

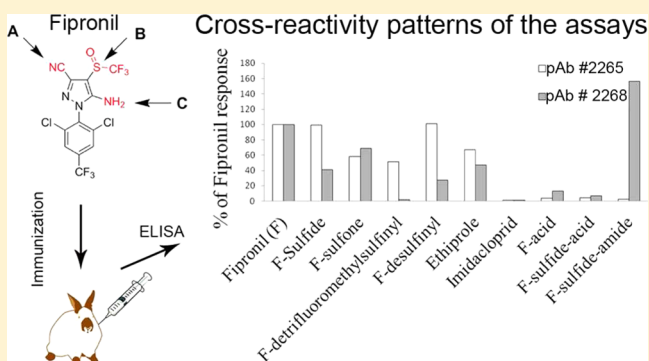
Development of an Immunoassay for the Detection of the Phenylpyrazole Insecticide Fipronil

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S Supporting Information

ABSTRACT: Phenylpyrazole insecticides such as fipronil have been used as replacements for organophosphates. The wide application of fipronil raises concern about environmental contamination and risk for fish, birds, and other nontargeted beings as well as human health. A sensitive, competitive indirect heterologous enzyme-linked immunosorbent assay (ELISA) was developed. Antibodies with different specificities to fipronil and its metabolites were produced. Two ELISAs having IC_{50} values of 0.58 ± 0.06 and 2.6 ± 0.4 ng/mL were developed. Design of different haptens and coating antigens resulted in two assays with distinct cross-reactivity patterns for structurally related compounds: 96, 38, and 101% versus 39, 1.4, and 25% for fipronil-sulfide, fipronil-detrifluoromethylsulfonyl, and fipronil-desulfinyl, respectively. Performance of the immunoassays was demonstrated by a recovery study from spiked water and human serum and urine matrices, giving recovery values in the range of 85–111% for different concentrations. The assays demonstrated good correlation in fipronil recovery with conventional LC-MS/MS analysis. The generic assay 2265 has the sensitivity to measure fipronil and its analogs in serum at levels relevant for exposure monitoring. The assays were used to analyze human urine samples obtained from exposure studies and serum samples from rats treated with a fipronil-containing diet.



INTRODUCTION

Fipronil is a highly effective broad-spectrum insecticide widely used for agricultural and nonagricultural purposes: soil injection,^{1–3} use on fruits, vegetables, coffee, rice, and other crops as well as for treatment of seeds.^{2,4} It is registered for use by lawn care and pest control operators to treat golf courses and food-handling establishments. It has also found application in topical pet care products. Acting as a neurotoxic GABAergic insecticide, fipronil disrupts normal nerve function by blocking the inhibitory gamma-aminobutyric acid type A (GABAA) receptor system of insects. Such inhibition results in excessive neuronal stimulation and death of the target insect.⁵ It has been shown that fipronil has higher affinity toward the insect GABAA receptor than to the human or other mammalian receptors.^{6–9} Despite the lower fipronil affinity to the native mammalian hetero-oligomeric receptor, a recent report showed fipronil had a high affinity to the human receptor subunit $\beta 3$ similar to that of the insect GABAA receptors.^{6,10} Human $\beta 3$ GABAA receptor is linked to neurodevelopmental disorders such as autism,^{11,12} Angelman syndrome,¹³ and epilepsy.¹³ Fipronil can induce some cytochrome P450s, and the in vitro cytotoxic effects of fipronil and its metabolites at high concentrations suggest the possibility of toxicity by non-neural mechanisms.^{7,14–16} Fipronil metabolites have also been shown to maintain bioactivity and toxicity in mammals, having 10-fold higher potency (for fipronil-desulfinyl, an environmental

metabolite) at the mammalian GABA-gated chloride channel, narrowing the selectivity between insects and mammals.⁷ Therefore, there is theoretical evidence of possible fipronil toxicity in humans independent of its neural target.

Human exposure may occur through interactions with pets both in the pet industry and at home. The principal risk to human health is likely to the brain and nervous system of young children and fetuses because exposures to the toxicants can alter organizational events in the developing brain.^{17–20} General symptoms of fipronil exposure are similar to those in rats, and in humans these include increased excitability, headache, dizziness, seizures, reduced food consumption, nausea, and vomiting.^{21,22} The U.S. Environmental Protection Agency (EPA) classified fipronil as a moderately toxic possible human carcinogen, with negligible risk for residential application.¹ According to the national survey, about 40% of American homes tested positive for the presence of fipronil (0.16 ng/cm² of the floor).²³ A number of case studies were registered with incidents generally of low to moderate severity, with few severe and lethal cases.¹ In contrast to possible risk from human exposure, the U.S. EPA identified a number of

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significant risks for the environment including acute and chronic risks to freshwater and marine invertebrates and fish species, acute lethal and reproduction risks to birds, and reproductive effects in insectivorous mammals.¹ To mitigate ecological risks, routine environmental monitoring could help in timely detection of environmental contamination, thus preventing at-risk species from exposure. Rapid detection tools could also be applied to monitoring of population exposure occurring at their homes, thus preventing the undesirable consequences of fipronil exposure.

Detection of fipronil residues (fipronil and metabolites) in environmental samples^{24,25} and body fluids^{26–29} is usually performed by well-established analytical techniques such as high-performance liquid chromatography (HPLC) or gas chromatography (GC) coupled to sensitive and highly selective mass detectors. Despite the advantage of being highly sensitive (LOQ 0.18–2.5 $\mu\text{g/L}$)^{26,28,29} and selective, instrumental methods require extensive sample preparation and cleanup procedures that become laborious, time-consuming, and expensive when a large number of samples have to be analyzed in monitoring studies. Immunoassay methods have been proven to be quantitative, relatively inexpensive, high-throughput methods of choice for large screening studies of environmental contaminants,^{30,31} pesticides,^{32–34} and their degradation products and biological metabolites.^{35,36} In the literature, only one publication³⁷ and one patent³ have been found on the development of a fipronil assay. However, in those studies authors used only one hapten to raise the antibody and assay development. The reported assays were not characterized for their robustness to matrix variables, such as pH, ionic strength, or effect of organic solvent on assay performance. In addition, they tested the cross-reactivity (CR) in their assays with different insecticides and pesticides, but these were only distantly structurally similar to fipronil.

