Elsevier Editorial System(tm) for Environment International
Manuscript Draft

Manuscript Number: ENVINT-D-14-01277R1

Title: Identification of fipronil metabolites by time-of-flight mass spectrometry for application in a human exposure study

Article Type: Research Paper

Keywords: Fipronil, LC/TOF, Biomarker, Human Exposure, Metabolism

Corresponding Author: Dr. Mark J. Strynar, Ph.D.

Corresponding Author's Institution: U.S. EPA/ORD/NERL

First Author: Rebecca L McMahen, MS

Order of Authors: Rebecca L McMahen, MS; Mark J. Strynar, Ph.D.; Sonia Dagnino, Ph.D; David W Herr, Ph.D; Virginia C Moser, Ph.D; Stavros Garantziotis, Ph.D; Erik M Andersen, MPH; Danielle L Freeborn; Larry McMillan, BS; Andrew B Lindstrom, Ph.D.

Abstract: Fipronil is a phenylpyrazole insecticide commonly used in residential and agricultural applications. To understand more about the potential risks for human exposure associated with fipronil, urine and serum from dosed Long Evans adult rats (5 and 10 mg/kg bw) were analyzed to identify metabolites as potential biomarkers for use in human biomonitoring studies. Urine from treated rats was found to contain seven unique metabolites, two of which had not been previously reported—M4 and M7 which were putatively identified as a nitroso compound and an imine, respectively. Fipronil sulfone was confirmed to be the primary metabolite in rat serum. The fipronil metabolites identified in the respective matrices were then evaluated in matched human urine (n=84) and serum (n=96) samples from volunteers with no known pesticide exposures. Although no fipronil or metabolites were detected in human urine, fipronil sulfone was present in the serum of approximately 25% of the individuals at concentrations ranging from 0.1-4 ng/mL. These results indicate that many fipronil metabolites are produced following exposures in rats and that fipronil sulfone is a useful biomarker in human serum. Furthermore, human exposure to fipronil may occur regularly and require more extensive characterization.

Dear Editor,

I would like to submit the manuscript entitled "Identification of fipronil metabolites by time-of-flight mass spectrometry for application in a human exposure study" for consideration for publication in Environment International. This study demonstrates how advanced time-of-flight mass spectrometry techniques can be used to more fully describe the metabolism of xenobiotic compounds in dosed-animal studies and how this knowledge can be applied in human biomonitoring investigations to make relevant conclusions about human exposures to emerging compounds of concern. The compound of interest in this study is fipronil, a commonly used insecticide that has led to concerns due to the associated effects in rodents which include thyroid disruption, endocrine disruption, and neurotoxic effects. There are many possible routes for human exposure to fipronil since it is used in many applications (veterinary, agricultural, and residential pest control) and has found to be a contaminant in soil, water, and house dust. The results presented within the manuscript describe the approach for the identification of potential biomarkers of exposure to fipronil using dosed rodents, including two new metabolites, and provide the first report on human biomarker measurements in the general population. I believe that the content of the manuscript would fit well into the scope of your journal. Suggested external reviewers include:

1. Paul Lioy, Ph.D. Professor and Vice Chair Department of Environmental Medicine

UMDNJ - Robert Wood Johnson Medical School

Email: plioy@eohsi.rutgers.edu

Phone: 732-445-0155

2. Antonia Calafat, Ph.D. Distinguished Consultant at Center for Disease Control (CDC)

Email: Antonia.calafat@cdc.hhs.gov

Phone: 770-488-7891

3. Dana Boyd Barr, Ph.D. Research Professor Rollins School of Public Health Emory University

Email: dbbarr@emory.edu

Phone: 404-727-9605

Correspondence regarding this article can be sent to Dr. Mark Strynar[b], whose contact information is listed below. Other authors include Rebecca McMahen[a] (mcmahen.rebecca@epa.gov (919)541-1071), Sonia Dagnino[a] (Dagnino.sonia@gmail.com),

David Herr[c] (herr.david@epa.gov (919)541-0380), Virginia Moser[c] (moser.ginger@epa.gov (919)541-5075), Stavros Garantziotis[d] (garanziotis@niehs.nih.gov), Erik Andersen[b] (eanderse4@gmail.com), Danielle Lyke[c] (lyke.danielle@epa.gov (919)541-4339), Larry McMillan[e] (mcmillian.larry-rtp@epa.gov (919)541-5657), and Andy Lindstrom[b] (lindstrom.andrew@epa.gov (919)541-0551). I thank you for your consideration of this manuscript for publication.

Dr. Mark Stynar
*Strynar.mark@epa.gov
(919)541-1071

[a]ORISE fellow at the United States Environmental Protection Agency, National Exposure Research Laboratory, Research Triangle Park, North Carolina
[b]United States Environmental Protection Agency, National Exposure Research Laboratory, Research Triangle Park, North Carolina
[c]National Health and Environmental Effects Research Lab, U.S. Environmental Protection Agency, Research Triangle Park, North Carolina
[d]National Institute for Environmental and Health Sciences, Research Triangle Park, North Carolina
[e]National Caucus and Center on Black Aged Employee, U.S. Environmental Protection Agency, National Exposure Research Laboratory, Research Triangle Park, North Carolina

Identification of fipronil metabolites by time-of-flight mass spectrometry for application in a human exposure study

Rebecca. L. McMahen[a], *Mark J. Strynar*[b]*, *Sonia Dagnino*[a], *David W. Herr*[c], *Virginia C. Moser*[c], *Stavros Garantziotis*[d], *Erik M. Andersen*[b], *Danielle F. Lyke*[c], *Larry McMillan*[e], *Andrew B. Lindstrom*[b]

[a]ORISE fellow at the United States Environmental Protection Agency, National Exposure Research Laboratory, Research Triangle Park, North Carolina

[b]United States Environmental Protection Agency, National Exposure Research Laboratory, Research Triangle Park, North Carolina

[c]National Health and Environmental Effects Research Lab, U.S. Environmental Protection Agency, Research Triangle Park, North Carolina

[d]National Institute for Environmental and Health Sciences, Research Triangle Park, North Carolina

[e]National Caucus and Center on Black Aged Employee, U.S. Environmental Protection Agency, National Exposure Research Laboratory, Research Triangle Park, North Carolina

*CORRESPONDING AUTHOR:

Phone: (919)541-3706

Address: 109 TW Alexander Dr. Durham, NC 27705

Email: strynar.mark@epa.gov

ABSTRACT

Fipronil is a phenylpyrazole insecticide commonly used in residential and agricultural applications. To understand more about the potential risks associated with fipronil, dosed Long Evans rats were evaluated for metabolites to develop a set of biomarkers for use in human exposure studies. Urine from treated rats was found to contain seven unique metabolites, two of which had not been previously reported. Fipronil sulfone was confirmed to be the primary metabolite in rat serum. The fipronil metabolites identified in the respective matrices were then evaluated in matched human urine and serum samples from volunteers with no known pesticide exposures. Although no fipronil or metabolites were detected in human urine, fipronil sulfone was present in the serum of approximately 25% of the individuals at concentrations ranging from 0.1-4 ng/mL. These results are comparable to results from an exposure study of workers in a fipronil production facility. These results indicate that many fipronil metabolites are produced following exposures in rats and that fipronil sulfone could be a useful biomarker in human serum. Furthermore, human exposure to fipronil may occur regularly and require more extensive characterization.

Keywords: Fipronil, LC/TOF, Biomarker, Human Exposure, Metabolism

ABBREVIATIONS

DI: Deionized

ESI: electrospray ionization

GABA: gamma-aminobutyric acid

HPLC: high performance liquid chromatography

LC: liquid chromatography

LOQ: limit of quantitation

MS: mass spectrometry

NIEHS: National Institute for Environmental Health Sciences

QC: quality control

Q-TOF: quadrupole time-of-flight

% RSD: Percent Relative Standard Deviation

SPE: solid phase extraction

TOF: time-of-flight

UPLC: ultra performance liquid chromatography

US EPA: United States Environmental Protection Agency

WWTP: waste water treatment plant

1. INTRODUCTION

Fipronil (Figure 1) is a phenylpyrazole broad-spectrum insecticide that is registered for use in residential settings including ant baits and gels, cockroach baits and gels, and termite control products; veterinary applications such as spot treatment flea and tick control products for dogs and cats; ornamental turf applications such as fire ant control; and agricultural applications such as pest control on potato crops[1]. When initially produced, fipronil was the first insecticide to act

by targeting the gamma-aminobutyric acid (GABA) receptor and has favorable selective toxicity towards insects rather than mammals[2-4].

A 1997 report indicated that 480 tons of fipronil were produced per year by Rhone Poulenc,[5] and between 1998 and 2008 it was reported that usage averaged 150,000 pounds of active ingredient per 1.5 million acres[1]. Widespread fipronil use has led to contamination of water and soil (1-158 ng/L of parent or environmental degradate) in several states including, but not limited to Alabama, Georgia, California, Louisiana, and Indiana[6, 7]. Perhaps as a result of this contamination, fipronil has been implicated as one of the chemicals associated with the colony bee collapse[8].

Because little was found in the peer-reviewed literature about the disposition of fipronil, Cravedi et al. (2013) performed a thorough study on the metabolism, distribution, and elimination of fipronil in rats that showed fipronil is primarily converted to fipronil sulfone (M1 Figure 1), a metabolite which was stored mainly in adipose tissue and adrenals[9]. Fipronil's association with thyroid disruption[10], endocrine disruption[11], and neurotoxic effects[12] in rats has also led to a growing concern about the potential for human health effects in the last decade.

The effects of acute human exposure to fipronil include headache, dizziness, vomiting, and seizures[9, 10]. Information on the effects of chronic exposure is limited, but the US EPA has classified fipronil as a possible human carcinogen based on data that shows an increase of thyroid follicular cell tumors in both sexes of the rat[13]. Vidau et al. (2011) also concluded that fipronil has the potential to cause apoptosis by uncoupling oxidative phosphorylation at relatively low concentrations (5-10 μM) in human cell lines[14]. A case of acute human self-poisoning with fipronil has demonstrated that fipronil levels can remain elevated in serum for days after exposure, and that fipronil sulfone was the primary metabolite[15]. A previous study also

showed that fipronil sulfone is the predominant metabolite in human liver microsomes via cytochrome P-450 oxidation[16]. Very little is known about human exposure to fipronil in the general population. One occupational exposure study of workers at a fipronil production facility reports a mean fipronil sulfone serum level of 7.79 ng/mL[17].

There is little published on fipronil in humans[14, 15, 17] and no data from the general population. This may be because human samples can be difficult to obtain and analyze. They often have significant matrix effects due to high concentrations of endogenous chemicals, making the identification of metabolites difficult. Therefore, we used a unique workflow where dosed animal samples were used to develop a set of potential serum/urine biomarkers using time-of-flight mass spectrometry. Serum and urine samples from human subjects with no known exposures were then analyzed via targeted screening for the putative fipronil biomarkers to characterize fipronil exposure in humans from the general population.

2. MATERIALS AND METHODS

Chemicals. Unlabeled fipronil (5-amino-1-[2,6-dichloro-4-(trifluoromethyl)-phenyl]-4-(trifluoromethylsulfinyl)-1-H-pyrazole-3-carbonitrile, >99%) and its metabolites: fipronil sulfone (5-amino-1-[2,6-dichloro-4-(trifluoromethyl)-phenyl]-4-[(trifluoromethyl)sulfonyl]-1*H*-pyrazole-3-carbonitrile, >99%), fipronil sulfide (5-amino-1-[2,6-dichloro-4-(trifluoromethyl)-phenyl]-4-[(trifluoromethyl)thio]-1*H*-pyrazole-3-carbonitrile, 98%), fipronil amide (5-amino-1-[2,6-dichloro-4-(trifluoromethyl)-phenyl]-4-[(trifluoromethyl)thio]-1*H*-pyrazole-3-carboxamide, >99%), and monochloro fipronil (5-amino-1-[2-chloro-4-(trifluoromethyl)-phenyl]-4-[(trifluoromethyl)sulfinyl]-1*H*-pyrazole-3-carbonitrile, >97%) were procured as solid analytical standards from the pesticide repository through the US EPA (Fort Meade, MD, USA). These five analytical standards were prepared as a mixture in acetonitrile and used for all subsequent

matrix-matched standard curves. The internal standard fipronil des-F_3 (see supporting information for structure) (5-amino-1-[2,6-dichloro-4-(trifluoromethyl)-phenyl]-4-(methylsulfinyl)-1-H-pyrazole-3-carbonitrile, 99%, 0.1 ng/uL in Acetonitrile) was ordered from Crescent Chemical Company (Islandia, NY, USA).

Acetonitrile and methanol (B&J Brand HighPurity Solvent) were purchased from Honeywell Burdick & Jackson (Muskegon, MI,USA) and ammonium acetate from Sigma Aldrich (St. Louis, MO, USA). Deionized water was generated in house from a Barnsted Easypure UV/UF (Dubuque, IA, USA) coupled with activated charcoal and ion exchange resin canisters.

2.1 Animals. This study was part of an investigation of the neurotoxic effects of fipronil in rodents[18, 19]. The animal facility is accredited by the American Association for Accreditation of Laboratory Animal Care International, and all protocols were approved by the National Health and Environmental Effects Research Laboratory Institutional Animal Care and Use Committee at the United States Environmental Protection Agency. Male Long Evans rats (60-90 days old) were acquired from Charles River Laboratories (Wilmington, MA). Animal husbandry details are provided in the Supporting Information. Animals were dosed repeatedly by oral gavage at either 5 (low dose) or 10 (high dose) mg/kg with fipronil suspended in corn oil (1 mL/kg) every 24 hours for two weeks. Control rats were gavaged with corn oil only. Six hours after the 14th dose, rats were euthanized. Trunk blood was collected in tubes without anticoagulant and stored on ice for 1-1.5 h.. The samples were centrifuged at 1300 × g for 30 min. at 4° C. The serum was collected, frozen on dry ice, and stored at -80 ^{o}C until analysis. Urine was collected in a syringe either from voids on a clean table or via bladder puncture and transferred to a micro-centrifuge tube, immediately frozen on dry ice, and stored at -80 °C until analysis.

2.2 Human Samples. Matched human urine (*n*=84) and serum (*n*=96) samples, from individuals with no known fipronil exposure, were collected by the National Institute for Environmental and Health Sciences (NIEHS protocol number 10-E-0063) between April and June 2011. The human samples were simply a sample of convenience and were not meant to be representative of a specific population. The urine collected was a spot sample and was not concentrated or representative of a specific sampling period. Volunteers were anonymous, and no personally identifiable information was provided. The samples were from male and female volunteers of various ethnicities between 19 and 73 years of age who live in the Raleigh-Durham area of North Carolina (Table 1). Although 100 volunteers participated in the study, several urine and serum samples were not included due to an insufficient volume for analysis.

Table 1. Human demographic data.

	Sex		Age				Race			
	Male	Female	19-33	34-48	48-62	62-76	Asian	Black	White	Other
%	30	70	29	30	33	8	3	32	63	2

2.3 Extraction Protocols. Samples were extracted in a manner that optimized recovery and reproducibility while reducing matrix interference. Animal samples were small volumes that did not require solid phase extraction (SPE). However, a protocol involving SPE was performed with the human samples to reduce matrix interference. Sample extraction protocols for biologicals are described below. More information on methods development for human samples can be found in the Supporting Information. Rat serum samples were first analyzed by liquid chromatography/time-of-flight mass spectrometer (LC/TOF-MS) in order to identify any metabolites. Human samples were then analyzed by liquid chromatography/triple-quadrupole mass spectrometer (LC/triple-quad) for quantification of metabolites for which analytical

156 standards were possessed. LC/Q-TOF was used for structure elucidation of unknown
157 metabolites.

158 **2.4 Rat serum.** Rat serum (25 µL) was denatured with 100 µL of 0.1 M formic acid and
159 precipitated with 1 mL of a cold acetonitrile solution spiked with the internal standard (fipronil
160 des-F_3, 25 ng). The sample was then centrifuged for 5 minutes at 12500 × *g*. An aliquot of the
161 supernatant was mixed 50:50 with 10 mM ammonium acetate buffer, and analyzed via LC/TOF
162 and LC/triple-quad. *n*=9 for high dose (10 mg/kg/day) ; *n*=10 for low dose (5 mg/kg/day); and
163 *n*=11 for control animals, which were treated with vehicle. Quantitation was performed for
164 fipronil and fipronil sulfone. The results of the quantitation are shown in the supporting
165 information.

166 **2.5 Rat urine.** Rat urine (100 uL) was precipitated with 900 µL of cold acetonitrile and
167 centrifuged for 8 minutes at 12500 × *g*. An aliquot of the supernatant was extracted and mixed
168 50:50 with 10 mM ammonium acetate buffer before LC/MS analysis. *n*=3 for high dose (10
169 mg/kg/day); *n*=4 for low dose (5 mg/kg/day); and *n*=3 for control animals. Quantitation was only
170 performed for the fipronil sulfone metabolite, as standards were not available for other
171 metabolites. Quantitation specifics can be found in the Supporting Information. Fipronil sulfone
172 concentrations in rat urine were used to approximate the relative concentrations of the other
173 observed metabolites.

174 **2.6 Human serum.** Human serum (200 µL) was denatured with 20 uL of a 0.1 M formic acid
175 solution spiked with internal standard (fipronil des-F_3, 5 ng) and precipitated with 2 mL of cold
176 acetonitrile. The sample was centrifuged for 10 minutes at 12500 × *g* and concentrated using
177 solid phase extraction (SPE) using an Oasis 3cc HLB cartridge (Waters Corporation, Milford,
178 MA) SPE cartridges were conditioned with 3 mL of methanol and 3 mL of ultrapure water,

179 samples were loaded, washed with 3 mL of 95:5 water/acetonitrile solution, then eluted with 3
180 mL of acetonitrile. The eluate was evaporated under N_2 at 40° C until approximately 200 µL
181 remained. The concentrated solution was mixed 50:50 with 10 mM ammonium acetate buffer
182 and analyzed via LC/TOF and LC/triple-quad (*n*=96). In order to determine the concentration of
183 compounds of interest, a seven-point matrix-matched (blank calf serum-Life Technologies-
184 Gibco®, Grand Island, NY) extracted standard curve from 0.1-50 ng/mL, along with a method
185 blank (DI water) and a matrix blank was run with the human serum samples. The lowest value on
186 the standard curve (0.1 ng/mL) was considered the lower limit of quantitation (LLOQ).

187 **2.7 Human urine.** Human urine (5-12 mL) was precipitated with 1 mL of acetonitrile and
188 concentrated using the SPE method described above with an Oasis 6cc HLB cartridge with the
189 exception that cartridges were conditioned with 5 mL of methanol and 5 mL of ultrapure water,
190 samples were loaded, washed with 5 mL of 95:5 water/acetonitrile solution, then eluted with 5
191 mL of acetonitrile. The eluate was evaporated under N_2 at 40° C until approximately 1 mL
192 remained. The concentrated solution was mixed 50:50 with 10 mM ammonium acetate buffer in
193 an LC vial and analyzed by LC-TOF/MS (*n*=84). Note that several urine samples were excluded
194 due to insufficient volume.

195 **2.8 Analytical Instrumentation.** Targeted analyses (LC/triple-quad) were carried out using an
196 Agilent 1100 HPLC (Agilent Technologies, Palo Alto, CA) interfaced with a Sciex 3000 triple
197 quadrupole mass spectrometer (Applied Biosystems/MDS Sciex, Foster City, CA) fitted with an
198 electrospray ionization source (ESI) operated in the negative ionization mode. Compounds
199 contained in the LC/triple-quad method (fipronil, fipronil sulfone, fipronil sulfide, fipronil
200 amide, and monochloro fipronil) were optimized on a compound specific basis. Information
201 regarding transitions are included in the Supporting Information.

The HPLC system consisted of a Phenomenex Luna C18 column (50 x 3 mm, 5 µm; Torrance, CA, USA) with a Security-guard guard column (Phenomenex). The method consisted of the following: 0.4 mL/min flow rate which increased to 0.75 mL/min at time=2 min; temperature: 30 °C; mobile phases – A: ammonium acetate buffer (0.2 mM) and DI water:methanol (95:5, v/v), and B: ammonium acetate buffer (0.2 mM) and acetonitrile:DI water (95:5, v/v); gradient: 0-2 min 50% A and 50% B; 2.1-4 min, a linear gradient from 50:50 A:B to 10:90 A:B; 4-6 min 10% A and 90% B; 6.1-10 min re-equilibration to 50% A and 50% B.

Non-targeted analyses (LC/TOF) were carried out using an Agilent 1100 HPLC (Agilent Technologies, Palo Alto, CA) interfaced with an Agilent 6210 Time-of-Flight (TOF) mass spectrometer fitted with an electrospray ionization source operated in the negative ionization mode at 120 Volts. Any drift in the mass accuracy of the TOF was continuously corrected by infusion of two reference compounds (purine [*m/z* = 119.0363] and hexakis (1H, 1H, 3H-tetrafluoropropoxy) phosphazene [*m/z* = 966.0007]) via dual-ESI sprayer.

The HPLC method consisted of a Zorbax Eclipse Plus C18 column (2.1 × 50 mm, 3.5 um; Agilent Technologies, Palo Alto, CA) fitted with a Phenomenex guard column (Torrance, CA). The method consisted of the following: 0.2 mL/min flow rate; at 30 °C; mobile phases: A: ammonium formate buffer (0.4 mM) and DI water:methanol (95:5 v/v), and B: ammonium formate (0.4 mM) and methanol:DI water (95:5 v/v); gradient: 0-5 min a linear gradient from 50:50 A:B to 100% B; 5-15 min, 100% B; 15-18 min re-equilibration to 50% A and 50% B.

2.9 Identification of Spectral Features. The TOF-MS system has proprietary software that can be used in non-targeted analyses to help identify compounds that are specific to a treatment group or a specific experimental condition. For example, to identify potential biomarkers of fipronil exposure, control and dosed animal samples are analyzed, and molecular features

225 (identifiable peaks) were first extracted according to user specified criteria (e.g., minimum peak
226 height, area count). The two groups of extracted features were then compared using The Mass
227 Profiler software, which singles out only those compounds that are found in the dosed group.
228 This collection of compounds can be thought to represent either the parent compound,
229 metabolites of the parent, or specific biological responses that are attributable to the treatment
230 administered.

231 The exact monoisotopic mass of each of these "treatment only" features was then used to
232 generate a ranked list of possible chemical formulae for each unknown. The numerical ranking
233 is based on the difference between the calculated and measured mass, the isotopic abundance and
234 the isotope spacing. If authentic standards are available, the identity of a proposed feature can be
235 confirmed using chromatographic retention time, exact monoisotopic mass, and isotopic
236 distribution.

237 Fipronil is an interesting and somewhat unique compound because it contains six fluorine
238 atoms and two chlorine atoms, that result in a significant negative mass defect (435.93869 Da,
239 with the $[M-H]^-$ ion seen in negative ionization mode being 434.9314 *m/z*) which is preserved in
240 most of its metabolic products to the extent that the F and Cl atoms are retained[20]. Moreover, the
241 isotopic spacing between the Cl isotopes (^{35}Cl [75.77%] and ^{37}Cl [24.23%]) leads to a distinctive
242 isotopic pattern that aids greatly with identification (SI Figure 2). Both of these characteristics
243 were useful in identifying fipronil-related metabolites.

244 Metabolites that were identified using the LC/TOF instrument described above were then
245 investigated further using an Agilent 1200 HPLC system fitted with a 6250 quadrupole time-of-
246 flight mass spectrometer (Q-TOF) (Agilent Technologies, Palo Alto, CA) using the same LC

247 conditions as previously described. The LC/Q-TOF allowed fragmentation at various collision
248 energies of metabolites of interest which helped with structure elucidation.

249 **2.10 Quality Assurance/Control.** For each analysis, method and matrix blanks were
250 evaluated for contamination or background levels of the compounds of interest. Three randomly
251 chosen samples were replicated in each quantitative experiment to ensure consistency within the
252 data sets. Parent-daughter ratios should be consistent, and ratio monitoring is a robust way to
253 confirm the presence of a specific compound. Therefore, in the targeted screening of samples,
254 the ratio between the primary and secondary parent-daughter transition was monitored to confirm
255 the presence of each compound in the MS method. High and low concentration quality control
256 (QC) samples containing the fipronil mixture of five analytical standards described in the
257 *Chemicals* section were run with each batch of human serum samples. These samples were
258 included to ensure analytical precision and accuracy.

259 **2.11 Statistics.** GraphPad Prism version 6.0 was used for statistical analyses of the fipronil
260 sulfone concentrations in human serum with respect to race, age, and gender. Normality was
261 tested using the Shapiro-Wilk normality test. Values were not normally distributed; therefore, we
262 used nonparametric statistics (Mann-Whitney test for comparison of ranks, and Spearman
263 correlation analysis) for all analyses. Statistics for gender and race differences were based on a
264 non-detect/detect designation of “0” and “1”, respectively. Values that were below the LOQ
265 (below the lowest curve point) were replaced with LOQ/2 for the Spearman Correlation analysis.
266 All tests were carried out at the 95% confidence level.

267 3. RESULTS

268 **3.1 Quality Assurance/Control.** All lab prepared target and non-target analysis blanks and
269 control samples were below the LLOQ for compounds of interest in all experiments. All

replicates for all experiments had a mean standard error of <15% for all replicates and ensured reliable data. For all targeted analyses, the ion ratios between the primary and secondary parent-daughter transitions were consistent for all standard compounds (mean ± 20%) and confirmed analytical precision. All QC samples (high and low) were 100% ± 15% of the nominal values.

3.2 Urine from Treated Rodents. The urine from rodents treated for 14 days with fipronil was analyzed for biomarkers of exposure via non-targeted analysis. As described above, molecular features (significant chromatographic peaks) were extracted from analytical runs of both dosed and control animals, and The Mass Profiler software was used to isolate those features that were unique to the dosed animals. The most plausible candidate biomarkers were those compounds with the signature isotope pattern of two chlorine atoms (SI Figure 2) and/or significant negative mass defects indicative of fluorine and chlorine atoms. Seven high abundance peaks fitting these criteria were identified, and the exact monoisotopic mass of each was used to generate a ranked list of plausible formulae and corresponding structures. We ultimately assigned tentative compound identity according to known metabolic pathways (e.g., oxidation, sulfation, glucuronidation), the retention of negative mass defect and/or the isotopic pattern associated with chlorine, and consistency with results from previous studies. Information on the seven metabolites can be found in Table 2. Four of the compounds (M1, M2, M3, M5, and M6) were identified in previous studies[9, 21], whereas two more (M4 and M7) are reported for the first time in this study (Figure 1). It should be noted that the spectral feature observed for the glucuronide conjugate (M6) splits into two chromatographic peaks, most likely meaning that the glucuronide molecule adds to both the oxygen and the nitrogen atom (see Figure 1). We were unable to differentiate which peak corresponded to which structure, but one was formed preferentially. However, this spectral feature is not observed for the sulfate conjugate (M5).

To better characterize the structures of metabolites M4 and M7, the fragmentation patterns of the parent metabolites were analyzed via LC/Q-TOF. No useful information was gained about metabolite M4; however, the fragmentation pattern of metabolite M7 helped to predict a plausible structure. M7 structural information could be gleaned from looking at the exact masses of molecular fragments originating from the parent molecule. For example, if the mass of a CO_2 group is observed in the fragmentation pattern, it can be assumed that the molecule likely contained a carboxylic acid. Spectral information regarding this fragmentation pattern can be found in the Supporting Information (SI Figure 3).

Fipronil sulfone (M1) was confirmed by an authentic standard that has the same retention time, monoisotopic mass, ion fragmentation pattern, and isotope spacing. Rats in the 5 mg/kg/day dose-group had median concentrations of fipronil sulfone of 25.4 (± 18.7) ng/mL, while the 10 mg/kg/day group had 31.9 (± 13.2) ng/mL (SI Figure 1). If the fipronil sulfone concentrations are used to generate estimated relative response factors for other metabolites that do not have standards (assuming that all respond similarly within the TOF-MS), we estimate the relative concentrations of fipronil metabolites in dosed-rodent urine to be M6>M4>M5>M3>M7>M1>M2. The estimated concentrations of M2 and M6 are 30 and 2,000 ng/mL respectively.

Table 2. LC/TOF characteristics of putative metabolites in rat urine.

