Supporting Information

Quantitative Detection of Fipronil and Fipronil-Sulfone in Sera of Black-Tailed Prairie Dogs and Rats after Oral Exposure to Fipronil by Camel Single-Domain Antibody-Based Immunoassays

Kai Wang, Natalia Vasylieva, Debin Wan, David A. Eads, Jun Yang, Tyler Tretten, Bogdan Barnych, Ji Li, Qing X. Li, Shirley J. Gee, Bruce D. Hammock, Ting Xu

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Reagents

Incomplete Freund's adjuvant, thyroglobulin (Thy), bovine serum albumin (BSA), 3,3′,5,5′-tetramethylbenzidine (TMB), polyethylene glycol 8000 (PEG 8000), isopropyl-β-D-thiogalactopyranoside (IPTG) and imidazole were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Mouse anti-M13 phage mAb-horseradish peroxidase (HRP) was from GE Healthcare (Piscataway, NJ). The phagemid vector pComb3X was a generous gift from Dr. Barbas (The Scripps Research Institute, La Jolla, CA). Electrocompetent cells of *E. coli* ER2738 were acquired from Lucigen Corporation (Middleton, WI). All restriction enzymes, T4 DNA ligase and M13KO7 helper phage were bought from New England Biolabs, Inc. (Ipswich, MA). The goat anti-HA tag IgG and HRP conjugate was purchased from Abcam (Cambridge, MA). HisPur Ni-NTA resin and Nunc MaxiSorp flat-bottom 96 well microtiter plates were purchased from Thermo Fisher Scientific Inc. (Rockford, IL). All the pesticide standards were purchased from the Institute for the Control of Agrochemicals, Ministry of Agriculture and Rural Affairs, China.

Selection of VHHs against fipronil and fipronil-sulfone

One well of a microtiter plate was coated overnight with 100 μL of H2-BSA or H3-BSA (10 μg mL^{-1}) at 4 ^{o}C, and additional four wells with 100 μL of 3% BSA in coating buffer. The plate was blocked with 1% BSA in PBS (0.01 mol L^{-1} phosphate, 0.137 mol L^{-1} NaCl, 3 mmol L^{-1} KCl, pH 7.5) for 1 h at ambient temperature. A 100 μL aliquot of phage-display VHH library was added into the first well with 5% methanol (MeOH) and incubated for 2 h with gentle shaking at ambient temperature. After washing 10 times with PBST, this well was eluted with 100 μL of fipronil (500 ng mL^{-1}) in PBS containing 5% MeOH for 1 h at ambient temperature with shaking. The eluent

was transferred in equal aliquots to the next four BSA-coated wells to remove phage-VHH that bind non-specifically. Then the eluent was collected for the determination of phage titer and phage amplification. The phage eluent was amplified with addition of the M13KO7 helper phage (1×10^{12} cfu mL^{-1}) for the next round of panning. The entire panning process was repeated 3 times, except the concentrations of coating antigen and fipronil to elute the VHH phage were decreased gradually. The concentrations of H2-BSA or H3-BSA for the 2nd, 3rd and 4th panning were 4, 2 and 1 μg mL^{-1}, respectively. Meanwhile, the concentrations of fipronil were decreased to 100, 10 and 2 ng mL^{-1}, respectively.

After the 4th round of panning, 80 clones were randomly selected from the plate derived from H2-BSA or H3-BSA panning and tested for their binding affinity to fipronil (100 ng mL^{-1}) by a competitive phage ELISA. Finally, eight clones (F1–F8) showing high binding capacity with fipronil were identified as positive clones. Each positive clone was further tested for the binding capacity with fipronil and fipronil-sulfone by a competitive phage ELISA. The clones F1 and F6 showing the highest sensitivity to fipronil and fipronil-sulfone, respectively, were selected for the rest of this study. VHHs F1 and F6 were expressed and purified according to the methods reported previously.[1]

