Supporting Information

Development of an Immunoassay for the Detection of the Phenylpyrazole Insecticide Fipronil

*Natalia Vasylieva[1], Ki Chang Ahn[1†], Bogdan Barnych[1], Shirley J. Gee[1], and Bruce D. Hammock[1]**

[1] Department of Entomology and UCD Comprehensive Cancer Center, University of California Davis, Davis, California 95616, United States

[†] Current address: PTRL West Inc. 625B Alfred Nobel Drive, Hercules, CA 94547

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Chemicals and instruments. All chemicals were of analytical grade and were purchased either from Fisher Scientific Co. (Chicago, Il) or from Sigma-Aldrich Co (St. Louis. MO) unless otherwise stated. Goat anti-rabbit IgG-horseradish peroxidase (HRP) conjugate was purchased from Abcam (Boston, MA). Fipronil sulfide was purchased from AccuStandard (New Haven, CT). Fipronil detrifluoromethylsulfinyl (FPN-deSOCF_3) was from ACCELA ChemBio Ink. (San Diego, CA). ELISA absorbances were spectrophotometrically read with a microplate reader (Molecular Devices, Sunnyvale, CA) at wavelength 450 nm. All synthesized compounds were analyzed by thin layer chromatography on precoated silica gel aluminium plates (Millipore, TLC silica gel 60 F254) using solvent system methanol/dichloromethane/acetic acid (1:9:0.1), v/v/v), with UV visualization. ^{1}H and ^{13}C NMR spectra were recorded with a Varian VNMR-600 spectrometer. Electrospray mass spectra of haptens in negative (MS-ESI^{-}) mode were recorded by the Waters 2795 LC coupled to the Micromass LCT ToF mass spectrometer. Melting points were determined on an OptiMelt melting point apparatus.

Buffers. All buffers and water solutions were prepared with ultrapure deionized water; phosphate-buffered saline (PBS, 10mM, pH 7.5); wash buffer PBST (PBS containing 0.05% Tween 20); coating buffer (14 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.8); blocking buffer (1% BSA in PBST); substrate buffer (0.1 M sodium citrate/acetate buffer, pH 5.5). Substrate solution contained 0.2 mL of 0.6% TMB (in dimethyl sulfoxide, DMSO w/v), 0.05 mL of 1% H_2O_2 in 12.5mL of substrate buffer. Stop solution was 2M H_2SO4.

Nomenclature. The nomenclature of haptens was obtained from Chembiodraw Ultra 13.0 (CambridgeSoft, Cambridge, MA).

Hapten Synthesis. Scheme 1 of the main article and Table S1 list a library of designed and synthesized fipronil haptens.

Hapten 1 [6-((3-Cyano-5-(2,6-dichloro-4-(trifluoromethyl)phenyl)-2-((trifluoromethyl)sulfinyl)cyclopenta-1,3-dien-1-yl)amino)-6-oxohexanoic acid] (BDH-297-7). The mixture of fipronil (100 mg, 0.23 mmol) and 6-(*tert*-butoxy)-6-oxohexanoic acid (70.7 mg, 0.35 mmol) was disolved in 3 mL of dry dichloromethane (DCM) containing a catalytic amount of 4-(dimethylamino)pyridine (DMAP) and dicyclohexyl carbodiimide (DCC, 71.1 mg, 0.35 mmol). It was stirred for 3d, then 0.5 mL of trifluoroacetic acid (TFA) was added and stirred an additional 10-20 min. Precipitated dicyclohexylurea (DCU) was removed by filtration and DCM was evaporated to dryness. The residue was dissolved in acetone, DCU was again removed and the acetone extract evaporated. The resulting product was purified by silica gel chromatography with a mixture of ethyl acetate and hexane (gradient 1:3 to 1:2, v/v) to give 42 mg (yield 42%) of a fine white crystalline powder. Melting point 139.6-143.7 °C (141.4°C). Compound gave a single spot on TLC, Rf 0.615. ^{1}H NMR (600 MHz, methanol-d4) δ 8.14 (s, 1H), 8.13 (s, 1H), 2.37 (td, J = 7.1, 1.9 Hz, 2H), 2.27 (t, J = 7.2 Hz, 2H), 1.62 (m, 2H), 1.56 (m, 2H). ^{13}C NMR (151 MHz, methanol-d4) δ 176.99, 173.10, 141.56, 137.23, 136.96, 136.29, 136.20, 136.06, 130.54, 128.29, 128.03, 128.00, 127.98, 127.96, 127.89, 127.87, 127.85, 127.82, 127.69, 126.29, 126.05, 124.48, 123.80, 122.66, 120.85, 111.65, 35.87, 34.36, 25.53, 25.26. MS-TOF m/z calcd for $[M-H]^-$=562.99; observed, 563.00.