Metabolite	Retention Time (mins)	Predicted Formula of parent	Score of Predicted Formula	$[M-H]^-$ Measured Mass *(m/z)*	$[M-H]^-$ Calculated Mass *(m/z)*	Δ ppm	Monoisotopic Mass *(m/z)*
M1 (Fipronil Sulfone)	7.57	$C_{12}H_4Cl_2F_6N_4O_2S$	99.63	450.9266	450.9263	0.67	451.9336
M2	7.3	$C_9H_4Cl_2F_3N_3$	93.50	279.9665	279.9662	1.07	280.9734
M3	1.62	$C_{11}H_4O_2N_4Cl_2F_3$	99.53	350.9667	350.9669	0.43	351.9742
M4	5.38	$C_{10}H_4Cl_2F_3N_3O_2$	98.63	323.9565	323.9560	1.54	324.9633
M5	1.4	$C_{11}H_5Cl_2F_3N_4O_4S$	99.38	414.9290	414.9288	0.48	415.9361
M6	1.39	$C_{17}H_{13}Cl_2F_3N_4O_7$	98.74	511.0036	511.0041	0.98	512.0113
M7	5.38	$C_{11}H_3Cl_2F_3N_4O$	98.93	332.9564	332.9563	0.30	333.9563

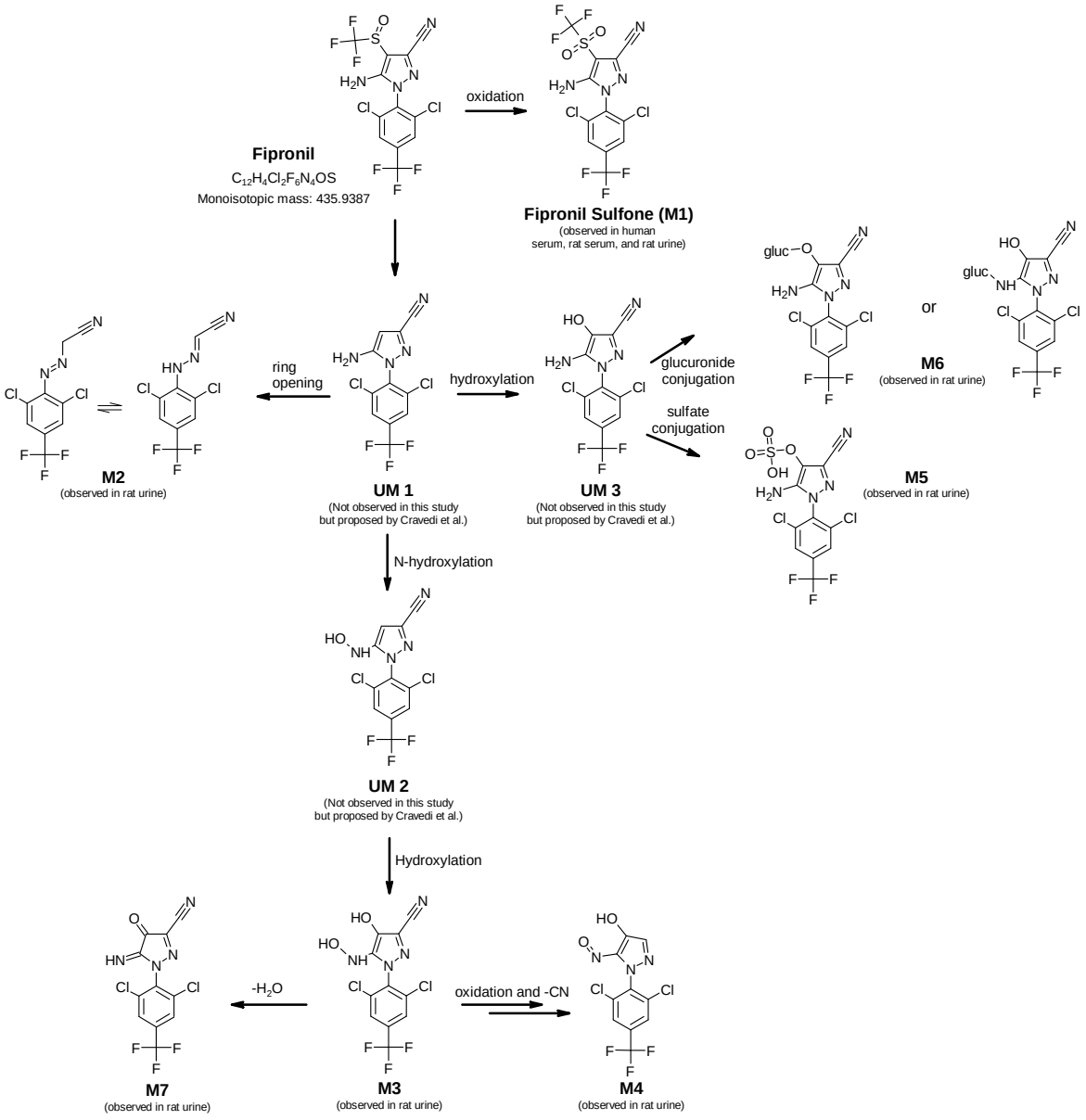


Figure 1. Proposed metabolic pathway of fipronil in the rat. M4 and M7 are proposed structures based on MS data, isotope distributions, and exact mass. M1, M2, M3, M5, and M6 were identified in rat urine. Unobserved metabolites labeled (UM) were not identified but are likely intermediates.

3.3 Serum from treated rodents. The serum from treated rats was analyzed for all suspected biological metabolites via LC-TOF to evaluate the presence of possible serum biomarkers. In our analysis we detected no additional metabolites other than small amounts of un-metabolized fipronil and fipronil sulfone which had been previously identified by several groups [4, 22]. Quantitative data for fipronil and fipronil sulfone in rat serum can be found in the Supporting Information.

3.4 Human urine. Urine samples from 100 volunteer North Carolina residents with no known exposures to fipronil or other pesticides were examined for M1-M7 (identified in rodent urine) and for all other plausible fipronil adducts or derivatives using the methods described above. No parent fipronil or any plausible metabolites were found in the human urine samples.

3.5 Human serum. Matched human serum samples were analyzed for the metabolites observed in rat serum (fipronil and fipronil sulfone) by a targeted approach (LC/triple-quad, LOQ = 0.1 ng/mL). Only trace amounts of the parent fipronil were found in the human blood samples. However, fipronil sulfone (the putative biomarker identified in the rodent study) was detected in approximately 25% of the samples, at levels ranging from 0.1 to 4 ng/mL [mean = 0.2 (±0.6) ng/mL] (Figure 2).

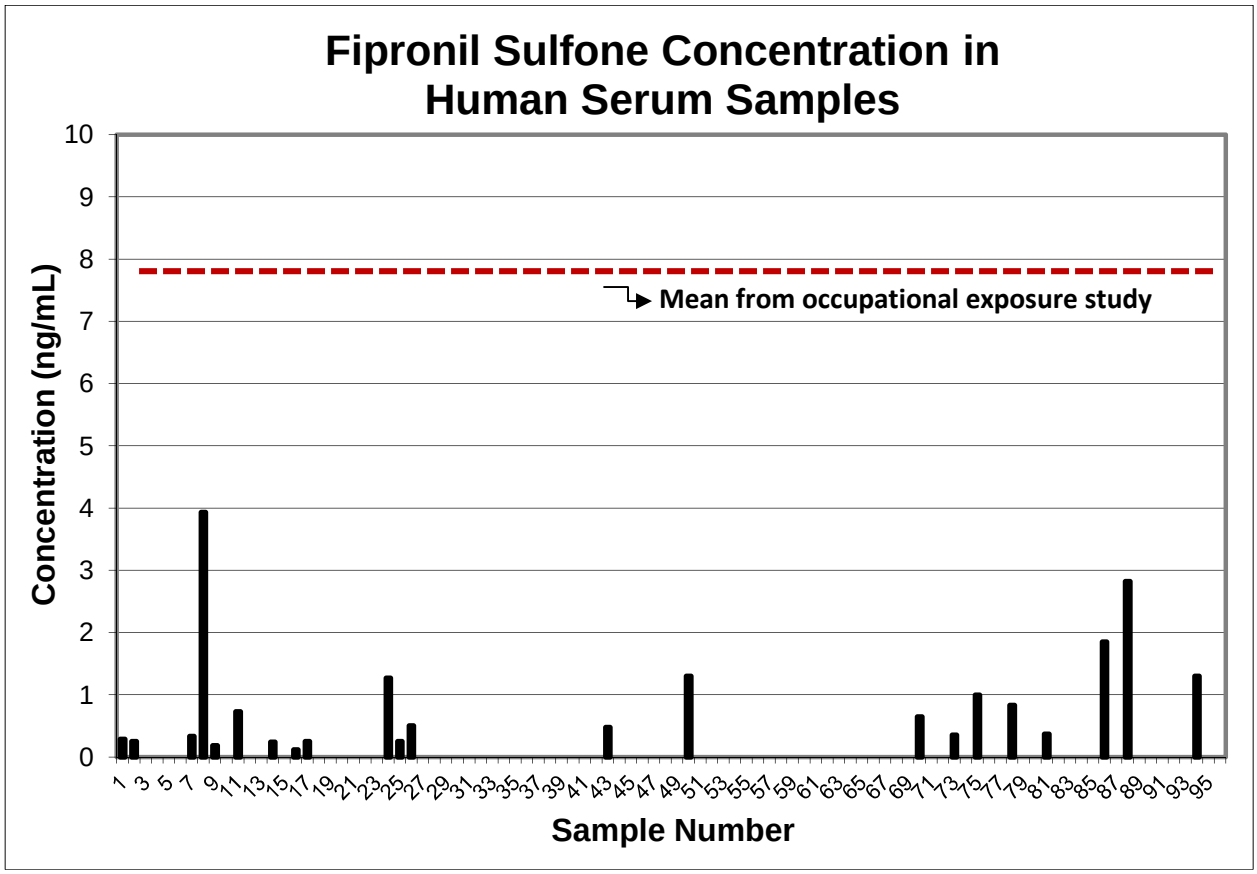


Figure 2 shows fipronil sulfone concentrations in human serum*. The red dotted line represents the mean (7.79 ng/mL) observed in an occupational exposure study.

**n* = 96, four samples were excluded due to insufficient volume.

3.6 Statistical Analyses. Statistics (for the human serum data) showed that race and age may have some impact on the level of fipronil sulfone in human serum. A slight positive correlation was found for increasing age and fipronil sulfone level (Spearman r = 0.21 and p = 0.042). Caucasians had median fipronil sulfone levels that were significantly higher than in African Americans (p < 0.0001 and Mann-Whitney U = 556) (the Asian and "other" categories were excluded from statistical analyses because there were too few samples). However, no significant difference was found between males and females (p = 0.99 and Mann-Whitney U = 959.5). Information regarding statistics can be found in the Supporting Information.

4. DISCUSSION

This study demonstrates how advanced time-of-flight mass spectrometry techniques can be used to more fully describe the metabolism of xenobiotic compounds in treated-animal studies and how this knowledge can be applied in human biomonitoring studies to make relevant conclusions about human exposures to emerging compounds of concern. Our specific goal was to use the biomarkers identified from the dosed rodent work in the analysis of a set of human biological samples to characterize the rate of fipronil exposure in the general population.

In describing the metabolism of fipronil in rodents, our results were largely consistent with previous studies,[9, 21, 23] while also extending what is known about the basic metabolic process. Two novel metabolites observed in rat urine in this study which were not seen by Cravedi et al. (2013) can be attributed to differences in study design. Specifically, our Long Evans rats were dosed (5 or 10 mg/kg/day) for 14 days then sacrificed 6 hours after the last dose. In contrast, Cravedi et al. (2013) dosed acutely at 10 mg/kg and collected urine and serum every 24 h. over a 72 h. period[9]. Differences between rat strain or length of dosing regimen may have made it possible to identify different products of fipronil metabolism, such as the pyrazole ring opened products or the highly oxidized heteroaromatic amine derivatives.

The proposed metabolic pathway in the rat and compound structures can be found in Figure 1. We propose that a new metabolite (M7) in rat urine, an imine, results from the loss of water from metabolite M3, which is a fipronil metabolite that is hydroxylated at both the carbon and the nitrogen. We also identified what is hypothesized to be nitroso compound (M4). We believe that M3 and M4 are formed from an unobserved hydroxyl amine intermediate (UM 2). The hydroxyl

amine (M3) has been identified in this and in previous studies[9], but to our knowledge this is the first report of a nitroso metabolite of fipronil in rat urine. Although the structure for metabolite M4 is only putative, heterocyclic aromatic amines are known to undergo biological oxidation to form nitroso compounds. This process is mediated by cytochrome P-450 and NADPH[24]. Many heterocyclic amines are known carcinogens,[25-29] due to their ability to be hydroxylated and then form DNA adducts. The observation of N-hydroxylated fipronil metabolites in this and other rodent studies warrants further investigation of fipronil metabolism in humans and the resulting effects.

Noninvasive biomarkers like those present in urine, exhaled breath, hair, fingernails, etc. are optimal for use in human studies, and one intention of this study was to explore whether any of the urinary metabolites found in the rats could be used as biomarkers of exposure in humans. Studies with human liver microsomes have shown that fipronil is metabolized to fipronil sulfone *in vitro*, and Mohamed et al. (2004) have identified fipronil sulfone as a metabolite in humans acutely exposed to high doses[15, 16]. ENREF 13 Aside from these, no publications comment on the disposition of fipronil in humans. In this study we analyzed human urine samples for any of the metabolites identified as possible biomarkers in rat urine. The absence of fipronil and its metabolites in the human urine samples was undoubtedly related to many factors. To start with, it is possible that most human elimination of these materials occurs via the feces, as is the case with rodents[13, 23]. Secondly, and perhaps more importantly, our study subjects were essentially volunteers from the Raleigh-Durham area of North Carolina with no known exposures to fipronil and/or any other similar pesticides. Identification of small amounts of unknown chemicals in urine from populations with no known exposure can be difficult due to the large amount of endogenous compounds found in the matrix. A more effective strategy would be to work with a

group of individuals with higher exposure levels (preferably occupationally) to determine human urinary metabolites. Despite negative findings with the human urine samples, 25% of the serum samples contained measureable amounts of fipronil sulfone (range 0.1 – 4 ng/mL), providing clear evidence that humans are regularly exposed to fipronil. Interestingly the highest concentrations found in this study were only half of the occupationally exposed worker serum levels reported[17].

The general population likely shares specific exposure routes. One of the most likely routes of exposure is contact with pets that have received applications of fipronil (i.e. Frontline® Plus) or have had contact with indoor/outdoor applications around the home. Notably, Morgan et al. (2008) concluded that family pets can act as vehicles for human exposure to the organophosphorous insecticides, such as diazinon[30]. Specifically, fipronil is widely used to control residential insect pests such as termites and fire ants outdoors where pets frequent, leading to transport of the material indoors. Furthermore, many flea and tick topical products contain approximately 10% fipronil and are applied directly to the skin and fur of dogs and cats, leading to human exposure to fipronil through direct contact with their pets. Dyk et al. (2012) used a fluorescent indicator to show that these fipronil residues are easily transferred from pets to humans by way of direct contact for one week following application[31]. According to estimates from the American Humane Association, up to 46% and 39% of US households keep dogs and cats, respectively. Use of fipronil containing products with these animals could conceivably result in some measurable human exposures. Ongoing efforts in our lab (data not shown) are investigating domestic indoor sources of exposure that may be important, since local waste water treatment plant (WWTP) effluent is shown to contain fipronil and metabolites.

Statistical analysis showed that higher concentrations of fipronil sulfone in human serum was correlated with increasing age and that people who self-identified as Caucasians had median concentrations of fipronil sulfone in serum that was significantly higher than those who self-identified as African Americans. The difference in the two races was particularly interesting, since the *p* value was very small and noticeable trends can be seen in the data with respect to detects and non-detects (see Supporting Information). No conclusions can be drawn from these observations because the sample set was from a relatively small subset of the population and no metadata was known. However, factors such as race or socioeconomic status have been found to influence exposure rates for other chemical classes[32-34].

While the target of fipronil is insects, the two trifluorormethyl groups of fipronil may increase the compound's absorption and distribution upon accidental exposure by humans. Approximately 20-25% of drugs produced in the pharmaceutical industry contain at least one strategically incorporated fluorine atom (usually in the form of either one fluorine atom or a trifluoromethyl group) because fluorine can significantly impact lipophilicity and improve the bioavailability of orally administered drugs. Several studies have shown that the addition of fluorine, the most electronegative element, can decrease the pKa and therefore basicity of surrounding functional groups[35, 36]. Although the effect is not always predictable, this decreased basicity stabilized molecules in the harsh acidic conditions of the stomach and increases bioavailability[37, 38]. Another factor that affects the absorption and distribution of a molecule is lipophilicity. Compounds usually enter into cell membranes via passive transport (although active transport is an alternate mechanism). Passive transport requires that the molecule is able to permeate the cell membrane, but also avoid entrapment by the lipid bilayer. The electron withdrawing capabilities of fluorine can, in some cases, be incorporated to tune a compound's

lipophilicity and ease passive transport into cells[39-41]. Fipronil's presence in human serum demonstrated that the chemical is, in fact, absorbed by humans. Further, Hainzl et. al (1996) found that fipronil lost almost all activity in neurotoxicity studies on mice without the trifluoromethylsulfinyl functional group.[2] Metabolites of fipronil have also been found in many rat tissues, including brain cells[2, 4, 9], demonstrating that even highly selective membranes are somewhat permeable to these chemicals. The fluorinated functional groups may increase fipronil's potency as an insecticide; however, they may also increase absorption and distribution of the potentially toxic compound in non-target organisms, such as humans. Considering that fipronil has been associated with endocrine disruption, neurotoxicity, and carcinogenicity[11-14], accidental exposure and increased bioavailability may be problematic.

In conclusion, previously reported metabolites in rat urine and serum were confirmed, and two novel urinary metabolites have been proposed. The putative biomarkers determined in the rodent study were used in human serum analysis, where fipronil sulfone was found in approximately 25% of serum samples from a random population of North Carolina residents. Serum fipronil levels in our study suggest that human exposure to fipronil may be common, and comparable to occupationally exposed workers. Matched urine was also analyzed, but no fipronil or any of its metabolites were identified, which suggests that urine may not be an appropriate matrix for biomonitoring populations with no known exposure to fipronil. More extensive characterization of the metabolites produced in humans exposed to higher levels of fipronil, as well as the effects from low but chronic exposure to fipronil is needed. Further investigations are also necessary to describe the sources of fipronil exposure and identify rates of exposure in other populations.

AUTHOR CONTRIBUTIONS

462 The manuscript was written through equal contributions of all authors. All authors have given
463 approval to the final version of the manuscript.

464 ## ACKNOWLEDGMENTS

465 This research was supported by an appointment to the Research Participation Program at the
466 National Exposure Research Laboratory administered by the Oak Ridge Institute for Science
467 Education through an interagency agreement between the U.S. Department of Energy and the
468 U.S. Environmental Protection Agency. We thank Agilent Technologies for providing us with
469 the LC/TOF mass spectrometer under a CRADA (#437-07) that was used to investigate
470 metabolic products. We also thank Michael Hays of US EPA who kindly allowed the use of his
471 LC/Q-TOF mass spectrometer for further structure elucidation, Benny Pyke of Arizona State
472 University for discussions on detection of fipronil in human urine, and Matthew Stiegel for help
473 with statistics.

474
475
476

477 ## DISCLAIMER

478 This article will be reviewed in accordance with the policy of the National Exposure Research
479 Laboratory, U.S. Environmental Protection Agency, and approved for publication. Approval
480 does not signify that the contents necessarily reflect the view and policies of the Agency, nor
481 does mention of trade names or commercial products constitute endorsement or recommendation
482 for use.

483

484 REFERENCES

485

486 1. Brassard, D.; Haddad, S.; Hill, E., US EPA BEAD Chemical Profile for Registration
487 Review: Fipronil (PC Code: 129121). In 2011.

488 2. Hainzl, D.; Casida, J. E., Fipronil insecticide: Novel photochemical desulfinylation with
489 retention of neurotoxicity. *Proceedings of the National Academy of Sciences* **1996,** *93*, 12764-
490 12767.

491 3. Ikeda, T.; Nagata, K.; Kono, Y.; Yeh, J. Z.; Narahashi, T., Fipronil modulation of
492 GABAA receptor single-channel currents. *Pest Management Science* **2004,** *60*, (5), 487-492.

493 4. Hainzl, D.; Cole, L. M.; Casida, J. E., Mechanisms for Selective Toxicity of Fipronil
494 Insecticide and Its Sulfone Metabolite and Desulfinyl Photoproduct. *Chemical Research in*
495 *Toxicology* **1998,** *11*, 1529-1535.

496 5. Rhône-Poulenc Agro to boost fipronil production. *Agrow* **1997,** *294*, 17.

497 6. *Trends in Concentrations of Pesticides in Urban Streams in the United States, 1992-*
498 *2008*; USGS.

499 7. Gunasekara, A. S.; Truong, T.; Goh, K. S.; Spurlock, F.; Tjeerderma, R. S.,
500 Environmental fate and toxicology of fipronil. *Jounal of Pesticide Science* **2007,** *32*, 189-199.

501 8. Erickson, B. E., Europe to ban fipronil pesticide to protect bees. *Chemical & Engineering*
502 *News Archive* **2013,** *91*, (29), 21.

503 9. Cravedi, J. P.; Delous, G.; Zalko, D.; Viguie, C.; Debrauwer, L., Disposition of fipronil in
504 rats. *Chemosphere* **2013,** *93*, 2275-2283.

505 10. Tingle, C. C. D.; Rother, J. A.; Dewhurst, C. F.; Lauer, S.; King, W. J., Fipronil:
506 Environmental Fate, Ecotoxicology, and Human Health Concerns. *Reviews of Environmental*
507 *Contamination and Toxicology* **2003,** *176*, 1-66.

508 11. Ohi, M.; Dalsenter, P. R.; Andrade, A. J. M.; Nascimento, A. J., Reproductive adverse
509 effects of fipronil in Wistar rats. *Toxicology Letters* **2004,** *146*, (2), 121-127.

510 12. Raquel, P.; Tercariol, G.; Godinho, A. F., Behavioral effects of acute exposure to the
511 insecticide fipronil. *Pesticide Biochemistry and Physiology* **2011,** *99*, 221-225.

512 13. *New Pesticide Fact Sheet for Fipronil*; US EPA: 1996.

513 14. Vidau, C.; Gonzalez-Polo, R. A.; Niso-Santano, M.; Gomez-Sanchez, R.; Bravo-San
514 Pedro, J. M.; Pizarro-Estrella, E.; Blasco, R.; Brunet, J. L.; Belzunces, L. P.; Fuentes, J. M.; ,

Fipronil is a powerful uncoupler of oxidative phosphorylation that triggers apoptosis in human neuronal cell line SHSY5Y. *NeuroToxicology* **2011,** *32*, 935-943.

15. Mohamed, F.; Senarathna, L.; Percy, A.; Abeyewardene, M.; Eaglesham, G.; Cheng, R.; Azher, S.; Hittarage, A.; Dissanayake, W.; Sheriff, M. H.; Davies, W.; Buckley, N. A.; M., E., Acute human self-poisoning with the N-phenylpyrazole insecticide fipronil--a GABAA-gated chloride channel blocker. *Jounal of Toxicology. Clinical Toxicology* **2004,** *42*, 955-963.

16. Tang, J. A.; Usmani, K.; Hodgson, E.; Rose, R. L., In vitro metabolism of fipronil by human and rat cytochrome P450 and its interactions with testosterone and diazepam. *Chemico-Biological Interactions* **2004,** *147*, (3), 319-329.

17. Herin, F.; Boutet-Robinet, E.; Levant, A.; Dulaurent, S.; Manika, M.; Galatry-Bouju, F.; Caron, P.; Soulat, J.-M., Thyroid function tests in persons with occupational exposure to fipronil. *Thyroid* **2011,** *21*.

18. Lyke, D. F.; McDaniel, K. L.; Moser, V. C.; Herr, D. W., Effects produced by single and repeated dosages of fipronil on the EEG of Long-Evans rats. *The Toxicologist CD-An Official Journal of the Society of Toxicology* **2011,** *120*, 290.

19. Herr, D. W.; Lyke, D. F.; McMahen, R.; Strynar, M.; Hedge, J. M.; McDaniel, K. L.; Moser, V. C., Changes in rat serum bioindicators after single or repeated dosages of fipronil. *The Toxicologist CD - An Official Journal of the Society of Toxicology* **2014,** *138*, (1), 490.

20. Xie, T.; Liang, Y.; A, J.; Hao, H.; Liu, L.; Zheng, X.; Dai, C.; Zhou, Y.; Guan, T.; Liu, Y.; Xie, L.; Wang, G., Post acquisition data processing techniques for lipid analysis by quadrupole time-of-flight mass spectrometry. *Journal of Chromatography B* **2012,** *905*, (0), 43-53.

21. In *Pesticide Residues in Food. Fipronil: Residue Evaluation 2001.*, Joint Meeting on Pesticide Residues in Food

22. Lacroix, M. Z.; Puel, S.; Toutain, P. L.; Viguie, C., Quantification of fipronil and its metabolite fipronil sulfone in rat plasma over a wide range of concentrations by LC/UV/MS. *Jounal of Chromatography B* **2010,** *878*, 1934-1938.

23. In *Pesticide Residues in Food. Fipronil: Residue evaluation 1997.*, Joint Meeting on Pesticide Residues in Food.

24. Kim, D.; Guengerich, F. P., CYTOCHROMEP450 ACTIVATION OFARYLAMINES ANDHETEROCYCLIC AMINES. *Annual Reviews in Pharmacology and Toxicology* **2005,** *45*, 27-49.

25. Snyderwine, E. G.; Turesky, R. J.; Turteltaub, K. W.; Davis, C. D.; Sadrieh, N.; Schut, H. A. J.; Nagao, M.; Sugimura, T.; Thorgeirsson, U. P.; Adamson, R. H.; Thorgeirsson, S. S., Metabolism of food-derived heterocyclic amines in nonhuman primates. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis* **1997,** *376*, (1–2), 203-210.

551 26. Eisenbrand, G.; Tang, W., Food-borne heterocyclic amines. Chemistry, formation,
552 occurrence, and biological activities. A literature review. *Toxicology* **1993,** *12*, 1-82.

553 27. Pezdirc, M.; Zegura, B.; Filipic, M., Genotoxicity and induction of DNA damage
554 responsive genes by food-borne heterocyclic aromatic amines in human hepatoma HepG2 cells.
555 *Food Chem. Toxicol.* **2013,** *59*, 386-394.

556 28. Nagao, M.; Sugimura, T., Carcinogenic factors in food with relevance to colon cancer
557 development. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*
558 **1993,** *290*, (1), 43-51.

559 29. Schut, H. A. J.; Snyderwine, E. G., DNA adducts of heterocyclic amine food mutagens:
560 implications for mutagenesis and carcinogenesis. *Carcinogenesis* **1999,** *20*, (3), 353-368.

561 30. Morgan, M. K.; Stout, D. M.; Jones, P. A.; Barr, D. B., An observational study of the
562 potential for human exposures to pet-borne diazinon residues following lawn applications.
563 *Environmental Research* **2008,** *107*, 336-342.

564 31. Dyk, M. B.; Liu, Y.; Chen, Z.; Vega, H.; Krieger, R. I., Fate and distribution of fipronil
565 on companion animals and in their indoor residences following spot-on flea treatments. *Journal*
566 *of Environmental Science and Health, Part B* **2012,** *47*, 913-924.

567 32. Hertz-Picciotto, I.; Cassady, D.; Lee, K.; Bennett, D.; Ritz, B.; Vogt, R., Study of Use of
568 Products and Exposure-Related Behaviors (SUPERB): study design, methods, and demographic
569 characteristics of cohorts. *Environmental Health* **2010,** *9*, (1), 54.

570 33. Zota, A. R.; Rudel, R. A.; Morello-Frosch, R. A.; Brody, J. G., Elevated House Dust and
571 Serum Concentrations of PBDEs in California: Unintended Consequences of Furniture
572 Flammability Standards? *Environmental Science & Technology* **2008,** *42*, (21), 8158-8164.

573 34. Windham, G. C.; Pinney, S. M.; Sjodin, A.; Lum, R.; Jones, R. S.; Needham, L. L.; Biro,
574 F. M.; Hiatt, R. A.; Kushi, L. H., Body burdens of brominated flame retardants and other
575 persistent organo-halogenated compounds and their descriptors in US girls. *Environmental*
576 *Research* **2010,** *110*, (3), 251-257.

577 35. van Niel, M. B.; Collins, I.; Beer, M. S.; Broughton, H. B.; Cheng, S. K. F.; Goodacre, S.
578 C.; Heald, A.; Locker, K. L.; MacLeod, A. M.; Morrison, D.; Moyes, C. R.; O'Connor, D.; Pike,
579 A.; Rowley, M.; Russell, M. G. N.; Moyes, C. R.; O'Connor, D.; Pike, A.; Rowley, M.; Russell,
580 M. G. N.; Sohal, B.; Stanton, J. A.; Thomas, S.; Verrier, H.; Watt, A. P.; Castro, J. L.,
581 Fluorination of 3-(3-(piperidin-1-yl)propyl)indoles and 3-(3-(piperazin-1-yl)propyl)indoles gives
582 selective human 5-HT1D receptor ligands with improved pharmacokinetic profiles. *Journal of*
583 *Medicinal Chemistry* **1999,** *42*, (12), 2087-2104.

584 36. Rowley, M.; Hallett, D. J.; Goodacre, S.; Moyes, C.; Crawforth, J.; Sparey, T. J.; Patel,
585 S.; Marwood, R.; Patel, S.; Thomas, S.; Hitzel, L.; O'Connor, D.; Szeto, N.; Castro, J. L.;
586 Hutson, P. H.; MacLeod, A. M., 3-(4-fluoropiperidin-3-yl)-2-phenylindoles as high affinity,
587 selective, and orally bioavailable h5-HT2A receptor antagonist. *Journal of Medicinal Chemistry*
588 **2001,** *44*, (10), 1603-1614.

589 37. Chambers, R. D., *Fluorine in Organic Chemistry*. Blackwell Publishing: Oxford, 2000.

590 38. Morgenthaler, M.; Schweizer, E.; Hoffmann-Ro, A.; Martin, R. E.; Jaeschke, G.;
591 Wagner, B.; Fischer, H.; Bendels, S.; Zimmerli, D.; Schneider, J.; Diederich, F.; M., K.; Mu, K.,
592 Predicting properties and tuning physicochemical in lead optimization: Amine basicities.
593 *ChemMedChem* **2007,** *2*, 1100.

594 39. Purser, S.; Moore, P. R.; Swallow, S.; Gouverneur, V., Fluorine in medicinal chemistry.
595 *Chemical Society Reviews* **2008,** *37*, (2), 320-330.

596 40. Smart, B. E., Fluorine Substituent Effects (on bioactivity). *Journal of Fluorine Chemistry*
597 **2001,** *109*, (1), 3-11.

598 41. Smith, D. A.; van de Waterbeemd, H.; Walker, D. K., Methods and Principles in
599 Medicinal Chemistry, vol 31: Pharmacokinetics and Metabolism in Drug Design. *Wiley-VCH*
600 **2006,** *31*.

601

602

603 4. SUPPORTING INFORMATION

604 **5.1 Rodents** were housed in polycarbonate cages containing heat-treated hardwood chip
605 bedding. Access to food (Purina 5008 Chow; Lab Diet/PMI Nutrition International, Richmond,
606 IN) and tap water was provided *ad libitum*. Animals were allowed to acclimate to their
607 surroundings in the animal colony for 5-7 days before beginning any tests. The animal colony
608 was maintained at a temperature of 22 ± 2 ^{o}C, with humidity at 40 ± 20%, and a 12:12 hr
609 light:dark cycle (light on at 6:00 a.m.).

610 **5.2 Recovery Experiment for Fipronil in Dosed-rat Serum.** Standard fipronil (200 ng) was
611 added to a vial containing blank rat serum (100 µL), along with 100 µL of 0.1 M formic acid and
612 1 mL of cold acetonitrile. The solution was centrifuged at 12,500 × g, and the supernatant was
613 extracted. In a separate vial, blank rat serum was directly spiked with fipronil standard (200 ng).

