MEASUREMENT OF THE INHIBITION OF CYTOCHROME P450 ENZYMES BY PYRETHROID PESTICIDES

By

NICOLE RENKEL

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Brian Buckley

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ABSTRACT OF THE THESIS

Measurement of the Inhibition of Cytochrome P450
Enzymes by Pyrethroid Pesticides

By NICOLE RENKEL

Thesis Director: Brian Buckley

Pyrethroids are a class of semi-volatile organic compounds (SVOCs) ubiquitously used as pesticides. These compounds are known to be metabolized by hydrolytic, oxidative, and conjugative means. The oxidative pathway is performed by cytochrome P450 enzymes (CYPs), which exhibit an overlap in having pyrethroids as substrates. This metabolic overlap produces a risk for enzyme inhibition, providing the focus of this study. It was hypothesized that pyrethroids would inhibit CYPs because metabolism of pyrethroids is shared across these critical pathways. Wistar rat liver microsomes (RLMs) were incubated with a spectrophotometric substrate, 7-ethoxyresorufin (7-ER), which is metabolized to resorufin via ethoxyresorufin O-deethylation (EROD), to measure CYP1A activity. In order to investigate potential gender differences in CYP profiles, all treatments were replicated in untreated male and female rat liver microsomes (RLMs). Measurement of EROD in male RLMs produced a K_m of 9.83 ± 5.56 μM and a V_{max} of 0.44 ± 0.14 nmol/min/mg protein, while female RLMs produced a K_m of 7.84 ± 0.79 μM and a V_{max} of 0.46 ± 0.02 nmol/min/mg protein. Co-incubation of 7-ER with permethrin or cypermethrin were used to determine inhibition concentrations. Upon addition of permethrin, male

RLMs experienced up to 20% reduction in reaction velocity, while the female RLMs experienced up to 30% reduction. Male RLMs produced less than 10% reduction in reaction velocity when treated with cypermethrin, while the female RLMs produced up to 20% reduction. Subsequent incubations used combinations of permethrin and cypermethrin to determine if there was an additive effect. Combinations produced reaction velocities with non-significant reductions when compared to control, all were within 10%, in both the male or female RLMs. Pyrethroids inhibited CYP1A activity as measured by EROD and the female RLMs seem to be more sensitive to this inhibition. Despite co-administration of permethrin and cypermethrin, even at the highest individual inhibition concentrations, the inhibition produced was not additive, disproving the hypothesis.

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LIST OF ABBREVIATIONS

CYP	Cytochrome P450
SVOCs	Semi-volatile organic compounds
DDT	Dichloro-diphenyl-trichloroethane
LD50	Lethal dose to 50% of population
ppb	Parts per billion
ppm	Parts per million
ADI	Acceptable daily intake
3-PBA	3-phenoxybenzoic acid
CE	Carboxylesterase
DBCA	*Cis* 3-(2,2-dibromovinyl)-2,2-dimethylcyclopropane carboxylic acid
NADPH	Energetic cofactor for enzymes
GC	Gas chromatography
ITMS	Ion trap mass spectrometry
MS/MS	Tandem mass spectrometry
m/z	Mass-to-charge ratio
EIM	Extracted Ion Monitoring
SIS	Selective Ion Storage
RLM	Rat liver microsome
Q ions	Quantification and qualification ions
SIS	Selective Ion Storage
S/N	Signal-to-noise ratio
DL	Detection limit
LOQ	Limit of quantification
CV	Coefficient of variance
7-ER	7-ethoxyresorufin

1.0 GENERAL INTRODUCTION

1.1 Pyrethroid Development

Pyrethroids are synthetic semi-volatile organic compounds (SVOCs) and analogs of pyrethrins; originally derived from *Christanthenum* extract (examples in Figure 1.1). The instability of the natural pyrethrins lead to the synthesis of photostable synthetic pyrethroids, which have the same insecticidal potency and low mammalian toxicity (Casida and Quistad, 1998, DeMicco, et al., 2010, Du, et al., 2013, Nasuti, et al., 2003, Scollon, et al., 2009, Tange, et al., 2014). Though there are many compounds that fall under the heading of pyrethroids, two classes exist, type I and type II, and they are differentiated by the presence of a cyano group at the α carbon and mechanisms of action (ATSDR, 2003).

Pyrethroids are an attractive alternative to organochlorine pesticides, such as dichloro-diphenyl-trichloroethane (DDT), which both bioaccumulate and biomagnify in living organisms and systems (Nasuti, et al., 2003). They are also more popular than organophosphate or carbamate pesticides, both of which alter essential acetylcholinesterase functionality and have longer half-lives (ATSDR, 2003, ATSDR, 1997, ATSDR, 2003, Sheets, 2000). Due to the phase-out of other pesticide classes, pyrethroids have become widely used worldwide in residential, agricultural, medicinal, and commercial settings (DeMicco, et al., 2010, Scollon, et al., 2009, van den Berg, et al., 2012, Willemin, et al., 2015, Yang, et al., 2009).

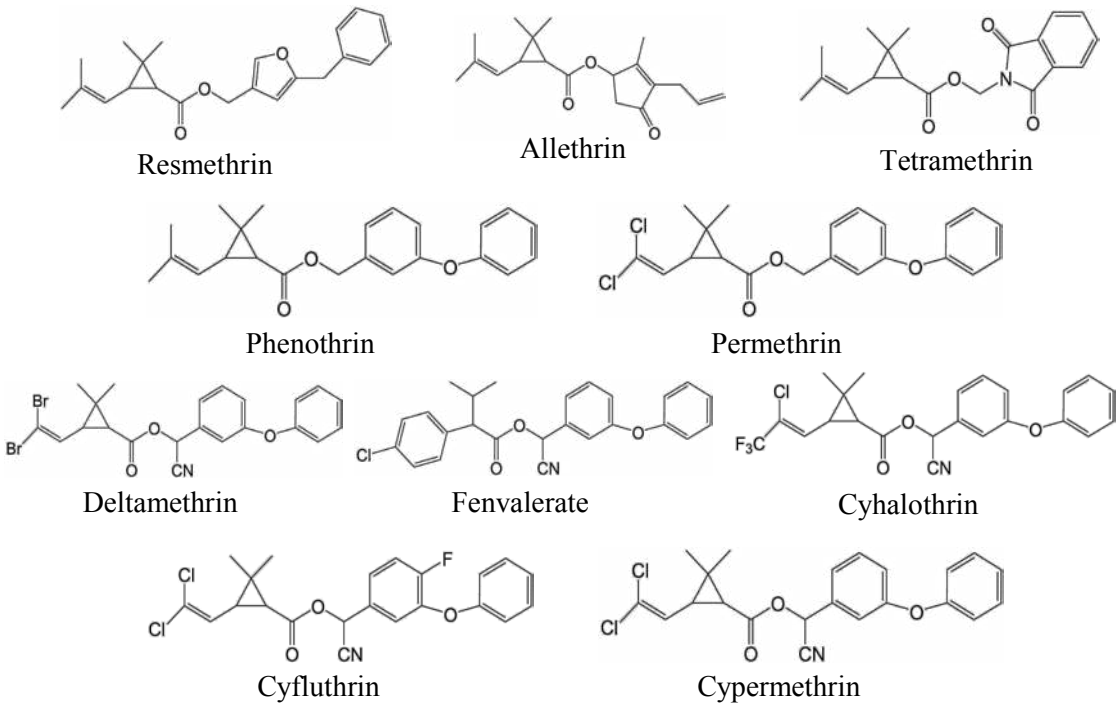


Figure 1.1 Chemical structures of ten pyrethroids. The five pyrethroids in the top half are Type I pyrethroids, while the five in the bottom half are Type II pyrethroids.

