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FACULTY OF PHARMACY

Assessment of fipronil exposure in humans after ectoparasiticide application on household pets

Ocena narażenia na fipronil u ludzi po zastosowaniu preparatu przeciw pasożytom zewnętrznym u zwierząt domowych

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DOCTORAL DISSERTATION

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ABSTRACT

Fipronil (FIP) is a phenylpyrazole insecticide widely used as a veterinary drug to treat and prevent ectoparasite infestation (e.g., ticks and fleas) on cats and dogs. Because FIP-based drugs are applied topically and often administered repeatedly by pet owners during the season, there is a considerable risk of exposure for people living with pets. Some studies suggest negative effects of FIP on human health, so there is a need to evaluate the exposure associated with such scenario. In urine, the parent compound and its oxidized derivative, fipronil-sulfone (FIP-sulfone) are used as biomarkers of FIP exposure. However, since urinary excretion of these compounds is low and they are also found in the environment, fipronil-hydroxy (FIP-hydroxy), hitherto only detected in rats, was recently proposed as an alternative. In environmental studies, apart from FIP and FIP-sulfone, fipronil-amide (FIP-amide), fipronil-desulfinyl (FIP-desulfinyl), and fipronil-sulfide (FIP-sulfide) are often detected. FIP and its (bio)transformation products are collectively known as fiproles (FIPs). For tracking external personal exposure, silicone wristbands (SWBs) have recently been proposed as a promising approach.

This study had four goals. The first was to develop and validate a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for determination of FIPs (including FIP-hydroxy) at trace levels in human urine. Secondly, another LC-MS/MS method for quantification of environmentally relevant FIPs in SWBs needed to be developed and validated. As a third step, a longitudinal study of human exposure to FIP both before and after application of FIP-based ectoparasiticide on household pets was conducted. Based on these results, the final goal could be reached: characterization of the human exposure as well as the health risk associated with the use of FIP-containing ectoparasiticide on household pets.

For urinalysis, a method based on solid phase extraction (SPE) and LC-MS/MS was developed. FIP-hydroxy could not be included in the method due to mismatch of mass signal observed between the standard and the theoretical mass spectrum. Other FIPs, however, were successfully quantified at pg/mL or ng/mL levels. For these analytes, satisfactory accuracy, precision, and other method performance parameters were achieved. Matrix effects were minimized using isotopically labeled internal standards and a matrix-matched calibration.

The analytical method for environmentally relevant FIPs in SWBs was based on solid-liquid extraction, followed by cleanup using freezing-out and liquid-liquid extraction. Again, LC-MS/MS was used for separation and detection and ng/g levels of analytes could be quantified. Before method validation, the sample preparation process was extensively optimized. Additional methodological experiments provided further insight into use of SWBs as a personal sampling technology.

In the human exposure study, the participants were asked to collect urine samples and wear SWBs both before and after they applied a FIP-based veterinary product on their pet(s). Stationary silicone wristbands were placed in the participants' environments for comparison. One person used a product that did not contain FIP, acting as a negative control. Detection rates of FIPs in urine collected before ectoparasiticide application were low and similar to these observed in general population of other countries. After the use of FIP-based medication on household pets, the detection rates and levels of FIP and

FIP-sulfone in urine increased significantly. In contrast, other FIPs were rarely detected. The urinary levels of both FIP and FIP-sulfone were still elevated at the last day of experiment, when another dose of FIP could be applied, suggesting a risk of accumulation of FIPs in humans after repeated use. No FIPs were detected in the negative control. Levels of FIPs in personal SWBs collected in the present study also increased dramatically following the use of FIP-based ectoparasiticide on pets. Apart from FIP and FIP-sulfone, other FIPs were frequently detected, especially FIP-desulfinyl and FIP-sulfide, providing insight into environmental fate of FIP following its use on household pets. Low levels of FIPs were observed both before and after application in the negative control. In contrast to personal SWBs, low concentrations of FIPs were observed in stationary SWBs, suggesting the key role of dermal transfer in human exposure. Additionally, the comparison of median urinary levels of FIPs in urine and sum of FIPs in personal SWBs during the week following FIP application revealed strong correlation between these matrices. This observation shows relevance of SWBs for estimation of average exposure and/or selection of the most exposed individuals. Finally, the dose reconstruction investigation followed by risk assessment revealed that the health risk associated with FIP exposure in the studied population was low. However, further research is necessary to evaluate the risk in other populations, such as small children or veterinary staff.

STRESZCZENIE

Fipronil to insektycyd z grupy fenylopirazoli szeroko stosowany jako lek weterynaryjny w profilaktyce i leczeniu inwazji pasożytów zewnętrznych (np. pcheł i kleszczy) u psów i kotów. Jako że podanie leków bazujących na fipronilu jest zewnętrzne i wykonywane wielokrotnie w trakcie sezonu, często samodzielnie przez właścicieli zwierzęcia, istnieje znaczące ryzyko narażenia na fipronil wśród osób posiadających zwierzęta domowe. Niektóre badania sugerują, że ekspozycja ludzi na ten związek wiąże się z negatywnymi skutkami zdrowotnymi. Istnieje więc potrzeba oceny narażenia na fipronil w takim scenariuszu. Biomarkerami narażenia na ten ksenobiotyk w moczu jest związek macierzysty oraz jego utleniona pochodna, sulfon fipronilu. Są one jednak wydalane z moczem w niewielkich ilościach, a ponadto występują one również w środowisku. Z tego powodu zaproponowano alternatywny biomarker, hydroksyfipronil, który dotychczas wykryto jednak tylko u szczurów. W badaniach środowiskowych oprócz fipronilu i sulfonu wykrywa się często również amid fipronilu, desulfinylofipronil oraz siarczek fipronilu. Fiprole to nazwa obejmująca fipronil oraz produkty jego (bio)transformacji. Jednym z obiecujących narzędzi do oceny osobistego narażenia zewnętrznego są opaski silikonowe.

Niniejsze badanie miało cztery cele. Pierwszym z nich był rozwój i walidacja metody ilościowego oznaczania śladowych ilości fiproli w moczu, w tym także hydroksyfipronilu, bazując na chromatografii cieczowej sprzężonej z tandemową spektrometrią mas (LC-MS/MS). Drugim zadaniem było opracowanie i walidacja kolejnej metody opartej o LC-MS/MS, tym razem w celu oznaczania w opaskach silikonowych pochodnych fipronilu istotnych w analizie środowiskowej. Trzecim celem było długookresowe badanie ekspozycji ludzi na fipronil zarówno przed jak i po zastosowaniu u zwierząt domowych leku przeciwpasożytniczego zawierającego ten związek. W oparciu o wyniki tej części zrealizowano ostatnią część badania: ocenę ekspozycji oraz ryzyka zdrowotnego ludzi wiążącego się ze stosowaniem u zwierząt preparatu zawierającego fipronil.

Do analizy próbek moczu opracowano metodę bazującą na ekstrakcji do fazy stałej oraz LC-MS/MS. Nie udało się włączyć hydroksyfipronilu do metody z powodu rozbieżności między sygnałem masowym wzorca a widmem oczekiwanym. Pozostałe fiprole można było oznaczyć ilościowo w stężeniach rzędu pg/ml lub ng/ml. Dla tych analitów wykazano zadowalającą wartość dokładności, precyzji oraz innych parametrów walidacyjnych. Efekt matrycowy ograniczono wykorzystując wzorce wewnętrzne znakowane izotopowo oraz kalibrację w matrycy zgodnej z matrycą próbki badanej.

Metoda analityczna do oznaczania fiproli środowiskowych w opaskach silikonowych bazowała na ekstrakcji ciecz-ciało stałe, po której następowało oczyszczenie uzyskanego ekstraktu poprzez wymrażanie oraz ekstrakcję ciecz-ciecz. W tym przypadku również posłużono się LC-MS/MS jako techniką separacji oraz detekcji, co umożliwiło ilościowe oznaczanie stężeń analitów rzędu ng/g opaski. Przed walidacją metody przeprowadzono szereg badań optymalizacyjnych. Dodatkowe badania metodologiczne przyczyniły się do poszerzenia wiedzy nt. wykorzystania opasek silikonowych jako osobistych próbników.

W badaniu ekspozycji u ludzi poproszono uczestników o zbieranie próbek moczu oraz noszenie opasek silikonowych zarówno przed jak i po podaniu preparatu przeciw-

pasożytniczego zawierającego fipronil zwierzętom domowym. W mieszkaniach uczestników rozlokowano również opaski stacjonarne. Kontrolą negatywną była jedna osoba, która zaaplikowała swojemu zwierzęciu produkt bez fipronilu. W próbkach zebranych przed podaniem leku weterynaryjnego fiprole były wykrywane rzadko, podobnie jak w badaniach populacji generalnej z innych krajów. W próbkach zebranych po aplikacji częstość detekcji oraz poziomy fipronilu oraz sulfonu fipronilu znacząco wzrosły; inne fiprole wciąż wykrywano rzadko. Stężenia fipronilu oraz sulfonu w moczu były podwyższone również w ostatnim dniu prowadzenia eksperymentu, gdy możliwe było podanie zwierzęciu kolejnej dawki, co sugeruje ryzyko kumulacji fiproli u ludzi po wielokrotnym zastosowaniu. Nie wykryto natomiast fiproli w próbkach pozyskanych w ramach kontroli negatywnej. Stężenia fiproli w osobistych opaskach silikonowych zebranych po aplikacji preparatu przeciwpasożytniczego były również znacząco wyższe niż w próbnikach noszonych przed. Oprócz fipronilu oraz sulfonu fipronilu w opaskach często wykrywano również inne pochodne, zwłaszcza desulfinylofipronil oraz siarczek fipronilu, co dostarcza nowych informacji o losie fiproli w środowisku po podaniu związku macierzystego zwierzęciu domowemu. W opaskach z kontroli negatywnej oznaczono śladowe ilości analitów zarówno przed, jak i po podaniu leku. W przeciwieństwie do osobistych próbników, w opaskach stacjonarnych oznaczono niskie stężenia fiproli, co sugeruje, że narażenie u ludzi ma miejsce głównie drogą dermalną. Ponadto mediany stężeń sumy fiproli w moczu z pierwszego tygodnia po aplikacji wykazywały silną korelację z sumą fiproli w opaskach silikonowych z tego samego okresu. Pokazuje to przydatność tych próbników w szacowaniu średniej ekspozycji i/lub wskazaniu osób o najwyższym narażeniu. Ostatnią częścią badania była rekonstrukcja dawki, po której przeprowadzono ocenę ryzyka zdrowotnego. Wykazano, że w badanej populacji ryzyko zdrowotne związane z narażeniem na fipronil w badanej populacji było niskie. Niezbędne są jednak dalsze badania by przeprowadzić podobną ocenę wśród innych grup, takich jak małe dzieci czy personel weterynaryjny.

LIST OF ABBREVIATIONS

3PBA	3-phenoxybenzoic acid
4OH3PBA	4-Hydroxy-3-phenoxybenzoic acid
AAS	Active air sampling
ADI	Acceptable daily intake
AOEL	Acceptable operator exposure level
APVMA	Australian Pesticides and Veterinary Medicines Authority
ARfD	Acute reference dose
BPS	Bisphenol S
bw	Body weight
CAS	Chemical Abstract Service
cis-DCCA	*cis*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylic acid
CV	Coefficient of variation
DBS	Dried blood spots
DI	Daily intake
DPhP	Diphenyl phosphate
dSPE	Dispersive solid phase extraction
EI	Electron ionization
EPA	Environmental Protection Agency
ESI	Electrospray ionization
EU	European Union
FAO	Food and Agriculture Organization of the United Nations
FIP	Fipronil
FIP-amide	Fipronil-amide
FIP-desulfinyl	Fipronil-desulfinyl
FIP-dtfms	Fipronil-detrifluoromethylsulfinyl
FIP-hydroxy	Fipronil-hydroxy
FIP-sulfide	Fipronil-sulfide
FIP-sulfone	Fipronil-sulfone
FIPs	Fiproles
GABA	*γ*-Aminobutyric acid
GC-MS	Gas chromatography-mass spectrometry
GEE	Generalized estimating equations
HQC	High concentration quality control
ICC	Intraclass correlation coefficient
IMI	Imidacloprid

IMZ	Imazalil-despropenyl
IS	Internal standard
LC-MS(/MS)	Liquid chromatography-(tandem) mass spectrometry
LC_{50}	Median lethal concentration
LD_{50}	Median lethal dose
LLOQ	Lower limit of quantification
LOD	Limit of detection
LQC	Low concentration quality control
MDA	Malondialdehyde
MRM	Multiple reaction monitoring
NOAEL	No observed adverse effect level
NOEL	No observed effect level
NZW	New Zealand White
PDMS	Polydimethylsiloxane
PER	permethrin
PFAS(s)	Per- and polyfluoroalkyl substance(s)
PYRs	Pyrethroid metabolites
rpm	Revolutions per minute
QC	Quality control
SG	Specific gravity
SIM	Selected ion monitoring
SPE	Solid phase extraction
SWB(s)	Silicone wristband(s)
T3	Triiodothyronine
T4	Thyroxine
TIC	Total ion current
trans-DCCA	*trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylic acid
TSH	Thyroid-stimulating hormone
TWA	Time-weighted average
UK	United Kingdom
ULOQ	Upper limit of quantification
USA	United States of America
VAMS	Volumetric absorptive microsampling
W0, 1, …, 4	Week 0, 1, …, 4 (of the human exposure study)
WHO	World Health Organization

1 INTRODUCTION

According to the World Health Organization (WHO), approximately 24% of the global burden of disease are attributed to environmental factors, including use of chemicals (Prüss-Üstün & Corvalän, 2007). Many of these factors can be modified through informed policy. A reasonable management of environmental risk factors associated with diseases can therefore help protecting human health (Prüss-Ustün et al., 2017).

Still, chemicals are part of our environment and play an important role in our daily lives. In many cases, however, their effect on the population health is unknown. Purposes of their use, as well as physicochemical and toxicological properties vary greatly, making risk assessment a considerable challenge. The exposure, both to a parent compound or its derivatives, may occur via various routes, further complicating the picture (Prüss-Ustün et al., 2011). The biologically active chemicals are of particular concern, since they are designed to alter functions of living organisms and may cause harm in humans via primary (intended) and secondary (alternative) effects. One of the compounds belonging to that group is fipronil (FIP) (Casida & Durkin, 2013).

1.1 FIPRONIL: AN OVERVIEW

1.1.1 Properties and use

FIP (chemical name: (±)-5-amino-1-[2,6-dichloro-4-(trifluoromethyl)phenyl]-4-trifluoromethyl)sulfinyl]-1H-pyrazole-3-carbonitrile, Chemical Abstract Service (CAS) Registry Number: 120068-37-3) (Figure 1.1) is a broad-spectrum phenylpyrazole insecticide and veterinary drug developed by Rhône-Poulenc between 1985 and 1987 (Tingle et al., 2000). Under the revised definition of per- and polyfluoroalkyl substances (PFASs) (OECD, 2021), FIP belongs to that group as well (EMA, 2023). FIP is a neutral compound (K. A. Lewis et al., 2016) that contains an asymmetrical sulfoxide moiety (Teicher et al., 2003), but is used as a racemic mixture (Gunasekara et al., 2007; X. Li et al., 2020). Its vapor pressure is low (2.8×10^{-9} mmHg at 25°C) and it dissolves well in polar organic solvents, such as acetone or methanol, yet poorly in water and hexane; its logP is equal to 3.5 (FAO/WHO, 2002).

FIP was first marketed in 1993 for use on field and horticultural crops against multiple insect pests (Gunasekara et al., 2007). Registration for other uses followed – it has been approved for flea and tick control on household pets (mainly cats and dogs) in multiple countries by the end of 1995. It was also registered and used for cockroach and ant control. As a result, FIP has been introduced to all major markets in the world, including the European Union (EU), the United States of America (USA), China, and Brazil. Due to its worldwide commercial success, it was the „flagship" insecticide of Rhône-Poulenc until its fusion with another company in 2000 (Tingle et al., 2000). Ultimately, FIP has been introduced in over 80 countries for use in more than 100 crops (Salgado et al., 2019). After being registered for wide range of applications, the use of FIP has been gradually restricted from late 2000's onwards, mainly due to growing evidence of its high toxicity towards bees and aquatic organisms (EFSA, 2013; Z. Liu, Chen, Lyu, Li, et al., 2022). An outline of FIP legal status in selected countries/regions is provided below.

In the EU, FIP use on crops has been limited in 2013 (The European Commission, 2013) only to be banned altogether in 2016 (The European Commission, 2016). A spectacular example of its misuse was the “fipronil incident”, which took place in 2016-2017 and involved illegal use of FIP on poultry farms, mainly in Belgium and the Netherlands (Focker et al., 2021; Gerletti et al., 2020; Reich & Triacchini, 2018). FIP was never approved for such application in the EU (Reich & Triacchini, 2018). On the other hand, FIP approval for use as a biocide in the EU expired as late as September 2023 (EMA, 2023). The latter fact can be observed in changes in products available on the market. For instance, after the phase-out of FIP-based Goliath Gel, used as an indoor bait insecticide against cockroaches (The European Commission, 2011), Goliath Gel New was introduced into the market. In this product, FIP had been replaced by clothianidin, a neonicotinoid insecticide (BASF, no date). In consequence of the aforementioned regulations, after September 2023, FIP is only approved for veterinary use in the EU.

In the USA, a FIP-based product for use on rice was voluntarily withdrawn from the market by manufacturer in 2004 (US EPA, 2004). Over a few years preceding this decision, several papers regarding negative effect of FIP-treated rice seed use on local wildlife had been published (Mize et al., 2008). In 2010, to mitigate ecological risks associated with FIP application (US EPA, 2011b), the Environmental Protection Agency (EPA) cancelled FIP use for corn in-furrow and corn seed treatment (US EPA, 2010). Today, FIP is approved in the USA for veterinary use, residential pest control, termite treatment, lawn care, and heavily restricted range of agricultural applications (US EPA, 2011b). It should be noted, however, that individual states may place greater restrictions than those enforced at federal level (Donley, 2019). For instance, FIP is not approved for any agricultural use in California (Teerlink et al., 2017). A nationwide longitudinal study of FIP occurrence in wastewater and sewage sludge suggests veterinary use and urban pest control act as major sources of environmental FIP contamination (Sadaria et al., 2019).

In 2009, use of FIP was restricted in China to veterinary medicine, residential hygiene, corn seed coating, and export products (Shi et al., 2021; Yan et al., 2023). Recently, FIP is manufactured there mostly for indoor application (Shi et al., 2020). However, illicit use of FIP poses a problem in Chinese agriculture (S. Liang et al., 2019; Z. Liu, Chen, Lyu, Li, et al., 2022).

Over the years, other phenylpyrazole insecticides were developed and marketed: ethiprole, pyriprole, and flufiprole (Figure 1.1). Ethiprole was introduced in 2005 by Bayer CropScience as an insecticide used in rice fields (Schnatterer, 2012), though mainly in Southeast Asia (US EPA, 2011a). Pyriprole, on the other hand, has been registered in the EU by Novartis in 2006 as an ectoparasiticide against ticks and fleas on dogs (Novartis, 2020). Finally, in 2009, Dalian Raiser Pesticide Company (J. Li et al., 2016) introduced flufiprole in China (K. A. Lewis et al., 2016) for use in rice and vegetables (J. Gao et al., 2020). FIP, however, remains the most important compound of the group (Schnatterer, 2012).

At this point, an important distinction should be made. As mentioned above, these compounds are collectively known as phenylpyrazoles. Another term, fiproles (FIPs) is also in use, but its meaning is ambiguous (Herin et al., 2011). Some authors (Casida &

Durkin, 2015; Cochran et al., 2015; Tingle et al., 2003) use it interchangeably with phenylpyrazoles, while other (Cryder et al., 2019; EMA, 2023; Perkins et al., 2024; Sadaria et al., 2017; Teerlink et al., 2017; Zhang et al., 2023) define FIPs as FIP and its (bio)transformation products. In this work, the latter definition is used. This distinction, however, is not clear-cut. For instance, flufiprole has been shown to degrade to FIP in the environment (J. Gao et al., 2020).

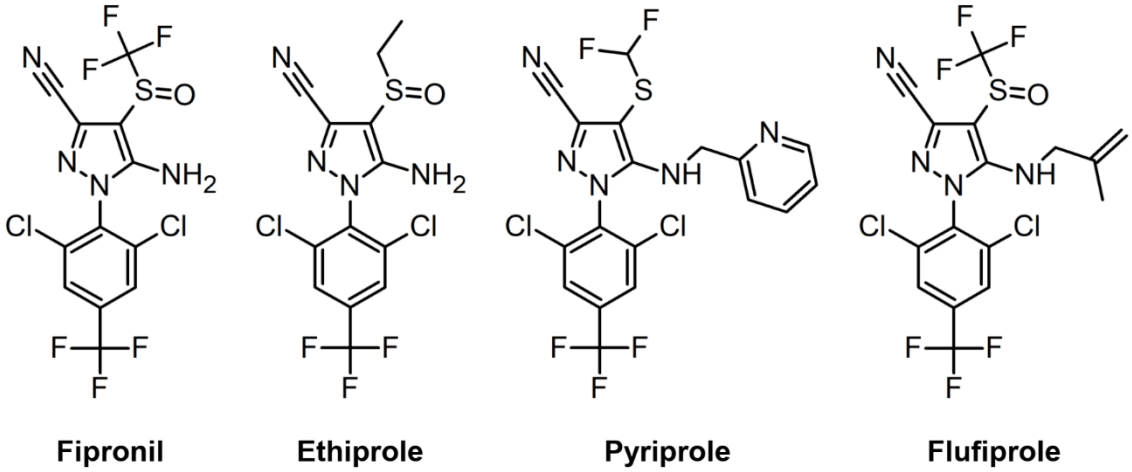

Figure 1.1 Structure of fipronil and three other phenylpyrazole insecticides – ethiprole, pyriprole, and flufiprole.

1.1.2 Mechanism of action

γ-Aminobutyric acid (GABA) is the main inhibitory neurotransmitter, both in arthropods and mammals (Casida & Durkin, 2013). It is released from a presynaptic neuron after stimulation and binds to postsynaptic $GABA_A$-receptors, which contain ligand-gated chloride channels. After binding GABA, $GABA_A$-receptors open the channel and cause hyperpolarization of postsynaptic membrane, inhibiting neuron excitation (Ozoe et al., 2009). FIP non-competitively blocks that process, leading to excessive neural depolarization, paralysis, and death of an insect (Gunasekara et al., 2007). In humans, the receptor usually consist of various combinations of *α*, *β*, and *γ* subunits, whereas several different subunits comprise insect GABA-receptors (Casida & Durkin, 2013). The structural differences in GABA receptors probably contribute to FIP's selectivity towards arthropods (Cole et al., 1993). FIP shares this mode of action with the classical organochlorine insecticides, such as lindane or endosulfan, as has been shown in experiments with cyclodiene-resistant *Drosophila* (Casida & Durkin, 2013). FIP, however, is also a potent blocker of insect glutamate-gated chloride channels. As these receptors are not expressed in vertebrates, this fact may also contribute to FIP's selectivity towards arthropods. However, the role of glutamate-gated chloride channels in FIP insecticidal action is not clearly understood (Ozoe et al., 2009).

1.1.3 Toxicity towards non-target organisms

Despite its high selectivity towards arthropods, FIP is also biologically active in other organisms, including mammals. Following acute exposure, the median lethal dose (LD_{50}, rat, oral) and median lethal concentration (LC_{50}, rat, inhalation) were 97 mg/kg body weight (bw) and 0.39 mg/L, respectively. In the case of dermal exposure, a LD_{50} value of 354 mg/kg bw was established for rabbits. Prolonged exposure studies were

conducted as well. Based on 1-year dog and 90-day rat and dog studies, a no observed adverse effect level (NOAEL) was set at 0.35 mg/kg bw/day. For repeated dermal exposure, a 21-day rabbit study was conducted. As a result, a dermal no observed effect level (NOEL) was established for rabbits (5 mg/kg bw/day). In all species, signs of neurological disturbance were observed. Additionally, hepatomegaly and hepatocyte enlargement were present in rats and dogs, whereas thyroid follicular hypertrophy/hyperplasia was observed only in rats (EFSA, 2006).

In line with the animal studies, case reports of acute FIP exposure in humans also mention symptoms associated with nervous system, such as headache, dizziness, agitation, and seizures (Chodorowski & Anand, 2004; Fung et al., 2003; S. J. Lee et al., 2010; Mohamed et al., 2004). Based on the aforementioned NOAEL value, the acceptable operator exposure level (AOEL) was set at 0.0035 mg/kg bw/day (safety factor of 100) (EFSA, 2006).

Long-term effects of oral FIP exposure were investigated in a 2-year rat study. Deleterious effects were observed in the liver, thyroid and kidneys. Moreover, convulsive episodes appeared in a dose-related manner. The toxicologically relevant NOAEL was 0.019 mg/kg bw/day and on that basis an Acceptable Daily Intake (ADI) was established for humans (0.0002 mg/kg bw/day; safety factor of 100) (EFSA, 2006). A more detailed description on long-term effects of FIP exposure on laboratory animals was provided in a recent evaluation carried out by Food and Agriculture Organization of the United Nations (FAO) and WHO (FAO/WHO, 2022). In a 54-week chronic toxicity study, CD-1 mice were exposed to daily dose of FIP equal to 0.1-60 ppm (five levels). The animals exposed to the highest dose died before the end of the experiment or were euthanized due to poor body condition. In all mice from this group, high relative liver weights were observed; some of males had convulsions at the beginning of the experiment. At the end of the study, increased relative liver weight compared to the control group was observed in males exposed to 0.5 ppm of FIP or more, whereas in females this effect occurred at 30 ppm level. Additionally, chronic exposure to FIP at 30 ppm resulted in reduced body weight gain. A similar study was conducted on Sprague Dawley rats (52 weeks, FIP dose 0.5-300 ppm, four levels of exposure). In contrast to the previous study, animals were observed for an additional 13-week recovery phase after the cessation of treatment. Following the exposure period, reduction of body weight gain compared to the controls was observed in two the most exposed groups (30 and 300 ppm); this effect was not reversed after the recovery period. Symptoms suggesting neurotoxicity appeared at exposure at or above 1.5 ppm but were not present during the recovery phase. The examples include convulsions, irritability, aggression, excessive vocalization and salivation. In this study, many laboratory parameters were tracked, including those related to thyroid function. Serum thyroxine (T4) levels were reduced in all groups exposed to FIP, but the effect was reversible. For other thyroid biomarkers, the results were less consistent – serum triiodothyronine (T3) increase was observed during recovery phase in females exposed to the two highest doses, whereas elevated thyroid-stimulating hormone (TSH) levels occurred in males at 30 and 300 ppm and in females at 300 ppm. The biochemical effects described above were accompanied by increased thyroid weights at 30 and 300 ppm FIP exposure level. In the case of the latter group, this effect did not fully resolve by the

end of the recovery period. At this exposure level, an increased incidence of thyroid follicular neoplasia was also observed (FAO/WHO, 2022).

Few papers investigated effects of FIP exposure on thyroid function in humans. In an occupational exposure study conducted in France (Herin et al., 2011), serum level of fipronil-sulfone (FIP-sulfone), one of FIP metabolites, showed weak negative correlation with TSH. No associations were observed for FIP. In a study on infants in South Korea, level of FIP-sulfone in cord blood was negatively correlated with T3 concentration in the same matrix (Y. A. Kim et al., 2019). Further research is necessary to investigate the effect of FIP on thyroid function in humans.

Several studies focused on genotoxicity of FIP. *In vitro* investigations included bacterial reverse mutation tests and chromosome aberration studies, among others. The vast majority of results were negative; the only positive results were obtained at cytotoxic concentrations. In the case of *in vivo* tests, most of them were based on mouse micronucleus assay. The results from all included studies were negative, despite dose levels being close to LD_{50}. Therefore, it was concluded that FIP can be considered non-genotoxic (FAO/WHO, 2022).

In a 78-week carcinogenicity study in CD-1 mice, malignant hepatocellular carcinomas were observed at the highest FIP dose in males. However, its incidence was within the range observed for control, so the neoplasms were determined as unrelated to FIP administration. In a 90-week oncogenicity study in Sprague Dawley rats, a statistically significant increased incidence of thyroid follicular neoplasia occurred in animals fed at the largest dose (300 ppm). However, this effect was determined to be of limited relevance to humans (FAO/WHO, 2022).

To determine reproductive toxicity of FIP, Sprague Dawley rats were fed FIP continuously for two generations. At 30 and 300 ppm levels, increased liver and thyroid weights were observed in both adult generations. Reduced fertility was observed in the second generation (83% compared to 100% in controls) at the 300 ppm dose. At the same exposure level, convulsions and reduced body weight gain was observed in the offspring of both generations (FAO/WHO, 2022).

In a developmental toxicity study, a group of presumably pregnant Sprague Dawley rats were administered FIP via oral gavage. Low maternal toxicity was observed and the litter was not affected. Similar study was conducted in New Zealand White (NZW) rabbits. Although there was a statistically significant decrease in maternal body weight, no toxicity was observed in the litter (FAO/WHO, 2022). Basing on rat developmental neurotoxicity data, the Acute Reference Dose (ARfD) was determined for humans (0.009 mg/kg bw; safety factor of 100) (EFSA, 2006).

Neurotoxicity of FIP was investigated in several animal studies. In an acute neurotoxicity study, dosing at 50 mg/kg bw to Sprague Dawley rats lead to reduced body weight gain, abnormal gait, fine tremors, head bobbing, decreased hind leg splay, convulsions, and other clinical signs of neurotoxicity; convulsion-associated deaths were also observed. A 90-day repeated dose neurotoxicity study (at lower FIP levels) only showed reversible reduction of body weight gain and exaggerated responses to external stimuli. A similar study conducted in dogs showed mild and reversible signs of neurotoxicity after repeated administration of FIP at 20 mg/kg bw dose. The symptoms included

underactivity, stiffness, convulsions, head nodding, abnormal gait, tremors, and other; no neuroanatomical abnormalities were observed (FAO/WHO, 2022).

Several *in vivo* and *in vitro* studies showed the potential of FIP to induce oxidative stress. In Wistar rats treated with 5 and 10 mg/kg bw of FIP, increase of reactive oxygen species levels in sperm was observed. In Swiss mice, the 10 mg/kg bw dose of FIP caused elevated levels of malondialdehyde (MDA, a known product of lipid peroxidation) in the liver; the increase was reversible by vitamin C or E. *In vitro* studies on human neuroblastoma (SH-SY5Y) and rat pheochromocytoma (PC12) cells also showed induction of MDA levels following exposure to FIP (X. Wang et al., 2016).

1.1.4 Pharmacokinetics and biotransformation

Data on FIP pharmacokinetics and biotransformation in humans is scarce. Experiments on Sprague Dawley rats showed limited, dose-dependent bioavailability of FIP following oral administration (34 and 53% at the highest and the lowest dose, respectively) (FAO/WHO, 2022). In another study on Sprague Dawley rats, oral FIP bioavailability ranged from 12 to 19%; again, a reverse correlation with dose was observed. In both studies, low FIP bioavailability probably resulted from extensive first-pass effect (Chang & Tsai, 2020; FAO/WHO, 2022). In contrast, FIP was well absorbed in NZW rabbits exposed to FIP via the same route (estimated absorption: 80%) (FAO/WHO, 2022). In a recent study on FIP pharmacokinetics in dogs, rapid absorption was observed (dos Santos et al., 2020), but the bioavailability was not determined. Following dermal application, 0.4% of the dose was absorbed in rats within 24 h post-dose (Cochran et al., 2015). In another rat study, the bioavailability via this route was estimated to be less than 3% (FAO/WHO, 2022).

Once absorbed, FIP is widely distributed in the tissues. Studies on rodents and rabbits exposed to radiolabeled FIP showed that it was highly concentrated in fat. Other tissues with considerable radioactivity were thyroid, liver, and kidneys (FAO/WHO, 2022). Transplacental transfer of FIP was shown in an intervention study in rats (Chang & Tsai, 2020) and an observational study in humans (Y. A. Kim et al., 2019). FIP was also detected in breast milk (Z. Liu, Chen, Lyu, Wu, et al., 2022).

FIP is extensively metabolized in mammals. If administered orally, considerable biotransformation occurs before reaching the systemic circulation (first-pass effect). More extensive metabolism was observed in rats compared to other laboratory animals (FAO/WHO, 2022). Additionally, FIP was shown to induce xenobiotic metabolizing enzymes in rats (Caballero et al., 2015) and human hepatocytes (Das et al., 2006). An outline of FIP metabolism in mammals is shown in Figure 1.2. Only a small fraction of unchanged FIP is excreted in urine (Cravedi et al., 2013). Due to the high lipophilicity of FIPs (see below) and their considerable enterohepatic circulation, they are excreted in substantial amounts with feces and have potential for bioaccumulation (Cravedi et al., 2013; FAO/WHO, 2002, 2022; Tonnelier et al., 2012).

FIP-sulfone is the main FIP metabolite (Figure 1.2). It is produced through oxidation of the sulfoxide moiety of FIP. Its formation was observed in rats, rabbits, mice (FAO/WHO, 2022; Hainzl & Casida, 1996), and in humans as well (Mohamed et al., 2004). FIP-sulfone was also the only FIP metabolite produced by human cytochrome P450 *in vitro* (Tang et al., 2004). Compared to the parent compound, FIP-sulfone was

shown to have higher affinity towards murine GABA receptors in the central nervous system (Hainzl et al., 1998) and higher potency to block ligand-gated chloride channels in GABA receptors in rats (X. Zhao et al., 2005). Therefore, this reaction can be considered a metabolic activation process (Cravedi et al., 2013). Additionally, FIP-sulfone was shown to persist longer in studied animals than FIP (FAO/WHO, 2022). Indeed, FIP-sulfone exhibits higher logP compared to the parent compound (3.8 *vs.* 3.5, respectively) (FAO/WHO, 2002) and was recognized as the main bioaccumulative fiprole (Cravedi et al., 2013). Consequently, researchers studying human exposure to FIP frequently include both parent compound and FIP-sulfone in chemical assays (Chen et al., 2022), the detection rate for the latter being higher in several studies (Herin et al., 2011; Y. A. Kim et al., 2019; McMahen et al., 2015; Shi et al., 2021). FIP-sulfone was also the main fiprole detected in breast milk (Z. Liu, Chen, Lyu, Wu, et al., 2022) and cord blood (Y. A. Kim et al., 2019). It was also found in substantial amounts in rat brain after oral FIP administration (Cravedi et al., 2013). These findings highlight wide distribution and efficient permeation of FIP-sulfone through biological barriers.

Fipronil-sulfide (FIP-sulfide; sometimes referred to as fipronil-thioether) (Figure 1.2) is a product of FIP reduction. It exhibits similar lipophilicity to FIP and FIP-sulfone (logP = 3.7). It was observed in feces of rats and rabbits, as well as in conjugated form in rat urine (FAO/WHO, 2002, 2022). Toxicological profile of FIP-sulfide is similar to FIP, with less pronounced neurotoxicity in animal studies (FAO/WHO, 2022). It is not detected in human biomonitoring studies (Chen et al., 2022) or only trace amounts are observed (Z. Liu, Chen, Lyu, Wu, et al., 2022).

Oxidation of cyano moiety of FIP leads to fipronil-amide formation (FIP-amide; also known as fipronil-carboxamide) (Figure 1.2). FIP-amide is less lipophilic than the aforementioned FIPs (Raveton et al., 2006). It was detected in small amounts in feces and urine of rats and rabbits orally exposed to FIP. FIP-amide is also less neurotoxic – its affinity towards rat brain $GABA_A$-receptor is over 20 times lower than the parent compound. Further oxidation of FIP-amide leading to formation of carboxy derivative was observed in rats. This metabolite also exhibits low neurotoxicity (FAO/WHO, 2022).

Detrifluoromethylsulfinylation or detrifluoromethylsulfonylation of FIP or FIP-sulfone, respectively, leads to formation of fipronil-detrifluoromethylsulfinyl (FIP-dtfms) (Figure 1.2). This metabolite also lacks the neurotoxicity of the aforementioned FIPs (Hainzl & Casida, 1996). FIP-dtfms undergoes extensive further metabolism, and products thereof are more readily excreted with urine compared to FIP and FIP-sulfone (McMahen et al., 2015). Apart from FIP-dtfms conjugates with glucuronide and sulfate (FAO/WHO, 2022), ring-opened products were observed in rat urine (McMahen et al., 2015) (Figure 1.2), and an oxidized derivative, referred to as fipronil-hydroxy (FIP-hydroxy) (Vasylieva et al., 2017).

If FIP-hydroxy was found in human urine, it could be an excellent biomarker of human exposure. Firstly, due to extensive urinary excretion (McMahen et al., 2015; Vasylieva et al., 2017), it could be detected in large quantities in urine, which is the preferred matrix in human biomonitoring (see section 1.3.1). Secondly, it was reported in the literature that all the other FIPs mentioned above can also be formed in the environment (see next section) (FAO/WHO, 2022; Hirashima et al., 2023). Thus, their presence

in urine could result from external contamination, which would not be possible in the case of FIP-hydroxy.

FIP-hydroxy can be further oxidized in rats to hydroxyamine and imine product (Figure 1.2). However, these compounds were formed in smaller quantities compared to FIP-hydroxy (McMahen et al., 2015).

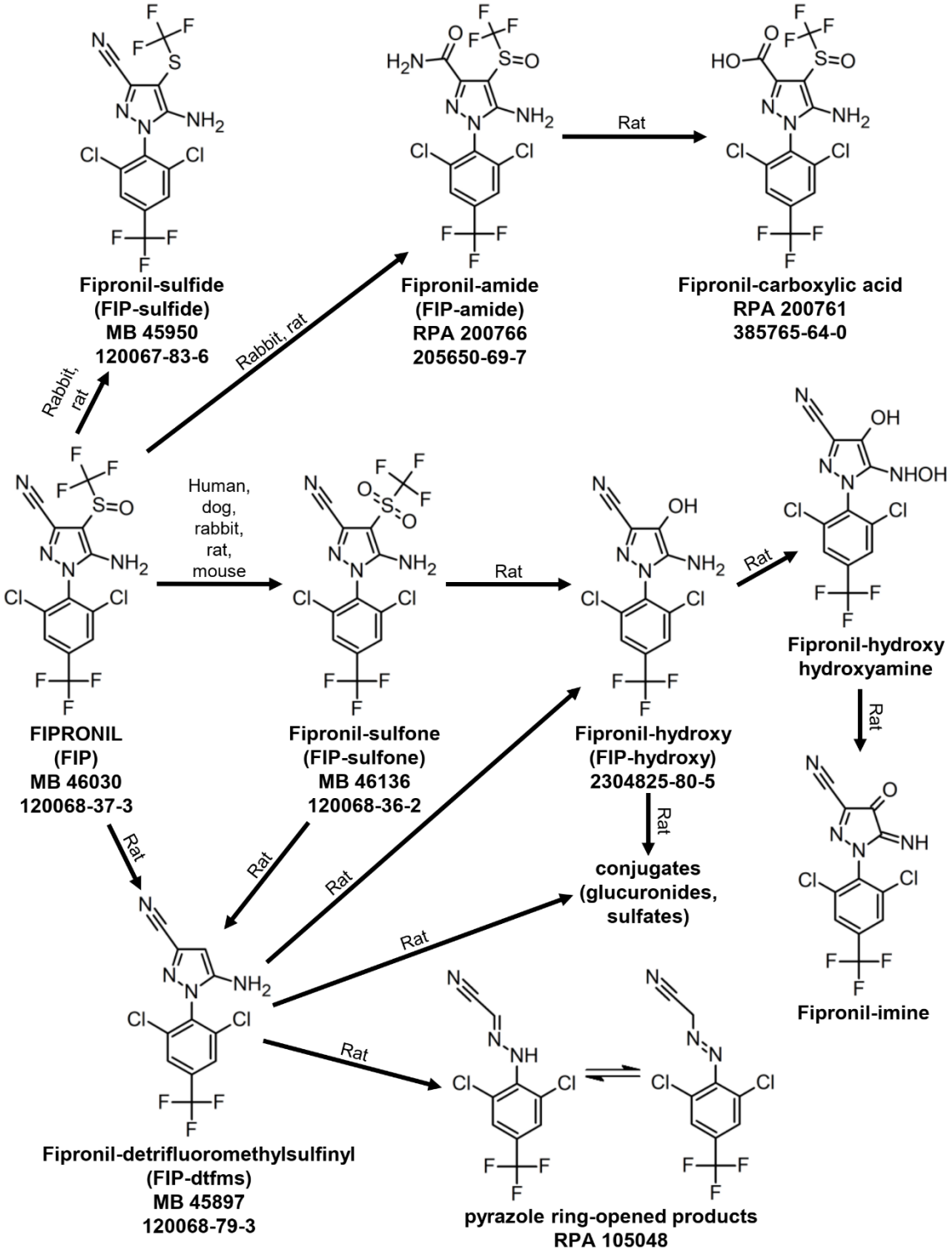

Figure 1.2 Outline of in vivo *fipronil metabolism in mammals basing on* Cravedi et al., 2013; FAO/WHO, 2002, 2022; McMahen et al., 2015; Vasylieva et al., 2017. *After the compound name, the abbreviation used throughout this work, code name, and CAS registry number is provided, if available.*

1.1.5 Environmental fate

In the environment, FIP may undergo many degradation pathways, depending on the nature of the physical, chemical and biological factors that FIP is subjected to (Figure 1.3). Many products of environmental FIP degradation have also been observed *in vivo* and their toxicity is described in the previous section.