Both the supernatant and the control sample were mixed 50:50 with 10 mM ammonium acetate buffer and analyzed via LC/triple-quad. The fipronil recovery rate was 98%.

5.3 Recovery Experiment for Fipronil in Spiked Human Serum. A standard mix of fipronil metabolites (10 ng each metabolite) was added to a vial containing blank calf serum (200 µL), along with 25 µL of a 0.1 M formic/internal standard solution (fipronil des-F_3, 10 ng) and 2 mL of acetonitrile. The solution was centrifuged at 12,500 × g and was extracted onto an Oasis 3cc HLB solid phase extraction cartridge. The solid phase extraction method consisted of conditioning the cartridge with 3 mL of methanol followed by 3 mL of DI water; loading the sample; washing with 3 mL of 95:5 water:acetonitrile; and eluting with 3 mL of acetonitrile. The samples were evaporated under N_2 at 40 °C until 200 µL remained. In a separate vial (the control sample), only 200 µL of blank calf serum, 25 µL of the 0.1 M formic acid/internal standard solution and 2 mL of acetonitrile was added (no fipronil or metabolites), and this vial was also carried through the procedure, just as the experimental sample. The control sample was spiked with the standard mix of fipronil metabolites (10 ng/metabolite) after evaporation. All the samples were prepared 50:50 with 10 mM ammonium acetate buffer, and analyzed via LC/triple-quad (*n*=3). The results are shown below in SI Table 1.

SI Table 1. Human serum recovery experiment results.

Compound	Average % Recovery (± %RSD)
Fipronil	82 ±2.4
Fipronil sulfone	83 ±3.6
Fipronil sulfide	84 ±3.6
Fipronil amide	82 ±7.3
Monochloro fipronil	85 ±3.5

5.4 Recovery Experiment for Fipronil in Spiked Human Urine. A standard mix of fipronil metabolites (400 ng/metabolite) was added to a vial containing 10 mL of blank human urine and 1 mL of acetonitrile/internal standard solution (fipronil des-F_3, 33 ng). The solution was extracted onto an Oasis 6cc HLB solid phase extraction cartridge. The solid phase extraction method was the same as for human serum, except the elution step used 5 mL instead of 3 mL of acetonitrile. The solution was evaporated under N_2 at 40 °C until 1 mL remained. In the control sample, 10 mL of blank human urine and 1 mL acetonitrile were added (no fipronil or metabolites), and this vial was also carried through the procedure, just as the experimental samples. After evaporation the control sample was spiked with the standard fipronil metabolite mixture (400 ng/metabolite). All samples were prepared 50:50 with 10 mM ammonium acetate buffer and analyzed via LC/triple-quad (n=3). The results are shown below in SI Table 2.

SI Table 2. Human urine recovery experiment results.

Compound	Average % Recovery (± %RSD)
Fipronil	103 ±5.8
Fipronil sulfone	100 ±10
Fipronil sulfide	99 ±7.0
Fipronil amide	104 ±3.8
Monochloro fipronil	101 ±5.0

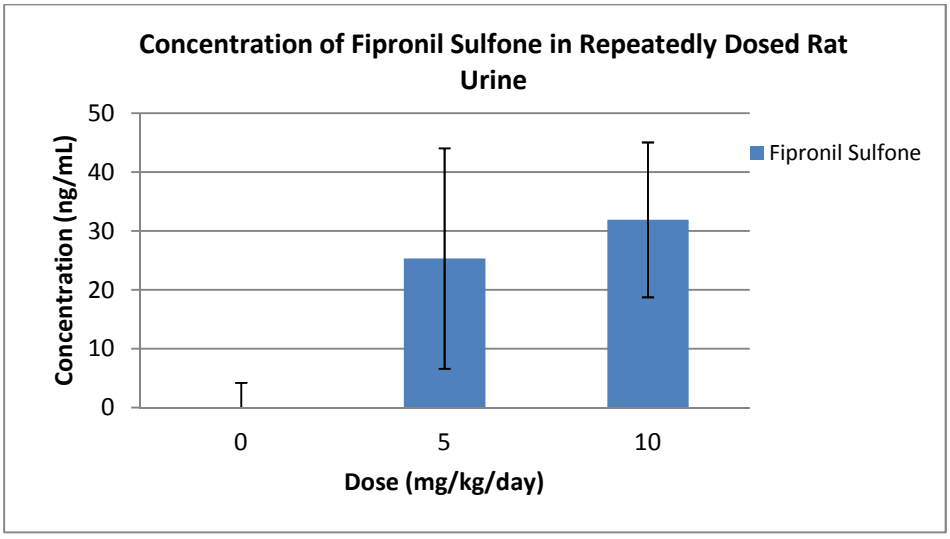
5.5 Quantitation of fipronil and fipronil sulfone in the serum of treated rodents. Rat serum (25 µL) was denatured with 100 µL of 0.1 M formic acid and precipitated with 1 mL of a cold acetonitrile solution spiked with the internal standard (fipronil des-F_3, 25 ng). The sample was then centrifuged for 5 minutes at 12500 × *g*. An aliquot of the supernatant was mixed 50:50 with 10 mM ammonium acetate buffer, and analyzed via LC/TOF and LC/triple-quad. *n*=9 for high dose (10 mg/kg/day) ; *n*=10 for low dose (5 mg/kg/day); and *n*=11 for control animals, which were treated with vehicle. To determine the concentration of compounds of interest, a nine-point matrix-matched extracted standard curve from 10-5000 ng/mL, a method blank (DI water), and a matrix blank (blank rat serum) was run with the rat serum samples via LC/triple-quad. The lowest value on the standard curve (10 ng/mL) was considered the lower limit of quantitation (LOQ). The results of the quantitation are shown in SI Table 3.

SI Table 3. Descriptive statistics and select percentiles for fipronil and fipronil sulfone in rat serum.

Compound	Dose Conc. (mg/kg bw)	LOQ (ng/mL)	%<LOQ	Min	5%	25%	50%	75%	95%	Max
Fipronil	Control	10	91	<LOQ	<LOQ	<LOQ	<LOQ	0.419	13.8	10.1
Sulfone	Control	10	91	<LOQ	0.133	1.01	1.65	2.12	8.12	13.3
Fipronil	5	10	0	4.83	4.98	5.52	8.82	11.9	12.9	13.7
Sulfone	5	10	0	2120	2147	2250	2465	2573	2630	2630
Fipronil	10	10	0	6.03	6.53	8.07	11.7	17.0	26.6	29.3
Sulfone	10	10	0	2,880	2,952	3,110	3,670	3,990	4,180	4,280

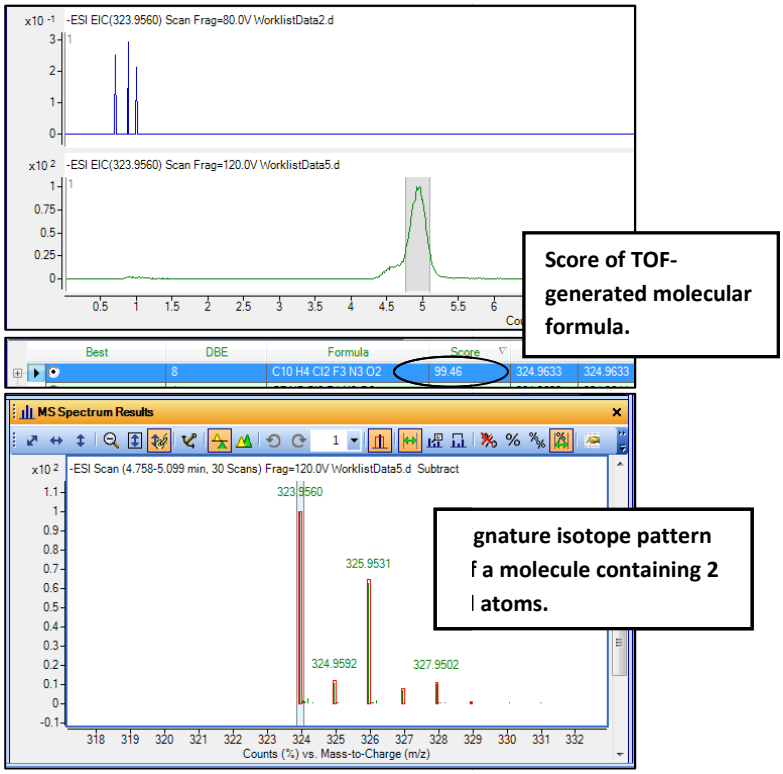
671 **5.6 Quantitation of fipronil sulfone in the urine of treated rodents.** Rat urine (100 µL) was
672 treated with 900 µL of cold acetonitrile. The sample was then centrifuged for 8 minutes at
673 12,500 × *g*, prepared 50:50 with 10mM ammonium acetate buffer, and analyzed via LC/triple-
674 quad. $n = 2$ for high dose (10 mg/kg/day); $n = 4$ for low dose (5 mg/kg/day); and $n = 6$ for
675 control animals. In order to determine concentration of compounds of interest, a seven-point
676 extracted standard curve prepared in DI water from 10-5000 ng/mL, along with a method blank
677 (DI water) was run with the experimental rat urine samples. SI Figure 1 shows median fipronil
678 sulfone concentrations for rodents dosed with fipronil. The high dose group had a median
679 concentration of 32 ±13 ng/mL fipronil sulfone, while the low dose group had 25 +/-19 ng/mL
680 and the control animals had 0 ±4 ng/mL.

681 The LC/triple quad used for the quantitation of fipronil sulfone was a Waters Acquity
682 ultraperformance liquid chromatography system coupled with a Waters Quatro Premier XE triple
683 quadrupole mass spectrometer (UPLC-MS/MS; Waters Corporation). A 20-µL aliquot of each
684 sample was injected onto an Acquity UPLC BEH C18 column (1.7 µm, 2.1 × 50 mm; Waters
685 Corporation) that was maintained at 50 ºC. The mobile phase consisted of solvent A: 2 mM
686 ammonium acetate buffer with 5% methanol and solvent B: acetonitrile at a flow rate of 400
687 µL/min, starting with 75% solvent A for 30 s and then increasing to 90% solvent B at 3.5 min
688 and 100% solvent B at 3.6 min and held for 0.9 min. At 4.6 min the gradient was returned to
689 60% solvent A and held until 6.0 min. Electrospray negative ionization was used in the mass
690 spectrometer source. The capillary voltage was set at negative 0.4 kV, and the source
691 temperature was 150 ºC. The primary transition used for quantitation was 451.2 - 244.0 *m/z*, and
692 two other transitions were monitored for confirmation, 451.2 to 281.9 *m/z* and 451.2 to 414.9
693 *m/z*.



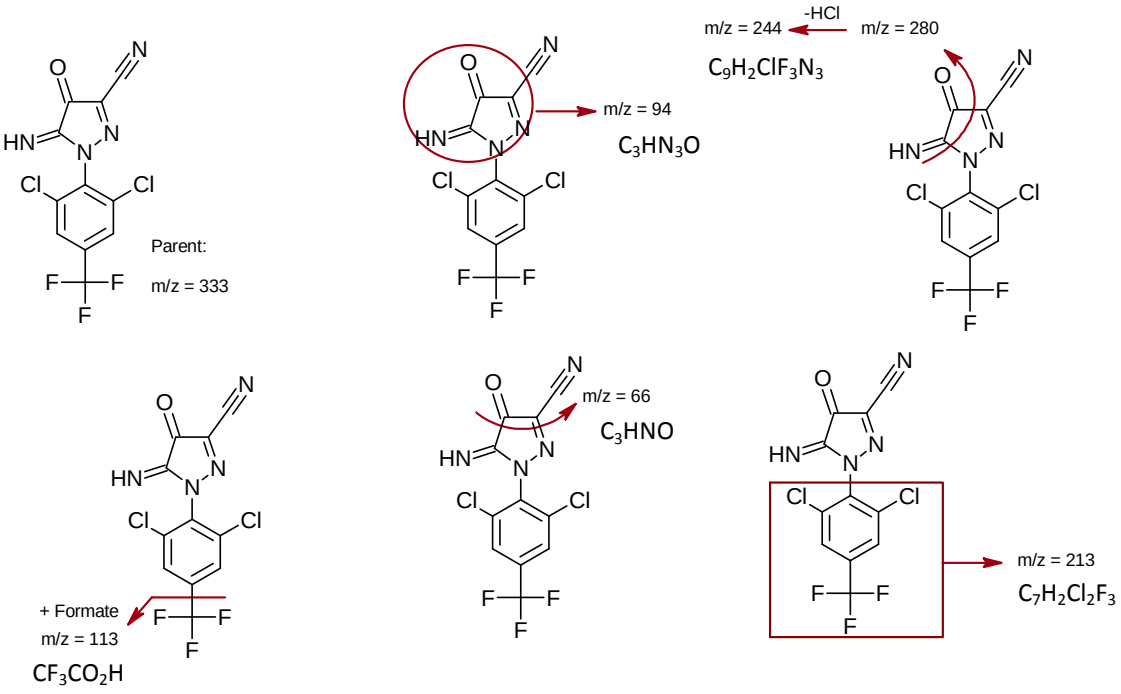
SI Figure 1. Median fipronil sulfone concentration in rat urine.

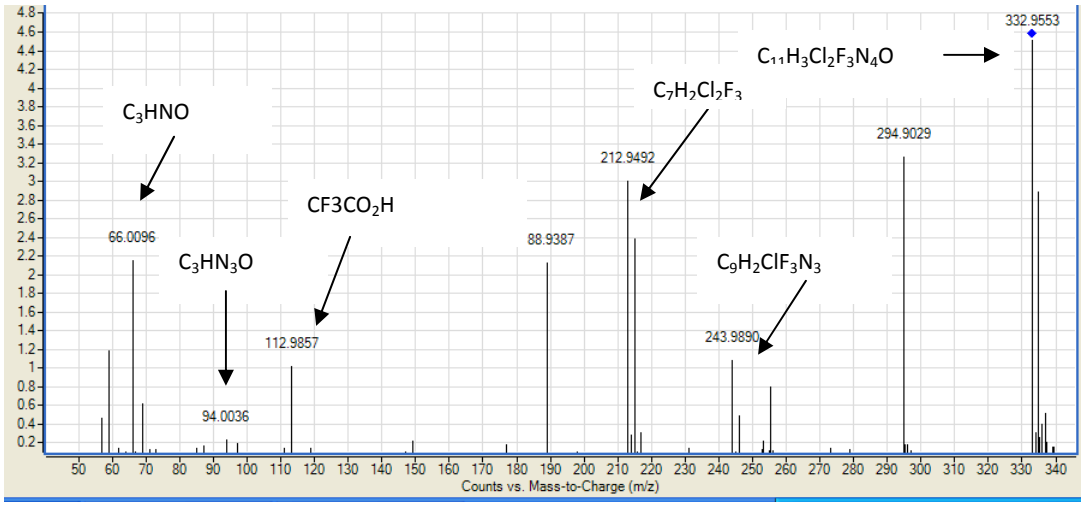
5.7 Time-of-flight mass spectrometry scoring and isotope patterns.



SI Figure 2 shows the spectral pattern of a molecule containing 2 chlorine atoms. Note that 323.9560 *m/z* is the most abundant isotope, 325.9531 *m/z* contains one ^{37}Cl, and 327.9502*m/z* contains two ^{37}Cl. The 324.9592 *m/z* contains one ^{13}C. The numerical ranking for formula generated for compound (M4) is shown. The top extracted ion chromatograph (Worklist Data 2) shows a control animal sample and the absence of a peak for M4.

5.8 Metabolite M7 in rat urine





SI Figure 3 shows the fragmentation pattern of Metabolite M7 in the LC/Q-TOF. The red circles/boxes show the fragment and the spectrum at the bottom shows the peaks corresponding to the fragments.

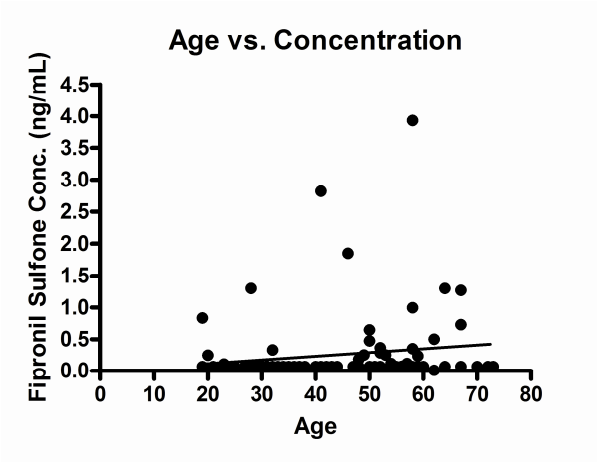
5.9 Statistical Data. Statistical analyses of the human serum data was performed. Rank comparisons for gender and race was done by a Mann-Whitney test. A Spearman Correlation analysis was also used to evaluate the relationship between age and concentration of fipronil sulfone in serum.

Gender: A two-tailed unpaired Mann-Whitney test was performed to compare the ranks between genders. The gender were not significantly different (P=0.99 and Mann-Whitney U=959.5). SI Table 4 shows the number of detects and non-detects for each gender.

SI Table 4. Number of detects and non-detects for the genders.

Gender	Detects	Non-Detects	Number of Samples
Male	7	12	29
Female	17	67	67

Age: A two-tailed Spearman Correlation analysis was performed to evaluate whether there was a relationship between age and concentration of fipronil sulfone. The correlation between age and concentration was significant (Spearman r = 0.21 and p = 0.0418). SI Figure 5 shows the linear regression.



SI Figure 5. Correlation between age and concentration of fipronil sulfone.

Race: A nonparametric analysis of the mean fipronil sulfone concentrations of the sample of people who self-identified as either Caucasian (*n*=62) or African American (*n*=34) origin was performed. The Asian and "other" categories were excluded because there were not enough samples in those categories for statistical analyses. According to a two-tailed unpaired Mann-Whitney test, there was found to be a significant difference in the ranks of the concentrations between the two races (p = <0.0001 and Mann-Whitney U=556). The number of detects and non-detects in each group is shown in SI Table 5. There were a large number of detects in the Caucasian category, but only one detect in the African American category.

SI Table 5 shows the number of detects vs. non-detects for each race.

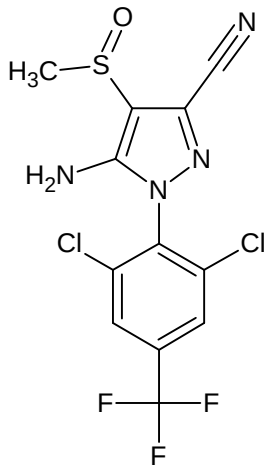
Race	Detects	Non-Detects	Number of Samples
Caucasian	22	39	61
African American	1	29	30
Asian	1	2	3
Other	0	2	2

5.10 Transitions in LC/triple quad method. SI Table 6 below lists the parent to daughter transitions which were monitored in the Agilent 1100 LC/triple quad method.

SI Table 6. LC/triple quad parent-daughter transitions.

Compound	Transition	Parent	Daughter
Fipronil	1°	434.9	329.8
Fipronil	2°	434.9	249.9
Fipronil	3°	434.9	277.8
Fipronil sulfone	1°	451.1	415.0
Fipronil sulfone	2°	451.1	281.9
Fipronil sulfone	3°	451.1	243.9
Fiproni sulfide	1°	418.9	382.8
Fiproni sulfide	2°	418.9	261.7
Fiproni sulfide	3°	418.9	313.8
Fipronil amide	1°	452.9	347.7
Fipronil amide	2°	452.9	303.8
Fipronil amide	3°	452.9	271.9
Monochloro fipronil	1°	401.1	283.9
Monochloro fipronil	2°	401.1	295.9
Monochloro fipronil	3°	401.1	331.9
Fipronil des F3	1°	387.2	281.9
Fipronil des F3	2°	387.2	331.0
Fipronil des F3	3°	387.2	351.0

5.11 SI Figure 6 shows fipronil des-F_3 which was used as an internal standard for analytical methods due to its similarity in structure to fipronil. The structure is shown below.



Molecular Formula: $C_{12}H_7Cl_2F_3N_4OS$
Monoisotopic Mass: 381.966971 Da
[M-H]-: 380.959694 Da

Dr. Alcock,

Thank you for the comments on our recently submitted manuscript entitled: "Identification of fipronil metabolites by time-of-flight mass spectrometry for application in a human exposure study" (Manuscript ID: ENVINT-D-14-01277). We appreciated the reviewers' careful reading and criticism of this manuscript, and we thank them for their considerable efforts to help improve this submission. We have made a substantial revision of this work by responding to the reviewers' comments. A point-by-point response to each of the comments follows:

Reviewer 1:

Summary

This manuscript discusses a new analytical method that was developed to identify seven different metabolites of fipronil in the serum and urine samples of male, adult rats. This method was subsequently used to identify the same metabolites in the serum and urine samples of adults from the general population. This is an important manuscript as no published biomonitoring data exist on the nonoccupational exposures of humans to fipronil. In my review, I found the methodology to be quite good, however, the manuscript needs to be written more clearly in several sections, particularly providing more specific details (as described below). I have the following suggested comments to improve the quality of this manuscript.

We appreciate that the reviewer recognizes the importance of the work. In an effort to address his/her concerns about the clarity and specificity of several of the sections, we have made corrections throughout the manuscript, which are highlighted in blue.

Abstract - Provide more detailed information (i.e., number of rats, actual doses, adult rats were used, number of human serum and urine samples, year of studies [rats/human]). Specify the aims/objectives of the manuscript. As this is a methods focused paper, I suggest that you list the actual seven metabolites that you identified in rat serum/urine if space permits (or at least the two newly identified metabolites). Suggest removing the following sentence (line 32) "These results are comparable to the results from an exposure study of workers in a fipronil production facility" (statement is vague and belongs more in the discussion section).

As to provide more detailed information on the dosed rodent study, we cited the Freeborn et al. manuscript that contains all pertinent details for which the reviewer asked. However, for the reviewer:

The study was performed from 10/10/12 – 11/20/12.

How many rats (total and by group) were used in this experiment?

0 mg/kg/day: 11 rats; 5 mg/kg/day: 10 rats; 10 mg/kg/day: 9 rats

Space doesn't permit listing the actual seven metabolites identified in the abstract, but we did incorporate the two that were newly identified. We added more details for the dosed rodent and human studies, and, as suggested, we removed line 32. We also expanded the discussion section to cover the comparison of this study with the occupational exposure study in more detail (lines 390-397 in the revised manuscript).

Introduction section - This section does not flow well and needs better organization and more specific details:

We have made some changes, and hopefully the introduction flows better now.

Lines 65 -71: Is there a newer citation of how many tons of fipronil are manufactured in the US or worldwide? The current one cites a 20 year old (1997) report. Make it clear that you are discussing levels of fipronil in only the US, worldwide or both. Suggest deleting the sentence "Perhaps as a result of this contamination, fipronil has been implicated as one of the chemicals associated with colony bee collapse". Instead suggest adding more information, including citations, on identified sources and potential routes of human exposures to fipronil in this paragraph

We cited the most recent reference for production volume that could be found, and we had also cited a more recent EPA report from 2011, which covers from 1998 to 2008. We also altered the text to indicate this (line 66 in the revised manuscript).

Line 72- Suggest first discussing the one case of human poisoning with fipronil and that fipronil sulfone was identified as the primary metabolite (mentioned in Line 83), then discuss the only recent study by Cravedi et al (2013) that examined the toxicokinetics of fipronil in rats administered a single oral dose of 10 mg/kg body weight.

We disagree with the reviewer. We feel as though the introduction is better organized by first discussing information pertaining to rat studies and then information about known human studies. We organized our study in this way, by first dosing rodents, looking for metabolites, and then analyzing human serum and urine for those metabolites.

Line 88- This sentence needs more details about the study by Herin et al, 2011 "One occupational exposure study of workers at a fipronil production facility reports a mean fipronil sulfone level of 7.79 ng/mL." - For example, how many workers, was this a cross sectional study, and year/ location of study.

We added some additional information: the number of workers in the study and the mean and standard deviation of fipronil sulfone in human serum (lines 89-90).

Line 97 (last paragraph) -As mentioned for the abstract, list the specific aims/objectives of this work.
The specific aims of the study were included in the introduction: "The specific objectives of the study were to develop a unique workflow where dosed animal samples were used to identify potential serum/urine biomarkers via time-of-flight mass spectrometry which were subsequently evaluated in serum and urine of a group of volunteers from North Carolina to assess exposure." (lines 93-97 in the revised manuscript)

Line 125 - States that the "Animals were dosed repeatedly by oral gavage at either 5 or 10 mg/kg with fipronil….. Did you based this on the weight of individual rats? Suggest removing the word "repeatedly".

Per the reviewer's suggestions, line 125 was changed to "daily". This is important from the toxicological perspective. The rats were weighed daily, and dosing occurred based on the weight of individual animals.

Lines 127-131 - How much trunk blood and urine were collected from each rat? When was the rat study performed? How many rats (total and by group) were used in this experiment?

The Moser et al. paper has all of these details (reference 19). However, for the reviewer, 2 ml of trunk blood was collected for serum. Urine varied by rat (some had none), since the method of collection was by bladder puncture. See earlier comment for when the rodent study was performed and number of rats in the study.

Line 142 - Suggest adding an additional row "number" of subjects for Table 1 and moving it to the results section or alternatively write out this information in a paragraph in the results section.

As the number of subjects is 100, we did not incorporate an additional row in the demographic Table 1, since the rows for percent and number would be repetitive. The number of participants was added to the table header (line 143) for clarification.

Lines 158 & Line 166: For rat serum and rat urine-- Unclear why you had different number of animals for each matrix and by treatment group (5 and 10 mg/kg and control). Were some of the rats dropped (i.e., died) from the experiment?

Details on numbers of rats per group and those dropped are in the Freeborn et al. paper and are beyond the scope of this work. We only used urine and serum for metabolite identification purposes.

Line 174: For human serum - specify here actual number of samples that were analyzed. List here the actual chemicals analyzed in this matrix.

Details on the number of serum samples were already provided in the manuscript, but they were moved to the beginning of the paragraph for clarification (line 173). We also added a sentence to clarify that all chemicals for which standards were possessed and that were in the methods section were included in the mass spec method (line 181).

Line 187: For human urine - specify the number of samples analyzed. Why wasn't the same volume of urine used per sample to analyze for the target chemicals ("5-12 mL" were used)? List the actual chemicals analyzed in this matrix.

Details on the number of urine samples were already provided in the manuscript, but they were moved to the beginning of the paragraph for clarification (line 187). And, as above we added clarification that all chemicals in the methods section were included in the mass spec method (lines 193-194). As for the volume used, we used the volume of urine we received, which differed among samples.

Line 341: Since fipronil sulfone was detected in only 25% of the samples, it is not appropriate to conduct more advanced statistical analysis (i.e., Mann-Whitney/Spearman Correlations) of all human serum data when 75% of the data are censored.

We agree with the reviewer. We have altered the manuscript by removing the statistics sections and discussing only range and trends in number of detects (lines 332-333 and 411 to 414). Table 3 in the revised manuscript was moved from the supporting information to the body of the manuscript. We also added a section on observations in our sample subset, specific to Caucasians (lines 420-423).

Discussion section (Line 395)- Should mention some limitations of this study. In particular, several studies have shown measureable levels of fipronil degradates, include fipronil sulfone, in environmental media. It is possible that some of the measureable levels of fipronil sulfone in the human serum samples could have originated from the preformed metabolite (e.g., fipronil sulfone). So, it may or may not be a useful biomarker?

We thank the reviewer for this suggestion and agree with the reviewer. Line 414-420 were added to discuss study limitations, specifically the limited specificity of fipronil sulfone as a biomarker.

Reviewer #2:

This manuscript describes a LC/TOF-MS method to develop a set of potential serum/urine biomarkers of fipronil exposure and a quantitative LC/MS method to estimate human fipronil exposure. The manuscript is clear; the results for metabolites identification are well described and discussed. However, as the authors explain in discussion (lines 389-391), searching urinary fipronil biomarkers in human known to be exposed to fipronil will be more relevant than human with no known fipronil exposure. Moreover, the results and statistical analysis obtained in human sample are too weak to be published Environment International.

We appreciate the comments from Reviewer 2. Our responses are highlighted in red.

While we agree with the reviewer on most of this point, the samples were samples of convenience. It would be much more applicable to conduct this study in an occupationally exposed cohort as the reviewer suggests, to identify urinary metabolites. However, we are mostly interested in assessing exposure in the general population, and as cited, some occupational exposure work has already been done.

We have altered the statistical treatment of our data per the suggestion of both reviewers. See comments above and below for specifics.

Major concerns:
A proper validation of the quantitation methods for both human and rat samples are lacking (linearity, accuracy, repeatability, reproductibility, validation of LOQ). There is no way to know if the announced LOQ is statistically significant from the blank sample. Moreover 9% of rat controls are contaminated with fipronil and fipronil sulfone (see SI table 3) impeding of the data in particular for low concentration.

For clarification, the human samples had different purposes. The rodent samples were only to identify metabolites. Quantitation of the rodent sample was determine the best biomarker candidates based on concentration. However, as suggested by the reviewer, we added r-squared values and validated the LLOQ for the human samples (lines 250-254 and 267-270).

In addition, the contamination of fipronil and fipronil sulfone was in only one of the control rat serum samples at the LLOQ (10 ng/mL). Again, these samples were specifically used to identify metabolites for our purposes. SI table 3 was changed to better show the data.

The human population is not big enough and not well documented so that seems not reasonable to make statistical analysis. If the objectives were to determine whether the biomarkers identified in rats are suitable for human biomonitoring survey, working with subject known to be exposed with fipronil (pet groomers, gardeners…) will be more relevant to search potential fipronil biomarkers in urine and consequently to make statistics on age, race and gender

We agree with the reviewer and made changes, which were also suggested by reviewer 1, specific to the statistics.

These were samples of convenience. Our objective was to analyze serum and urine samples from people in the general population in order to characterize exposure. Of course, an occupational exposure study would be useful for identifying urinary metabolites, but we didn't have access to these types of samples.