Insecticidal potency and efficiency is provided by receptor site specificity within insect species (DeMicco, et al., 2010, Du, et al., 2013). Additionally, the inability of insects to biotransform pyrethroids effectively allows the biologically active neurotoxic parent molecules to exhibit their insecticidal properties (Godin, et al., 2007, Lavado, et al., 2014, Nakamura, et al., 2007, Scollon, et al., 2009, Tange, et al., 2014, Yang, et al., 2009). The most commonly used pyrethroids include λ-cyhalothrin, deltamethrin, cypermethrin, bifenthrin, and α-cypermethrin (Kaneko, 2011). Current formulations are made with single pyrethroids, such as Flea, Tick and Lice Killer with Odor Neutralizer from HotShot®, or mixtures, like Hero® from FMC Corporation, possibly leading to diminished metabolic capacity along shared detoxification pathways (ATSDR, 2003).

1.2 Routes of Exposure

Humans are exposed to pyrethroids by all common routes (Figure 1.2). Dietary, especially fruits, vegetables, and drinking water, and non-dietary pathways, including hand to mouth, are the most prominent routes for ingestion (ATSDR, 2003, Attfield, et al., 2014, Barr, et al., 2010, Lu, et al., 2000, Riederer, et al., 2008). Dermal absorption of pyrethroids can be observed, albeit dependent on species and vehicle (ATSDR, 2003, Attfield, et al., 2014, Barr, et al., 2010, Kaneko, 2011, Lu, et al., 2000, Riederer, et al., 2008). Examples of dermal exposure include: occupational and accidental exposure from pesticide application; accidental exposure to treated surfaces or pesticide sinks; during medicinal and veterinary treatment of lice, scabies, or fleas; or from clothes and furniture exposed to pyrethroids (ATSDR, 2003, Attfield, et al., 2014, Barr, et al., 2010, Lu, et al., 2000, Riederer, et al., 2008). Due to their semi-volatile nature, pyrethroids can be found in agricultural drift and

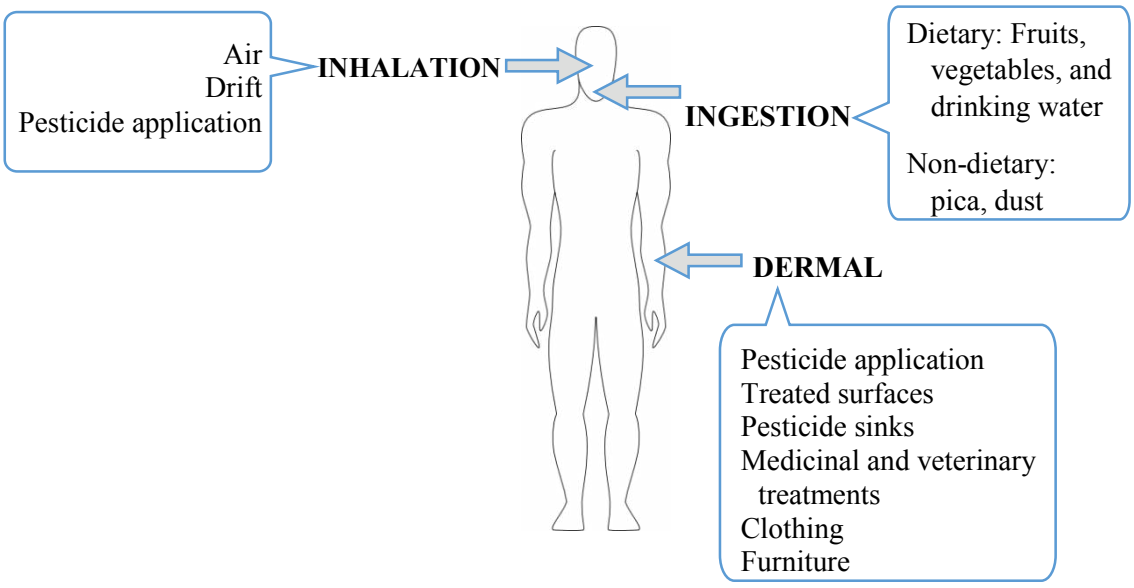


Figure 1.2 The routes of pyrethroids exposure. Each route, inhalation, ingestion, and dermal contact, has several exposure scenarios that people can experience.

the air in the vicinity of applications, all of which lead to inhalation exposure (ATSDR, 2003, Attfield, et al., 2014, Barr, et al., 2010, Lu, et al., 2009, Lu, et al., 2000, Naeher, et al., 2010, Riederer, et al., 2008, Trunnelle, et al., 2014, Wielgomas, 2013).

1.3 Targets and Effects

The primary target of action for pyrethroids is the α subunit of voltage-gated sodium (Na^+) channels, which experience altered functioning with some stereospecificity (Anand, et al., 2006, Bass, et al., 2004, Casida and Quistad, 1998, DeMicco, et al., 2010, Kaneko, 2011, Nakamura, et al., 2007, Nasuti, et al., 2003, Yang, et al., 2009). A plethora of secondary toxic effects (Enan and Matsumura, 1993, Kaneko, 2011, Lavado, et al., 2014, Nakamura, et al., 2007, Nasuti, et al., 2003, Ray and Fry, 2006, Tange, et al., 2014, Yang, et al., 2009), ranging from chromosomal damage (Ray and Fry, 2006) to hormonal effects (Tange, et al., 2014), have been investigated in the last two decades.

Nerve impulse transmission is affected by pyrethroid binding to the sodium channels, primarily in axons within the peripheral and central nervous systems (Tange, et al., 2014). Type I pyrethroids prolong the initial opening of the channel and induce repetitive firing of action potentials, sending electrical pulses along neurons (Du, et al., 2013, Nasuti, et al., 2003). Though they also keep the channel open, type II pyrethroids cause the membrane to depolarize, decreasing the ability to generate action potentials over time (Du, et al., 2013, Nasuti, et al., 2003). Alterations to neuron membrane potentials are extremely detrimental due to the importance of action potentials. The clinical manifestations, known as T syndrome and CS syndrome, are named after their primary symptom(s). The symptomatic

manifestations of type II pyrethroids corroborate the increased potency of the cyano group (DeMicco, et al., 2010, Sheets, 2000, Yang, et al., 2009) (Table 1.1). Type I pyrethroid intoxication induces T syndrome, named for fine body and progressive whole body tremor (ATSDR, 2003, Soderlund, et al., 2002). CS syndrome, usually seen with type II pyrethroid intoxication, is named for salivation and progressive choreoathetosis, which is a combination of irregular migrating contractions, twisting, and writhing. This syndrome also manifests with coarse tremor, clonic seizures, pawing, and burrowing in mammalian studies (DeMicco, et al., 2010, Nasuti, et al., 2003, Scollon, et al., 2009, Soderlund, et al., 2002, Yang, et al., 2009).

In general, acute poisonings have been observed with additional symptoms: headache, dizziness, skin and nose irritation, and nausea (Nakamura, et al., 2007, Tange, et al., 2014). In dermal exposure, the primary effect is paresthesia, denoted by continuous tingling or pricking, and burning sensations at the site of contact (Narahashi, 1996). Effects are reversible, dissipating in roughly 24 hours, and can be treated with topical vitamin E or oil (Narahashi, 1996, Ray and Fry, 2006).