Oral administration of fipronil for black-tailed prairie dogs

All animals were handled in a humane and appropriate manner during the following trial. In April 2017, 6 adult black-tailed prairie dogs were live-trapped from a colony in Boulder, Colorado and trialed for fipronil safety at the U. S. Fish and Wildlife Service's National Black-Footed Ferret Conservation Center (NBFFCC), Carr, Colorado. A trial of fipronil safety on prairie dogs was completed from 10 April through 14 April 2017. The 6 adult prairie dogs were randomly categorized into 3 sets of 2 and were individually assigned unique alphanumeric codes for identification (A through F). The prairie dogs were placed in communal bins furnished with a nestbox and plastic tubing as places of refuge. Each bin had a 1.3 cm layer of pine shavings as bedding material and was treated with DeltaDust® to ensure flea extermination and to inhibit flies. Prairie dogs in each bin had access to 2 water bottles that were filled with clean water and refilled ad libitum. Grain bait (either fipronil-treated or untreated control) was presented to each prairie dog in a food dish near the nestbox. Each of the three pairs received a different quantity or variety of grain bait: prairie dogs in pair A/B each received ½ cup of grain containing 0.005% (50 mg kg^{-1}) systemic fipronil (Scimetrics Ltd. Corp., Wellington, CO) which was actually determined to be 54.9 ± 1.5 mg kg^{-1} by Poché et al.,[2] pair C/D each received ¼ cup of fipronil grain, and pair E/F each received ½ cup of untreated control grain. The grain was weighed before it was provided to prairie dogs on 10 April and reweighed every day during the trial.

During the trial, the prairie dogs consumed 5–63 g of grain and appeared in good health. On average, the prairie dogs consumed 10.5 ± 8.91 g of grain per day. Regarding the 4 prairie dogs provided with fipronil grain, the animals were indexed to have consumed 35–63 g ($\bar{x}$ = 48 ±13.3 g) of grain bait containing 1.92–3.46 mg ($\bar{x}$ = 2.64 ± 0.73 mg) of fipronil.

Collection of black-tailed prairie dog serum

On the final day of the feeding trial blood was collected from the 6 prairie dogs. In order to acquire adequate blood samples, and because this was a terminal study, intracardiac phlebotomies were performed on the prairie dogs while they were anesthetized. Prairie dogs were individually anesthetized with 4.0% isoflurane and laid on their dorsal side. Blood was collected with the use of 22G hypodermic needles attached to 10 mL syringes. After acquisition, blood samples were immediately transferred to Covidien Corvac ™ integrated serum separator tubes and spun in a centrifuge for five minutes to separate serum from the remainder of the sample. Samples were then frozen at −20°C while still in serum separator tubes. The prairie dogs were humanly euthanized with carbon dioxide while still under anesthesia and stored frozen on site for future use in carnivore safety trials.

Analysis of fipronil and its metabolites by LC-MS-MRM

The analysis of fipronil and its metabolites was performed on a Waters Acquity UPLC system, coupled to Xevo TQ-S Triple Quadrupole LC-MS. LC-MS-MRM conditions are shown in Table S2–S5. The 200 nM of 12-(3-cyclohexyl-ureido)-dodecanoic acid (CUDA) in methanol was used to account for ion suppression in mass spectrometry. All data were acquired and processed using Masslynx 4.1 software with TargetLynx.

Table S1. Immunization protocol of camel

Day	H2-Thy[a] (mL)	H3-Thy[a] (mL)	FIA[b] (mL)	Multi-point injection site
1st	0.5	0.5	1.0	Nape of the neck
14th	0.5	0.5	1.0	Nape of the neck
28th	0.5	0.5	1.0	Nape of the neck
42nd	0.5	0.5	1.0	Nape of the neck
56th	0.5	0.5	1.0	Nape of the neck

[a]Concentration of H2-Thy or H3-Thy: 2.0 mg mL^{-1}

[b]FIA: Freund's incomplete adjuvant

Table S2. LC condition

Column	Kinetex C18, 1.7μm, 2.1×100mm
Column Tem.	45℃
Injection Volume	5μL
Mobile Phase	Mobile phase A: Deionized water containing 0.1% glacial acetic acid
	Mobile phase B: Acetonitrile containing 0.1% glacial acetic acid
Flow Rate	0.4mL min^{-1}
Timed Events Enabled	0min, flow state sets up to waste
	1.5min, flow state sets up to LC
	10min, flow state sets up to waste

Table S3. LC gradient

Time (min)	B (%)
0	25
0.5	25
10	70
11	100
11.1	25
12.0	25

Table S4. Mass spectrometric source parameters

Acquisition Parameters	ESI, negative mode, MRM
Capillary (kV)	3
Source Offset (V)	50
Source Temperature (°C)	150
Desolvation Temperature (°C)	500
Cone Gas Flow (L/Hr)	150
Desolvation Gas Flow (L/Hr)	1000
Collision Gas Flow (mL min^{-1})	0.15
Nebuliser Gas Flow (Bar)	7.0