In this study, we developed an immunoassay to fipronil and the class of fipronil metabolites. Our effort was directed to improve the sensitivity of the immunoassay compared to one published by Liu et al.³⁷ by applying careful design of immunizing and coating antigen haptens. We also studied the selectivity of resulting assays by testing not only compounds generally similar to fipronil but also those closely structurally related molecules, chiefly environmental and biological metabolites. The resulting assays were optimized, characterized, and validated with spike–recovery studies from fortified water and human serum and urine matrices. The recovery values were also compared to conventional LC-MS/MS analysis. Finally, the developed assay was applied to the analysis of real urine samples from a human exposure study.

MATERIALS AND METHODS

Information concerning chemicals and instruments, buffers, hapten synthesis, immunization and antiserum preparation, reagents and assay buffer optimization, CR, and human serum matrix effect is detailed in the [Supporting Information](#).

Preparation of Immunogens and Coating Antigens.

Haptens with a reactive carboxylic acid group were conjugated to proteins by a sulfo-*N*-hydroxysuccinimide (NHS, haptens 1–4) method and haptens with an amine group ($-\text{NH}_2$) were conjugated by the diazotization method (haptens 5 and 6). Haptens 1–4 ([Table S1](#)) were conjugated to thyroglobulin (Thy) for immunogen preparation. Haptens 1–6 were conjugated to bovine serum albumin (BSA) and conalbumin (CON) for coating antigen screening ([Table S1](#)). The

conjugation protocols are detailed in the [Supporting Information](#).

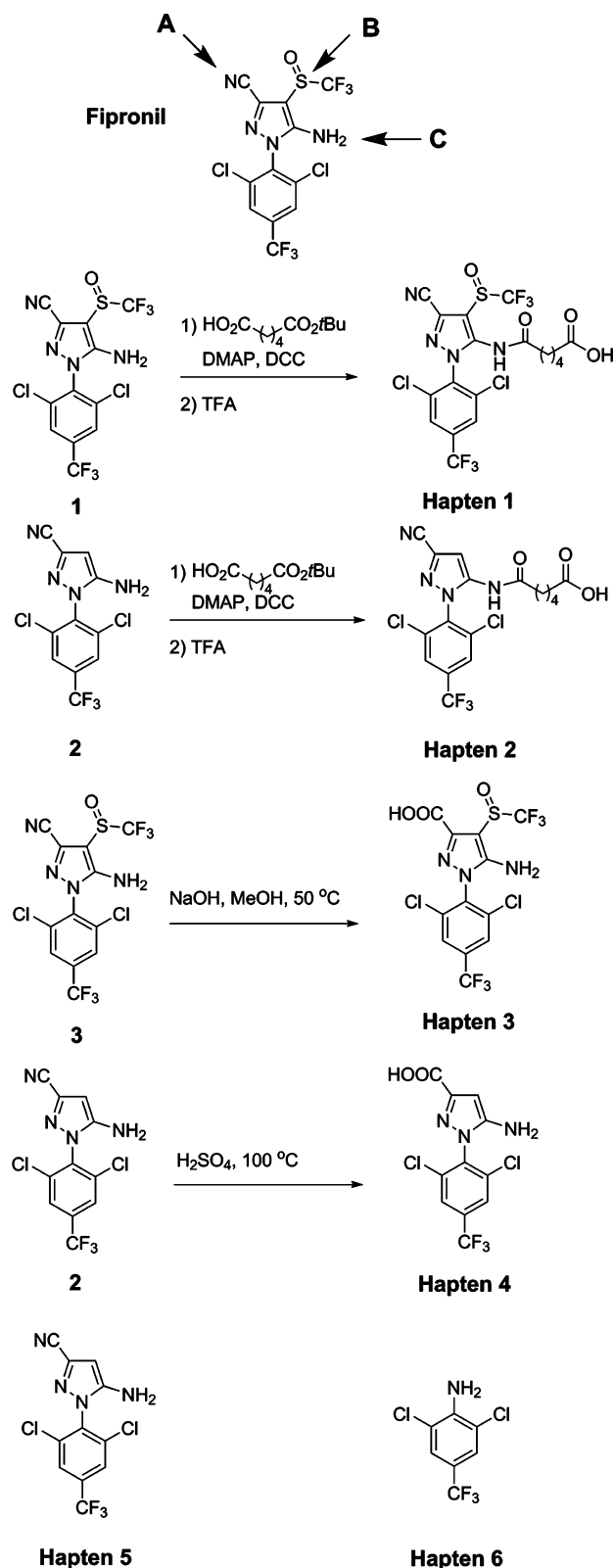
Indirect Competitive ELISA. Plates were coated with the optimal concentration of antigen diluted in coating buffer (100 $\mu\text{L/well}$). After incubation for 1 h at room temperature (RT), the solution was replaced with blocking buffer (200 $\mu\text{L/well}$), and plates were incubated overnight at 4 °C or for 1–4 h at RT. Plates were washed with PBST three times prior to sample loading. Sample solutions in assay buffer were loaded in the first row of the coated plate (in duplicate or triplicate) and diluted in subsequent rows preloaded with assay buffer (50 $\mu\text{L/well}$). An equal volume of anti-fipronil antiserum diluted in PBS was added. The plate was incubated for 1 h at RT and then washed five times with wash buffer. Goat anti-rabbit IgG–HRP conjugate was added at 100 $\mu\text{L/well}$ in a 1/10 000 dilution as instructed by manufacturer. The plate was incubated for 1 h at RT and washed five times. Substrate solution was added (100 $\mu\text{L/well}$) and was left to develop color for about 10 min. The reaction was stopped by addition of 2 M H_2SO_4 (50 $\mu\text{L/well}$), and absorbance was read at 450 nm. SigmaPlot 11.0 software was used for curve fitting and data analysis.