Fipronil-desulfinyl (FIP-desulfinyl; Figure 1.3) is the main product of FIP photodegradation (Hainzl & Casida, 1996; Raveton et al., 2006). It was shown to be similarly neurotoxic to both mammals and arthropods as the parent compound (Hainzl & Casida, 1996). With logP equal to 3.4 (FAO/WHO, 2002), it also shares similar lipophilicity to FIP. Although FIP-desulfinyl is not formed *in vivo* (FAO/WHO, 2022), substantial quantities of this compound were found in breast milk collected during nationwide study on Chinese women (Z. Liu, Chen, Lyu, Wu, et al., 2022). The cause of this unexpected finding was not discussed in that paper. In the author's opinion, this surprising result might have been caused by exposure to FIP-desulfinyl formed in the environment.

FIP photodegradation may also result in formation of FIP-sulfone, FIP-dtfms, and FIP-sulfide (Figure 1.3). However, these compounds are formed in relatively small quantities compared to FIP-desulfinyl. Additionally, FIP-sulfone was postulated by Bobé et al., 1998 and Hirashima et al., 2023 to degrade into FIP-desulfinyl. All these photodegradates can be further decomposed under sunlight – firstly to open-ring pyrazole compounds, then to aniline derivatives, and, eventually, to inorganic species.

FIP oxidation, either caused by microbial activity or well-aerated environment, leads to FIP-sulfone formation in soil (Figure 1.3). FIP reduction to FIP-sulfide can also be caused by microorganisms. This process, however, indirectly relies on moisture content of the environment, which strongly affects microbial activity and creates more reducing environment, favoring formation of FIP-sulfide at expense of FIP-sulfone (Ying & Kookana, 2002).

At alkaline conditions (pH ≥ 9) FIP decomposes into FIP-amide (Figure 1.3). The rate of degradation increases in direct relationship to increase in pH and follows pseudo-first-order kinetics (Bobé et al., 1998). Main properties of FIP-amide were briefly described in the previous section.

Finally, fipronil-monochloro derivative was detected in a study on environmental fate of FIP (Figure 1.3). However, mechanism of its formation is unknown (Starr et al., 2016).

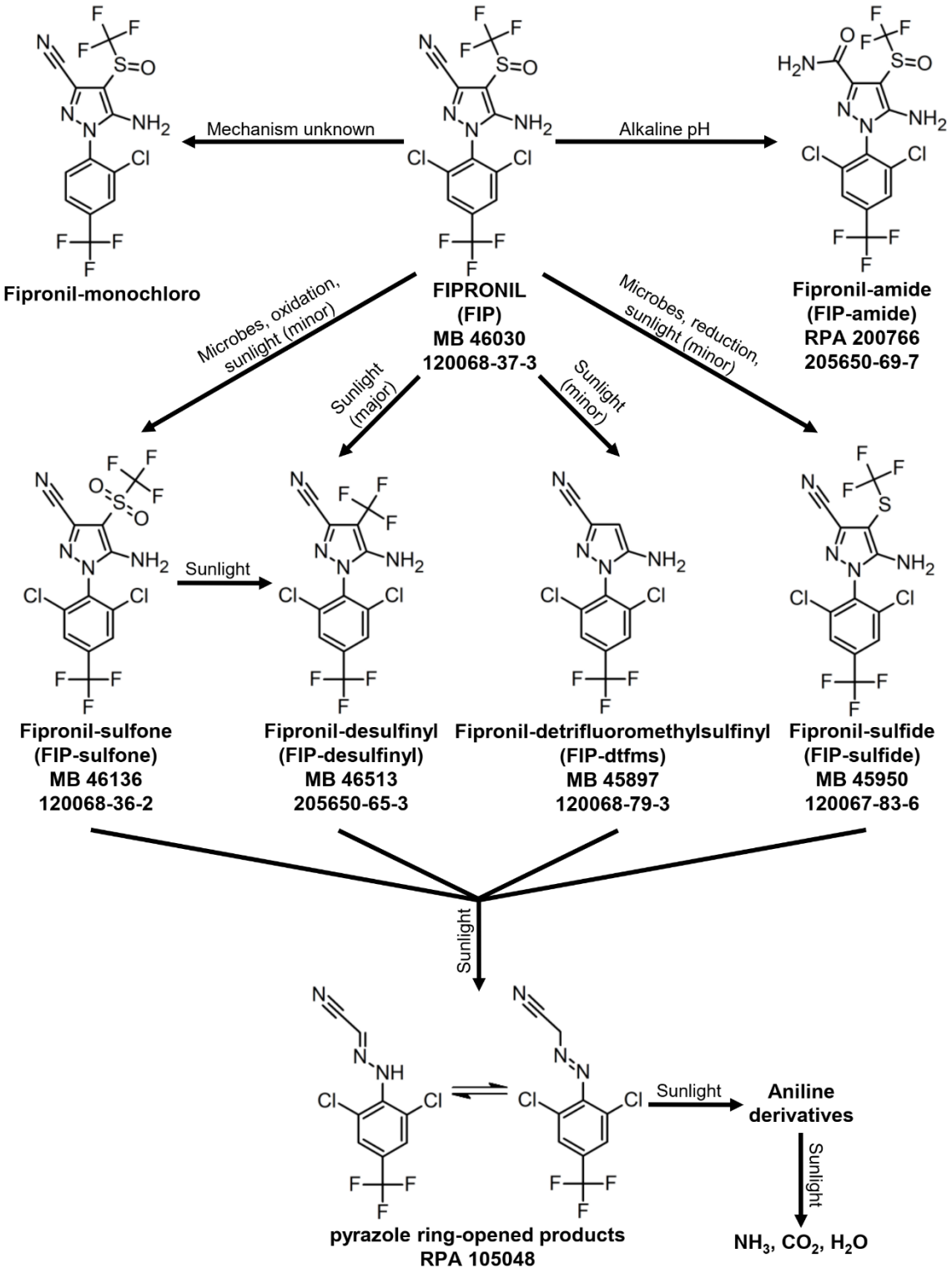

Figure 1.3 Selected degradation pathways of fipronil in the environment basing on Bobé et al., 1998; Hainzl & Casida, 1996; Hirashima et al., 2023; Raveton et al., 2006; Starr et al., 2016; Ying & Kookana, 2002. *For the photodegradates, the distinction between minor and major products is made. Following the compound name, the abbreviation, code number, and CAS registry number is provided, if available.*

1.2 PET OWNERSHIP AND EXTERNAL PARASITE TREATMENT

Cats and dogs were integral part of human communities as early as 9,000 years ago. Initially kept for pragmatic reasons, both became more valued as companions over time (Walsh, 2009). Indeed, owning a cat or a dog as a pet has many positive effects on psychological health of their owners. Additionally, since dogs need to be walked, they can facilitate social contacts between people and increase physical activity of their caregivers (D. L. Wells, 2009). Therefore, it is not surprising that pet ownership is common in many countries. In 2020, 24% and 25% of EU households were estimated to own at least one cat or one dog, respectively (FEDIAF, 2021). In the USA, these animals are even more popular. According to a 2022 survey, 32% and 45% of American households own cats and/or dogs, respectively (AVMA, 2024). Pet ownership is less frequent among Chinese population, although it is rapidly increasing in recent years (Dan Wu et al., 2024). In 2020, 15% and 18% of Chinese households owned a cat or a dog, respectively (L. Lewis & White, 2024). Globally, more than half of the global population is estimated to have a pet, with dog and cat being the two most popular choices (GfK Intelligence, 2016).

Infestation by external parasites (ectoparasites), such as fleas and ticks, negatively affects the health and well-being of a companion animal. Ectoparasites may transmit pathogens which cause vector-borne diseases, often more dangerous than the infestation itself. Some of ectoparasites are known to transfer to humans. Additionally, many pathogens carried by ectoparasites can also induce diseases in humans, posing a health risk to people who have contact with infected pet. Therefore, proper ectoparasite management in companion animals is important for public health as well (Curet Bobey, 2015). For cats and dogs, veterinary associations as well as individual practitioners often recommend flea and tick control administration 12 months a year, although the length of that period may depend on many factors, such as outdoor activity of the pet and seasonality of parasites' activity (Lavan et al., 2020, 2021; Perkins & Goulson, 2023).

Medicines used to treat and prevent ectoparasite infestations in animals are known as ectoparasiticides (C. Wells & Collins, 2022). They are often applied on a pet by their owners themselves (Driver et al., 2010; Perkins & Goulson, 2023). To improve pets' welfare and caregiver's convenience, these products are available in topical formulations, such as spot-on solutions and collars, as well as oral forms, like chewable tablets (Lavy et al., 2022; Selzer & Epe, 2021).

Spot-on products are usually a concentrated solution of an active substance in an organic solvent (Lavy et al., 2022). For easy application, the volume of a single dose is low (typically 1-2 mL) (Witchey-Lakshmanan, 1999) and it is usually packaged in a pipette (EMA & CVMP, 2018). The entire dose is released in a single timepoint by dripping the applicator content onto a spot on the animal's skin between the shoulder blades (Driver et al., 2010). Then the drug, such as FIP, spreads rapidly over the skin and forms a depot in sebaceous glands (Brayden, 2003). The drug sequestrated in the glands is slowly released back onto the skin over time, providing extended release and long-lasting insecticidal effect (Jennings et al., 2002; Lavy et al., 2022). In the EU, FIP is the most popular active substance used in spot-on products (EMA, 2023); they are also popular

in the United Kingdom (UK) (Perkins & Goulson, 2023) and the USA (Lavan et al., 2021). The typical treatment interval is 4 weeks (EMA, 2023).

Ectoparasiticidal collars have a long history of use against pests in companion animals. Today, the technology relies on incorporation of an active ingredient into a polymeric resin and its slow diffusion onto the fur of an animal once it starts wearing the product (Rothen-Weinhold et al., 2000). Collars provide a more homogenous release of the active substance than spot-on products and longer duration of activity, often within the 4-6 months range (EMA, 2023). However, in the EU, FIP-based collars are only approved for use in Bulgaria (EMA, 2024).

Recently, a discovery of isoxazoline insecticides allowed to develop ectoparasiticides administered orally. Such products offer improved convenience and limited exposure of humans to the active ingredient compared to topical formulations (Selzer & Epe, 2021), but require the parasite to feed on the animal to get exposed to the drug (Roeber & Webster, 2021), which involves a risk of vector-borne disease transmission (Curet Bobey, 2015).

Integrating the estimates on pet ownership, as well as survey and market data on ectoparasiticide use, FIP is applied annually over 1.6 million times in the UK (C. Wells & Collins, 2022) and above 41 million times in the European Economic Area (EMA, 2023). Sales reports from veterinary clinics (Lavan et al., 2020) and the state of California (Teerlink et al., 2017) suggests frequent FIP use in the USA as well. Since people have extensive contact with their pets, exposure to active ingredients of ectoparasiticidal products, such as FIP, seems inevitable (Driver et al., 2010). Aforementioned data on FIP use on pets suggests that the exposure may be widespread in many populations. Appropriate scientific tools need to be used in order to determine the magnitude and route of that exposure.

1.3 EXPOSURE ASSESSMENT

Exposure assessment is a tool to measure and characterize exposures in studied populations (Nieuwenhuijsen et al., 2015). It is critical for many research fields in environmental health science, allowing to understand exposures among groups and individuals, as well as search for appropriate remedies, including changing human behaviors and legislation. In the past, studies often tracked exposure to a single or a few chemicals. Today, research tends to include many exposures occurring simultaneously, in line with the concept of exposome, which considers the totality of exposures from conception to death (Vandenberg et al., 2023).

Source-oriented exposure assessment focuses on emissions records and ambient concentrations. The exposure is estimated basing on human behavior patterns. One major flaw of this method is the discrepancy between ambient concentration of a pollutant and the exposure of a given person, especially when indoor exposure is important. Other approaches are closer to an individual (Vandenberg et al., 2023).

In point-of-contact exposure assessment, the measurements are made at the barrier between the individual and the environment using personal monitoring devices. When combined with contact duration, the personal monitoring data can be used to estimate exposure. Since these devices do not capture dietary route, they can be used to

discriminate between dietary and non-dietary exposure (Vandenberg et al., 2023). Personal monitoring devices can also be used to identify the source of exposure (Hammel et al., 2016; Quintana et al., 2020).

Dose reconstruction method is based on chemical quantitation in biological matrices (biomonitoring) and use of toxicokinetic models. This approach allows to aggregate exposures from all routes and sources, even if they are unknown. However, if the biomonitoring and/or toxicokinetic data is limited, the resulting exposure estimate may be inaccurate or uncertain (Vandenberg et al., 2023).

As outlined above, there are several approaches to exposure assessment. The analytical tools needed for personal exposure assessment are described in more detail below.

1.3.1 Biological monitoring

Biological monitoring (biomonitoring) is the measurement of chemicals in human tissues or fluids, such as blood or urine (LaKind et al., 2019). It is capable of tracking exposure from all routes and its results can be directly related to the internal dose, which then can be linked to health effects (Needham et al., 2007). Although it often requires expensive and sophisticated methods of instrumental analysis (Angerer et al., 2007) and interpretation of collected data is complex (Alves et al., 2014), biomonitoring is the primary tool for human exposure assessment; urine and blood are the matrices most commonly used (Aylward et al., 2014).

Urine

The most abundant components of urine, apart from water, are urea, electrolytes, and metabolic waste products (Muscat et al., 2011). Urine is widely used in biological monitoring due to non-invasive sampling procedure and relatively higher concentration of non-persistent chemicals compared to other matrices (Verner et al., 2020). Additionally, it can be obtained in large quantities and be reliably collected by participants themselves (Šulc et al., 2022).

Urine production rate, however, may vary significantly both within and between individuals (Panuwet et al., 2016) and it largely depends on fluid intake prior to urination (Cone et al., 2009). Therefore, it is important to take dilution variability into consideration. The correction is often done using measurements of urinary creatinine or specific gravity (SG) (D. B. Barr, Wilder, et al., 2005; Cone et al., 2009; Kuiper et al., 2021). Creatinine is a spontaneously formed byproduct of creatine metabolism in muscles. The rate of creatinine formation is relatively constant, but several factors – apart from hydration status – were found to affect it, such as muscle mass, gender, age, and diet. Creatinine adjustment of the analyte concentration is usually performed by simply dividing the latter by the creatinine concentration in the same sample (D. B. Barr, Wilder, et al., 2005). SG is a relative density of a substance compared to water. A recent large-scale study on National Health and Nutrition Examination Survey data showed that SG, although also influenced by gender, body composition and other factors, is likely preferable to creatinine as an indicator of urine dilution (Kuiper et al., 2021). Studies suggesting equivalence of SG and creatinine adjustment (Muscat et al., 2011) as well as superiority of the latter

(Gaines et al., 2010) can also be found. The SG-corrected results are usually obtained using the Levine-Fahy equation (Cone et al., 2009; Levine & Fahy, 1945):

$$C_{adjusted} = C_{measured} \times \frac{SG_{mean} - 1}{SG_{measured} - 1}$$

where $C_{adjusted}$ is analyte concentration corrected for SG, $C_{measured}$ represents the uncorrected concentration, SG_{mean} is an average SG for a given population, and $SG_{measured}$ is an SG of a sample.

Blood

Blood, although less popular in exposure assessment than urine, is also frequently used for this purpose (Polkowska et al., 2004). Either whole blood or its components, such as plasma or serum, can be analyzed (Holland et al., 2005). Exposure assessment using blood has certain advantages. Since blood is in direct contact with all organs, it is representative of all the chemicals present in the body at a given timepoint (Wallace et al., 2016). Additionally, the amount of blood is similar among adults, which makes comparisons and body burden calculations easier. However, the lipid content of blood, which is important for lipophilic analytes, is subject to postprandial variation. Normalization to blood lipid content can be used to limit both intra- and interindividual variation resulting from sampling time (D. B. Barr, Wang, et al., 2005).

Biological monitoring using blood is limited by several obstacles. In contrast to urine, blood collection is an invasive procedure which causes discomfort, requires trained staff and controlled environment, and generates infectious waste. The first two disadvantages are particularly important in exposure assessment because the study subjects are often healthy individuals, who are more reluctant to such burdensome procedures than patients in a clinical setting. The personal and infrastructure requirements associated with blood sampling significantly increase research cost and impede studies on remote populations (Wallace et al., 2016). Blood microsampling techniques, such as dried blood spots (DBS) or volumetric absorptive microsampling (VAMS) are less invasive and do not require trained staff. However, small sample volume obtained using these techniques (ca. 50 µL and 10-30 µL for DBS and VAMS, respectively) negatively affects assay sensitivity (Protti et al., 2019; Wallace et al., 2016). Nonpersistent chemicals are often found in blood at lower concentrations than in urine, making the latter matrix even more preferable (D. B. Barr, Wang, et al., 2005). Despite the disadvantages associated with sampling, blood is still the preferred matrix for exposure assessment to persistent pollutants, such as polychlorinated biphenyls, due to their poor urinary excretion (Polkowska et al., 2004).

Variability of biomarker concentration

As discussed above, properties of biological matrix used for exposure assessment are important. However, other factors, such as properties of a chemical of interest and characteristics of exposure (route, frequency, duration) also significantly affect the magnitude of exposure (Aylward et al., 2014). Since so many factors may affect the quantified concentration, considerable research effort has been done to determine reproducibility of biomonitoring results (Roggeman et al., 2022). One of the parameters used for reproducibility evaluation is the intraclass correlation coefficient (ICC) (Pleil et al., 2018). ICC

is the ratio of between-person variation to total variance in the studied population (Aylward et al., 2014). ICC can range from 0 to 1; the higher the value, the higher the reproducibility (less variability) (Roggeman et al., 2022). Value below 0.4, between 0.4 and 0.75, and equal to or above 0.75 correspond to poor, fair to good, and excellent reproducibility, respectively (Rosner, 2016). For rapidly excreted chemicals, such as bisphenol A, the ICC values are often low and variable between studies, depending on the biomarker selection, urinary dilution correction and sampling strategy used (range for bisphenol A: 0.04-0.60) (Roggeman et al., 2022). For FIPs in biofluids, the only attempt for ICC calculation was made by Faÿs et al., 2020. The ICCs for urinary FIP and FIP-sulfone in that study were 0.35 and 0.37, respectively, indicating high variability of observed concentrations. In consequence, repeated sampling is necessary to obtain a reliable estimate of exposure.

1.3.2 Environmental monitoring

Environmental monitoring can be defined as “gathering, assessing and reporting environmental information obtained through continuous or periodic sampling, observation and analysis of both natural variation or changes and anthropogenic pressures and their effects on humans and the environment” (UN, 2003). Contrary to biomonitoring, environmental monitoring measures external exposure (Weis et al., 2005) and can be used to identify the source and route of exposure (Lioy, 1990). In this regard, environmental and biological monitoring may be considered complementary to each other.

Environmental monitoring may utilize ambient media, such as outdoor air or groundwater, or the media progressively closer to an individual – microenvironmental or personal (Lioy, 1995). The media or sensors that are closer to a person tend to reflect the individual exposure better than ambient measurements (Vandenberg et al., 2023). Microenvironmental media are sampled in a confined space; the examples include indoor dust or indoor air. Personal measurements involve use of personal monitors worn by a study subject (Lioy, 1995). The classic examples of personal monitors include portable active and passive air samplers (B. Wang et al., 2016), whereas silicone wristbands (SWBs) are one of the emerging technologies in this field (Wacławik et al., 2022).

Silicone wristbands

SWBs (Figure 1.4A) are passive sampling devices used as personal monitors since 2014 (O’Connell et al., 2014). Commonly known as a fashion accessory, SWBs showed great potential for tracking dermal and inhalation exposure to pesticides, flame retardants, polycyclic aromatic hydrocarbons, and other organic environmental contaminants (Wacławik et al., 2022). For instance, Bergmann et al., 2018 developed a qualitative method for 1550 chemicals in SWBs, whereas Doherty et al., 2020 used SWBs to simultaneously quantitate chemicals which logP value spanned over nine orders of magnitude. Wide range of chemicals sequestrated using these samplers stems from properties of polydimethylsiloxane (PDMS) – the material SWBs are made of.

PDMS is a silicone polymer consisting of $Si(CH_3)_2O$ units (Seethapathy & Górecki, 2012). Although silicones with substituents other than methyl group are known (Martin et al., 2016), PDMS is so popular that many authors use terms “PDMS” and “silicone” interchangeably (Wacławik et al., 2022) and this pattern is followed in this work. Passive sampling using PDMS relies on absorption of chemicals into the polymer phase which is

driven by van der Waals interactions, hydrogen bonding, and other phenomena. The higher affinity of an analyte for the polymer material, the easier its sequestration (ter Laak et al., 2008). Additionally, the flexibility of siloxane backbone facilitates permeation of chemicals into the PDMS (Seethapathy & Górecki, 2012). In consequence, PDMS is a versatile hydrophobic material capable of linear absorption of lipophilic organic substances over a wide concentration range (ter Laak et al., 2008). For instance, PDMS has been shown to effectively extract organic compounds with logP ranging from 2 to 7 (Martin et al., 2016; ter Laak et al., 2008). In another paper, an even wider range of hydrophobicity is suggested (logP 3-10) for sampling in water (Vrana et al., 2005).

As devices made of homogenous material, SWBs are single-phase passive samplers (Salim & Górecki, 2019). Passive air sampling relies on free flow of analyte molecules from the sampling matrix into the receiving phase (Ouyang & Pawliszyn, 2007). At the same time, elimination of the analyte from the sampler may also take place. Considering the balance between these two phenomena, the uptake of chemicals into the sampler can be divided into three stages: linear, curvilinear, and equilibrium. During the linear stage, analyte concentration in the sampler is negligible. Therefore, the change in the concentration in the sampler is driven only by uptake, which linearly depends on the length of sampling period and the concentration in the sampling matrix. Samplers that sequestrate a given analyte in a linear fashion throughout the entire sampling period are known as kinetic samplers (Bartkow et al., 2005). Such samplers provide the time-weighted average (TWA) concentration of the analyte, i.e. the average concentration over the time of exposure, using a single sampler (Salim & Górecki, 2019). After a certain amount of an analyte has been captured in the sampler, the elimination process can no longer be ignored. In consequence, the uptake rate becomes slower and the process reaches the curvilinear stage. Once the sequestration and elimination of analyte reach a constant rate, the uptake attains the equilibrium. The process of analyte diffusion within the phase, which follows the uptake step, is governed by Fick's First Law (Bartkow et al., 2005).

As the name implies, SWBs are usually worn on a wrist (Figure 1.4B), therefore they capture inhalation and dermal exposure simultaneously (Wacławik et al., 2022). In a few studies, a piece of SWB was worn as a lapel (J. A. Craig et al., 2019; O'Connell et al., 2014) or a brooch (S. Wang et al., 2019), acting as an air sampler. Carried across many microenvironments, SWBs provide the TWA of exposures taking place during deployment period, which usually lasts 5 or 7 days. However, its length may range from hours to even weeks, thanks to convenience of SWBs which are small, lightweight, and do not require power source. For the same reasons, SWBs are affordable and easy to use, making them a great tool for large-scale exposure studies even among sensitive populations, such as children or the elderly. SWBs can also be used as stationary samplers (Figure 1.4C) (Wacławik et al., 2022).

Disadvantages and knowledge gaps regarding use of SWBs in exposure assessment should also be mentioned. As an emerging technology, SWBs suffer from poor standardization of used materials and deployment protocols. In addition, a scientific basis for fully quantitative interpretation is still yet to be developed. For instance, there is considerable body of literature on air sampling theory using passive samplers (such as SWBs; see above) but, to the author's knowledge, the scientific framework for analyte

sequestration via direct contact is lacking. Since SWBs simultaneously capture both inhalation and dermal exposure, determination of the main route of exposure may be difficult if no additional data is available (Wacławik et al., 2022). PDMS, although versatile, may be too lipophilic to effectively sample ionic or highly polar species (Lohmann et al., 2012; Villaverde-de-Sáa et al., 2012). Furthermore, as shown by Anderson et al., 2017, O'Connell et al., 2014 and Wacławik et al., 2025, the impurities present in raw PDMS material warrant their removal prior to use, which takes time and resources. There are also several knowledge gaps related to sample preparation and instrumental analysis of SWBs. In many papers, the samplers were rinsed with water and/or organic solvent after deployment to remove bound particles but no data on potential analyte loss was provided. Furthermore, many authors reported no sample cleanup other than solvent exchange and/or filtration (Wacławik et al., 2022), which poses a risk of contamination to the system used for instrumental analysis (Han et al., 2016; Rajski et al., 2013). At the same time, methodological papers focused on sample preparation of SWBs are scarce; Wacławik et al., 2025 is a rare example. Finally, very few papers reported using liquid chromatography-(tandem) mass spectrometry (LC-MS(/MS)) (Wacławik et al., 2022), which is often preferred over gas chromatography-mass spectrometry (GC-MS) for analysis of currently used pesticides (Masiá et al., 2014; Mol et al., 2008) and veterinary drugs (Kaufmann et al., 2008), including FIP (Alder et al., 2006).

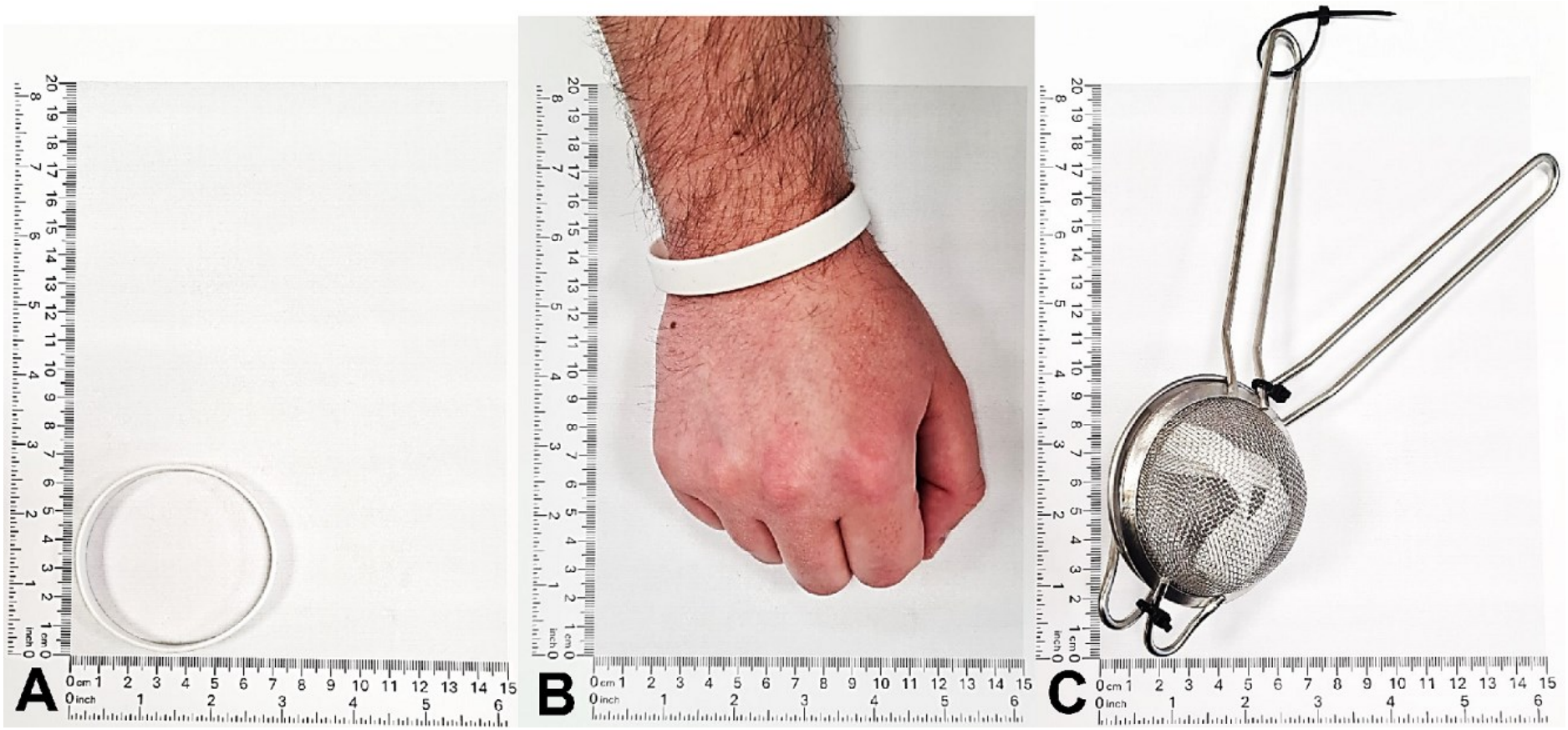

Figure 1.4 A silicone wristband (A), a silicone wristband worn during an exposure assessment study (B), and a silicone wristband in a stainless steel housing to be used as a stationary sampler (C).

Other environmental media

Personal passive air samplers share many features with SWBs described above. However, as a group, the former are much more diverse in terms of design and sorbent materials being used (Namieśnik et al., 2005). Additionally, the sorbent is often located inside a sampler body which prevents its direct contact with surrounding surfaces (Kot-Wasik et al., 2007). In consequence, these samplers only capture inhalation exposure, whereas SWBs track both inhalation and dermal routes. This difference may play important role in certain scenarios, such as exposure to poorly volatile chemicals.

In contrast, personal active air samplers share few similarities with SWBs (Wacławik et al., 2022). Since active air samplers operate in the equilibrium region of the uptake process (see previous section), they provide data on analyte concentration at the moment of sample collection, rather than the TWA (Salim & Górecki, 2019). Additionally, these devices rely on forced, constant airflow through the sampling medium. In consequence, active air sampling (AAS) devices require a pump and power source, which makes them prone to failure, cumbersome, expensive (B. Wang et al., 2016) and impractical in long term studies (Bohlin et al., 2007). However, the forced airflow allows to sample chemicals more efficiently, which makes AAS more suitable for short-term studies (National Research Council, 2012), such as exposure assessment during a single 8 hour shift. Similarly to typical passive air sampling described above, AAS can only track inhalation exposure (Bohlin et al., 2007).

Hand wipes are another tool available for personal exposure assessment. These sampling media are used by trained operators to remove chemicals from the skin of study subjects by applying external force. Examples of hand wipes include surgical pads and sponges. The medium used for sampling can be dry or wetted with water, organic solvents, or mixture thereof (Brouwer et al., 2000). Hand wipes provide information mostly on dermal exposure, but can provide insight into chemicals in vapor and particle phase, (S. Wang et al., 2019), as well as possible hand-to-mouth contact (Stapleton et al., 2008). Thanks to low cost and ease of use, hand wipes are widely used in exposure assessment, also in occupational setting. However, they reflect only very recent exposure (Watkins et al., 2012). At the same time, hand wipes are not suitable for repeated sampling since they may irritate skin surfaces and place burden on study participants. Finally, the sample collection process is prone to between-operator variability (Brouwer et al., 2000).

In contrast to the previously described media, indoor dust is not a personal exposure tool. As mentioned earlier, it can be classified as a microenvironmental medium (Lioy, 1995) which, if collected from enclosed spaces where study participants spend a considerable amount of time, may be used to estimate their exposure to chemicals of interest (Weisskopf & Webster, 2017). The sample collection step is short and usually involves vacuuming, brushing or brooming the floors and/or other relevant surfaces (Whitehead et al., 2011). The variety of methodologies used to collect indoor dust hinder comparisons of different studies (Watkins et al., 2012). Sample processing, such as sieving, also has a considerable impact on obtained results (Mercier et al., 2011). Finally, levels of chemicals in indoor dust represent a long-term average, making it useful mainly in assessment of retrospective exposure (Whitehead et al., 2011).

2 RESEARCH AIMS

The general aim of the hereby thesis was to investigate the use of FIP-containing ectoparasiticides on household pets as a source of human exposure to FIP. To achieve this goal, the following aims had to be fulfilled:

- **Aim 1**: To develop and validate an LC-MS/MS method for assessment of FIP exposure at trace levels in human urine. Not only FIP but also its widely known derivatives (FIPs: FIP-amide, FIP-desulfinyl, FIP-dtfms, FIP-sulfide, FIP-sulfone) were included. Additionally, the usefulness of a novel urinary biomarker of FIP exposure, FIP-hydroxy, was investigated.
- **Aim 2**: To develop and validate an LC-MS/MS method for determination of environmentally relevant FIPs in SWBs and investigate selected methodological aspects associated with use of SWBs as an environmental monitoring tool.
- **Aim 3**: To conduct a longitudinal study of human exposure to FIP before and after application of FIP-containing ectoparasiticide on household pets.
- **Aim 4**: To determine the magnitude and route(s) of human exposure to FIP as well as the health risk associated with its use on household pets.

3 METHODS

3.1 RESOURCES

The instruments, equipment, software, materials, and reagents used in this work are listed below.

3.1.1 Instrumentation

1) Liquid chromatography-tandem mass spectrometry system, including:
    a) Two 212-LC dual-piston pumps (Varian, Walnut Creek, CA, USA)
    b) ProStar 420 autosampler (Varian, Walnut Creek, CA, USA)
    c) High pressure mixer (Varian, Walnut Creek, CA, USA)
    d) Heated column compartment (Dionex, Sunnyvale, CA, USA)
    e) 320-MS triple quadrupole mass spectrometer with electrospray interface (Varian, Walnut Creek, CA, USA)
    f) Calypso 2G/1.BR nitrogen/air generator (F-DGSI, Evry, France)
    g) HS 602 rotary vane pump (Varian Vacuum Technologies, Turin, Italy)
    h) HS 652 rotary vane pump (Varian Vacuum Technologies, Turin, Italy)
    i) Delta P-6 Premium uninterruptible power supply (Delta, Taipei, Taiwan)
    j) Personal computer for system management and data acquisition (Dell, Round Rock, TX, USA)

3.1.2 Equipment

1) DMT-2500 multi-tube vortex mixer (Miu Instruments, Hangzhou, China)
2) Yellowline TTS2 vortex (IKA, Staufen, Germany)
3) Sorvall ST 16R centrifuge (Thermo Scientific, Waltham, MA, USA)
4) ED 53 drying oven (WTB Binder, Tuttlingen, Germany)
5) Multipette E3 handheld electronic dispenser (Eppendorf, Hamburg, Germany)
6) Research plus single-channel pipettes (Eppendorf, Hamburg, Germany)
7) HLP 10 demineralizer (Hydrolab, Straszyn, Poland)
8) M-525 series II muffle furnace (Ney, Barkmeyer Division, Yucaipa, CA, USA)
9) Vacuum drying system for solid phase extraction (SPE) cartridges
    a) MZ 2C NT diaphragm pump (Vacuubrand, Wertheim, Germany)
    b) SDS024M vacuum SPE dryer (Kamush, Gdańsk, Poland)
10) Accublock D1200 digital dry bath (Labnet International, Edison, NJ, USA)
11) PAL-10S pocket refractometer (Atago, Tokyo, Japan)
12) FiveEasy FE20 pH meter (Mettler-Toledo, Schwerzenbach, Switzerland)
13) PS 4500.R2 precision balance (Radwag, Radom, Poland)
14) WPS 110/C/2 precision balance (Radwag, Radom, Poland)
15) TS400D precision balance (Ohaus, Florham Park, NJ, USA)

16) AP110S analytical balance (Ohaus, Florham Park, NJ, USA)
17) U-505 ultrasonic bath (Ultron, Dywity, Poland)

3.1.3 Software

1) MS Workstation version 6.9.3 (Varian, Walnut Creek, CA, USA)
2) Microsoft Excel 365 (Microsoft, Redmond, USA)
3) Statistica version 13.3 (Tibco Software, Palo Alto, USA)
4) GraphPad Prism version 8.0.2 (GraphPad Software, San Diego, CA, USA)
5) R version 4.4.1 (R Foundation for Statistical Computing, Vienna, Austria)

3.1.4 Materials

1) Bond Elut Plexa 30 mg extraction cartridges (Agilent Technologies, Santa Clara, CA, USA)
2) Oasis HLB 3 cc (60 mg) extraction cartridges (Waters, Drinagh, Ireland)
3) Centrifugal filters, 0.2 µm, 500 µL, modified nylon (VWR, Leuven, Belgium)
4) Wide neck bottles, polypropylene (Kautex, Bonn, Germany)
5) Duran Pure bottles, borosilicate glass (DWK Life Sciences, Wertheim, Germany)
6) Volumetric flasks, class A, borosilicate glass (VWR, Leuven, Belgium)
7) Measuring cylinders, class A, borosilicate glass (VWR, Leuven, Belgium)
8) Short thread vials, 1.5 mL, amber borosilicate glass (La-Pha-Pack, Langerwehe, Germany)
9) Short thread polypropylene caps with silicone/polytetrafluoroethylene septa (La-Pha-Pack, Langerwehe, Germany)
10) Microinserts for short thread vials, 12 mm top, borosilicate glass (La-Pha-Pack, Langerwehe, Germany)
11) Microinserts for short thread vials, 15 mm top, borosilicate glass (La-Pha-Pack, Langerwehe, Germany)
12) Pyrex disposable culture tubes, 15-415, 16×100 mm, borosilicate glass (Corning, Corning, NY, USA)
13) Screw thread caps, 15-415, phenolic (Sun-Sri, Rockwood, TN, USA)
14) Test tubes, without rim, 16×100 mm, soda glass (VWR, Leuven, Belgium)
15) Test tubes, without rim, 12×75 mm, soda glass (VWR, Leuven, Belgium)
16) Pipette tips, polypropylene (Sarstedt, Nümbrecht, Germany)
17) Combitips Advanced, 1 mL (Eppendorf, Hamburg, Germany)
18) Disposable transfer pipets, 4.6 mL, low-density polyethylene (VWR, Leuven, Belgium)
19) Scintillation vials, 20 mL, high-density polyethylene (Sarstedt, Nümbrecht, Germany)
20) Urine collection cups, 500 ml, polypropylene (Sarstedt, Nümbrecht, Germany)
21) Urine containers, 2 L, polyethylene (Sarstedt, Nümbrecht, Germany)
22) Glass syringe, 1000 µL, polytetrafluoroethylene tip, luer lock, gas-tight (Trajan Scientific, Ringwood, Australia)

23) ACE Excel 3 SuperC18 HPLC analytical column, 75×3.0 mm ID (Advanced Chromatography Technologies, Aberdeen, Scotland)
24) ACE Excel UHPLC pre-column filter (Advanced Chromatography Technologies, Aberdeen, Scotland)
25) Uniguard guard cartridge holder (Thermo Scientific, Rockwood, TN, USA)
26) Hypersil Gold 3 µm drop-in guard cartridge (Thermo Scientific, Rockwood, TN, USA)
27) Smart spatulas, 210 mm length, disposable (LevGo, Berkeley, CA, USA)
28) Weigh boats, diamond, polystyrene (VWR, Leuven, Belgium)
29) Surgical scalpel handle No. 4 (Swann-Morton, Sheffield, England)
30) Surgical blades, carbon steel, No. 24 (Swann-Morton, Sheffield, England)
31) Conical tubes, 15 mL, polypropylene (Deltalab, Barcelona, Spain)
32) Aluminium foil, 30 µm (VWR, Leuven, Belgium)

3.1.5 Reagents

1) Nitrogen, technical grade (Oxygen, Gdańsk, Poland)
2) Argon BIP (Air Products, Allentown, PA, USA)
3) β-Glucuronidase from *Helix pomatia*, type HP-2, low sulfatase activity (Sigma-Aldrich, Burlington, MA, USA)
4) Acetic acid, glacial (Sigma-Aldrich, Burlington, MA, USA)
5) Sodium acetate, anhydrous (POCH, Gliwice, Poland)
6) Formic acid pro analysis (Sigma-Aldrich, Burlington, MA, USA)
7) Formic acid for LC-MS, LiChropur (Supelco, Bellefonte, PA, USA)
8) Ethyl acetate for GC-MS, SupraSolv (Supelco, Bellefonte, PA, USA)
9) Acetonitrile hypergrade for LC-MS, LiChrosolv (Supelco, Bellefonte, PA, USA)
10) Methanol hypergrade for LC-MS, LiChrosolv (Supelco, Bellefonte, PA, USA)
11) Water for chromatography, LC-MS grade, LiChrosolv (Supelco, Bellefonte, PA, USA)
12) Extran MA 01 detergent (Supelco, Bellefonte, PA, USA)
13) C18 endcapped bulk sorbent (Agilent Technologies, Santa Clara, CA, USA)
14) QuEChERS EMR-Lipid sorbent (Agilent Technologies, Santa Clara, CA, USA)
15) Primary secondary amine (PSA) bulk sorbent (Agilent Technologies, Santa Clara, CA, USA)
16) Supel QuE Z-Sep bulk sorbent (Supelco, Bellefonte, PA, USA)
17) Supel QuE Z-Sep+ bulk sorbent (Supelco, Bellefonte, PA, USA)
18) C30 Selectrasorb endcapped bulk sorbent (UCT, Bristol, PA, USA)
19) Acetonitrile for HPLC, gradient grade (VWR, Leuven, Belgium)
20) n-Hexane, Baker analyzed pesticide reagent (Avantor Performance Materials, Gliwice, Poland)
21) Ethyl acetate pro analysis (Sigma-Aldrich, Burlington, MA, USA)

3.2 QUANTIFICATION OF FIPROLES IN HUMAN URINE

3.2.1 Analytical standards

Standards of FIPs used in this study are listed below (Table 3.1). FIP-hydroxy was kindly provided by prof. Bruce Hammock, University of California Davis, USA. Other compounds were included in the developed method as well and are provided in a separate table (Table 3.2). The latter, however, will not be discussed further unless it is important for the decisions made during method development.