Table S5. MRM parameters

Compounds	Q1	Q3	Dwell (secs)	CV(V)	CE(V)
Fipronil-detrifluoromethyl-sufinyl	319.08	262.97	0.015	24	20
Fipronil-detrifluoromethyl-sufinyl	319.08	283.0	0.015	24	12
Fipronil-hydroxy	335.08	252.96	0.015	44	18
Fipronil-detrifluoromethyl-sufinyl-acid	338.08	212.9	0.015	30	18
Fipronil-desulfinyl	387.07	350.98	0.015	38	10
Fipronil-sulfide	418.97	216.91	0.015	38	26
Fipronil	434.97	249.95	0.015	38	26
Fipronil	434.97	329.91	0.015	38	14
Fipronil-sulfide-amide	436.98	287.98	0.015	2	30
Fipronil-sulfide-amide	436.98	400.89	0.015	2	12
Fipronil-sulfide-acid	438.04	288.83	0.015	2	18
Fipronil-sulfone	450.96	281.94	0.015	38	26
Fipronil-sulfone	450.96	414.93	0.015	38	14
Fipronil-acid	454.03	252.94	0.015	26	20
Fipronil-acid	454.03	256.97	0.015	26	30
CUDA[a]	339.30	214.20	0.015	22	38

[a] CUDA is used as IS to account for signal response in mass spectrometry.

Table S6. Serum titer and its binding capacity to fipronil by ELISAs based on coating antigens H2-BSA and H3-BSA.

Booster immunization	Titer of serum		Inhibition by fipronil (100 ng mL^{-1})	
	H2-BSA	H3-BSA	H2-BSA	H3-BSA
3th	4.0×10^4	2.0×10^4	57%	50%
4th	2.0×10^6	5.0×10^5	75%	58%
5th	4.0×10^6	1.0×10^6	82%	70%

Table S7. Recognition of VHHs F1 and F6 to different coating haptens. The concentrations of all VHHs and coating antigens were 0.1 and 1.0 μg mL^{-1} (100 μL/well), respectively.

VHH	Carrier protein	OD (450 nm)				
		H1	H2	H3	H4	H5
F1	BSA	1.72	1.93	0.32	0.25	1.55
	CON	1.66	2.21	0.24	0.22	1.68
F6	BSA	<0.1	<0.1	2.13	0.83	<0.1
	CON	<0.1	<0.1	1.97	0.79	<0.1

Table S8. Selection of pairs of VHH/coating antigen

VHH	Coating antigen	A_0[a]	IC_{50} of fipronil (ng mL^{-1})
F1	H1-BSA	0.91	8.7
	H1-CON	0.97	6.8
	H2-BSA	1.21	**4.2**
	H2-CON	1.15	6.6
	H5-BSA	1.17	15
	H5-CON	0.92	17
VHH	Coating antigen	A_0	IC_{50} of fipronil-sulfone (ng mL^{-1})
F6	H3-BSA	1.17	18
	H3-CON	1.18	**11**
	H4-BSA	0.75	25
	H4-CON	0.82	28

[a]A_0, maximum signal

Table S9. Recovery of fipronil and fipronil-sulfone co-spiked in control sera determined by LC-MS

Species	Spiked levels in sera (ng mL^{-1})		Concentration detected by LC-MS (ng mL^{-1}), n = 3 mean±SD		Recovery (CV)[a], %	
	Fipronil	Fipronil-sulfone	Fipronil	Fipronil-sulfone	Fipronil	Fipronil-sulfone
Prairie dog	100	100	94.4±2.1	101±3.1	94 (2.2)	101 (3.0)
	400	400	346±5.3	346±7.6	87 (1.5)	87 (2.2)
	2000	2000	1723±35	1880±28	86 (2.0)	94 (1.5)
Rat	100	100	81.4±1.7	97.5±4.2	81 (2.0)	98 (4.3)
	400	400	348±6.8	358±4.5	87 (2.0)	90 (1.3)
	2000	2000	1945±33	1725±26	97 (1.7)	86 (1.5)

[a]Coefficient of variation

Table S10. Fipronil and fipronil-sulfone levels in real sera of rodents determined by LC-MS and ELISAs. Black-tailed prairie dogs #E and #F and rats #1–#5 were control animals (CNTR) and the remains were orally administered with fipronil.