Immunoassay Validation. To evaluate the performance of the fipronil immunoassays, a series of three experiments were performed: (A) Recovery from fortified samples measured by immunoassay (from industrial tap water and human serum and urine). (B) Correlation of recovery values obtained by immunoassay and LC-MS/MS. (C) Immunoassay application to analysis of real samples. An extensive validation was performed only for generic assay #2265 because it could be applied for the detection of fipronil-like analytes in environmental samples as well as in human biofluids. The CR of assay #2268 for fipronil-sulfide-amide makes this assay more suitable for environmental analysis. Thus, assay #2268 was only characterized for a water matrix. The immunoassay was used to analyze urine samples obtained from people exposed to fipronil during application of flea treatment to their companion animals. Urine samples were kindly provided by Dr. Krieger from the University of California, Riverside, and detailed information on the exposure study is available from Dyk et al.³⁸ The immunoassays were also used for quantification of total concentration of fipronil and its metabolites in the serum of rats treated with fipronil-containing diet. Serum samples were provided by Dr. Strynar from the U.S. EPA, and details on animal experiments are available from McMahan et al.³⁹ Sample preparation for validation studies and analysis of samples from exposure studies are detailed in the [Supporting Information](#).

RESULTS AND DISCUSSION

Hapten Design. Fipronil is a small molecule; thus, it does not elicit an immune response by itself. To be immunogenic, it is conjugated to a carrier protein of high molecular weight (i.e., Thy) in a particular orientation so that key functional groups of the target molecule are most effectively exposed. Antibodies are generally formed to the part of the molecule that is the most distal from the protein.⁴⁰ It is generally accepted that a linker arm of 3–5 carbon atoms⁴¹ is the most efficient distance of hapten from carrier protein; it is neither too short for the hapten to be shielded by the protein nor too long for it to allow the hapten to fold back into the lipophilic core of the protein.

Immunizing haptens were designed to expose structural determinants A–C ([Scheme 1](#)) of the fipronil molecule to produce antibodies selective to these particular patterns of analyte. Haptens were synthesized from fipronil through

Scheme 1. Synthesis Routes of Designed Haptens^a

^aHaptens 1 and 2 were obtained by linker attachment to the amine group. Haptens 3 and 4 were obtained by hydrolysis of the nitrile group. A, B, and C refer to structural determinants exposed for antibody production.

modification of the amine group by attachment of a linker arm containing a carboxylic group (hapten 1) and by hydrolysis of

the nitrile group to a carboxylic group (hapten 3, Scheme 1). In this way, pairs of structural determinants A and B or B and C were exposed for recognition and antibody production. Two other immunizing haptens (haptens 2 and 4) were synthesized by similar reactions starting from the fipronil analogue missing the SOCF₃ group (compound 2, Scheme 1). Haptens 1 and 3 were designed to produce fipronil-selective antibodies, whereas haptens 2 and 4 targeted more class-selective recognition.

A number of publications have shown that in competitive immunoassays the sensitivity of the assay is greatly increased when the hapten in the coating antigen is different from the immunogen.^{40,42–44} Therefore, additional haptens were used in the development of heterologous immunoassays (haptens 5 and 6). Fipronil analogue, compound 2 (Scheme 1), and 2,6-dichloro-4-(trifluoromethyl)aniline were attached to protein without additional modification through the amine group. Thereby, heterology was achieved by altering parent structure (haptens 2 and 4–6) or by altering parent structural determinants (haptens 1 and 3). Haptens 5 and 6 also used a different coupling chemistry (diazotization).

Coating Antigen Screening. Each immunogen was used for immunization of three rabbits. Sera from 12 rabbits were screened in a three-point competitive format against six haptens conjugated with BSA and CON (data not shown). The serum/coating antigen pairs showing good inhibition with fipronil were then tested in a full competitive format (Table S2, selected data). As expected, the IC₅₀ values for homologous assays were generally higher than the IC₅₀ values for heterologous assays. For example, in the homologous competitive assay with serum 2265, the IC₅₀ value was 54.2 μg/L (2-BSA), whereas the IC₅₀ was 2.1 μg/L in the heterologous assay (5-CON). Among the most successful combinations of sera/coating antigens, having high assay sensitivity, sufficiently high maximum signal, good signal-to-noise ratio, and slope values around 0.5–1, pairs of #2265/5-CON and #2268/1-CON were chosen for the following studies because these assays had the highest sensitivity.

Assay Optimization. Because fipronil has only moderate solubility in aqueous solutions (about 2 mg/L), the influence of organic solvent concentration in assay buffer on assay sensitivity was evaluated. The organic solvent in assay buffer is also necessary to keep hydrophobic analytes in solution and prevent their nonspecific binding on the plastic containers. Only methanol was assessed because methanol is often used in sample preparation due to its being less volatile than acetonitrile yet still easy to evaporate. It is an appropriate solvent when downstream LC/MS analysis is required. PBS buffer containing an increasing amount of methanol was tested in both assays with sera 2265 and 2268 (Figures S1A and S2A). There was no significant effect on the serum 2268-based assay sensitivity with IC₅₀ around 3.5 μg/L. However, the maximum absorbance was concentration-dependent, and a 20% decrease in maximum absorbance value was observed in the assay with 40% methanol in the buffer. In the 2265 assay, the IC₅₀ values were very close, 0.49 and 0.51 μg/L, respectively, when methanol was present at 10 or 20% in PBS buffer (prior to antibody addition). However, in PBS containing 40% methanol the sensitivity of the assay decreased dramatically with an IC₅₀ at 9.0 μg/L, probably because of protein denaturation by the organic solvent.