Hapten 2 [6-((3-Cyano-5-(2,6-dichloro-4-(trifluoromethyl)phenyl)cyclopenta-1,3-dien-1-yl)amino)-6-oxohexanoic acid] (BDH-297-32). The mixture of 4-amino-3-(2,6-dichloro-4-(trifluoromethyl)phenyl)cyclopenta-1,4-diene-1-carbonitrile (FPN-deSOCF$_3$) (50mg, 0.16 mmol) and 6-(*tert*-butoxy)-6-oxohexanoic acid (38.4 mg, 0.19 mmol) was dissolved in 3 mL of dry DCM containing a catalytic amount of DMAP and DCC (49.44 mg, 0.24 mmol). It was stirred for 3d, then 0.5 mL of TFA was added and stirred an additional 10-20 min. Precipitated DCU was removed by

filtration and DCM was evaporated to dryness. The residue was dissolved in acetone, DCU was again removed and the acetone extract evaporated. The resulting product was purified by silica gel chromatography with a mixture of ethyl acetate and hexane (gradient 1:5 to 1:1, v/v) to give 46.7 mg (yield 93.4%) of a fine white crystalline powder. Melting point 173.2-175.7 °C (174.0°C). The compound gave single spot on TLC, Rf 0.64. ^{1}H NMR (600 MHz, methanol-d4) δ 8.06 (s, 2H), 7.13 (s, 1H), 2.34 (t, J = 7.2 Hz, 2H), 2.27 (t, J = 7.3 Hz, 2H), 1.63 (m, 2H), 1.56 (m, 2H). ^{13}C NMR (151 MHz, methanol-d4) δ 177.03, 173.13, 141.53, 137.45, 137.18, 135.84, 135.61, 135.39, 135.16, 128.42, 127.60, 126.39, 124.58, 122.76, 120.95, 114.22, 103.32, 36.23, 34.43, 25.84, 25.36. MS-TOF m/z calcd for $[M-H]^-$=447.03; observed, 447.17.

Hapten 3 [4-Amino-3-(2,6-dichloro-4-(trifluoromethyl)phenyl)-5-((trifluoromethyl)sulfinyl)cyclopenta-1,4-diene-1-carboxylic acid] (BDH-297-43). Fipronil (500 mg) was dissolved in 3.5 mL of acetone and adjusted dropwise with NaOH to pH 12. After incubation at 50 °C for 24 h, 30 mL of MeOH was added maintaining pH 12. It was stirred at 90 °C until a transparent solution was obtained, then acidified with 6 M HCl. The precipitate was removed by filtration. The filtrate was extracted three times with ethyl acetate. Extracted fractions were combined and concentrated by solvent evaporation. The resulting oily residue was dissolved in a small amount of methanol and re-crystallized from ethyl acetate/hexane giving a fine crystalline powder with light yellow color. Melting point 116.0-139.1 °C (119.4°C). The compound gave three spots on TLC with Rf_1 0.33 for the main product. Two other spots were of low intensity compared to the first one, with Rf_2 0.625 and Rf_3 0.833. The third spot had the same Rf as fipronil tested on the same TLC plate suggesting that it corresponds to residues of the starting material. ^{1}H NMR (600 MHz, methanol-d4) δ 8.06 (s, 2H). ^{13}C NMR (151 MHz, methanol-d4) δ 163.08, 153.86, 145.10, 138.14, 137.88, 136.32, 136.03, 135.80, 135.57, 135.34, 130.98, 128.74, 127.73, 127.70, 127.68,

127.66, 127.65, 127.64, 127.61, 127.59, 126.50, 126.44, 124.63, 124.25, 122.83, 121.02. MS-TOF m/z calcd for $[M-H]^-$=453.93; observed, 454.16.