	LC-MS (μg mL^{-1})		ELISA[a] (μg mL^{-1}), n=3		ELISA (μg mL^{-1}), n=3	
Samples	Fipronil	Fipronil-sulfone	Fipronil equivalent	Fipronil-sulfone equivalent	Fipronil[b]	Fipronil-sulfone[c]
Black-tailed prairie dog						
A	0.19±0.02	0.90±0.09	0.42±0.03	0.96±0.10	0.22±0.01	0.90±0.10
B	0.54±0.07	1.61±0.08	0.93±0.06	1.80±0.08	0.57±0.05	1.63±0.07
C	0.35±0.01	1.43±0.10	0.76±0.03	1.60±0.13	0.44±0.00	1.47±0.13
D	0.19±0.03	0.58±0.09	0.33±0.06	0.66±0.11	0.20±0.04	0.60±0.10
E(CNTR)	ND[d]	ND	ND	ND	ND	ND
F(CNTR)	ND	ND	ND	ND	ND	ND
Average	**0.32**	**1.13**	**0.61**	**1.25**	**0.36**	**1.15**
Rat						
1(CNTR)	ND	ND	ND	ND	ND	ND
2(CNTR)	ND	ND	ND	ND	ND	ND
3(CNTR)	ND	ND	ND	ND	ND	ND
4(CNTR)	ND	ND	ND	ND	ND	ND
5(CNTR)	ND	ND	ND	ND	ND	ND
6	0.62±0.02	2.21±0.08	1.15±0.05	2.34±0.14	0.68±0.02	2.14±0.13
7	0.64±0.01	1.92±0.07	1.06±0.06	2.36±0.27	0.58±0.00	2.19±0.27

8	0.47±0.01	2.19±0.17	1.08±0.03	2.31±0.08	0.61±0.01	2.13±0.08
9	0.41±0.02	1.47±0.04	0.79±0.07	1.60±0.13	0.47±0.04	1.46±0.12
10	0.51±0.01	1.02±0.03	0.73±0.04	1.21±0.12	0.50±0.01	1.07±0.12
11	0.42±0.01	2.08±0.14	0.94±0.04	2.22±0.15	0.48±0.01	2.07±0.15
12	0.33±0.01	1.35±0.11	0.71±0.03	1.48±0.11	0.41±0.01	1.36±0.11
Average	**0.49**	**1.75**	**0.92**	**1.93**	**0.53**	**1.77**

[a]Fipronil and fipronil-sulfone equivalents were detected by the F1/H2-BSA ELISA and the F6/H3-CON ELISA, respectively.

[b]Fipronil concentation = Fipronil equivalent by ELISA × 1.068 − Fipronil-sulfone equivalent by ELISA × 0.235

[c]Fipronil-sulfone concentation = Fipronil-sulfone equivalent by ELISA × 1.068 − Fipronil equivalent by ELISA × 0.31

[d]ND, not detectable

Figure S1. The amino acid sequences of VHHs F1–F8. The dots indicate amino acid residues identical to VHH F1.

