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

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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 IC₅₀ values of 0.58 ± 0.06 and 2.6 ± 0.4 ng/mL were developed. Design of different hapten containers 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-sulfoxide, 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, use on fruits, vegetables, coffee, rice, and other crops as well as for treatment of seeds. 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. It has been shown that fipronil has higher affinity toward the insect GABAₐ receptor than to the human or other mammalian receptors. 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 β₃ similar to that of the insect GABAₐ receptors. Human β₃ GABAₐ receptor is linked to neurodevelopmental disorders such as autism, Angelman syndrome, and epilepsy. 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. 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. 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. 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. The U.S. Environmental Protection Agency (EPA) classified fipronil as a moderately toxic possible human carcinogen, with negligible risk for residential application. According to the national survey, about 40% of American homes tested positive for the presence of fipronil (0.16 ng/cm² of the floor). A number of case studies were registered with incidents generally of low to moderate severity, with few severe and lethal cases. In contrast to possible risk from human exposure, the U.S. EPA identified a number of

Received: February 25, 2015
Revised: July 15, 2015
Accepted: July 21, 2015
Published: July 21, 2015
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 \(^6\) 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 \(\mu\)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, \(^5,31\) pesticides. \(^32\)−\(^34\) and their degradation products and biological metabolites. \(^5,35,36\) In the literature, only one publication \(^17\) and one patent \(^1\) 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. \(^17\) by applying careful design of immunizing and coating antigen hapten. 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.

\section*{Materials and Methods}

Information concerning chemicals and instruments, buffers, hapten synthesis, immunization and antisera preparation, reagents and assay buffer optimization, CR, and human serum matrix effect is detailed in the Supporting Information.

**Preparation of Immunogens and Coating Antigens.**

Haptenss with a reactive carboxylic acid group were conjugated to proteins by a sulfo-N-hydroxysuccinimide (NHS, hapten 1−4) method and hapten with an amine group (−NH\(_2\)) were conjugated by the diazotization method (hapten 5 and 6). Hapten 1−4 (Table S1) were conjugated to thyroglobulin (Thy) for immunogen preparation. Hapten 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 \(\mu\)L/well). After incubation for 1 h at room temperature (RT), the solution was replaced with blocking buffer (200 \(\mu\)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 \(\mu\)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 \(\mu\)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 \(\mu\)L/well) and was left to develop color for about 10 min. The reaction was stopped by addition of 2 M H\(_2\)SO\(_4\) (50 \(\mu\)L/well), and absorbance was read at 450 nm. SigmaPlot 11.0 software was used for curve fitting and data analysis.

**Immunosassay 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-sulphide-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 McMahen et al. \(^39\) Sample preparation for validation studies and analysis of samples from exposure studies are detailed in the Supporting Information.

\section*{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 atoms \(^34\) 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 hapten was designed to expose structural determinants A−C (Scheme 1) of the fipronil molecule to produce antibodies selective to these particular patterns of analyte. Haptenss were synthesized from fipronil through
Scheme 1. Synthesis Routes of Designed Haptens

A
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\begin{align*}
\text{Fipronil} & \\
\text{NC-S-Cl} & \rightarrow \text{NC-S-CF}_3 \\
\text{Cl} & \rightarrow \text{Cl} \\
\text{N-H} & \rightarrow \text{N-H} \\
\text{O-CO}_2 & \rightarrow \text{O-CO}_2 \\
\text{Bu} & \rightarrow \text{Bu} \\
\text{DMAP, DCC} & \implies \text{Hapten 1} \\
\text{TFA} & \implies \text{Hapten 2}
\end{align*}
\]

B
\[
\begin{align*}
\text{NC-S-Cl} & \rightarrow \text{NC-S-CF}_3 \\
\text{Cl} & \rightarrow \text{Cl} \\
\text{N-H} & \rightarrow \text{N-H} \\
\text{O-CO}_2 & \rightarrow \text{O-CO}_2 \\
\text{Bu} & \rightarrow \text{Bu} \\
\text{DMAP, DCC} & \implies \text{Hapten 3} \\
\text{TFA} & \implies \text{Hapten 4}
\end{align*}
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C

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"Haptens 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.
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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 (haptons 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 haptons 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. Therefore, additional haptons were used in the development of heterologous immunoassays (haptons 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 (haptons 2 and 4–6) or by altering parent structural determinants (haptons 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 haptons 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 μg/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 0.14–2.2 μg/L of fipronil in assay buffer and an IC$_{50}$ value of 0.58 ± 0.06 μg/L (tested in triplicate for 8 days) for 2265 (Figure 1) and a linear range (IC$_{20–80}$) of 0.54–12.6 μg/L of fipronil in assay buffer and IC$_{50}$ value of 2.6 ± 0.4 μg/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 μg/L for 2265 and 2268, respectively. The sensitivity of the assays is comparable

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 linear 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. 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 μg/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 μg/L for the pAb and between 0.07 and 485 μg/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. They demonstrated that fipronil detection occurs almost linearly through the range of concentrations from 5 to 80 μg/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.