1.4 Insect Susceptibility

Insects are extremely susceptible to pyrethroid action due to their highly sensitive Na^+ channels (Yang, et al., 2009) and lower capacity for biotransformation (Nakamura, et al., 2007). Depending on the pyrethroid the LD50 for insects ranges from 0.015 – 0.25 ng/mg. However, when a synergist, such as piperonyl butoxide, is added the LD50 drops at least

Table 1.1 Symptoms of pyrethroid intoxication. (ATSDR, 2003, DeMicco, et al., 2010, Kaneko, 2011, Nasuti, et al., 2003, Scollon, et al., 2009, Soderlund, et al., 2002, Yang, et al., 2009).

Type I Pyrethroids	Type II Pyrethroids
Fine body tremor	Salivation
Progressive whole body tremor	Progressive choreoathetosis
Behavioral arousal	Coarse tremor
Aggressive sparring	Clonic seizures
Increased startled response	Pawing
Prostration	Burrowing
Increased stimulus response	

10-fold (Elliott, 1971). When the insects are in the larval stage, this value drops lower still to 1 ppb (Bradbury and Coats, 1989). This could result from their low capacity for metabolism and makes young animals more likely to encounter neurodevelopmental effects as demonstrated in neonatal rat and zebrafish studies (DeMicco, et al., 2010, Nakamura, et al., 2007, Sheets, 2000). Pyrethroids are highly toxic to aquatic life, with LC50s as low as 0.5 ppb, but do not seem to have a similar effect on avian species (DeMicco, et al., 2010, Miyamoto, 1976).

1.5 Human Exposure and Vulnerability

The acceptable daily intake (ADI) for technical grade permethrin, as set by the World Health Organization (WHO), is 0.05 mg/kg body weight/day, which is 1000-fold higher than the recorded average daily intake of 36 – 71 ng/kg/day (ATSDR, 2003). The ADI for cypermethrin is 0.02 mg/kg body weight/day, which is about half of permethrin. Those that apply pyrethroids, including farmers, applicators, veterinarians and pet groomers, and home gardeners, are at much greater risk for exposure and toxicity. Matrices from applicators around the world have been analyzed to estimate their exposure: urine of Japanese agricultural workers applying permethrin showed 1-5 ng/mL of the metabolite 3-phenoxybenzoic acid (3-PBA), handheld and aerial applicators show an estimated total of 46 – 1140 mg/kg with dermal exposure, and inhalation rate for workers is estimated at 4 µg/hour (ATSDR, 2003).

While mammalian species are relatively unaffected by pyrethroids, *in utero* exposure produced changes in many neurological facets, including neurochemistry, behavior,

learning, and motor activity (Riederer, et al., 2008). An NHANES study found an average of 0.4 ng 3-phenoxybenzoic acid (3-PBA) per mg creatinine in the urine samples of children age 6-10 (Riederer, et al., 2008). Toddlers and children are also vulnerable to heightened negative health outcomes from pyrethroids due to: unique surface interactions; childhood behaviors, including exploration, pica, and hand-to-mouth; and physiological functions, including underdeveloped detoxification mechanisms, can generate greater toxicity (Attfield, et al., 2014, Buckley, et al., 2000, Freeman, et al., 2001, Gurunathan, et al., 1998, Reed, et al., 1999, Riederer, et al., 2008, Xue, et al., 2007).

1.6 Metabolism

Although acutely toxic to insects, mammals generally metabolize pyrethroids into putatively non-toxic metabolites. Pyrethroids are metabolized via Phase I and Phase II pathways; Phase I reactions are thought to include esterases in humans which hydrolyze (Godin, et al., 2007, Nakamura, et al., 2007, Willemin, et al., 2015, Yang, et al., 2009), or cytochrome P450 enzymes that oxidize (Godin, et al., 2007, Nakamura, et al., 2007, Scollon, et al., 2009, Tange, et al., 2014, Willemin, et al., 2015, Yang, et al., 2009). Phase II metabolism conjugates pyrethroids with molecules, such as thiocyanate or cholesterol esters, leaving them to be rapidly excreted (Kaneko, 2011). In rodents and humans the parent molecules are rendered biologically inactive, albeit at different rates and through different species specific isozymes (Godin, et al., 2007, Godin, et al., 2006, Kaneko, 2011, Nakamura, et al., 2007, Scollon, et al., 2009, Tange, et al., 2014, Willemin, et al., 2015, Yang, et al., 2009).

1.6.1 Hydrolysis

Some pyrethroids have been observed to be preferentially metabolized via hydrolysis, a phase I pathway, by carboxylesterases (CEs) (Lavado, et al., 2014). Permethrin, for example, undergoes extensive hydrolysis in rats, mice, and humans when in the *trans* configuration, but limited hydrolysis in the *cis* configuration (Godin, et al., 2006) (Figure 1.3). CEs, found in the liver and small intestine, break the ester linkage leading to separation of the acid and alcohol moieties and possible abatement of the neurological effects associated with pyrethroids (Anand, et al., 2006, Lavado, et al., 2014). Metabolism by the human isozymes, hCE1 and hCE2, has been shown to vary as much as 14 fold due to differences in the rate of hydrolysis (Yang, et al., 2009). Although studies investigated acetylcholinesterase and butyrylcholinesterase for their hydrolytic capacity, neither was found to be a likely contributor to pyrethroid metabolism, potentially due to their small, selective active sites (Ross, et al., 2006).

The major metabolites of pyrethroids have been shown to be the products of ester hydrolysis (Figure 1.4) and are generally regarded as non-toxic (Kaneko, 2011). Metabolites can be specific to one parent molecule, such as DBCA is to deltamethrin, though most are non-specific, such as 3-PBA (Table 1.2).

1.6.2 Oxidation

Alternative Phase I metabolism of pyrethroids leads to oxidation by cytochrome P450 enzymes (CYPs). During this process, CYPs such as CYP2C9 and CYP3A4 alter positions

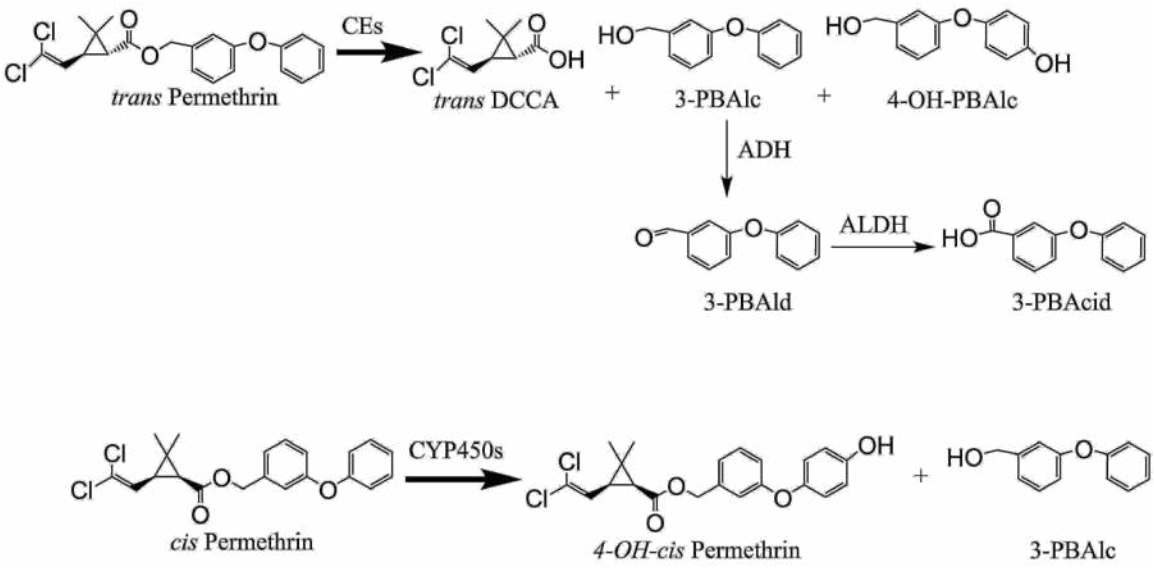


Figure 1.3 Permethrin metabolic pathways. Permethrin can be metabolized by both hydrolytic and oxidative mechanisms. However, the confirmation dictates which metabolic path will be taken. *Cis* permethrin prefers the oxidative path, while *trans* permethrin prefers the hydrolytic path.