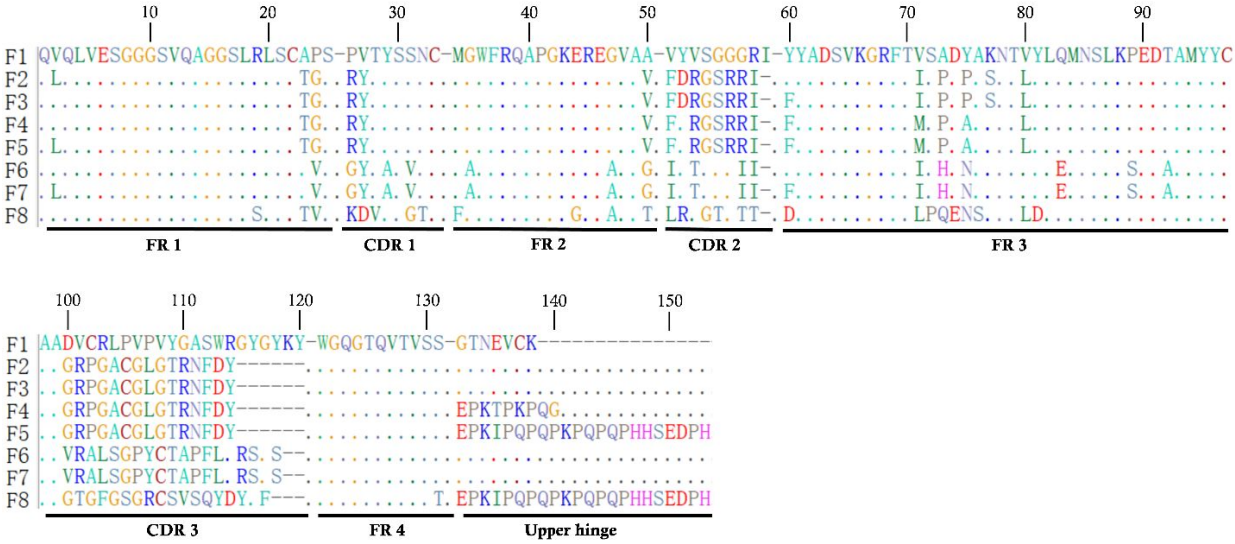


Figure S2. SDS-PAGE gel image of purified F1 (lane 1) and F6 (lane 2) under reduced conditions. Molecular weight markers (M) are shown and their sizes are indicated.

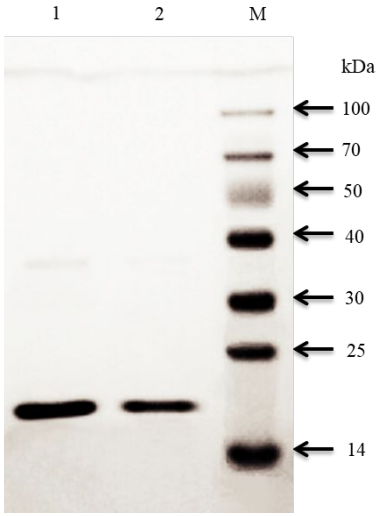


Figure S3. Effects of methanol (A), pH (B) and NaCl (C) on the F1/H2-BSA ELISAs for fipronil.