Hapten 4 [4-Amino-3-(2,6-dichloro-4-(trifluoromethyl)phenyl)cyclopenta-1,4-diene-1-carboxylic acid] (297-53). FPN-de$SOCF_3$ (50 mg) was dissolved in 1 mL of 50% H_2SO_4 in water and heated at 100°C over night. The reaction mixture was then diluted with 100 mL of deionized water and extracted twice with ethyl acetate. Extracts were combined and concentrated. CCl_4 was added to remove water traces. The residue was re-crystallized from CCl_4/hexane giving 40 mg (yield 80%) of a fine white crystalline powder. Melting point 221.2-222.8 °C (221.8°C). The compound gave two spots on TLC with Rf_1 0.33 for the main product and Rf_2 0.63 for the second spot of faint intensity. ^{1}H NMR (600 MHz, methanol-d4) δ 7.97 (s, 2H). ^{13}C NMR (151 MHz, methanol-d4) δ 165.31, 151.37, 147.00, 138.12, 137.88, 135.22, 134.99, 134.76, 134.54, 127.34, 127.32, 127.30, 127.27, 126.54, 124.74, 122.93, 121.12. MS-TOF m/z calcd for $[M-H]^-$=337.98; observed, 338.28.

Preparation of immunogens and coating antigens.

Sulfo-N-hydroxysuccinimide Method. Haptens 1-4 were coupled covalently with available amines of the carrier protein. Each hapten (0.06 mmol) was dissolved in 3 mL of dry dimethylformamide (DMF) with sulfo-NHS (0.072 mmol) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC, 0.07 mmol). After the mixture was stirred overnight at room temperature (RT), the active ester was added slowly to a solution of protein (25 mg in 6 mL borate buffer 0.05 M at pH8) with vigorous stirring for 30 min at RT, followed by slow stirring for 3h at 4°C to complete the conjugation. The conjugates were dialyzed (6000-8000 MW) against PBS for 36 hours at 4°C with a buffer change every 12 h and stored at -20°C until use. The EDC conjugation may lead to formation of an antigenic urea on the protein, resulting in antibody development toward it. This may have undesirable consequences for assay development if the coating antigen was obtained using a similar

conjugation reaction. An alternative conjugation approach should be used in the synthesis of the coating antigen.

Diazotization Method. Haptens 5 and 6 were covalently conjugated to tyrosine moieties of the protein. Each hapten (0.10 mmol) was dissolved in 4 drops of ethanol and treated with 1 mL of 1M HCl. The resulting solution was stirred in an ice bath while 0.5 mL of 0.2 M sodium nitrite was added dropwise. DMF (0.4 mL) was added, and the solution was stirred for 10 min and then divided into two aliquots. One aliquot was added to a bovine serum albumin (BSA) solution, and the other to a conalbumin (CON) solution (25mg of protein in 6 mL of ice-cold borate buffer 0.2 M, pH 9 and 1 mL DMF). The reaction mixtures were cooled in an ice bath and stirred for 30 min. The pH of the yellow solution was adjusted to 7 with 1M NaOH, dialyzed and stored as described above.

Immunization and antiserum preparation. The immunization procedure followed the protocol reported previously[1]. In brief, three female New Zealand white rabbits were immunized for each immunogen (Table S1). The final bleed was collected after about 3 months of booster injections every 2 weeks following the first immunization. Blood was collected in test tubes and allowed to clot. Serum was obtained by centrifugation, stored at -20°C, and used without purification.