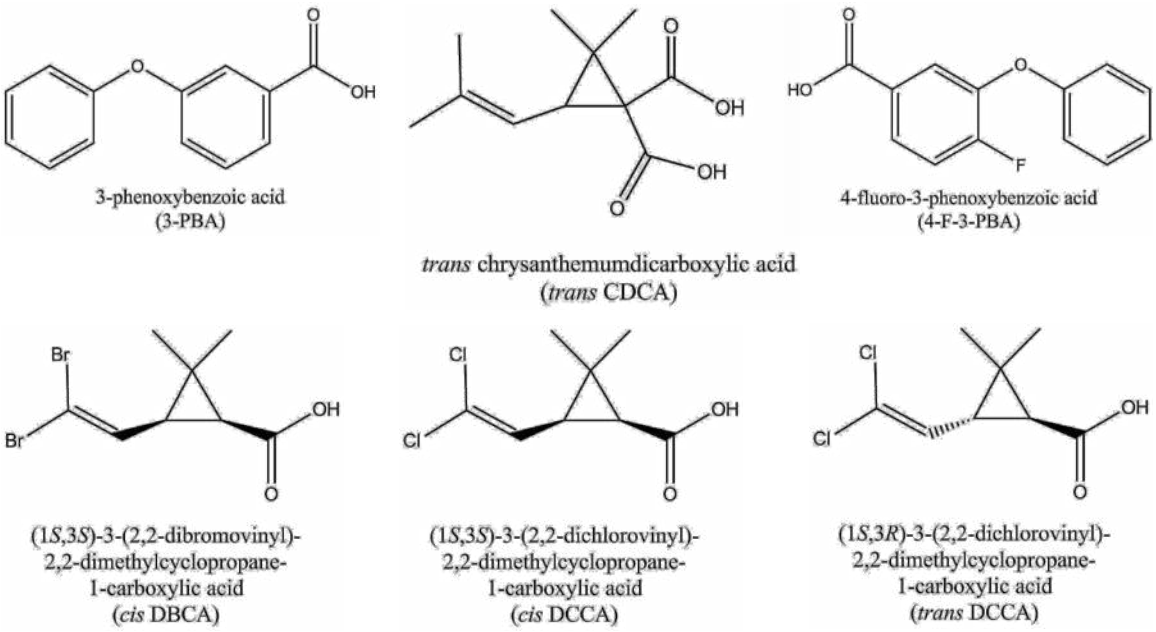


Figure 1.4 Chemical structures of the known hydrolytic pyrethroid metabolites.

Table 1.2 Origin of hydrolytic metabolites. From left to right, the metabolites are chrysanthemumdicarboxylic acid (CDCA), 3-phenoxybenzoic acid (3-PBA), 4-fluoro-3-phenoxybenzoic acid (4-F-3-PBA), 3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid (DCCA), and 3-(2,2-dibromovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid (DBCA).

Pyrethroid	CDCA	3-PBA	4-F-3-PBA	DCCA	DBCA
Allethrin (Barr, et al., 2010)	x				
Resmethrin (Barr, et al., 2010)	x				
Tetramethrin (Barr, et al., 2010)	x				
Phenothrin (ATSDR, 2003)	x	x			
Cyhalothrin (ATSDR, 2003)		x			
Permethrin (Barr, et al., 2010, Ross, et al., 2006, Tange, et al., 2014, Willemin, et al., 2015)		x		x	
Cyfluthrin (Barr, et al., 2010)		x		x	
Cypermethrin (Barr, et al., 2010)			x	x	
Deltamethrin (Anand, et al., 2006, Barr, et al., 2010)		x			x

in the alcohol moiety of pyrethroids (Anand, et al., 2006, Godin, et al., 2007, Kaneko, 2011, Tange, et al., 2014, Willemin, et al., 2015). Alternatively, aromatic hydroxylation can occur, as is the case with deltamethrin where the 2', 4', or 5' position can be hydroxylated (Anand, et al., 2006) (Figure 1.5). These enzymes can vary in specificity due to the size of the active site (Johnson and Stout, 2005). CYP2C19 has been shown to be the most active in metabolizing permethrin, though it is not always the most abundant CYP (Lavado, et al., 2014, Scollon, et al., 2009). The broad substrate range of CYPs allows for multiple enzymes to metabolize a single xenobiotic, as is the case with pyrethroid-metabolizing CYPs.

1.6.3 Conjugation

Phase II conjugation primarily results in increased hydrophilic compounds, though occasionally increases the lipophilicity of compounds. Hydrophilic pyrethroid conjugates, most commonly glucuronides, sulfates, and amino acid conjugates, are easily excreted via urine because of their hydrophilicity (Kaneko, 2011). In contrast, lipophilic pyrethroid conjugates, including cholesterol esters, glyceride, and bile acid conjugates, display a longer retention time leading to a slower excretion (Kaneko, 2011). Certain conjugates are pyrethroid specific, such as thiocyanate for cypermethrin, deltamethrin, and fenvalerate; sulfonic acid for tetramethrin; and cholesterol ester for fenvalerate. 3-phenoxybenzoic acid, one of the major pyrethroid metabolites, has a broad range of amino acid conjugate capability that is also species dependent (Kaneko, 2011).