An increase in ionic strength resulted in a slight improvement of 2268 assay sensitivity, but the maximum absorbance was negatively affected (Figure S2B). In contrast, ionic strength

had a dramatic effect on the 2265 assay, leading to a 10-fold increase in IC_{50} value and about a 50% decrease in maximum absorbance (Figure S1B). These results suggest that the binding interaction between antibody and analyte/coating antigen is gradually suppressed in solutions with high ionic strength.

There was a slight change in sensitivity upon pH change in the range of 6.5–9.5, with the lowest IC_{50} values at pH 8.5 for both the 2265 and the 2268 assays (Figures S1C and S2C). Similar to IC_{50} values, the maximum absorbance also decreased constantly as pH increased for the 2265 assay, but no remarkable change was observed for the 2268 assay. The pH was held at 7.5 for the following experiments.

The optimized ELISAs used coating-antigen–antibody pairs 5-CON/2265 and 1-CON/2268 at coating concentrations of 1 $\mu\text{g/mL}$. The coated plate was blocked with 1% BSA. The analyte was loaded in assay buffer containing 10% MeOH in PBS, pH 7.5. Sera dilutions were 1:6000 and 1:8000 in PBS, respectively, after addition to the plate. The heterologous assay had a linear range (IC_{20-80}) of 0.14–2.22 $\mu\text{g/L}$ of fipronil in assay buffer and an IC_{50} value of 0.58 ± 0.06 $\mu\text{g/L}$ (tested in triplicate for 8 days) for 2265 (Figure 1) and a linear range

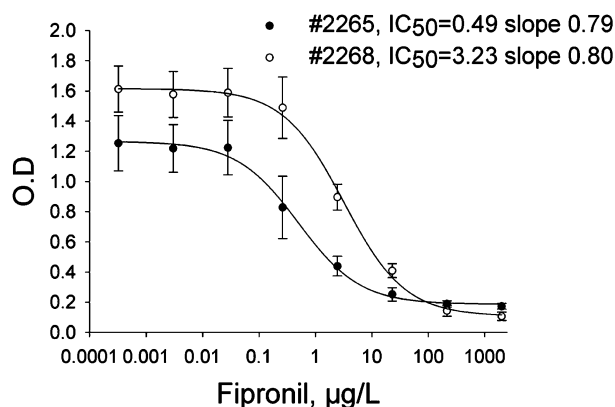


Figure 1. Fipronil competition curves with serum from rabbits 2265 and 2268 in 10 mM PBS assay buffer at pH 7.5 containing 10% MeOH. Reagent concentrations: coating antigen, 1 $\mu\text{g/mL}$ (5-CON/2265 and 1-CON/2268); anti-fipronil serum, 1/6000 and 1/8000, respectively; and goat anti-rabbit IgG-HRP, 1/10 000. Data points are the mean and standard deviation over 3 days.

(IC_{20-80}) of 0.54–12.6 $\mu\text{g/L}$ of fipronil in assay buffer and IC_{50} value of 2.6 ± 0.4 $\mu\text{g/L}$ (tested in triplicate for 8 days) for 2268 (Figure 1). The LOD in the buffer was determined from the IC_{10} value and estimated at 0.06 and 0.22 $\mu\text{g/L}$ for 2265 and 2268, respectively. The sensitivity of the assays is comparable with those of instrumental LC- and GC-MS/MS methods being in the range of 0.18–2.5 $\mu\text{g/L}$.^{26,29}

The overall assay optimization data suggest that the binding properties of the serum obtained from rabbit 2268 are less affected by changes of sample matrix, thus giving a more robust assay. The two assays significantly differ in metrological characteristics, whereas assay 2265 gives a very low limit of detection and high sensitivity, the 2268 assay has a wider analytical range and better signal-to-noise ratio.

Only a few references to a fipronil immunoassay could be found in the literature. Liu et al.³⁷ developed poly- and monoclonal antibodies using a homologous hapten, a derivative of fipronil-sulfone, for preparation of the immunogen and coating antigen. The assay had lower sensitivity compared to

those described in this paper, having IC_{50} values of 18.0 and 6.0 $\mu\text{g/L}$ for polyclonal (pAb) and monoclonal (mAb) antibodies, respectively. However, the linear range of detection for the reported assays was wider. Based on IC_{10-90} , the assay was linear between 0.07 and 203 $\mu\text{g/L}$ for the pAb and between 0.07 and 485 $\mu\text{g/L}$ for the mAb. Another assay based on a mAb aimed to detect the active ingredients of a termite insecticide, including fipronil, was patented by Miyake et al.³ They demonstrated that fipronil detection occurs almost linearly through the range of concentrations from 5 to 80 $\mu\text{g/L}$. On the basis of comparison with these two publications, it appears that the assay described here demonstrates very good sensitivity to fipronil. Careful design of the immunogen and a heterologous approach for coating antigen selection allowed the development of a high-sensitivity assay using polyclonal serum that is much easier to obtain compared to the laborious procedure of monoclonal antibody production.

