deltamethrin at pH values ranging from 5.0 to 8.0. However, ionic strength strongly influenced ELISA performance. A higher salt concentration in the assay system resulted in lower optical densities (OD). The OD values at salt concentrations of 4× and 8× PBS decreased by 54 and 70%, respectively, from the OD value at a salt concentration 1× PBS. 2× PBS had the lowest I_{50} (21.9 μg/L), followed by 4× PBS (22.3 μg/L) and then 1× PBS (30.0 μg/L) and 8× PBS (34.7 μg/L). Salt concentrations can affect antibody binding, and for this assay, a salt concentration slightly higher than the commonly used PBS improved assay performance.

The optimized deltamethrin ELISA used 0.06 μg/mL of coating antigen **13**−BSA, antibody 732 at a dilution of 1/22000, deltamethrin in 40% methanol/PBS buffer, pH value of 7, and PBS concentration of 2×. The I_{50} value of this assay was 17.5 ± 3.6 μg/L with a limit of quantitation (LOQ) of 1.1 ± 0.5 μg/L in buffer. The LOQ was estimated as the concentration that corresponded to the absorbance of the control (zero concentration of analyte minus 3 times the standard deviation of the control) (*29*).

Cross-Reactivities. The optimized ELISA system (Ab732/**13**−BSA) had the highest CR with cypermethrin (37.3%) followed by permethrin (9.5%), cyfluthrin (8.5%), phenothrin (6.8%), and bioresmethrin (2.6%). Because the antibodies were made to a hapten attached to the protein through the phenoxybenzyl group, it was expected that the ability of the antibody to distinguish among pyrethroids would be less with substitutions in the alcohol portion of the pyrethroid molecule, but still quite good in the acid portion of the pyrethroid molecule. The antibody recognizes substitutions on the ethenyl as Br > Cl > CH_3 as demonstrated by decreasing CR for deltamethrin versus cypermethrin and permethrin versus phenothrin showing the predicted high selectivity. Because esfenvalerate, fenvalerate, and fluvalinate do not cross-react (at 5 μg/mL), it is clear that a cyclopropane ring is important to binding. However, this crosw-reactivity decreases with the loss of the α-cyano group (cypermethrin vs permethrin), showing that both the cyclopropane ring and the presence of the α-cyano group are important determinants to binding. In addition, the antibody is less selective in the alcohol portion of the molecule as bioresmethrin can also cross-react.

Although the CT of bioresmethrin (1*R-trans*) is low, it is interesting that the *trans*-bioresmethrin isomer shows stronger CR than the *cis*-/*trans*-resmethrin mixture. This is in contrast to the data from **Table 1** that indicate that the antibody binding is directed more toward the 1*R-cis* configuration. For example, deltamethrin, which has a 1*R-cis* configuration, can inhibit the assay more when the coating antigen is 1*R-trans* (haptens **12** vs **13** and **10** vs **11**, **Table 1**).

Among those pyrethroids that show high cross-reactivity, cypermethrin and cyfluthrin are actively used in the agricultural field (*30*). Therefore, cypermethrin will probably be an important interference when these assays are used for real world sample measurement. This problem can be addressed by (1) using the assay as a screening tool, knowing that other pyrethroids were used in the area to be examined; (2) separating the pyrethroids chromatographically prior to analysis (*31*, *32*); or (3) utilizing multiple immunoassays of varying selectivity for pyrethroids

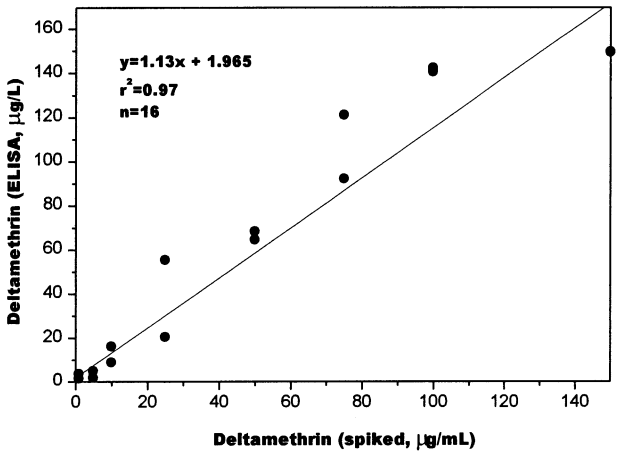

Figure 3. Relationship between deltamethrin spiked into water and determined by ELISA in a blind fashion. Water samples were from Putah Creek and were analyzed following SPE as indicated under Materials and Methods.

and analyzing the resulting data by a pattern recognition system to identify the compounds (*33*−*36*).

Analysis of Spiked Water Sample. To study the spike recovery, river water from Putah Creek (Davis, CA) was spiked with four different concentrations of deltamethrin (0, 0.2, 1, and 10 ppb) and analyzed in a blind fashion by ELISA. Putah Creek is an oxbow lake with high levels of particulates, nitrogen salts, and algae. The samples were extracted using SPE. No matrix effect was measured when a 200 mL water sample was extracted with SPE, eluted into 1 mL of methanol, and analyzed in the ELISA following a 4× dilution (concentration factor of 50). Recoveries were 89 ± 14.3, 115 ± 13.0, and 102 ± 27.5% for 0.2, 1.0, and 10 μg/L, respectively. A good correlation between nominal and ELISA measurements of deltamethrin (r^2 = 0.97) was obtained for spiked water samples from 0.2 to 150 μg/L (**Figure 3**). The results demonstrate that these assays are suitable for the detection of trace levels of deltamethrin in water.

This study is part of a comprehensive effort to develop immunoassays for classes of pyrethroids (types I and II), specific pyrethroid compounds (permethrin, bioallethrin, fenpropathrin, and esfenvalerate), and their indicator metabolites and environmental degradation products [phenoxybenzoic acid and (*S*)-fenvalerate acid]. These assays, along with those developed in other laboratories, can be used for rapid screening or for accurate, precise, and sensitive determination of specific compounds. Alternatively, the assays can be used in a tiered approach where bioassay and/or class selective pyrethroid assays are followed by compound selective assays (*35*). Finally, as a complement to traditional analytical methods, this relatively simple, sensitive, and highly selective immunochemical assay could play an important role in environmental contamination studies and monitoring.

ABBREVIATIONS USED

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

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