Reagent optimization. Two homologous hapten-protein (BSA, CON) conjugates and 5 heterologous hapten-protein conjugates each having two, BSA and CON, carrier proteins. 10 heterologous haptens in total (Table S1) were screened as potential coating antigens for each serum by three point competitive ELISA at 0, 50 and 5000 µg/L fipronil concentration. Selected coating antigens were used to obtain 8-point full competition curves to determine and compare assay sensitivities (Table S2). In further assay development, the dilution of serum from rabbit #2265 was

optimized in checkerboard titration with coating antigen 5-CON, and serum #2268 with coating antigen 1-CON.

Assay buffer optimization. The tolerance of the ELISA to changing parameters of the assay buffer was tested. **(a) pH.** The effect of pH on assay sensitivity was determined by analyzing competition curves of fipronil diluted in PBS buffer adjusted to pH 6.5, 7.5, 8.5 and 9.5 and containing 10% methanol. **(b) Ionic strength.** The effect of ionic strength on assay sensitivity was evaluated by analyzing standard curves of fipronil diluted in 10 mM, 20 mM, 30 mM, 40 mM PBS (pH 7.5) and each containing 10% methanol. **(c) Methanol.** The effect of organic solvent on assay sensitivity was studied by analyzing standard solutions of fipronil prepared in PBS containing 10, 20 or 40% of methanol (prior to addition of antibody in the well).

Cross-reactivity (CR). The selectivity of antiserum obtained from rabbits #2265 and #2268 was evaluated by analyzing standard solutions of fipronil and structurally similar compounds. The CR was calculated as (IC_{50} of fipronil/ IC_{50} of tested compound) x100.

Human serum matrix effect. The effect of matrix on the 2265 assay performance was evaluated using human serum spiked with fipronil-sulfone, a major metabolite of fipronil identified in serum, together with parent compound. Competition curves were obtained by serial dilution of analyte in PBS (10% methanol), serum diluted in PBS (10% methanol) or in neat serum, to give assay buffer containing 0, 10%, 50% or 100% human serum respectively (prior to addition of antibody in the well).

Sample preparation for recovery study. A) Recovery from fortified samples measured by immunoassay. Commercial human serum (Aldrich), urine from healthy volunteers and industrial tap water were spiked with fipronil, fipronil-sulfone (human serum) and fipronil desulfinyl (tap

water) standard solutions at concentrations of 10, 20 and 50 µg/L. Samples were vortexed, diluted 10 times with PBS containing 10% methanol and analyzed with the 2265 assay. The calibration curve was prepared by serial dilution of standard solutions of analytes in blank matrix diluted 10 times with assay buffer. The calibration curve was measured in duplicate; each sample was measured on 3-5 days in triplicate each. The calibration data were fitted to a four-parameter logistic equation. **B) Correlation of recovery values obtained by immunoassay and LC-MS/MS.** Industrial tap water was collected from the University of California at Davis; urban water was collected from Putah Creek an oxbow lake in Davis; urine was collected from healthy volunteers. Since the LC-MS/MS technique does not allow direct injection of unextracted samples into the instrument, even diluted, liquid-liquid extraction (LLE) of analyte from matrix was used. We chose LLE for its simplicity. This approach has been applied for the past 20 years at the Swedish National Food Administration (NFA) for analysis of hundreds of pesticides and their metabolites in a single extraction.[2]

Samples were spiked at a range of 2-50 ng/mL. An aliquot 2 mL of each sample was extracted with ethyl acetate 3 times. The organic layers were combined and 100 µL of 25% glycerol was added to trap fipronil and other possible extracted components. Extracts were evaporated to dryness under mild nitrogen stream and dissolved in 2 mL methanol. Water extracts were diluted 5 times with PBS and analyzed with the 2268 assay. Urine extracts were diluted 10 times with PBS and analyzed with the 2265 assay. The calibration curve was prepared by serial dilution of standard solutions of analyte in blank matrix diluted 5 or 10 times with PBS for 2268 and 2265 assays, respectively. Methanol extracts were analyzed by LC-MS/MS.