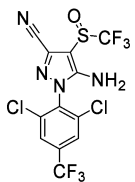
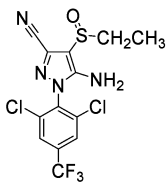
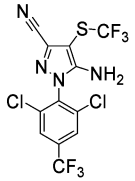
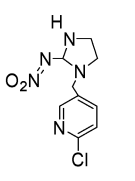
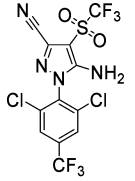
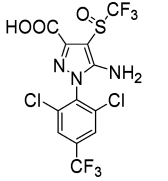
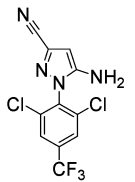
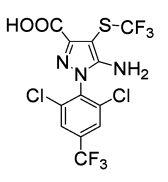
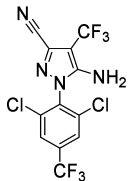
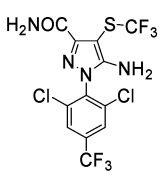
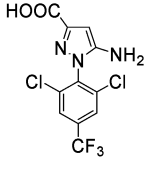
Cross-Reactivity. A range of fipronil analogues with modified substituents were purchased or synthesized to be used in CR studies to determine the specific epitope of hapten against which the antibody was raised and how the structure of immunogen may alter the selectivity of the developed antiserum. The main reactions to prepare fipronil congeners were based on methods previously described. Analytical data of the resulting compounds conformed to published information.^{45–47}

Antiserum 2265 was raised against a hapten exposing the nitrile group while attached to the protein through the amine moiety (hapten 2). Thus, resulting antibodies are very selective to the presence of the nitrile group in the analyte. All fipronil analogues containing the nitrile group strongly inhibited the assay, giving CR in the range of 50–100% compared to fipronil (compounds 2–5, Table 1). In contrast, the analytes missing the nitrile group poorly competed with coating antigen, and their CR hardly exceeded 4%. This remaining activity may come from antibody selectivity to the skeletal structure of the substituted phenylpyrazole.

The binding pocket seems to be more complex in antibodies from serum 2268 raised against a hapten having $SOCF_3$ and the amine group exposed while attached to the protein through –CONH linkage of fipronil-acid (hapten 3). The developed serum selectively recognized $SOCF_3$. This hypothesis is supported by the decrease in CR of compounds with modified substituents at position B of the fipronil molecule: $SOCF_3 > SO_2CF_3 > SOC_2H_5 \approx SCF_3 > CF_3 > H$ (compounds 1 > 3 > 6 \approx 2 > 5 > 4, Table 1) corresponding to 100 > 71 > 43 \approx 39 > 25 > 1.4%. However, the serum had even stronger selectivity toward the amide group at the adjacent position because specificity to fipronil-sulfide-amide was higher than to fipronil (CR 157%, compound 10; Table 1), despite having a sulfide group instead of a $SOCF_3$. Interestingly, assay 2268 was only slightly inhibited by compounds mimicking fipronil and fipronil-sulfide with the nitrile group substituted by a carboxyl group (compounds 8 and 9). Taking into account that the nitrile group was not exposed for recognition and antibody production, the results obtained suggest that nitrogen as a part of either –CONH– or nitrile group occupies a specific place in the binding pocket of the antibody used in the assay. The role of the fipronil amine group (structural determinant C) in antiserum recognition was not explored.

These effects demonstrate that hapten design had a significant impact on the resulting antiserum selectivity. The careful hapten design and successful organic synthesis allow

Table 1. Cross-Reactivity of Fipronil Antiserum to Structurally Related Compounds

| Compound | Structure | #2265, % (n) ^a | #2268, % (n) | Compound | Structure | #2265, % | #2268, % |
|--|---|------------------------------|-----------------|--|---|-------------|-------------|
| 1. Fipronil ^b |  | 100 (19) | 100 (7) | 6. Ethiprole (insecticide) |  | 61±15 | 43±13 |
| 2. Fipronil-sulfide (environment. photoproduct) |  | 96±49 (6) | 39±11 (4) | 7. Imidacloprid (insecticide) |  | <1 | <1 |
| 3. Fipronil-sulfone (environment. photoproduct, biometabolite) |  | 60±26 (8) | 71±14 (4) | 8. Fipronil-acid (environment. metabolite) |  | 4.2±1.5 | 13±2.1 |
| 4. Fipronil-deSOCF ₃ ^c (environment. photoproduct, biometabolite) |  | 38±14 (6) | 1.4±0.2 (4) | 9. Fipronil-sulfide-acid (environment. metabolite) |  | 4.4±2.4 | 6.9±3.3 |
| 5. Fipronil-desulfinyl (environment. photoproduct) |  | 101±23 (7) | 25±3 (4) | 10. Fipronil-sulfide-amide (environment. metabolite) |  | 2.6±0.7 | 157±109 |
| | | | | 11. Fipronil-deSOCF ₃ -acid (environment. metabolite) |  | <1 | <1 |

^aCross-reactivity was calculated as (IC₅₀ of fipronil/IC₅₀ of the tested compound) × 100. ^bIC₅₀ of fipronil was 1.4 ± 0.5 nmol/L (#2265) and 6.8 ± 1.6 nmol/L (#2268). ^cdeSOCF₃ refers to detrifluoromethylsulfinyl. (n) represents the number of days CR experiment was performed, in triplicate wells. For compounds 6–11, n = 3 days, in triplicate wells.

construction of a library of diverse but closely related chemicals that could be manipulated to produce antibodies with desired characteristics: target-, group-, or class-specific reagents. It is not always necessary to have highly selective assays. For example, a number of organophosphorus pesticides are used for residential and agricultural purposes. These compounds have led to numerous poisonings of nontarget species, including human fatalities. The specific assays for individual compounds of this large class are less efficient for pesticide control and screening.^{48,49} In contrast to selective tests, the antibodies used

in screening assays should be cross-reactive in order to detect the analyte in a risk cup. In the literature, it has been shown that considered manipulation of the hapten structure gives useful highly cross-reactive assays for rapid screening of dangerous substances and related compounds.^{50,51} High CR is usually achieved when the immunizing antigen structure combines the analyte main body and some of its structural determinants common to all compounds aimed to be recognized. In our study, serum 2265 recognizes not only fipronil but also metabolites of fipronil which contain the nitrile