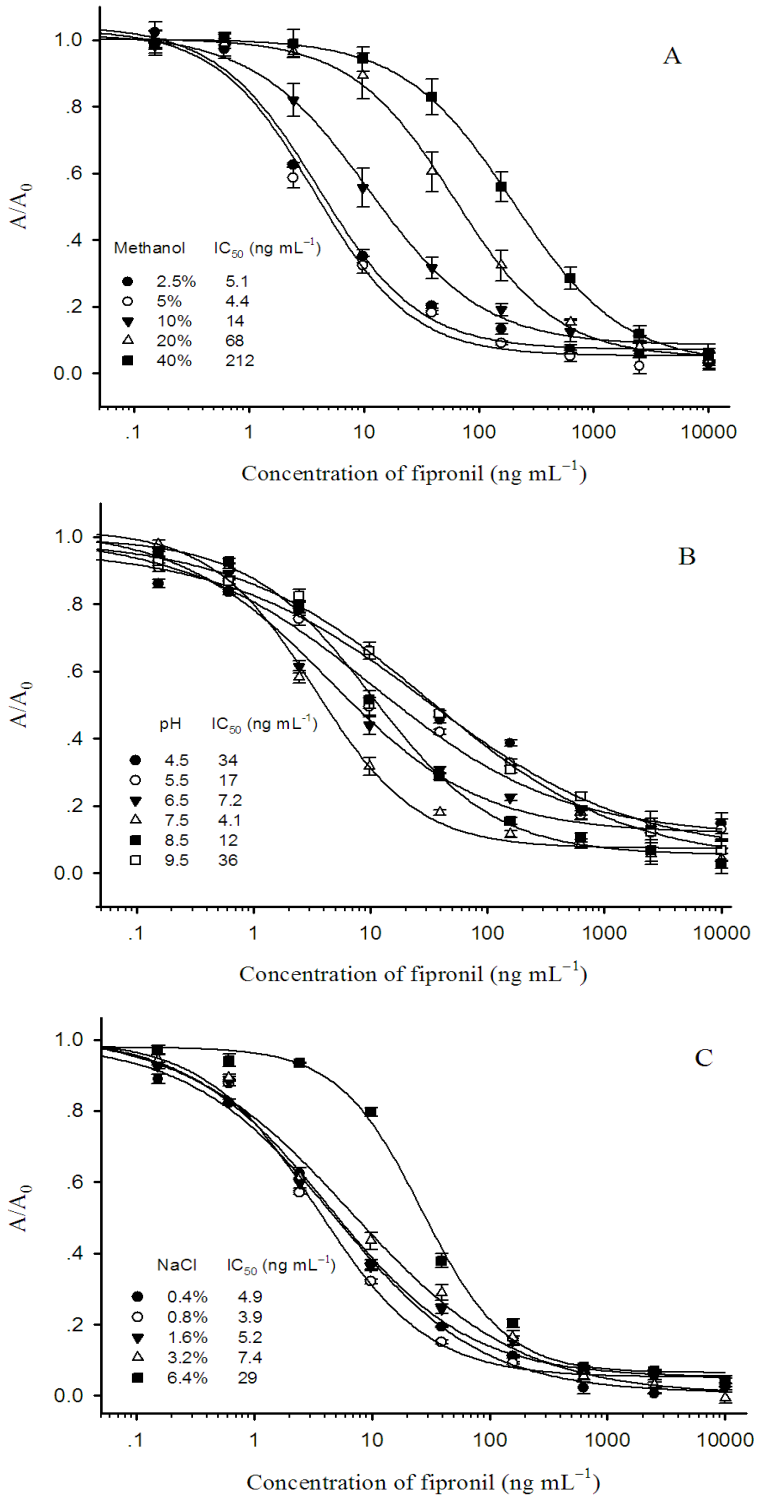
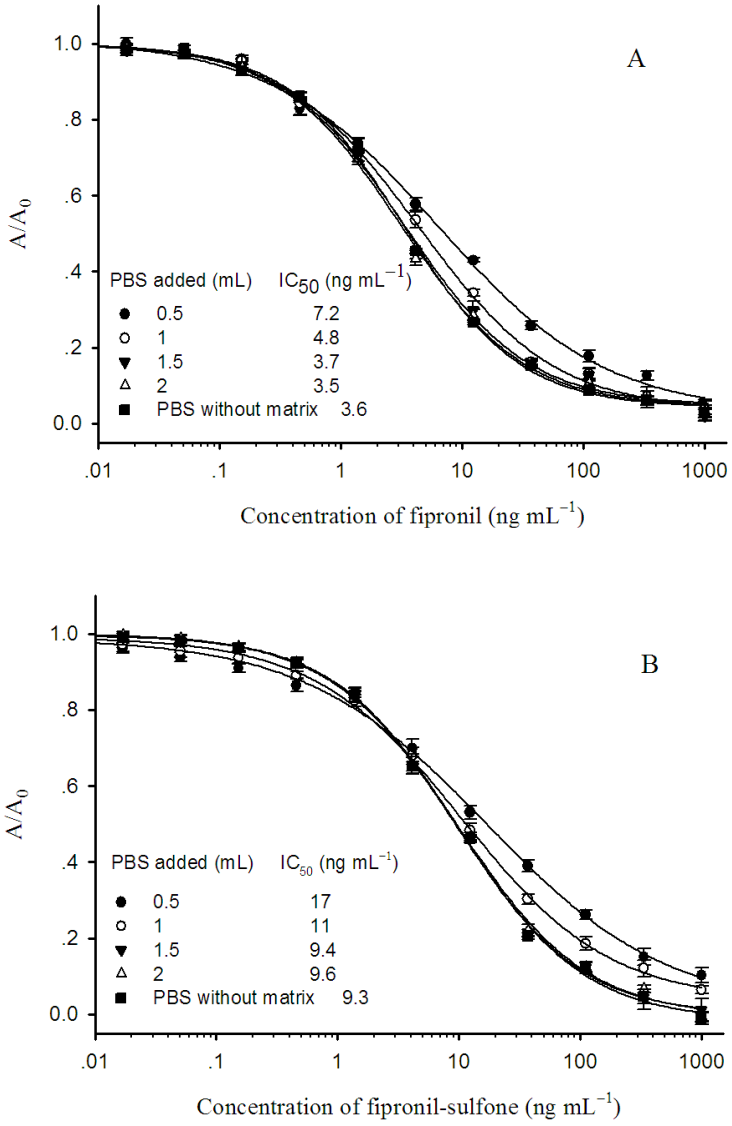


Figure S4. Matrix effects of prairie dog serum on ELISAs for fipronil (A) and fipronil-sulfone (B). The extracts from prairied dogs were constituted by adding different volume of PBS containing 5% methanol to dissolve the residues.



Reference

(1) Wang, J.; Bever, C. S.; Majkova, Z.; Dechant, J. E.; Yang, J.; Gee, S. J.; Xu, T.; Hammock, B. D. *Anal. Chem.* **2014**, *86*(16), 8296–8302.

(2) Poché, D. M.; Hartman, D.; Polyakova, L.; Poché, R. M. *J. Vector Ecol.* **2017**, *42*(1), 171–177.