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 antibodies 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$_2$CF$_3$ > SOCl$_2$H$_3$ ≈ SCF$_3$ > CF$_3$ > H (compounds 1 > 3 > 6 ≈ 2 > 5 > 4, Table 1) corresponding to 100 > 71 > 43 ≈ 39 > 25 > 14%. However, the serum had even stronger selectivity toward the amide group at the adjacent position because specificity to fipronil-sulphide-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-sulphide 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
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. 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. 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>
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<tr>
<th>Compound</th>
<th>Structure</th>
<th>% (n)</th>
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<th>Compound</th>
<th>Structure</th>
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<th>%</th>
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<tbody>
<tr>
<td>1. Fipronil</td>
<td>![Fipronil Structure]</td>
<td>100 (19)</td>
<td>100 (7)</td>
<td>6. Ethiprole</td>
<td>![Ethiprole Structure]</td>
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<td>3. Fipronil-</td>
<td>![Fipronil-sulfone Structure]</td>
<td>60±26 (8)</td>
<td>71±14 (4)</td>
<td>8. Fipronil-acid</td>
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<td>4. Fipronil-</td>
<td>![Fipronil-deSOCF3 Structure]</td>
<td>38±14 (6)</td>
<td>1.4±0.2 (4)</td>
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<td>5. Fipronil-</td>
<td>![Fipronil-desulfanyl Structure]</td>
<td>101±23 (7)</td>
<td>25±3 (4)</td>
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<td>![Fipronil-deSOCF3-acid Structure]</td>
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Cross-reactivity was calculated as (IC_{50} of fipronil/IC_{50} of the tested compound) × 100. IC_{50} of fipronil was 1.4 ± 0.5 nmol/L (#2265) and 6.8 ± 1.6 nmol/L (#2268). deSOCF3 refers to de trifluoromethylsulfonyl. (n) represents the number of days CR experiment was performed, in triplicate wells. For compounds 6–11, n = 3 days, in triplicate wells.
group and variable substituents at the adjacent position. Indeed, such broad selectivity appears because the antibodies were raised against hapten 2, where the nitro 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-desulfanyl 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 495 μg/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 μg/L), followed by a subsequent decrease in sensitivity with an increase of serum matrix portion in the assay buffer (IC₅₀ 3.37 μg/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 matrices, 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 μg/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 μg/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,50 it appears that fipronil is present in very low

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

<table>
<thead>
<tr>
<th>spiked conc. (μg/L)</th>
<th>industrial water</th>
<th>serum</th>
<th>urine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>fipronil, μg/L (% recovered)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.5</td>
<td>1.8 ± 0.2 (73.3 ± 9.6)</td>
<td>2.3 ± 0.6 (93.1 ± 22.7)</td>
<td>2.14 ± 0.28 (85 ± 11)</td>
</tr>
<tr>
<td>1</td>
<td>0.9 ± 0.05 (85.5 ± 5.5)</td>
<td>1.0 ± 0.1 (103.4 ± 13.3)</td>
<td>0.90 ± 0.20 (90 ± 20)</td>
</tr>
<tr>
<td>0.5</td>
<td>0.5 ± 0.0 (91.3 ± 11.6)</td>
<td>0.6 ± 0.1 (111 ± 11)</td>
<td>0.48 ± 0.0 (96 ± 9)</td>
</tr>
<tr>
<td></td>
<td>fipronil-sulfone</td>
<td>fipronil-desulfonyl</td>
<td></td>
</tr>
<tr>
<td>2.5</td>
<td>2.6 ± 0.4 (105.8 ± 17.3)</td>
<td>2.4 ± 0.23 (96.4 ± 9.3)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1.2 ± 0.1 (116.8 ± 9.1)</td>
<td>1.0 ± 0.09 (104.4 ± 9.0)</td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>0.6 ± 0.2 (118 ± 47)</td>
<td>0.5 ± 0.04 (107 ± 8)</td>
<td></td>
</tr>
</tbody>
</table>

*Assay conditions: coating antigen (5-CON), 1 μg/mL; anti-fipronil serum #2265, 1/6000; goat anti-rabbit IgG-HRP, 1/10 000. **Spiked 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. *In human serum. **In industrial water. Values are the mean ± standard deviation (n ≥ 3 days).*
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 μg/L and toxicity of fipronil for aquatic animals is generally above 10 μg/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-desulfanyl 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-desulfanyl.

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 urine were fortuitously 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-desulfanyl.