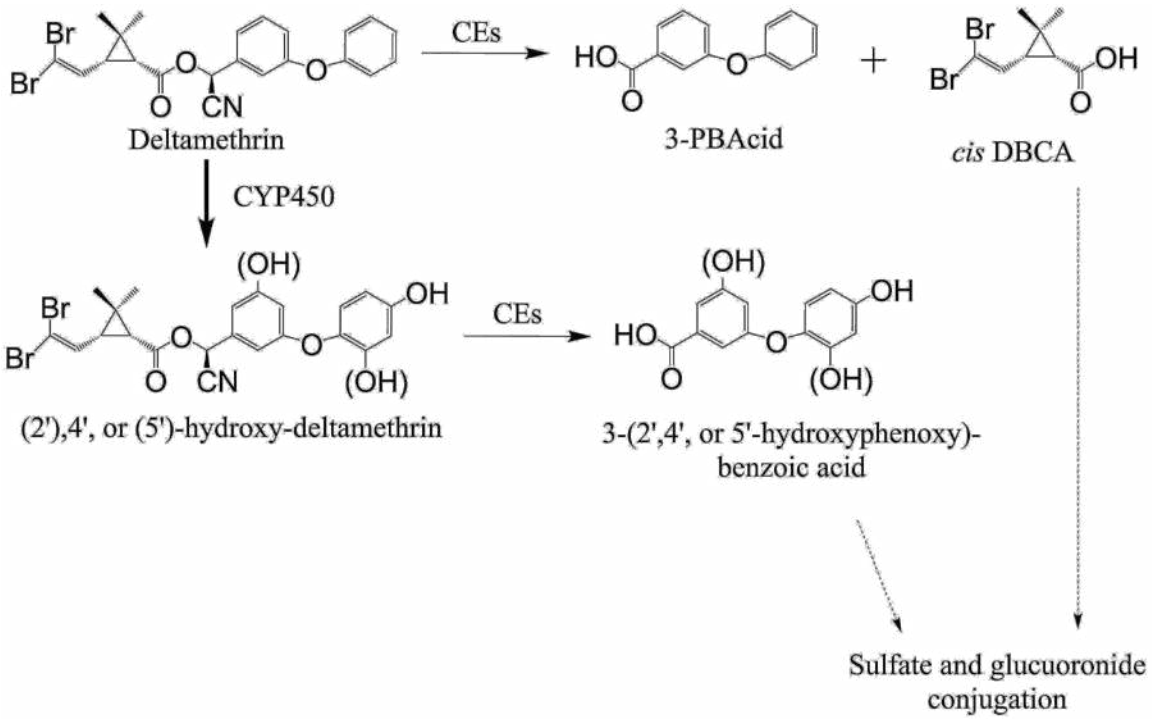


Figure 1.5 Deltamethrin metabolic pathways. Deltamethrin can also follow both hydrolytic and oxidative pathways. The oxidative path is favored, and the oxidative metabolite can be further hydrolyzed and then conjugated to further enhance excretion.

1.6.4 Metabolic Preferences

Pyrethroids have shown metabolic preference by type, configuration, species, and gender. Type I pyrethroids usually have a higher rate of hydrolysis than type II pyrethroids, due to sterics and other physicochemical properties (Yang, et al., 2009). Type II pyrethroids can be metabolized via both Phase I pathways (hydrolysis and oxidation) but have the lowest overall rate (Scollon, et al., 2009).

Trans isomers of type I pyrethroids are more rapidly metabolized, primarily by hydrolysis, while their *cis* counterparts undergo a slower, usually oxidative metabolism. Resmethrin is a good example of this: the *trans* isomer can be 8-14 times more rapidly hydrolyzed than the *cis* isomer, and it exhibits a 70-80% oxidative rate of *cis* (Figure 1.6). However, co-incubation with TEPP, an organophosphate insecticide, shifts metabolism of resmethrin to the oxidative pathway (Soderlund, 2015, Ueda, et al., 1975).

In vitro and *in vivo* studies have shown large differences in rates of hydrolysis and oxidation across rodent and human exposures, possibly due to enzymatic and physical differences (Godin, et al., 2007, Godin, et al., 2006) (Tables 1.3 and 1.4). However, microsomes extracted from rats, mice, and humans have similar rates of hydrolysis (Godin, et al., 2006). This discrepancy may be due to pathway or enzyme preference between species. For example, cypermethrin was shown to be metabolized independent of the presence of NADPH in human liver microsomes, indicating a hydrolytic pathway; however, cypermethrin is only metabolized via oxidation in rat liver microsomes (Scollon, et al., 2009). Human CYP2C8, 2C19, and 3A5 were found to metabolize esfenvalerate and

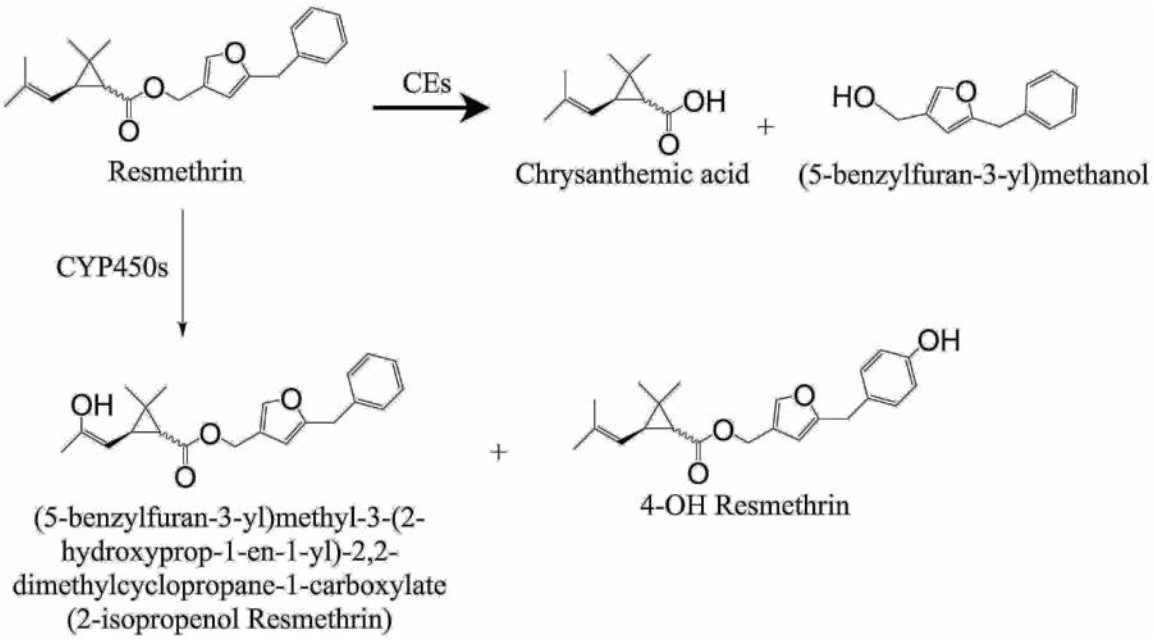


Figure 1.6 Resmethrin metabolic pathways. Resmethrin prefers the hydrolytic pathway, though oxidation is possible.

Table 1.3 Contribution of human CYP isozymes to pyrethroid metabolism.

Pyrethroid	1A1	1A2	2B6	2C8	2C9*1	2C9*2	2C9*3	2C19	3A4
Resmethrin (Scollon, et al., 2009)				x	x	x		**M**	x
Cyhalothrin (Scollon, et al., 2009)	*m*	*m*	*m*	*m*	*m*		*m*	**M**	x
Permethrin (Scollon, et al., 2009)	x	x		*m*	x	*m*		**M**	x
Cyfluthrin (Scollon, et al., 2009)	*m*	x		*m*	x			**M**	x
Cypermethrin (Scollon, et al., 2009)		x		x		*m*	*m*	**M**	x
Deltamethrin (Godin, et al., 2007)				x				**M**	*m*

M = major contributor (>60%), x = moderate contributor (20-60%), *m* = minor contributor (<20%)

Table 1.4 Contribution of rat CYP isozymes to pyrethroid metabolism.

Pyrethroid	1A1	1A2	2A1	2B1	2C6	2C11	2C12	2C13	2D1	2D2	3A1	3A2
Resmethrin (Scollon, et al., 2009)	**M**	x	x		**M**	**M**		*m*		*m*	x	x
Cyhalothrin (Scollon, et al., 2009)	**M**				x	x			x			x
Permethrin (Scollon, et al., 2009)	x	x			**M**	**M**					x	
Cyfluthrin (Scollon, et al., 2009)	**M**		*m*		x		*m*		*m*		x	x
Cypermethrin (Scollon, et al., 2009)	**M**	*m*	x	*m*	x	**M**			*m*	*m*	x	x
Deltamethrin (Godin, et al., 2007)	x	**M**			x	*m*						x

M = major contributor (>60%), x = moderate contributor (20-60%), *m* = minor contributor (<20%)

deltamethrin, although, 2C9 only metabolizes esfenvalerate (Godin, et al., 2007), further indicating some enzyme specificity. Alternatively, general interspecies differences may play a role in the study discrepancies. For example, rodents have a higher metabolic rate than humans and possess a larger CYP content in their liver, allowing for a faster clearance rate (Scollon, et al., 2009).