Enzymatic hydrolysis of human urine. Urine aliquots (5 mL) were hydrolyzed using a solution containing 60 µL (85000/7500 U/mL) of β-glucuronidase/sulfatase (Sigma Chemical Co., St. Louis, MO) and 0.2 mL of 1 M ammonium acetate buffer at pH 5.5. The reaction was left overnight at 37

°C under gentle mixing. Hydrolyzed samples were diluted 1:1 with 20 mM PBS containing 20% MeOH and analyzed with assay 2265 without further treatment. The calibration curve was prepared using blank urine treated using the same protocol.

Rat serum preparation for immunoassay analysis. Serum samples, with expected high concentration of fipronil and its metabolites, from rats having fipronil-containing diet, were diluted 1000 times, and 25 μL was loaded in the well containing 25 μL of assay buffer, in triplicate. Serum from control animals was diluted 100 times and 50 μL of diluted sample was load on the plate. Additional 50 μL of antibody solution was added in the each well, resulting in the final dilution of 4000 and 200 times for treated and control samples, respectively. The control samples were diluted only 100 times to ensure that there was no fipronil in the controls and also verify that there was no matrix effects at this dilution, then certainly the 1000 dilution would not give it. The calibration curve was prepared in assay buffer without any blank matrix.

LC-MS/MS Analysis. Samples fortified with fipronil were extracted according to the procedure described earlier. The extracts were used in parallel recovery studies using the LC-MS/MS method and immunoassay. Thereby no surrogate standard was used during extraction. The obtained recovery values were compared between ELISA and LC-MS/MS and relatively to theoretical spiked concentrations.

Chromatographic separation was performed using a Waters Acquity Ultra Performance LC system equipped with a 2.1x50 mm Acquity UPLC BEH C18 1.7 μm column held at 40°C. A solvent system consisted of water with 0.1% formic acid (solvent A) and methanol with 0.1% formic acid (solvent B) and the following gradient was applied (for solvent B): the initial solvent B fraction at 5% (t=0) was held for 0.15 min, followed by linear gradient to 35% over 0.15-0.33 min; then by linear gradient to 90% over 0.33-2.0 min; linear gradient to 100% over 2.0-2.5 min and held at 100% for 0.5 min to t=3.0 min, before returning to 95:5 water:methanol at 3.01. The column was

allowed to equilibrate for 2 min for a total run time of 5.0 min. The flow rate was maintained at 0.35mL/min. The injection volume was 10 μL. The samples were kept at 15 °C in the autosampler.

Fipronil was detected on a Quattro Premier tandem quadrupole mass spectrometer (Waters). Nitrogen gas flow rates were fixed with a cone gas flow of 50 L/H and a desolvation gas flow of 1000 L/h. Electrospray ionization was performed in positive mode with a capillary voltage fixed at 0.75 kV using a source temperature of 120 °C and a desolvation temperature of 300 °C. A transition of 436.9>368 m/z for fipronil was monitored using a cone voltage of 35 V and a collision voltage of 20 V. The current LC-MS/MS detection method was adapted from methods described in the literature.[3,4]

Table S1. Structure of Haptens and Hapten-Protein Conjugates

Analyte	Hapten	Hapten-Protein conjugate	Rabbit	Hapten	Hapten-Protein conjugate	Rabbit
Fipronil		1-Thy 1-CON 1-BSA	#2262 #2263 #2264	*	4-Thy 4-CON 4-BSA	#2271 #2272 #2273
		2-Thy 2-CON 2-BSA	#2265 #2266 #2267		5-BSA 5-CON	
	*	3-Thy 3-CON 3-BSA	#2268 #2269 #2270		6-BSA 6-CON	

*position of attachment to protein

Table S2. Competitive ELISA Results for Homologous and Heterologous Coating Antigens

rabbit	Immunogen	coating antigen[a]	dilution		curve parameters[d]			
			coat. antigen, μg/mL	antiserum	A	B	C	D
#2263	1-Thy	1-CON[b]	1	1/6000	0.62	0.60	22.8	0.00
#2265	2-Thy	2-BSA[b]	0.01	1/256000	1.02	0.60	54.2	0.11
		5-CON[c]	1	1/6000	1.07	0.62	2.1	0.01
#2266	2-Thy	5-CON[c]	1	1/6000	0.71	0.42	1.6	0.04
#2268	3-Thy	1-BSA[c]	1	1/6000	1.11	0.91	11	0.01
		1-CON[c]	1	1/6000	0.88	1.13	9.5	0.01
		2-BSA[c]	1	1/12000	0.75	1.10	16.2	0.01
#2272	4-Thy	5-CON[c]	1	1/6000	0.74	1.06	77.7	0.01

[a]See Table S1 for structures. [b]Homologous (hapten same as immunogen). [c]Heterologous (hapten different from immunogen). [d]A, maximum absorbance; B, slope; C, IC_{50}; D, minimum absorbance.