There is not assessment of interspecies variability of fipronil metabolism either quantitative or qualitative. Knowing that such variability exists as shown in rat and sheep (Leghait et al. Toxicol Lett. 2010 May 4;194(3):51-7.), information should be provided to ascertain the fact that rat is relevant to human.

Interspecies variability is not relevant to the scope of this study. No changes were made.

Minor Comments:

Line 72: Leghait et al works about fipronil thyroid disruption and hepatic effects on metabolism in rats should be mentioned (Toxicology. 2009 Jan 8;255(1-2):38-44). Moreover authors should specify that hepatic metabolism is not well documented in the literature.

Again, this is interesting but, beyond the scope of the paper.

Line 92: Authors should generalize this sentence to "biological matrix" leads to "matrix effect due to high concentration of endogenous chemicals", endogenous compounds are not only interfering in human samples but also in animal serum samples.

We agree with the reviewer that this is true in human samples; however, in dosed animal samples there is so much chemical of interest relative to the endogenous chemicals that matrix is generally not an issue. No changes were made.

Line 125: 5 mg/kg/Day cannot be considered as a low dose of fipronil, author should correct by (dose 1 or lowest dose) for 5 mg/kg/Day and (dose 2 or highest dose) for 10 mg/kg/Day. Authors should explain why they used these 2 doses.

Per the reviewer's suggestions, throughout the paper as appropriate we changed low to "lowest" and high to "highest" dose for 5 and 10 mg/kg bw, respectively. We cite the Freeborn et al. paper for specifics on why these doses were chosen. Further discussion on this matter is beyond the scope of the paper.

Line 133: Is there a questionnaire for human sample collection to know if the individuals were in contact with pets or gardening during the previous weeks? Authors should document how was evaluated the "no known fipronil exposure". Information can be crucial for discussion about human fipronil sulfone exposure. Because fipronil sulfone is a persistent metabolite in the organism (half time life estimated to 200 Hr).

Although this information would be nice to have, no questionnaire came with these samples, as they were samples of convenience. This would be a good idea for a follow-up study on fipronil source identification though.

What we mean by "no known exposure" is that we do not know what the study participants were exposed to, as we don't know occupation, habits, hobbies, etc.

As suggested by the reviewer, information on half-life *in rodents* from the manuscript by Mohamed et al. was added to the introduction on line 74.

Line 188: Why working with 6 cc cartridges for urine sample (5-12 mL), which need high volume loadings, instead of 3 cc cartridges which have been developed for serum samples?

No changes were made, but for the reviewer:

We had a clogging issue, since urine was diluted in 20 mL of DI water, and we needed a bigger cartridge.

§ 3.5 (line 333): Fipronil sulfone is the main serum metabolite of fipronil in human or rodent. It is well described in the literature and as so can be considered as a "known" biomarker.

As the reviewer suggested, the word putative was removed from line 331. However, the only human data available are two studies, one on occupationally exposed workers and one on an acute poisoning. This is the first study to demonstrate this biomarker's utility in samples from the general population.

§ 4 (line 303-308): Authors should explain the use of these 2 dosing regimen for urinary metabolites identification. Roques et al. works (Toxicol Sci. 2012 Dec;130(2):444-5), concluded that a dosing regimen of 1.5 mg/kg/D of fipronil for 14 days increase fipronil biotransformation rate into sulfone (Toxicol Sci. 2012 Dec;130(2):444-5). Authors should have considered this information to establish their dosing regimen. Comparing urinary metabolites obtained with a high dose (5 or 10 mg/kg/D for 14 days) and a low dose (estimated from mean occupational exposure study, for example) would be more judicious to evaluate the relevance of identified urinary biomarkers of fipronil regarding reported exposure scheme in human.

As above, this is beyond the scope of the study, since no toxicology work was undertaken in this effort, and relevant citations are given. The reviewer clearly has an in depth understanding of the fipronil literature, and we thank him/her for the additional information. Dosed animal studies are generally above human exposure levels, so extrapolation is always an issue.

SI table 3: This table is useless and very difficult to understand. Moreover, there are inconstancies between 95% column and Max column (line 1and 4).

We altered the table (SI Table 3 in the revised manuscript line 519) to make it more useful. Thank you for pointing out the inconsistencies.

SI Table 4: the column "Numbers of samples" do not correspond to the sum of the "detects" and "non-detects" column.

The previous SI table 4 was corrected and moved to the body of the text (Table 3 line 335 in the revised manuscript). Thank you for pointing this out.

--

EDITOR COMMENTS:

I strongly agree with Reviewer #2's comment on method validation. Please provide more details in the revised manuscript.

In responding to the comments of the reviewers and the editor we included new information on the validation of the method (r-squared and signal-to-noise ratio of blanks compared to the lowest working standard curve sample). High and low QA/QC samples were already in the text for rodent and human samples to address precision validation questions. In addition the text discussed replicate precision (~5-10% of sample) that were run in each analytical batch. As a whole this data shows good precision and accuracy for both rodent and human samples. However, due to the scope of our study, the method validation in human samples was more pertinent as we used rodent samples for metabolite discovery exclusively.

Please use <mu>L instead of uL throughout the text Line 384: reference missing (ENREF 13)

Suggestions as noted were changed. Thank you for your careful consideration of this work.

Additional changes:

After careful consideration of the reviewer's comments on our statistical treatment of the data, we altered Figure 2 and included median estimated concentration rather than mean concentration of the Herin et al., study. We noted the data in the occupationally exposed worker study was log-normally distributed, and thus the mean was not appropriate. We used a recent publication from Pleil et al., 2014 (reference added) for this calculated median concentration using the published values in Herin et al. Some additional text was added to the manuscript (line 390-397) addressing this change.

Identification of fipronil metabolites by time-of-flight mass spectrometry for application in a human exposure study

Rebecca.L. McMahen[a], *Mark J. Strynar*[b]*, *Sonia Dagnino*[a], *David W. Herr*[c], *Virginia C. Moser*[c], *Stavros Garantziotis*[d], *Erik M. Andersen*[b], *Danielle L. Freeborn*[c], *Larry McMillan*[e], *Andrew B. Lindstrom*[b]

[a]ORISE fellow at the United States Environmental Protection Agency, National Exposure Research Laboratory, Research Triangle Park, North Carolina

[b]United States Environmental Protection Agency, National Exposure Research Laboratory, Research Triangle Park, North Carolina

[c]National Health and Environmental Effects Research Lab, U.S. Environmental Protection Agency, Research Triangle Park, North Carolina

[d]National Institute for Environmental and Health Sciences, Research Triangle Park, North Carolina

[e]National Caucus and Center on Black Aged Employee, U.S. Environmental Protection Agency, National Exposure Research Laboratory, Research Triangle Park, North Carolina

*CORRESPONDING AUTHOR:

Phone: (919)541-3706

Address: 109 TW Alexander Dr. Durham, NC 27705

Email: strynar.mark@epa.gov

ABSTRACT

Fipronil is a phenylpyrazole insecticide commonly used in residential and agricultural applications. To understand more about the potential risks for human exposure associated with fipronil, urine and serum from dosed Long Evans adult rats (5 and 10 mg/kg bw) were analyzed to identify metabolites as potential biomarkers for use in human biomonitoring studies. Urine from treated rats was found to contain seven unique metabolites, two of which had not been previously reported—M4 and M7 which were putatively identified as a nitroso compound and an imine, respectively. Fipronil sulfone was confirmed to be the primary metabolite in rat serum. The fipronil metabolites identified in the respective matrices were then evaluated in matched human urine (*n*=84) and serum (*n*=96) samples from volunteers with no known pesticide exposures. Although no fipronil or metabolites were detected in human urine, fipronil sulfone was present in the serum of approximately 25% of the individuals at concentrations ranging from 0.1-4 ng/mL. These results indicate that many fipronil metabolites are produced following exposures in rats and that fipronil sulfone is a useful biomarker in human serum. Furthermore, human exposure to fipronil may occur regularly and require more extensive characterization.

Keywords: Fipronil, LC/TOF, Biomarker, Human Exposure, Metabolism

ABBREVIATIONS

DI: Deionized

ESI: electrospray ionization

GABA: gamma-aminobutyric acid

GSD: geometric standard deviation

HPLC: high performance liquid chromatography

LC: liquid chromatography

LLOQ: lower limit of quantitation

MS: mass spectrometry

NIEHS: National Institute for Environmental Health Sciences

QC: quality control

Q-TOF: quadrupole time-of-flight

% RSD: Percent Relative Standard Deviation

SD: standard deviation

SPE: solid phase extraction

TOF: time-of-flight

UPLC: ultra performance liquid chromatography

US EPA: United States Environmental Protection Agency

1. INTRODUCTION

Fipronil (Figure 1) is a phenylpyrazole broad-spectrum insecticide that is registered for use in residential settings as part of ant and cockroach baits and gels and termite control products; veterinary applications such as spot treatment flea and tick control products for dogs and cats; ornamental turf applications such as fire ant control; and agricultural applications such as pest control on potato crops[1]. When initially produced, fipronil was the first insecticide to act by

63 targeting the gamma-aminobutyric acid (GABA) receptor and has favorable selective toxicity
64 towards insects rather than mammals[2-4].

65 A 1997 report indicated that 480 tons of fipronil were produced per year by Rhone Poulenc,[5]
66 and a more recent EPA report indicated that between 1998 and 2008 usage averaged 150,000
67 pounds of active ingredient per 1.5 million acres[1]. Widespread fipronil use has led to
68 contamination of water and soil (1-158 ng/L of parent or environmental degradate) in several
69 states including, but not limited to Alabama, Georgia, California, Louisiana, and Indiana[6, 7].
70 Perhaps as a result of this contamination, fipronil has been implicated as one of the chemicals
71 associated with the colony bee collapse[8].

72 Because little was found in the peer-reviewed literature about the disposition of fipronil,
73 Cravedi et al. (2013) performed a thorough study on the metabolism, distribution, and
74 elimination of fipronil in rats and showed that fipronil is primarily converted to fipronil sulfone
75 (M1 Figure 1), a more persistent metabolite (estimated half-life is 208 hours in rodents)[9] which
76 was stored mainly in adipose tissue and adrenals[10]. In addition, fipronil has been associated with
77 thyroid disruption[11], endocrine disruption[12], and neurotoxic effects[13] in rats which has led to
78 concern about the potential for human health effects in the last decade.

79 The effects of acute human exposure to fipronil include headache, dizziness, vomiting, and
80 seizures[9, 10]. Information on the effects of chronic exposure is limited, but the US EPA has
81 classified fipronil as a possible human carcinogen based on data that shows an increase of
82 thyroid follicular cell tumors in both sexes of the rat[14]. Vidau et al. (2011) also concluded that
83 fipronil has the potential to cause apoptosis by uncoupling oxidative phosphorylation at
84 relatively low concentrations (5-10 µM) in human cell lines,[15] and a case of acute human self-
85 poisoning with fipronil has demonstrated that fipronil levels can remain elevated in serum for

days after exposure, and that fipronil sulfone was the primary metabolite[9]. A previous study also showed that fipronil sulfone is the predominant metabolite in human liver microsomes via cytochrome P-450 oxidation[16].

Although, one occupational exposure study of workers (*n*=159) at a fipronil production facility reports a mean fipronil sulfone serum level of 7.8 (SD = 7.7) ng/mL,[17] very little is known about human exposure to fipronil in the general population[9, 15, 17]. This may be because human samples can be difficult to obtain and analyze due to high concentrations of endogenous chemicals and significant matrix effects which make the identification of metabolites difficult. The specific objectives of the study were to develop a unique workflow where dosed animal samples were used to identify potential serum/urine biomarkers via time-of-flight mass spectrometry which were subsequently evaluated in serum and urine of a group of volunteers from North Carolina to assess exposure.

MATERIALS AND METHODS

2.1 Chemicals. Unlabeled fipronil (5-amino-1-[2,6-dichloro-4-(trifluoromethyl)-phenyl]-4-(trifluoromethylsulfinyl)-1-H-pyrazole-3-carbonitrile, >99%) and its metabolites: fipronil sulfone (5-amino-1-[2,6-dichloro-4-(trifluoromethyl)-phenyl]-4-[(trifluoromethyl)sulfonyl]-1*H*-pyrazole-3-carbonitrile, >99%), fipronil sulfide (5-amino-1-[2,6-dichloro-4-(trifluoromethyl)-phenyl]-4-[(trifluoromethyl)thio]-1*H*-pyrazole-3-carbonitrile, 98%), fipronil amide (5-amino-1-[2,6-dichloro-4-(trifluoromethyl)-phenyl]-4-[(trifluoromethyl)thio]-1*H*-pyrazole-3-carboxamide, >99%), and monochloro fipronil (5-amino-1-[2-chloro-4-(trifluoromethyl)-phenyl]-4-[(trifluoromethyl)sulfinyl]-1*H*-pyrazole-3-carbonitrile, >97%) were procured as solid analytical standards from the pesticide repository through the US EPA (Fort Meade, MD, USA). These five

analytical standards were prepared as a mixture in acetonitrile and used for all subsequent matrix-matched standard curves. The internal standard fipronil des-F_3 (see supporting information for structure) (5-amino-1-[2,6-dichloro-4-(trifluoromethyl)-phenyl]-4-(methylsulfinyl)-1-H-pyrazole-3-carbonitrile, 99%, 0.1 ng/µL in Acetonitrile) was ordered from Crescent Chemical Company (Islandia, NY, USA).

Acetonitrile and methanol (B&J Brand HighPurity Solvent) were purchased from Honeywell Burdick & Jackson (Muskegon, MI,USA) and ammonium acetate from Sigma Aldrich (St. Louis, MO, USA). Deionized water was generated in house from a Barnsted Easypure UV/UF (Dubuque, IA, USA) coupled with activated charcoal and ion exchange resin canisters.

2.2 Animals. This study was part of an investigation of the neurotoxic effects of fipronil in rodents[18, 19]. The animal facility is accredited by the American Association for Accreditation of Laboratory Animal Care International, and all protocols were approved by the National Health and Environmental Effects Research Laboratory Institutional Animal Care and Use Committee at the United States Environmental Protection Agency. Male Long Evans rats (60-90 days old) were acquired from Charles River Laboratories (Wilmington, MA). Animal husbandry details are provided in the Supporting Information. Animals were dosed daily by oral gavage at either 5 (lowest dose) or 10 (highest dose) mg/kg with fipronil suspended in corn oil (1 mL/kg) every 24 hours for two weeks. Control rats were gavaged with corn oil only. Six hours after the 14th dose, rats were euthanized. Trunk blood (2 mL) was collected in tubes without anticoagulant and stored on ice for 1-1.5 h.. The samples were centrifuged at 1300 × *g* for 30 min. at 4° C. The serum was collected, frozen on dry ice, and stored at -80 ^{o}C until analysis. Urine was collected in

a syringe either from voids on a clean table or via bladder puncture and transferred to a micro-centrifuge tube, immediately frozen on dry ice, and stored at -80 °C until analysis.

2.3 Human Samples. Matched human urine (*n*=84) and serum (*n*=96) samples, from individuals with no known fipronil exposure, were collected by the National Institute for Environmental and Health Sciences (NIEHS protocol number 10-E-0063) between April and June 2011. The human samples were simply a sample of convenience and were not meant to be representative of a specific population. The urine collected was a spot sample and was not concentrated or representative of a specific sampling period. Volunteers were anonymous, and no personally identifiable information was provided. The samples were from male and female volunteers of various ethnicities between 19 and 73 years of age who live in the Raleigh-Durham area of North Carolina (Table 1). Although 100 volunteers participated in the study, several urine and serum samples were not included due to an insufficient volume for analysis.

Table 1. Human demographic data for the 100 volunteers.

	Sex		Age				Race			
	Male	Female	19-33	34-48	48-62	62-76	Asian	Black	White	Other
%	30	70	29	30	33	8	3	32	63	2

2.4 Extraction Protocols. Samples were extracted in a manner that optimized recovery and reproducibility while reducing matrix interference. Animal samples were small volumes that did not require solid phase extraction (SPE). However, a protocol involving SPE was performed with the human samples to reduce matrix interference. Sample extraction protocols for biologicals are described below. More information on methods development for human samples can be found in the Supporting Information. Rat serum samples were first analyzed by liquid chromatography/time-of-flight mass spectrometer (LC/TOF-MS) in order to identify any metabolites. Human samples were then analyzed by liquid chromatography/triple-quadrupole

mass spectrometer (LC/triple-quad) for quantification of metabolites for which analytical standards were possessed. LC/quadrupole/time-of-flight mass spectrometry (LC/Q-TOF) was used for structure elucidation of unknown metabolites.

2.5 Rat serum. Rat serum (25 µL) was denatured with 100 µL of 0.1 M formic acid and precipitated with 1 mL of a cold acetonitrile solution spiked with the internal standard (fipronil des-F_3, 25 ng). The sample was then centrifuged for 5 minutes at 12500 × *g*. An aliquot of the supernatant was mixed 50:50 with 10 mM ammonium acetate buffer, and analyzed via LC/TOF and LC/triple-quad. *n*=9 for highest dose (10 mg/kg/day) ; *n*=10 for low dose (5 mg/kg/day); and *n*=11 for control animals, which were treated with vehicle. Quantitation was performed for fipronil and fipronil sulfone. The results of the quantitation are shown in the supporting information.

2.6 Rat urine. Rat urine (100 µL) was precipitated with 900 µL of cold acetonitrile and centrifuged for 8 minutes at 12500 × *g*. An aliquot of the supernatant was extracted and mixed 50:50 with 10 mM ammonium acetate buffer before LC/MS analysis. *n*=3 for highest dose (10 mg/kg/day); *n*=4 for low dose (5 mg/kg/day); and *n*=3 for control animals. Quantitation was only performed for the fipronil sulfone metabolite, as standards were not available for other metabolites. Quantitation specifics can be found in the Supporting Information. Fipronil sulfone concentrations in rat urine were used to approximate the relative concentrations of the other observed metabolites.

2.7 Human serum. Human serum (200 µL; *n*=96) was denatured with 20 µL of a 0.1 M formic acid solution spiked with internal standard (fipronil des-F_3, 5 ng) and precipitated with 2 mL of cold acetonitrile. The sample was centrifuged for 10 minutes at 12,500 × *g* and concentrated using solid phase extraction (SPE) using an Oasis 3cc HLB cartridge (Waters

Corporation, Milford, MA). SPE cartridges were conditioned with 3 mL of methanol and 3 mL of ultrapure water, samples were loaded, washed with 3 mL of 95:5 water/acetonitrile solution, then eluted with 3 mL of acetonitrile. The eluate was evaporated under N_2 at 40° C until approximately 200 µL remained. The concentrated solution was mixed 50:50 with 10 mM ammonium acetate buffer and analyzed via LC/TOF and LC/triple-quad for all compounds listed in the chemical section. In order to determine the concentration of compounds of interest, a seven-point matrix-matched (blank calf serum-Life Technologies-Gibco®, Grand Island, NY) extracted standard curve from 0.1-50 ng/mL, along with a method blank (DI water) and a matrix blank was run with the human serum samples. The lowest value on the standard curve (0.1 ng/mL) was considered the lower limit of quantitation (LLOQ).

2.8 Human urine. Human urine (5-12 mL; *n*=84) was precipitated with 1 mL of acetonitrile and concentrated using the SPE method described above with an Oasis 6cc HLB cartridge with the exception that cartridges were conditioned with 5 mL of methanol and 5 mL of ultrapure water, samples were loaded, washed with 5 mL of 95:5 water/acetonitrile solution, then eluted with 5 mL of acetonitrile. The eluate was evaporated under N_2 at 40° C until approximately 1 mL remained. The concentrated solution was mixed 50:50 with 10 mM ammonium acetate buffer in an LC vial and analyzed by LC-TOF/MS (*n*=84) for all compounds listed in the chemicals section, as well as for any unknown metabolites. Note that several urine samples were excluded due to insufficient volume.

2.9 Analytical Instrumentation. Targeted analyses (LC/triple-quad) were carried out using an Agilent 1100 HPLC (Agilent Technologies, Palo Alto, CA) interfaced with a Sciex 3000 triple quadrupole mass spectrometer (Applied Biosystems/MDS Sciex, Foster City, CA) fitted with an electrospray ionization source (ESI) operated in the negative ionization mode. Compounds

contained in the LC/triple-quad method (fipronil, fipronil sulfone, fipronil sulfide, fipronil amide, and monochloro fipronil) were optimized on a compound specific basis. Information regarding transitions are included in the Supporting Information.

The HPLC system consisted of a Phenomenex Luna C18 column (50 x 3 mm, 5 µm; Torrance, CA, USA) with a Security-guard guard column (Phenomenex). The method consisted of the following: 0.4 mL/min flow rate which increased to 0.75 mL/min at time=2 min; temperature: 30 °C; mobile phases – A: ammonium acetate buffer (0.2 mM) and DI water:methanol (95:5, v/v), and B: ammonium acetate buffer (0.2 mM) and acetonitrile:DI water (95:5, v/v); gradient: 0-2 min 50% A and 50% B; 2.1-4 min, a linear gradient from 50:50 A:B to 10:90 A:B; 4-6 min 10% A and 90% B; 6.1-10 min re-equilibration to 50% A and 50% B.

Non-targeted analyses (LC/TOF) were carried out using an Agilent 1100 HPLC (Agilent Technologies, Palo Alto, CA) interfaced with an Agilent 6210 Time-of-Flight (TOF) mass spectrometer fitted with an electrospray ionization source operated in the negative ionization mode at 120 Volts. Any drift in the mass accuracy of the TOF was continuously corrected by infusion of two reference compounds (purine [m/z = 119.0363] and hexakis (1H, 1H, 3H-tetrafluoropropoxy) phosphazene [m/z = 966.0007]) via dual-ESI sprayer.

The HPLC method consisted of a Zorbax Eclipse Plus C18 column (2.1 × 50 mm, 3.5 um; Agilent Technologies, Palo Alto, CA) fitted with a Phenomenex guard column (Torrance, CA). The method consisted of the following: 0.2 mL/min flow rate; at 30 °C; mobile phases: A: ammonium formate buffer (0.4 mM) and DI water:methanol (95:5 v/v), and B: ammonium formate (0.4 mM) and methanol:DI water (95:5 v/v); gradient: 0-5 min a linear gradient from 50:50 A:B to 100% B; 5-15 min, 100% B; 15-18 min re-equilibration to 50% A and 50% B.

2.10 Identification of Spectral Features. The TOF-MS system has proprietary software that can be used in non-targeted analyses to help identify compounds that are specific to a treatment group or a specific experimental condition. For example, to identify potential biomarkers of fipronil exposure, control and dosed animal samples are analyzed, and molecular features (identifiable peaks) were first extracted according to user specified criteria (e.g., minimum peak height, area count). The two groups of extracted features were then compared using The Mass Profiler software, which singles out only those compounds that are found in the dosed group. This collection of compounds can be thought to represent either the parent compound, metabolites of the parent, or specific biological responses that are attributable to the treatment administered.

The exact monoisotopic mass of each of these "treatment only" features was then used to generate a ranked list of possible chemical formulae for each unknown. The numerical ranking is based on the difference between the calculated and measured mass, the isotopic abundance and the isotope spacing. If authentic standards are available, the identity of a proposed feature can be confirmed using chromatographic retention time, exact monoisotopic mass, and isotopic distribution.

Fipronil is an interesting and somewhat unique compound because it contains six fluorine atoms and two chlorine atoms, that result in a significant negative mass defect (435.93869 Da, with the $[M-H]^-$ ion seen in negative ionization mode being 434.9314 *m/z*) which is preserved in most of its metabolic products to the extent that the F and Cl atoms are retained[20]. Moreover, the isotopic spacing between the Cl isotopes (^{35}Cl [75.77%] and ^{37}Cl [24.23%]) leads to a distinctive isotopic pattern that aids greatly with identification (SI Figure 2). Both of these characteristics were useful in identifying fipronil-related metabolites.

247 Metabolites that were identified using the LC/TOF instrument described above were then
248 investigated further using an Agilent 1200 HPLC system fitted with a 6250 quadrupole time-of-
249 flight mass spectrometer (Q-TOF) (Agilent Technologies, Palo Alto, CA) using the same LC
250 conditions as previously described. The LC/Q-TOF allowed fragmentation at various collision
251 energies of metabolites of interest which helped with structure elucidation.

252 **2.11 Quality Assurance/Control.** For each analysis, method and matrix blanks were
253 evaluated for contamination or background levels of the compounds of interest. The LLOQ was
254 determined as the concentration of the lowest working standard, which back-predicted within
255 30% of a theoretical value. The LLOQ in the quantitative human serum experiments was
256 validated by calculating signal-to-noise ratios for the 451-415 *m/z* transition relative to a method
257 blank. R-squared values for all quantitative procedures were monitored to ensure predictability.
258 Three randomly chosen samples were replicated in each quantitative experiment to ensure
259 consistency within the data sets. Parent-daughter ratios should be consistent, and ratio
260 monitoring is a robust way to confirm the presence of a specific compound. Therefore, in the
261 targeted screening of samples, the ratio between the primary and secondary parent-daughter
262 transition was monitored to confirm the presence of each compound in the MS method. High and
263 low concentration quality control (QC) samples containing the fipronil mixture of five analytical
264 standards described in the *Chemicals* section were run with each batch of human serum samples
265 to ensure analytical precision and accuracy.
266

267 3. RESULTS

268 **3.1 Quality Assurance/Control.** All lab prepared target and non-target analysis blanks and
269 control samples were below the respective LLOQ for compounds of interest in all experiments.

Validation of the LLOQ in the human serum quantitative experiments showed that the lowest curve point differed from the method blank (signal-to-noise ratio for method blank = 3 ± 1; signal-to-noise ratio for 0.1 ng/mL standard = 20 ± 12). All r-squared values were greater than 0.99, which ensured predictability. All replicates for all experiments had a relative standard deviation of <15%. For all targeted analyses, the ion ratios between the primary and secondary parent-daughter transitions were consistent for all standard compounds and those observed in unknown samples (ion ratio mean ± 20%). All QC samples (high and low) were 100% ± 15% of the nominal values.

3.2 Urine from Treated Rodents. The urine from rodents treated for 14 days with fipronil was analyzed for biomarkers of exposure via non-targeted analysis. As described above, molecular features (significant chromatographic peaks) were extracted from analytical runs of both dosed and control animals, and The Mass Profiler software was used to isolate those features that were unique to the dosed animals. The most plausible candidate biomarkers were those compounds with the signature isotope pattern of two chlorine atoms (SI Figure 2) and/or significant negative mass defects indicative of fluorine and chlorine atoms. Seven high abundance peaks fitting these criteria were identified, and the exact monoisotopic mass of each was used to generate a ranked list of plausible formulae and corresponding structures. We tentatively assigned compound identity according to known metabolic pathways (e.g., oxidation, sulfation, glucuronidation), the retention of negative mass defect and/or the isotopic pattern associated with chlorine, and consistency with results from previous studies. Information on the seven metabolites can be found in Table 2. Four of the compounds (M1, M2, M3, M5, and M6) were identified in previous studies[10, 21], whereas two more (M4 and M7) are reported for the first time in this study (Figure 1). It should be noted that the spectral feature observed for the

293 glucuronide conjugate (M6) splits into two chromatographic peaks, most likely meaning that the
294 glucuronide molecule adds to both the oxygen and the nitrogen atom (see Figure 1). We were
295 unable to differentiate which peak corresponded to which structure, but one was formed
296 preferentially. However, this spectral feature is not observed for the sulfate conjugate (M5).

297 To better characterize the structures of metabolites M4 and M7, the fragmentation patterns of
298 the parent metabolites were analyzed via LC/Q-TOF. No useful information was gained about
299 metabolite M4; however, the fragmentation pattern of metabolite M7 helped to predict a
300 plausible structure. M7 structural information could be gleaned from looking at the exact masses
301 of molecular fragments originating from the parent molecule. For example, if the mass of a CO_2
302 group is observed in the fragmentation pattern, it can be assumed that the molecule likely
303 contained a carboxylic acid. Spectral information regarding this fragmentation pattern can be
304 found in the Supporting Information (SI Figure 3).

305 Fipronil sulfone (M1) was confirmed by an authentic standard that has the same retention time,
306 monoisotopic mass, ion fragmentation pattern, and isotope spacing. Rats in the 5 mg/kg/day
307 dose-group had mean concentrations of fipronil sulfone of 24.1 (SD = 18.7) ng/mL, while the 10
308 mg/kg/day group had 31.9 (SD = 13.1) ng/mL (SI Figure 1). If the fipronil sulfone
309 concentrations are used to generate estimated relative response factors for other metabolites that
310 do not have standards (assuming that all respond similarly within the TOF-MS), we estimate the
311 relative concentrations of fipronil metabolites in dosed-rodent urine to be
312 M6>M4>M5>M3>M7>M1>M2. The estimated concentrations of M2 and M6 are 30 and 2,000
313 ng/mL respectively.

314 **Table 2.** LC/TOF characteristics of putative metabolites in rat urine.