Solely in terms of oxidation, gender differences have also been established in rats. The three most relevant CYP families to pyrethroid metabolism are 1A, 2C, and 3A. According to data in Long-Evans or Sprague-Dawley rats, males and females both have 1A1 enzymes present in roughly equal proportions, though 1A2 is five-fold higher in females (Ryan and Levin, 2013). CYP2C has many sex-specific enzymes: 2C6, 2C11, and 2C13 are male dominant, while 2C12 is female dominant (Ryan and Levin, 2013). As for 3A, females have not shown expression of either 3A1 or 3A2, but males show expression for 3A2 (Ryan and Levin, 2013) (Table 1.5). Given most of the data are for specific rat strains later in their lifetime, changes from birth are important to investigate. One such study showed females do express 3A2 at birth and after 60 days, hepatic content becomes suppressed by hormones (Kato and Yamazoe, 1992). The human orthologs of the rat CYPs (Table 1.5) do not necessarily show similar abundance as determined from pooled male and female Caucasian and Japanese patients, but prove correlations can be made.

1.7 Regulation of Pyrethroid Metabolism

1.7.1 mRNA Induction

Table 1.5 Difference in cytochrome P450 profiles for enzymes relevant to pyrethroid metabolism.

Rat Liver Microsomes (Ryan and Levin, 2013)			Human Liver Microsomes	
Cytochrome P450	**Male Abundance**	**Female Abundance**	**Cytochrome P450 ortholog (Zerbino, et al., 2018)**	**Abundance (Zanger and Schwab, 2013)**
1a1	10	10	1A1	< 3
1a2	10	50	1A2	17.7 – 65
2c6	300	200	2C9	18 – 116
2c11	300	ND	2C18	NA
2c12	ND	150	NA	NA
2c13	100	ND	NA	NA
3a1	ND	ND	3A4	58 – 146
3a2	50	ND	3A5	3.5 – 4

Induction of CYP mRNA can occur ubiquitously throughout the body (Nishimura, et al., 2003). Although mRNA of pyrethroid-metabolizing CYPs are detectable in all major tissues, the actual proteins are only expressed in a few tissues, ultimately influencing where xenobiotics can be metabolized. Since oxidation is a major metabolizing pathway for pyrethroids, induction of CYP mRNA is a major mechanism leading to pyrethroid metabolism and subsequent detoxification. Pyrethroids act not only as substrates for the CYPs, but also as inducers, which generates a feed-forward loop (Abass, et al., 2012, Lemaire, et al., 2004).

Liver mRNA induction has been investigated for some pyrethroids to explain the mechanism to some degree, typically by using luciferase assays and RT-PCR to measure mRNA while Western blot analysis is used to determine protein levels (Table 1.6). While hepatocytes seem to give lower readings than cell lines due to the ability of the hepatocytes to metabolize pyrethroids via hydrolytic and oxidative means, h1A2, 2A6, and 2B6 all seem to undergo moderate mRNA induction upon exposure, roughly 1-8 fold, regardless of matrix origin. However, h3A4 is highly inducible, with mRNA levels up 40 fold from cypermethrin exposure as determined by RT-PCR analysis (Abass, et al., 2012, Lemaire, et al., 2004). Despite the up-regulation in mRNA, protein expression is regulated by a multitude of factors, making a seemingly simple mechanism rather complex (Zanger and Schwab, 2013). Some pyrethroids may induce CYPs specific to their own metabolism, leading to an increased capacity for metabolism and potentially eliminating their toxicity (Abass, et al., 2012, Lemaire, et al., 2004).

Table 1.6 CYP induction by pyrethroids in cell culture.

Pyrethroid	CYP(s) induced, Fold induction	Culture	Dose
Cypermethrin	3A4, 2 2B6, 2	Human Hepatocytes (Lemaire, et al., 2004)	10 μM
	1A2, 1 - 3 2A6, 2 - 5 2B6, 3 - 7 3A4, 8 - 40	HepaRG line (Abass, et al., 2012)	10 μM, 50 μM
Fenvalerate	3A4, 2 2B6, 2	Human Hepatocytes (Lemaire, et al., 2004)	10 μM
	1A2, 1.5 - 3.5 2A6, 3 - 6 2B6, 3 - 5 2A4, 17 - 21	HepaRG line (Abass, et al., 2012)	10 μM, 50 μM
α - Cypermethrin	1A2, 3 2A6, 8 2B6, 5 2A4, 3 - 18	HepaRG line (Abass, et al., 2012)	10 μM, 50 μM
Deltamethrin	1A2, 2 - 3 2A6, 3 - 5 2B6, 4 – 6 3A4, 8 - 17	HepaRG line (Abass, et al., 2012)	10 μM, 50 μM
	3A4, 2	Primary Human Hepatocytes (Yang, et al., 2009)	10 μM
λ - Cyhalothrin	1A2, 2 2A6, 2 - 4 2B6, 3 - 5 2A4, 13 - 27	HepaRG line (Abass, et al., 2012)	10 μM, 50 μM
	3A4, 3.5	Primary Human Hepatocytes (Yang, et al., 2009)	10 μM
Permethrin	3A4, 2	Primary Human Hepatocytes (Yang, et al., 2009)	10 μM
Tetramethrin	3A4, 2	Primary Human Hepatocytes (Yang, et al., 2009)	10 μM

1.7.2 Inhibition

Metabolic inhibition can potentiate adverse effects by the parent molecule. For example, carboxylesterases (CEs) may have a significant impact on the rate of pyrethroid metabolism (Choi, et al., 2004). Some CE inhibitors caused altered toxicity due to reduced hydrolytic rates of pyrethroid metabolism (Ross, et al., 2006). Chlorpyrifos oxon, a reactive organophosphate pesticide metabolite, irreversibly inhibits hydrolysis of *trans* permethrin. DEET inhibits *trans* permethrin hydrolysis up to almost 50%, blocking a major pathway of metabolism (Choi, et al., 2004).

Piperonyl butoxide, a common pesticide additive, is a synergist (ATSDR, 2003) that selectively inhibits insect CYPs, preventing oxidative pyrethroid metabolism; increasing the toxicity and effectiveness of pyrethroid pesticides (Anand, et al., 2006).

Permethrin has self-inhibitory effects. The *cis* configuration inhibits the metabolism of the *trans* configuration by at least 40% but the reverse is not the case, suggesting competitive inhibition of the cis isomer in the presence of trans (Scollon, et al., 2009). Additionally, inhibitors of h3A4 and h2C19 produced a switch to CEs or other non-inhibited CYPs (Lavado, et al., 2014). One study found that co-incubation of the isomers decreased the metabolic rate by increasing residency time and showed a reduction in *trans* clearance by over 60% in human and rat microsomes (Willemin, et al., 2015).

1.8 Research Hypothesis and Techniques

With the all information on metabolism and pyrethroids in mind, the hypothesis that combining two pyrethroids would generate an inhibitory effect on metabolism emerged. This thesis initially focused on optimizing an analytical gas chromatographic – mass spectrometric (GC-MS) method that would be applicable to two GC-MS instruments with different ionization sources and using it to study the metabolism of pyrethroids.

The recovery repeatability was not predictive enough in this matrix to study the kinetics of pyrethroid metabolism. Therefore, the analytical method was transitioned to the use of spectrophotometry, which is highly suited to investigating kinetics. The metabolism of a CYP-specific substrate, 7-ethoxyresorufin (7-ER), could be monitored and would indicate the acute impact of two pyrethroids on the enzymatic activity of CYP1A. This altered the hypothesis to combining two pyrethroids with a CYP-specific substrate would produce an additive inhibitory effect on metabolism of the substrate.