Table 3.1 Standards of fiproles used during development of analytical method for quantification of fiproles in urine.

Full compound name	Abbreviation	CAS number	Manufacturer	Catalog number
Analytes				
Fipronil	FIP	120068-37-3	Sigma-Aldrich	46451
Fipronil-amide	FIP-amide	205650-69-7	TRC Canada	D436240
Fipronil-desulfinyl	FIP-desulfinyl	205650-65-3	Sigma-Aldrich	41865
Fipronil-detrifluoromethylsulfinyl	FIP-dtfms	120068-79-3	TRC Canada	F342220
Fipronil-hydroxy	FIP-hydroxy	2304825-80-5	UC Davis	NA
Fipronil-sulfide	FIP-sulfide	120067-83-6	Sigma-Aldrich	34520
Fipronil-sulfone	FIP-sulfone	120068-36-2	Sigma-Aldrich	32333
Internal standards				
Fipronil-$^{13}C_4$	FIP-$^{13}C_4$	NA	Sigma-Aldrich	79157
Fipronil-detrifluoromethylsulfinyl-$^{13}C_2{}^{15}N_2$	FIP-dtfms-$^{13}C_2{}^{15}N_2$	NA	TRC Canada	F342222

Table 3.2 Other analytes and internal standards included in the method for urinalysis.

Full compound name	Group	Abbreviation	CAS number	Manufacturer	Catalog number	Parent compound(s)
Analytes						
Bisphenol S	Other	BPS	80-09-1	Sigma-Aldrich	43034	
Boscalid-5-hydroxy	Pesticides and related compounds	BOS-OH	661463-87-2	Sigma-Aldrich	28001	Boscalid
3-(2-Chloro-2-(4-chlorophenyl)vinyl)-2,2-dimethylcyclopropanecarboxylic acid	Pesticides and related compounds	CPhCA	88419-72-1	abcr	AB438398	Flumethrin
N,N-Diethyl-*meta*-toluamide	Pesticides and related compounds	DEET	134-62-3	Instytut Przemysłu Organicznego	IPO 922	
Diphenyl phosphate	Organophosphate flame retardants	DPhP	838-85-7	Aldrich	850608	Triphenyl phosphate, other
4-Fluoro-3-phenoxybenzoic acid	Pesticides and related compounds	4F3PBA	77279-89-1	abcr	AB531857	Flumethrin, cyfluthrin
4'-Hydroxy-3-phenoxybenzoic acid	Pesticides and related compounds	4OH3PBA	35065-12-4	Rousel Uclaf	RU46606	Permethrin, cypermethrin
Imazalil-despropenyl	Pesticides and related compounds	IMZ-OH	24155-42-8	Sigma-Aldrich	Y0000137	Imazalil
Imidacloprid	Pesticides and related compounds	IMI	138261-41-3	Instytut Przemysłu Organicznego	IPO 297	
Imidacloprid-5-hydroxy	Pesticides and related compounds	IMI-OH	155802-61-2	Witega	PS201	Imidacloprid
Perfluorooctanoic acid	Other	PFOA	335-67-1	Aldrich	171468	
Tebuconazole-*tert*-butylhydroxy	Pesticides and related compounds	TEB-OH	212267-64-6	Sigma-Aldrich	72843	Tebuconazole
Internal standards						
Bisphenol S-D_8	Other	BPS-D_8	NA	TRC Canada	B447392	
Imidacloprid-D_4	Pesticides and related compounds	IMI-D_4	1015855-75-0	TRC Canada	I274992	
3-Phenoxybenzoic acid-$^{13}C_6$	Pesticides and related compounds	3PBA-$^{13}C_4$	NA	Cambridge Isotope Laboratories	CLM-4542	

3.2.2 Method development

Final protocol

All reusable glassware used during method development and sample preparation was thoroughly cleaned using ultrasonic bath and laboratory detergent, then rinsed with methanol, and baked in a muffle furnace at 350°C for 4 hours; single-use glassware was baked in the furnace as well.

In the final method, 5 mL aliquots of urine samples in glass screw cap tubes were spiked with an internal standard (IS) mixture and incubated overnight at 37°C with 1250 µL of β-glucuronidase type HP-2 from *Helix pomatia* dispersed in 1 M acetate buffer pH 5.0 (glucuronidase and sulfatase activity: 300 and 3 U/mL buffer, respectively); the process was stopped the next day by addition of 750 µL of formic acid. The deconjugation procedure described above is an adapted protocol used elsewhere (Klimowska & Wielgomas, 2018). Following mixing and centrifugation, the supernatants were loaded on Bond Elut Plexa 30 mg cartridges preconditioned sequentially with 1 mL of ethyl acetate, 1 mL of 1% formic acid in methanol (*v*/*v*), and 1 mL of 1% formic acid in water (*v*/*v*). After loading, the cartridges were washed with 1 mL of 1% formic acid (*v*/*v*) in 15% methanol (*v*/*v*) and dried for 30 minutes in an SPE dryer connected to a vacuum pump. Analytes were eluted with 4×250 µL of ethyl acetate, similarly to Klimowska et al., 2023, and then carefully evaporated at 40°C under nitrogen stream. The reconstitution comprised of several steps. Firstly, 40 µL of methanol and 10 µL of water were added to the tube. After addition of each of the solvents, the content was vortexed at 2000 rpm. Then, to prevent loss of the extract, the tubes were centrifuged for 2 minutes at 1500 rpm. Finally, the entire extract was transferred to centrifugal filters and centrifuged for 3 minutes at 14000 *g*. The content of centrifugal tubes was then transferred to amber glass vials containing glass microinserts and injected into an LC-MS/MS system.

The outline of the procedure is shown in a figure below; description of optimization experiments follows.

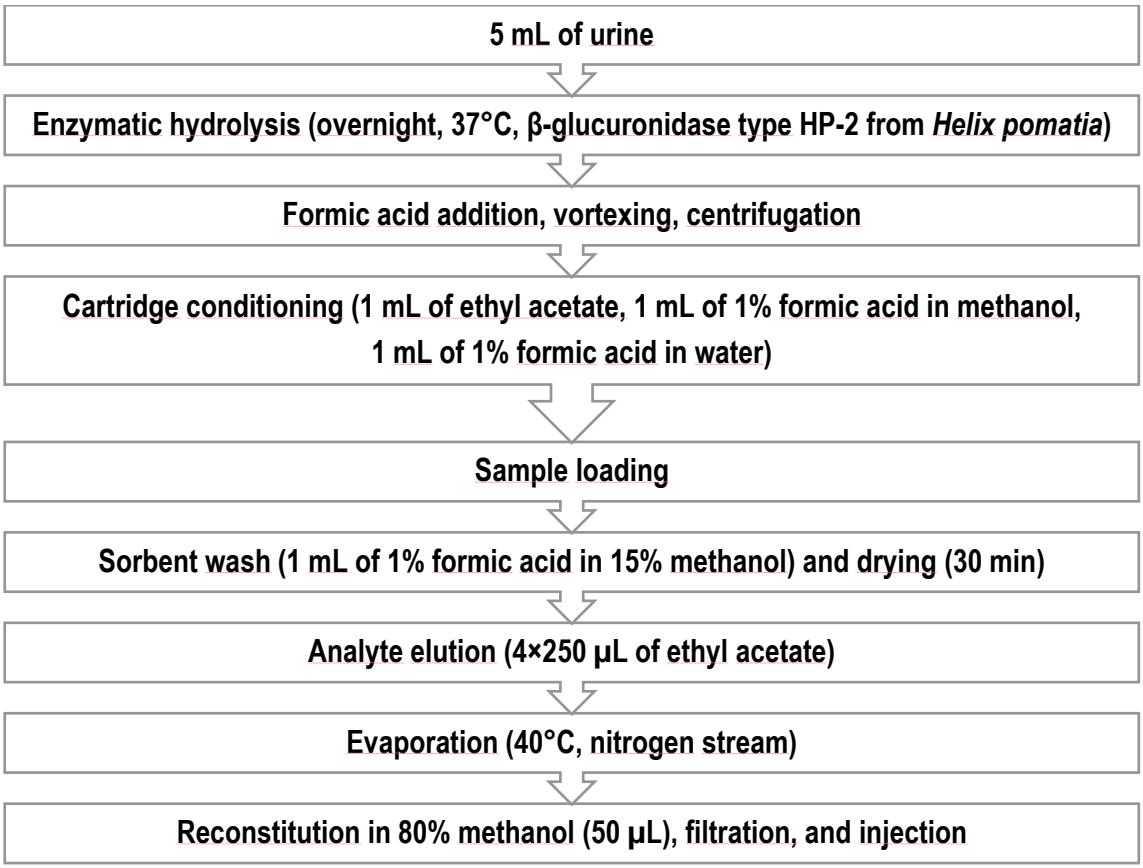

Figure 3.1 Workflow of the final sample preparation procedure for urine samples.

Filtration loss experiment

The goal of a filtration process is to remove particles present in the extract which could clog the tubing and/or the column of the LC system. It is typically the final step of sample preparation (Michlig et al., 2024). However, wrong choice of the filter material and/or filtration conditions may cause substantial analyte loss (Hebig et al., 2014; Michlig et al., 2024). The process of binding analytes to filter material may involve van der Waals forces, hydrophobic interactions, hydrogen bonding, electrostatic interactions, or other phenomena. Organic content of the solution is one of the key parameters affecting this process (Michlig et al., 2024). Since the filtration step was present in all optimization experiments, the investigation on the effect of solvent composition on analyte loss during filtration was conducted first. Nylon was used as filter membrane material; 60%, 80%, and 100% methanol (*v*/*v*) were investigated as solvent compositions. To cover a wide range of lipophilicity, mixture of three compounds in acetonitrile was prepared: imidacloprid (IMI; 80 ng/mL; logP 0.57) (NIH, 2024), FIP (2 ng/mL; logP 3.5) (FAO/WHO, 2002), and *trans*-permethrin (*trans*-PER; 160 ng/mL; logP 6.5) (NIH, 2024). 100 µL of the mixture was added to glass tubes and evaporated. The reconstitution was performed as follows: first, methanol was added and the tube content was mixed, then water was transferred into the tube followed by short vortexing. For each methanol percentage, three replicates were prepared and filtered. Since solvent composition of a sample injected into LC system is known to affect peak shape and height (VanMiddlesworth & Dorsey, 2012), three unfiltered samples with the same methanol content acted as a reference for every tested percentage (100% recovery).

Extraction cartridge selection

Choice of SPE sorbent strongly affects the amount of analyte extracted and the cleanliness of the sample extract (Waters, 2014). To select optimal stationary phase for SPE, Bond Elut Plexa 30 mg and Oasis HLB 60 mg extraction cartridges were compared. Pooled urine used for this experiment was split in half; one was left unchanged, whereas the other was spiked with standard mixture at 20 pg/ml for FIP, FIP-desulfinyl, FIP-sulfide, and FIP-sulfone, 100 pg/ml for FIP-amide, 500 pg/ml for FIP-dtfms, and 1000 pg/ml for FIP-hydroxy; other analytes, mentioned in Table 3.2, were added as well. Three aliquots of spiked and unspiked urine were loaded on each type of the cartridges that were preconditioned with 1 mL (Bond Elut Plexa 30 mg) or 2 mL (Oasis HLB 60 mg) of 1% formic acid in methanol (*v*/*v*) and 1% formic acid in water (*v*/*v*). Additionally, a single reagent and urine blank for both cartridges was prepared, so eight samples in total were run per sorbent. The washing step was performed using 1% formic acid (*v*/*v*) in 5% methanol (*v*/*v*); again, 1 mL of washing solution was added to Plexa, and 2 mL to Oasis cartridges. After drying step, the analytes were eluted using 1 mL (Plexa) or 2 mL (Oasis) of ethyl acetate; at that moment, mixture of analytes corresponding to 100% recovery was added to three unspiked urine extracts per sorbent. After evaporation of ethyl acetate under nitrogen stream, the dry residue was reconstituted using 80% methanol (*v*/*v*) and injected into the LC-MS/MS system.

Washing step optimization

After sorbent selection, the washing procedure was optimized. Ideally, this step allows to remove interferences without analyte loss (Waters, 2014). 1% formic acid (*v*/*v*) in 0, 5, 10, 15, 20, and 25% methanol (*v*/*v*) were tested (n = 3). The same (un)spiked urine as in the previous section was used, and the rest of the SPE procedure remained unchanged.

3.2.3 Liquid chromatography conditions

A list of LC settings is provided in Table 3.3.

Table 3.3 The parameters of liquid chromatography system used for urine analysis.

Liquid chromatograph part	Parameter name	Parameter value	
Pumping system	Mobile phase A composition	0.5 mM ammonium formate buffer pH 3 in water:methanol 9:1 (*v*/*v*)	
	Mobile phase B composition	0.5 mM ammonium formate buffer pH 3 in methanol	
	Flow rate (mL/min)	0.4	
	Mixer volume (µL)	150	
	Gradient program	Time (min:sec)	%B
		0:00	5
		12:00	100
		17:00	100
		17:01	5
		20:00	5
Autosampler	Temperature	Ambient	
	Injection volume (µL)	10	
	Needle wash solvent composition	Water:methanol:acetonitrile:isopropanol 1:1:1:1 (*v*/*v*/*v*/*v*)	

Liquid chromatograph part	Parameter name	Parameter value
Column compartment	Temperature (°C)	40
	Column	ACE Excel 3 SuperC18, 75×3.0 mm

3.2.4 Mass spectrometry conditions

A list of general mass spectrometry parameters is provided below (Table 3.4).

Table 3.4 Settings of mass spectrometer used for quantitation of fiproles and other analytes in urine.

Mass spectrometer part	Parameter name	Parameter value
Interface	Ionization type	Electrospray
	Needle voltage (V)	5000, -4500[1]
	Spray shield voltage (V)	600, -600[1]
	Nebulizing gas	Nitrogen, air[1]
	Nebulizing gas pressure (psi)	60
	Drying gas	Nitrogen
	Drying gas pressure (psi)	42
	Drying gas temperature (°C)	220
	Housing temperature (°C)	50
Mass analyzer	Type	Triple quadrupole
	Manifold temperature (°C)	40
	Collision gas	Argon
	Collision gas pressure (mTorr)	2.4
	Mass resolution (amu)	0.7
Detector	Detector voltage (V)	1500

[1] For positive and negative ionization, respectively.

Analyte- and IS-specific mass spectrometry conditions were determined by infusing a 100 ng/mL methanolic solution of the given compound into the mass spectrometer using a built-in syringe pump. Precursor m/z and capillary voltage were optimized in scan mode, whereas product ions *m*/*z* values and collision energies were selected in product scan and multiple reaction monitoring (MRM) mode, respectively (Table 3.5). The compound concentration was adjusted if needed (the 10-1000 ng/mL range was sufficient for all of them). Later, retention times were determined using LC conditions provided above (Table 3.3) in order to divide the data acquisition time of mass spectrometer into segments. In the case of FIP-hydroxy, additional LC-MS/MS experiments were necessary.

Table 3.5 Compound-specific parameters of fiproles analysis using LC-MS/MS urinalysis.

Full compound name	Abbreviation	Retention time (min)	Precursor ion	Precursor m/z	Capillary voltage (V)	Product ions m/z[1]	Collision energy (V)[2]
Analytes							
Fipronil	FIP	10.96	$[M-H]^-$	435.0	-70	329.7, 249.6, 277.6	15, 26, 27
Fipronil-amide	FIP-amide	9.26	$[M-H]^-$	453.0	-70	347.8, 271.9, 303.8	15, 41, 25
Fipronil-desulfinyl	FIP-desulfinyl	10.77	$[M-H]^-$	387.0	-50	350.9, 281.9, 330.8	12, 30, 28
Fipronil-detrifluoromethylsulfinyl	FIP-dtfms	9.14	$[M-H]^-$	319.0	-70	282.9, 262.8	8, 20
Fipronil-sulfide	FIP-sulfide	11.12	$[M-H]^-$	419.0	-70	261.8, 313.9, 382.9	26, 18, 11
Fipronil-sulfone	FIP-sulfone	11.34	$[M-H]^-$	451.0	-70	281.9, 243.8, 414.9	25, 44, 17
Internal standards							
Fipronil-$^{13}C_4$	FIP-$^{13}C_4$	10.97	$[M-H]^-$	439.0	-70	334.0, 250.9, 321.9	15, 26, 24
Fipronil-detrifluoromethylsulfinyl-$^{13}C_2{}^{15}N_2$	FIP-dtfms-$^{13}C_2{}^{15}N_2$	9.13	$[M-H]^-$	323.0	-70	287.0, 184.9	8, 28

[1] First ion is the quantifier, the other are the qualifiers (in increasing m/z order).

[2] For product ions, respectively.

3.2.5 Fipronil-hydroxy

As mentioned in the Introduction (section 1.1.4, Figure 1.2), FIP-hydroxy is a promising candidate for urinary biomarker of human FIP exposure. However, it was only detected in rats so far (Vasylieva et al., 2017). After an analytical standard was kindly provided by prof. Bruce Hammock from UC Davis, a series of experiments was conducted in order to investigate FIP-hydroxy potential for human biomonitoring. Since the precursor m/z observed during infusion of FIP-hydroxy standard into the mass spectrometer did not match the expected values, additional LC-MS(/MS) analyses of FIP-hydroxy were performed. LC conditions were the same as in Table 3.3. Scans were run in both positive and negative mode (m/z range 100-920) at 70 V capillary voltage. Several peaks were observed in negative mode and the MS/MS parameters were determined separately for peaks selected for further analysis.

3.2.6 Method validation

The goal of method validation is to ensure that the assay performance is sufficient and the results it provides are reliable. Here, the validation process was conducted basing on ICH M10 guideline on bioanalytical method validation and study sample analysis (EMA, 2022), with exception of the matrix effect investigation, which was carried out following the procedure described in EMA, 2011. A detailed description of performed experiments is provided below.

Selectivity

Selectivity as a capability of an analytical method to differentiate and measure the analyte(s) despite the presence of interferences was assessed by injecting several blank samples from separate sources. The acceptable threshold was less or equal to 20% of

the analyte response at the lower limit of quantification (LLOQ) level and no more than 5% of the IS response in the LLOQ sample.

Internal standard selection and matrix effect

Matrix effect, understood as a change of analyte response due to interferences present in the matrix, was thoroughly investigated. Seven different lots of urine were prepared in triplicate (SG range 1.006-1.031, determined refractometrically) and fortified post-extraction at 80 pg/mL level for FIP, FIP-desulfinyl, FIP-sulfide, and FIP-sulfone, 400 pg/mL for FIP-amide, and 2000 pg/mL for FIP-dtfms. Internal standards were added as well (200 pg/mL for FIP-$^{13}C_4$ and 5000 pg/mL for FIP-dtfms-$^{13}C_2{}^{15}N_2$). In parallel, three repetitions of analyte and IS mixture in pure solvent were prepared. Following analysis, matrix factor was calculated as the ratio of analyte peak area in blank matrix to analyte peak area in pure solvent and expressed in %. A result equal to 100% indicates no matrix effect, while results below indicate signal suppression, and values above 100% reflect signal enhancement. IS-normalized matrix factor was measured by dividing the matrix factor of a given analyte by the matrix factor of the IS. The coefficient of variation (CV) not greater than 15% for IS-normalized matrix factor was considered satisfactory. For each fiprole, both FIP-related ISs were tested; the one that provided the lowest CV of IS-normalized matrix factor was chosen for routine analysis.

Linearity and lower limit of quantification

LLOQ is “the lowest amount (concentration) of an analyte in a sample that can be quantitatively determined using a method with predefined precision and accuracy" (EMA, 2022). Linearity is the assumption that the signal-concentration relationship for given analyte(s) forms a straight line (Araujo, 2009). To test this premise, calibration curves were prepared by spiking blank urine matrix at 10 calibration levels for all analytes. Blank and zero samples (blank sample spiked with IS) were run in parallel. The calibration range was based on pre-validation studies on assay sensitivity and expected concentrations in real samples. The curves were prepared and run in quadruplicate over four-days period. For LLOQ determination, the acceptable accuracy of each standard was ±20% of nominal concentration; for other levels, accuracy within ±15% was considered sufficient. Minimum 75% of the samples at each calibration level had to meet the aforementioned criteria. For each analyte, curve fitting and weighting were assessed using the MS Workstation software. The linearity was monitored using coefficient of determination (R^2); value above 0.9900 was considered acceptable.

Accuracy and precision

Accuracy can be defined as the closeness of the measurement to the nominal value, whereas precision is the degree of agreement between a series of measurements (EMA, 2022). The quality control (QC) samples were prepared from a single source of blank matrix at two concentration levels: low (LQC) and high (HQC). Within-run accuracy and precision were determined by analyzing 5 replicates at both concentration levels in a single run; 15 replicates over three days were run and combined to assess between-run accuracy and precision. In both experiments, accuracy within ±15% of nominal concentration and precision (measured as CV) less or equal to 15% were considered acceptable.

In non-validation runs, usually consisting of 48 samples, two LQC and HQC samples were run. At least three had to be within ±15% of the nominal values for the run to be accepted.

Carry-over

Carry-over is a change of measured analyte concentration due to its residues from the previous injection still being present in the system. It was assessed by blank solvent analysis that directly followed injection of a sample at the highest calibration level. The maximum acceptable carry-over was 20% of LLOQ for the analytes and 5% of the response for the internal standards.

Dilution integrity

This experiment was performed in order to determine whether sample dilution affected the accuracy and precision of the results. The same urine lot as used for the accuracy and precision experiments was spiked with analytes at level eight times higher than the upper limit of quantification (ULOQ) and prepared normally in five replicates. Before instrumental analysis, the samples were diluted eightfold with blank solvent. Accuracy and precision were determined and acceptance criteria were the same as for LQC and HQC samples (see above).

Stability

In biomonitoring studies, the samples may undergo many processes before analysis, such as shipping, preparation, or short- and long-term storage (Ye et al., 2007). Therefore, analyte stability, understood as a lack of analyte degradation in a given matrix during defined storage conditions (EMA, 2022), is another important validation parameter (Kruve et al., 2015). Several stability studies were conducted. In all cases, the samples were run in triplicates. The accuracy needed to be within 15% of nominal concentration and precision (CV) less than or equal to 15% for the result to be accepted. Firstly, a 24-hour autosampler stability at room temperature was performed. The LQC and HQC urine samples prepared according to the final protocol (see section 3.2.2) were injected at t = 0 and t = 24 h. Secondly, LQC and HQC samples were used to determine a 30-day and 12-month storage stability at -20°C. Before the 12-month stability study was conducted, the stability of standard mixture used to prepare calibration curves at the same storage conditions was investigated.

Recovery

Recovery describes the extraction efficiency of an analytical process and is reported as a fraction of the known amount of analyte carried through the sample extraction and processing steps of the method (EMA, 2022). It was evaluated by comparing analyte peak area of three pre- and post-extraction spiked urine samples. The nominal concentrations were as follows: 20 pg/mL for FIP, FIP-desulfinyl, FIP-sulfide and FIP-sulfone, 100 pg/mL for FIP-amide, and 500 pg/mL for FIP-dtfms.

3.3 QUANTIFICATION OF FIPROLES IN SILICONE WRISTBANDS

3.3.1 Analytical standards

A list of FIP-related standards used in this study is provided below (Table 3.6). A table of other compounds included in the method development follows (Table 3.7).

Table 3.6 Standards of fiproles used in method development for silicone wristbands. NA, not assignable

Full compound name	Abbreviation	CAS number	Manufacturer	Catalog number
Analytes				
Fipronil	FIP	120068-37-3	Sigma-Aldrich	46451
Fipronil-amide	FIP-amide	205650-69-7	TRC Canada	D436240
Fipronil-desulfinyl	FIP-desulfinyl	205650-65-3	Sigma-Aldrich	41865
Fipronil-detrifluoromethylsulfinyl	FIP-dtfms	120068-79-3	TRC Canada	F342220
Fipronil-sulfide	FIP-sulfide	120067-83-6	Sigma-Aldrich	34520
Fipronil-sulfone	FIP-sulfone	120068-36-2	Sigma-Aldrich	32333
Internal standards				
Fipronil-$^{13}C_4$	FIP-$^{13}C_4$	NA	Sigma-Aldrich	79157
Fipronil-detrifluoromethylsulfinyl-$^{13}C_2{}^{15}N_2$	FIP-dtfms-$^{13}C_2{}^{15}N_2$	NA	TRC Canada	F342222
Fipronil-sulfone-$^{13}C_2{}^{15}N_2$	FIP-sulfone-$^{13}C_2{}^{15}N_2$	NA	TRC Canada	F342217

Table 3.7 Standards of other analytes and internal standards used during method development for silicone wristbands.

Full compound name	Group	Abbreviation	CAS number	Manufacturer	Catalog number	Parent compound(s)
Analytes						
Acetamiprid	Pesticides and related compounds	ACE	160430-64-8	Dr Ehrenstorfer	C10013000	
Atrazine	Pesticides and related compounds	ATZ	1912-24-9	abcr	AB171119	
Avobenzone	Pharmaceuticals and personal care products	AVO	70356-09-1	RTC	PHR1073	
Bisphenol S	Other	BPS	80-09-1	Sigma-Aldrich	43034	
Boscalid	Pesticides and related compounds	BOS	188425-85-6	Instytut Przemysłu Organicznego	IPO 924	
n-Butyl paraben	Pharmaceuticals and personal care products	nBuP	94-26-8	RTC	P500022	
Carbendazim	Pesticides and related compounds	CBDZ	10605-21-7	Instytut Przemysłu Organicznego	IPO 095	
Cortisol	Pharmaceuticals and personal care products	COR	50-23-7	Sigma-Aldrich	H4001	
Cotinine	Pharmaceuticals and personal care products	COT	486-56-6	Sigma	C5923	Nicotine
Cresyl diphenyl phosphate	Organophosphate flame retardants	CrDPhP	26444-49-5	Fluka	32957	
Crylenic acid	Pharmaceuticals and personal care products	CA	10380-41-3	TRC Canada	C979373	Octocrylene
Cypermethrin	Pesticides and related compounds	CYP	52315-07-8	Sigma-Aldrich	36128	
Deltamethrin	Pesticides and related compounds	DEL	52918-63-5	Rousel Uclaf	0B0188B3	
cis-(2,2-Dibromovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid	Pesticides and related compounds	DBCA	53179-78-5	Rousel Uclaf	RU23441	Deltamethrin
(1R,3R)-3-(2,2-Dichloroethenyl)-2,2-dimethylcyclopropane-1-carboxylic acid	Pesticides and related compounds	*cis*-DCCA	59042-49-8	TRC Canada	P287700	Permethrin, cypermethrin
(1S,3R)-3-(2,2-Dichloroethenyl)-2,2-dimethylcyclopropane-1-carboxylic acid	Pesticides and related compounds	*trans*-DCCA	59042-50-1	TRC Canada	P287705	Permethrin, cypermethrin
N,N-Diethyl-*meta*-toluamide	Pesticides and related compounds	DEET	134-62-3	Instytut Przemysłu Organicznego	IPO 922	
Diphenyl phosphate	Organophosphate flame retardants	DPhP	838-85-7	Aldrich	850608	Triphenyl phosphate, other

Full compound name	Group	Abbreviation	CAS number	Manufacturer	Catalog number	Parent compound(s)
Ethyl paraben	Pharmaceuticals and personal care products	EtP	120-47-8	Fluka	PHR1011	
2-Ethylhexyl diphenyl phosphate	Organophosphate flame retardants	EHDPhP	1241-94-7	Fluka	34064	
Imazalil	Pesticides and related compounds	IMZ	35554-44-0	Sigma-Aldrich	32007	
Imidacloprid	Pesticides and related compounds	IMI	138261-41-3	Instytut Przemysłu Organicznego	IPO 297	
Isobutyl paraben	Pharmaceuticals and personal care products	iBuP	4247-02-3	Aldrich	715077	
Methyl paraben	Pharmaceuticals and personal care products	MeP	99-76-3	Supelco	47889	
Nicotine	Pharmaceuticals and personal care products	NIC	54-11-5	Aldrich	18,637-6	
Octocrylene	Pharmaceuticals and personal care products	OC	6197-30-4	Sigma-Aldrich	PHR1083	
Oxybenzone	Pharmaceuticals and personal care products	BP-3	131-57-7	Fluka	59647	
cis-Permethrin	Pesticides and related compounds	*cis*-PER	52645-53-1[1]	Sigma-Aldrich	45614	
trans-Permethrin	Pesticides and related compounds	*trans*-PER	52645-53-1[1]	Sigma-Aldrich	45614	
Piperonyl butoxide	Pesticides and related compounds	PBO	51-03-6	Instytut Przemysłu Organicznego	IPO 571	
Pirimicarb	Pesticides and related compounds	PIR	23103-98-2	Dr Ehrenstorfer	C16250000	
Propyl paraben	Pharmaceuticals and personal care products	PrP	94-13-3	Sigma-Aldrich	P53357	
Tebuconazole	Pesticides and related compounds	TEB	107534-96-3	Supelco	32013	
Tri-n-butyl phosphate	Organophosphate flame retardants	TnBP	126-73-8	Aldrich	240494	
Triclocarban	Pharmaceuticals and personal care products	TCC	101-20-2	Sigma-Aldrich	05666	
Triethyl phosphate	Organophosphate flame retardants	TEP	78-40-0	Aldrich	538728	
Triphenyl phosphate	Organophosphate flame retardants	TPhP	115-86-6	Aldrich	241288	
Tris(2-butoxyethyl) phosphate	Organophosphate flame retardants	TBOEP	78-51-3	Aldrich	130591	
Tris(2-chloroethyl) phosphate	Organophosphate flame retardants	TCEP	115-96-8	Aldrich	119660	
Tris(1-chloro-2-propyl) phosphate	Organophosphate flame retardants	TCPP	13674-84-5	Fluka	32952	
Tris(1,3-dichloro-2-propyl) phosphate	Organophosphate flame retardants	TDCPP	13674-87-8	Fluka	32951	

Full compound name	Group	Abbreviation	CAS number	Manufacturer	Catalog number	Parent compound(s)
Internal standards						
Bisphenol S-D_8	Other	BPS-D_8	NA	TRC Canada	B447392	
n-Butyl paraben-D_9	Pharmaceuticals and personal care products	nBuP-D_9	121904-65-2	TRC Canada	B693602	
Cotinine-D_3	Pharmaceuticals and personal care products	COT-D_3	110952-70-0	Aldrich	610577	
Diphenyl phosphate-D_{10}	Organophosphate flame retardants	DPhP-D_{10}	1477494-97-5	TRC Canada	D492002	
Imidacloprid-D_4	Pesticides and related compounds	IMI-D_4	1015855-75-0	TRC Canada	I274992	
Methyl paraben-D_4	Pharmaceuticals and personal care products	MeP-D_4	362049-51-2	TRC Canada	M325663	
Nicotine-D_4	Pharmaceuticals and personal care products	NIC-D_4	350818-69-8	Sigma-Aldrich	N-048	
Octocrylene-D_{15}	Pharmaceuticals and personal care products	OC-D_{15}	NA	Sigma-Aldrich	00609	
Oxybenzone-D_5	Pharmaceuticals and personal care products	BP-3-D_5	1219798-54-5	Fluka	73875	
cis-Permethrin-D_5	Pesticides and related compounds	*cis*-PER-D_5	110952-70-0[1]	Sigma-Aldrich	74567	
trans-Permethrin-D_5	Pesticides and related compounds	*trans*-PER-D_5	110952-70-0[1]	Sigma-Aldrich	74567	
Propyl paraben-D_7	Pharmaceuticals and personal care products	PrP-D_7	1246820-92-7	TRC Canada	P838287	
Triphenyl phosphate-$^{13}C_6$	Organophosphate flame retardants	TPhP-$^{13}C_6$	NA	TRC Canada	T808993	

[1] Refers to a mixture of *cis*- and *trans*- isomers.

3.3.2 Method development

Pre-deployment cleanup

As mentioned earlier (see section 1.3.2), the impurities present in raw SWBs material warrant their cleaning before use (Anderson et al., 2017; O'Connell et al., 2014; Wacławik et al., 2025). SWBs were cleaned prior to deployment according to the protocol described in Wacławik et al., 2025. Briefly, up to 10 SWBs (weighing approximately 5 g each) were put in a glass jar containing 500 mL of ethyl acetate:n-hexane mixture (1:1, *v*/*v*) and mixed on a mechanical shaker for 30 minutes at 1200 rpm. This step was performed three times; each wash was performed with a fresh solvent. As a second step, the SWBs were washed twice with 500 mL of ethyl acetate:methanol mixture (1:1, *v*/*v*). The mixing conditions were the same. Again, for each wash, a fresh batch of solvent was used. Finally, the SWBs were transferred onto aluminum foil, air-dried overnight under a fume hood and stored at -20°C in individual self-sealing polyethylene bags before use.

Final protocol

Similarly to the urine method, all reusable glassware was thoroughly washed using ultrasonic bath, laboratory detergent, organic solvents and muffle furnace (for details, see section 3.2.2). Single-use glassware was baked in the furnace as well.

Following deployment, the entire pre-cleaned SWBs (see previous section) were cut into small pieces using disposable equipment. Then, 0.5 ± 0.025 g samples were accurately weighed in glass screw cap tubes, spiked with internal standard solution and subjected to ultrasound-assisted extraction with two 2.5 mL volumes of ethyl acetate, as described in Wacławik et al., 2025. Each extraction lasted 15 minutes. Extracts were combined in rimless tubes and evaporated under nitrogen stream at 40°C. As a result of extensive optimization (see next sections), the following sample cleanup protocol was developed. The dry residue was redissolved in 0.5 mL of acetonitrile, transferred to 1.5 mL eppendorf tubes and put in a freezer (-20°C) overnight. After freezing-out, the eppendorf tubes were centrifuged (15,000 rpm, 4 min, -10°C), and 0.3 mL of supernatant was transferred to a new glass screw cap tube. Then, 0.7 mL of acetonitrile was added to the samples followed by 1 mL of acetonitrile-saturated hexane. The samples were shaken for 5 minutes at 1500 rpm and centrifuged at 4000 rpm for 2 minutes. After the hexane layer was discarded, the extraction was performed again with fresh solvent. Following the removal of the second portion of acetonitrile-saturated hexane, the acetonitrile layer was transferred to another rimless tube and evaporated to dryness at 40°C using nitrogen stream. The dry residue was redissolved in 80 µL of methanol and vortexed for a few seconds at 2000 rpm. Then, 20 µL of water was added and the tubes were vortexed again. After centrifugation at 1500 rpm for 2 minutes, the entire extract was transferred to centrifugal filter and centrifuged for 3 minutes at 14000 *g*. Finally, the filtrates were transferred to amber glass vials containing glass microinserts and injected into the LC-MS/MS system. The outline of the final procedure is provided below.

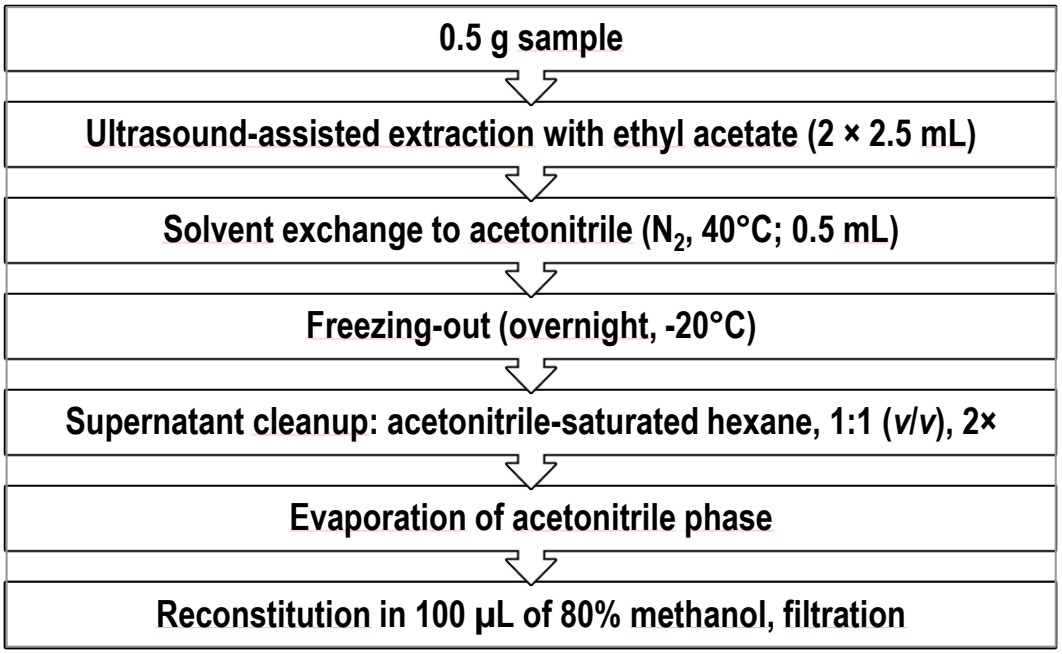

Figure 3.2 Workflow of final sample preparation procedure for silicone wristbands.

Sample cleanup optimization

The sample injected into the analytical instrument should contain as few interferences as possible and be compatible with the intended analytical method (Majors, 2013). At the same time, there is a clear trend towards development of methods capable of quantifying multiple analytes within a single run (Mol et al., 2008). This tendency is particularly relevant in exposure assessment, where exposures to many chemicals occur simultaneously (Rappaport, 2011). Therefore, a considerable effort was made to develop a versatile method of SWBs sample preparation suitable for an LC-MS/MS instrument.

The crude ethyl acetate extract from deployed wristbands was obtained as written in the final protocol, following the procedure reported in Wacławik et al., 2025. Then, several matrix removal possibilities were tested, all preceded by solvent exchange to 1 mL acetonitrile, overnight freezing-out at -20°C and transfer of supernatant (0.4 mL) for further cleanup: a dispersive solid phase extraction (dSPE) using 20 or 60 mg of sorbent (either C18, C30, PSA, Z-sep, Z-sep+, or EMR-Lipid), and, alternatively, liquid-liquid extraction with acetonitrile-saturated hexane. The latter procedure is also known as acetonitrile-hexane partitioning (García-Reyes et al., 2007). In dSPE, the acetonitrile supernatant was vortexed with 20 or 60 mg of sorbent in eppendorf tubes for three minutes at 1500 rpm. In the case of EMR-Lipid, 100 µl of water was added to acetonitrile phase before dSPE, as recommended by the manufacturer (Agilent, 2019). Then, after centrifugation for 2 minutes at 15,000 rpm, half of supernatant volume was transferred to glass tubes and evaporated under nitrogen stream. In liquid-liquid extraction, 0.6 mL of acetonitrile was added to the supernatant before addition of 1 mL of acetonitrile-saturated hexane. Then the samples were shaken for 5 minutes at 1500 rpm and centrifuged at 4000 rpm for 2 minutes. After the hexane layer was discarded, the extraction was performed again with fresh solvent. 0.5 mL of the acetonitrile phase was transferred to rimless glass tubes and evaporated under nitrogen stream. In all cases, the dry residue was

redissolved in 80 µL of methanol and vortexed for a few seconds at 2000 rpm. Then, 20 µL of water was added and the tubes were vortexed again. After centrifugation at 1500 rpm for 2 minutes, the entire extract was transferred to centrifugal filter and centrifuged for 3 minutes at 14000 *g*. Finally, the filtrate was transferred to amber glass vials containing glass microinserts and injected into the LC-MS/MS system. In total, 13 approaches were taken into consideration. For each option, six samples were prepared: one reagent blank, one deployed wristband blank, two samples spiked pre-extraction, and two replicates spiked post-extraction. Nominal concentration was 1 ng/g wristband for FIP, FIP-desulfinyl, FIP-sulfide and FIP-sulfone, and 5 ng/g for FIP-amide and FIP-dtfms. In the case of samples fortified pre-extraction, the raw ethyl acetate extract was spiked, not the wristband itself. In this experiment, the extraction efficiency and its repeatability were the main considered factors.