Metabolite	Retention Time (mins)	Predicted Formula of parent	Score of Predicted Formula	$[M-H]^-$ Measured Mass *(m/z)*	$[M-H]^-$ Calculated Mass *(m/z)*	Δ ppm	Monoisotopic Mass *(m/z)*
M1 (Fipronil Sulfone)	7.57	C12H4Cl2F6N4O2S	99.63	450.9266	450.9263	0.67	451.9336
M2	7.3	C9H4Cl2F3N3	93.50	279.9665	279.9662	1.07	280.9734
M3	1.62	C11H4O2N4Cl2F3	99.53	350.9667	350.9669	0.43	351.9742
M4	5.38	C10H4Cl2F3N3O2	98.63	323.9565	323.9560	1.54	324.9633
M5	1.4	C11H5Cl2F3N4O4S	99.38	414.9290	414.9288	0.48	415.9361
M6	1.39	C17H13Cl2F3N4O7	98.74	511.0036	511.0041	0.98	512.0113
M7	5.38	C11H3Cl2F3N4O	98.93	332.9564	332.9563	0.30	333.9563

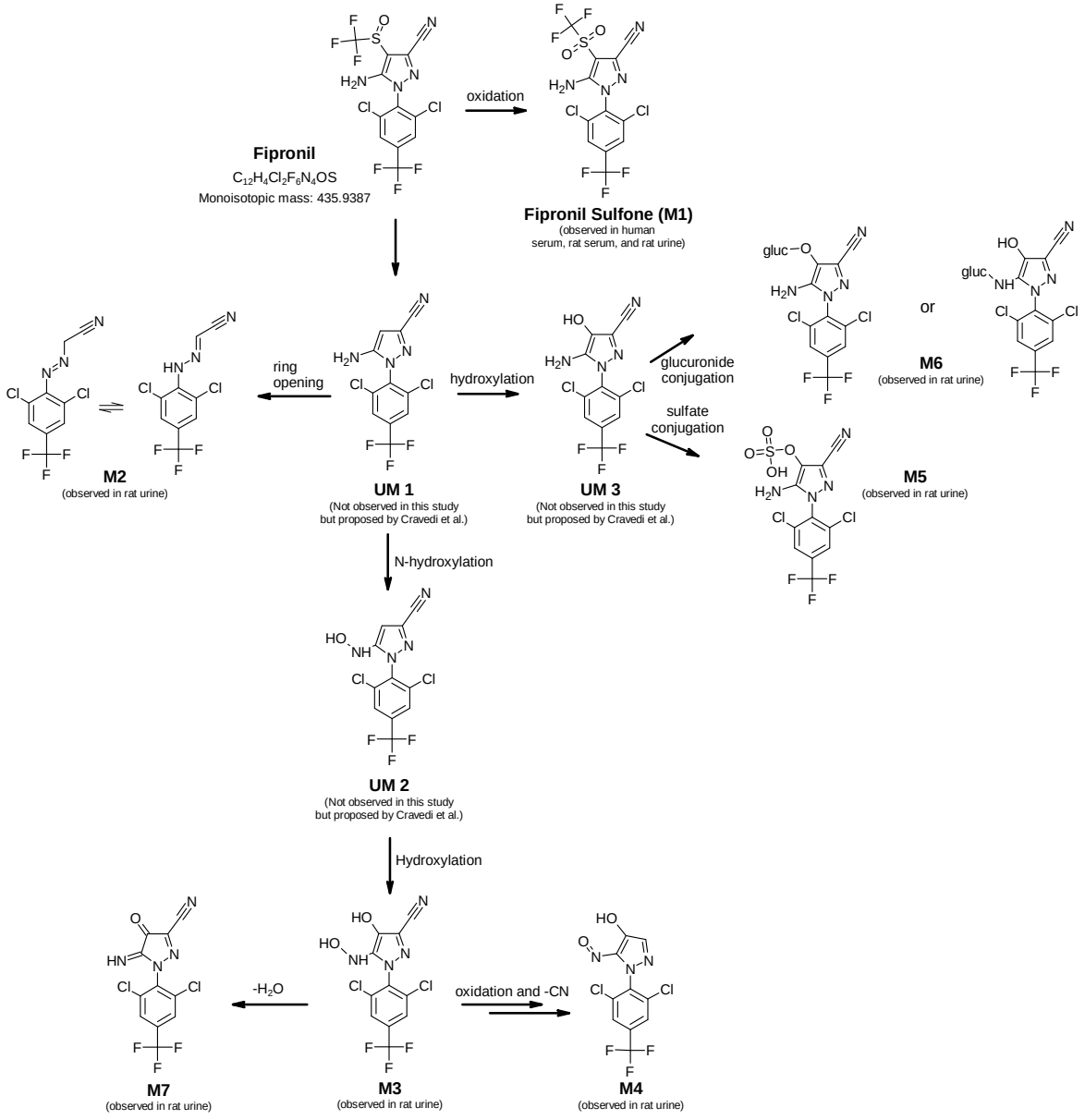


Figure 1. Proposed metabolic pathway of fipronil in the rat. M4 and M7 are proposed structures based on MS data, isotope distributions, and exact mass. M1, M2, M3, M5, and M6 were identified in rat urine. Unobserved metabolites labeled (UM) were not identified but are likely intermediates.

3.3 Serum from treated rodents. The serum from treated rats was analyzed for all suspected biological metabolites via LC-TOF to evaluate the presence of possible serum biomarkers. In our analysis we detected no additional metabolites other than small amounts of un-metabolized fipronil and fipronil sulfone which had been previously identified by several groups [4, 22]. Quantitative data for fipronil and fipronil sulfone in rat serum can be found in the Supporting Information.

3.4 Human urine. Urine samples (*n*=84) from volunteer North Carolina residents with no known exposures to fipronil or other pesticides were examined for M1-M7 (identified in rodent urine) and for all other plausible fipronil adducts or derivatives using the methods described above. No parent fipronil or any plausible metabolites were found in the human urine samples.

3.5 Human serum. Matched human serum samples (*n*=96) were analyzed for the metabolites observed in rat serum (fipronil and fipronil sulfone) by a targeted approach (LC/triple-quad, LOQ = 0.1 ng/mL). Only trace amounts of the parent fipronil were found in the human blood samples. However, fipronil sulfone (the biomarker identified in the rodent study) was detected in approximately 25% of the samples, at levels ranging from 0.1 to 3.9 ng/mL (Figure 2). Table 3 describes general trends in the data in terms of detects vs. non-detects.

Table 3 shows the number of detects vs. non-detects for each gender and race.

Gender	Detects	Non-Detects	Number of Samples
Male	7	12	29
Female	17	67	67
Race	**Detects**	**Non-Detects**	**Number of Samples**
Caucasian	22	39	61
African American	1	29	30
Asian	1	2	3
Other	0	2	2

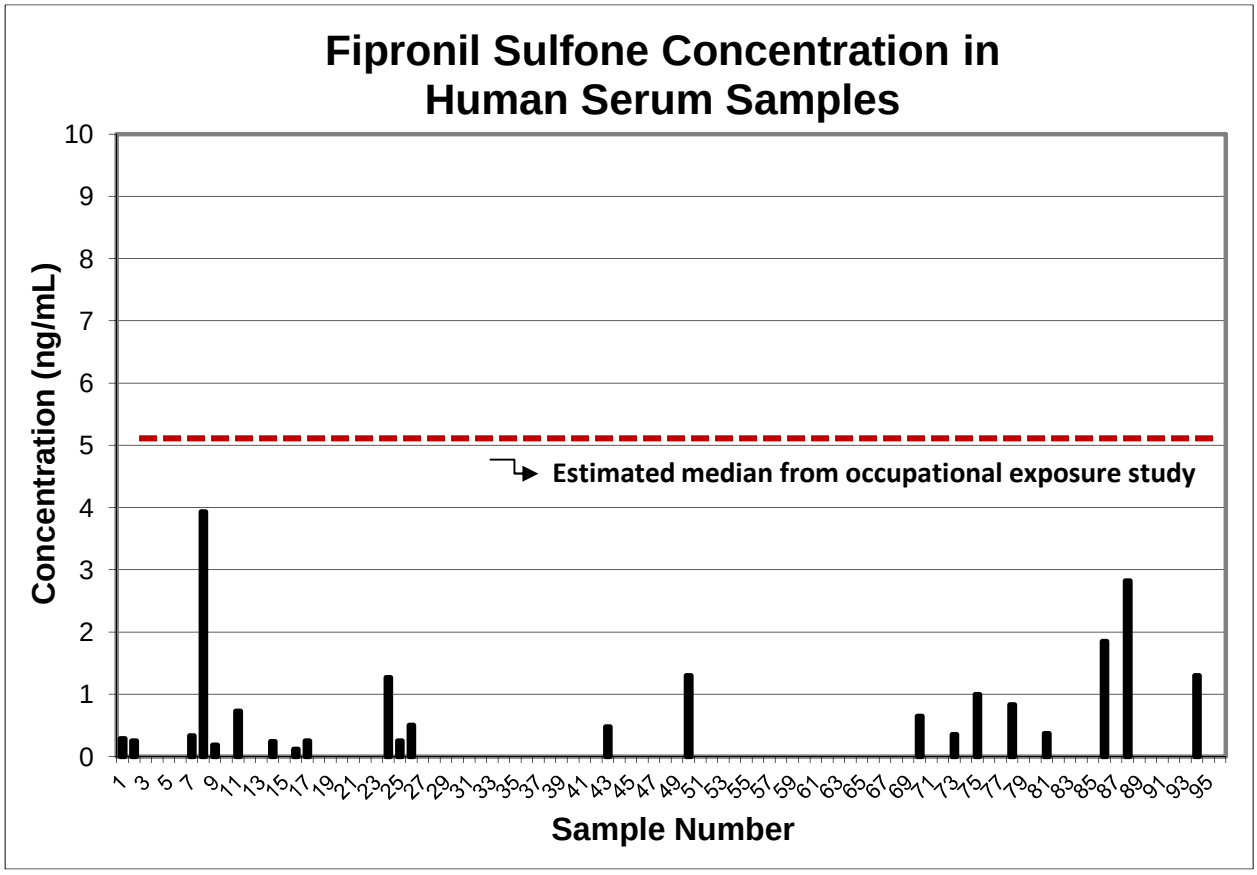


Figure 2 shows fipronil sulfone concentrations in human serum*. The red dotted line represents the median calculated from an occupational exposure study[17, 23].

**n* = 96, four samples were excluded due to insufficient volume.

4. DISCUSSION

This study demonstrates how advanced time-of-flight mass spectrometry techniques can be used to more fully describe the metabolism of xenobiotic compounds in treated-animal studies and how this knowledge can be applied in human biomonitoring studies to make relevant conclusions about human exposures to emerging compounds of concern. Our specific goal was to use the biomarkers identified from the dosed rodent work in the analysis of a set of human biological samples to characterize the rate of fipronil exposure in the general population.

In describing the metabolism of fipronil in rodents, our results were largely consistent with previous studies,[10, 21, 24] while also extending what is known about the basic metabolic process. Two novel metabolites observed in rat urine in this study which were not seen by Cravedi et al. (2013) can be attributed to differences in study design. Specifically, our Long Evans rats were dosed (5 or 10 mg/kg/day) for 14 days then sacrificed 6 hours after the last dose. In contrast, Cravedi et al. (2013) dosed acutely at 10 mg/kg and collected urine and serum every 24 h. over a 72 h. period[10]. Differences between rat strain or length of dosing regimen may have made it possible to identify different products of fipronil metabolism, such as the pyrazole ring opened products or the highly oxidized heteroaromatic amine derivatives.

The proposed metabolic pathway in the rat and compound structures can be found in Figure 1. We propose that a new metabolite (M7) in rat urine, an imine, results from the loss of water from metabolite M3, which is a fipronil metabolite that is hydroxylated at both the carbon and the nitrogen. We also identified what is hypothesized to be nitroso compound (M4). We believe that M3 and M4 are formed from an unobserved hydroxyl amine intermediate (UM 2). The hydroxyl amine (M3) has been identified in this and in previous studies[10], but to our knowledge this is the first report of a nitroso metabolite of fipronil in rat urine. Although the structure for metabolite M4 is only putative, heterocyclic aromatic amines are known to undergo biological oxidation to

form nitroso compounds. This process is mediated by cytochrome P-450 and NADPH[25]. Many heterocyclic amines are known carcinogens,[26-30] due to their ability to be hydroxylated and then form DNA adducts. The observation of N-hydroxylated fipronil metabolites in this and other rodent studies warrants further investigation of fipronil metabolism in humans and the resulting effects.

Noninvasive biomarkers like those present in urine, exhaled breath, hair, fingernails, etc. are optimal for use in human studies, and one intention of this study was to explore whether any of the urinary metabolites found in the rats could be used as biomarkers of exposure in humans. Studies with human liver microsomes have shown that fipronil is metabolized to fipronil sulfone *in vitro*, and Mohamed et al. (2004) have identified fipronil sulfone as a metabolite in humans acutely exposed to high doses[9, 16]. Aside from these, no publications comment on the disposition of fipronil in humans. In this study we analyzed human urine samples for any of the metabolites identified as possible biomarkers in rat urine. The absence of fipronil and its metabolites in the human urine samples was undoubtedly related to many factors. To start with, it is possible that most human elimination of these materials occurs via the feces, as is the case with rodents[14, 24]. Secondly, and perhaps more importantly, our study subjects were essentially volunteers from the Raleigh-Durham area of North Carolina with no known exposures to fipronil and/or any other similar pesticides. Identification of small amounts of unknown chemicals in urine from populations with no known exposure can be difficult due to the large amount of endogenous compounds found in the matrix. A more effective strategy would be to work with a group of individuals with higher exposure levels (preferably occupationally) to determine human urinary metabolites. Despite negative findings with the human urine samples, 25% of the serum samples

contained measureable amounts of fipronil sulfone (range 0.1 – 4 ng/mL), providing clear evidence that humans are regularly exposed to fipronil.

We compared our results to those from a study by Herin et al. where the serum from workers in a fipronil production facility was measured for fipronil and fipronil sulfone. The median serum concentration from the occupational exposure study was calculated from the mean (μ) and standard deviation (σ) provided via a method by Pleil et al.[23] where the geometric mean is used to estimate the median which is equal to $\mu/[1 + 0.5 \times(\sigma/\mu)^2]$. Interestingly, the maximum concentration observed in this study (3.9 ng/mL) was only slightly less than the calculated median of 5.2 (± GSD = 2.4) ng/mL for the occupationally exposed workers[17] (see Figure 2).

The general population likely shares specific exposure routes. One of the most likely routes of exposure is contact with pets that have received applications of fipronil (i.e. Frontline® Plus) or have had contact with indoor/outdoor applications around the home. Notably, Morgan et al. (2008) concluded that family pets can act as vehicles for human exposure to the organophosphorous insecticides, such as diazinon[31]. Specifically, fipronil is widely used to control residential insect pests such as termites and fire ants outdoors where pets frequent, leading to transport of the material indoors. Furthermore, many flea and tick topical products contain approximately 10% fipronil and are applied directly to the skin and fur of dogs and cats, leading to human exposure to fipronil through direct contact with their pets. Dyk et al. (2012) used a fluorescent indicator to show that these fipronil residues are easily transferred from pets to humans by way of direct contact for one week following application[32]. According to estimates from the American Humane Association, up to 46% and 39% of US households keep dogs and cats, respectively. Use of fipronil containing products with these animals could conceivably result in some measurable human exposures. Ongoing efforts in our lab (data not shown) are

investigating domestic indoor sources of exposure that may be important, since local WWTP effluent is shown to contain fipronil and metabolites.

Although we felt the study was well-designed, it did have a few limitations. First, the fipronil sulfone metabolite may not be a specific biomarker for fipronil exposure, since it is known that it can undergo photochemical degradation[2] and its presence has been documented in environmental media by several reports,[7, 33] thus one could be exposed to either fipronil or the degradate. In addition our sample size was relatively small (n=100). Furthermore, the number of detects was less than 30% of the total sample; which did not warrant a statistical analysis. More work is needed on a larger and more diverse sample before further conclusions can be drawn. Worth mentioning, however, was that approximately 92% of fipronil sulfone detections in human serum were from Caucasians, which represented only 63% of our samples. This result suggests that discrepancies between ethnicities may be present.

While the target of fipronil is insects, the two trifluorormethyl groups of fipronil may increase the compound's absorption and distribution upon accidental exposure by humans. Approximately 20-25% of drugs produced in the pharmaceutical industry contain at least one strategically incorporated fluorine atom (usually in the form of either one fluorine atom or a trifluoromethyl group) because fluorine can significantly impact lipophilicity and improve the bioavailability of orally administered drugs. Several studies have shown that the addition of fluorine, the most electronegative element, can decrease the pKa and therefore basicity of surrounding functional groups[34, 35]. Although the effect is not always predictable, this decreased basicity stabilized molecules in the harsh acidic conditions of the stomach and increases bioavailability[36, 37]. Another factor that affects the absorption and distribution of a molecule is lipophilicity. Compounds usually enter into cell membranes via passive transport (although

active transport is an alternate mechanism). Passive transport requires that the molecule is able to permeate the cell membrane, but also avoid entrapment by the lipid bilayer. The electron withdrawing capabilities of fluorine can, in some cases, be incorporated to tune a compound's lipophilicity and ease passive transport into cells[38-40]. Fipronil's presence in human serum demonstrated that the chemical is, in fact, absorbed by humans. Further, Hainzl et. al (1996) found that fipronil lost almost all activity in neurotoxicity studies on mice without the trifluoromethylsulfinyl functional group.[2] Metabolites of fipronil have also been found in many rat tissues, including brain cells[2, 4, 10], demonstrating that even highly selective membranes are somewhat permeable to these chemicals. The fluorinated functional groups may increase fipronil's potency as an insecticide; however, they may also increase absorption and distribution of the potentially toxic compound in non-target organisms, such as humans. Considering that fipronil has been associated with endocrine disruption, neurotoxicity, and carcinogenicity[12-15], accidental exposure and increased bioavailability may be problematic.

In conclusion, previously reported metabolites in rat urine and serum were confirmed, and two novel urinary metabolites have been proposed. The putative biomarkers determined in the rodent study were used in human serum analysis, where fipronil sulfone was found in approximately 25% of serum samples from a convenient sample of North Carolina residents. Serum fipronil levels in our study suggest that environmental exposures to fipronil may be common, but likely lower than occupational exposures. Matched urine was also analyzed, but no fipronil or any of its metabolites were identified, which suggests that urine may not be an appropriate matrix for biomonitoring populations with no known exposure to fipronil. More extensive characterization of the metabolites produced in humans exposed to higher levels of fipronil, as well as the effects

462 from low but chronic exposure to fipronil is needed. Further investigations are also necessary to
463 describe the sources of fipronil exposure and identify rates of exposure in other populations.
464
465
466 5. SUPPORTING INFORMATION

467 **5.1 Rodents** were housed in polycarbonate cages containing heat-treated hardwood chip
468 bedding. Access to food (Purina 5008 Chow; Lab Diet/PMI Nutrition International, Richmond,
469 IN) and tap water was provided *ad libitum*. Animals were allowed to acclimate to their
470 surroundings in the animal colony for 5-7 days before beginning any tests. The animal colony
471 was maintained at a temperature of 22 ± 2 ^{o}C, with humidity at 40 ± 20%, and a 12:12 hr
472 light:dark cycle (light on at 6:00 a.m.).

473 **5.2 Recovery Experiment for Fipronil in Dosed-rat Serum.** Standard fipronil (200 ng) was
474 added to a vial containing blank rat serum (100 µL), along with 100 µL of 0.1 M formic acid and
475 1 mL of cold acetonitrile. The solution was centrifuged at 12,500 × g, and the supernatant was
476 extracted. In a separate vial, blank rat serum was directly spiked with fipronil standard (200 ng).
477 Both the supernatant and the control sample were mixed 50:50 with 10 mM ammonium acetate
478 buffer and analyzed via LC/triple-quad. The fipronil recovery rate was 98%.

479 **5.3 Recovery Experiment for Fipronil in Spiked Human Serum.** A standard mix of fipronil
480 metabolites (10 ng each metabolite) was added to a vial containing blank calf serum (200 µL),
481 along with 25 µL of a 0.1 M formic/internal standard solution (fipronil des-F_3, 10 ng) and 2 mL
482 of acetonitrile. The solution was centrifuged at 12,500 × g and was extracted onto an Oasis 3cc
483 HLB solid phase extraction cartridge. The solid phase extraction method consisted of

conditioning the cartridge with 3 mL of methanol followed by 3 mL of DI water; loading the sample; washing with 3 mL of 95:5 water:acetonitrile; and eluting with 3 mL of acetonitrile. The samples were evaporated under N_2 at 40 °C until 200 µL remained. In a separate vial (the control sample), only 200 µL of blank calf serum, 25 µL of the 0.1 M formic acid/internal standard solution and 2 mL of acetonitrile was added (no fipronil or metabolites), and this vial was also carried through the procedure, just as the experimental sample. The control sample was spiked with the standard mix of fipronil metabolites (10 ng/metabolite) after evaporation. All the samples were prepared 50:50 with 10 mM ammonium acetate buffer, and analyzed via LC/triple-quad (*n*=3). The results are shown below in SI Table 1.

SI Table 1. Human serum recovery experiment results.

Compound	Average % Recovery (± %RSD)
Fipronil	82 ±2.4
Fipronil sulfone	83 ±3.6
Fipronil sulfide	84 ±3.6
Fipronil amide	82 ±7.3
Monochloro fipronil	85 ±3.5

5.4 Recovery Experiment for Fipronil in Spiked Human Urine. A standard mix of fipronil metabolites (400 ng/metabolite) was added to a vial containing 10 mL of blank human urine and 1 mL of acetonitrile/internal standard solution (fipronil des-F_3, 33 ng). The solution was extracted onto an Oasis 6cc HLB solid phase extraction cartridge. The solid phase extraction method was the same as for human serum, except the elution step used 5 mL instead of 3 mL of acetonitrile. The solution was evaporated under N_2 at 40 °C until 1 mL remained. In the control

sample, 10 mL of blank human urine and 1 mL acetonitrile were added (no fipronil or metabolites), and this vial was also carried through the procedure, just as the experimental samples. After evaporation the control sample was spiked with the standard fipronil metabolite mixture (400 ng/metabolite). All samples were prepared 50:50 with 10 mM ammonium acetate buffer and analyzed via LC/triple-quad (*n*=3). The results are shown below in SI Table 2.

SI Table 2. Human urine recovery experiment results.

Compound	Average % Recovery (± %RSD)
Fipronil	103 ±5.8
Fipronil sulfone	100 ±10
Fipronil sulfide	99 ±7.0
Fipronil amide	104 ±3.8
Monochloro fipronil	101 ±5.0

5.5 Quantitation of fipronil and fipronil sulfone in the serum of treated rodents. Rat serum (25 µL) was denatured with 100 µL of 0.1 M formic acid and precipitated with 1 mL of a cold acetonitrile solution spiked with the internal standard (fipronil des-F_3, 25 ng). The sample was then centrifuged for 5 minutes at 12500 × *g*. An aliquot of the supernatant was mixed 50:50 with 10 mM ammonium acetate buffer, and analyzed via LC/TOF and LC/triple-quad. *n*=9 for highest dose (10 mg/kg/day) ; *n*=10 for lowest dose (5 mg/kg/day); and *n*=11 for control animals, which were treated with vehicle. To determine the concentration of compounds of interest, a nine-point matrix-matched extracted standard curve from 10-5000 ng/mL, a method blank (DI water), and a matrix blank (blank rat serum) was run with the rat serum samples via LC/triple-quad. The lowest value on the standard curve (10 ng/mL) was considered the lower limit of quantitation (LLOQ). The results of the quantitation are shown in SI Table 3.

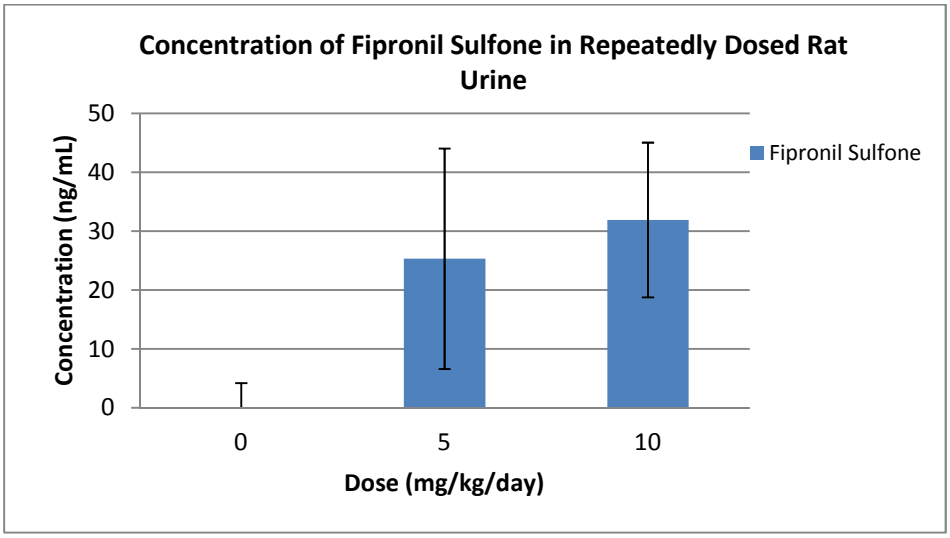
SI Table 3. Mean, standard deviation, and 95% confidence interval for fipronil and fipronil sulfone in rat serum.

Compound	Dose Conc. (mg/kg bw)	LOQ (ng/mL)	Mean (ng/mL)	St. Dev.	95% Conf. Int.
Fipronil	control	10	1.0 (<LLOQ)	3.0	1.8
Sulfone	control	10	2.5 (<LLOQ)	3.7	2.2
Fipronil	5	10	8.9 (<LLOQ)	3.4	2.1
Sulfone	5	10	2424	193.3	119.8
Fipronil	10	10	13.9	7.8	5.1
Sulfone	10	10	3548	511.9	334.4

5.6 Quantitation of fipronil sulfone in the urine of treated rodents. Rat urine (100 µL) was treated with 900 µL of cold acetonitrile. The sample was then centrifuged for 8 minutes at 12,500 × *g*, prepared 50:50 with 10mM ammonium acetate buffer, and analyzed via LC/triple-quad. $n = 2$ for highest dose (10 mg/kg/day); $n = 4$ for lowest dose (5 mg/kg/day); and $n = 6$ for control animals. In order to determine concentration of compounds of interest, a seven-point extracted standard curve prepared in DI water from 10-5000 ng/mL, along with a method blank (DI water) was run with the experimental rat urine samples. SI Figure 1 shows median fipronil sulfone concentrations for rodents dosed with fipronil. The highest dose group had a mean concentration of 31.9 (SD = 13.1) ng/mL fipronil sulfone, while the lowest dose group had 24.1 (SD = 18.7) ng/mL and the control animals had mean concentrations below the LLOQ.

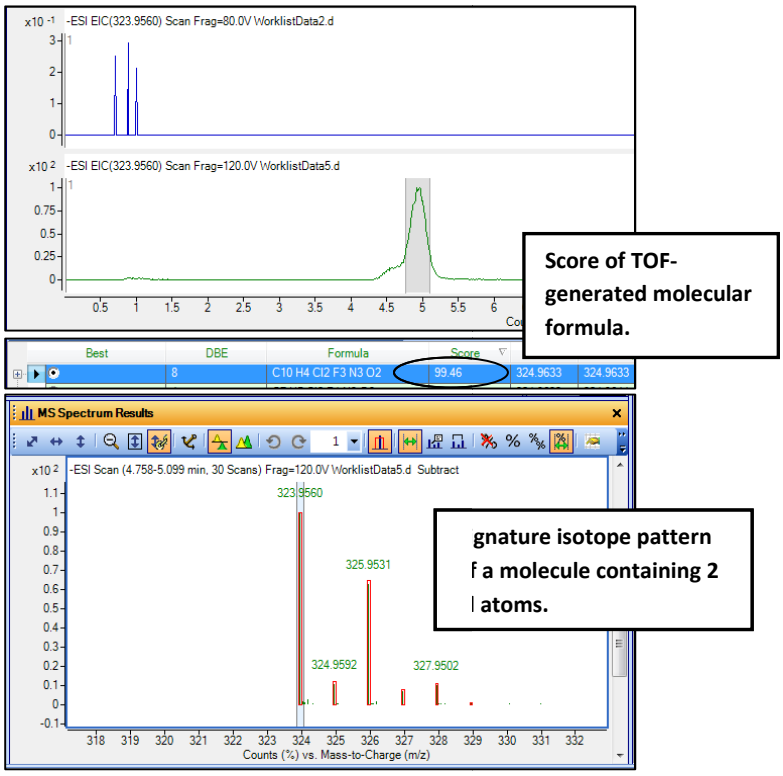
The LC/triple quad used for the quantitation of fipronil sulfone was a Waters Acquity ultraperformance liquid chromatography system coupled with a Waters Quatro Premier XE triple quadrupole mass spectrometer (UPLC-MS/MS; Waters Corporation). A 20-µL aliquot of each sample was injected onto an Acquity UPLC BEH C18 column (1.7 µm, 2.1 × 50 mm; Waters Corporation) that was maintained at 50 ºC. The mobile phase consisted of solvent A: 2 mM ammonium acetate buffer with 5% methanol and solvent B: acetonitrile at a flow rate of 400 µL/min, starting with 75% solvent A for 30 s and then increasing to 90% solvent B at 3.5 min

and 100% solvent B at 3.6 min and held for 0.9 min. At 4.6 min the gradient was returned to 60% solvent A and held until 6.0 min. Electrospray negative ionization was used in the mass spectrometer source. The capillary voltage was set at negative 0.4 kV, and the source temperature was 150 ºC. The primary transition used for quantitation was 451.2 - 244.0 *m/z*, and two other transitions were monitored for confirmation, 451.2 to 281.9 *m/z* and 451.2 to 414.9 *m/z*.



SI Figure 1. Median fipronil sulfone concentration in rat urine.

5.7 Time-of-flight mass spectrometry scoring and isotope patterns.



SI Figure 2 shows the spectral pattern of a molecule containing 2 chlorine atoms. Note that 323.9560 *m/z* is the most abundant isotope, 325.9531 *m/z* contains one ^{37}Cl, and 327.9502*m/z* contains two ^{37}Cl. The 324.9592 *m/z* contains one ^{13}C. The numerical ranking for formula generated for compound (M4) is shown. The top extracted ion chromatograph (Worklist Data 2) shows a control animal sample and the absence of a peak for M4.