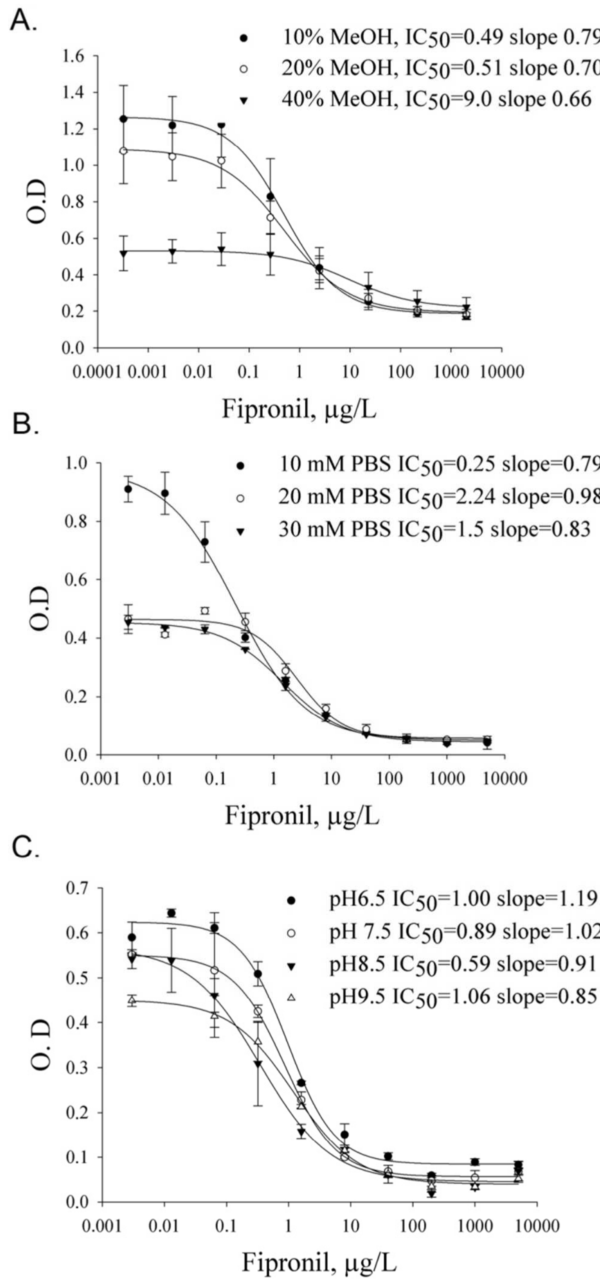

Figure S1. Fipronil competition curves with serum from rabbit 2265 in assay buffers of (A) increasing concentration of methanol (in triplicates per day, 3 days); (B) varying ionic strength and (C) pH (in triplicates on 1 day). Reagent concentrations: coating antigen (5-CON) 1µg/mL; anti-fipronil serum (1/6000); goat anti-rabbit IgG-HRP (1/10000).