For the most promising approaches, an additional experiment, focused on cleanup efficiency, was performed. The matrix removal efficiency investigation was carried out for the following cleanup procedures: freezing-out prior to dSPE with 60 mg of either C18, C30, or EMR-lipid sorbent, and freezing-out followed by LLE with acetonitrile-saturated hexane. 20 µL of final extracts of reagent blank and deployed wristband blank from the previous experiment was subjected to nitrogen blowdown evaporation and redissolved in 100 µL of ethyl acetate. Additionally, “no cleanup” and “freezing-out only” deployed wristband blank samples were prepared as follows. The raw ethyl acetate extract from deployed wristbands was evaporated and reconstituted in 0.5 mL of acetonitrile. For the “no cleanup” sample, the overnight freezing-out step was omitted. After centrifugation for 2 minutes at 15,000 rpm, 0.2 mL of acetonitrile extracts was transferred to glass tubes, evaporated again and dissolved in methanol and water, as described in the previous experiment. Finally, 20 µL of the final extract was solvent-exchanged to 100 µL of ethyl acetate. All samples prepared for this experiment were injected into a GC-MS system (see section 3.3.3). The total ion current (TIC) chromatograms were obtained and integrated to calculate the matrix removal for each procedure according to the following formula (Agilent, 2019):

$$Matrix\ removal\ (\%) = \left(\frac{Peak\ area_{no\ cleanup} - Peak\ area_{deployed\ wristband\ blank}}{Peak\ area_{no\ cleanup} - Peak\ area_{reagent\ blank}}\right) \times 100$$

Post-deployment rinse experiment

In many papers, the particles present on the surface of SWBs after deployment (debris, sebum, etc.) is removed by soaking the entire SWB in solvents, so that only the analytes sequestrated by the SWB material are analyzed (Wacławik et al., 2022). Due to a wide array of chemicals included in the developed method, a decision whether to include a water rinse at the beginning of sample preparation required a separate investigation on potential losses of analytes during that process.

Six precleaned pieces of unused SWB (0.5 g each) were directly spiked with 2 ng of FIP, FIP-sulfide, and FIP-sulfone, 10 ng of FIP-desulfinyl, 40 ng of FIP-amide, and 80 ng of FIP-dtfms in acetonitrile using a piston pipette and left under a fume hood overnight for the solvent to evaporate completely. Then, three of the pieces were subjected to twofold vortexing with 1 mL of water for a few seconds; for the other three, this step was omitted. Thereafter, all six of the samples were prepared following the final protocol (see

above). As a reference, additional three samples of unused SWBs were prepared and spiked after the extraction.

Variability assessment

As stated in the final protocol (see earlier), only after the fragmentation of entire wristband a 0.5 g aliquot was weighed and subjected to extraction. However, in some papers (Hammel et al., 2020; Levasseur et al., 2021) a piece of the whole wristband was used for analysis without prior cutting into small fragments. Therefore, a silent assumption was made that the analytes are uniformly distributed throughout the entire wristband. The validity of such approach has been tested in this experiment.

Three separate, equidistant 0.5 g pieces (Figure 3.3) were cut from 19 wristbands that were previously deployed for 7 days. Each wristband was worn by a different person. Then, all 57 pieces were prepared for LC-MS/MS analysis using the procedure described in the “Final protocol” section. To investigate variability of the results, CVs were calculated for each triplet.

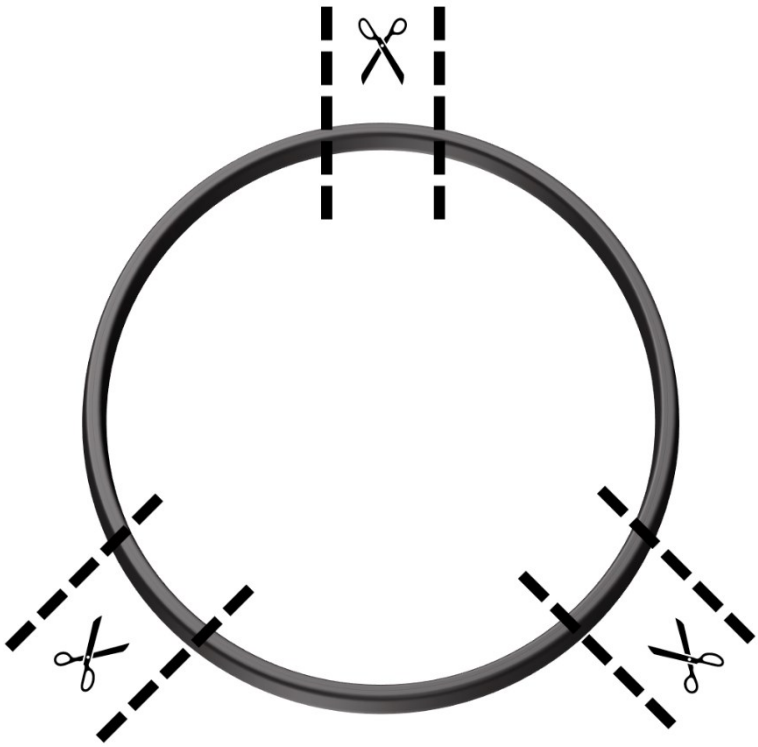

Figure 3.3 Sampling method for homogeneity test of silicone wristbands.

Fipronil degradation during deployment – simulation study

FIP is known to degrade in the environment (Simon-Delso et al., 2015). For instance, exposure to sunlight, both in field and laboratory setting, leads to the formation of FIP-desulfinyl (major product), as well as FIP-sulfone, FIP-sulfide, and FIP-dtfms (minor products) (Hainzl & Casida, 1996). Therefore, it was relevant to investigate the fate of FIP sampled by an SWB during simulated 7-day deployment.

Nine 0.5 g pieces of SWB were spiked with 1 µL of fipronil stock solution (1 mg/mL in acetonitrile) and left to dry under the fume hood. Then, the pieces of wristband were put on a roof of the Faculty of Pharmacy building, exposed to the sunlight, along with three field blanks, which were unspiked pieces of SWB, precleaned in the same batch as the spiked ones. Three spiked samples, along with one field blank, were removed immediately (t = 0 h). Another three spiked samples along with a single blank were removed after 24 h, and the remaining were collected after 168 h. The field experiment was conducted August 10th to 17th, 2022. Weather conditions throughout the study were comparable to previous years (Table 3.8). All samples were stored at -20°C until analysis. FIP and other fiproles were quantitated according to the final protocol (section 3.3.2).

Table 3.8 Selected weather conditions during simulation study and comparison with previous years.

	August 2018	August 2019	August 2020	Study period
Average temperature (°C)	20.2	19.9	20	21.2
Average irradiation (W/m^2/day)	5442	5126	5387	5589

August 2018-2020: Direct Normal Irradiation, PVGIS-5 geo-temporal irradiation database, 54.382°N, 18.624°E, European Commission, https://re.jrc.ec.europa.eu/pvg_tools/en/

Study period: Agency of Regional Air Quality Monitoring Foundation (personal communication)

3.3.3 Gas chromatography-mass spectrometry conditions

The conditions for gas chromatography-mass spectrometry analyses are provided below (Table 3.9). Electron ionization (EI) was used to produce ionic species.

Table 3.9 Gas chromatography-mass spectrometry conditions used for the analysis of silicone wristbands.

System part	Parameter name	Parameter value	
Autosampler	Injection volume (µL)	1	
	Needle wash solvent	Ethyl acetate	
Injector	Injection mode	Splitless	
	Temperature (°C)	290	
Column compartment	Temperature program	Time (min:sec)	Temperature (°C)
		0:00	60
		1:00	60
		25:00	300
		41:00	300
	Column	Phenomenex Zebron ZB-5MS plus, 30 m × 0.25 mm ID × 0.25 µm film thickness + 10 m guard column	
	Carrier gas	Helium	
	Carrier gas flow (mL/min)	1	
Mass spectrometer	Mass analyzer type	Ion trap	

System part	Parameter name	Parameter value
	Scan range (m/z)	85-650
	Scan time (s/scan)	0.6
	Electron multiplier voltage (V)	1500
	Multiplier offset (V)	0
	Emission current (µA)	40
	Data acquisition segment (min)	4.3-41.0

3.3.4 Liquid chromatography conditions

The liquid chromatography settings used for SWBs analysis are provided below (Table 3.10).

Table 3.10 Settings for liquid chromatography separation used during analysis of silicone wristbands.

Liquid chromatograph part	Parameter name	Parameter value	
Pumping system	Mobile phase A composition	0.5 mM ammonium formate buffer pH 3 in water:methanol 9:1 (*v*/*v*)	
	Mobile phase B composition	0.5 mM ammonium formate buffer pH 3 in methanol	
	Flow rate (mL/min)	0.4	
	Mixer volume (µL)	150	
	Gradient program	Time (min:sec) 0:00 12:00 20:00 20:01 23:00	%B 5 100 100 5 5
Autosampler	Temperature	Ambient	
	Injection volume (µL)	10	
	Needle wash solvent composition	methanol:acetonitrile:isopropanol:acetone 1:1:1:1 (*v*/*v*/*v*/*v*)	
Column compartment	Temperature (°C)	40	
	Column	ACE Excel 3 SuperC18, 75×3.0 mm	

3.3.5 Mass spectrometry conditions

The general mass spectrometry conditions were the same as in urine analysis (see section 3.2.4, Table 3.4). Analyte-specific settings are provided below (Table 3.11).

Table 3.11 Compound-specific parameters of fiproles quantitation in silicone wristbands using LC-MS/MS.

Full compound name	Abbreviation	Retention time (min)	Precursor ion	Precursor m/z	Capillary voltage (V)	Product ions m/z[1]	Collision energy (V)[2]
Analytes							
Fipronil	FIP	10.96	$[M-H]^-$	435.0	-70	329.7, 249.6, 277.6	15, 26, 27
Fipronil-amide	FIP-amide	9.27	$[M-H]^-$	453.0	-70	347.8, 271.9, 303.8	15, 41, 25
Fipronil-desulfinyl	FIP-desulfinyl	10.76	$[M-H]^-$	387.0	-50	350.9, 281.9, 330.8	12, 30, 28
Fipronil-detrifluoromethylsulfinyl	FIP-dtfms	9.12	$[M-H]^-$	319.0	-70	282.9, 262.8	8, 20
Fipronil-sulfide	FIP-sulfide	11.11	$[M-H]^-$	419.0	-70	261.8, 313.9, 382.9	26, 18, 11
Fipronil-sulfone	FIP-sulfone	11.32	$[M-H]^-$	451.0	-70	281.9, 243.8, 414.9	25, 44, 17
Internal standards							
Fipronil-$^{13}C_4$	FIP-$^{13}C_4$	10.97	$[M-H]^-$	441.0	-70	336.0, 324.0	15, 24
Fipronil-detrifluoromethylsulfinyl-$^{13}C_2{}^{15}N_2$	FIP-dtfms-$^{13}C_2{}^{15}N_2$	9.13	$[M-H]^-$	323.0	-70	287.0, 185.0	8, 28
Fipronil-sulfone-$^{13}C_2{}^{15}N_2$	FIP-sulfone-$^{13}C_2{}^{15}N_2$	11.32	$[M-H]^-$	457.0 455.0	-76	287.6 249.6[3]	27.0 39.5

[1] First ion is the quantifier, the other are the qualifiers (in increasing m/z order).

[2] For product ions, respectively.

[3] For the first and second precursor ion, respectively.

3.3.6 Method validation

The method developed for SWBs analysis was validated basing on M10 ICH guideline on bioanalytical method validation and study sample analysis (EMA, 2022), unless stated otherwise.

Selectivity

Selectivity is the ability of the method to distinguish the analyte from other constituents of the sample (EMA, 2022). To assess it, several blank samples from independent sources were prepared in accordance to the final protocol and injected into the LC-MS/MS system. Analytical responses corresponding to no more than 20% of LLOQ response for analytes and at or below 5% of the IS response were considered acceptable.

Internal standard selection and matrix effect

Similarly to the method developed for urine, this assay also relied on mass spectrometric detection. Therefore, the matrix effect had to be assessed, preferably over a wide range of analyte concentrations. The relative matrix effect was investigated according to the methodology described in Matuszewski et al., 2003. Five calibration curves were prepared in deployed wristbands and one in solvent. The samples were spiked with analytes and internal standards at the end of sample preparation. Curve concentration range (ng/g wristband) was 1-50 for FIP, FIP-sulfide, and FIP-sulfone, 5-250 for FIP-desulfinyl, 20-1000 for FIP-amide, and 40-2000 for FIP-dtfms. To quantitate the relative matrix effect, calibration curves were constructed in each deployed wristband lot for every analyte using all three FIP-related internal standards. Then, the %CV was used to

measure the scatter of slopes for all obtained calibration curves. Among the internal standards tested, the one that that provided the least variable slopes of calibration curves in different matrix lots was chosen for a given analyte.

Linearity and lower limit of quantification

In this experiment, the method sensitivity (the lowest analyte concentration measured with predetermined accuracy and precision) (EMA, 2023) and linearity (the ability to generate a straight line relationship between the analyte response and its concentration) (Araujo, 2009) were investigated. The calibration curves consisted of ten levels prepared by spiking blank, fragmented 0.5 g SWB aliquots directly before extraction. For LLOQ, the accuracy of the calibration standards had to fall within ±20% of nominal value. Additionally, the precision (expressed as %CV) was required not to exceed 20%. Three calibration curves over three independent runs were prepared and analyzed. For each analyte, the curve fit and weighting were assessed using the MS Workstation software. The linearity was monitored using R^2; value above 0.9900 was considered sufficient.

Accuracy and precision

Accuracy was defined as the degree of closeness of the measured concentration to the nominal value, whereas precision was used to measure the variability within a series of measurements (EMA, 2022). The QC samples (LQC and HQC) were prepared by spiking the pre-cleaned, cut and weighed wristband samples (0.5 ± 0.025 g) right before extraction with ethyl acetate. Within-run accuracy and precision was assessed by analyzing 6 samples at both concentration levels in a single run. To determine between-run parameters, 18 replicates were prepared and analyzed over three separate runs. Accuracy within ±15% of nominal concentration and precision (measured as CV) less or equal to 15% were considered acceptable.

Non-validation runs typically consisted of 48 samples. In such runs, two LQC and two HQC samples were included. For at least three of them, accuracy within 85-115% had to be achieved for the run to be considered valid.

Carry-over

Carry-over is the presence of analyte signal from a preceding analysis (EMA, 2023). It was determined by analyzing blank sample after the calibration standard at ULOQ level. Maximum acceptable carry-over was 20% of LLOQ for the analytes and 5% of the response for the internal standards.

Dilution integrity

Dilution integrity is an assessment of sample dilution procedure conducted in order to confirm that the dilution step does not affect the assay performance (EMA, 2023). Five pre-cleaned, cut and weighed wristband samples were spiked with FIPs levels corresponding to thirty times the ULOQ and prepared according to the final protocol. Before instrumental analysis, the samples were diluted thirtyfold with blank solvent. Accuracy and precision were determined and acceptance criteria were the same as for LQC and HQC samples (see above).

Stability

Stability is a measure of analyte's intactness in a defined matrix under specific storage conditions for a given period of time (EMA, 2023). In the case of SWBs, only a 24 h autosampler stability study at the room temperature was performed. Six replicates per QC level were prepared and injected into the LC-MS/MS system at t = 0 and t = 24 h. The requirements for accuracy and precision were the same as above.

Recovery

Recovery is an extraction efficiency metric of an analytical process (EMA, 2023). Five precleaned pieces of unused SWB (0.5 ± 0.025 g each) were directly spiked with 2 ng of FIP, FIP-sulfide, and FIP-sulfone, 10 ng of FIP-desulfinyl, 40 ng of FIP-amide, and 80 ng of FIP-dtfms in acetonitrile and left under a fume hood overnight for the solvent to dry. Then the samples were prepared according to the final protocol along with five samples that were spiked at the same nominal level, but at the end of sample preparation process. Recovery was calculated as a ratio of the averaged analytical signal from the samples spiked before extraction to the mean analytical signal of samples fortified at the end of the procedure; the results were reported as a percentage.

3.4 HUMAN EXPOSURE STUDY

3.4.1 Enrollment of participants

A convenience sample of 15 volunteers living in 6 households, each owning at least one cat or dog, was enrolled in 2020-2021. All participants resided in Gdańsk Metropolitan Area during the entire study period and reported no use of FIP on their pets within a year before enrollment. After the study protocols and goals were explained, written consent was obtained from the participants and materials necessary for the experiment were provided during home visits. The study obtained approval of the Medical University of Gdańsk Bioethics Committee for Scientific Research (Resolution No. NKBBN/535/2020).

3.4.2 Study outline

The study had a longitudinal design and, due to the intervention present in the study, it consisted of two main parts: *before* application of ectoparasiticide on a pet (week 0, W0) and *after* (week 1-4; W1-4) (Figure 3.4). At the beginning of W0, the participants filled a questionnaire regarding the key sociodemographic and lifestyle factors (including hitherto pesticide use and pet treatments), as well as household characteristics and relevant pet features, such as body weight and fur length. During the entire W0, the participants collected three spot urine samples at the time of their choice and wore a precleaned SWB on a dominant hand. Study subjects were asked to wear the SWB continuously, including bathing and sleeping. Additionally, a precleaned stationary wristband in a steel cage was hanged in the most frequently used room of the household at a height of approximately 200 cm above floor level, out of reach of pets. After collection, urine samples were weighted, immediately transferred to two high-density polyethylene scintillation vials and frozen. Similarly, after 7 days of deployment, both individual and stationary wristbands were put back by the participants into zip lock bags and stored at -20°C. Date and time of biological and environmental samples collection was recorded in a provided log book. Week 1 (W1) of the study began with ectoparasiticide application

on the pet(s) (Figure 3.4), which was performed by the participants themselves within four weeks since W0. Before the application, a researcher provided the participants with a suitable ectoparasiticide product, encouraged the participants to read the instructions, and answered the questions, if any. In all cases, the medication contained FIP in a form of spot-on solution (Frontline Combo and Frontline Tri-Act for cats and dogs, respectively; Merial, Lyon, France), except for household #6, where a Frexin pet collar was deployed (LAB, Jaworzno, Poland). Since the collar did not contain FIP, this household acted as a negative control. In most cases, the applied product also contained permethrin, a pyrethroid insecticide. On the first day of W1, the participants were asked to apply the product on their pet(s) in the morning and collect all urine samples that day. Also, right after application (and washing hands, in case of the person applying the product), the participants wore a new precleaned SWB and hanged another one indoors at the same location as in W0. On the following 6 days, they were expected to collect one spot urine sample per day. The SWBs were deployed continuously for 7 days. Two (W2) and four weeks (W4) after application, the study subjects collected a single spot urine sample. Similarly to W0, all biological and environmental samples collected on weeks 1-4 were stored at -20°C until collected by a researcher, who transported it under wet ice to the laboratory freezer set at -20°C as well.

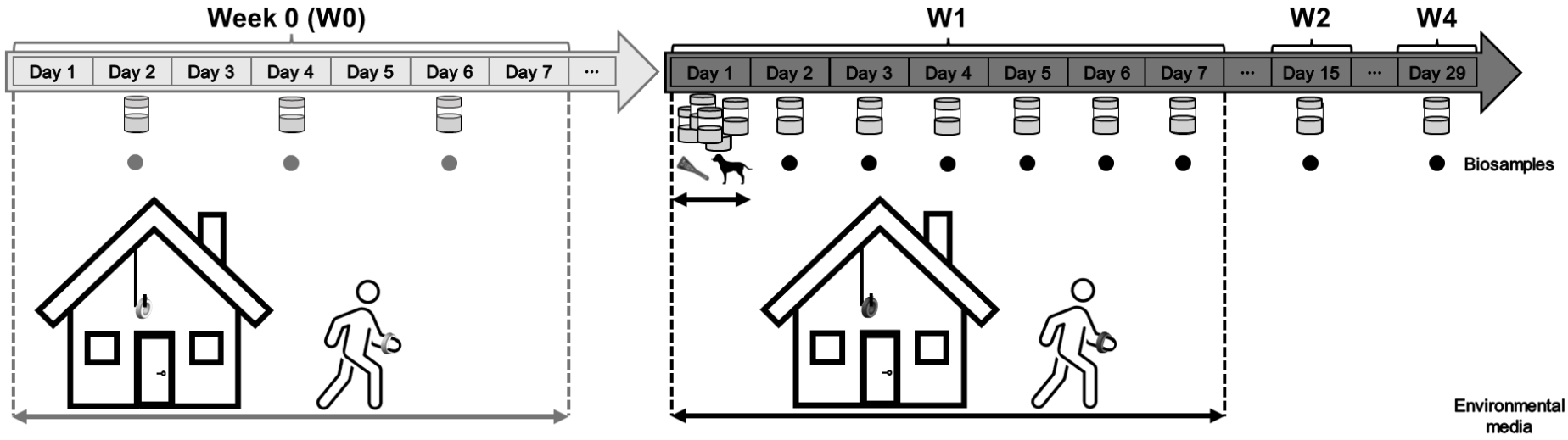

Figure 3.4 Design of experiment on human exposure to fipronil associated with use of ectoparasiticides on household pets.

3.4.3 Laboratory analysis

Urine samples and SWBs were analyzed according to the final protocols described in detail in sections 3.2.2 and 3.3.2, respectively. All devices, materials and reagents used for this study are listed in section 3.1. Every analytical run consisted of a reagent blank, blank sample, zero sample, at least two LQC and HQC samples (at the same levels as in validation experiments), and up to 41 real samples. Accuracy and precision of QC samples were closely monitored to ensure validity of the results. Reagent blanks and zero samples were included in every run to prevent false positives.

3.4.4 Data handling and statistical analysis

The software used for data handling and statistical treatment of the results is listed in section 3.1.3.

To take into account the variability of urine dilution (Panuwet et al., 2016), SG was measured in all collected samples using a handheld refractometer. SG-adjusted urinary concentration was calculated using the Levine-Fahy equation (see section 1.3.1). The average SG for studied population was 1.018.

Descriptive statistics for urinary concentrations were calculated using both SG-corrected and uncorrected values. Detection rates and selected quantiles were calculated. For analytes detected in ≥50% of the samples, the arithmetic and geometric mean, as well as standard deviation were also calculated, preceded by imputation of the LLOQ/√2 value for the results below LLOQ (Hornung & Reed, 1990). Due to the intervention present in the study, these statistics were calculated separately for samples collected before and after ectoparasiticide application.

Before further statistical analysis, a different approach was applied for the treatment of results below LLOQ. Although limit of detection (LOD) was not determined in a validation study, it can be estimated using the following equation (Hecht et al., 2018):

$$LOD = 3.3 \times \frac{SD(LLOQ)}{S}$$

where LOD denotes limit of detection, SD(LLOQ) is the standard deviation of analyte response ratio to an internal standard at LLOQ level, and S corresponds to the slope of the line between the mean analyte response ratio to an internal standard at LLOQ level and the origin (0,0). After calculation of LODs for all analytes, the results between LOD and LLOQ were used as reported, and for analytes detected in ≥50% of the samples, values below LOD were replaced by LOD/√2. Then, mass concentrations were converted to molar equivalents and summed to produce total FIPs. In the case of comparisons before and after the application, medians of results from W0 and W1 were used.

Correlations between variables within the same study period were computed using Spearman's rang correlation coefficient (r_s); in that case, the non-detects were omitted. r_s value of 0.20-0.39 denoted a weak association; values falling within range of 0.40-0.59, 0.60-0.79, and 0.80-1.00 were considered a moderate, strong, and very strong correlation, respectively (Dixon et al., 2018). In selected cases, coefficient of determination and linear regression equation were also provided.

Generalized estimating equations (GEE) were used for statistical investigation of temporal changes in FIPs levels (Ballinger, 2004). Developed by K. Y. Liang and Zeger, 1986, GEE are suitable for analysis of clustered, longitudinal data where responses are not normally distributed. In the case of this study, several samples were collected from each participant (Figure 3.4), so the results were not considered independent. Additionally, some level of correlation can be expected within each household (same dose, same animal, same environment). Therefore, this approach offers advantages over classical hypothesis testing methods, such as paired *t*-test (Burton et al., 1998). Each of the household was considered a separate "cluster", and the fact that repeated measurements were taken within subjects was included into the model as well. Wald test was used to investigate statistical significance of observed trends. Statistical analysis based on GEE was performed using the *geepack* package (Halekoh et al., 2006) of R Statistical Software (R Core Team, 2024).

In the case of SWBs, the exact weights of samples were recorded and the results were normalized to 1 g of sample after analysis. Similarly to the urine samples, summary statistics were computed (in ng/g wristband) using LLOQ/√2, if needed. For further statistical analysis, imputation was not necessary. Again, for assessment of ectoparasiticide impact on FIPs levels, mass concentrations were converted to molar equivalents and summed to produce total FIPs. Temporal changes in FIPs levels, as well as correlations within the same study period (before or after application) were investigated in the same fashion as for urinary concentrations.

3.4.5 Dose reconstruction and risk assessment

Since the highest human exposure to FIP was expected shortly after the ectoparasiticide application (EMA & CVMP, 2018), the participants were asked to use the product in the morning and collect all urine samples that day (see section 3.4.2). Basing on the biomarker levels in these samples, an estimate of the ingested FIP dose can be made and used in human risk assessment. However, since no FIP toxicokinetic data is available for humans, dose reconstruction is a difficult task. To overcome the data limitations and explore a wider range of possibilities, two scenarios were used to assess FIP exposure on the day of ectoparasiticide application.

In the first scenario ("FIPs-based"), a simple daily intake (DI) estimation is used for dose reconstruction (Figure 3.5). It is a stochastic approach where continuous exposure and steady-state conditions are assumed. The latter means that the intake and elimination rates of the chemical are considered equal (Angerer et al., 2011; Gurusankar et al., 2017). Consequently, basing on urine volume adjustment, the exposure can be calculated using a simple equation:

$$DI_V = \frac{C_{volumetric} \times V_{24h} \times M_p}{bw \times FUE}$$

where DI_V denotes DI estimate using urine volume (µg/kg bw/day), $C_{volumetric}$ is the concentration of a chemical (µmoles/L), V_{24h} corresponds to estimated 24h urine volume, M_p denotes molecular weight of parent compound, whereas FUE is the fractional urinary excretion (Gurusankar et al., 2017). Here, the product of the concentration of the chemical and the estimated 24h urine volume was replaced by molar sum of FIP and FIP-sulfone in individual urine samples collected on the day of application. FIP-sulfone was

included as it is the main FIP residue and its toxicity is similar to the parent compound (EFSA, 2006). These two compounds were also included in FIP dose estimation performed by the German Federal Institute for Risk Assessment while estimating consumer exposure to FIP associated with the "fipronil incident" (Bundesinstitut für Risikobewertung, 2017). The FUE is a proportion of the administered dose excreted with urine (Gurusankar et al., 2017). It is the only chemical-specific pharmacokinetic parameter used in this model (Angerer et al., 2011). FUE values are usually determined during controlled human dosing experiment (Gurusankar et al., 2017) and they may vary greatly for different pairs of chemicals and their respective biomarkers. For instance, an average FUE for monoisononyl phthalate, an urinary biomarker of exposure to diisononyl phthalate, is as low as 0.02 (Koch & Angerer, 2007). In contrast, the FUE for bisphenol A (both parent compound and urinary biomarker) is equal to 1 (Krishnan et al., 2010). Inter-individual variability may be observed as well (Sandborgh-Englund et al., 2006). To the author's knowledge, no human dosing experiment study has been performed so far for FIP. Consequently, animal data needed to be used as an estimate. Several studies on FIP pharmacokinetics in rats using radiolabeled compound have been conducted. Although none of them are published, they are summarized in reviews carried out by government agencies, such as the Australian Pesticides and Veterinary Medicines Authority (APVMA) (APVMA, 2009) or international bodies, like FAO/WHO (FAO/WHO, 2002, 2022). In the studies described in these reviews, the FUE of FIP measured as radioactivity ranged from 0.0085 to 0.293. The former value was used to calculate the ingested dose for two reasons. Firstly, as mentioned earlier, more extensive FIP metabolism was observed in rats compared to other laboratory animals (FAO/WHO, 2022). Secondly, the radioactivity measurement used in the aforementioned studies encompasses all excreted metabolites, so the urinary excretion is probably overestimated in comparison to the present study, where only FIP and FIP-sulfone are taken into account as urinary biomarkers of FIP exposure.

The second scenario, based on pyrethroid metabolites (PYRs), relies on the fact that the spot-on products applied on the pets often contained not only FIP, but also permethrin (section 3.4.2) (Figure 3.5). The permethrin/FIP molar ratio was constant in all spot-on products (8.437), regardless of the dose. In contrast to FIP, toxicokinetics of permethrin in humans is well known, including the FUE values (Ratelle et al., 2015). Therefore, the DI estimation model described in the previous paragraph may be applied easily. A GC-MS method for permethrin urinary metabolites, such as 3-phenoxybenzoic acid (3PBA) and *cis*/*trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylic acid (*cis*/*trans*-DCCA) was used to determine permethrin exposure; the results are the subject of a separate publication (Wacławik et al., under review). The same analytical method was also used in several preceding papers (Rodzaj et al., 2021; Wielgomas, 2013; Wielgomas & Piskunowicz, 2013). The FUE values used for DI calculation were 0.46 and 0.36 for 3PBA and the sum of cis/trans-DCCA, respectively (Ratelle et al., 2015). Assuming that the absorption of permethrin and FIP are the same, the ingested dose of FIP can be calculated by dividing the permethrin DI_V (obtained using either 3PBA or the sum of *cis*- and *trans*-DCCA) by the permethrin/FIP molar ratio in the spot-on products. This model also presumes that ectoparasiticide application is the only significant source of

pyrethroid metabolites found in urine. This approach to FIP dose reconstruction will hereafter be referred to as the "PYRs-based" scenario.

After calculation of the FIP DIs using either the FIPs- or PYRs-based scenario, human health risk assessment was performed by comparing the results to reference doses: ADI (0.0002 mg/kg bw/day) and ARfD (0.009 mg/kg bw) (EFSA, 2006).

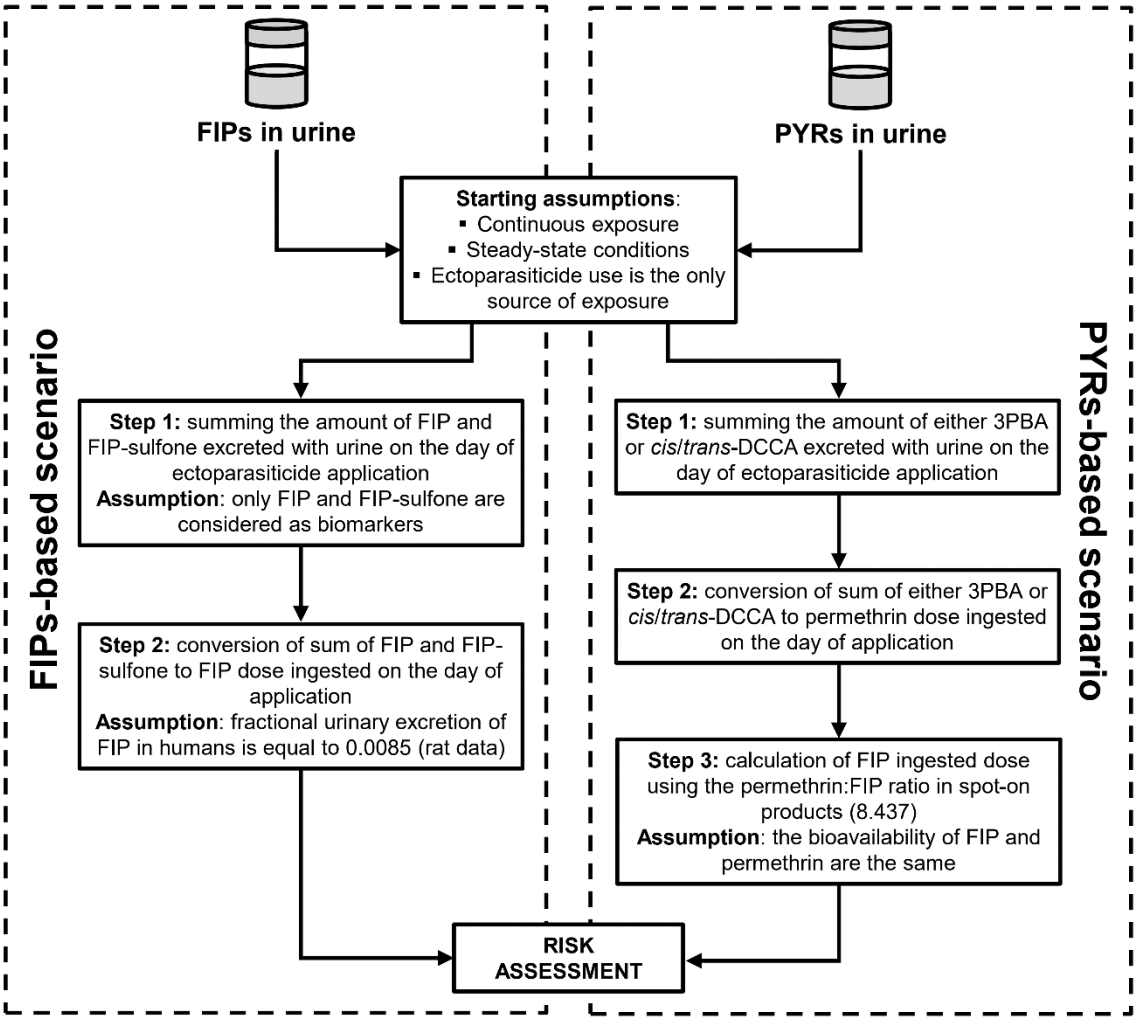

Figure 3.5 The two workflows used for ingested dose estimation. 3PBA, 3-phenoxybenzoic acid; cis/trans-*DCCA,* cis/trans-*3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylic acid; FIP, fipronil; FIPs, fiproles; PYRs, pyrethroid metabolites.*

4 RESULTS AND DISCUSSION

4.1 METHOD FOR DETERMINATION OF FIPROLES IN URINE

4.1.1 Development

The final protocol is described in section 3.2.2. Flow-through SPE was chosen as a sample preparation technique because it is an exhaustive extraction method and, in consequence, it provides maximum sensitivity (Mirnaghi et al., 2013). Some of the steps of sample preparation were adapted from other methods developed in the laboratory. The deconjugation procedure was already used in Klimowska & Wielgomas, 2018, whereas ethyl acetate was demonstrated to be optimal elution solvent in Klimowska et al., 2023. Incubation with glucuronidase/sulfatase was included due to animal data suggesting that some FIPs can be excreted with urine as conjugates (see section 1.1.4). Additionally, several non-fiprole analytes included in the method, such as bisphenol S (BPS) and hydroxy-tebuconazole are known to be extensively metabolized in humans into conjugated forms (Grandin et al., 2017; Mercadante et al., 2014). FIP-hydroxy is not included in this paragraph due to reasons described in section 4.1.2.

Filtration loss experiment

The filtration process may cause substantial analyte loss (Hebig et al., 2014; Michlig et al., 2024). Since the organic content of the solution being filtered is one of the main factors affecting this process, an experiment was performed to select the optimal solvent composition. The results of optimization of solvent composition before filtration are shown below (Figure 4.1). IMI, FIP and *trans*-PER were used as model compounds for the experiments. Although *trans*-PER was not among the analytes quantitated in urine, it was included in this experiment to take into account the compounds more lipophilic than FIP. As shown in Figure 4.1, solvent composition was not an important factor for recovery of IMI and FIP (average recovery within 96-116%). However, a statistically significant loss was observed for *trans*-PER at 60% methanol (77% average recovery; *t*-test, $p = 0.0183$) compared to the unfiltered reference. No statistically significant loss was observed in case of 80% or 100% methanol. The loss of *trans*-PER at the lowest methanol content was not unexpected, as the lipophilic analytes are generally more strongly retained by filter membranes if the water content of the extract is high (Michlig et al., 2024). At the same time, lower methanol content in the final extract would be beneficial to the peak shape of early-eluting analytes (VanMiddlesworth & Dorsey, 2012). As a compromise, 80% methanol was used to redissolve evaporated extracts before filtration.

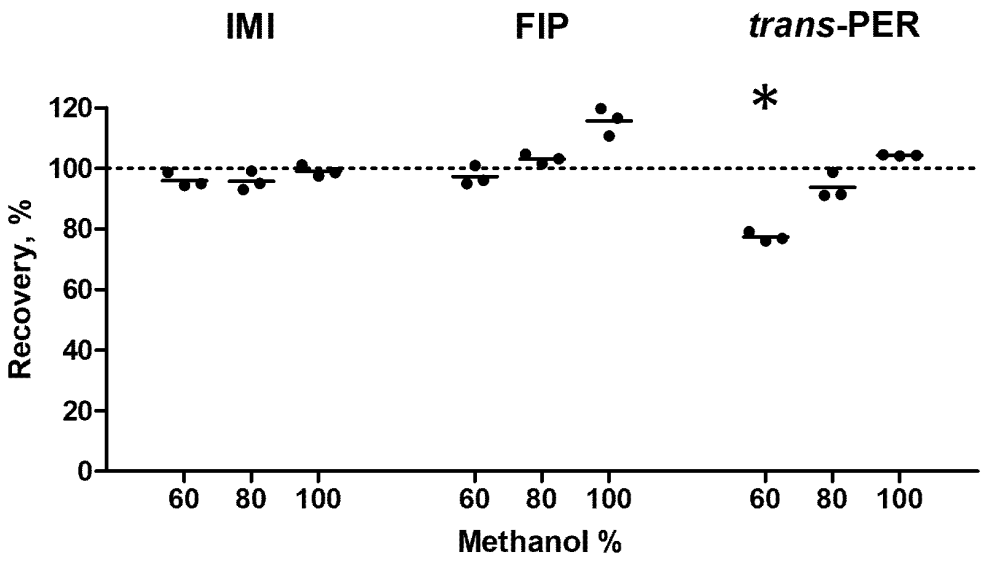

*Figure 4.1 Analyte loss due to filtration. A dashed line at 100% recovery was added for reference. An asterisk denotes a statistically significant difference (*p < *0.05).*

Extraction cartridge selection

Careful consideration of the stationary phase used during SPE procedure is necessary for efficient sample preparation (Waters, 2014). The comparison of tested sorbents is shown in Figure 4.2. Extraction efficiency for FIPs was close to 100% and similar for both tested sorbents (Figure 4.2A). The only exception was FIP-amide, with average extraction efficiency equal to 75% and 176% for Bond Elut Plexa and Oasis HLB, respectively. While the efficiency for Bond Elut Plexa was still acceptable, the result for the other sorbent was rather surprising. Since the experiment was performed using spiked urine, signal enhancement in the electrospray of mass spectrometer might have occurred due to presence of coeluting interferents in the extracts obtained using Oasis HLB, especially given that the eluting strength of wash solvent was low (1% formic acid (*v*/*v*) in 5% methanol (*v*/*v*)).

In the case of non-fiprole analytes, the differences are clear (Figure 4.2B). The average extraction efficiency of Oasis HLB for BPS, diphenyl phosphate (DPhP), 4-hydroxy-3-phenoxybenzoic acid (4OH3PBA), and imazalil-despropenyl (IMZ-OH) was at or below 3%, despite the relatively high sorbent mass (60 mg). On the other hand, satisfactory efficiency was obtained using Bond Elut Plexa 30 mg cartridges for BPS and 4OH3PBA (106% and 102%, respectively), but not for DPhP and IMZ-OH (15% and 29%, respectively). Yet, in all cases, significantly better results were obtained with Bond Elut Plexa compared to HLB Oasis. Therefore, the former was chosen to be used in the final protocol. The discrepancies between tested cartridges may stem from different chemistries of sorbent material. While the Oasis HLB sorbent is described by the manufacturer as a copolymer of divinylbenzene and N-vinylpyrrolidone (Waters, 2014), Agilent describes Bond Elut Plexa as “divinylbenzene-based” (Agilent, 2012). Additionally, Agilent provides data suggesting more uniform particle size for the Bond Elut Plexa material compared to Oasis HLB (Agilent, 2011). However, further research would be necessary to provide a definitive answer to this question.