572

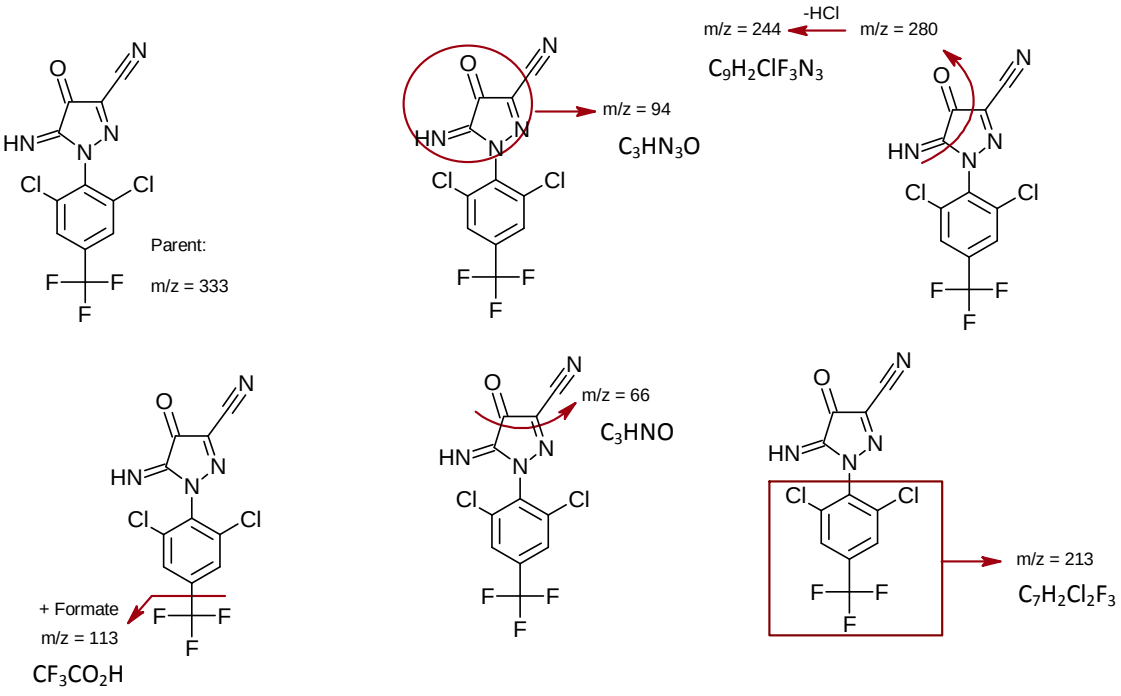
573

574

575

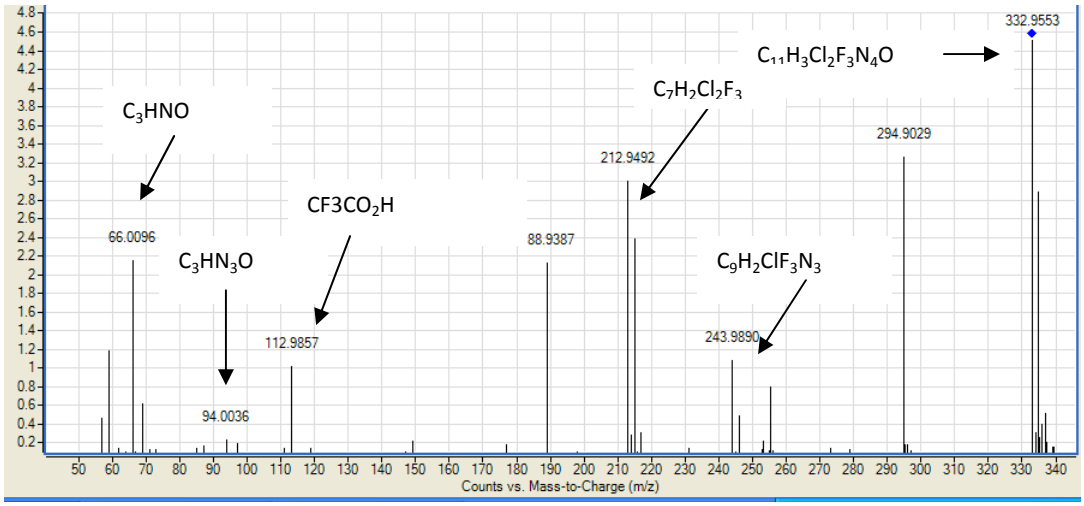
576 **5.8 Metabolite M7 in rat urine**

577



578

579



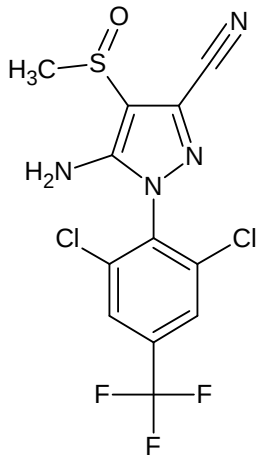
SI Figure 3 shows the fragmentation pattern of Metabolite M7 in the LC/Q-TOF. The red circles/boxes show the fragment and the spectrum at the bottom shows the peaks corresponding to the fragments.

5.9 Transitions in LC/triple quad method. SI Table 6 below lists the parent to daughter transitions which were monitored in the Agilent 1100 LC/triple quad method.

SI Table 6. LC/triple quad parent-daughter transitions.

Compound	Transition	Parent	Daughter
Fipronil	1°	434.9	329.8
Fipronil	2°	434.9	249.9
Fipronil	3°	434.9	277.8
Fipronil sulfone	1°	451.1	415.0
Fipronil sulfone	2°	451.1	281.9
Fipronil sulfone	3°	451.1	243.9
Fiproni sulfide	1°	418.9	382.8
Fiproni sulfide	2°	418.9	261.7
Fiproni sulfide	3°	418.9	313.8
Fipronil amide	1°	452.9	347.7
Fipronil amide	2°	452.9	303.8
Fipronil amide	3°	452.9	271.9
Monochloro fipronil	1°	401.1	283.9
Monochloro fipronil	2°	401.1	295.9
Monochloro fipronil	3°	401.1	331.9
Fipronil des F3	1°	387.2	281.9
Fipronil des F3	2°	387.2	331.0
Fipronil des F3	3°	387.2	351.0

5.10 SI Figure 6 shows fipronil des-F_3 which was used as an internal standard for analytical methods due to its similarity in structure to fipronil. The structure is shown below.



Molecular Formula: $C_{12}H_7Cl_2F_3N_4OS$
Monoisotopic Mass: 381.966971 Da
[M-H]-: 380.959694 Da

AUTHOR CONTRIBUTIONS

The manuscript was written through equal contributions of all authors. All authors have given approval to the final version of the manuscript.

ACKNOWLEDGMENTS

This research was supported by an appointment to the Research Participation Program at the National Exposure Research Laboratory administered by the Oak Ridge Institute for Science Education through an interagency agreement between the U.S. Department of Energy and the U.S. Environmental Protection Agency. We thank Agilent Technologies for providing us with the LC/TOF mass spectrometer that was used to investigate metabolic products. We also thank Michael Hays of US EPA who kindly allowed the use of his LC/Q-TOF mass spectrometer for further structure elucidation, Benny Pyke of Arizona State University for discussions on detection of fipronil in human urine, and Matthew Stiegel, Jon Sobus, and Peter Egeghy for help with data analysis.

DISCLAIMER

This article will be reviewed in accordance with the policy of the National Exposure Research Laboratory, U.S. Environmental Protection Agency, and approved for publication. Approval does not signify that the contents necessarily reflect the view and policies of the Agency, nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

REFERENCES

1. Brassard, D.; Haddad, S.; Hill, E., US EPA BEAD Chemical Profile for Registration Review: Fipronil (PC Code: 129121). In 2011.

2. Hainzl, D.; Casida, J. E., Fipronil insecticide: Novel photochemical desulfinylation with retention of neurotoxicity. *Proceedings of the National Academy of Sciences* **1996,** *93*, 12764-12767.

3. Ikeda, T.; Nagata, K.; Kono, Y.; Yeh, J. Z.; Narahashi, T., Fipronil modulation of GABAA receptor single-channel currents. *Pest Management Science* **2004,** *60*, (5), 487-492.

4. Hainzl, D.; Cole, L. M.; Casida, J. E., Mechanisms for Selective Toxicity of Fipronil Insecticide and Its Sulfone Metabolite and Desulfinyl Photoproduct. *Chemical Research in Toxicology* **1998,** *11*, 1529-1535.

5. Rhône-Poulenc Agro to boost fipronil production. *Agrow* **1997,** *294*, 17.

6. *Trends in Concentrations of Pesticides in Urban Streams in the United States, 1992-2008*; USGS.

7. Gunasekara, A. S.; Truong, T.; Goh, K. S.; Spurlock, F.; Tjeerderma, R. S., Environmental fate and toxicology of fipronil. *Jounal of Pesticide Science* **2007,** *32*, 189-199.

8. Erickson, B. E., Europe to ban fipronil pesticide to protect bees. *Chemical & Engineering News Archive* **2013,** *91*, (29), 21.

9. Mohamed, F.; Senarathna, L.; Percy, A.; Abeyewardene, M.; Eaglesham, G.; Cheng, R.; Azher, S.; Hittarage, A.; Dissanayake, W.; Sheriff, M. H.; Davies, W.; Buckley, N. A.; M., E., Acute human self-poisoning with the N-phenylpyrazole insecticide fipronil--a GABAA-gated chloride channel blocker. *Jounal of Toxicology. Clinical Toxicology* **2004,** *42*, 955-963.

10. Cravedi, J. P.; Delous, G.; Zalko, D.; Viguie, C.; Debrauwer, L., Disposition of fipronil in rats. *Chemosphere* **2013,** *93*, 2275-2283.

11. Tingle, C. C. D.; Rother, J. A.; Dewhurst, C. F.; Lauer, S.; King, W. J., Fipronil: Environmental Fate, Ecotoxicology, and Human Health Concerns. *Reviews of Environmental Contamination and Toxicology* **2003,** *176*, 1-66.

12. Ohi, M.; Dalsenter, P. R.; Andrade, A. J. M.; Nascimento, A. J., Reproductive adverse effects of fipronil in Wistar rats. *Toxicology Letters* **2004,** *146*, (2), 121-127.

13. Raquel, P.; Tercariol, G.; Godinho, A. F., Behavioral effects of acute exposure to the insecticide fipronil. *Pesticide Biochemistry and Physiology* **2011,** *99*, 221-225.

14. *New Pesticide Fact Sheet for Fipronil*; US EPA: 1996.

15. Vidau, C.; Gonzalez-Polo, R. A.; Niso-Santano, M.; Gomez-Sanchez, R.; Bravo-San Pedro, J. M.; Pizarro-Estrella, E.; Blasco, R.; Brunet, J. L.; Belzunces, L. P.; Fuentes, J. M.; ,

659 Fipronil is a powerful uncoupler of oxidative phosphorylation that triggers apoptosis in human
660 neuronal cell line SHSY5Y. *NeuroToxicology* **2011,** *32*, 935-943.

661 16. Tang, J. A.; Usmani, K.; Hodgson, E.; Rose, R. L., In vitro metabolism of fipronil by
662 human and rat cytochrome P450 and its interactions with testosterone and diazepam. *Chemico-*
663 *Biological Interactions* **2004,** *147*, (3), 319-329.

664 17. Herin, F.; Boutet-Robinet, E.; Levant, A.; Dulaurent, S.; Manika, M.; Galatry-Bouju, F.;
665 Caron, P.; Soulat, J.-M., Thyroid function tests in persons with occupational exposure to fipronil.
666 *Thyroid* **2011,** *21*.

667 18. Freeborn, D. L.; McDaniel, K. L.; Moser, V. C.; Herr, D. W., Use of
668 electroencephalography (EEG) to assess CNS changes produced by pesticides with different
669 modes of action: effects of permethrin, deltamethrin, fipronil, imidacloprid, carbaryl, and
670 triadimefon. *Toxicology and Applied Pharmacology*, (doi:10.1016/j.taap.2014.11.011), in press.

671 19. Moser, V. C.; Stewart, N.; Lyke, D. F.; Crooks, J.; MacMillan, D. K.; Hedge, J. M.;
672 Wood, C. E.; McMahen, R. L.; Strynar, M. J.; Herr, D. W., Assessment of serum biomarkers in
673 rats after exposure to pesticides of different chemical classes. *Toxicology and Applied*
674 *Pharmacology*, (doi:10.1016/j.taap.2014.11.016), in press.

675 20. Xie, T.; Liang, Y.; A, J.; Hao, H.; Liu, L.; Zheng, X.; Dai, C.; Zhou, Y.; Guan, T.; Liu,
676 Y.; Xie, L.; Wang, G., Post acquisition data processing techniques for lipid analysis by
677 quadrupole time-of-flight mass spectrometry. *Journal of Chromatography B* **2012,** *905*, (0), 43-
678 53.

679 21. In *Pesticide Residues in Food. Fipronil: Residue Evaluation 2001.*, Joint Meeting on
680 Pesticide Residues in Food

681 22. Lacroix, M. Z.; Puel, S.; Toutain, P. L.; Viguie, C., Quantification of fipronil and its
682 metabolite fipronil sulfone in rat plasma over a wide range of concentrations by LC/UV/MS.
683 *Jounal of Chromatography B* **2010,** *878*, 1934-1938.

684 23. Pleil, J. D.; Sobus, J. R.; Stiegel, M. A.; Hu, D.; Oliver, K. D.; Olenick, C.; Strynar, M.
685 J.; Clark, M.; Madden, M. C.; Funk, W. E., Estimating Common Parameters of Lognormally
686 Distributed Environmental and Biomonitoring Data: Harmonizing Disparate Statistics From
687 Publications. *Journal of Toxicology and Environmental Health, Part B* **2014,** *17*, 341-368.

688 24. In *Pesticide Residues in Food. Fipronil: Residue evaluation 1997.*, Joint Meeting on
689 Pesticide Residues in Food.

690 25. Kim, D.; Guengerich, F. P., CYTOCHROMEP450 ACTIVATION OFARYLAMINES
691 ANDHETEROCYCLIC AMINES. *Annual Reviews in Pharmacology and Toxicology* **2005,** *45*,
692 27-49.

693 26. Snyderwine, E. G.; Turesky, R. J.; Turteltaub, K. W.; Davis, C. D.; Sadrieh, N.; Schut, H.
694 A. J.; Nagao, M.; Sugimura, T.; Thorgeirsson, U. P.; Adamson, R. H.; Thorgeirsson, S. S.,

Metabolism of food-derived heterocyclic amines in nonhuman primates. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis* **1997,** *376*, (1–2), 203-210.

27. Eisenbrand, G.; Tang, W., Food-borne heterocyclic amines. Chemistry, formation, occurrence, and biological activities. A literature review. *Toxicology* **1993,** *12*, 1-82.

28. Pezdirc, M.; Zegura, B.; Filipic, M., Genotoxicity and induction of DNA damage responsive genes by food-borne heterocyclic aromatic amines in human hepatoma HepG2 cells. *Food Chem. Toxicol.* **2013,** *59*, 386-394.

29. Nagao, M.; Sugimura, T., Carcinogenic factors in food with relevance to colon cancer development. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis* **1993,** *290*, (1), 43-51.

30. Schut, H. A. J.; Snyderwine, E. G., DNA adducts of heterocyclic amine food mutagens: implications for mutagenesis and carcinogenesis. *Carcinogenesis* **1999,** *20*, (3), 353-368.

31. Morgan, M. K.; Stout, D. M.; Jones, P. A.; Barr, D. B., An observational study of the potential for human exposures to pet-borne diazinon residues following lawn applications. *Environmental Research* **2008,** *107*, 336-342.

32. Dyk, M. B.; Liu, Y.; Chen, Z.; Vega, H.; Krieger, R. I., Fate and distribution of fipronil on companion animals and in their indoor residences following spot-on flea treatments. *Journal of Environmental Science and Health, Part B* **2012,** *47*, 913-924.

33. Ensminger, M.; Budd, R.; Kelley, K.; Goh, K., Pesticide occurrence and aquatic benchmark exceedances in urban surface waters and sediments in three urban areas of California, USA, 2008–2011. *Environ Monit Assess* **2013,** *185*, (5), 3697-3710.

34. van Niel, M. B.; Collins, I.; Beer, M. S.; Broughton, H. B.; Cheng, S. K. F.; Goodacre, S. C.; Heald, A.; Locker, K. L.; MacLeod, A. M.; Morrison, D.; Moyes, C. R.; O'Connor, D.; Pike, A.; Rowley, M.; Russell, M. G. N.; Moyes, C. R.; O'Connor, D.; Pike, A.; Rowley, M.; Russell, M. G. N.; Sohal, B.; Stanton, J. A.; Thomas, S.; Verrier, H.; Watt, A. P.; Castro, J. L., Fluorination of 3-(3-(piperidin-1-yl)propyl)indoles and 3-(3-(piperazin-1-yl)propyl)indoles gives selective human 5-HT1D receptor ligands with improved pharmacokinetic profiles. *Journal of Medicinal Chemistry* **1999,** *42*, (12), 2087-2104.

35. Rowley, M.; Hallett, D. J.; Goodacre, S.; Moyes, C.; Crawforth, J.; Sparey, T. J.; Patel, S.; Marwood, R.; Patel, S.; Thomas, S.; Hitzel, L.; O'Connor, D.; Szeto, N.; Castro, J. L.; Hutson, P. H.; MacLeod, A. M., 3-(4-fluoropiperidin-3-yl)-2-phenylindoles as high affinity, selective, and orally bioavailable h5-HT2A receptor antagonist. *Journal of Medicinal Chemistry* **2001,** *44*, (10), 1603-1614.

36. Chambers, R. D., *Fluorine in Organic Chemistry*. Blackwell Publishing: Oxford, 2000.

37. Morgenthaler, M.; Schweizer, E.; Hoffmann-Ro, A.; Martin, R. E.; Jaeschke, G.; Wagner, B.; Fischer, H.; Bendels, S.; Zimmerli, D.; Schneider, J.; Diederich, F.; M., K.; Mu, K.,

731 Predicting properties and tuning physicochemical in lead optimization: Amine basicities.
732 *ChemMedChem* **2007,** *2*, 1100.

733 38. Purser, S.; Moore, P. R.; Swallow, S.; Gouverneur, V., Fluorine in medicinal chemistry.
734 *Chemical Society Reviews* **2008,** *37*, (2), 320-330.

735 39. Smart, B. E., Fluorine Substituent Effects (on bioactivity). *Journal of Fluorine Chemistry*
736 **2001,** *109*, (1), 3-11.

737 40. Smith, D. A.; van de Waterbeemd, H.; Walker, D. K., Methods and Principles in
738 Medicinal Chemistry, vol 31: Pharmacokinetics and Metabolism in Drug Design. *Wiley-VCH*
739 **2006,** *31*.

740

Identification of fipronil metabolites by time-of-flight mass spectrometry for application in a human exposure study

Rebecca.L. McMahen[a], *Mark J. Strynar*[b]*, *Sonia Dagnino*[a], *David W. Herr*[c], *Virginia C. Moser*[c], *Stavros Garantziotis*[d], *Erik M. Andersen*[b], *Danielle L. Freeborn*[c], *Larry McMillan*[e], *Andrew B. Lindstrom*[b]

[a]ORISE fellow at the United States Environmental Protection Agency, National Exposure Research Laboratory, Research Triangle Park, North Carolina

[b]United States Environmental Protection Agency, National Exposure Research Laboratory, Research Triangle Park, North Carolina

[c]National Health and Environmental Effects Research Lab, U.S. Environmental Protection Agency, Research Triangle Park, North Carolina

[d]National Institute for Environmental and Health Sciences, Research Triangle Park, North Carolina

[e]National Caucus and Center on Black Aged Employee, U.S. Environmental Protection Agency, National Exposure Research Laboratory, Research Triangle Park, North Carolina

*CORRESPONDING AUTHOR:

Phone: (919)541-3706

Address: 109 TW Alexander Dr. Durham, NC 27705

Email: strynar.mark@epa.gov

ABSTRACT

Fipronil is a phenylpyrazole insecticide commonly used in residential and agricultural applications. To understand more about the potential risks for human exposure associated with fipronil, urine and serum from dosed Long Evans adult rats (5 and 10 mg/kg bw) were analyzed to identify metabolites as potential biomarkers for use in human biomonitoring studies. Urine from treated rats was found to contain seven unique metabolites, two of which had not been previously reported—M4 and M7 which were putatively identified as a nitroso compound and an imine, respectively. Fipronil sulfone was confirmed to be the primary metabolite in rat serum. The fipronil metabolites identified in the respective matrices were then evaluated in matched human urine (*n*=84) and serum (*n*=96) samples from volunteers with no known pesticide exposures. Although no fipronil or metabolites were detected in human urine, fipronil sulfone was present in the serum of approximately 25% of the individuals at concentrations ranging from 0.1-4 ng/mL. These results indicate that many fipronil metabolites are produced following exposures in rats and that fipronil sulfone is a useful biomarker in human serum. Furthermore, human exposure to fipronil may occur regularly and require more extensive characterization.

Keywords: Fipronil, LC/TOF, Biomarker, Human Exposure, Metabolism

ABBREVIATIONS

DI: Deionized

ESI: electrospray ionization

GABA: gamma-aminobutyric acid

GSD: geometric standard deviation

HPLC: high performance liquid chromatography

LC: liquid chromatography

LLOQ: lower limit of quantitation

MS: mass spectrometry

NIEHS: National Institute for Environmental Health Sciences

QC: quality control

Q-TOF: quadrupole time-of-flight

% RSD: Percent Relative Standard Deviation

SD: standard deviation

SPE: solid phase extraction

TOF: time-of-flight

UPLC: ultra performance liquid chromatography

US EPA: United States Environmental Protection Agency

1. INTRODUCTION

Fipronil (Figure 1) is a phenylpyrazole broad-spectrum insecticide that is registered for use in residential settings as part of ant and cockroach baits and gels and termite control products; veterinary applications such as spot treatment flea and tick control products for dogs and cats; ornamental turf applications such as fire ant control; and agricultural applications such as pest control on potato crops[1]. When initially produced, fipronil was the first insecticide to act by

targeting the gamma-aminobutyric acid (GABA) receptor and has favorable selective toxicity towards insects rather than mammals[2-4].

A 1997 report indicated that 480 tons of fipronil were produced per year by Rhone Poulenc,[5] and a more recent EPA report indicated that between 1998 and 2008 usage averaged 150,000 pounds of active ingredient per 1.5 million acres[1]. Widespread fipronil use has led to contamination of water and soil (1-158 ng/L of parent or environmental degradate) in several states including, but not limited to Alabama, Georgia, California, Louisiana, and Indiana[6, 7]. Perhaps as a result of this contamination, fipronil has been implicated as one of the chemicals associated with the colony bee collapse[8].

Because little was found in the peer-reviewed literature about the disposition of fipronil, Cravedi et al. (2013) performed a thorough study on the metabolism, distribution, and elimination of fipronil in rats and showed that fipronil is primarily converted to fipronil sulfone (M1 Figure 1), a more persistent metabolite (estimated half-life is 208 hours in rodents)[9] which was stored mainly in adipose tissue and adrenals[10]. In addition, fipronil has been associated with thyroid disruption[11], endocrine disruption[12], and neurotoxic effects[13] in rats which has led to concern about the potential for human health effects in the last decade.

The effects of acute human exposure to fipronil include headache, dizziness, vomiting, and seizures[9, 10]. Information on the effects of chronic exposure is limited, but the US EPA has classified fipronil as a possible human carcinogen based on data that shows an increase of thyroid follicular cell tumors in both sexes of the rat[14]. Vidau et al. (2011) also concluded that fipronil has the potential to cause apoptosis by uncoupling oxidative phosphorylation at relatively low concentrations (5-10 μM) in human cell lines,[15] and a case of acute human self-poisoning with fipronil has demonstrated that fipronil levels can remain elevated in serum for

days after exposure, and that fipronil sulfone was the primary metabolite[9]. A previous study also showed that fipronil sulfone is the predominant metabolite in human liver microsomes via cytochrome P-450 oxidation[16].

Although, one occupational exposure study of workers (*n*=159) at a fipronil production facility reports a mean fipronil sulfone serum level of 7.8 (SD = 7.7) ng/mL,[17] very little is known about human exposure to fipronil in the general population[9, 15, 17]. This may be because human samples can be difficult to obtain and analyze due to high concentrations of endogenous chemicals and significant matrix effects which make the identification of metabolites difficult. The specific objectives of the study were to develop a unique workflow where dosed animal samples were used to identify potential serum/urine biomarkers via time-of-flight mass spectrometry which were subsequently evaluated in serum and urine of a group of volunteers from North Carolina to assess exposure.

MATERIALS AND METHODS

2.1 Chemicals. Unlabeled fipronil (5-amino-1-[2,6-dichloro-4-(trifluoromethyl)-phenyl]-4-(trifluoromethylsulfinyl)-1-H-pyrazole-3-carbonitrile, >99%) and its metabolites: fipronil sulfone (5-amino-1-[2,6-dichloro-4-(trifluoromethyl)-phenyl]-4-[(trifluoromethyl)sulfonyl]-1*H*-pyrazole-3-carbonitrile, >99%), fipronil sulfide (5-amino-1-[2,6-dichloro-4-(trifluoromethyl)-phenyl]-4-[(trifluoromethyl)thio]-1*H*-pyrazole-3-carbonitrile, 98%), fipronil amide (5-amino-1-[2,6-dichloro-4-(trifluoromethyl)-phenyl]-4-[(trifluoromethyl)thio]-1*H*-pyrazole-3-carboxamide, >99%), and monochloro fipronil (5-amino-1-[2-chloro-4-(trifluoromethyl)-phenyl]-4-[(trifluoromethyl)sulfinyl]-1*H*-pyrazole-3-carbonitrile, >97%) were procured as solid analytical standards from the pesticide repository through the US EPA (Fort Meade, MD, USA). These five

analytical standards were prepared as a mixture in acetonitrile and used for all subsequent matrix-matched standard curves. The internal standard fipronil des-F_3 (see supporting information for structure) (5-amino-1-[2,6-dichloro-4-(trifluoromethyl)-phenyl]-4-(methylsulfinyl)-1-H-pyrazole-3-carbonitrile, 99%, 0.1 ng/µL in Acetonitrile) was ordered from Crescent Chemical Company (Islandia, NY, USA).

Acetonitrile and methanol (B&J Brand HighPurity Solvent) were purchased from Honeywell Burdick & Jackson (Muskegon, MI,USA) and ammonium acetate from Sigma Aldrich (St. Louis, MO, USA). Deionized water was generated in house from a Barnsted Easypure UV/UF (Dubuque, IA, USA) coupled with activated charcoal and ion exchange resin canisters.

2.2 Animals. This study was part of an investigation of the neurotoxic effects of fipronil in rodents[18, 19]. The animal facility is accredited by the American Association for Accreditation of Laboratory Animal Care International, and all protocols were approved by the National Health and Environmental Effects Research Laboratory Institutional Animal Care and Use Committee at the United States Environmental Protection Agency. Male Long Evans rats (60-90 days old) were acquired from Charles River Laboratories (Wilmington, MA). Animal husbandry details are provided in the Supporting Information. Animals were dosed daily by oral gavage at either 5 (lowest dose) or 10 (highest dose) mg/kg with fipronil suspended in corn oil (1 mL/kg) every 24 hours for two weeks. Control rats were gavaged with corn oil only. Six hours after the 14th dose, rats were euthanized. Trunk blood (2 mL) was collected in tubes without anticoagulant and stored on ice for 1-1.5 h.. The samples were centrifuged at 1300 × *g* for 30 min. at 4° C. The serum was collected, frozen on dry ice, and stored at -80 ^{o}C until analysis. Urine was collected in

a syringe either from voids on a clean table or via bladder puncture and transferred to a micro-centrifuge tube, immediately frozen on dry ice, and stored at -80 °C until analysis.

2.3 Human Samples. Matched human urine (*n*=84) and serum (*n*=96) samples, from individuals with no known fipronil exposure, were collected by the National Institute for Environmental and Health Sciences (NIEHS protocol number 10-E-0063) between April and June 2011. The human samples were simply a sample of convenience and were not meant to be representative of a specific population. The urine collected was a spot sample and was not concentrated or representative of a specific sampling period. Volunteers were anonymous, and no personally identifiable information was provided. The samples were from male and female volunteers of various ethnicities between 19 and 73 years of age who live in the Raleigh-Durham area of North Carolina (Table 1). Although 100 volunteers participated in the study, several urine and serum samples were not included due to an insufficient volume for analysis.

Table 1. Human demographic data for the 100 volunteers.

	Sex		Age				Race			
	Male	Female	19-33	34-48	48-62	62-76	Asian	Black	White	Other
%	30	70	29	30	33	8	3	32	63	2

2.4 Extraction Protocols. Samples were extracted in a manner that optimized recovery and reproducibility while reducing matrix interference. Animal samples were small volumes that did not require solid phase extraction (SPE). However, a protocol involving SPE was performed with the human samples to reduce matrix interference. Sample extraction protocols for biologicals are described below. More information on methods development for human samples can be found in the Supporting Information. Rat serum samples were first analyzed by liquid chromatography/time-of-flight mass spectrometer (LC/TOF-MS) in order to identify any metabolites. Human samples were then analyzed by liquid chromatography/triple-quadrupole

mass spectrometer (LC/triple-quad) for quantification of metabolites for which analytical standards were possessed. LC/quadrupole/time-of-flight mass spectrometry (LC/Q-TOF) was used for structure elucidation of unknown metabolites.

2.5 Rat serum. Rat serum (25 µL) was denatured with 100 µL of 0.1 M formic acid and precipitated with 1 mL of a cold acetonitrile solution spiked with the internal standard (fipronil des-F_3, 25 ng). The sample was then centrifuged for 5 minutes at 12500 × *g*. An aliquot of the supernatant was mixed 50:50 with 10 mM ammonium acetate buffer, and analyzed via LC/TOF and LC/triple-quad. *n*=9 for highest dose (10 mg/kg/day) ; *n*=10 for low dose (5 mg/kg/day); and *n*=11 for control animals, which were treated with vehicle. Quantitation was performed for fipronil and fipronil sulfone. The results of the quantitation are shown in the supporting information.

2.6 Rat urine. Rat urine (100 µL) was precipitated with 900 µL of cold acetonitrile and centrifuged for 8 minutes at 12500 × *g*. An aliquot of the supernatant was extracted and mixed 50:50 with 10 mM ammonium acetate buffer before LC/MS analysis. *n*=3 for highest dose (10 mg/kg/day); *n*=4 for low dose (5 mg/kg/day); and *n*=3 for control animals. Quantitation was only performed for the fipronil sulfone metabolite, as standards were not available for other metabolites. Quantitation specifics can be found in the Supporting Information. Fipronil sulfone concentrations in rat urine were used to approximate the relative concentrations of the other observed metabolites.