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 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 antisera 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 μg/L (n = 6 days) for fipronil-sulfone, fipronil-sulfide, fipronil-detrifluoromethylsulfanyl, and fipronil-desulfanyl, 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. McMahen 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 bioluids 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/MS46 |
|-----------------|------------------|-----------------|-----------------|------------------|
| analyte         | spike            | ELISA (A)       | LC-MS/MS (B)    | ratio (A/B)      |
|                 |                  | Industrial Water| Urban Runoff Water| Urine Extract |
| 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             |
|                 | 9.9              | 10.2 ± 0.8      | 8.1 ± 0.2       | 1.3             |
|                 | 4.8              | 5.1 ± 1.0       | 4.2 ± 0.3       | 1.2             |
|                 | 9.9              | 10.6 ± 4.0      | 8.0 ± 1.2       | 1.3             |
|                 | 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             |

For recovery studies from water, assay 2268: coating antigen (1-CON), 1 μg/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 μg/mL; anti-fipronil serum, 1/6000; and goat anti-rabbit IgG-HRP, 1/10 000. Values are the mean ± standard deviation (n = 3 days).
recognition behavior of antibodies was achieved by using different haptenos, 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 ± 0.4 μg/L. The assays have the sensitivity to measure fipronil and its analogous for medical screening where toxicity of fipronil for aquatic animals is above 10 μg/L and for exposure monitoring (toxicity of fipronil for aquatic animals is above 10 μg/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 immunoochemical 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.

## ACKNOWLEDGMENTS

This work was supported by National Institute of Environmental Health Sciences, Superfund Research Program, P42 ES04699, and the National Institute for Occupational Safety and Health Western Regional Center for Agricultural Health Science, U50 OH07550. The research was also supported by the CounterACT Program, National Institutes of Health Office of the Director, and the National Institute of Neurological Disorders and Stroke, grant no. U54 NS079202. We are thankful to Dr. McMahen and Dr. Syrnyan for providing rat serum samples and LC/QQQ data and to Dr. Krieger for human urine samples.

### REFERENCES


Supporting Information

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

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Number of pages: 16
Number of Tables: 3
Number of Figures: 3
**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 d trifluoromethylsulfinyl (FPN-deSO\textsubscript{CF\textsubscript{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. \textsuperscript{1}H and \textsuperscript{13}C NMR spectra were recorded with a Varian VNMR-600 spectrometer. Electrospray mass spectra of haptens in negative (MS-ESI\textsuperscript{H}) 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\textsubscript{2}CO\textsubscript{3}, 35 mM NaHCO\textsubscript{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\textsubscript{2}O\textsubscript{2} in 12.5mL of substrate buffer. Stop solution was 2M H\textsubscript{2}SO\textsubscript{4}.

**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 dissolved 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-deSCOF\(_3\)) (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-d$_4$) 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-d$_4$) 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. MSH 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-d$_4$) δ 8.06 (s, 2H). $^{13}$C NMR (151 MHz, methanol-d$_4$) 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,
Hapten 4 [4-Amino-3-(2,6-dichloro-4-(trifluoromethyl)phenyl)cyclopenta-1,4-diene-1-carboxylic acid] (297-53). FPN-deSO\textsubscript{3} (50 mg) was dissolved in 1 mL of 50% H\textsubscript{2}SO\textsubscript{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\textsubscript{4} was added to remove water traces. The residue was re-crystallized from CCl\textsubscript{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\textsubscript{1} 0.33 for the main product and Rf\textsubscript{2} 0.63 for the second spot of faint intensity.

\textsuperscript{1}H NMR (600 MHz, methanol-d4) δ 7.97 (s, 2H).
\textsuperscript{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.

\textit{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.

*Diazoization 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 (25 mg 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. 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-C0N, and serum #2268 with coating antigen 1-C0N.

**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 \( \frac{IC_{50} \text{ of fipronil}}{IC_{50} \text{ of tested compound}} \times 100 \).

**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.

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
℃ 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 0.35 mL/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.\textsuperscript{3,4}
<table>
<thead>
<tr>
<th>Analyte</th>
<th>Hapten</th>
<th>Hapten-Protein conjugate</th>
<th>Rabbit</th>
<th>Hapten</th>
<th>Hapten-Protein conjugate</th>
<th>Rabbit</th>
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<td><img src="image3" alt="Fipronil 1-CON" /> #2263</td>
<td><img src="image4" alt="Fipronil 1-BSA" /> #2264</td>
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<td><img src="image6" alt="Fipronil 4-CON" /> #2272</td>
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*position of attachment to protein
### Table S2. Competitive ELISA Results for Homologous and Heterologous Coating Antigens

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<tr>
<th>Rabbit</th>
<th>Immunogen</th>
<th>coating antigen&lt;sup&gt;a&lt;/sup&gt;</th>
<th>dilution</th>
<th>curve parameters&lt;sup&gt;d&lt;/sup&gt;</th>
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<td></td>
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<td>coat. antigen, µg/mL antiserum</td>
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<td>A</td>
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<tr>
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<td>1-Thy</td>
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<td>0.74</td>
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<sup>a</sup>See Table S1 for structures. <sup>b</sup>Homologous (hapten same as immunogen). <sup>c</sup>Heterologous (hapten different from immunogen). <sup>d</sup>A, maximum absorbance; B, slope; C, IC<sub>50</sub>; 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 (5HCON) 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-HCON) 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

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<tr>
<th>Compound</th>
<th>Structure</th>
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<th>% CR</th>
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<td>2. TETS&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>3. PTZ&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>(drug)</td>
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<tr>
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<td>6. Picrotoxin</td>
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<sup>a</sup> Tetramethylenedisulfotetramine, <sup>b</sup> 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