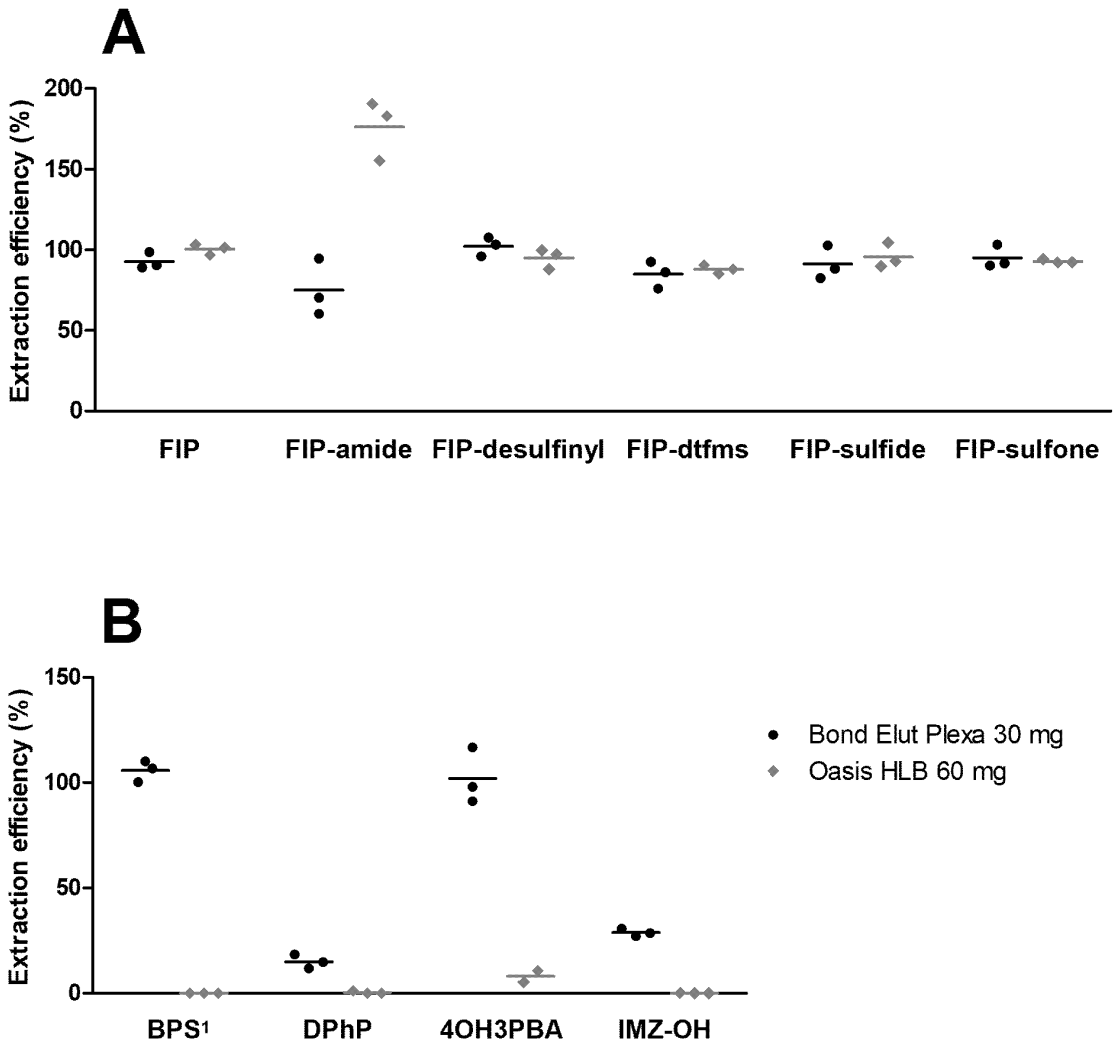

Figure 4.2 Comparison of extraction efficiencies for fiproles (A) and selected other analytes (B) obtained using Bond Elut Plexa 30 mg and Oasis HLB 60 mg.

[1] Due to the presence of unlabeled bisphenol S in the matrix, a deuterated analog was used to determine extraction efficiency.

Washing step optimization

After selecting the SPE cartridge, the sorbent wash conditions were optimized in order to wash away as many interferences as possible without losing the compounds of interest. The formic acid concentration (1%, *v*/*v*) was kept constant, while methanol percentages ranging from 0% to 25% were tested. Figure 4.3 depicts the results. Only small differences were observed for FIPs; however, the recovery of IMZ-OH was strongly affected by the wash solution composition. Consequently, the analytical response of this compound was used as a benchmark for this experiment. The average IMZ-OH signal decreased by 60% after methanol percentage increase from 15% to 20% (Figure 4.3). The result was statistically significant (*t*-test, $p = 0.0004$). Therefore, 1% formic acid (*v*/*v*) in 15% methanol (*v*/*v*) was selected for the final protocol.

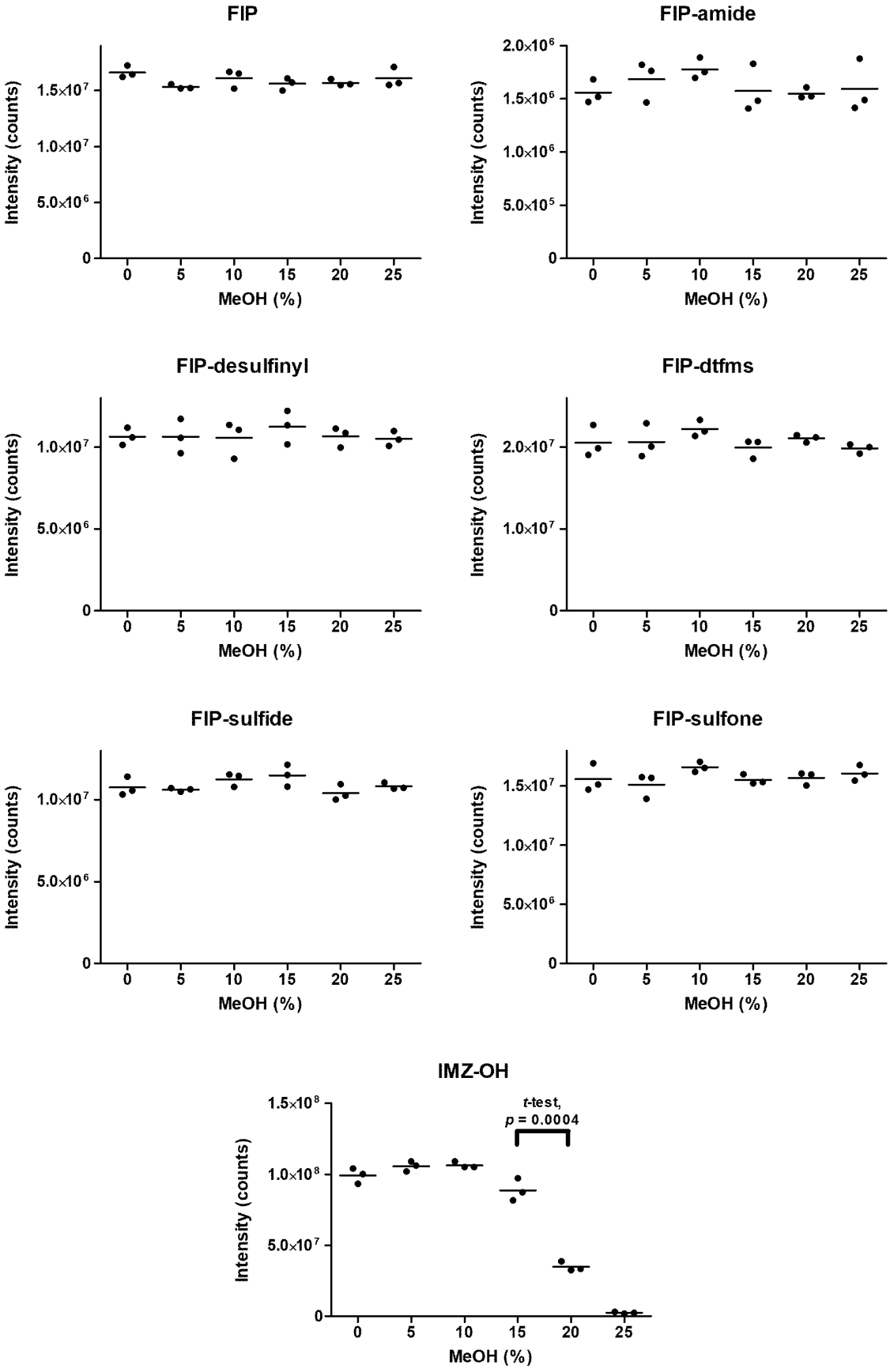

Figure 4.3 Effect of wash solution composition used during SPE procedure on analytical signal of selected compounds.

4.1.2 Fipronil-hydroxy

The total ion chromatogram in negative mode of FIP-hydroxy standard donated by prof. Bruce Hammock from UC Davis is shown below (Figure 4.4). Several peaks were observed instead of a single one. Since FIP-hydroxy is a hydroxylated derivative of FIP-dtfms, its retention time should be shorter than both FIP-dtfms and FIP. Only one peak, marked with an "X" on Figure 4.4, matched that criterion.

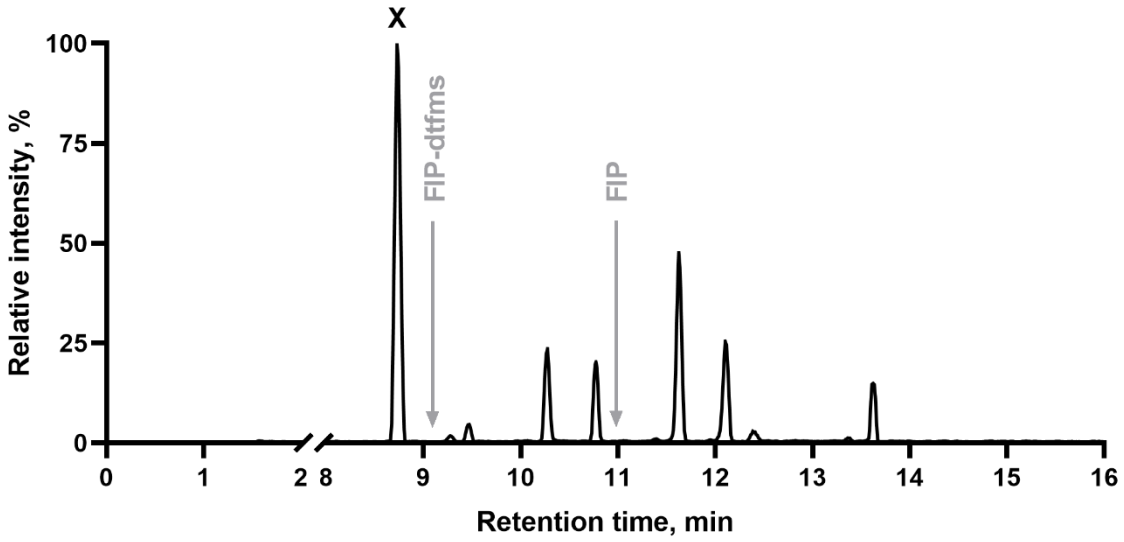

Figure 4.4 Total ion chromatogram of FIP-OH standard in negative ionization. Peak that was subject to further investigation is marked by "X". Retention times for FIP-dtfms and FIP are indicated by grey arrows.

For that peak, a mass spectrum in negative mode was obtained (Figure 4.5, left). However, it did not match a theoretical spectrum calculated using molecular formula of FIP-hydroxy: $C_{11}H_5Cl_2F_3N_4O$ (Figure 4.5, right). The mismatch might have been caused by coelution with another compound or partial in-source oxidation of hydroxy group, possibly to an imine derivative (see Figure 1.2). At the same retention time, m/z 381 and 383 in ratio corresponding to presence of two chlorine atoms were also observed (Figure 4.5, left). These m/z match values expected for a formate adduct of FIP-hydroxy and were used as a proof of FIP-hydroxy formation in rats in Vasylieva et al., 2017. Since the mobile phase used in present study contained formate ions (Table 3.3), such adducts could be formed he as well. However, the discrepancy between the m/z values observed for pseudomolecular ions and potential adducts makes drawing certain conclusions difficult. Concerns about the stability of FIP-hydroxy have already been raised in the paper proposing its use for biomonitoring (Vasylieva et al., 2017). To rule out the possibility of FIP-hydroxy degradation during storage, a new FIP-hydroxy standard was ordered from one of the largest manufacturers of analytical standards in the industry. However, after repeated attempts, the company failed to synthesize the product.

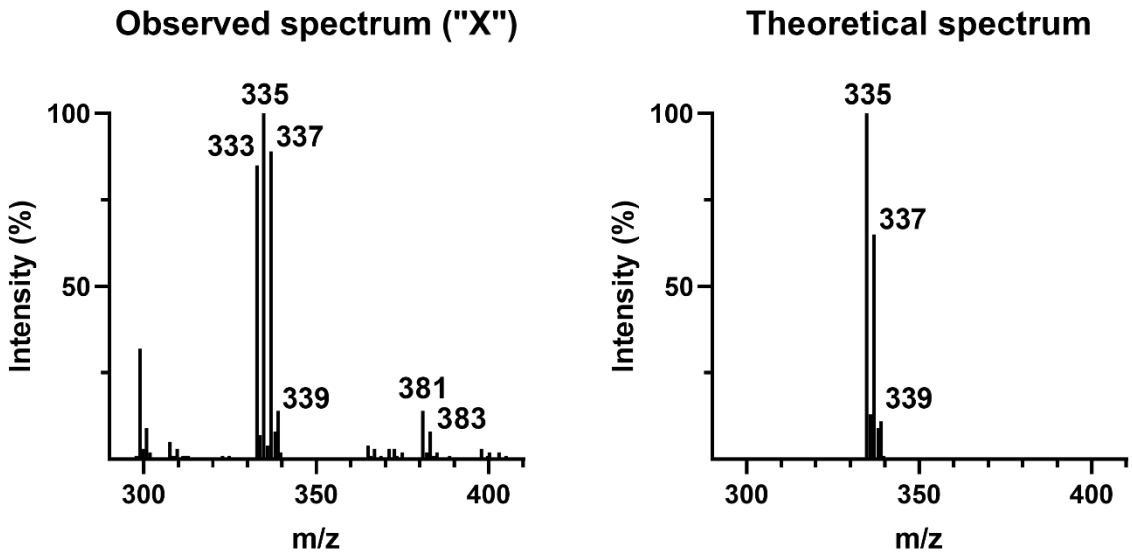

Figure 4.5 Spectrum of peak "X" (left) and theoretical spectrum expected for FIP-OH (right).

Finally, MS/MS conditions for m/z 335 (Figure 4.5, left) were optimized and 42 samples with quantifiable FIP(s) levels were screened for transitions obtained. Additionally, a transition in positive mode, used by Vasylieva et al., 2017, was also included. The samples were prepared according to the final protocol (section 3.2.2). None of the samples tested were positive for FIP-hydroxy. Consequently, FIP-hydroxy was not included in the method.

Failure to establish FIP-hydroxy as a urinary biomarker of FIP exposure in humans may stem from several reasons. Firstly, FIP-hydroxy has only been found in rats so far (Cravedi et al., 2013; Vasylieva et al., 2017), and both quantitative and qualitative differences in metabolism between rats and humans are widely reported in the literature (Caldwell et al., 2004; Cao et al., 2006; Indorf et al., 2021; Martignoni et al., 2006). Although a comparative study of human and rat liver microsomes stresses the qualitative similarity between FIP metabolism in both species (Tang et al., 2004), only one metabolite, FIP-sulfone, was taken into account in that study. Secondly, the poor stability of the standard itself shown above suggests that even if FIP-hydroxy is produced in human body and excreted with urine, it might have degraded during sample storage and/or preparation. Finally, assay sensitivity may have affected the results. It turns out that the trifluoromethylsulfinyl moiety of FIP molecule plays an important role in ionization efficiency in electrospray ion source. For instance, at the same molar concentration, FIP generates signal approximately 30 times higher than FIP-dtfms, which is deprived of that functional group (data not shown). A similar pattern can be expected for FIP-hydroxy, making it undetectable despite being excreted with urine. Since several other urinary metabolites have been reported in laboratory animals (Cravedi et al., 2013; McMahen et al., 2015), searching for a different urinary biomarker might yield better results in the future.

4.1.3 Validation

Selectivity

For all analytes, a satisfactory selectivity was achieved. As an example, chromatograms of FIP and FIP-$^{13}C_4$ in spiked and unspiked urine samples are shown in Figure 4.6 below.

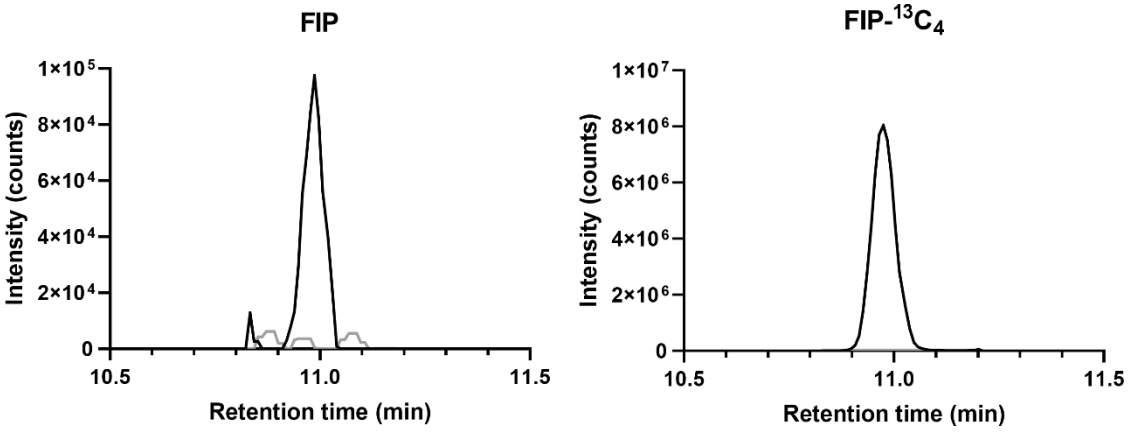

Figure 4.6 Signal of FIP and FIP-$^{13}C_4$ in spiked (black line) and unspiked (grey line) samples. In the spiked samples, the signals correspond to concentrations equal to 1 pg/mL and 200 pg/mL for FIP and FIP-$^{13}C_4$, respectively. Only the quantitative transitions are shown for clarity.

Internal standard selection and matrix effect

Mass spectrometric methods are prone to matrix effects, which may reduce their accuracy, precision, and robustness (Panuwet et al., 2016). Therefore, the presence and the magnitude of matrix effects were carefully evaluated. Absolute matrix effects (matrix factors) for FIPs are provided in Table 4.1. Additionally, SGs for all urine lots are shown. In all cases, signal suppression was observed compared to pure solvent. The signal intensity for all analytes was inversely proportional to SG of the urine sample (the r_s range from -0.8829 to -0.9643, $p \leq 0.015$). These observations were not unexpected as samples with higher SG contain more matrix components which contribute to matrix effects (Panuwet et al., 2016). According to Ferrer Amate et al., 2010, the uncorrected matrix effect within 80% and 120% corresponds to soft matrix effect, whereas matrix effects between 50% and 79% and between 121% and 150% can be considered medium signal suppression and enhancement, respectively; finally, values below 49% and above 151% indicate strong matrix effect. As shown in Table 4.1, all analytes were subject to strong ion suppression (average matrix factor ranging from 10% to 46%), despite the negative electrospray (ESI) mode being used to ionize FIPs, which is less susceptible to matrix effects (Lehotay et al., 2010; Panuwet et al., 2016) and background noise (Liigand et al., 2017) compared to positive mode. This can be attributed to the fact that urine samples were concentrated one hundred times during sample preparation with relatively little cleanup (wash solvent used during SPE contained only 15% methanol), resulting in high amounts of interferents being injected into the LC-MS/MS system. However, the gain in sensitivity resulting from sample concentration was still higher than the signal suppression resulting from matrix effect, especially for FIP and FIP-sulfone, which were considered the most important analytes in this method. The two FIP-related analytes which suffered the most from ion suppression were FIP-amide and FIP-dtfms (matrix factor 10% and 17%, respectively). This might be related to shorter retention times of these compounds compared to other FIPs (Table 3.5), which may have caused coelution with components of hydrophilic urine matrix.

Table 4.1 Matrix factors for FIPs included in the method for urinalysis.

Urine lot number	Urine SG	Analyte (nominal concentration, pg/mL); matrix factor, %					
		FIP (80)	FIP-amide (400)	FIP-desulfinyl (80)	FIP-dtfms (2000)	FIP-sulfide (80)	FIP-sulfone (80)
1	1.006	71	19	67	38	53	61
2	1.010	48	11	40	19	37	31
3	1.011	52	14	34	18	37	41
4	1.017	48	10	36	15	36	35
5	1.026	34	5	30	9	5	28
6	1.028	39	6	32	10	27	28
7	1.031	26	5	21	7	12	20
Mean		46	10	37	17	30	35

The IS-corrected matrix factors along with their CVs are provided in Table 4.2. Unsurprisingly, the two analytes that exhibited average IS-corrected matrix factors closest to 100% (corresponding to no matrix effect) were FIP and FIP-dtfms, for both of which a labeled internal standard was included in the method. In the case of other FIPs, both FIP-$^{13}C_4$ and FIP-dtfms-$^{13}C_2{}^{15}N_2$ were tested; Table 4.2 only shows the results with IS that better compensated the matrix effect. Average IS-corrected matrix factors for other FIPs ranged from 57% to 75%. CVs of IS-corrected matrix factors, which reflect the relative matrix effect (Raposo & Barceló, 2021), are given in the last row of the table. For all FIPs, the CVs were satisfactory (≤15%), except for FIP-sulfide (35%). In consequence, matrix-matched external calibration was used for quantitative analysis.

Table 4.2 IS-corrected matrix factors and their respective CVs for FIPs quantitated in urine.

Urine lot number	Urine SG	Analyte (nominal concentration, pg/mL); IS-corrected matrix factor, %					
		FIP (80)[1]	FIP-amide (400)[2]	FIP-desulfinyl (80)[1]	FIP-dtfms (2000)[2]	FIP-sulfide (80)[1]	FIP-sulfone (80)[1]
1	1.006	95	57	89	114	71	80
2	1.010	93	62	78	110	72	60
3	1.011	90	80	58	105	64	70
4	1.017	95	72	70	110	71	68
5	1.026	93	65	82	111	15	76
6	1.028	91	69	73	106	62	66
7	1.031	95	78	76	116	41	72
Mean		93	69	75	110	57	70
CV		2	11	12	3	35	9

[1] FIP-$^{13}C_4$ was used as internal standard.

[2] FIP-dtfms-$^{13}C_2{}^{15}N_2$ was used as internal standard.

Linearity and lower limit of quantification

The results of experiments on assay linearity and sensitivity are shown in Table 4.3. LLOQs ranged from 0.5 pg/mL (FIP-sulfone) to 5 pg/mL (FIP-desulfinyl) and 200 pg/mL (FIP-dtfms). For FIP, LLOQ of 1 pg/mL was established. These values are among the lowest reported in urine, despite rigorous criteria used for LLOQ determination (see section 3.2.6) and usage of three MS/MS transitions for all FIPs except FIP-dtfms (Table 3.5). In comparison, Faÿs et al., 2020 reported similar limits of detection (LODs), equal to 1 pg/mL and 10 pg/mL for FIP and FIP-sulfone, respectively; in that paper, however, relaxed approach for method sensitivity determination was used, with LOD defined as "the lowest concentration that was detected in the samples analyzed during this study". Using similar approach, Hardy et al., 2021 reported LODs for FIP and FIP-sulfone as low

as 0.9 and 3.7 pg/mL, respectively. In other works, where sensitivity was assessed basing on signal-to-noise ratio (S/N), quantitation limits ranged from 0.1 pg/mL (B. Gao et al., 2022) to 2.5 pg/mL (Shi et al., 2021) for FIP, FIP-desulfinyl, and FIP-sulfone. It should be noted, however, that S/N-based approaches for sensitivity determination in LC-MS are sometimes contested (AB Sciex, 2010; Evard et al., 2016).

High sensitivity of the developed method can be attributed to high concentration factor achieved during SPE-based sample preparation (section 3.2.2). This approach, although laborious and requiring 5 mL of sample, proved useful for determination of trace levels of FIPs.

Table 4.3 Internal standard selection, linearity data and sensitivity obtained for fiproles during validation of analytical method for urine.

Analyte	IS	LLOQ (pg/mL)	Linear range[1] (pg/mL)	Regression equation	Curve fit	Curve weighting	Regression coefficient (R^2)
FIP	FIP-$^{13}C_4$	1	1-200 (1600)	1.2291x + 0.0013	Linear	1/x	0.9996
FIP-amide	FIP-dtfms-$^{13}C_2{}^{15}N_2$	50	50-5000	0.0003x - 0.0004	Linear	1/x	0.9948
FIP-desulfinyl	FIP-$^{13}C_4$	5	5-1000 (8000)	0.9155x + 0.0027	Linear	1/x	0.9988
FIP-dtfms	FIP-dtfms-$^{13}C_2{}^{15}N_2$	200	200-40000 (320000)	0.8586x - 0.0021	Linear	1/x	0.9995
FIP-sulfide	FIP-$^{13}C_4$	1	1-200 (1600)	0.8579x + 0.0007	Linear	1/x	0.9992
FIP-sulfone	FIP-$^{13}C_4$	0.5	0.5-100 (800)	1.3612x + 0.0053	Linear	1/x	0.9995

[1] Values in brackets are quantifiable after 8-fold dilution.

Accuracy and precision

The results of validation studies on method accuracy and precision are shown in Table 4.4. At both concentration levels, accuracy and precision were at acceptable levels (100% ±15% and ≤15%, respectively).

Table 4.4 Accuracy and precision investigation for FIPs in urine at LQC and HQC level.

Analyte	Nominal concentration (pg/mL)	Intra-run accuracy (n = 5, %)	Inter-run accuracy (n = 15, %)	Intra-run precision (n = 5, CV, %)	Inter-run precision (n = 15, CV, %)
LQC					
FIP	3	97	93	7	12
FIP-amide	150	95	88	7	13
FIP-desulfinyl	15	99	97	6	8
FIP-dtfms	600	100	98	9	6
FIP-sulfide	3	102	96	10	14
FIP-sulfone	1.5	103	88	10	13
HQC					
FIP	50	99	97	4	7
FIP-amide	2500	96	95	7	6
FIP-desulfinyl	250	100	103	4	4
FIP-dtfms	10000	102	102	3	3
FIP-sulfide	50	95	99	7	11
FIP-sulfone	25	97	100	7	14

Carry-over

Basing on comparison of analytes and IS signal in blank solvent after injection of a sample at the highest calibration level, no carry-over was detected (data not shown).

Dilution integrity

Dilution integrity was assessed after eightfold sample dilution to widen the concentration range of the assay. The results are shown in Table 4.5. The acceptance criteria were the same as for accuracy and precision studies (see above). Dilution integrity was successfully demonstrated for all FIPs except FIP-amide.

Table 4.5 Dilution integrity for FIPs quantitated in urine (eightfold dilution, n = 5).

Analyte	Accuracy (%)	Precision (CV, %)
FIP	104	4
FIP-amide	-	-
FIP-desulfinyl	100	5
FIP-dtfms	99	4
FIP-sulfide	114	4
FIP-sulfone	93	4

Stability

As mentioned in section 3.2.6, the analytical runs usually consisted of 48 samples. With each analysis taking 20 minutes (Table 3.3), it may take as long as 16 hours from the end of sample preparation until sample injection into LC-MS/MS system. Therefore, an autosampler stability study at room temperature was conducted. To ensure a safety margin, samples were stored in an autosampler for 24 hours instead of 16 hours. The results are provided in Table 4.6. Satisfactory accuracy and precision at both LQC and HQC levels were achieved.

Storage stability data is essential for meaningful biomonitoring (Leng et al., 1997). Since such data for FIPs is lacking, so a long-term stability study of FIPs in urine at -20°C was conducted. Two timepoints were selected: 30 days and 12 months. Results for the periods studied are provided in Table 4.6. In all cases, FIPs were found to be stable at both LQC and HQC concentration levels. In parallel, 12-month stability of standard mixture used in the study was determined. All FIPs were found to be stable in solvent (data not shown).

Table 4.6 Results of stability study for FIPs quantitated in urine.

Analyte	LQC			HQC		
	Nominal concentration (pg/mL)	Accuracy (n = 3, %)	Precision (n = 3, CV, %)	Nominal concentration (pg/mL)	Accuracy (n = 3, %)	Precision (n = 3, CV, %)
24-h						
FIP	3	90	5	50	90	1
FIP-amide	150	89	6	2500	91	6
FIP-desulfinyl	15	94	11	250	106	4
FIP-dtfms	600	94	5	10	105	2
FIP-sulfide	3	91	13	50	92	0
FIP-sulfone	1.5	89	12	25	89	4
30-day						
FIP	3	89	1	50	90	4
FIP-amide	150	105	11	2500	93	6
FIP-desulfinyl	15	93	3	250	102	2
FIP-dtfms	600	97	1	10	105	3
FIP-sulfide	3	90	10	50	92	4
FIP-sulfone	1.5	86	9	25	89	5

Analyte	LQC			HQC		
	Nominal concentration (pg/mL)	Accuracy (n = 3, %)	Precision (n = 3, CV, %)	Nominal concentration (pg/mL)	Accuracy (n = 3, %)	Precision (n = 3, CV, %)
12-month						
FIP	3	96	0	50	89	1
FIP-amide	150	102	8	2500	99	3
FIP-desulfinyl	15	98	14	250	90	3
FIP-dtfms	600	98	1	10	99	2
FIP-sulfide	3	95	12	50	85	5
FIP-sulfone	1.5	85	3	25	86	10

Recovery

Results of recovery study are shown in Table 4.7. Recoveries ranged from 75% (FIP-amide) to 102% (FIP-desulfinyl), falling within commonly accepted 70-120% range (EURL, 2019).

Table 4.7 Recovery of FIPs from urine.

Analyte	Nominal concentration (pg/mL)	Recovery (n = 3, %)
FIP	20	93
FIP-amide	100	75
FIP-desulfinyl	20	102
FIP-dtfms	500	85
FIP-sulfide	20	91
FIP-sulfone	20	95

4.1.4 Strengths and limitations

The analytical method developed for urinalysis had a few advantages. High sensitivity and selectivity as well as wide linear range are the main features of this method. For instance, FIP and FIP-sulfone could be quantified at levels as low as 1 and 0.5 pg/mL, respectively. Additionally, limited sample cleanup allowed to include several other analytes in the method.

The method developed here also had several drawbacks. Large sample volume was needed for analysis (5 mL). Since urine is excreted in large amounts (J. R. Barr et al., 1999), collecting sufficient volume usually did not pose a problem on its own. It did, however, prolong the sample preparation process and cause heavy contamination of electrospray source of mass spectrometer. Thorough cleaning of interface using tissue wetted with methanol was necessary after each analytical run. However, the source was robust and no unplanned downtime was needed. Additionally, the use of SPE cartridges and centrifugal filters generate considerable cost, which may pose a problem in large-scale studies.

4.2 METHOD FOR DETERMINATION OF FIPROLES IN SILICONE WRISTBANDS

4.2.1 Development

The final procedure is described in section 3.3.2. The protocol for predeployment cleanup and for SWBs extraction with ethyl acetate after deployment were already developed in Wacławik et al., 2025 and were not further optimized here. Initial attempts at method development showed that the use of ethyl acetate for SWBs extraction leads to co-extraction of lipid species present on skin surface (Figure 4.7). Similar observations

were made by Bergmann et al., 2018. However, ethyl acetate is known to cause swelling of SWBs material (PDMS) which is useful for extracting the analytes from the matrix (J. N. Lee et al., 2003). Additionally, the capability of ethyl acetate to extract a wide spectrum of analytes from solid material was already shown during development of SPE procedure for urine analysis, where ethyl acetate was used as eluting solvent (section 3.2.2). Moreover, ethyl acetate-based solid-liquid extraction is the most frequently used approach for post-deployment extraction of SWBs (Wacławik et al., 2022). Finally, the goal of this part of the project was to develop a method that could be used not only for FIPs analysis, but also for the quantitation of a broader spectrum of organic contaminants. Therefore, ethyl acetate was kept as a solvent for SWBs extraction and the main focus of sample cleanup optimization was efficient removal of lipophilic interferences sequestrated from the skin while maintaining satisfactory recovery of target compounds. Since SWBs are still an emerging technology in exposure assessment (Wacławik et al., 2022), additional methodological studies were conducted as well.

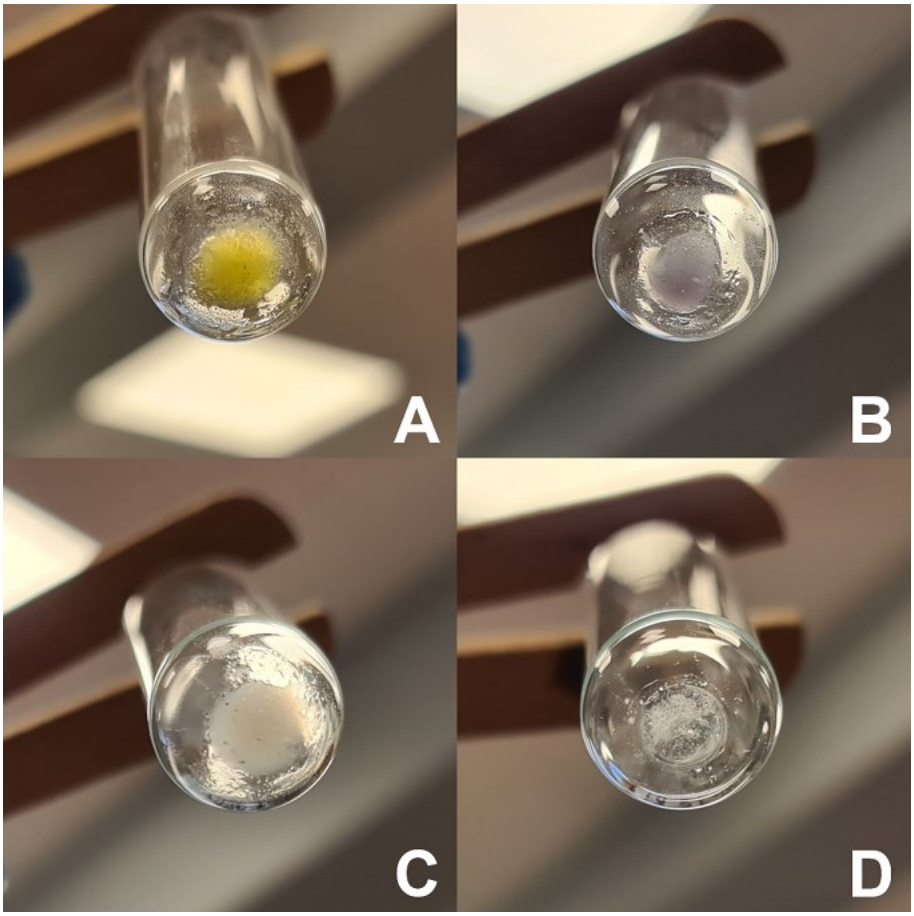

Figure 4.7 Dry residue after evaporation of ethyl acetate extract of used (A-C) and unused (D) wristbands. Note the oily residue in used samplers. All wristbands were in the same color (white).

Sample cleanup optimization

The selection of solvent for reconstitution of dry residue left after evaporation of the ethyl acetate extract (Figure 4.7) was based entirely on literature data. It was presumed that such a solvent should be more hydrophilic than ethyl acetate to limit the dissolution of lipophilic interferents. Out of the commonly used solvents, three were taken

into consideration: methanol, acetonitrile, and acetone. Methanol is an effective, universal solvent, widely used in laboratory work (E. Chambers et al., 2007). However, in a comparative study by Mol et al., 2008, where these three solvents were tested during generic extraction method development for food matrices, including fat-rich food products like egg and meat, methanol produced the least favorable results in terms of matrix effect and recovery; acetonitrile was chosen there as the best overall compromise. Additionally, data from blood lipidomics studies in humans show that methanol dissolves lipids slightly better than acetonitrile (Bang et al., 2014; Höring et al., 2022) which, in this case, is a disadvantage. A metabolomics study, although on agricultural products, showed a similar pattern when acetone and acetonitrile were compared (Sugitate et al., 2012). In fact, acetonitrile is known as a poor solvent for highly nonpolar fats (Lehotay et al., 2005). Finally, there is extensive literature showing that acetonitrile is an effective solvent for extraction of variety of chemicals, including pesticides and other organic contaminants, from diverse matrices, especially as a part of the quick, easy, cheap, effective, rugged, and safe (QuEChERS) methodology (Anastassiades et al., 2003; González-Curbelo et al., 2015; L. Kim et al., 2019; Madej et al., 2018; Perestrelo et al., 2019; Rejczak & Tuzimski, 2015). On the basis of data outlined above, acetonitrile was chosen as a solvent used to redissolve the dry residue after evaporation of raw ethyl acetate extract. Following reconstitution, the acetonitrile phase was subjected to numerous tests regarding its purification, as described in section 3.3.2. All of these tests were preceded by the freezing-out step. The results of these experiments are provided below.

The efficiency of the 13 tested cleanup procedures for extraction of FIPs and selected other analytes is shown in Tables 4.8 and 4.9, respectively. It should be noted that in the case of samples spiked before extraction, it was the pooled ethyl acetate extract that was fortified with analytes, not the SWBs themselves; therefore, these values should not be confused with method recovery. Taking into account the prior sample preparation steps, the use of 20 and 60 mg of sorbent during dSPE corresponds to 100 and 300 mg of sorbent per gram of sample, respectively, which is in line with other methods used for quantitation of organic contaminants in lipid-rich matrices, such as palm oil (Sobhanzadeh et al., 2012), avocado (Rajski et al., 2013), olives (Cunha et al., 2007), and olive oil (Cunha et al., 2007; Polgár et al., 2012). Extraction efficiency of FIPs was similar regardless of sample cleanup method and close to 100%, with the exception of FIP-dtfms, where results between 60% and 70% were most frequently observed.

Table 4.8 Extraction efficiency of fiproles from SWBs, % (%CV), n = 2. Results with extraction efficiencies outside the range of 70-120% and/or CVs exceeding 15% are shown in bold.

Method / Analyte	C18		C30		PSA		Z-sep		Z-sep+		EMR-Lipid		Hexane
	20 mg	60 mg	20 mg	60 mg	20 mg	60 mg	20 mg	60 mg	20 mg	60 mg	20 mg	60 mg	
FIP	117 (8)	**121 (8)**	120 (2)	88 (1)	91 (3)	104 (3)	101 (2)	102 (1)	96 (3)	98 (6)	89 (2)	89 (2)	90 (3)
FIP-amide	90 (7)	116 (4)	118 (4)	84 (3)	93 (3)	95 (3)	98 (4)	92 (0)	95 (1)	91 (1)	83 (3)	83 (0)	96 (2)
FIP-desulfinyl	115 (2)	119 (3)	111 (4)	82 (4)	95 (3)	104 (2)	109 (4)	100 (4)	98 (6)	103 (13)	86 (0)	82 (3)	100 (1)
FIP-dtfms	76 (5)	**62 (9)**	96 (3)	**65 (1)**	**66 (2)**	**68 (4)**	86 (6)	**68 (15)**	72 (8)	**58 (8)**	**60 (5)**	**61 (1)**	71 (8)
FIP-sulfide	114 (7)	112 (9)	118 (2)	86 (1)	93 (2)	104 (0)	103 (0)	103 (3)	89 (5)	98 (8)	93 (1)	86 (0)	88 (0)
FIP-sulfone	115 (9)	116 (15)	116 (1)	82 (1)	93 (2)	97 (1)	107 (4)	107 (0)	85 (6)	99 (12)	93 (4)	80 (1)	88 (0)

Much more striking differences were observed in the case of some non-fiprole analytes (Table 4.9). For instance, almost the entire amount of bisphenol S spiked before

extraction was lost when PSA and Z-sep+ were used for dSPE; a sharp drop in extraction efficiency was also observed when 60 mg of Z-sep was used instead of 20 mg. This is not unexpected, since PSA is known to remove polar compounds (Perestrelo et al., 2019), whereas Z-sep and Z-sep+ contain zirconium dioxide, which is amphoteric and can bind both acidic and basic species (Rajski et al., 2013). The estimated pK_a of bisphenol S is 7.4-8.0 (Nejumal et al., 2023), so it can be considered a very weak acid. Significant loss of carbendazim, nicotine and oxybenzone was also observed in the case of both zirconia-based sorbents, probably due to the same reasons as bisphenol S. Some surprising observations were also made. dSPE with C18 and C30, as well as LLE with n-hexane performed poorly for nicotine (extraction efficiency range: 6-40%). In turn, for *cis*-permethrin, extraction efficiencies as high as 139% and 152% in case of 20 mg C18 and 20 mg Z-sep, respectively, were observed. While it is difficult to explain the results for nicotine, in the case of *cis*-permethrin, such results may be caused by small number of repeated measurements for each method tested (n = 2). The relatively low signal obtained for *cis*-permethrin after LLE with n-hexane can be attributed to its high affinity towards aliphatic hydrocarbons. Finally, dSPE based on EMR-Lipid sorbent provided good recoveries for all analytes. Due to the reasons described above, dSPE procedures based on PSA, Z-sep and Z-sep+ sorbents were not taken into account in further tests.

Table 4.9 Extraction efficiency (CV) of selected other analytes from SWBs, n = 2. Results with extraction efficiencies outside the range of 70-120% and/or CVs exceeding 15% are shown in bold. NA, not assignable.