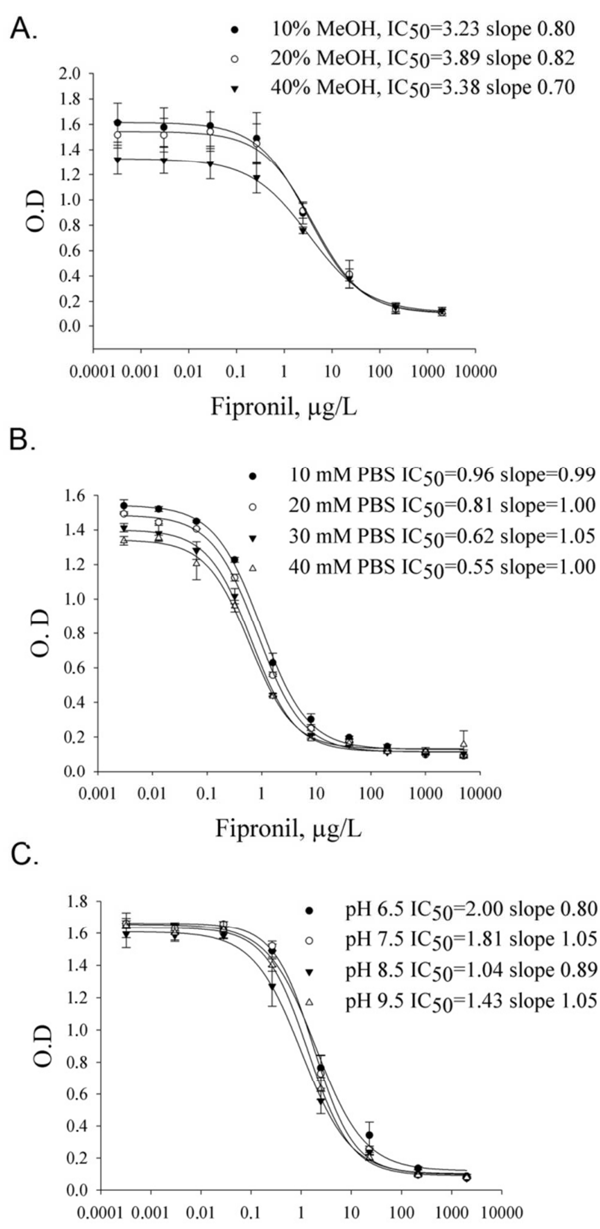

Figure S2. Fipronil competition curves with serum from rabbit 2268 in assay buffers of (A) increasing concentration of methanol (in triplicates per day, 3 days); (B) varying ionic strength and (C) pH (in triplicates on 1 day). Reagent concentrations: coating antigen (1-CON) 1µg/mL; anti-fipronil serum (1/8000); goat anti-rabbit IgG-HRP (1/10000).

Table S3. Cross Reactivity of Fipronil Antiserum to GABA antagonists and Cage Convulsants

Compound	Structure	#2265, 2268 % CR
1. Fipronil (insecticide)		100
2. TETS[a] *(rodenticide)*		ND
3. PTZ[b] *(drug)*		ND
4. Toxaphene *(insecticide)*		ND
5. Heptachlor *(insecticide)*		ND
6. Picrotoxin *(natural poison)*		ND

a Tetramethylenedisulfotetramine, *b* pentylenetetrazol.

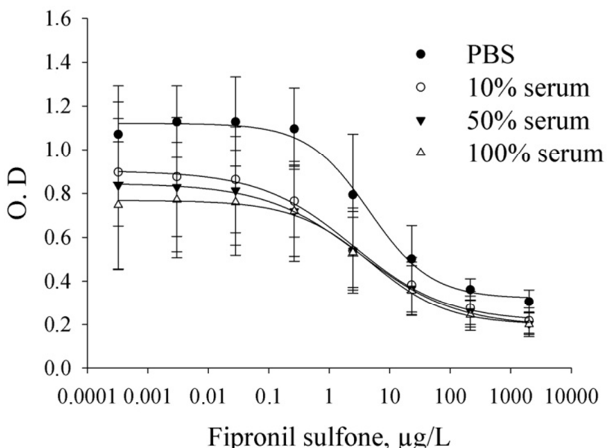

Figure S3. Matrix effect evaluation for human serum spiked with fipronil-sulfone, the major fipronil metabolite in serum (n=3 days, in triplicate each). Serum 2265 was diluted in PBS containing 10% methanol; assay buffer consisted of human serum diluted in PBS, except the experiment where the assay buffer was 100% human serum (serum matrix % is before addition of antibody in the well).

References

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