2.0 AIM ONE: Quantification of pyrethroid insecticides by internal and external ionization mode ion trap GC-MS (GC-ITMS)

2.1 Abstract

Pesticides are of global concern and pyrethroids are one of the most commonly used classes. Analytical methods using chromatographic – mass spectrometric instruments have been published for several matrices, including water and food, for the parent compounds. Ion trap mass spectrometers have more recently been designed as a possible improvement over quadrupole based instruments, and have either an external or an internal ionization source. The differences between the ionization source efficiencies have not been previously reported. In this study a suite of pyrethroids were analyzed to determine differences in sensitivity and detection, comparing both source types. Though the external source showed many benefits, including greater sensitivity and lower background, the internal source may be needed depending on the target analytes, such as fenvalerate which was not detected on the external source.

2.2 Introduction

Pyrethroids are a class of synthetic semi-volatile organic compounds, or SVOCs, derived from pyrethrins and *Chrisanthenum* extract. This class of compounds is commonly used as insecticides in agricultural, household, and commercial products due to their insecticidal potency and low mammalian toxicity (Casida and Quistad, 1998, DeMicco, et al., 2010, Du, et al., 2013, Nasuti, et al., 2003, Scollon, et al., 2009, Tange, et al., 2014). Given the photostability, limited dermal absorption, and non-persistent nature in the environment,

pyrethroids are less toxic than other pesticide classes, such as organophosphates and carbamates, which have been shown to produce severe health issues (Nasuti, et al., 2003, Sheets, 2000). Pyrethroid usage is quite diverse but globally relevant, and residues have been found in environmental media around the world, including water sources, sediment, and soils (Tang, et al., 2018).

Many analytical methods have been implemented to analyze pyrethroids in a plethora of matrices. Separation has traditionally been performed with gas chromatography, though liquid chromatography has also been investigated (Chen and Chen, 2007). Detection has been achieved with quadrupole mass spectrometry (MS) (Feo, et al., 2010, Gullick, et al., 2014, Ramesh and Ravi, 2004), tandem MS (MS/MS) (Esteve-Turrillas, et al., 2005), high resolution MS (HRMS) (Barr, et al., 2002), and electron capture detection (ECD) (Wang, et al., 2009). Some studies have debated whether a liquid or gas chromatograph is the best suited instrument for pyrethroid analysis (Alder, et al., 2006, Paradis, et al., 2014), though no final conclusion had been drawn. Methods have been developed for both the parent compounds and metabolites in blood fractions, including serum (Barr, et al., 2002, Ramesh and Ravi, 2004), plasma (Barr, et al., 2002, Gullick, et al., 2014, Perez, et al., 2010), and cord blood (Corrion, et al., 2005); food items (Chen and Chen, 2007, Esteve-Turrillas, et al., 2005, González-Rodríguez, et al., 2008, Okihashi, et al., 2005, Paradis, et al., 2014); water (Feo, et al., 2010, Hladik, et al., 2008, Serôdio and Nogueira, 2005, Wang, et al., 2009); urine (Leng, et al., 1997); and tissue matrices (Gullick, et al., 2014, Zhu, et al., 2014).

With ion trap mass spectrometers, the goal is to produce greater sensitivity by trapping more ions. One advantage of the trap is that the ion preparation can be adjusted to increase selectivity, in other words, the capability of not only tandem MS (MS^2) but MS^n. The purpose of a mass spectrometer is to measure the mass-to-charge ratio (*m/z*) of the charged ions of molecules by separating them by *m/z*, to identify and quantify each analyte. With an internal ionization source, compounds coming out of the transfer line go directly to the trap to be both ionized and fragmented. With an external ionization source, ionization occurs outside of the trap and after ions are guided into the trap, they are fragmented. Regardless of ionization source, once ions are inside the trap, they are ejected into the multiplier to be detected. The external source was developed in hopes of reducing background and producing greater sensitivity.

The ability of one method to be used for a suite of pyrethroids within a variety of matrices is important, especially with small sample volumes, as it allows for the determination and quantification in limited samples, such as neonatal blood.

2.3 Experimental

In brief, solvent standards containing ten pyrethroids (Table 2.1) were separated under duplicate chromatographic conditions and injected into similar ITMS instruments, one with an internal and one with an external ionization source, with all differences amongst the pyrethroid analytes noted. To demonstrate applicability to samples with more complicated matrices, samples from microsomal enzyme assays were analyzed.

2.3.1 Materials

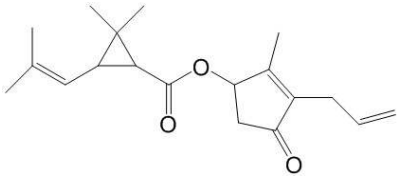
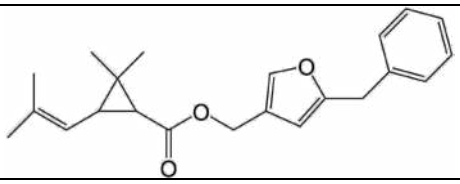
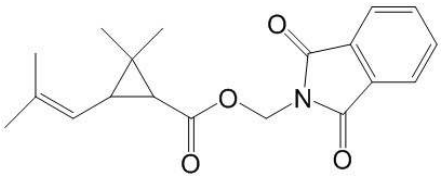
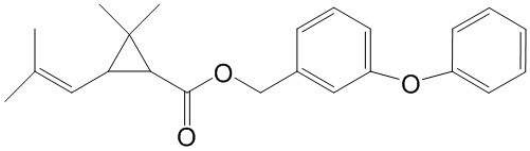
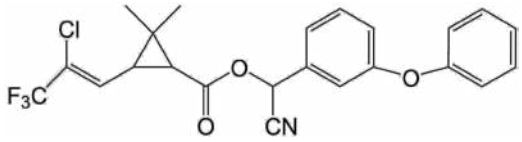
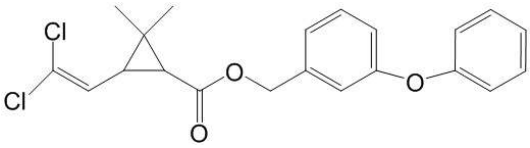
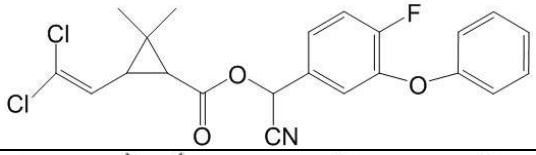
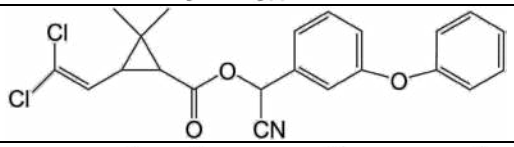
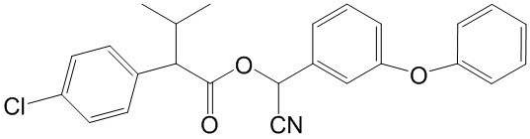
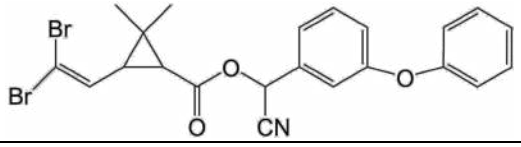
All pyrethroid standards were purchased from ChemService (West Chester, PA). *Pyrethrins and Pyrethroids Mixture #1* contained fenvalerate (200 µg/mL), cyfluthrin, phenothrin, and tetramethrin (400 µg/mL, acetonitrile), and *Pyrethrins and Pyrethroids Mixture #2* contained allethrin, permethrin, and resmethrin (400 µg/mL, acetonitrile). Additionally, deltamethrin, cyfluthrin, and lambda-cyhalothrin were purchased as individual native standards (100 µg/mL, acetonitrile).