Method / Analyte	C18		C30		PSA		Z-sep		Z-sep+		EMR-Lipid		Hexane
	20 mg	60 mg	20 mg	60 mg	20 mg	60 mg	20 mg	60 mg	20 mg	60 mg	20 mg	60 mg	
Bisphenol S	101 (3)	108 (0)	111 (1)	102 (1)	**1 (32)**	**0 (NA)**	81 (2)	**18 (2)**	**4 (1)**	**0 (NA)**	83 (0)	82 (2)	86 (0)
Carbendazim	101 (3)	93 (4)	97 (0)	85 (2)	79 (0)	**56 (1)**	**28 (4)**	**24 (3)**	**13 (3)**	**4 (3)**	86 (1)	81 (1)	84 (3)
Ethyl paraben	84 (11)	89 (0)	100 (4)	112 (2)	**57 (6)**	**32 (6)**	105 (4)	80 (10)	**26 (13)**	**9 (2)**	96 (2)	105 (5)	87 (9)
Nicotine	**12 (11)**	**6 (17)**	**32 (10)**	**40 (18)**	78 (9)	**77 (25)**	**0 (NA)**	**0 (NA)**	**0 (NA)**	**0 (NA)**	77 (2)	75 (3)	**37 (5)**
Oxybenzone[1]	92 (2)	86 (11)	105 (6)	96 (6)	82 (9)	73 (14)	**15 (4)**	**4 (100)**	**0 (NA)**	**0 (NA)**	102 (11)	106 (8)	91 (8)
cis-Permethrin[1]	**139 (25)**	91 (3)	**87 (21)**	108 (6)	100 (2)	96 (5)	**152 (20)**	87 (1)	75 (14)	87 (4)	72 (14)	104 (2)	**46 (2)**

[1] Due to presence of unlabeled analyte in the matrix, a deuterated analog was used to determine extraction efficiency.

No cleanup method for raw SWB extract before LC-MS/MS analysis, other than normal-phase SPE, or solvent exchange followed by filtration, has been hitherto described in the literature (Wacławik et al., 2022). Bearing in mind the difficulties associated with ion source contamination encountered during urine analysis, it was a top priority to develop a simple method of sample preparation for SWBs that is capable of efficient matrix removal. Therefore, for selected methods that were previously tested for extraction efficiency, an additional experiment for the final extract purity determination was conducted (see section 3.3.2). Such study is often performed by gravimetric analysis of dry residues before and after the cleanup procedure (Cunha et al., 2007; Han et al., 2016), but due to the small sample weights used here, such approach was not feasible. The results of sample cleanup investigation based on areas integrated in TIC GC-MS chromatograms are shown in Table 4.10.

Table 4.10 Efficiencies of matrix removal obtained using selected cleanup techniques of SWBs.

Analyte	Matrix removal (%)	
	vs. no cleanup	*vs*. freezing-out only
dSPE, C18, 60 mg	62	35
dSPE, C30, 60 mg	2	-85
dSPE, EMR-Lipid, 60 mg	32	-6
LLE, 2×1 mL n-hexane	70	47

It is evident that dSPE using C30 and EMR-lipid sorbent provided little to no cleanup (Table 4.10). Matrix removal compared to no cleanup and freezing-out only (all results will be given in that order) was 2% and -85% for C30, and 32% and -6% for EMR-Lipid. In contrast, 70% and 47% matrix removal was achieved using LLE with n-hexane, whereas 62% and 35% cleanup was obtained with the C18 sorbent. The results reported above correlated with visual appearance of final extracts after water addition. In the case of samples prepared with C30 and EMR-Lipid, the extracts became cloudy; extracts after dSPE with C18 were somewhat opaque; finally, the use of LLE with n-hexane lead to almost transparent extracts. GC-MS chromatograms showing improvement in sample cleanup after LLE with n-hexane compared to no cleanup and freezing-out only are provided in Figures 4.8 and 4.9, respectively.

Negative values of matrix removal observed in the case of dSPE with C30 and EMR-Lipid were probably caused by sorbent contamination. dSPE salts are strong adsorbents that are not preconditioned before extraction, in contrast to conventional SPE; as a result, higher background signals can be observed (van der Vegt et al., 2022). Sorbent contamination has also been reported in other studies (Capdeville & Budzinski, 2011; Stiles et al., 2007), but no papers referring specifically to EMR-Lipid or C30 material were found.

Regarding the cleanup determination experiment, an interesting comparison can be made with results obtained by H. Zhao et al., 2022. In that paper, a method for determination of 107 pesticides and their relevant metabolites in animal fat was developed. Sample preparation consisted of extraction with acetonitrile followed by freezing-out and a final cleanup step, for which acetonitrile-hexane partitioning, as well as dSPE using either PSA, C18, neutral alumina, basic alumina or Florisil were considered. Cleanup efficiency was determined gravimetrically. The best fat removal was obtained for neutral alumina method, with over 50% of fatty acids removed. It was followed by basic alumina, Florisil, PSA, acetonitrile-hexane partitioning, and C18. Therefore, similarly to the results from Table 4.10, LLE with n-hexane was shown to provide better cleanup compared to C18 (32% and 24%, respectively). In the present study, PSA was not included in sample cleanup test performed for SWBs due to significant loss of several analytes during extraction (Table 4.9). Neutral alumina, which gave best results in H. Zhao et al., 2022 was not used in present study at all. However, considering its high affinity towards hydroxyl groups, aromatic structures and negatively charged compounds (H. Zhao et al., 2022), a significant loss of certain analytes, such as bisphenol S, would have probably occurred.

Taking into consideration both the extraction efficiency of target compounds and the matrix removal percentage, freezing-out followed by LLE with n-hexane was chosen as a final cleanup method for SWBs. During optimization studies it proved to be a simple,

efficient, and inexpensive approach for sample preparation of SWBs, suitable for a multiresidue method. Since the first use of acetonitrile-hexane partitioning by Jones and Riddick, 1952, it has been employed in many studies as a straightforward technique for sample treatment for analysis of pesticides in fatty matrices, mainly food (García-Reyes et al., 2007; Liem et al., 1992; Madej et al., 2018). Several regulatory bodies utilized acetonitrile-hexane partitioning as a purification step in their analytical methods. For instance, it has been included in procedures published by Food and Drug Administration (FDA, 1999), EPA (US EPA, 1992), and Association of Official Analytical Chemists (AOAC, 1980), although the latter used petroleum ether as a hydrocarbon solvent. Similarly to other LLE techniques, the main disadvantages of acetonitrile-hexane partitioning are the use of toxic solvents and challenging automation (Madej et al., 2018). Insufficient cleanup of food samples has also been reported (Walters, 1990). The former problem has been limited by miniaturization: 1 mL of acetonitrile and 2 mL of n-hexane were needed for preparation of a single sample. Sample cleanup was satisfactory (70% matrix removal compared to no cleanup) and the relatively low recovery for *cis*-permethrin (Table 4.9) suggests that a more rigorous procedure would lead to further loss of that analyte and sensitivity deterioration.

Following cleanup, the extract needed to be evaporated and redissolved in appropriate solvent in order to preconcentrate the sample and ensure its compatibility with the chromatographic system. As mentioned earlier, methanol was used as an organic modifier in the instrumental analysis (section 3.3.4). In addition, experiments performed during method development for urine analysis revealed that 80% methanol efficiently dissolves a wide array of chemicals (section 3.2.2 and 4.1.1). Thus, this mixture was chosen to redissolve the dry residue before the chromatographic separation without further optimization.

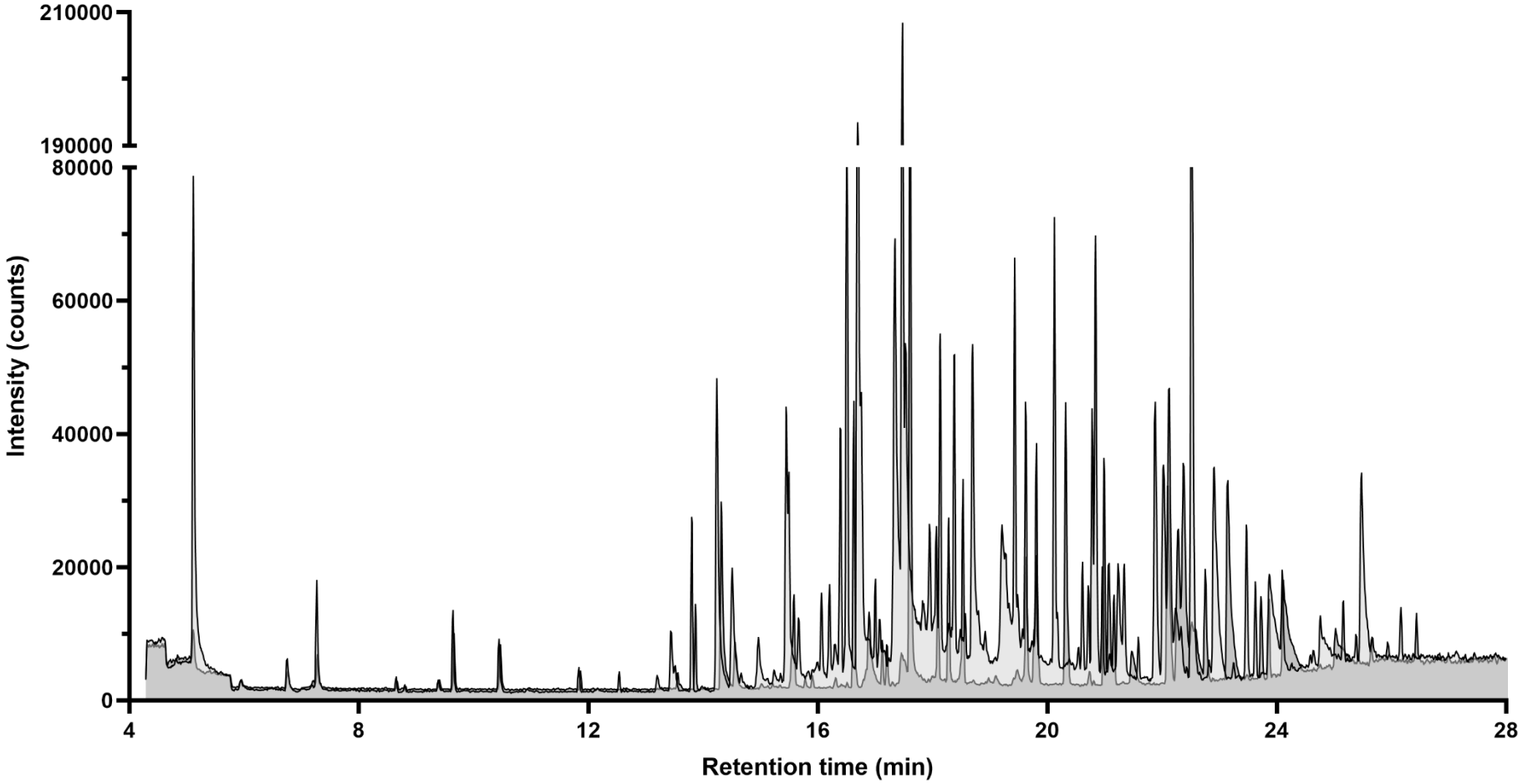

Figure 4.8 Comparison of TIC chromatogram of sample without cleanup (black line, light grey fill) and sample after freezing-out and LLE with n-hexane (grey line, dark grey fill).

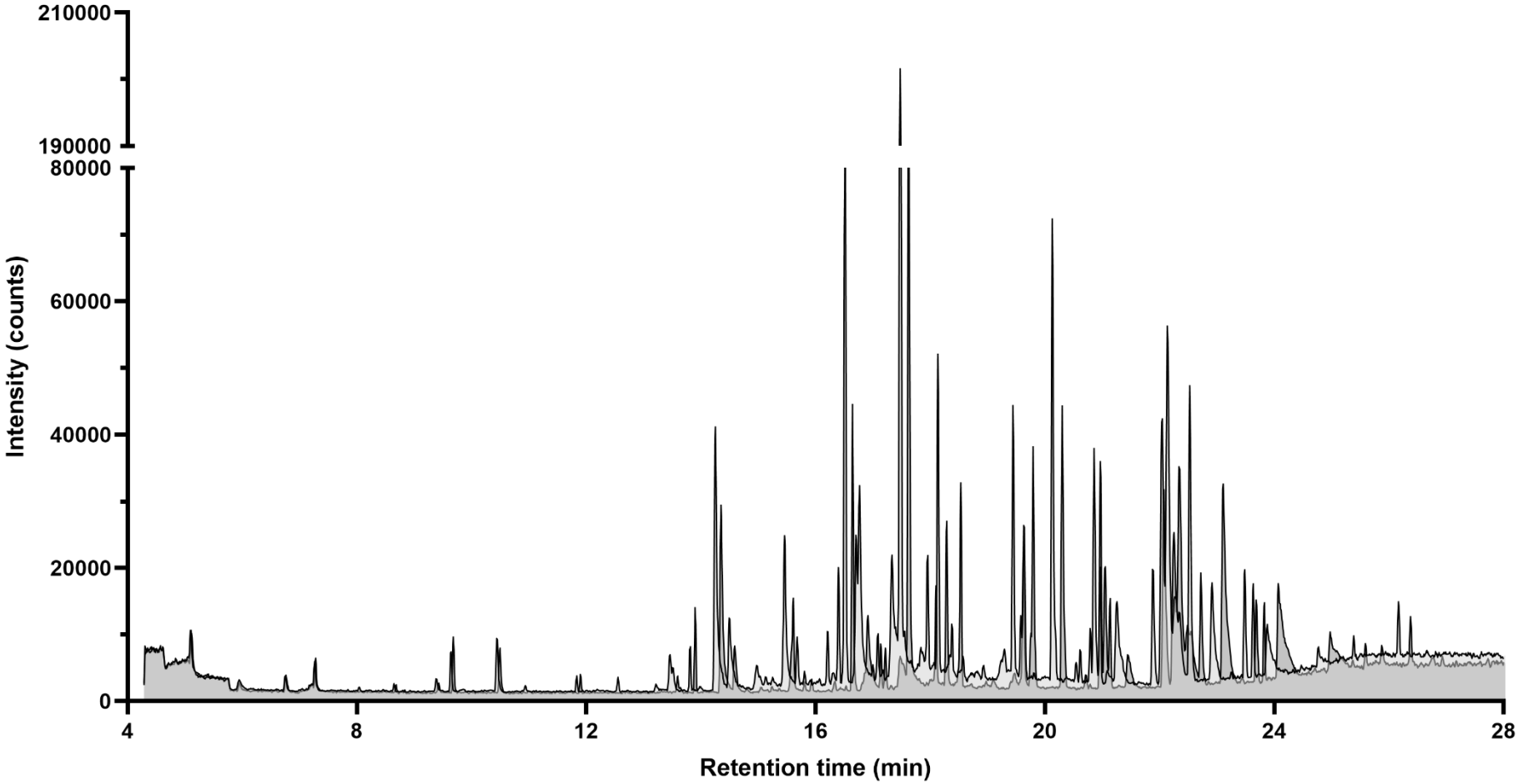

Figure 4.9 Comparison of TIC chromatogram of sample after freezing-out (black line, light grey fill) and sample cleaned up using freezing-out and LLE with n-hexane (grey line, dark grey fill).

Post-deployment rinse experiment

In this test, the impact of post-deployment rinsing step on the recovery of compounds of interest was determined. Recoveries for samples with and without water rinse step before extraction are shown in Table 4.11. Rinsing with isopropanol, which is also frequently performed after SWB deployment (Wacławik et al., 2022), was not tested. For all FIPs, the water rinse was associated with a decrease in recovery. The most striking drop was observed for FIP-amide (from 67% to 36%) and FIP-dtfms (from 61% to 37%). Since these compounds are the least lipophilic among FIPs included in the method (see retention times in Table 3.11), the obtained data align with what could be expected based on the physicochemical properties of the analytes. Therefore, in contrast to many methods published (Wacławik et al., 2022), it was decided not to rinse the SWBs after deployment. It should be noted that some authors adopted a similar approach (Nguyen et al., 2020; Y. Wang et al., 2020), although no experimental data to support it was provided. This choice, however, can be backed by the results from experiments employing other sampling techniques for FIPs and compounds of similar lipophilicity. For instance, Deziel et al., 2011 tested cotton wipes wetted with deionized water for FIP sampling efficiency from stainless steel surfaces. The yield was 7-9%, depending on the analyte concentration; these values correspond to losses observed in present study (Table 4.11). In another study, isopropanol-wetted wipes allowed for considerable FIP recovery (> 47%) even from vinyl surfaces (Willison et al., 2023). Additionally, while investigating dermal exposure to organophosphate flame retardants, (X. Liu et al., 2017) found that washing hands significantly decreases levels of these pollutants collected using skin wipes, whereas (Hoffman et al., 2015; Stapleton et al., 2014) found inverse correlation between hand washing frequency and amount of organophosphate flame retardants in hand wipes, indirectly supporting that claim. Finally, hand washing has been used as a sampling technique on its own in studies focused on exposure to pesticides via dermal route (Fenske et al., 1999; Kuster et al., 2022).

Table 4.11 Analyte recovery with and without rinsing of SWBs before extraction.

Analyte	Recovery (%)	
	With rinsing (n = 3)	Without rinsing (n = 3)
FIP	75	81
FIP-amide	36	67
FIP-desulfinyl	78	84
FIP-dtfms	37	61
FIP-sulfide	73	79
FIP-sulfone	65	77

Variability assessment

The variability of analyte concentrations throughout a sampler was determined by analyzing three separate pieces of 19 SWBs. FIPs were chosen for this experiment not only because they were the main analytes in this method, but also due to their physicochemical properties. FIPs are non-volatile (Gunasekara et al., 2007), and therefore more likely to be sampled via direct contact, rather than sequestrated from the air as in typical passive air sampling. It was assumed that such compounds may exhibit a more heterogenous distribution throughout the sampler. In this way, the results obtained here may be considered a worst-case scenario.

The results, expressed as CVs of FIP and FIP-sulfone concentrations obtained from analyses of three separate pieces of each of the 19 SWBs tested are shown in Figure 4.10A and 4.10B, respectively. The CVs ranged from 3% to 26% (median: 13%) for FIP and from 3% to 53% (median: 13%) for FIP-sulfone. Since a 15% CV is a commonly accepted threshold for the assay itself (EMA, 2022; FDA, 2018), the variability observed for both FIPs can be considered acceptable. However, for FIP-sulfone, the results were more scattered (Figure 4.10B). Since FIP-sulfone is a photodegradation product (Simon-Delso et al., 2015), uneven exposure of the SWB surface to sunlight may have contributed to observed dispersion. No clear association between observed CV and average concentration was found (Figure 4.10).

In a similar experiment, Levasseur et al., 2022 separately analyzed three separate pieces (approximately 1 g each) taken from 10 randomly selected SWBs and calculated CVs for compounds detected in each sample, which included several polycyclic hydrocarbons, flame retardants, pesticides, and plasticizers. Although FIPs were not included in that experiment, the average variability for all analytes was 9%, which is close to the value obtained here (13%). The highest CV was reported for one of the flame retardants (39%). No clear trends were identified.

Although this was just a preliminary study, it already showed that analysis of a piece of SWB without prior homogenization may lead to some bias. However, a more thorough investigation is needed to fully and accurately quantify the error resulting from sampling.

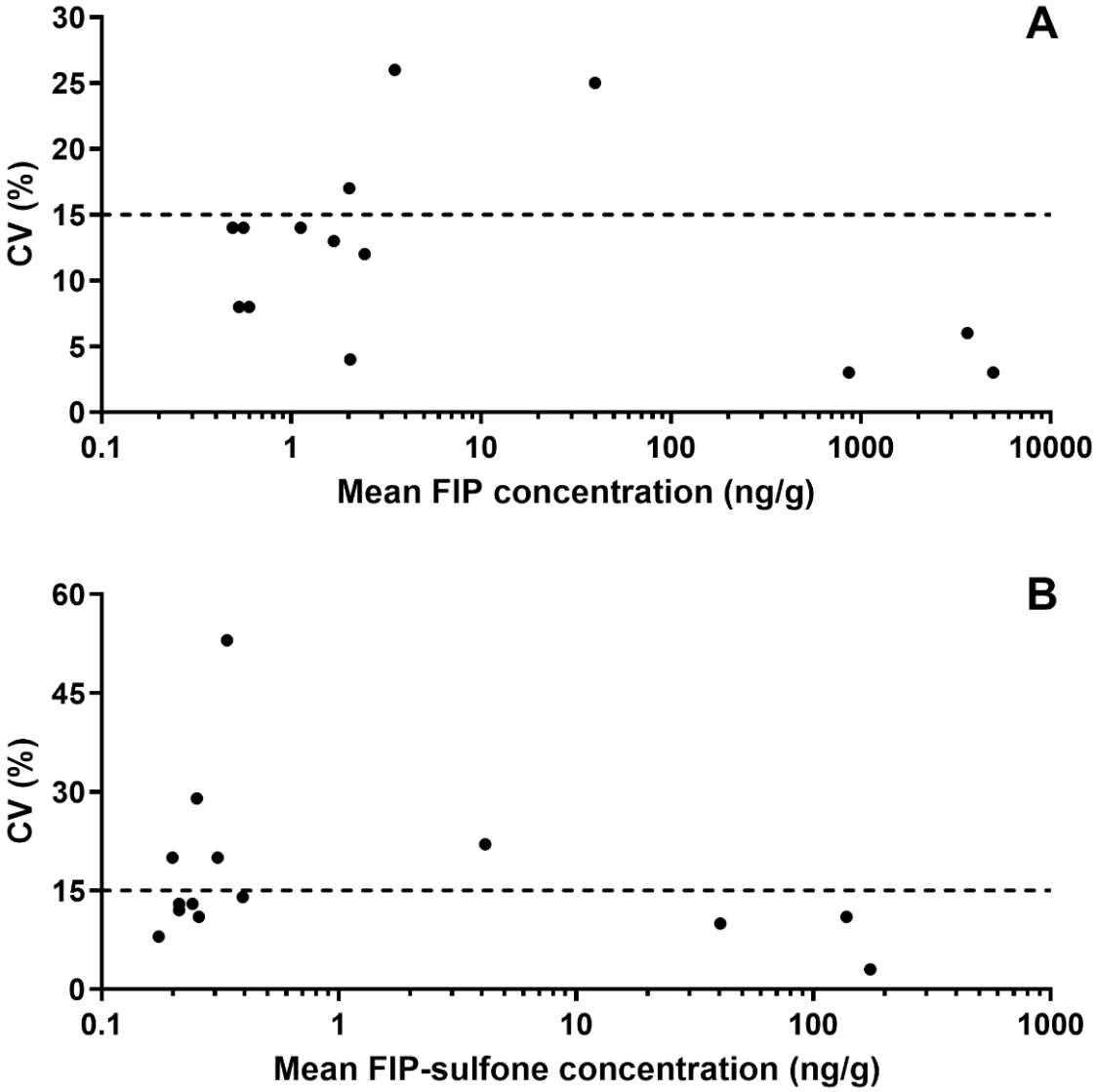

Figure 4.10 Variability of FIP (A) and FIP-sulfone (B) in tested triplets of wristband pieces plotted against their average concentration.

Fipronil degradation during deployment – simulation study

This experiment was performed in order to assess the fate of FIP spiked on SWBs during simulated 7-day deployment period. The results are shown in Figure 4.11. At the beginning, FIP corresponded to 93% of the molar sum of FIPs measured; FIP-sulfone and FIP-sulfide constituted 6% and 1%, respectively. This result was anticipated, since FIP-sulfone and FIP-sulfide are known impurities of fipronil, mentioned in the European Pharmacopoeia (Council of Europe, 2019). After 24 hours, the molar sum of FIPs decreased by 32%. While the amounts of FIP-sulfone and FIP-sulfide increased only slightly, FIP-desulfinyl appeared (6% of total FIPs at t = 0 h) along with trace amounts of FIP-dtfms. In turn, FIP accounted for only 52% of the initial sum of FIPs at this timepoint. Finally, after 168 hours, only 47% of what was at time 0 h could be found in the samples. Nearly half of it was FIP-desulfinyl and FIP-sulfone (10% and 9%, respectively). The amount of FIP-dtfms remained constant, whereas the signal for FIP-sulfide slightly decreased. FIP-amide, which is formed during environmental hydrolysis (Simon-Delso et

al., 2015), was not detected in any sample. Also, no FIPs were detected in field blanks at any timepoint of the study.

The study was performed in summer (section 3.3.2) as for FIP, a compound prone to photodegradation, it was considered a worst-case scenario. The results show that a significant amount of degradation products may be formed during SWB deployment. The loss of total FIPs over time may have been caused by two processes: the formation of derivatives not covered by the analytical method and the evaporation of FIPs into the surrounding air. Indeed, numerous new photodegradates of FIP that were not included in this study were described recently, albeit in an aquatic environment, in a study by Hirashima et al., 2023. As mentioned earlier, FIP volatility is low (Gunasekara et al., 2007) and similar behavior can be expected for its derivatives. Nevertheless, FIPs evaporation during the study period cannot be ruled out. Most likely, both phenomena contributed to the observed temporal trends, with photodegradation being a major factor.

The experiment had some limitations. For instance, it is not known whether spiking the surface with FIP dissolved in acetonitrile accurately mimics the real-life scenario. Additionally, no samples unexposed to sunlight were prepared, therefore the role of sunlight in FIP degradation was confounded with other factors, such as temperature or oxidation. Nevertheless, this study clearly showed that not only the parent compound, but its relevant degradates should be included in the method to provide a more complete picture of external exposure.

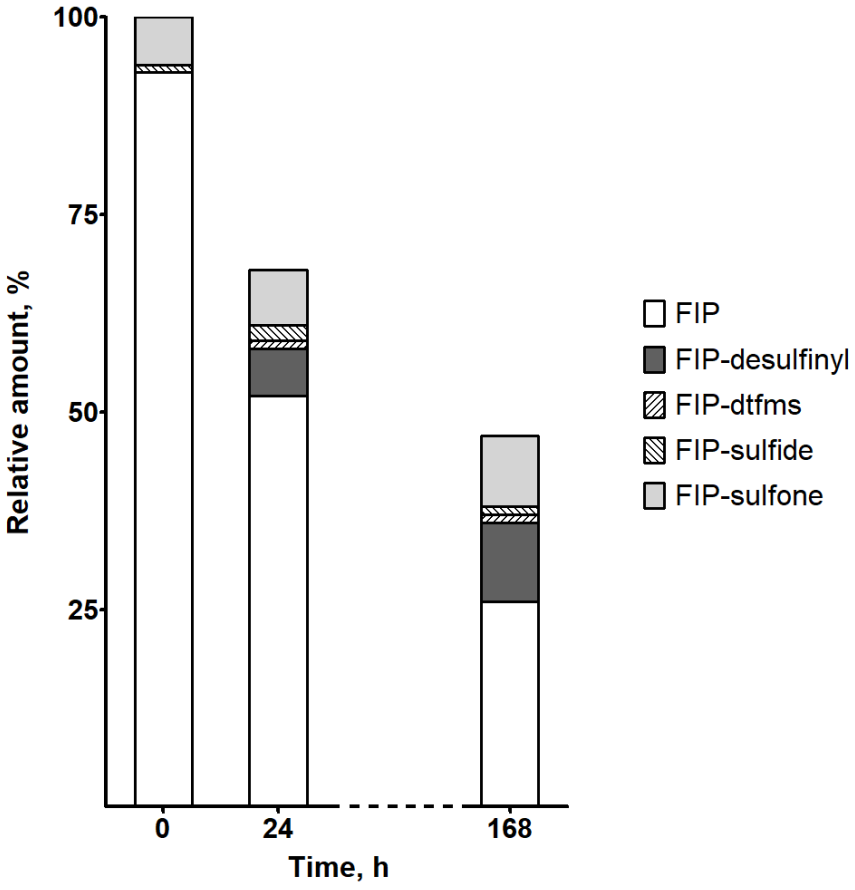

Figure 4.11 Relative amounts of FIPs during simulation study of outdoor stability of FIP spiked on SWBs. All amounts relative to molar sum of FIPs at t = 0h. FIP-amide was omitted (not detected).

4.2.2 Validation

Selectivity

For all analytes, the method selectivity was confirmed. As an example, a comparison of chromatograms of a blank sample and a sample spiked at the LLOQ level (FIP-sulfone) or with the amount routinely used for quantitative analysis (FIP-sulfone-$^{13}C_2{}^{15}N_2$) is shown in Figure 4.12.

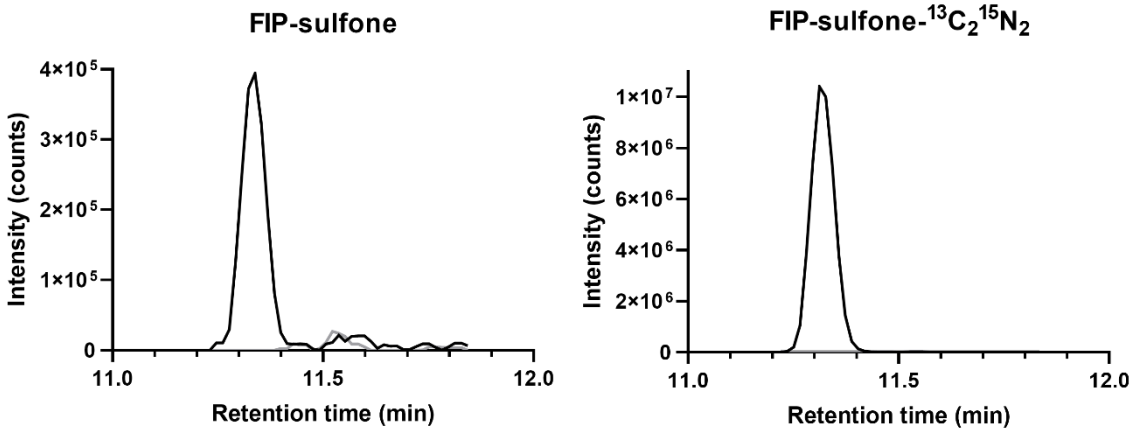

Figure 4.12 Signal of FIP-sulfone and FIP-sulfone-$^{13}C_2{}^{15}N_2$ in spiked (black line) and unspiked (grey line) samples of SWBs. In the spiked samples, the signals correspond to concentrations equal to 0.1 ng/g and 9.6 ng/g for FIP-sulfone and FIP-sulfone-$^{13}C_2{}^{15}N_2$, respectively. Only the quantitative transitions are shown for clarity.

Internal standard selection and matrix effect

The results of the relative matrix effect investigation for FIPs are shown in Table 4.12 and, for FIP only, in Figure 4.13. The absolute matrix effect was not investigated. For the final analyte-internal standard pairs, a relative matrix effect of 1% to 6% was achieved and was considered satisfactory. In nearly all cases, the matrix effect could be effectively compensated for using either FIP-$^{13}C_4$ or FIP-sulfone-$^{13}C_2{}^{15}N_2$ as an internal standard. FIP-dtfms was a notable exception: neither FIP-$^{13}C_4$ or FIP-sulfone-$^{13}C_2{}^{15}N_2$ could effectively minimize the response variability of this analyte associated with matrix effect (CVs 31% and 29%, respectively). For this analyte, only FIP-dtfms-$^{13}C_2{}^{15}N_2$ did provide acceptable CVs. The reverse was also true: FIP-dtfms-$^{13}C_2{}^{15}N_2$ did not compensate for matrix effect of any other analyte (Table 4.12).

As the name implies, FIP-dtfms is deprived of the trifluoromethylsulfinyl moiety, which seemingly plays an important role in the ionization process (see section 4.1.2). Since the compounds with lower ionization efficiency are more prone to the ionization suppression (Liigand et al., 2018), this difference in structure may explain the observed discrepancy in relative matrix effect. This reasoning is strongly supported by the results of relative matrix effect experiment for FIP-amide which, despite having similar retention time to FIP-dtfms (9.27 and 9.12 min, respectively), behaved similarly to the other FIPs (Table 4.12).

Table 4.12 Relative matrix effect for FIPs in SWBs (matrix slopes CV, %, n = 5). Results for optimal analyte-internal standard pairs are in bold.

Analyte \ IS	FIP-$^{13}C_4$	FIP-dtfms-$^{13}C_2{}^{15}N_2$	FIP-sulfone-$^{13}C_2{}^{15}N_2$
FIP	**1**	27	6
FIP-amide	**6**	21	8
FIP-desulfinyl	5	27	**4**
FIP-dtfms	31	**5**	29
FIP-sulfide	**6**	24	7
FIP-sulfone	4	24	**4**

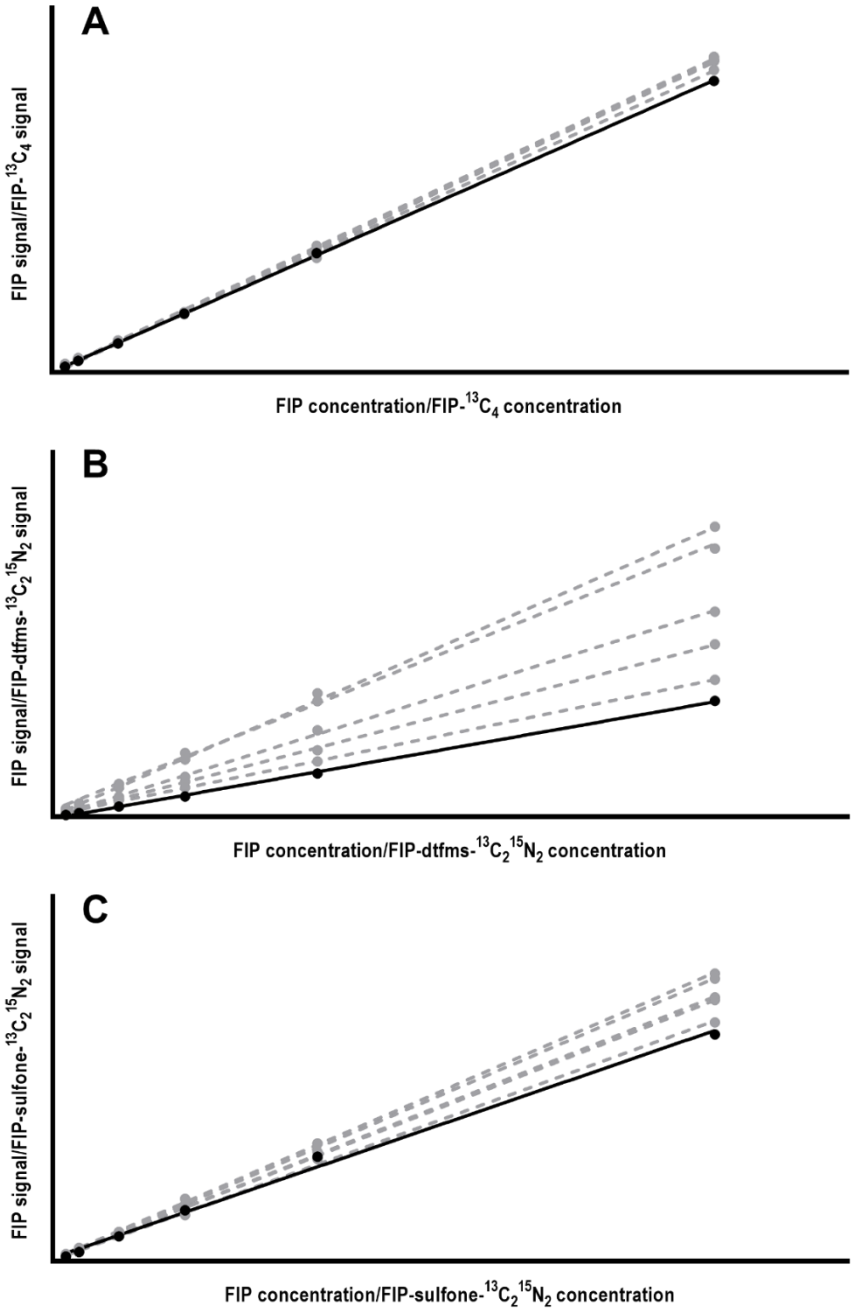

Figure 4.13 Matrix effect correction of FIP in SWBs using FIP-$^{13}C_4$ (A), FIP-dtfms-$^{13}C_2{}^{15}N_2$ (B), and FIP-sulfone-$^{13}C_2{}^{15}N_2$ (C). Grey dashed lines represent calibration curves in 5 different lots of wristband samples; a calibration curve in blank solvent (black solid line) was added for reference. Note the difference in relative matrix effect. Units and values for both axes were omitted for clarity.

Linearity and lower limit of quantification

The results of the method linearity investigation are shown in Table 4.13. LLOQs determined for FIPs ranged from 0.1 (FIP, FIP-sulfone) to 8 ng/g for FIP-dtfms and were below even the lowest detection limits reported in the literature (Aerts et al., 2018; Harley et al., 2019; Mu et al., 2024). Since high variability in concentrations in real samples was expected, a wide linear range of calibration curves was obtained whenever possible. The range was further extended in a separate dilution integrity study (see below). The linearity, calculated using R^2, exceeded 0.9900 in all cases.

Table 4.13 Sensitivity and linearity data for FIPs determination in SWBs.

Analyte	IS	LLOQ, ng/g	Linear range[1], ng/g	Regression equation	Curve fit	Curve weighting	Regression coefficient, R^2
FIP	FIP-$^{13}C_4$	0.1	0.1-200 (6000)	1.9403x + 0.0006	Linear	1/x	0.9994
FIP-amide	FIP-$^{13}C_4$	2	2-4000	0.1298x + 0.0077	Linear	$1/x^2$	0.9995
FIP-desulfinyl	FIP-sulfone-$^{13}C_2{}^{15}N_2$	0.5	0.5-125 (3750)	0.3067x + 0.0042	Linear	$1/x^2$	0.9938
FIP-dtfms	FIP-dtfms-$^{13}C_2{}^{15}N_2$	8	8-8000	0.8263x - 0.0006	Linear	1/x	0.9998
FIP-sulfide	FIP-$^{13}C_4$	0.2	0.2-25 (750)	2.7577x + 0.0170	Linear	1/x	0.9952
FIP-sulfone	FIP-sulfone-$^{13}C_2{}^{15}N_2$	0.1	0.1-200 (6000)	2.2384x – 0.0007	Linear	1/x	0.9991

[1] The values in brackets are quantifiable after 30-fold dilution.

Accuracy and precision

The results of validation experiments on accuracy and precision at the LQC and HQC levels are shown in Table 4.14. At both levels tested, accuracy and precision were within the expected values for all analytes.

Table 4.14 Accuracy and precision for FIPs in SWBs at LQC and HQC level.

Analyte	Nominal concentration (ng/g)	Intra-run accuracy (n = 6, %)	Inter-run accuracy (n = 18, %)	Intra-run precision (n = 6, CV, %)	Inter-run precision (n = 18, CV, %)
LQC					
FIP	1	94	97	3	3
FIP-amide	20	103	92	2	12
FIP-desulfinyl	5	105	114	6	8
FIP-dtfms	40	92	96	5	6
FIP-sulfide	1	96	101	2	6
FIP-sulfone	1	92	96	5	6
HQC					
FIP	10	98	99	2	3
FIP-amide	200	93	91	10	10
FIP-desulfinyl	50	97	98	7	9
FIP-dtfms	400	97	97	3	3
FIP-sulfide	10	108	107	4	5
FIP-sulfone	10	104	101	2	5

Carry-over

No carry-over was observed in a blank sample injected directly after a sample at the highest calibration level (data not shown).

Dilution integrity

To broaden the range of quantifiable concentrations of FIPs, a 30-fold dilution integrity study was conducted. The results are shown in Table 4.15. For FIP-amide and

FIP-dtfms, accuracy and precision were outside the required values (accuracy within 85-115% of expected value and precision ≤15%), so the linear range was not extended.

Table 4.15 Results of 30-fold dilution integrity study for FIPs in SWBs.

Analyte	Accuracy (n = 5, %)	Precision (n = 5, CV, %)
FIP	85	4
FIP-amide	-	-
FIP-desulfinyl	87	11
FIP-dtfms	-	-
FIP-sulfide	109	7
FIP-sulfone	114	14

Stability

Since non-validation runs normally consisted of 48 runs (section 3.3.6) and each analysis lasted 23 minutes (section 3.3.4), over 18 hours were needed to analyze all samples. Therefore, a 24 h autosampler stability study was conducted and the results are shown in Table 4.16. For all analytes, satisfactory stability at both the LQC and HQC levels was demonstrated.

Table 4.16 Results of 24 h autosampler stability study for FIPs quantified in SWBs.

Analyte	LQC			HQC		
	Nominal concentration (ng/g)	Accuracy (n = 6, %)	Precision (n = 6, CV, %)	Nominal concentration (ng/g)	Accuracy (n = 6, %)	Precision (n = 6, CV, %)
FIP	1	99	4	10	97	3
FIP-amide	20	98	6	200	85	10
FIP-desulfinyl	5	104	3	50	92	9
FIP-dtfms	40	97	2	400	97	3
FIP-sulfide	1	99	3	10	100	4
FIP-sulfone	1	93	7	10	95	4

Recovery

The results of the recovery study for FIPs are shown in Table 4.17. Except for FIP-dtfms, recoveries within the 70-120% range were obtained. Note that the recoveries in this experiment are at least a few percentage points lower than extraction efficiencies observed during method optimization (Table 4.8). The observed differences can be attributed to the fact that in recovery study the pieces of SWBs were directly spiked with analytes, whereas in the optimization study, the fortification occurred later, right before crude ethyl acetate extract evaporation. The losses resulted from the incomplete transfer of extract to another tube for solvent exchange, as well as the swelling of the SWBs material (PDMS) due to absorption of ethyl acetate.