2.7 Human serum. Human serum (200 µL; *n*=96) was denatured with 20 µL of a 0.1 M formic acid solution spiked with internal standard (fipronil des-F_3, 5 ng) and precipitated with 2 mL of cold acetonitrile. The sample was centrifuged for 10 minutes at 12,500 × *g* and concentrated using solid phase extraction (SPE) using an Oasis 3cc HLB cartridge (Waters

Corporation, Milford, MA). SPE cartridges were conditioned with 3 mL of methanol and 3 mL of ultrapure water, samples were loaded, washed with 3 mL of 95:5 water/acetonitrile solution, then eluted with 3 mL of acetonitrile. The eluate was evaporated under N_2 at 40° C until approximately 200 µL remained. The concentrated solution was mixed 50:50 with 10 mM ammonium acetate buffer and analyzed via LC/TOF and LC/triple-quad for all compounds listed in the chemical section. In order to determine the concentration of compounds of interest, a seven-point matrix-matched (blank calf serum-Life Technologies-Gibco®, Grand Island, NY) extracted standard curve from 0.1-50 ng/mL, along with a method blank (DI water) and a matrix blank was run with the human serum samples. The lowest value on the standard curve (0.1 ng/mL) was considered the lower limit of quantitation (LLOQ).

2.8 Human urine. Human urine (5-12 mL; *n*=84) was precipitated with 1 mL of acetonitrile and concentrated using the SPE method described above with an Oasis 6cc HLB cartridge with the exception that cartridges were conditioned with 5 mL of methanol and 5 mL of ultrapure water, samples were loaded, washed with 5 mL of 95:5 water/acetonitrile solution, then eluted with 5 mL of acetonitrile. The eluate was evaporated under N_2 at 40° C until approximately 1 mL remained. The concentrated solution was mixed 50:50 with 10 mM ammonium acetate buffer in an LC vial and analyzed by LC-TOF/MS (*n*=84) for all compounds listed in the chemicals section, as well as for any unknown metabolites. Note that several urine samples were excluded due to insufficient volume.

2.9 Analytical Instrumentation. Targeted analyses (LC/triple-quad) were carried out using an Agilent 1100 HPLC (Agilent Technologies, Palo Alto, CA) interfaced with a Sciex 3000 triple quadrupole mass spectrometer (Applied Biosystems/MDS Sciex, Foster City, CA) fitted with an electrospray ionization source (ESI) operated in the negative ionization mode. Compounds

contained in the LC/triple-quad method (fipronil, fipronil sulfone, fipronil sulfide, fipronil amide, and monochloro fipronil) were optimized on a compound specific basis. Information regarding transitions are included in the Supporting Information.

The HPLC system consisted of a Phenomenex Luna C18 column (50 x 3 mm, 5 µm; Torrance, CA, USA) with a Security-guard guard column (Phenomenex). The method consisted of the following: 0.4 mL/min flow rate which increased to 0.75 mL/min at time=2 min; temperature: 30 °C; mobile phases – A: ammonium acetate buffer (0.2 mM) and DI water:methanol (95:5, v/v), and B: ammonium acetate buffer (0.2 mM) and acetonitrile:DI water (95:5, v/v); gradient: 0-2 min 50% A and 50% B; 2.1-4 min, a linear gradient from 50:50 A:B to 10:90 A:B; 4-6 min 10% A and 90% B; 6.1-10 min re-equilibration to 50% A and 50% B.

Non-targeted analyses (LC/TOF) were carried out using an Agilent 1100 HPLC (Agilent Technologies, Palo Alto, CA) interfaced with an Agilent 6210 Time-of-Flight (TOF) mass spectrometer fitted with an electrospray ionization source operated in the negative ionization mode at 120 Volts. Any drift in the mass accuracy of the TOF was continuously corrected by infusion of two reference compounds (purine [m/z = 119.0363] and hexakis (1H, 1H, 3H-tetrafluoropropoxy) phosphazene [m/z = 966.0007]) via dual-ESI sprayer.

The HPLC method consisted of a Zorbax Eclipse Plus C18 column (2.1 × 50 mm, 3.5 um; Agilent Technologies, Palo Alto, CA) fitted with a Phenomenex guard column (Torrance, CA). The method consisted of the following: 0.2 mL/min flow rate; at 30 °C; mobile phases: A: ammonium formate buffer (0.4 mM) and DI water:methanol (95:5 v/v), and B: ammonium formate (0.4 mM) and methanol:DI water (95:5 v/v); gradient: 0-5 min a linear gradient from 50:50 A:B to 100% B; 5-15 min, 100% B; 15-18 min re-equilibration to 50% A and 50% B.

2.10 Identification of Spectral Features. The TOF-MS system has proprietary software that can be used in non-targeted analyses to help identify compounds that are specific to a treatment group or a specific experimental condition. For example, to identify potential biomarkers of fipronil exposure, control and dosed animal samples are analyzed, and molecular features (identifiable peaks) were first extracted according to user specified criteria (e.g., minimum peak height, area count). The two groups of extracted features were then compared using The Mass Profiler software, which singles out only those compounds that are found in the dosed group. This collection of compounds can be thought to represent either the parent compound, metabolites of the parent, or specific biological responses that are attributable to the treatment administered.

The exact monoisotopic mass of each of these "treatment only" features was then used to generate a ranked list of possible chemical formulae for each unknown. The numerical ranking is based on the difference between the calculated and measured mass, the isotopic abundance and the isotope spacing. If authentic standards are available, the identity of a proposed feature can be confirmed using chromatographic retention time, exact monoisotopic mass, and isotopic distribution.

Fipronil is an interesting and somewhat unique compound because it contains six fluorine atoms and two chlorine atoms, that result in a significant negative mass defect (435.93869 Da, with the $[M-H]^-$ ion seen in negative ionization mode being 434.9314 *m/z*) which is preserved in most of its metabolic products to the extent that the F and Cl atoms are retained[20]. Moreover, the isotopic spacing between the Cl isotopes (^{35}Cl [75.77%] and ^{37}Cl [24.23%]) leads to a distinctive isotopic pattern that aids greatly with identification (SI Figure 2). Both of these characteristics were useful in identifying fipronil-related metabolites.

Metabolites that were identified using the LC/TOF instrument described above were then investigated further using an Agilent 1200 HPLC system fitted with a 6250 quadrupole time-of-flight mass spectrometer (Q-TOF) (Agilent Technologies, Palo Alto, CA) using the same LC conditions as previously described. The LC/Q-TOF allowed fragmentation at various collision energies of metabolites of interest which helped with structure elucidation.

2.11 Quality Assurance/Control. For each analysis, method and matrix blanks were evaluated for contamination or background levels of the compounds of interest. The LLOQ was determined as the concentration of the lowest working standard, which back-predicted within 30% of a theoretical value. The LLOQ in the quantitative human serum experiments was validated by calculating signal-to-noise ratios for the 451-415 *m/z* transition relative to a method blank. R-squared values for all quantitative procedures were monitored to ensure predictability. Three randomly chosen samples were replicated in each quantitative experiment to ensure consistency within the data sets. Parent-daughter ratios should be consistent, and ratio monitoring is a robust way to confirm the presence of a specific compound. Therefore, in the targeted screening of samples, the ratio between the primary and secondary parent-daughter transition was monitored to confirm the presence of each compound in the MS method. High and low concentration quality control (QC) samples containing the fipronil mixture of five analytical standards described in the *Chemicals* section were run with each batch of human serum samples to ensure analytical precision and accuracy.

3. RESULTS

3.1 Quality Assurance/Control. All lab prepared target and non-target analysis blanks and control samples were below the respective LLOQ for compounds of interest in all experiments.

Validation of the LLOQ in the human serum quantitative experiments showed that the lowest curve point differed from the method blank (signal-to-noise ratio for method blank = 3 ± 1; signal-to-noise ratio for 0.1 ng/mL standard = 20 ± 12). All r-squared values were greater than 0.99, which ensured predictability. All replicates for all experiments had a relative standard deviation of <15%. For all targeted analyses, the ion ratios between the primary and secondary parent-daughter transitions were consistent for all standard compounds (mean ± 20%). All QC samples (high and low) were 100% ± 15% of the nominal values.

3.2 Urine from Treated Rodents. The urine from rodents treated for 14 days with fipronil was analyzed for biomarkers of exposure via non-targeted analysis. As described above, molecular features (significant chromatographic peaks) were extracted from analytical runs of both dosed and control animals, and The Mass Profiler software was used to isolate those features that were unique to the dosed animals. The most plausible candidate biomarkers were those compounds with the signature isotope pattern of two chlorine atoms (SI Figure 2) and/or significant negative mass defects indicative of fluorine and chlorine atoms. Seven high abundance peaks fitting these criteria were identified, and the exact monoisotopic mass of each was used to generate a ranked list of plausible formulae and corresponding structures. We tentatively assigned compound identity according to known metabolic pathways (e.g., oxidation, sulfation, glucuronidation), the retention of negative mass defect and/or the isotopic pattern associated with chlorine, and consistency with results from previous studies. Information on the seven metabolites can be found in Table 2. Four of the compounds (M1, M2, M3, M5, and M6) were identified in previous studies[10, 21], whereas two more (M4 and M7) are reported for the first time in this study (Figure 1). It should be noted that the spectral feature observed for the glucuronide conjugate (M6) splits into two chromatographic peaks, most likely meaning that the

293 glucuronide molecule adds to both the oxygen and the nitrogen atom (see Figure 1). We were
294 unable to differentiate which peak corresponded to which structure, but one was formed
295 preferentially. However, this spectral feature is not observed for the sulfate conjugate (M5).

296 To better characterize the structures of metabolites M4 and M7, the fragmentation patterns of
297 the parent metabolites were analyzed via LC/Q-TOF. No useful information was gained about
298 metabolite M4; however, the fragmentation pattern of metabolite M7 helped to predict a
299 plausible structure. M7 structural information could be gleaned from looking at the exact masses
300 of molecular fragments originating from the parent molecule. For example, if the mass of a CO_2
301 group is observed in the fragmentation pattern, it can be assumed that the molecule likely
302 contained a carboxylic acid. Spectral information regarding this fragmentation pattern can be
303 found in the Supporting Information (SI Figure 3).

304 Fipronil sulfone (M1) was confirmed by an authentic standard that has the same retention time,
305 monoisotopic mass, ion fragmentation pattern, and isotope spacing. Rats in the 5 mg/kg/day
306 dose-group had mean concentrations of fipronil sulfone of 24.1 (SD = 18.7) ng/mL, while the 10
307 mg/kg/day group had 31.9 (SD = 13.1) ng/mL (SI Figure 1). If the fipronil sulfone
308 concentrations are used to generate estimated relative response factors for other metabolites that
309 do not have standards (assuming that all respond similarly within the TOF-MS), we estimate the
310 relative concentrations of fipronil metabolites in dosed-rodent urine to be
311 M6>M4>M5>M3>M7>M1>M2. The estimated concentrations of M2 and M6 are 30 and 2,000
312 ng/mL respectively.

313 **Table 2.** LC/TOF characteristics of putative metabolites in rat urine.

Metabolite	Retention Time (mins)	Predicted Formula of parent	Score of Predicted Formula	$[M-H]^-$ Measured Mass *(m/z)*	$[M-H]^-$ Calculated Mass *(m/z)*	Δ ppm	Monoisotopic Mass *(m/z)*
M1 (Fipronil Sulfone)	7.57	C12H4Cl2F6N4O2S	99.63	450.9266	450.9263	0.67	451.9336
M2	7.3	C9H4Cl2F3N3	93.50	279.9665	279.9662	1.07	280.9734
M3	1.62	C11H4O2N4Cl2F3	99.53	350.9667	350.9669	0.43	351.9742
M4	5.38	C10H4Cl2F3N3O2	98.63	323.9565	323.9560	1.54	324.9633
M5	1.4	C11H5Cl2F3N4O4S	99.38	414.9290	414.9288	0.48	415.9361
M6	1.39	C17H13Cl2F3N4O7	98.74	511.0036	511.0041	0.98	512.0113
M7	5.38	C11H3Cl2F3N4O	98.93	332.9564	332.9563	0.30	333.9563

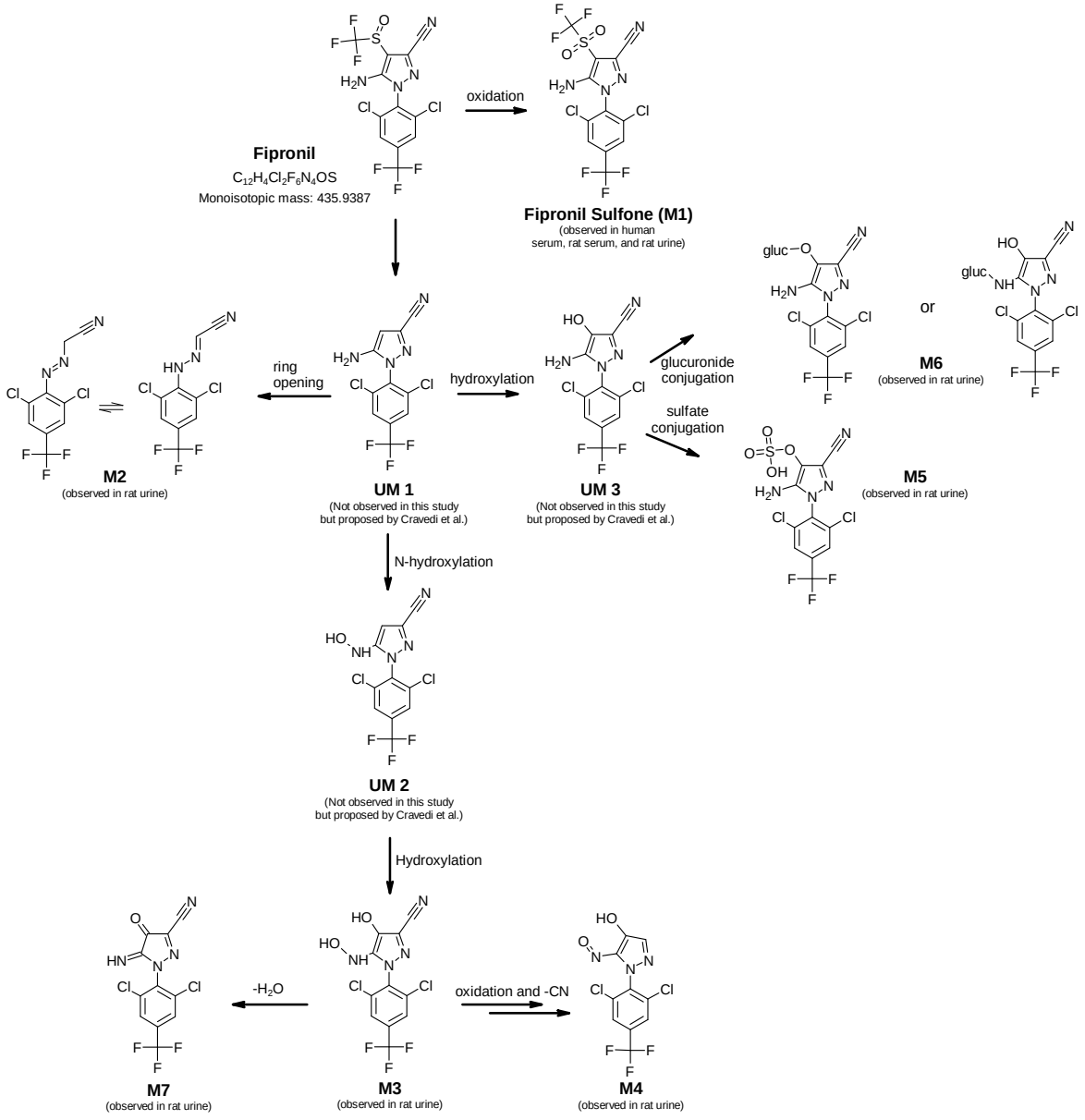


Figure 1. Proposed metabolic pathway of fipronil in the rat. M4 and M7 are proposed structures based on MS data, isotope distributions, and exact mass. M1, M2, M3, M5, and M6 were identified in rat urine. Unobserved metabolites labeled (UM) were not identified but are likely intermediates.

3.3 Serum from treated rodents. The serum from treated rats was analyzed for all suspected biological metabolites via LC-TOF to evaluate the presence of possible serum biomarkers. In our analysis we detected no additional metabolites other than small amounts of un-metabolized fipronil and fipronil sulfone which had been previously identified by several groups [4, 22]. Quantitative data for fipronil and fipronil sulfone in rat serum can be found in the Supporting Information.

3.4 Human urine. Urine samples (*n*=84) from volunteer North Carolina residents with no known exposures to fipronil or other pesticides were examined for M1-M7 (identified in rodent urine) and for all other plausible fipronil adducts or derivatives using the methods described above. No parent fipronil or any plausible metabolites were found in the human urine samples.

3.5 Human serum. Matched human serum samples (*n*=96) were analyzed for the metabolites observed in rat serum (fipronil and fipronil sulfone) by a targeted approach (LC/triple-quad, LOQ = 0.1 ng/mL). Only trace amounts of the parent fipronil were found in the human blood samples. However, fipronil sulfone (the biomarker identified in the rodent study) was detected in approximately 25% of the samples, at levels ranging from 0.1 to 3.9 ng/mL (Figure 2). Table 3 describes general trends in the data in terms of detects vs. non-detects.

Table 3 shows the number of detects vs. non-detects for each gender and race.

Gender	Detects	Non-Detects	Number of Samples
Male	7	12	29
Female	17	67	67
Race	**Detects**	**Non-Detects**	**Number of Samples**
Caucasian	22	39	61
African American	1	29	30
Asian	1	2	3
Other	0	2	2

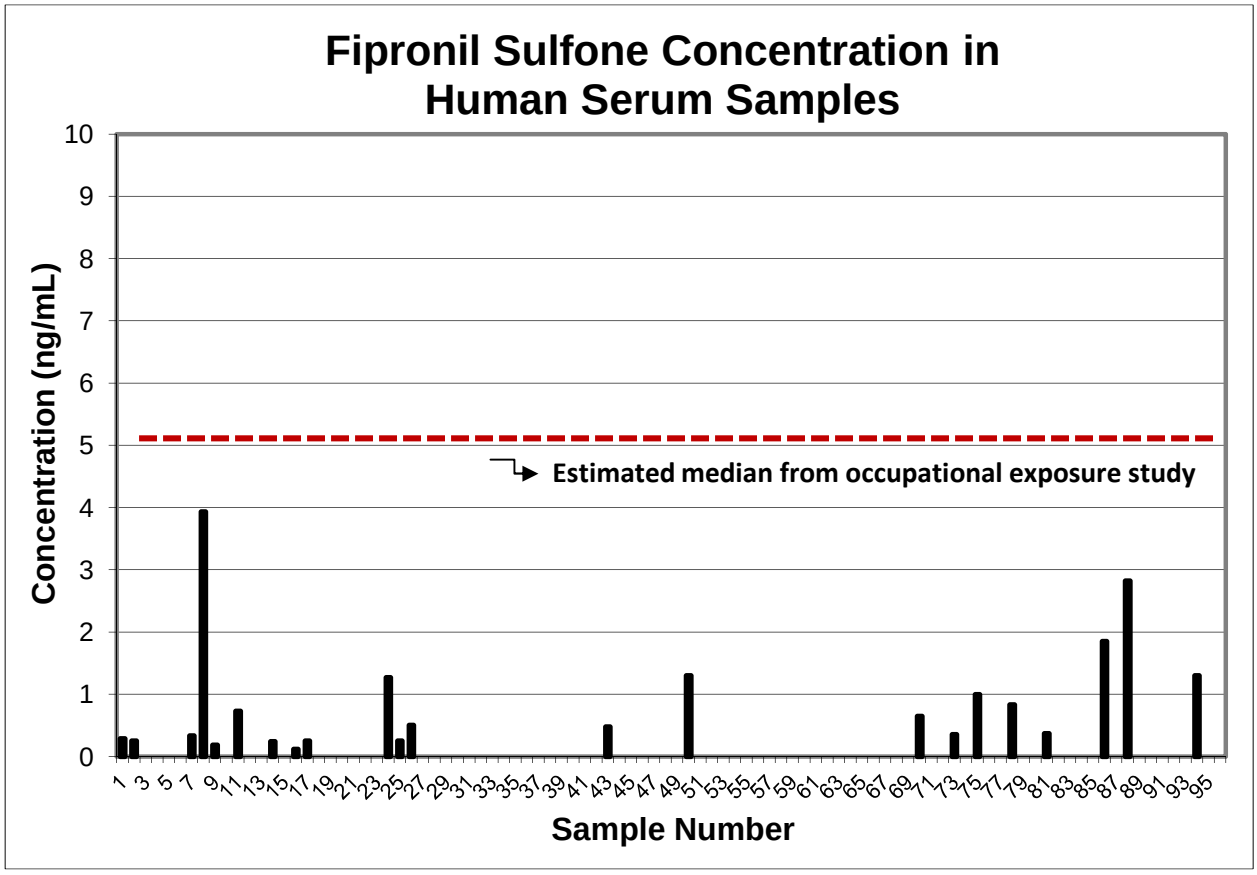


Figure 2 shows fipronil sulfone concentrations in human serum*. The red dotted line represents the median calculated from an occupational exposure study[17, 23].

**n* = 96, four samples were excluded due to insufficient volume.

4. DISCUSSION

348 This study demonstrates how advanced time-of-flight mass spectrometry techniques can be
349 used to more fully describe the metabolism of xenobiotic compounds in treated-animal studies
350 and how this knowledge can be applied in human biomonitoring studies to make relevant
351 conclusions about human exposures to emerging compounds of concern. Our specific goal was
352 to use the biomarkers identified from the dosed rodent work in the analysis of a set of human
353 biological samples to characterize the rate of fipronil exposure in the general population.

354 In describing the metabolism of fipronil in rodents, our results were largely consistent with
355 previous studies,[10, 21, 24] while also extending what is known about the basic metabolic process.
356 Two novel metabolites observed in rat urine in this study which were not seen by Cravedi et al.
357 (2013) can be attributed to differences in study design. Specifically, our Long Evans rats were
358 dosed (5 or 10 mg/kg/day) for 14 days then sacrificed 6 hours after the last dose. In contrast,
359 Cravedi et al. (2013) dosed acutely at 10 mg/kg and collected urine and serum every 24 h. over a
360 72 h. period[10]. Differences between rat strain or length of dosing regimen may have made it
361 possible to identify different products of fipronil metabolism, such as the pyrazole ring opened
362 products or the highly oxidized heteroaromatic amine derivatives.

363 The proposed metabolic pathway in the rat and compound structures can be found in Figure 1.
364 We propose that a new metabolite (M7) in rat urine, an imine, results from the loss of water from
365 metabolite M3, which is a fipronil metabolite that is hydroxylated at both the carbon and the
366 nitrogen. We also identified what is hypothesized to be nitroso compound (M4). We believe that
367 M3 and M4 are formed from an unobserved hydroxyl amine intermediate (UM 2). The hydroxyl
368 amine (M3) has been identified in this and in previous studies[10], but to our knowledge this is the
369 first report of a nitroso metabolite of fipronil in rat urine. Although the structure for metabolite
370 M4 is only putative, heterocyclic aromatic amines are known to undergo biological oxidation to

form nitroso compounds. This process is mediated by cytochrome P-450 and NADPH[25]. Many heterocyclic amines are known carcinogens,[26-30] due to their ability to be hydroxylated and then form DNA adducts. The observation of N-hydroxylated fipronil metabolites in this and other rodent studies warrants further investigation of fipronil metabolism in humans and the resulting effects.

Noninvasive biomarkers like those present in urine, exhaled breath, hair, fingernails, etc. are optimal for use in human studies, and one intention of this study was to explore whether any of the urinary metabolites found in the rats could be used as biomarkers of exposure in humans. Studies with human liver microsomes have shown that fipronil is metabolized to fipronil sulfone *in vitro*, and Mohamed et al. (2004) have identified fipronil sulfone as a metabolite in humans acutely exposed to high doses[9, 16]. Aside from these, no publications comment on the disposition of fipronil in humans. In this study we analyzed human urine samples for any of the metabolites identified as possible biomarkers in rat urine. The absence of fipronil and its metabolites in the human urine samples was undoubtedly related to many factors. To start with, it is possible that most human elimination of these materials occurs via the feces, as is the case with rodents[14, 24]. Secondly, and perhaps more importantly, our study subjects were essentially volunteers from the Raleigh-Durham area of North Carolina with no known exposures to fipronil and/or any other similar pesticides. Identification of small amounts of unknown chemicals in urine from populations with no known exposure can be difficult due to the large amount of endogenous compounds found in the matrix. A more effective strategy would be to work with a group of individuals with higher exposure levels (preferably occupationally) to determine human urinary metabolites. Despite negative findings with the human urine samples, 25% of the serum samples

393 contained measureable amounts of fipronil sulfone (range 0.1 – 4 ng/mL), providing clear
394 evidence that humans are regularly exposed to fipronil.

395 We compared our results to those from a study by Herin et al. where the serum from workers
396 in a fipronil production facility was measured for fipronil and fipronil sulfone. The median from
397 the occupational exposure study was calculated from the mean (μ) and standard deviation (σ)
398 provided via a method by Pleil et al.[23] where the geometric mean is used to estimate the median
399 which is equal to $\mu/[1 + 0.5 \times(\sigma/\mu)^2]$. Interestingly, the maximum concentration observed in this
400 study (3.9 ng/mL) was only slightly less than the calculated median of 5.2 (GSD = 2.4) ng/mL
401 for the occupationally exposed workers[17] (see Figure 2), where error is represented in terms of
402 the geometric standard deviation (GSD).

403 The general population likely shares specific exposure routes. One of the most likely routes of
404 exposure is contact with pets that have received applications of fipronil (i.e. Frontline® Plus) or
405 have had contact with indoor/outdoor applications around the home. Notably, Morgan et al.
406 (2008) concluded that family pets can act as vehicles for human exposure to the
407 organophosphorous insecticides, such as diazinon[31]. Specifically, fipronil is widely used to
408 control residential insect pests such as termites and fire ants outdoors where pets frequent,
409 leading to transport of the material indoors. Furthermore, many flea and tick topical products
410 contain approximately 10% fipronil and are applied directly to the skin and fur of dogs and cats,
411 leading to human exposure to fipronil through direct contact with their pets. Dyk et al. (2012)
412 used a fluorescent indicator to show that these fipronil residues are easily transferred from pets to
413 humans by way of direct contact for one week following application[32]. According to estimates
414 from the American Humane Association, up to 46% and 39% of US households keep dogs and
415 cats, respectively. Use of fipronil containing products with these animals could conceivably

result in some measurable human exposures. Ongoing efforts in our lab (data not shown) are investigating domestic indoor sources of exposure that may be important, since local WWTP effluent is shown to contain fipronil and metabolites.

Although we felt the study was well-designed, it did have a few limitations. First, the fipronil sulfone metabolite may not be a specific biomarker for fipronil exposure, since it is known that it can undergo photochemical degradation[2] and its presence has been documented in environmental media by several reports,[7, 33] thus one could be exposed to either fipronil or the degradate. In addition our sample size was relatively small (n=100). Furthermore, the number of detects was less than 30% of the total sample; which did not warrant a statistical analysis. More work is needed on a larger and more diverse sample before further conclusions can be drawn. Worth mentioning, however, was that approximately 92% of fipronil sulfone detections in human serum were from Caucasians, which represented only 63% of our samples. This result suggests that discrepancies between ethnicities may be present.

While the target of fipronil is insects, the two trifluorormethyl groups of fipronil may increase the compound's absorption and distribution upon accidental exposure by humans. Approximately 20-25% of drugs produced in the pharmaceutical industry contain at least one strategically incorporated fluorine atom (usually in the form of either one fluorine atom or a trifluoromethyl group) because fluorine can significantly impact lipophilicity and improve the bioavailability of orally administered drugs. Several studies have shown that the addition of fluorine, the most electronegative element, can decrease the pKa and therefore basicity of surrounding functional groups[34, 35]. Although the effect is not always predictable, this decreased basicity stabilized molecules in the harsh acidic conditions of the stomach and increases bioavailability[36, 37]. Another factor that affects the absorption and distribution of a molecule is

lipophilicity. Compounds usually enter into cell membranes via passive transport (although active transport is an alternate mechanism). Passive transport requires that the molecule is able to permeate the cell membrane, but also avoid entrapment by the lipid bilayer. The electron withdrawing capabilities of fluorine can, in some cases, be incorporated to tune a compound's lipophilicity and ease passive transport into cells[38-40]. Fipronil's presence in human serum demonstrated that the chemical is, in fact, absorbed by humans. Further, Hainzl et. al (1996) found that fipronil lost almost all activity in neurotoxicity studies on mice without the trifluoromethylsulfinyl functional group.[2] Metabolites of fipronil have also been found in many rat tissues, including brain cells[2, 4, 10], demonstrating that even highly selective membranes are somewhat permeable to these chemicals. The fluorinated functional groups may increase fipronil's potency as an insecticide; however, they may also increase absorption and distribution of the potentially toxic compound in non-target organisms, such as humans. Considering that fipronil has been associated with endocrine disruption, neurotoxicity, and carcinogenicity[12-15], accidental exposure and increased bioavailability may be problematic.

In conclusion, previously reported metabolites in rat urine and serum were confirmed, and two novel urinary metabolites have been proposed. The putative biomarkers determined in the rodent study were used in human serum analysis, where fipronil sulfone was found in approximately 25% of serum samples from a convenient sample of North Carolina residents. Serum fipronil levels in our study suggest that environmental exposures to fipronil may be common, but likely lower than occupational exposures. Matched urine was also analyzed, but no fipronil or any of its metabolites were identified, which suggests that urine may not be an appropriate matrix for biomonitoring populations with no known exposure to fipronil. More extensive characterization of the metabolites produced in humans exposed to higher levels of fipronil, as well as the effects

462 from low but chronic exposure to fipronil is needed. Further investigations are also necessary to
463 describe the sources of fipronil exposure and identify rates of exposure in other populations.
464
465
466 5. SUPPORTING INFORMATION

467 **5.1 Rodents** were housed in polycarbonate cages containing heat-treated hardwood chip
468 bedding. Access to food (Purina 5008 Chow; Lab Diet/PMI Nutrition International, Richmond,
469 IN) and tap water was provided *ad libitum*. Animals were allowed to acclimate to their
470 surroundings in the animal colony for 5-7 days before beginning any tests. The animal colony
471 was maintained at a temperature of 22 ± 2 ^{o}C, with humidity at 40 ± 20%, and a 12:12 hr
472 light:dark cycle (light on at 6:00 a.m.).