Table 2. Recovery of Fipronil and Fipronil Metabolites in Spiked Samples of Industrial Water, Human Serum and Urine Measured by Immunoassay^a

| spiked conc. ($\mu\text{g/L}$) ^b | fipronil, $\mu\text{g/L}$ (% recovered) | | |
|---|---|----------------------------------|-------------------------------|
| | industrial water | serum | urine |
| 2.5 | 1.8 \pm 0.25 (73.3 \pm 9.6) | 2.3 \pm 0.6 (93.1 \pm 22.7) | 2.14 \pm 0.28 (85 \pm 11) |
| 1 | 0.9 \pm 0.05 (85.5 \pm 5.5) | 1.0 \pm 0.1 (103.4 \pm 13.3) | 0.90 \pm 0.20 (90 \pm 20) |
| 0.5 | 0.5 \pm 0.0 (91.3 \pm 11.6) | 0.6 \pm 0.1 (111 \pm 11) | 0.48 \pm 0.0 (96 \pm 9) |
| | fipronil metabolites, $\mu\text{g/L}$ (% recovered) | | |
| | fipronil sulfone ^c | fipronil-desulfinyl ^d | |
| 2.5 | 2.6 \pm 0.4 (105.8 \pm 17.3) | 2.4 \pm 0.23 (96.4 \pm 9.3) | |
| 1 | 1.2 \pm 0.1 (116.8 \pm 9.1) | 1.0 \pm 0.09 (104.4 \pm 9.0) | |
| 0.5 | 0.6 \pm 0.2 (118 \pm 47) | 0.5 \pm 0.04 (107 \pm 8) | |

^aAssay conditions: coating antigen (5-CON), 1 $\mu\text{g/mL}$; anti-fipronil serum #2265, 1/6000; goat anti-rabbit IgG-HRP, 1/10 000. ^bSpiked concentrations indicated in the table are the final concentrations in the well after sample dilution prior to loading onto the plate and after addition of the antibody in the well. ^cIn human serum. ^dIn industrial water. Values are the mean \pm standard deviation ($n \geq 3$ days).

group and variable substituents at the adjacent position. Indeed, such broad selectivity appears because the antibodies were raised against hapten **2**, where the nitrile group was preserved in the structure, carrier protein was attached to the amine moiety, and the SOCF₃ structural determinant was missing. The results obtained were in strong accordance with literature reports.^{48,51}

Serum 2265, having broad-selectivity properties for closely related compounds, can be used for construction of unique biosensors or ELISA assays that will be able to detect fipronil-sulfone and fipronil in the blood of humans and animals to assess their exposure to the insecticide. The same assay/sensor could also be applied for rapid on-site monitoring of fipronil and fipronil-desulfinyl in environmental samples, thus assuring the real-time monitoring of environmental contamination.

In addition to fipronil analogues, we also tested a number of GABA-antagonists (Table S3). There are cases where the antibody raised to biologically active molecules will detect a group of different structures, all of which bind to the same receptor. In these cases, the antibody can act as a surrogate receptor for screening.^{52,53} In our case, the immunoassay failed to detect a variety of insecticides and cage convulsants acting on the GABA channel. Because these compounds bind to a series of diverse sites on the GABA-gated chloride channel of arthropods and vertebrate, CR was not expected.

Matrix Effect. Sample preparation is an important step in complex sample analysis influencing accuracy and reliable determination in many analytical methods. However, sample cleanup procedures are often time-consuming and laborious. Similar to other analytical techniques, immunoreactions employed in an ELISA may be altered by multiple components present in complex media. However, depending on the nature of the matrix and immunoassay characteristics the interference could be minimal so that preparation of a calibration curve in a similar matrix may decrease the error of analysis. The interference in ELISA could also be diminished by simple sample dilution. The effect of matrix on assay 2265 performance was evaluated in human serum (Figure S3). In this study, fipronil-sulfone was chosen as the analyte because it is the major metabolite of fipronil identified in serum. Because we wanted to use the generic assay for both analytes, other parameters of the assay were used as optimized for fipronil. Serum matrix had variable effects on the competition curve. When the assay was performed in 100% human serum (prior to antibody addition), the sensitivity did not change dramatically

compared to that of the assay conducted in buffer, with IC₅₀ values of 4.11 and 4.95 $\mu\text{g/L}$ (Figure S3), respectively. However, a constant decrease in maximum signal was observed when the content of serum matrix increased in assay buffer. Interestingly, the sensitivity of the assay increased in buffer containing serum matrix at 10% (IC₅₀ at 2.47 $\mu\text{g/L}$), followed by a subsequent decrease in sensitivity with an increase of serum matrix portion in the assay buffer (IC₅₀ 3.37 $\mu\text{g/L}$ at 50% matrix). It is possible that proteins present in the serum matrix help to decrease nonspecific binding in the assay, thus improving assay characteristics without affecting the desired immuno-recognition and binding. Because matrix suppressed the maximum signal even at 10-fold dilution, we decided to prepare the calibration curve in a 10% blank matrix of human serum.

Similar analysis was performed with urine matrix for sera 2265 and 2268. There was no significant effect of urine matrix on assay sensitivity. However, the signal intensity was again suppressed with increasing amount of urine matrix (data not shown). A 10-fold dilution of sample with assay buffer was chosen for further validation studies.

Validation in Various Samples. To evaluate the performance of the fipronil immunoassays developed to detect quantitatively the analyte in complex samples, we performed a spike–recovery analysis from different matrixes, including industrial water and human serum and urine. In case report studies, the concentration of fipronil and fipronil-sulfone in the serum of humans intoxicated with fipronil were reported to be up to 4000 $\mu\text{g/L}$ of plasma.²¹ Taking into account these data, human serum was fortified with known concentrations of fipronil or fipronil-sulfone at concentrations in the range of 10–50 μM (0.5–2.5 $\mu\text{g/L}$ in the well after dilutions). We aimed to study the recovery of low concentrations to estimate the influence of the matrix on the accuracy of quantification. Otherwise, with higher spiked concentrations the assay would require higher dilution of the sample, decreasing the amount of interfering matrix and facilitating quantitative analysis. We also evaluated concentrations of fipronil over a narrow range to validate the accuracy of the assay to distinguish close but different concentrations of the analyte in the matrix. Table 2 presents good recoveries ranging from 93 to 118% for both analytes at all concentrations tested.