Table 4.17 Results of FIPs recovery study in SWBs.

Analyte	Nominal concentration (ng/g)	Recovery (n = 5, %)
FIP	4	83
FIP-amide	80	75
FIP-desulfinyl	20	88
FIP-dtfms	160	57
FIP-sulfide	4	83
FIP-sulfone	4	82

4.2.3 Strengths and limitations

The method developed for the analysis of FIPs and other organic contaminants in SWBs, based on extraction with ethyl acetate followed by solvent exchange, freezing-out, acetonitrile-hexane partitioning and LC-MS/MS detection was shown to be a simple and efficient approach. It also offers considerable flexibility at the LLE step. For instance, Jones and Riddick, 1952 showed that for DDT, recovery ranging from 32% to 75% may be obtained depending on the volume ratio of acetonitrile and n-hexane. The use of other hydrocarbons, such as n-heptane or cyclohexane, may also be considered. All sample preparation steps mentioned above require very common chemicals and equipment, perhaps with the exception of centrifugal filters used at the end of sample preparation. This step can be omitted if pure methanol is used for reconstitution, but the peak shape of early-eluting compounds and extract purity may be affected; additionally, use of less lipophilic analytical column, such as C8, should be considered to avoid contamination buildup in the system. Method sensitivity for FIPs was satisfactory and good recoveries were obtained for a wide array of chemicals. In contrast to the method developed for urine (section 4.1.4), no visible buildup of sample material was observed in ESI ion source after analytical run completion.

Finally, the limitations of this method should also be addressed. Throughout the sample preparation procedure, solvent exchange is performed twice, limiting its use for determination of volatile compounds. The freezing-out step is cumbersome and requires that the samples are kept in a freezer overnight, significantly prolonging the procedure. This part of the procedure is planned to be improved in the future. Additionally, the LLE with n-hexane is performed twice, making the method somewhat laborious and hard to automate. Use of n-hexane and other solvents (ethyl acetate, acetonitrile, methanol) may pose a health risk if safety measures, such as fume hoods, are not introduced.

4.3 HUMAN EXPOSURE STUDY

4.3.1 Population characteristics

Main population characteristics are shown in Table 4.18. In total, 15 participants (8 women, 7 men) took part in the study. Their ages ranged from 15 to 63 and all were non-smokers except for participants from household no. 3. Most of the households were located in urban area, but households no. 1 and 2 were within 150 meters of agricultural lands. None of the participants were occupationally exposed to FIP or worked with companion animals. However, one participant from household no. 4 volunteered at an animal shelter. In households no. 2 and 4, the storage of FIP for residential pest control was reported. Additionally, in household no. 2, FIP had been applied on the pet included in the study, but the last application took place over a year before enrollment. Since the veterinary drug applied during the study on a pet from household no. 6 did not contain FIP, it was considered a negative control.

Table 4.18 Main characteristics of population recruited for FIP application study.

Household number	Number of participants	Age, years (range)	Residential area	Sampling season	Prior use of fipronil at home[1]	Prior use of fipronil on pets	Companion animal(s)	Ectoparasiticide application	
								Formulation	Active pharmaceutical ingredient(s) (dose, mg)
1	4	26-63	rural	summer	no	no	1 dog	spot-on	Fipronil (405.6) Permethrin (3028.8)
2	5	15-54	urban	spring	yes	yes[2]	1 dog	spot-on	Fipronil (270.4) Permethrin (2019.2)
3	2	27	suburban	fall	no	no	2 dogs	spot-on	Fipronil (135.2) Permethrin (1009.6) + Fipronil (270.4) Permethrin (2019.2)
4	2	29-31	urban	fall	yes	no	2 cats	spot-on	Fipronil (50) + Fipronil (50)
5	1	26	urban	summer	no	no	1 cat	spot-on	Fipronil (50)
6	1	27	urban	spring	no	no	1 dog	collar	Cypermethrin (1200) Deltamethrin (240)

[1] Excluding the use on household pets.

[2] Last application took place over a year before enrollment.

4.3.2 Fiproles in urine

FIPs listed in Table 3.5 were quantified using the method developed and validated according to description provided in sections 3.2.2 and 3.2.6. In total, 220 urine samples were collected. Summary statistics for samples collected during week 0 (before application) are shown in Table 4.19. Very low detection rates were observed for all FIPs, with 7% being the highest (FIP-sulfone). Also, low concentrations were observed at this stage, not exceeding 3 pg/mL. A striking exception was the only positive result for FIP-amide, where as much as 1421 pg/mL was quantified. Possible explanation for this outlier is the fact that the participant who provided the sample declared volunteer work at the animal shelter (see previous section). FIP-desulfinyl and FIP-dtfms were not detected in any sample. Also, no FIPs were found in negative control.

In urine samples collected post-application, the levels of FIPs skyrocketed (Table 4.19), except for negative control, where no FIPs were detected. Detection rate for FIP and FIP-sulfone was 67% and 36%, respectively, compared to only 5% and 7% before application. The 95th percentile (SG-adjusted) for these compounds post-application was 135.7 and 19.73 pg/mL, respectively. In contrast, in samples collected before application, the SG-adjusted 95th percentile for these FIPs was 1.379 and 2.192 pg/mL, respectively. Both before and after application, detection rate for FIP-amide was low; in three samples collected after FIP use, FIP-sulfide was quantitated. Again, FIP-desulfinyl and FIP-dtfms were not detected in any sample.

Table 4.19 Urinary concentrations of FIPs after application, negative control excluded (no FIPs detected). FIP-desulfinyl and FIP-dtfms were not detected in any sample.

SG-correction	Study period	N	N > LLOQ	% > LLOQ	AM	SD	GM	P50	P75	P90	P95	Max
FIP												
unadjusted	Before application	42	2	5	-	-	-	-	-	-	1.766	1.800
	After application	162	109	67	28.80	113.1	4.355	2.925	16.33	51.24	135.7	1358
adjusted	Before application	42	2	5	-	-	-	-	-	-	1.379	1.401
	After application	162	109	67	79.37	635.8	5.265	3.587	24.70	118.8	159.3	8109

SG-correction	Study period	N	N > LLOQ	% > LLOQ	AM	SD	GM	P50	P75	P90	P95	Max
FIP-amide												
unadjusted	Before application	42	1	2	-	-	-	-	-	-	-	1421
	After application	162	6	4	-	-	-	-	-	-	-	2129
adjusted	Before application	42	1	2	-	-	-	-	-	-	-	1212
	After application	162	6	4	-	-	-	-	-	-	-	1412
FIP-sulfide												
unadjusted	Before application	42	-	-	-	-	-	-	-	-	-	-
	After application	162	3	2	-	-	-	-	-	-	-	11.82
adjusted	Before application	42	-	-	-	-	-	-	-	-	-	-
	After application	162	3	2	-	-	-	-	-	-	-	70.55
FIP-sulfone												
unadjusted	Before application	42	3	7	-	-	-	-	-	-	2.549	2.771
	After application	162	59	36	-	-	-	-	3.612	10.42	19.73	82.50
adjusted	Before application	42	3	7	-	-	-	-	-	-	2.192	2.363
	After application	162	59	36	-	-	-	-	6.314	10.53	19.25	492.5

[1] SG, N, LLOQ, P50-95, and Max stand for specific gravity, number of samples, lower limit of quantification, 50th-95th percentile, and maximum, respectively.

A comparison of urinary FIPs levels observed in this study with results obtained by other researchers is shown in Table 4.20. Urinary data on FIP exposure in humans is scarce, so few comparisons can be made. Detection rates of FIP and FIP-sulfone before application were similar to these observed in general population of China (Shi et al., 2021) and among pregnant women in France (Hardy et al., 2021) and did not exceed 10%. Sensitivities of the analytical methods used in the present work and aforementioned papers were also comparable (Table 4.20). Much higher detection rates and concentrations were observed in present study after ectoparasiticide application and in a longitudinal urinary excretion variability study by Faÿs et al., 2020. Relatively high levels of FIPs in the latter can be attributed to the fact that nearly one-third of its participants had pets at home and applied ectoparasiticides on them, although no data was available on formulations and substances used. In the present study, detection rate for FIP and FIP-sulfone after application were 67% and 36%, respectively, whereas in Faÿs et al., 2020, FIP and FIP-sulfone were detected in 48% and 40% of the samples, respectively (Table 4.20). Proportions of these compounds, however, varied greatly between the two studies. The 75th percentile for FIP and FIP-sulfone after application were 16.33 and 3.612 pg/mL, respectively; in Faÿs et al., 2020, it was 1 and 50 pg/mL, respectively. In the present study, urine samples after FIP application on a pet were collected within hours and days; in Faÿs et al., 2020, it is not clear how much time passed from ectoparasiticide application to urine collection. Since FIP-sulfone may be also formed due to environmental degradation of FIP (section 1.1.5), it can be hypothesized that people are exposed to different profiles of FIPs, depending on time elapsed since application. This possibility is further explored in discussion on FIPs in SWBs (section 4.3.3). It should also be noted, however, that in Faÿs et al., 2020, the method sensitivity was markedly different for FIP and FIP-sulfone (1 pg/mL and 10 pg/mL, respectively).

Table 4.20 Comparison of FIPs levels observed in urine with recent studies (pg/mL, unadjusted concentrations).

Reference	Country	Sampling years	N[1]	Population	Age (years)	Method sensitivity[2]	DR[3] (%)	P50	P75	P95
FIP										
Present study	Poland	2020-2021	42	Before application	15-63	1	5	-	-	1.766
Present study	Poland	2020-2021	162	After application	15-63	1	67	2.925	16.33	135.7
(Faÿs et al., 2020)	France, Luxembourg	2018	805	Not defined[4]	22-71	1	48	-	1	NR
(Hardy et al., 2021)	France	2007	93	Pregnant women	NR	0.9	2	-	-	-
(Shi et al., 2021)	China	2020	39	General	22-51	2.5	-	-	-	-
(B. Gao et al., 2022)	EU	NR	25	Not defined	NR	0.1	8	-	-	NR
FIP-sulfone										
Present study	Poland	2020-2021	42	Before application	15-63	0.5	7	-	-	2.549
Present study	Poland	2020-2021	162	After application	15-63	0.5	36	-	3.612	19.73
(Faÿs et al., 2020)	France, Luxembourg	2018	805	Not defined[4]	22-71	10	40	-	50	NR
(Hardy et al., 2021)	France	2007	93	Pregnant women	NR	3.7	2	-	-	-
(Shi et al., 2021)	China	2020	39	General	22-51	2.5	10	-	-	NR
(B. Gao et al., 2022)	EU	NR	25	Not defined	NR	0.1	-	-	-	-

[1] Numbers presented here refer to number of samples, not participants.

[2] Various methodologies were used to determine this value for different studies.

[3] DR and NR stand for detection rate and “not reported”, respectively.

[4] Five out of 16 participants who took part in this study had pet(s) at home and reported treating them with ectoparasiticides.

Before further statistical analysis, the estimated urinary LODs were derived as described in section 3.4.4. The LODs were equal to 0.218, 2.286, 0.181, and 0.035 pg/mL for FIP, FIP-desulfinyl, FIP-sulfide, and FIP-sulfone, respectively. FIP-amide and FIP-dtfms were not detected in any additional sample, so their LODs are not reported here. Following use of aforementioned LODs, the percentage of samples without any detectable fiprole decreased from 47% to 41%. For the vast majority of these samples, the analytical signal of all analytes was indistinguishable from zero. If the concentration of FIP was below LOD, it was replaced by LOD/√2.

The relationship between FIP and FIP-sulfone, the most common FIPs measured in urine, is shown in Figure 4.14A. The correlation was strong (r_s = 0.6886) and statistically significant (p < 0.0001). Unfortunately, in the only study with comparable detection rates for both analytes in urine (Faÿs et al., 2020), no data on FIP and FIP-sulfone correlation was provided. Since in three households FIP was applied in parallel with permethrin, a pyrethroid insecticide, a correlation between post-application concentrations of FIPs and PYRs in these households was investigated. As shown in Figure 4.14B, only loose association was observed (r_s = 0.3866, p = 0.0002). Although the correlation was weaker than expected, there might be a good explanation for this. Firstly, permethrin and FIP have different pharmacokinetics – the former is quickly metabolized and rapidly excreted with urine (Ueyama et al., 2010), whereas the latter has potential for bioaccumulation (see section 1.1.4). Consequently, the temporal patterns of their respective biomarkers’ levels in urine may not overlap, despite simultaneous exposure. Secondly, pyrethroid exposure is common among many populations, including Polish (Klimowska et al., 2020; Rodzaj et al., 2021; Wacławik et al., 2025; Wielgomas et al., 2013), and dietary route is known to be a contributing factor, as shown in several intervention studies

(Baudry et al., 2021; Curl et al., 2019; Göen et al., 2017; Hyland et al., 2019). Therefore, pyrethroid exposure resulting from ectoparasiticide application on pets could be confounded by dietary exposure, negatively affecting the FIPs-PYRs correlation. In the case of FIP, exposure from other sources was negligible, as evident from low detection rates before application (Table 4.19).

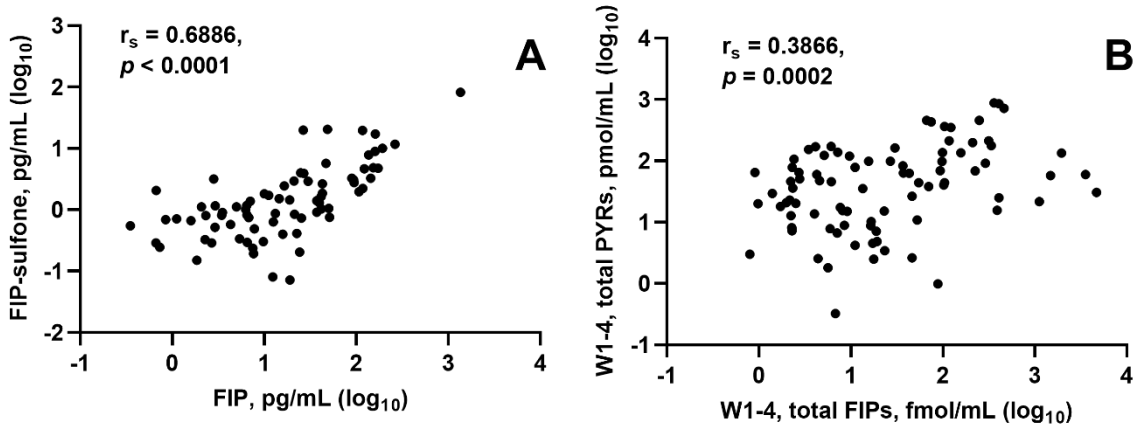

Figure 4.14 Correlations between urinary FIP and FIP-sulfone throughout the entire study (A), and between sum of pyrethroid metabolites (PYRs) measured in urine by Wacławik et al., under review, and sum of urinary FIPs measured in present study in samples collected after ectoparasiticide application (B). The latter correlation was calculated only for the households were FIP and permethrin were applied on a pet.

Changes of FIPs urinary levels over the entire study period are shown in Figure 4.15. To summarize the results in a single graph, the SG-adjusted mass concentrations of FIPs were replaced by molar concentrations and summed to produce total FIPs. Low levels of FIPs before application were followed by a rapid increase in measured FIPs shortly after ectoparasiticide use on pets, as expected (EMA & CVMP, 2018). Then, a steady decline towards the end of the sampling period was observed, although the baseline observed before application was not reached after 28 days, when another dose could be applied on the pet (Merial, 2009). This implies a risk of accumulation after repeated application. Overall, the profile shown in Figure 4.15 suggests significant contribution of FIP-based spot-on products use on pets to internal FIP exposure among humans living with them.

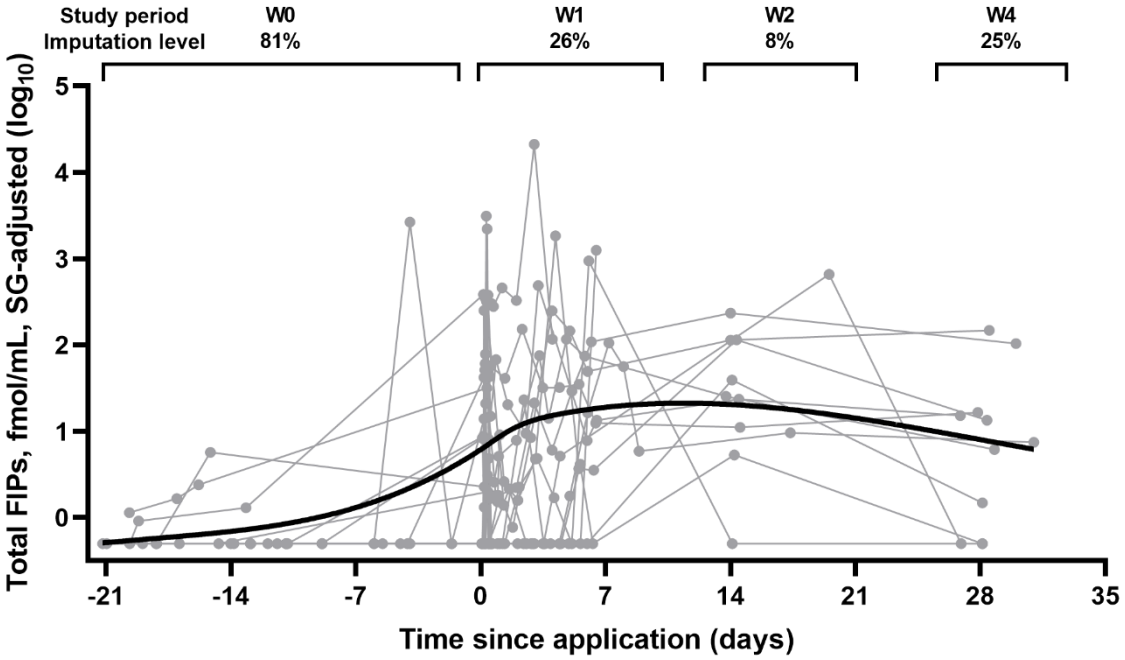

Figure 4.15 Temporal profiles of total FIPs levels throughout the study among participants who applied fipronil-based ectoparasiticides on their pets. The grey circles represent individual data points, whereas the black line is a smoothing spline fitted to the data; the latter should not be confused with a statistical model. Imputation level describes for what fraction of samples the value of LOD/√2 for FIP was imputed and no other analyte detected.

Urinary levels of FIPs measured in this study were already discussed with levels observed in a few cross-sectional studies (see Table 4.20 and the text that precedes it). A comparison with the only hitherto published longitudinal study on human exposure to FIP after ectoparasiticide application on household pets (Dyk et al., 2012) requires a separate discussion. In that study, conducted by the team from the University of California, Riverside, USA, the transferability of FIP from pets treated with FIP-based spot-on product to humans via direct contact was investigated using fluorescent tracer and cotton dosimeters (gloves and socks). Pet hair was also collected and analyzed. Additionally, an attempt was made to assess internal exposure using human biomonitoring. The latter part of that study comprised of collection and analysis of 2 pre-application and 12 post-application urine samples, either first morning voids or 24-h samples. Contrary to the results obtained here, no consistent changes in FIPs levels before and after ectoparasiticide use were observed in that study. In fact, the inconsistency was so glaring, that the authors decided not to report the quantitative results of urine biomonitoring. The observed discrepancy may stem from analytical aspects and other differences between the studies.

In Dyk et al., 2012, sample preparation involved acidic hydrolysis of 100 mL urine aliquots followed by pH adjustment and reversed-phase SPE. The evaporated eluate was redissolved in 250 µL of ethyl acetate, resulting in 400× sample preconcentration; 1 µL was injected in splitless mode into a GC-EI-MS system. The mass spectrometer was operated in a selected ion monitoring (SIM) mode, and instrumental LODs were 10-30 ng/mL. Assuming 100% recovery and no matrix effect – which, in the case of so high sample preconcentration and the use of SIM instead of MRM, is certainly an underestimate – the LODs of the entire method were 25-75 pg/mL. In the present study, FIP and

FIP-sulfone at levels ≥ 25 pg/mL were observed in less than 25% and 5% of the post-application samples, respectively. Possibly, that insufficient sensitivity of the analytical method used by Dyk et al., 2012 was the main reason why inconsistent profiles of urinary FIPs were observed after spot-on application. The limited number of urine samples collected in that study could have also compromised the results. Although sample collection time and study group description were not provided in that paper, other authors report heavy residential use of FIP in California in 2000s and early 2010s (Gan et al., 2012; Jiang et al., 2016; Lin et al., 2009), which, in turn, might have artificially elevated the baseline levels of FIPs in the studied population, masking the effect of spot-on product application.

To conclude, despite the differences between the results of the present study and the study by Dyk et al., 2012, they may not necessarily be contradictory. Further research, arguably using even more sensitive methods, is needed to better quantify the effect of ectoparasiticide use on pets on internal human exposure to FIP and its derivatives.

Statistical analysis based on the GEE model was used to investigate the causal relationship between the application of FIP-based spot-on product on pets and internal exposure to FIP in humans living with them. Urinary concentrations observed during W0 and W1 were averaged using medians. As shown in Figure 4.16, the differences between study period before application (W0) and the ones that followed were either significant (*vs*. W2, $p = 0.022$) or marginally significant (*vs*. W1, $p = 0.074$; *vs*. W4, $p = 0.065$) suggesting important role of FIP use on pets in FIPs burden among humans. Additionally, since urinary concentrations of FIPs were still relatively high at the end of experiment, where another application of FIP could take place (W4), the data suggests a risk of accumulation of FIPs in humans after repeated use.

Another phenomenon evident in Figure 4.16 is a considerable variability both within and between study participants, even though the results for W0 and W1 were averaged using the median. While some of the between-subject variability can be attributed to the large range of FIP doses applied during the experiment (50-405.6 mg per household; Table 4.18), the spread of data is much larger, suggesting other factors being involved. It is possible that patterns of pet-human interaction, as well as certain practices among pet owners, significantly contributed to observed levels of urinary FIPs. For instance, in an online survey among Portuguese pet owners (do Vale et al., 2021), over a half of the respondents reported sharing bed with the pet. In the same study, nearly 80% of pet owners admitted that they kiss their pets and/or the animals lick their faces. In a study conducted in Ireland, only 20% of respondents reported washing hands after every contact with their dog (Sherlock et al., 2023). Finally, hand-to-mouth behavior and contact between hand and *peri*-oral area, which occur both in children and adults (Cherrie et al., 2006; Gorman Ng et al., 2016; Stapleton et al., 2008; Wilson et al., 2021), can cause inadvertent ingestion of FIPs, leading to spikes in observed urinary concentrations. All the factors described above may have played a part in the magnitude of exposure observed here. Unfortunately, questions regarding such behavior were not included in a questionnaire provided to participants of the present study, so further studies are necessary to investigate the behavioral factors affecting FIP exposure.

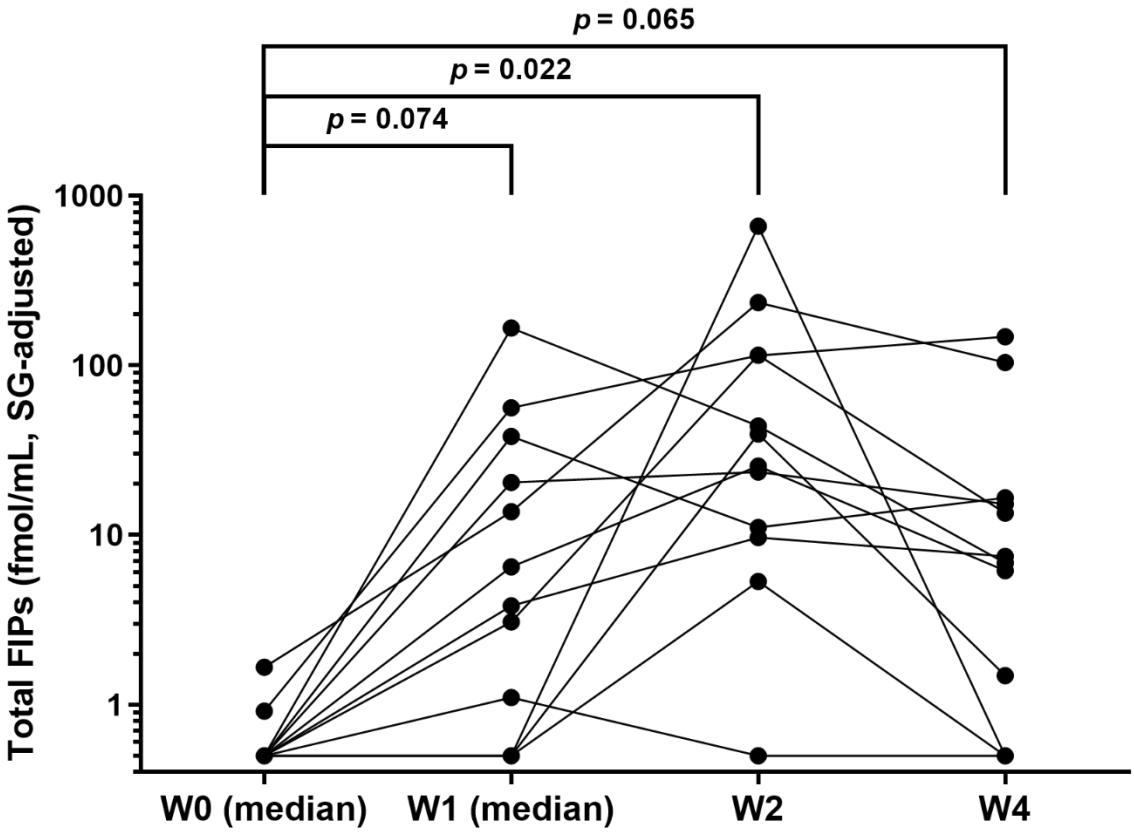

Figure 4.16 Changes of FIPs burden measured in urine of participants throughout study period. The ectoparasiticide was applied on household pets at the beginning of week 1 (W1). For W0 and W1, the results were averaged using median. The statistical significance of differences between study periods was determined using the GEE model (see text).

4.3.3 Fiproles in silicone wristbands

Concentrations of FIPs in stationary SWBs are presented in Table 4.21. In these samplers, only after the ectoparasiticide application were any analytes detected, and even then just FIP and FIP-sulfone could be quantitated and the concentrations did not exceed 10 ng/g. Similarly, in a study of Chinese rural community, where FIPs were sampled using stationary indoor SWBs (Mu et al., 2024), detection rates were very low (15% and 4% for FIP and FIP-sulfone, respectively).

Table 4.21 FIPs levels in stationary SWBs after application. No fiproles were quantified in any stationary SWB deployed before application.

Household No. / Analyte	1	2	3	4	5	6
Post-application (ng/g)						
FIP	-	9.631	6.018	1.675	0.248	-
FIP-sulfone	-	0.257	0.470	0.100	-	-

FIPs levels in personal SWBs are shown in Table 4.22. In contrast to results for urine (Table 4.19), high detection rates were observed for FIP and FIP-sulfone not only after, but also before ectoparasiticide application. However, the differences in concentrations are substantial – before application, the median values for FIP and FIP-sulfone were 1.885 and 0.678 ng/g, respectively; after FIP use, the medians were 650.4 and 123.6 ng/g, respectively, marking a rise of over two orders of magnitude. In parallel, a

significant increase in FIP-desulfinyl detection rate (21% vs. 77%, respectively) and levels was observed (90th percentile: 0.649 ng/g vs. 231.5 ng/g, respectively). Additionally, following the ectoparasiticide application, other FIPs were frequently detected, such as FIP-amide and FIP-sulfide (Table 4.22). FIP-dtfms was not detected in any sample.

Table 4.22 FIPs levels in individual SWBs before and after application. Results for negative control are not shown here; one SWB deployed post-application was lost. FIP-dtfms was not detected in any sample.

Study period	N	N > LLOQ[1]	% > LLOQ	AM	SD	GM	Min	P05	P10	P25	P50	P75	P90	P95	Max
FIP															
Before application	14	14	100	4.805	8.515	1.777	0.121	0.193	0.327	0.806	1.885	4.188	8.672	18.54	34.06
After application	13	13	100	1577	1662	593.7	2.796	52.99	119.2	263.9	650.4	2601	4217	4798	5144
FIP-amide															
Before application	14	-	-	-	-	-	-	-	-	-	-	-	-	-	-
After application	13	5	38	-	-	-	-	-	-	-	-	4.691	5.696	7.057	8.742
FIP-desulfinyl															
Before application	14	3	21	-	-	-	-	-	-	-	-	-	0.649	1.078	1.800
After application	13	10	77	129.0	325.0	7.542	-	-	-	-	4.874	45.53	231.5	649.4	1227
FIP-sulfide															
Before application	14	-	-	-	-	-	-	-	-	-	-	-	-	-	-
After application	13	12	92	46.84	54.84	13.12	-	-	1.043	5.015	19.75	78.29	140.8	153.9	157.7
FIP-sulfone															
Before application	14	14	93	1.520	1.574	0.882	-	0.119	0.169	0.271	0.678	2.357	3.817	4.190	4.858
After application	13	13	100	484.0	985.6	88.63	0.682	7.147	13.18	27.06	123.6	257.0	1052	2199	3723

[1] LLOQ, AM, SD, GM stand for lower limit of quantitation, arithmetic mean, standard deviation, and geometric mean, respectively, whereas P05-95 represent the respective percentiles.

High detection rate of FIP and FIP-sulfone observed before application was surprising, since for half of participants no potential sources were identified in a questionnaire (Table 4.18). These compounds were even quantified in negative control (data not shown). Similar observations, however, were made in studies on FIPs in house dust (Mahler et al., 2009; Shi et al., 2020; Starr et al., 2016). Recall bias or unknown sources of FIP may be responsible for this discrepancy. Nevertheless, a significant increase of FIPs levels in SWBs deployed after application, as well as sharp contrast between concentrations measured in stationary and personal SWBs suggest an important role of dermal route in human exposure.

A summary of FIPs concentrations measured in SWBs in this and other studies is provided in Table 4.23. Due to limited literature, few comparisons can be drawn. Before application, the levels observed for FIP, FIP-desulfinyl, and FIP-sulfone were similar to those observed among university employees in Belgium (Aerts et al., 2018). Detection rates, however, were much higher in the present study, with the exception of FIP-desulfinyl. These differences may, in part, have arisen from a shorter sampling period and a slightly higher limit of quantification in Aerts et al., 2018. Concentrations similar to FIP and FIP-sulfone before application were observed among Chinese farmers (Mu et al., 2024), which could be surprising. However, as mentioned in section 1.1.1, the use of FIP in Chinese agriculture is heavily restricted, and only 5% of participants of that study reported using FIP. FIPs levels observed in the present study after ectoparasiticide application were unprecedentedly high, with the notable exception of FIP-sulfide, which had

been quantified at similar levels in Latino children from North Carolina (Vidi et al., 2017) and at much higher levels among Latina adolescents from farmworker community of Monterey County, California (Harley et al., 2019) (median: 19.75 ng/g, 9.850 ng/g, and 90.3 ng/g, respectively; Table 4.23). In the case of FIP, FIP-desulfinyl, and FIP-sulfone, however, levels observed in our study were roughly two orders of magnitude higher than in hitherto published literature. A comprehensive comparison could have been made with a study on FIP levels in SWBs worn by pet owners (Wise et al., 2022), but only semi-quantitative results were provided in that paper. Nevertheless, the FIP detection rate in that study was 100%, regardless of reported FIP use, which is in line with the results presented here.

Table 4.23 Comparison of FIPs levels observed in individual SWBs with results from recent studies (ng/g).

Reference	Country	Data collection	Sampling period (days)	N	Population	Age (years)	Method sensitivity[1]	DR (%)[2]	P50	P75	P95
FIP											
Present study	Poland	2020-2021	7	14	Before application	15-63	0.1	100	1.885	4.188	18.54
Present study	Poland	2020-2021	7	13	After application	15-63	0.1	100	650.4	2601	4798
(Vidi et al., 2017)	USA	NR	7	10	Latino children	7-9	8.4	0	-	-	-
(Aerts et al., 2018)	Belgium	2016	5	30	University employees	24-72	0.2	33	-	3.825	45.45
(Harley et al., 2019)	USA	2016	7[3]	97	Adolescents[4]	14-16	NR[5]	10	-	-	NR
(Mu et al., 2024)	China	NR	28	21	Farmers	NR	1	56	2.1[6]	NR	NR
FIP-desulfinyl											
Present study	Poland	2020-2021	7	14	Before application	15-63	0.5	21	-	-	1.078
Present study	Poland	2020-2021	7	13	After application	15-63	0.5	77	4.874	45.53	649.4
(Vidi et al., 2017)	USA	NR	7	10	Latino children	7-9	-	-	-	-	-
(Aerts et al., 2018)	Belgium	2016	5	30	University employees	24-72	0.2	10	-	-	1.060
(Harley et al., 2019)	USA	2016	7[3]	97	Adolescents[4]	14-16	NR[5]	-	-	-	-
FIP-sulfide											
Present study	Poland	2020-2021	7	14	Before application	15-63	0.2	0	-	-	-
Present study	Poland	2020-2021	7	13	After application	15-63	0.2	92	19.75	78.29	153.9
(Vidi et al., 2017)	USA	NR	7	10	Latino children	7-9	6.0	50	9.850	35.25	108.1
(Aerts et al., 2018)	Belgium	2016	5	30	University employees	24-72	-	-	-	-	-
(Harley et al., 2019)	USA	2016	7[3]	97	Adolescents[4]	14-16	NR[5]	87	90.3	256.6	1015
FIP-sulfone											
Present study	Poland	2020-2021	7	14	Before application	15-63	0.1	93	0.678	2.357	4.190
Present study	Poland	2020-2021	7	13	After application	15-63	0.1	100	123.6	257.0	2199
(Vidi et al., 2017)	USA	NR	7	10	Latino children	7-9	5.7	10	-	-	18.92
(Aerts et al., 2018)	Belgium	2016	5	30	University employees	24-72	0.2	27	-	0.6	3.94
(Harley et al., 2019)	USA	2016	7[3]	97	Adolescents[4]	14-16	NR[5]	45	-	4.9	18.9
(Mu et al., 2024)	China	NR	28	21	Farmers	NR	1	69	1.4[6]	NR	NR

[1] Various methodologies were used to determine this value for different studies.

[2] DR and NR stand for detection rate and “not reported”, respectively, whereas P50-95 represent the respective percentiles.

[3] In that study, time-weighted concentrations were used (ng/g/day). Since SWBs were worn for 7 days, the results were multiplied by 7 to make comparisons easier.

[4] Members of farmworker community.

[5] Value not provided here because only instrumental limit of detection (in pg/µL) was mentioned in the paper.

[6] Geometric mean; since in that study the SWBs were worn for 28 days, the results was divided by 4 to make them comparable with other studies.

Distribution of selected FIPs before and after ectoparasiticide application in personal SWBs is shown in Figures 4.17A and 4.17B, respectively. The most prevalent FIP degradate in both study periods was FIP-sulfone (Table 4.22). FIP-sulfone is a known impurity of FIP, but so is FIP-sulfide (Council of Europe, 2019), which was not detected prior to application (Table 4.22). Environmental studies revealed that abiotic oxidation of FIP to FIP-sulfone is favored in low moisture conditions (Ying & Kookana, 2002) and accelerated in elevated temperature (Starr et al., 2016). Such conditions are common in indoor environment (Abbatt & Wang, 2020), in which people spend most of their time (Klepeis et al., 2001; Leech et al., 2002; Schweizer et al., 2007); also, all participants described their pets as living mostly/only indoors. Therefore, this finding was not surprising. However, taking into account the FIP stability during simulated SWB deployment (Figure 4.11), it cannot be ruled out that at least some FIP-sulfone quantified in personal SWBs originated from FIP degradation in SWBs. Such a process might have occurred both in outdoor and indoor environment, as FIP-sulfone is also a minor photodegradation product (Hainzl & Casida, 1996). Degradation during storage is rather unlikely – the samples were kept at -20°C both before and after collection by researchers (section 3.4.2), and FIP along with its degradates were shown to be stable at that temperature for 12-24 months (FAO/WHO, 2002).

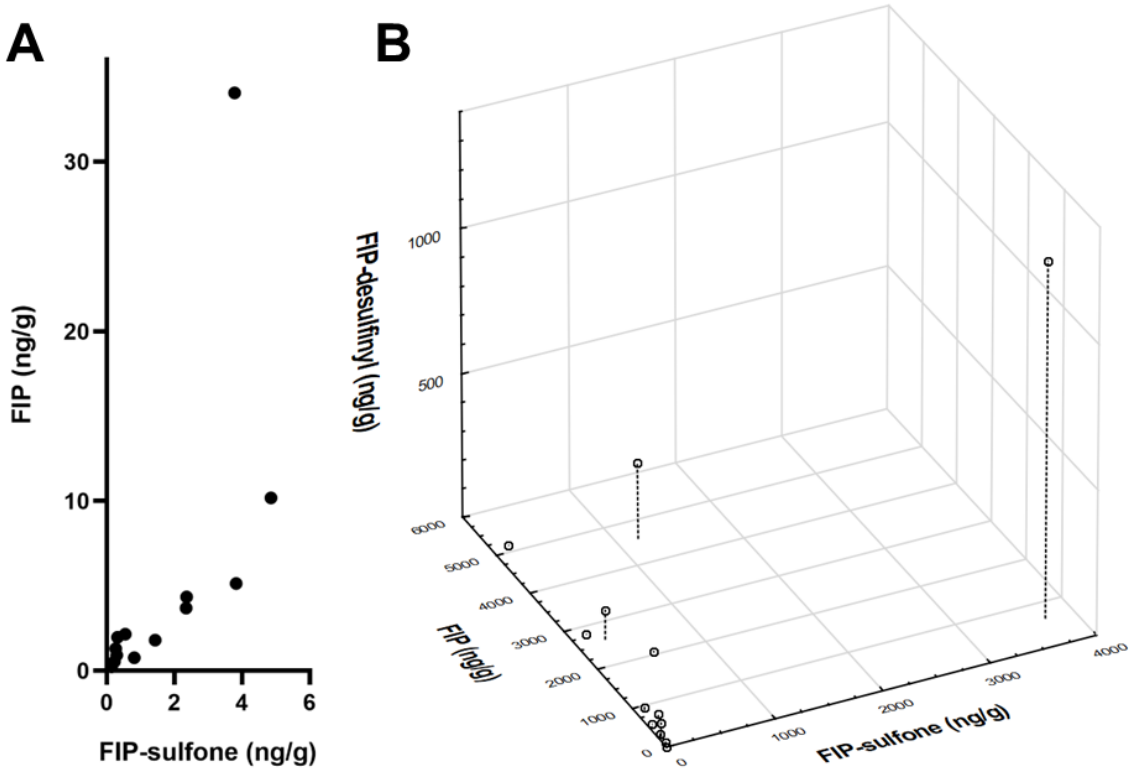

Figure 4.17 Scatterplot of FIP and FIP-sulfone in SWBs before application (A) and 3D scatterplot of selected FIPs in SWBs deployed post-application (B). The proportion of axes for FIP and FIP-sulfone are the same in both plots.

Closer investigation of FIP/FIP-sulfone ratio revealed an interesting pattern (Figure 4.18). In SWBs deployed before application, the median proportion between molar concentrations of these two FIPs was 2.042, whereas after application: 10.04. The change was statistically significant (p = 0.001) and occurred probably due to the sudden influx of

FIP (with only traces of FIP-sulfone) into the surrounding environment caused by application of spot-on product on pets. It would be beneficial to investigate these changes over a longer period of time; however, in this study, only two 7-day deployments of SWBs were planned for each participant. Interestingly, a FIP/FIP-sulfone ratio similar to one observed here before application was noted in a house dust study conducted in the USA (2.38) (Starr et al., 2016) and in Shenzhen, China in summer (2.125)[1] (Shi et al., 2020). Different ratios were observed in the latter study during winter (0.666) and in Wuhan (1.159) (Shi et al., 2020). Despite certain variability, all these values are rather small compared to the ratio observed in present study after application (10.04). In other studies on house dust (Mahler et al., 2009; Mu et al., 2024; Testa et al., 2019) paired data for comparison was not provided.