473 **5.2 Recovery Experiment for Fipronil in Dosed-rat Serum.** Standard fipronil (200 ng) was
474 added to a vial containing blank rat serum (100 µL), along with 100 µL of 0.1 M formic acid and
475 1 mL of cold acetonitrile. The solution was centrifuged at 12,500 × g, and the supernatant was
476 extracted. In a separate vial, blank rat serum was directly spiked with fipronil standard (200 ng).
477 Both the supernatant and the control sample were mixed 50:50 with 10 mM ammonium acetate
478 buffer and analyzed via LC/triple-quad. The fipronil recovery rate was 98%.

479 **5.3 Recovery Experiment for Fipronil in Spiked Human Serum.** A standard mix of fipronil
480 metabolites (10 ng each metabolite) was added to a vial containing blank calf serum (200 µL),
481 along with 25 µL of a 0.1 M formic/internal standard solution (fipronil des-F_3, 10 ng) and 2 mL
482 of acetonitrile. The solution was centrifuged at 12,500 × g and was extracted onto an Oasis 3cc
483 HLB solid phase extraction cartridge. The solid phase extraction method consisted of

conditioning the cartridge with 3 mL of methanol followed by 3 mL of DI water; loading the sample; washing with 3 mL of 95:5 water:acetonitrile; and eluting with 3 mL of acetonitrile. The samples were evaporated under N_2 at 40 °C until 200 µL remained. In a separate vial (the control sample), only 200 µL of blank calf serum, 25 µL of the 0.1 M formic acid/internal standard solution and 2 mL of acetonitrile was added (no fipronil or metabolites), and this vial was also carried through the procedure, just as the experimental sample. The control sample was spiked with the standard mix of fipronil metabolites (10 ng/metabolite) after evaporation. All the samples were prepared 50:50 with 10 mM ammonium acetate buffer, and analyzed via LC/triple-quad (*n*=3). The results are shown below in SI Table 1.

SI Table 1. Human serum recovery experiment results.

Compound	Average % Recovery (± %RSD)
Fipronil	82 ±2.4
Fipronil sulfone	83 ±3.6
Fipronil sulfide	84 ±3.6
Fipronil amide	82 ±7.3
Monochloro fipronil	85 ±3.5

5.4 Recovery Experiment for Fipronil in Spiked Human Urine. A standard mix of fipronil metabolites (400 ng/metabolite) was added to a vial containing 10 mL of blank human urine and 1 mL of acetonitrile/internal standard solution (fipronil des-F_3, 33 ng). The solution was extracted onto an Oasis 6cc HLB solid phase extraction cartridge. The solid phase extraction method was the same as for human serum, except the elution step used 5 mL instead of 3 mL of acetonitrile. The solution was evaporated under N_2 at 40 °C until 1 mL remained. In the control

sample, 10 mL of blank human urine and 1 mL acetonitrile were added (no fipronil or metabolites), and this vial was also carried through the procedure, just as the experimental samples. After evaporation the control sample was spiked with the standard fipronil metabolite mixture (400 ng/metabolite). All samples were prepared 50:50 with 10 mM ammonium acetate buffer and analyzed via LC/triple-quad (*n*=3). The results are shown below in SI Table 2.

SI Table 2. Human urine recovery experiment results.

Compound	Average % Recovery (± %RSD)
Fipronil	103 ±5.8
Fipronil sulfone	100 ±10
Fipronil sulfide	99 ±7.0
Fipronil amide	104 ±3.8
Monochloro fipronil	101 ±5.0

5.5 Quantitation of fipronil and fipronil sulfone in the serum of treated rodents. Rat serum (25 µL) was denatured with 100 µL of 0.1 M formic acid and precipitated with 1 mL of a cold acetonitrile solution spiked with the internal standard (fipronil des-F_3, 25 ng). The sample was then centrifuged for 5 minutes at 12500 × *g*. An aliquot of the supernatant was mixed 50:50 with 10 mM ammonium acetate buffer, and analyzed via LC/TOF and LC/triple-quad. *n*=9 for highest dose (10 mg/kg/day) ; *n*=10 for lowest dose (5 mg/kg/day); and *n*=11 for control animals, which were treated with vehicle. To determine the concentration of compounds of interest, a nine-point matrix-matched extracted standard curve from 10-5000 ng/mL, a method blank (DI water), and a matrix blank (blank rat serum) was run with the rat serum samples via LC/triple-quad. The lowest value on the standard curve (10 ng/mL) was considered the lower limit of quantitation (LLOQ). The results of the quantitation are shown in SI Table 3.

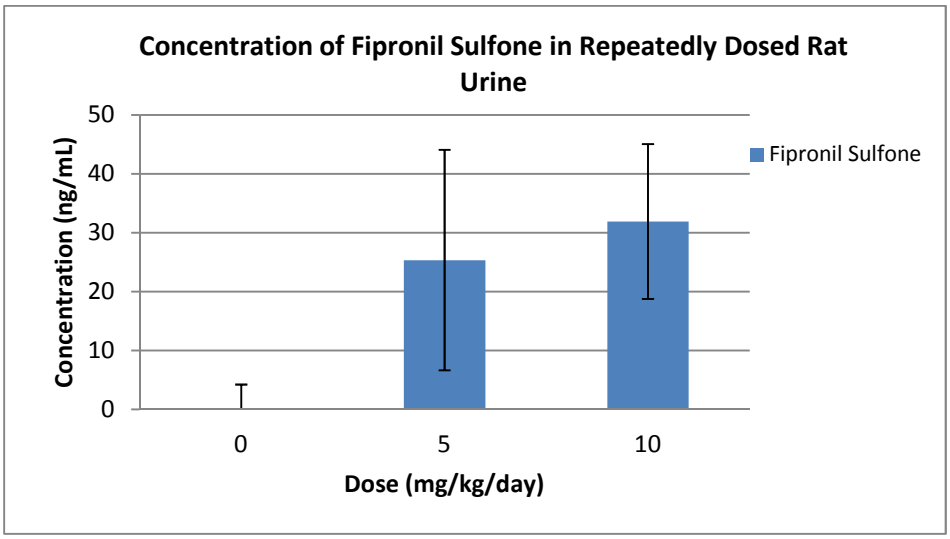
SI Table 3. Mean, standard deviation, and 95% confidence interval for fipronil and fipronil sulfone in rat serum.

Compound	Dose Conc. (mg/kg bw)	LOQ (ng/mL)	Mean (ng/mL)	St. Dev.	95% Conf. Int.
Fipronil	control	10	1.0 (<LLOQ)	3.0	1.8
Sulfone	control	10	2.5 (<LLOQ)	3.7	2.2
Fipronil	5	10	8.9 (<LLOQ)	3.4	2.1
Sulfone	5	10	2424	193.3	119.8
Fipronil	10	10	13.9	7.8	5.1
Sulfone	10	10	3548	511.9	334.4

5.6 Quantitation of fipronil sulfone in the urine of treated rodents. Rat urine (100 µL) was treated with 900 µL of cold acetonitrile. The sample was then centrifuged for 8 minutes at 12,500 × *g*, prepared 50:50 with 10mM ammonium acetate buffer, and analyzed via LC/triple-quad. $n = 2$ for highest dose (10 mg/kg/day); $n = 4$ for lowest dose (5 mg/kg/day); and $n = 6$ for control animals. In order to determine concentration of compounds of interest, a seven-point extracted standard curve prepared in DI water from 10-5000 ng/mL, along with a method blank (DI water) was run with the experimental rat urine samples. SI Figure 1 shows median fipronil sulfone concentrations for rodents dosed with fipronil. The highest dose group had a mean concentration of 31.9 (SD = 13.1) ng/mL fipronil sulfone, while the lowest dose group had 24.1 (SD = 18.7) ng/mL and the control animals had mean concentrations below the LLOQ.

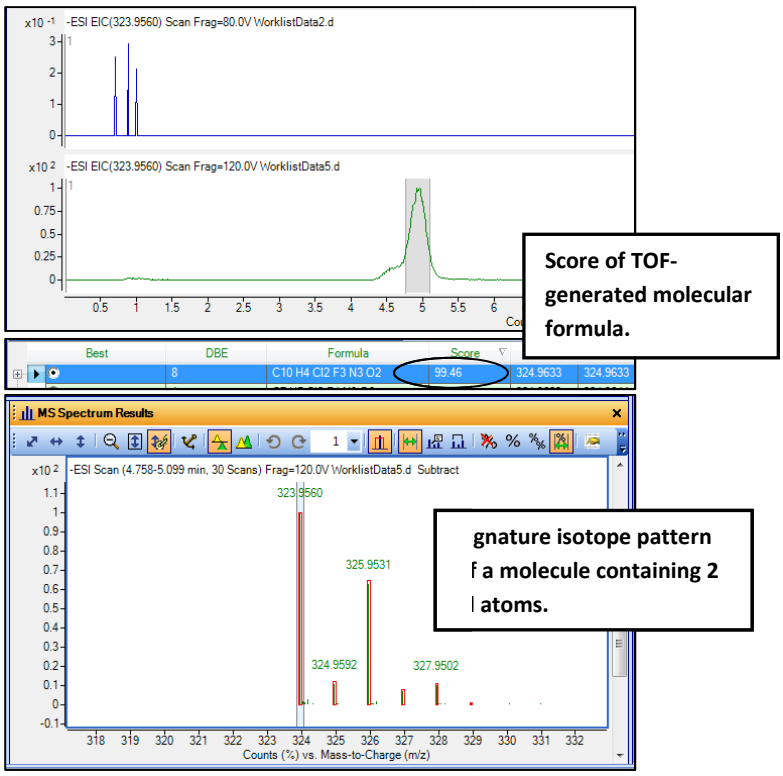
The LC/triple quad used for the quantitation of fipronil sulfone was a Waters Acquity ultraperformance liquid chromatography system coupled with a Waters Quatro Premier XE triple

quadrupole mass spectrometer (UPLC-MS/MS; Waters Corporation). A 20-µL aliquot of each sample was injected onto an Acquity UPLC BEH C18 column (1.7 µm, 2.1 × 50 mm; Waters Corporation) that was maintained at 50 ºC. The mobile phase consisted of solvent A: 2 mM ammonium acetate buffer with 5% methanol and solvent B: acetonitrile at a flow rate of 400 µL/min, starting with 75% solvent A for 30 s and then increasing to 90% solvent B at 3.5 min and 100% solvent B at 3.6 min and held for 0.9 min. At 4.6 min the gradient was returned to 60% solvent A and held until 6.0 min. Electrospray negative ionization was used in the mass spectrometer source. The capillary voltage was set at negative 0.4 kV, and the source temperature was 150 ºC. The primary transition used for quantitation was 451.2 - 244.0 *m/z*, and two other transitions were monitored for confirmation, 451.2 to 281.9 *m/z* and 451.2 to 414.9 *m/z*.



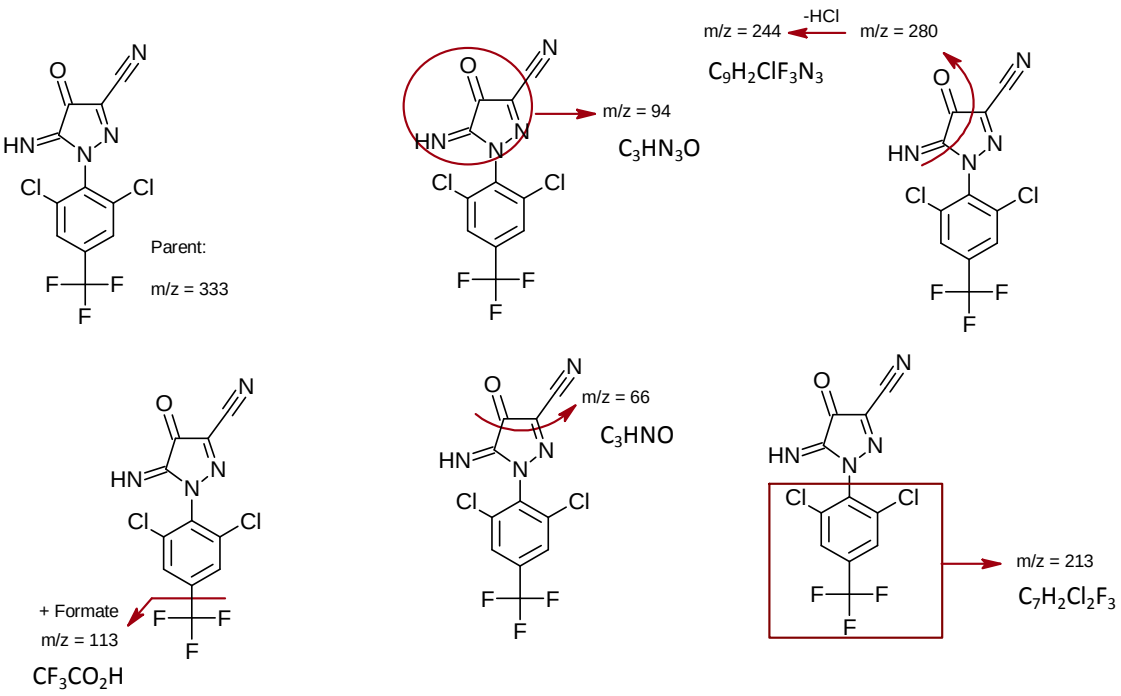
SI Figure 1. Median fipronil sulfone concentration in rat urine.

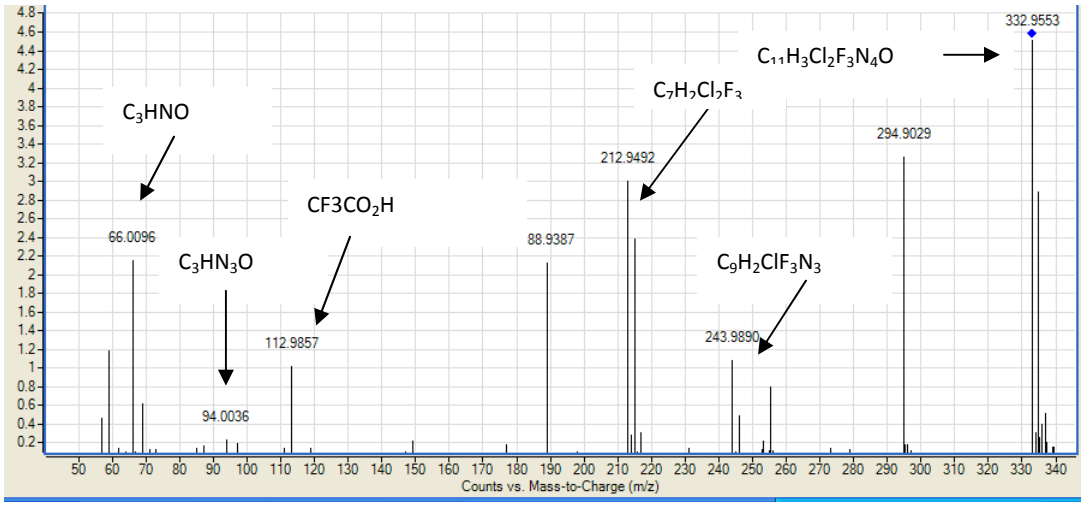
5.7 Time-of-flight mass spectrometry scoring and isotope patterns.



SI Figure 2 shows the spectral pattern of a molecule containing 2 chlorine atoms. Note that 323.9560 *m/z* is the most abundant isotope, 325.9531 *m/z* contains one ^{37}Cl, and 327.9502*m/z* contains two ^{37}Cl. The 324.9592 *m/z* contains one ^{13}C. The numerical ranking for formula generated for compound (M4) is shown. The top extracted ion chromatograph (Worklist Data 2) shows a control animal sample and the absence of a peak for M4.

5.8 Metabolite M7 in rat urine





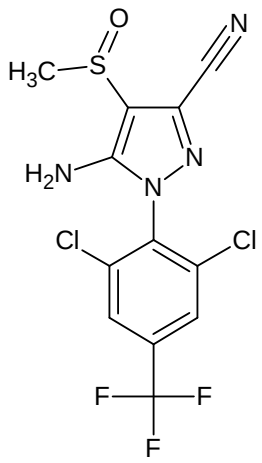
SI Figure 3 shows the fragmentation pattern of Metabolite M7 in the LC/Q-TOF. The red circles/boxes show the fragment and the spectrum at the bottom shows the peaks corresponding to the fragments.

5.9 Transitions in LC/triple quad method. SI Table 6 below lists the parent to daughter transitions which were monitored in the Agilent 1100 LC/triple quad method.

SI Table 6. LC/triple quad parent-daughter transitions.

Compound	Transition	Parent	Daughter
Fipronil	1°	434.9	329.8
Fipronil	2°	434.9	249.9
Fipronil	3°	434.9	277.8
Fipronil sulfone	1°	451.1	415.0
Fipronil sulfone	2°	451.1	281.9
Fipronil sulfone	3°	451.1	243.9
Fiproni sulfide	1°	418.9	382.8
Fiproni sulfide	2°	418.9	261.7
Fiproni sulfide	3°	418.9	313.8
Fipronil amide	1°	452.9	347.7
Fipronil amide	2°	452.9	303.8
Fipronil amide	3°	452.9	271.9
Monochloro fipronil	1°	401.1	283.9
Monochloro fipronil	2°	401.1	295.9
Monochloro fipronil	3°	401.1	331.9
Fipronil des F3	1°	387.2	281.9
Fipronil des F3	2°	387.2	331.0
Fipronil des F3	3°	387.2	351.0

5.10 SI Figure 6 shows fipronil des-F_3 which was used as an internal standard for analytical methods due to its similarity in structure to fipronil. The structure is shown below.



Molecular Formula: $C_{12}H_7Cl_2F_3N_4OS$
Monoisotopic Mass: 381.966971 Da
[M-H]-: 380.959694 Da

AUTHOR CONTRIBUTIONS

The manuscript was written through equal contributions of all authors. All authors have given approval to the final version of the manuscript.

ACKNOWLEDGMENTS

This research was supported by an appointment to the Research Participation Program at the National Exposure Research Laboratory administered by the Oak Ridge Institute for Science Education through an interagency agreement between the U.S. Department of Energy and the U.S. Environmental Protection Agency. We thank Agilent Technologies for providing us with

609 the LC/TOF mass spectrometer that was used to investigate metabolic products. We also thank
610 Michael Hays of US EPA who kindly allowed the use of his LC/Q-TOF mass spectrometer for
611 further structure elucidation, Benny Pyke of Arizona State University for discussions on
612 detection of fipronil in human urine, and Matthew Stiegel, Jon Sobus, and Peter Egeghy for help
613 with data analysis.

614

615 DISCLAIMER

616 This article will be reviewed in accordance with the policy of the National Exposure Research
617 Laboratory, U.S. Environmental Protection Agency, and approved for publication. Approval
618 does not signify that the contents necessarily reflect the view and policies of the Agency, nor
619 does mention of trade names or commercial products constitute endorsement or recommendation
620 for use.

621

622 REFERENCES

623

624 1. Brassard, D.; Haddad, S.; Hill, E., US EPA BEAD Chemical Profile for Registration
625 Review: Fipronil (PC Code: 129121). In 2011.

626 2. Hainzl, D.; Casida, J. E., Fipronil insecticide: Novel photochemical desulfinylation with
627 retention of neurotoxicity. *Proceedings of the National Academy of Sciences* **1996,** *93*, 12764-
628 12767.

629 3. Ikeda, T.; Nagata, K.; Kono, Y.; Yeh, J. Z.; Narahashi, T., Fipronil modulation of
630 GABAA receptor single-channel currents. *Pest Management Science* **2004,** *60*, (5), 487-492.

631 4. Hainzl, D.; Cole, L. M.; Casida, J. E., Mechanisms for Selective Toxicity of Fipronil
632 Insecticide and Its Sulfone Metabolite and Desulfinyl Photoproduct. *Chemical Research in*
633 *Toxicology* **1998,** *11*, 1529-1535.

634 5. Rhône-Poulenc Agro to boost fipronil production. *Agrow* **1997,** *294*, 17.

6. *Trends in Concentrations of Pesticides in Urban Streams in the United States, 1992-2008*; USGS.

7. Gunasekara, A. S.; Truong, T.; Goh, K. S.; Spurlock, F.; Tjeerderma, R. S., Environmental fate and toxicology of fipronil. *Jounal of Pesticide Science* **2007,** *32*, 189-199.

8. Erickson, B. E., Europe to ban fipronil pesticide to protect bees. *Chemical & Engineering News Archive* **2013,** *91*, (29), 21.

9. Mohamed, F.; Senarathna, L.; Percy, A.; Abeyewardene, M.; Eaglesham, G.; Cheng, R.; Azher, S.; Hittarage, A.; Dissanayake, W.; Sheriff, M. H.; Davies, W.; Buckley, N. A.; M., E., Acute human self-poisoning with the N-phenylpyrazole insecticide fipronil--a GABAA-gated chloride channel blocker. *Jounal of Toxicology. Clinical Toxicology* **2004,** *42*, 955-963.

10. Cravedi, J. P.; Delous, G.; Zalko, D.; Viguie, C.; Debrauwer, L., Disposition of fipronil in rats. *Chemosphere* **2013,** *93*, 2275-2283.

11. Tingle, C. C. D.; Rother, J. A.; Dewhurst, C. F.; Lauer, S.; King, W. J., Fipronil: Environmental Fate, Ecotoxicology, and Human Health Concerns. *Reviews of Environmental Contamination and Toxicology* **2003,** *176*, 1-66.

12. Ohi, M.; Dalsenter, P. R.; Andrade, A. J. M.; Nascimento, A. J., Reproductive adverse effects of fipronil in Wistar rats. *Toxicology Letters* **2004,** *146*, (2), 121-127.

13. Raquel, P.; Tercariol, G.; Godinho, A. F., Behavioral effects of acute exposure to the insecticide fipronil. *Pesticide Biochemistry and Physiology* **2011,** *99*, 221-225.

14. *New Pesticide Fact Sheet for Fipronil*; US EPA: 1996.

15. Vidau, C.; Gonzalez-Polo, R. A.; Niso-Santano, M.; Gomez-Sanchez, R.; Bravo-San Pedro, J. M.; Pizarro-Estrella, E.; Blasco, R.; Brunet, J. L.; Belzunces, L. P.; Fuentes, J. M.; , Fipronil is a powerful uncoupler of oxidative phosphorylation that triggers apoptosis in human neuronal cell line SHSY5Y. *NeuroToxicology* **2011,** *32*, 935-943.

16. Tang, J. A.; Usmani, K.; Hodgson, E.; Rose, R. L., In vitro metabolism of fipronil by human and rat cytochrome P450 and its interactions with testosterone and diazepam. *Chemico-Biological Interactions* **2004,** *147*, (3), 319-329.

17. Herin, F.; Boutet-Robinet, E.; Levant, A.; Dulaurent, S.; Manika, M.; Galatry-Bouju, F.; Caron, P.; Soulat, J.-M., Thyroid function tests in persons with occupational exposure to fipronil. *Thyroid* **2011,** *21*.

18. Freeborn, D. L.; McDaniel, K. L.; Moser, V. C.; Herr, D. W., Use of electroencephalography (EEG) to assess CNS changes produced by pesticides with different modes of action: effects of permethrin, deltamethrin, fipronil, imidacloprid, carbaryl, and triadimefon. *Toxicology and Applied Pharmacology*, (doi:10.1016/j.taap.2014.11.011), in press.

19. Moser, V. C.; Stewart, N.; Lyke, D. F.; Crooks, J.; MacMillan, D. K.; Hedge, J. M.; Wood, C. E.; McMahen, R. L.; Strynar, M. J.; Herr, D. W., Assessment of serum biomarkers in rats after exposure to pesticides of different chemical classes. *Toxicology and Applied Pharmacology*, (doi:10.1016/j.taap.2014.11.016), in press.

20. Xie, T.; Liang, Y.; A, J.; Hao, H.; Liu, L.; Zheng, X.; Dai, C.; Zhou, Y.; Guan, T.; Liu, Y.; Xie, L.; Wang, G., Post acquisition data processing techniques for lipid analysis by quadrupole time-of-flight mass spectrometry. *Journal of Chromatography B* **2012,** *905*, (0), 43-53.

21. In *Pesticide Residues in Food. Fipronil: Residue Evaluation 2001.*, Joint Meeting on Pesticide Residues in Food

22. Lacroix, M. Z.; Puel, S.; Toutain, P. L.; Viguie, C., Quantification of fipronil and its metabolite fipronil sulfone in rat plasma over a wide range of concentrations by LC/UV/MS. *Jounal of Chromatography B* **2010,** *878*, 1934-1938.

23. Pleil, J. D.; Sobus, J. R.; Stiegel, M. A.; Hu, D.; Oliver, K. D.; Olenick, C.; Strynar, M. J.; Clark, M.; Madden, M. C.; Funk, W. E., Estimating Common Parameters of Lognormally Distributed Environmental and Biomonitoring Data: Harmonizing Disparate Statistics From Publications. *Journal of Toxicology and Environmental Health, Part B* **2014,** *17*, 341-368.

24. In *Pesticide Residues in Food. Fipronil: Residue evaluation 1997.*, Joint Meeting on Pesticide Residues in Food.

25. Kim, D.; Guengerich, F. P., CYTOCHROMEP450 ACTIVATION OFARYLAMINES ANDHETEROCYCLIC AMINES. *Annual Reviews in Pharmacology and Toxicology* **2005,** *45*, 27-49.

26. Snyderwine, E. G.; Turesky, R. J.; Turteltaub, K. W.; Davis, C. D.; Sadrieh, N.; Schut, H. A. J.; Nagao, M.; Sugimura, T.; Thorgeirsson, U. P.; Adamson, R. H.; Thorgeirsson, S. S., Metabolism of food-derived heterocyclic amines in nonhuman primates. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis* **1997,** *376*, (1–2), 203-210.

27. Eisenbrand, G.; Tang, W., Food-borne heterocyclic amines. Chemistry, formation, occurrence, and biological activities. A literature review. *Toxicology* **1993,** *12*, 1-82.

28. Pezdirc, M.; Zegura, B.; Filipic, M., Genotoxicity and induction of DNA damage responsive genes by food-borne heterocyclic aromatic amines in human hepatoma HepG2 cells. *Food Chem. Toxicol.* **2013,** *59*, 386-394.

29. Nagao, M.; Sugimura, T., Carcinogenic factors in food with relevance to colon cancer development. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis* **1993,** *290*, (1), 43-51.

30. Schut, H. A. J.; Snyderwine, E. G., DNA adducts of heterocyclic amine food mutagens: implications for mutagenesis and carcinogenesis. *Carcinogenesis* **1999,** *20*, (3), 353-368.

31. Morgan, M. K.; Stout, D. M.; Jones, P. A.; Barr, D. B., An observational study of the potential for human exposures to pet-borne diazinon residues following lawn applications. *Environmental Research* **2008,** *107*, 336-342.

32. Dyk, M. B.; Liu, Y.; Chen, Z.; Vega, H.; Krieger, R. I., Fate and distribution of fipronil on companion animals and in their indoor residences following spot-on flea treatments. *Journal of Environmental Science and Health, Part B* **2012,** *47*, 913-924.

33. Ensminger, M.; Budd, R.; Kelley, K.; Goh, K., Pesticide occurrence and aquatic benchmark exceedances in urban surface waters and sediments in three urban areas of California, USA, 2008–2011. *Environ Monit Assess* **2013,** *185*, (5), 3697-3710.

34. van Niel, M. B.; Collins, I.; Beer, M. S.; Broughton, H. B.; Cheng, S. K. F.; Goodacre, S. C.; Heald, A.; Locker, K. L.; MacLeod, A. M.; Morrison, D.; Moyes, C. R.; O'Connor, D.; Pike, A.; Rowley, M.; Russell, M. G. N.; Moyes, C. R.; O'Connor, D.; Pike, A.; Rowley, M.; Russell, M. G. N.; Sohal, B.; Stanton, J. A.; Thomas, S.; Verrier, H.; Watt, A. P.; Castro, J. L., Fluorination of 3-(3-(piperidin-1-yl)propyl)indoles and 3-(3-(piperazin-1-yl)propyl)indoles gives selective human 5-HT1D receptor ligands with improved pharmacokinetic profiles. *Journal of Medicinal Chemistry* **1999,** *42*, (12), 2087-2104.

35. Rowley, M.; Hallett, D. J.; Goodacre, S.; Moyes, C.; Crawforth, J.; Sparey, T. J.; Patel, S.; Marwood, R.; Patel, S.; Thomas, S.; Hitzel, L.; O'Connor, D.; Szeto, N.; Castro, J. L.; Hutson, P. H.; MacLeod, A. M., 3-(4-fluoropiperidin-3-yl)-2-phenylindoles as high affinity, selective, and orally bioavailable h5-HT2A receptor antagonist. *Journal of Medicinal Chemistry* **2001,** *44*, (10), 1603-1614.

36. Chambers, R. D., *Fluorine in Organic Chemistry*. Blackwell Publishing: Oxford, 2000.

37. Morgenthaler, M.; Schweizer, E.; Hoffmann-Ro, A.; Martin, R. E.; Jaeschke, G.; Wagner, B.; Fischer, H.; Bendels, S.; Zimmerli, D.; Schneider, J.; Diederich, F.; M., K.; Mu, K., Predicting properties and tuning physicochemical in lead optimization: Amine basicities. *ChemMedChem* **2007,** *2*, 1100.

38. Purser, S.; Moore, P. R.; Swallow, S.; Gouverneur, V., Fluorine in medicinal chemistry. *Chemical Society Reviews* **2008,** *37*, (2), 320-330.

39. Smart, B. E., Fluorine Substituent Effects (on bioactivity). *Journal of Fluorine Chemistry* **2001,** *109*, (1), 3-11.

40. Smith, D. A.; van de Waterbeemd, H.; Walker, D. K., Methods and Principles in Medicinal Chemistry, vol 31: Pharmacokinetics and Metabolism in Drug Design. *Wiley-VCH* **2006,** *31*.

***Highlights (for review)**

Highlights for: Identification of fipronil metabolites by time-of-flight mass spectrometry for application in a human exposure study

- A fipronil dosed-rodent study was used for metabolite discovery in urine and serum
- Time-of-flight mass spectrometry was used for metabolite identification
- Identified metabolites were analyzed in 100 human serum and urine samples
- This is the first study to identify these biomarkers of fipronil in a general population
- Results showed 25% of human serum samples contained a fipronil metabolite