To our knowledge, there are few reports on fipronil monitoring in the environment. From the U.S. Geological Survey,⁵⁴ it appears that fipronil is present in very low

concentration in water and soil in a number of states. The concentration varies from very low to hundreds of nanograms per liter. However, from the same survey, fipronil concentrations may go up to micrograms per liter in spring, for example, when water is released from rice fields. Therefore, an ELISA could be used for monitoring downstream water released from farms. Because its sensitivity is around 0.5–1 $\mu\text{g/L}$ and toxicity of fipronil for aquatic animals is generally above 10 $\mu\text{g/L}$, immunoassay could be an appropriate environmental screening tool. In addition, the assay with serum 2265 detects environmental metabolites that are even more toxic. Similarly to the serum matrix, water samples were separately spiked with fipronil and fipronil-desulfinyl in the 10–50 μM range, and recoveries were assessed with assay 2265. No significant matrix effect was observed from industrial water. Table 2 demonstrates good recoveries ranging from 73 to 91% for fipronil and from 96 to 107% for fipronil-desulfinyl.

Finally, the metabolism of fipronil in mammals is being studied, and only limited data are available concerning humans. Despite the fact that up to now there are contradictory data on presence of parent compound in urine in animals, human urine remains a possible way of exposure monitoring. Recoveries from human urine matrix fortified with fipronil in the 10–50 μM range were 85–96% (Table 2).

In another series of experiments, a comparative study was performed to estimate the accuracy of the immunoassay compared to an instrumental method. Three matrices, including industrial tap water, urban runoff water, and human urine were fortified with fipronil, extracted, and analyzed blindly by LC-MS/MS and immunoassay. The assay 2265 was used to estimate recovery values from urine, and the assay 2268 was used to estimate these from water. As seen in Table 3, there was relatively good agreement between ELISA and LC-MS/MS data. In industrial water and human urine extracts, fipronil

concentrations detected by immunoassay were closer to theoretical spiked values than were those detected by LC-MS/MS. However, recoveries from urban runoff water were closer to theoretical values when detected by LC-MS/MS, with slight overestimation for certain spikes in immunoassay analysis. Overall, there is a linear correlation between data obtained by LC-MS/MS and ELISA with the ratio varying between 1.1 and 1.4. These overall data suggest that immunoassay based on antiserum 2265 (and 2268) could be directly used for quantitative monitoring of fipronil and fipronil metabolites in various matrices without any additional sample preparation, thus reducing analysis time, especially in the case of a large screening campaign, and reducing the cost of analysis.

Urine Samples from Exposure Studies. Dyk et al.³⁸ were looking for fipronil and its metabolites in urine of pet owners after they used Frontline insecticide in companion animals. Many urine samples from pet owners were collected prior to and after product application. Authors analyzed samples by LC-MS/MS before and after hydrolysis of possible bioconjugates of fipronil and its metabolites. The selected urine samples were also analyzed by a third independent laboratory using LC-MS/MS. We used selected urine samples to conduct the analysis using the fipronil immunoassay. Assay 2265 was chosen because it was the most sensitive to fipronil and a number of its metabolites. In the literature, there are limited data on fipronil metabolism in humans, and metabolites present in human urine have not been well-studied. Xenobiotics may be excreted in the urine in forms of glucuronide or sulfate conjugates of parent compound or its metabolites. To hydrolyze possible conjugates of fipronil, we used an enzymatic solution of β -glucuronidase/sulfatase. This method provides mild conditions for hydrolysis, decreasing the possibility of destroying the compound of interest by harsh acidic hydrolysis conditions. The assay showed nondetectable levels of compounds of interest (data not shown) with limits of detection of 0.05 ± 0.02 , 0.02 ± 0.01 , 0.04 ± 0.02 , and 0.07 ± 0.02 $\mu\text{g/L}$ ($n = 6$ days) for fipronil-sulfone, fipronil-sulfide, fipronil detrifluoromethylsulfinyl, and fipronil-desulfinyl, respectively. Our findings are similar to data published by Dyk et al.³⁸ They concluded that levels observed after Frontline application were not different from levels observed in preapplication urine samples and that a time/concentration trend was not observed.

Serum Samples from Dosed Rats. McMahan et al.³⁹ identified serum/urine biomarkers of fipronil exposure from dosed animal samples as potential biomarkers for use in human biomonitoring studies. The authors used LC/QQQ (triple quadrupole) mass spectrometry to identify possible fipronil derivatives present in biofluids and to quantify fipronil and fipronil-sulfone in rat serum. We used both immunoassays to quantify total concentration of fipronil and its metabolites in the selected serum samples. The data obtained by both assays are in very good correlation with LC/QQQ results (Table 4). The assays gave higher estimates compared to fipronil or fipronil-sulfone concentrations separately because compounds are cross-reactive in both assays. However, the values obtained by the assays are very close to the total fipronil detected with LC/QQQ. No significant difference was detected between methods using simple t test analysis with $p < 0.05$.