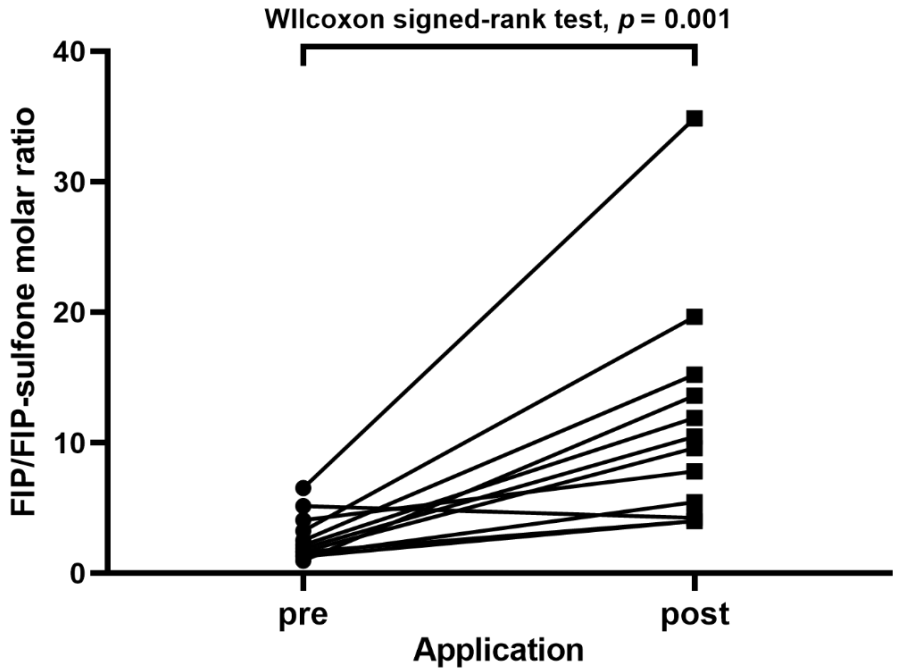

Figure 4.18 Comparison of FIP and FIP-sulfone ratio in SWBs before and after ectoparasiticide application. Pairs with at least one value missing were omitted.

In indoor environment, FIP and its derivatives are protected from important degradative factors, such as moisture, sunlight, and microbes (Starr et al., 2016). However, FIP-desulfinyl, a main FIP photodegradate (Caboni et al., 2003) was detected in SWBs, though mainly after ectoparasiticide application (Table 4.22). Additionally, FIP-sulfide was often quantified in SWBs deployed post-application. The latter was found, on average, in the amount corresponding to 2.3±1% of FIP amount in the same SWB. This value is quite close to the pharmacopeial acceptance criterion for FIP-sulfide content in FIP for veterinary use (1.5%) (Council of Europe, 2019). Therefore, it is suspected that most of the FIP-sulfide measured in SWBs stemmed from impurities present in spot-on products. Some of it could also have been formed in environment (Hainzl & Casida, 1996; Ying & Kookana, 2002) or directly on SWB, as shown in Figure 4.11. Finally, a study on transfer

[1] The FIP/FIP-sulfone ratios referring to Shi et al., 2020 were computed by the author basing on the data provided in the supplementary material to that article.

of FIP and its derivatives between pets and humans (Dyk et al., 2012) showed that amount of FIP-sulfide collected from pets fur and skin using hair brushing and gloves after 24 h since FIP-based ectoparasiticide application exceeded the amount of FIP-sulfide present in the medication. Additionally, considerable variability between animals was observed. These findings suggest that FIP-sulfide may be incorporated in fur or formed on the skin of a pet, although the short time between application and collection of samples where the highest amount of FIP-sulfide was observed suggests the latter. Overall, traces of FIP-sulfide present in spot-on products were probably the main source of this compound in SWBs, but formation in the environment contributed to observed levels as well.

FIP photodegradation, on the other hand, had probably been the main reason why FIP-desulfinyl was found in considerable amounts in some SWBs (Figure 4.17B). The highest concentrations of FIP-desulfinyl were observed in households No. 1 and 2, where FIP was applied on dogs in summer and spring of 2021, respectively. However, dogs tend to spend more time outdoors than cats (Carvelli et al., 2020; Prata, 2020), even if only for urination or defecation (E. C. Y. Lee & Devlin, 2022). More time spent outside, in parallel with warm and sunny weather, probably contributed to the formation of FIP-desulfinyl on dog skin and transfer to SWBs. No direct comparisons with SWBs collected during fall and winter could be made due to small sample sizes. Similarly to FIP-sulfide, FIP-desulfinyl was also measured on gloves and hair brushings collected after FIP application in greater amounts than can be expected from FIP contamination (Dyk et al., 2012). But, again, degradation of FIP already sequestrated in SWBs to FIP-desulfinyl cannot be outruled (Figure 4.11).

FIP-amide was also detected in SWBs deployed after application (Table 4.22), although the detection rate was low (38%) and concentrations did not exceed 9 ng/g. Since FIP-amide is formed as a result of FIP hydrolysis, preferably in alkaline conditions (Bobé et al., 1998) and study participants were asked to wear SWBs at all times (section 3.4.2), traces of FIP-amide might have formed from FIP residues on hands or SWBs due to contact with water and/or soap while washing hands or bathing. Further research is necessary to confirm or refute this hypothesis.

Finally, the correlations between FIP and its environmental degradates measured in SWBs both before and after application were investigated using Spearman's rank correlation coefficient. Due to the low detection rate prior to FIP use on pets, the only correlation investigated at that study period was between FIP and FIP-sulfone. The observed relationship was very strong and statistically significant ($r_s = 0.8726$, $p < 0.0001$). More correlations could be investigated in SWBs deployed after FIP application (Figure 4.19), but only three were statistically significant: between FIP and FIP-sulfone ($r_s = 0.7692$, $p = 0.003$), FIP and FIP-sulfide ($r_s = 0.9650$, $p < 0.0001$), and FIP-sulfone and FIP-sulfide ($r_s = 0.8252$, $p = 0.001$). Presence of trace amounts of FIP-sulfone and FIP-sulfide in FIP products used was probably the driving factor for these associations. More extensive FIP-sulfone formation in the environment might be responsible for its weaker correlation with FIP compared to FIP-sulfide (r_s 0.7692 *vs*. 0.9650, respectively). In contrast, no significant correlations were observed for FIP-desulfinyl. Since FIP-desulfinyl is formed at expense of FIP (Hainzl & Casida, 1996; Ngim & Crosby, 2001), an inverse

correlation with these compounds could be expected. However, the rate of FIP degradation into FIP-desulfinyl probably varied throughout the sampling period, resulting in poor and insignificant correlation observed overall (Figure 4.19).

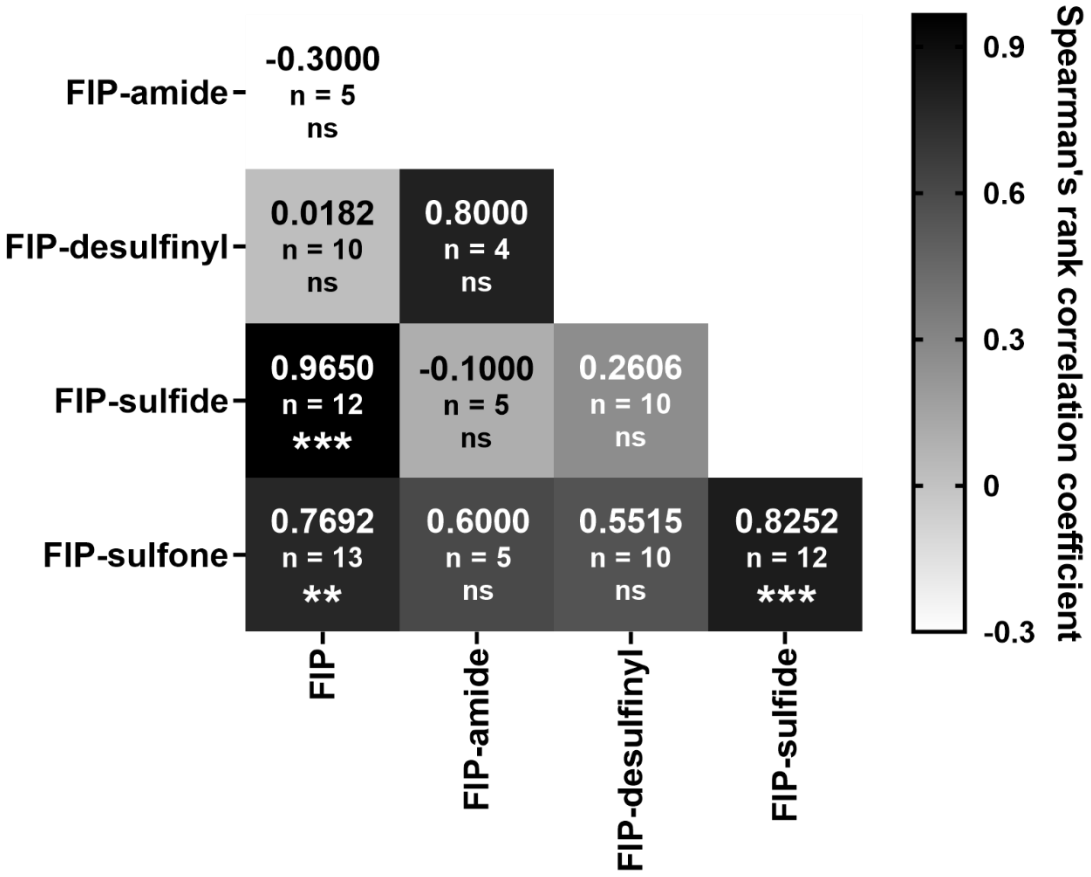

*Figure 4.19 Correlations between FIPs in SWBs collected post-application. In each box, the following information is pro-vided (top to bottom): strength of the association (Spearman's rank correlation coefficient), number of sample pairs, and statistical significance. ns, not significant; **,* $p \leq 0.01$*; ***,* $p \leq 0.001$*.*

The SWBs results presented above highlight the importance of monitoring both FIP and its degradates in studies employing SWBs, especially since some of them, like FIP-desulfinyl, did not correlate with parent compound. The need for comprehensive methods capable of tracking FIP and its degradates simultaneously has also been stressed in large-scale studies of surface water (Mahler et al., 2021; Stone et al., 2014) and sewage sludge (Sadaria et al., 2019), which shows an interesting parallel between personal and environmental exposure research.

In further analysis of SWBs results, molar sum of all degradates was used instead of comparing individual chemicals. The sums of FIPs observed in SWBs before and after ectoparasiticide application are shown in Figure 4.20. The median sum of FIPs skyrocketed from 5 to 2266 pmol/g. The GEE model was used to investigate statistical significance of observed changes ($p < 0.0001$). In all cases, FIP use on the pet was associated with increase of total FIPs on SWBs; the only decrease was seen in negative control (Figure 4.20, grey line), highlighting the important role of ectoparasiticide application on pets in FIPs levels in SWBs and their capability to capture dermal exposure.

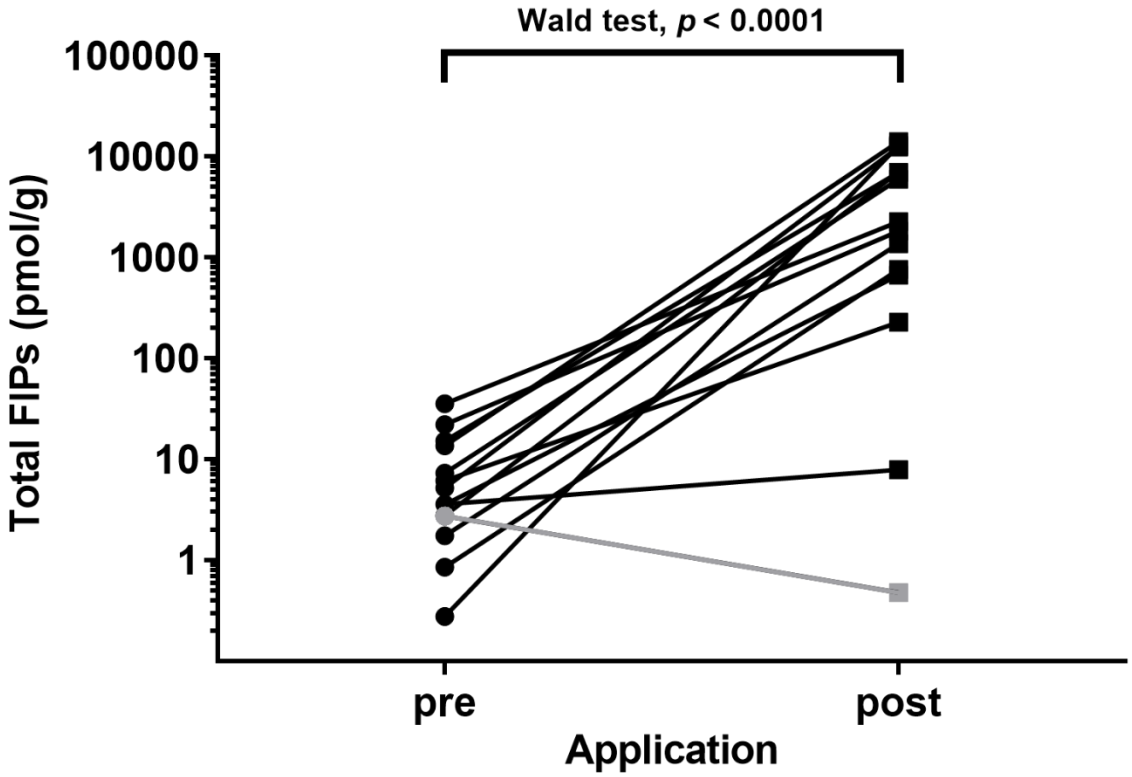

Figure 4.20 Comparison of FIPs levels before and after ectoparasiticide application. The negative control (no fipronil in applied product) is shown in grey and was not included in statistical test.

In parallel to FIP, pyrethroids were also applied on pets, if possible (Table 4.18). Changes in levels of pyrethroids within the studied population were the subject of a separate study (Wacławik et al., under review). Here, only the correlation between permethrin and the sum of FIPs measured in SWBs was investigated. Before application, the association was strong but marginally insignificant (r_s = 0.7714, n = 5, p = 0.072). After ectoparasiticide use, the correlation became very strong and statistically significant (r_s = 0.9515, n = 10, p < 0.001) (Figure 4.21), suggesting that permethrin and FIPs measured in SWBs originated from the same source. To further support that claim, the proportion between permethrin and FIPs quantitated in SWBs was investigated. As presented in Table 4.18, the mass ratio between permethrin and FIP in spot-on products was constant, regardless of the dose; it corresponded to a molar ratio equal to 8.437. In comparison, the proportion between molar concentrations of permethrin and FIPs measured in SWBs, expressed as a slope of a linear regression model, was 8.890 (Figure 4.21). This value corresponds to only a +5% error and confirms that the spot-on products used on pets were the source of analytes found in SWBs. The results described above demonstrate the usefulness of SWBs in identifying sources of non-dietary exposure to organic contaminants, as it was previously shown for organophosphate flame retardants (Hammel et al., 2016) and tobacco-specific compounds (Quintana et al., 2020).

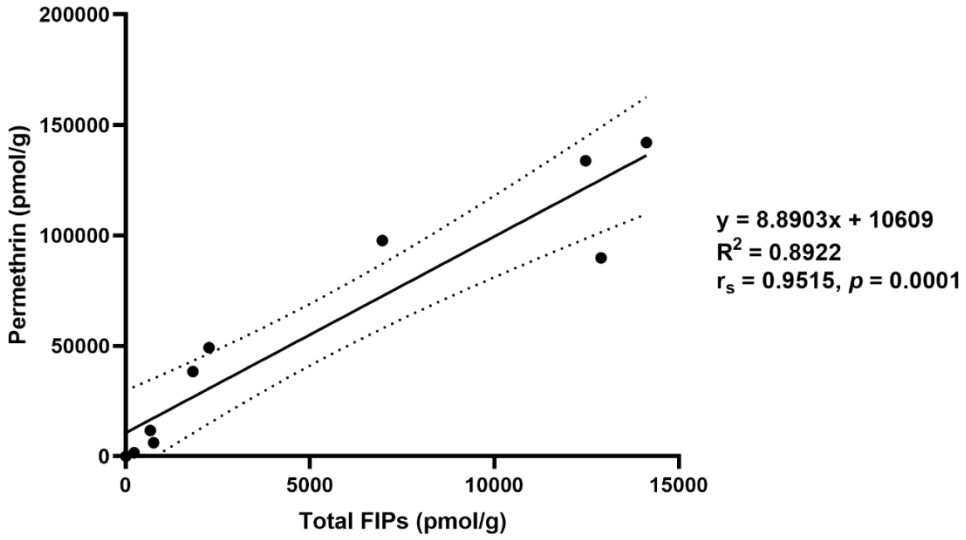

Figure 4.21 Correlation between permethrin and molar sum of fiproles in post-application wristbands of participants who applied an ectoparasiticide containing both fipronil and permethrin. The solid line represents the best-fit result of linear regression; the dotted lines show the 95% confidence interval.

4.3.4 Combining the biological and environmental monitoring

SWBs provide information on average, integrated exposure over the deployment period (Wise et al., 2020). Urine, in turn, reflects the exposure from several preceding hours, although for FIPs this period might be longer since it shows a moderate potential for bioaccumulation in simulation (Tonnelier et al., 2012) and animal studies (Cravedi et al., 2013). Therefore, the median sum of FIPs in urine was plotted against the sum of FIPs in SWBs during W1 to investigate the relationship between concentrations observed between these two matrices (Figure 4.22). The association was very strong (r_s = 0.8500, p = 0.0037) and loglinear (R^2 = 0.7737), although datapoints were relatively few (n = 9) due to limited sample size and extremely low concentrations of FIPs in urine. These results are a striking example of SWBs' relevance to the estimation of averaged internal exposure and/or selection of the most exposed individuals, provided that non-dietary routes are the driving factor.

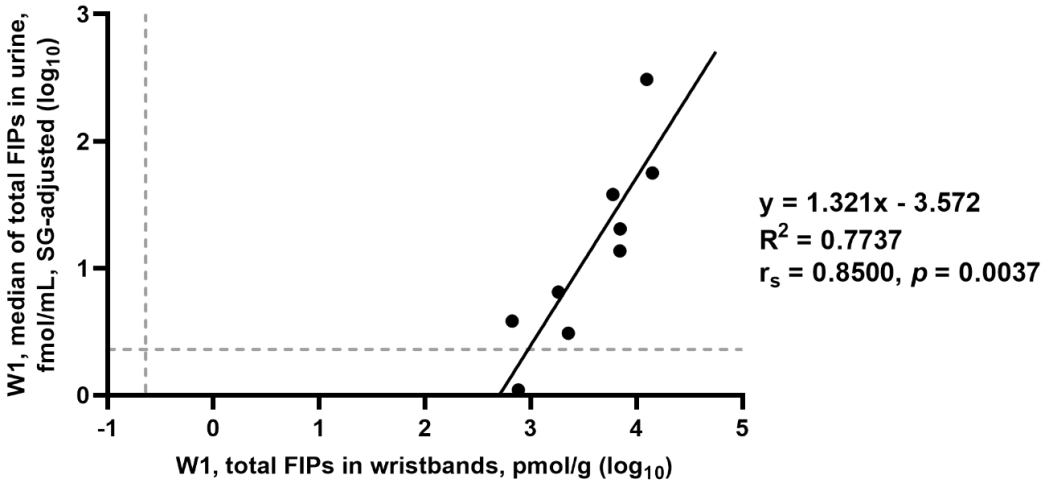

Figure 4.22 Correlation of median sum of FIPs in urine (fmol/mL) and in SWBs (pmol/g) during Week 1. Grey horizontal line represents log-transformed urinary LLOQ of FIP expressed in fmol/mL, whereas the vertical one shows LLOQ of FIP in SWBs (pmol/g, log-transformed). The datapoint below the horizontal line was obtained using the urinary concentrations between LOD and LLOQ.

4.3.5 Dose reconstruction and risk assessment

FIP exposure on the day of ectoparasiticide application in study participants who used a FIP-containing product on their pets, calculated using both FIPs-based and PYRs-based scenario, is shown in Figure 4.23. In the case of the latter approach, only the results basing on *cis*/*trans*-DCCA are shown and discussed, as they represented the worst-case scenario. It is also worth noting that although the permethrin/FIP ratio declared by the ectoparasiticide manufacturer was used in that model (8.437), the ratio observed in SWBs (8.890) would have given very similar results. This fact highlights the usefulness of SWBs in exposure modeling. The exposures shown in Figure 4.23 and discussed below may be underestimated due to no morning void samples collected from the day after ectoparasiticide application. Nevertheless, in author's opinion, the overall picture would have been similar.

Regardless of the dose reconstruction approach, a wide range of exposures was observed, spanning over two orders of magnitude. The FIPs-based scenario tended to produce lower estimates than the PYRs-based scenario, except for three participants. When only the participants with non-zero exposure to both FIPs and PYRs were considered, the median exposure was 1.46% ADI and 31.4% ADI for FIP-based and PYRs-based scenario, respectively (all values will be given in that order). Maximum exposure was 98% and 274% ADI (2% and 6% ARfD, respectively). Despite using independent data, the estimates from both scenarios were usually within the same order of magnitude. While it is difficult to determine which model produces results closer to the real exposure, it should be noted that the PYRs-based scenario is more informed since it uses human pharmacokinetic data and the known permethrin/FIP ratio in spot-on products, which was confirmed by SWBs analysis. At the same time, it still relies on assumption that the absorption of both insecticides is the same, while there is no human FIP data available to support this claim. On the other hand, the FIPs-based model is built on urinary levels of actual biomarkers of FIP exposure, which certainly is an advantage. However, this scenario heavily relies on the fraction of the dose excreted with urine in the rat, which greatly undermines its credibility. The use of human pharmacokinetic data would certainly improve this model.

The exposure data obtained here can be compared to the results of consumer health risk assessment associated with consumption of FIP-contaminated poultry products during the so-called "fipronil incident" of 2016-2017 (see section 1.1.1). According to the data of (Bundesinstitut für Risikobewertung, 2017), the maximum FIP intake for adults ranged from 2% to 51% ARfD, depending on food commodity and model selection. Interestingly, these values are similar to the maximum exposures observed here (2% and 6% ARfD, depending on the model). Taking into account the popularity of pet ownership in EU and other areas (see section 1.2), such exposure might be relatively common.

However, since the maximum observed exposure did not exceed 6% ARfD, the acute health risk in the studied population can be considered low. In several cases, the exposure was close to or more than 100% ADI, suggesting a chronic health risk. However, the samples used for risk assessment were collected on the day of ectoparasiticide application, which is supposed to be performed only once every four weeks. Therefore,

lower exposure can be expected on the days/weeks that followed. This assumption is confirmed by the urinary profiles of FIPs shown on Figure 4.15. Consequently, the chronic health risk after a single application can also be considered low. The same figure, however, shows that the concentrations of FIPs in urine on the 28th day since application did not reach the baseline observed at the beginning of experiment, suggesting possible accumulation of FIP burden after repeated application, as discussed in section 4.3.2. Further research is necessary to investigate the chronic health risk associated with repeated ectoparasiticide application.

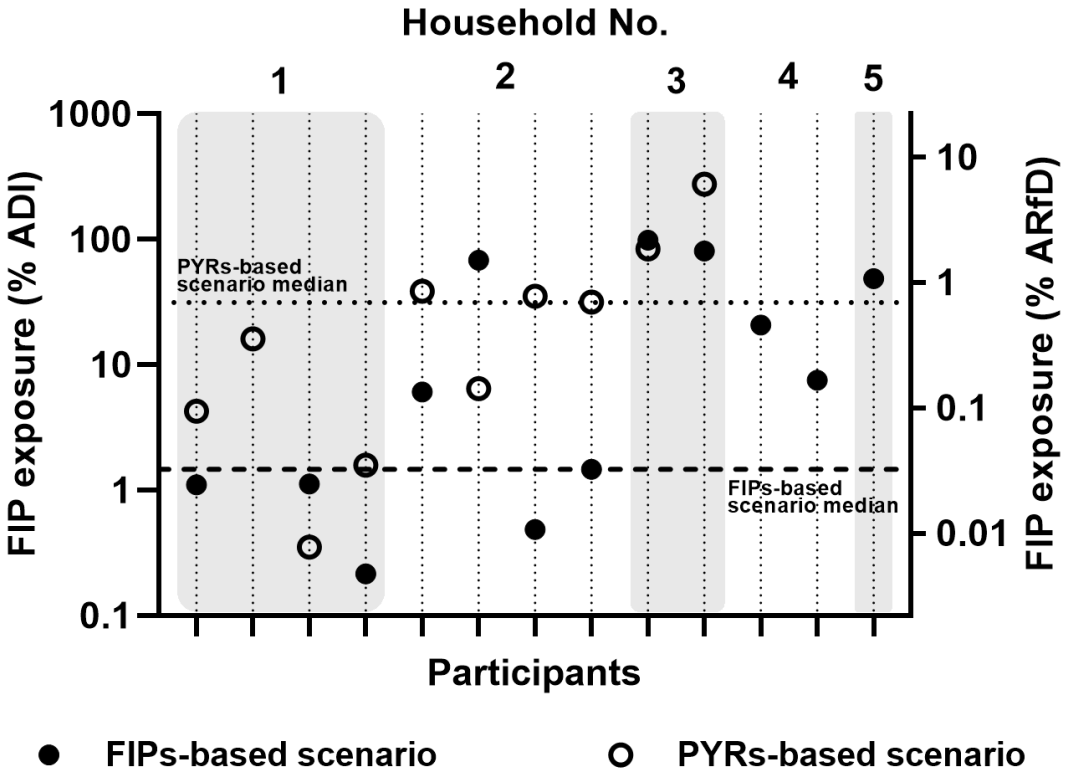

Figure 4.23 FIP exposure (relative to ADI and ARfD) in study participants on the day of ectoparasiticide application on household pets calculated using the FIPs-based and PYRs-based scenarios. The grey and white areas on the graph are only used to group participants living in the same household. In one participant from household no. 2, no FIPs or PYRs were detected, whereas in households no. 4 and 5 permethrin was not applied, so the PYRs-based exposure assessment to FIP was not conducted. The medians were calculated only for the participants with non-zero exposure in both scenarios. ADI, acceptable daily intake; ARfD, acute reference dose.

A correlation between both exposure scenarios is shown in Figure 4.24. Although both models used independent data, a strong association was observed between them (r_s = 0.6833, p = 0.0503). While it is difficult to tell which model gives better approximations of the real exposure (see discussion above), the correlation presented here shows that FIPs and PYRs quantified in urine originate from the same source – the ectoparasiticide application on household pets.

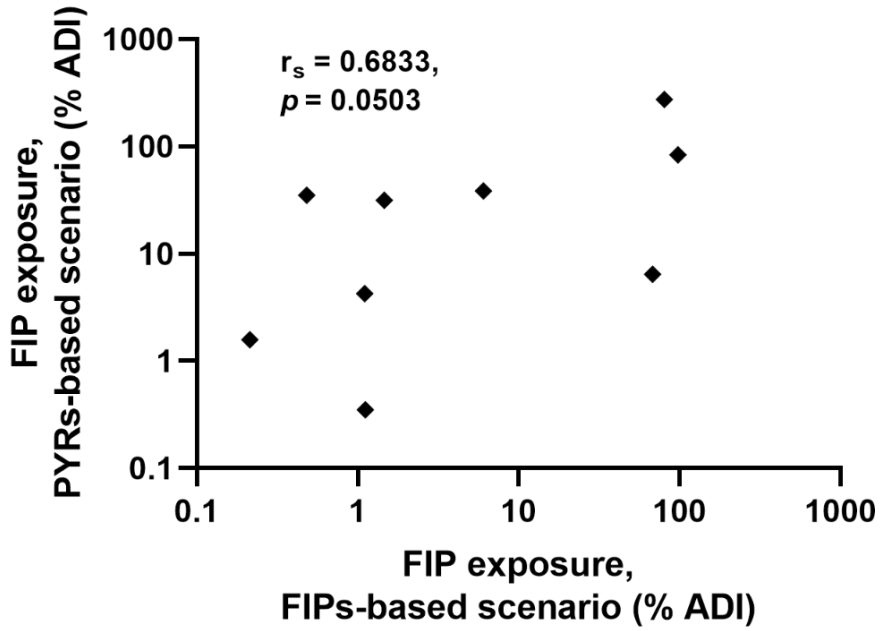

Figure 4.24 Correlation between the FIPs-based and PYRs-based scenarios. Datapoints with a zero value in either scenario were not included in the analysis.

4.3.6 Strengths and limitations

The study described here had several advantages. Simultaneous use of biological and environmental monitoring allowed to provide comprehensive data on the route and magnitude of FIP exposure. The high sensitivity of analytical methods for both matrices allowed to shed new light on the subject in comparison to hitherto published literature. Finally, the longitudinal design of the study, involving sample collection both before and after spot-on application, as well as the inclusion of negative control, allowed to demonstrate a causative link between ectoparasiticide use on pets and FIP exposure in humans.

The limitations of this study should also be addressed. Although several FIPs were included in the analytical method for SWBs, there are other FIP-related derivatives, such as FIP-monochloro, that were not included in this work, despite being detected in other environmental studies (Starr et al., 2016). In consequence, the picture presented here might not be complete. Furthermore, failure to establish FIP-hydroxy as a urinary biomarker of exposure (section 4.1.2) necessitated the use of parent compound (FIP) and its metabolite/environmental degradate (FIP-sulfone) as the biomarkers of internal exposure, which posed a risk of external contamination, especially during sample collection. Such a situation, although undesirable, is not uncommon. Human biomonitoring of bisphenols (Markham et al., 2010), pyrethroids (Sudakin, 2006), phthalates (Koch et al., 2003), and parabens (Moos et al., 2016) often faces the same challenges. Additionally, frequent non-detects for FIPs in urine along with a limited number of participants hindered some comparisons, particularly evident in marginally significant changes in urinary FIPs after ectoparasiticide application (Figure 4.16). Finally, the lack of human FIP toxicokinetics data necessitated establishing a model relying on animal data (FIPs-based scenario) or human pharmacokinetics of permethrin, which was applied in parallel to FIP in most cases (PYRs-based scenario).

5 BROADER CONTEXT AND FUTURE PERSPECTIVES

This work consisted of three main parts. The first one was devoted to the method development and validation for FIPs quantitation in urine. Solid phase extraction was used for sample cleanup and preconcentration followed by separation and detection by LC-MS/MS. The second part was focused on the method development for determination of FIPs in SWBs. Sample preparation workflow included solid-liquid extraction, followed by solvent exchange, freezing-out, and acetonitrile-hexane partitioning. Similarly to the method for urine, LC-MS/MS was used for analysis of the final extract. A validation study was conducted as well. The third, final part of the thesis used both analytical methods mentioned above to quantify human exposure to FIPs and conduct risk assessment associated with ectoparasiticide application on household pets. Insights into FIPs exposure routes as well as environmental fate were also provided. A discussion on the broader context of the main findings of this thesis and an outline of future perspectives are provided below.

The biomonitoring method included FIP, FIP-amide, FIP-desulfinyl, FIP-dtfms, FIP-sulfide, and FIP-sulfone (Table 3.5) as target compounds. Urine was chosen as a matrix since it can be collected in large quantities and in a non-invasive way (Yusa et al., 2015), which was very helpful in the human exposure study performed later on. An attempt was made to include FIP-hydroxy in the method as well. This compound is a promising urinary biomarker of FIP observed in considerable quantities in rats (Vasylieva et al., 2017). Since it is not formed in the environment, it could be used to unequivocally distinguish between internal and external exposure. In addition, relatively high urinary excretion of FIP-hydroxy could have made it easier to determine FIP exposure by urinalysis. However, chemical stability issues rendered this task impossible (see section 4.1.2). To the author's knowledge, there is still no published paper confirming applicability of FIP-hydroxy in human biomonitoring, despite several papers on human FIP exposure being published since 2017 (Chen et al., 2022).

Sample preparation of urine samples involved enzymatic hydrolysis followed by SPE, which is one of the typical approaches for trace urinalysis (Yusa et al., 2015). Despite large sample volume (5 mL) and use of exhaustive extraction technique, the achieved sensitivity turned out to be a limiting factor during the human exposure study. Therefore, a method transfer to a more sensitive instrument is planned in the future. Additionally, an oral FIP exposure study conducted on healthy volunteers should be conducted in order to investigate FIP toxicokinetics in humans and search for a more suitable urinary biomarker of FIP than FIP-sulfone or FIP itself. Finally, since FIP has been shown in several animal and in vitro studies to induce oxidative stress (X. Wang et al., 2016), it would be interesting to investigate a causal link between FIPs levels in biological matrix from human subjects and concentrations of oxidative stress biomarkers in the same samples; however, development of a separate analytical method would probably be necessary.

The same FIP-related target compounds were included in the analytical method for SWBs. In the hitherto published literature, LC-MS/MS is rarely used for SWBs analysis (Wacławik et al., 2022). To the author's knowledge, this is the first method for SWBs

that uses LC-MS/MS and provides efficient matrix removal (70% compared to no cleanup).

However, the sample preparation process can still be improved. For instance, omitting the freezing-out step would speed up the procedure significantly. Before that, its effect on sample cleanup efficiency will be determined. Additionally, the wide scope of the method will allow to add more analytes in the future, including the compounds not amenable for GC-MS analysis. Before that happens, an improved chromatographic separation is needed to maintain the same sensitivity of the instrumental method despite the addition of new analytes.

As the final part of the thesis, a human exposure and risk assessment study following FIP-based spot-on ectoparasiticide application on household pets was conducted. In the 1990s, the development of new formulations and active ingredients, including FIP, revolutionized the treatment of ectoparasite infestation in companion animals. Before their introduction to the market, a comprehensive indoor and outdoor treatment of animal's surrounding was necessary in order to control the ectoparasites. This approach was associated with increased risk of human and environmental exposure (Carlotti & Jacobs, 2000; Smith, 1995). Thanks to the aforementioned advances in therapy, the paradigm changed to animal treatment only (Rust, 2005). New approaches to ectoparasiticide application, such as the widespread use of spot-on products, warranted generation of new data on human risks involved (Driver et al., 2015). From the 2000s onward, several studies were conducted in order to determine the transferability and/or human exposure to insecticides present in topical products used on household pets (Bland et al., 2013; Boone et al., 2001, 2006; J. E. Chambers et al., 2007; M. S. Craig et al., 2005; Davis et al., 2008; Driver et al., 2014, 2015; Dyk et al., 2011; Gupta et al., 2005; Wrobel et al., 2023), including FIP (APVMA, 2009; Cochran et al., 2015; Dyk et al., 2012; Jennings et al., 2002). The growing body of literature allows to draw more generalized conclusions and discuss future needs in the field.

Human biomonitoring was performed in only a handful of such studies (J. E. Chambers et al., 2007; Davis et al., 2008; Driver et al., 2015; Dyk et al., 2011; Wrobel et al., 2023). Although not required either by American or European regulatory agencies when introducing a new product to the market (EMA & CVMP, 2018; EPA, 2012), the use of biomonitoring would provide the most accurate data on human exposure (Sobus et al., 2011). In the aforementioned biomonitoring studies, several different insecticides were used and the results were inconsistent, arguably due to their varied experimental design or shortcomings thereof. For instance, some of the studies recruited just one or a few participants (Dyk et al., 2011; Wrobel et al., 2023), so the statistical significance of the observed increases in biomarker levels after application was not investigated. In two other papers, a collar instead of a spot-on product was used (J. E. Chambers et al., 2007; Davis et al., 2008); drastically different kinetics of these two formulations make comparisons difficult. The other studies relied on environmental media, most commonly cotton gloves that were used to stroke the treated animal in a predefined manner. These experiments had more consistent design: the transferability of insecticide residues present on animals' fur was usually determined by petting the animal with cotton gloves a day after ectoparasiticide application and then once a week for a period of 28 days total; a sample collected before application was used as a baseline. A variety of active ingredients were

tested: chlorpyrifos, phosmet, selamectin, imidacloprid, tetrachlorvinphos, etofenprox, indoxacarb, cyphenothrin, and FIP. In all studies where a spot-on product was used (APVMA, 2009; Bland et al., 2013; M. S. Craig et al., 2005; Driver et al., 2014; Dyk et al., 2012; Gupta et al., 2005; Jennings et al., 2002), the peak levels of the insecticide transferred to a glove was observed within 24 hours since application. This observation is intuitive since spot-on products release the entire dose in a single timepoint (see section 1.2). It is also in line with the results of the biomonitoring study performed here, where a peak in urinary FIPs among study participants was observed on the day of application (see Figure 4.15). In the cotton gloves collected four weeks after applications, the levels of the active ingredients were substantially lower. The rate of decline, however, varied considerably between studies. For instance, in Jennings et al., 2002, the amount of FIP collected with gloves four weeks since ectoparasiticide use corresponded to 22% of the amount collected a day after application, suggesting a risk associated with repeated application in four-week intervals. Conversely, in Dyk et al., 2012, only 2% of the maximum amount was quantified in the gloves collected four weeks post-application, even though FIP was determined along with its environmental degradates in that study. These discrepancies may reflect the problems associated with using gloves as passive dosimeters, such as the variability and reproducibility of the sampling procedure (Boone et al., 2001, 2006). Interestingly, Dyk et al., 2012 was the only study on pet-human insecticide transferability published so far that monitored not only the parent compound, but also its relevant degradates. The amount of degradates relative to the parent compound observed in that study increased over time – in the case of dogs, the degradates corresponded to 11% of total FIPs collected on the gloves after 24 hours since application, whereas after four weeks this fraction increased to 36%. The results obtained in the present work using SWBs (section 4.3.3) also show that the degradates may represent a substantial proportion of the total FIPs. In consequence, both studies stress the importance of comprehensive analytical tools for reliable exposure assessment. However, the rate of the parent compound degradation may vary for different active substances; environmental factors, such as climate and the time of the year during application, can also be expected to affect this process.

Future directions related to the human exposure study should also be discussed. Since no human data on FIP pharmacokinetics are available, animal data had to be used in the dose reconstruction scenario relying on urinary FIPs (FIPs-based scenario; section 3.4.5). A volunteer study on FIP pharmacokinetics in humans would provide data on urinary excretion of FIPs, which would improve the accuracy of exposure assessment. Another matter to consider in the future is performing an exposure assessment experiment associated with ectoparasiticide use on household pets in other populations. In the present study, only adolescents and adults were recruited (Table 4.18) and the FIP exposure was low (Figure 4.23). However, taking into account that children may be more exposed to insecticides used in residential setting due to their hand-to-mouth activity, poor hygiene, underdeveloped detoxication system, and a higher surface area-to-volume ratio (Boone et al., 2006; Cochran et al., 2015; Davis et al., 2008), conducting a similar study in that age group seems warranted. Documents released by regulatory agencies strongly support this claim (EMA & CVMP, 2018; EPA, 2012). Development and efficiency assessment of additional safety measures to limit the exposure associated with

the use of ectoparasiticides should also be considered. For instance, wearing gloves during application is not explicitly recommended by the manufacturers (Dyk et al., 2011; Merial, 2009; Wrobel et al., 2023). This problem was not investigated here due to sample size limitations. Scarce data suggests that collars cause lower human exposure compared to other topical products (Dyk et al., 2011). However, the availability of FIP-containing collars for companion animals is poor (see Introduction). The currently observed shift towards animal treatment using systemic insecticides at the expense of topical formulations can be expected to diminish human exposure even further (Selzer & Epe, 2021), although literature is lacking to confirm that claim. Finally, another data gap that should be filled is the magnitude of exposure to insecticides among veterinarians and other veterinary staff, who work with or close to ectoparasiticide-treated companion animals on a daily basis. To the author's knowledge, no study on that subject has been published so far. Bearing in mind that many animals are handled in a veterinary practice on any given day, the occupational exposure may be much higher than observed in any study on pet owners.

6 SUMMARY

- An analytical method for FIPs determination in urine, based on sample cleanup and preconcentration using SPE followed by separation and detection with LC-MS/MS was developed. The method was validated and its performance parameters were satisfactory.
- A separate method for FIPs determination in SWBs was developed. Sample preparation workflow included solid-liquid extraction, followed by solvent exchange, freezing-out and acetonitrile-hexane partitioning. Similarly to urine method, LC-MS/MS was used for analysis of the final extract. The method parameters obtained during validation process were sufficient for its intended purpose.
- The two aforementioned methods were used to analyze the samples collected in a human exposure study, where FIP-based spot-on products were applied on their pets. Both urine and SWBs were collected before and after ectoparasiticide use.
- The urinalysis revealed a substantial effect of the application on levels of FIPs observed in comparison to concentrations observed before ectoparasiticide application. Two dose reconstruction approaches were used; both indicated a low risk of acute and chronic health effects in the studied group. However, the decline in FIPs levels after application was slow, posing a risk of accumulation after repeated use of the product.
- Analysis of SWBs highlighted the importance of dermal route in FIP exposure resulting from spot-on use on the pet. Moreover, the environmental fate of FIP following the application was investigated. Finally, using combined data for FIPs and co-applied permethrin, the spot-on products were identified as the main source of FIP exposure in the studied population.
- Comparison of FIPs levels in urine and SWBs showed close correlation, proving relevance of the latter for estimation of internal exposure and/or selection of the most exposed individuals.

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- Some results of this study were presented at the 12th International Symposium on Biological Monitoring in Occupational and Environmental Health (June 21-23, 2023, Porto, Portugal) and the 14th Scientific and Training Conference of the Polish Society of Toxicology (September 4-6, 2024, Poznań, Poland).
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