Two sensitive immunoassays were developed. One assay appeared to be selective to fipronil and its major metabolite fipronil-sulfone. Another assay demonstrated recognition of the class of structures closely related to fipronil. Such difference in

Table 3. Recoveries of Fipronil in Spiked Industrial and Urban Water and in Spiked Human Urine Samples: Comparison between Immunoassay and LC-MS/MS^a

| analyte | spike | ELISA (A) | LC-MS/MS (B) | ratio (A/B) |
|--------------------|-------|-----------------|----------------|-------------|
| Industrial Water | | | | |
| fipronil | 2.0 | 1.7 ± 0.4 | 1.7 ± 0.4 | 1.0 |
| | 29.1 | 30.8 ± 4.6 | 21.6 ± 1.7 | 1.4 |
| | 47.6 | 49.6 ± 7.3 | 35.3 ± 5.6 | 1.4 |
| | 9.9 | 10.2 ± 0.8 | 8.1 ± 0.2 | 1.3 |
| | 4.8 | 5.1 ± 1.0 | 4.2 ± 0.3 | 1.2 |
| Urban Runoff Water | | | | |
| fipronil | 29.1 | 39.5 ± 14.0 | 20.0 ± 4.9 | 2.0 |
| | 2.0 | 1.9 ± 0.8 | 1.8 ± 0.2 | 1.1 |
| | 47.6 | 65.5 ± 22.8 | 39.2 ± 8.4 | 1.7 |
| | 4.8 | 7.0 ± 2.5 | 4.4 ± 0.9 | 1.6 |
| | 9.9 | 10.6 ± 4.0 | 8.0 ± 1.2 | 1.3 |
| Urine Extract | | | | |
| fipronil | 10 | 9.6 ± 0.9 | 8.5 ± 0.6 | 1.1 |
| | 20 | 18.0 ± 3.9 | 14.7 ± 2.5 | 1.2 |
| | 25 | 22.7 ± 2.7 | 18.6 ± 3.0 | 1.2 |
| | 50 | 42.7 ± 5.5 | 38.5 ± 1.1 | 1.1 |

^aFor recovery studies from water, assay 2268: coating antigen (1-CON), 1 $\mu\text{g/mL}$; anti-fipronil serum, 1/8000; and assay buffer with 20% MeOH and goat anti-rabbit IgG-HRP, 1/20 000. For recovery studies from urine: coating antigen (5-CON), 1 $\mu\text{g/mL}$; anti-fipronil serum, 1/6000; and goat anti-rabbit IgG-HRP, 1/10 000. Values are the mean \pm standard deviation ($n = 3$ days).

Table 4. Detection of Fipronil and Fipronil-Sulfone in Rat Serum Samples: Comparison between Immunoassays and LC/QQQ-MS

| rat sample | LC/QQQ-MS ($\mu\text{g/mL}$) ^a | | | immunoassays ($\mu\text{g/mL}$) | |
|------------|---|----------------------|------|-----------------------------------|-------------------|
| | fipronil (A) | fipronil-sulfone (B) | A+B | #2265 | #2268 |
| 1 (CNTR) | <LOQ ^b | <LOQ ^c | <LOQ | <LOD ^d | <LOD ^e |
| 2 | 0.5 | 1.9 | 2.4 | 3.9 \pm 2.7 | 2.4 \pm 0.6 |
| 3 (CNTR) | <LOQ | <LOQ | <LOQ | <LOD | <LOD |
| 4 | 0.5 | 2.3 | 2.8 | 3.0 \pm 1.3 | 2.3 \pm 0.8 |
| 5 (CNTR) | <LOQ | <LOQ | <LOQ | <LOD | <LOD |
| 6 | 0.4 | 1.9 | 2.3 | 1.7 \pm 0.4 | 1.9 \pm 0.3 |
| 7 (CNTR) | <LOQ | <LOQ | <LOQ | <LOD | <LOD |
| 8 | 0.3 | 1.4 | 1.7 | 1.5 \pm 0.5 | 1.9 \pm 0.5 |
| 9 | 0.4 | 0.7 | 1.1 | 0.9 \pm 0.1 | 1.3 \pm 0.4 |
| 10 | 0.4 | 2.1 | 2.5 | 2.3 \pm 0.4 | 2.5 \pm 0.3 |
| 11 (CNTR) | <LOQ | <LOQ | <LOQ | <LOD | <LOD |
| 12 | 0.3 | 1.3 | 1.6 | 1.6 \pm 0.0 | 1.3 \pm 0.4 |

^aResults are provided by McMahan et al.³⁹ ^bLOQ = 10 ng/mL. ^cLOQ = 10 ng/mL, RSD is <15%. ^dLOD = 0.06 ng/mL. ^eLOD = 0.22 ng/mL. For ELISA, prior to addition to the plate, serum samples were diluted 1000 or 100 times for treated and control (CNTR) animals, respectively. $n = 2$ different days; no statistical difference with (A + B) was observed ($p < 0.05$).

recognition behavior of antibodies was achieved by using different haptens, exposing either the single nitrile structural determinant of fipronil (for generic assay) or the trifluoromethylsulfonyl and amine structural determinants. A heterologous format has proven to result in more sensitive assays with IC₅₀ values of 0.58 ± 0.06 and $2.6 \pm 0.4 \mu\text{g/L}$. The assays have the sensitivity to measure fipronil and its analogs relevant for medical screening where fipronil and fipronil-sulfone concentration in serum may be up to $4000 \mu\text{g/L}$ and for exposure monitoring (toxicity of fipronil for aquatic animals is above $10 \mu\text{g/L}$). The assays successfully demonstrated their accuracy and reliability when applied in spike–recovery studies and compared to established analytical techniques (LC-MS) in different matrices, providing a valuable tool for further development of rapid immunochemical screening methods. Developed assay 2265 might be used in screening studies whenever analyte is a fipronil-like molecule. Assay 2268 is more convenient for quantitative studies because the assay is more robust to changing experimental conditions and is thus the best choice for analysis of environmental samples.

■ ASSOCIATED CONTENT

■ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.5b01005.

Additional information including text, three tables, and three figures. (PDF)

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Notes

The authors declare no competing financial interest.

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