For microsomal enzyme samples, a permethrin native standard from ChemService was used. Control liver microsomes were pooled from 200 male or 100 female Wistar rats (XenoTech, Lenexa, KS). NADPH was purchased from Sigma-Aldrich (St. Louis, MO). Potassium monobasic and dibasic phosphates (Sigma-Aldrich) were combined to create potassium phosphate buffer. MilliQ element water was generated by an in-house system (MilliPore, Burlington, MA). Ethanol was purchased from Pharmaco-Aaper (Brookfield, CT).

2.3.2 Standards

A 10 µg/mL standard pyrethroid stock solution was prepared by combining the five native standards in pesticide residue grade toluene (VWR International, Radnor, PA). From the stock standard, 11 working standards (10, 25, 50, 75, 100, 250, 500, 750, 1000, 2500, and 5000 ng/mL) were prepared to define the range of the analytical method.

Table 2.1 Chemical structures of the pyrethroids utilized in this study.

Pyrethroid (Abbreviation)	Structure
Allethrin (Alle)	
Resmethrin (Res)	
Tetramethrin (Tetra)	
Phenothrin (Pheno)	
Cyhalothrin (Cyhalo)	
Permethrin (Perm)	
Cyfluthrin (Cyflu)	
Cypermethrin (Cyper)	
Fenvalerate (Fen)	
Deltamethrin (Delta)	

2.3.3 Sample preparation

To assess the sensitivity of the method with real world samples, enzyme assays were conducted and permethrin was extracted from them. These assays were performed in clear flat-bottomed 96-well plates (ThermoFisher, Waltham, MA). To assess pyrethroid metabolism, permethrin was used as a substrate. Reaction mixtures contained 50 mM potassium phosphate buffer (pH 7.4), 430 μM NADPH, and 10 μM permethrin. This mixture was heated at 37°C for 10 minutes, then initiated with 1 mg/mL of microsomes, creating a final volume of 125 μL. Mixtures were allowed to react for 20 minutes at 37°C, after which the addition of 125 μL cold ethanol was used to terminate the reaction and initiate protein precipitation. Samples were centrifuged at 14,000 *g* and 4°C for 25 minutes, then evaporated to dryness under nitrogen, reconstituted in 75 μL toluene, and analyzed by GC-ITMS.

2.3.4 Instrumental Analysis

Two GC-ITMS systems were utilized: a Varian CP-3800 gas chromatograph coupled to a Varian Saturn 2200 ion trap mass spectrometer using an internal ionization source, and an Agilent Technologies 7890B gas chromatograph coupled to an Agilent Technologies 240 ion trap mass spectrometer using an external ionization source. Both instrument systems were under the control of their own MS WorkStation software.

Unless otherwise noted, instrument parameters were identical and the ion preparation parameters are presented in Table 2.2. *In situ* purifier (ISP) grade helium (Airgas, Radnor, PA) was used as the carrier gas at a flow rate of 0.9 mL/min, which was automatically

Table 2.2 Selective Ion Storage (SIS) parameters.

Pyrethroid	Time Window (minutes)		Ion Window (*m/z*)	
	Internal	External	Internal	External
Alle	8.0 – 17.0	14.0 – 17.0	121 – 125 134 – 138	121 – 124 135 – 138
Res	17.0 – 21.2	17.0 – 20.0	121 – 130 141 – 145 162 – 173	121 – 130 142 – 144 169 – 173
Tetra Pheno		20.0 – 22.9	181 – 185	122 – 124 163 – 165
Cyhalo	21.2 – 23.0		139 – 143 179 – 183 195 - 199	180 – 184 196 – 198
Perm Cyflu Cyper Fen	23.0 – 27.0	22.9 – 27.5	161 – 174 179 – 185 195 – 228	147 – 151 161 – 171 180 – 185 196 – 228
Delta	27.0 – 29.0	27.5 – 29.8	170 – 174 251 - 257	251 - 256

controlled by the instruments. Splitless injections of pyrethroid standards and samples were made by CombiPAL autosamplers, injecting 1 μL sample volume into the inlet containing an Ultra Inert glass liner (Agilent Technologies, Santa Clara, CA, single taper with glass wool). The injectors ran a temperature program: initially 150°C, ramped to 280°C at 150°C/min and held for 12 minutes. The oven programs began at 90°C, holding for 2 minutes, then ramped to 200°C at 18°C/min, then ramped at 5°C/min to 300°C, and held at a final temperature of 300°C for 4.89 minutes. Compound separation was achieved using a DB-XLB microcapillary column (J&W, Agilent) with a 0.18 μm film thickness, 0.18 mm internal diameter, and 30 m length. Once the compounds were separated, the molecules were directed into the mass spectrometer. The external source mass spectrometer (Agilent 240) kept the transfer line, ion trap, manifold, and ion source at temperatures of 270°C, 200°C, 70°C, and 215°C, respectively, throughout each run. For the internal source mass spectrometer (Varian 2200), the transfer line, ion trap, and manifold were kept at 275°C, 225°C, and 70°C, respectively, throughout each run. The total analysis time was 33 minutes per injection.

General mode scans using a mass range of 40 to 450 *m/z* were employed first on each instrument to determine the retention times, and qualitative and quantitative ions (Q ions) for each pyrethroid. In this study, the quantitative ion was considered as the most abundant *m/z* per pyrethroid, while the qualitative ion was considered as the second most abundant *m/z*, which was unique to that pyrethroid. Subsequently, selective ion storage (SIS) mode was utilized on each mass spectrometer using more narrow ion windows that encapsulated

the Q ions of each pyrethroid, which increased selectivity and sensitivity for all pyrethroids (Table 2.2).

2.3.5 Data processing and analysis

Data from both instruments were processed using MS Data Review software (v 6.9.3, Varian), supplied with the GC-ITMS systems. Triplicate injections were made each day for three days and values were averaged per day. The lowest concentrations analyzed were at or below the detection limit (DL), defined below. Calibration curves were generated using 11 standards versus area under the curve response. Single isomer and combined isomer curves for each analyte were generated to assess their correlation.

For the purpose of this work, the DL was defined as one isomer of an analyte having a signal-to-noise ratio of at least three and the limit of quantification (LOQ) was defined as three times the DL. Repeatability is represented by the coefficient of variance (CV) for the areas of triplicate injections per concentration on a given day, while reproducibility is represented by the CV comparing the areas of a concentration across days.

2.4 Results

2.4.1 GC separation of compounds

Baseline separation of eight pyrethroids was accomplished on both instruments. However, fenvalerate co-eluted with the second isomer of cypermethrin on the internal source, and was not detected on the external source. The co-elution on the internal source was overcome using extracted ion monitoring (EIM), which is a software tool that allows for peak deconvolution using an analyte's Q ions. Given that the chromatography was

performed under the same columns and conditions on both the chromatography and mass spectrometry sides, the only point of departure in methodology was the ionization source.

General scan mode gas chromatographic separations were used to determine the retention time, quantitative and qualitative ions (Q ions), and additional ion fragments of the pyrethroids. Setting the first peaks as equal, the retention times of all analytes match up within 3% and all analytes were eluted within 15 minutes of the first analyte. The fragmentation patterns of the pyrethroid analytes were well correlated, though some differences occurred as seen in Table 2.3. These differences could be a result of voltage or ionization energies.

2.4.2 Increasing sensitivity by utilizing a different ion preparation mode

After the general scans identified the preliminary parameters, SIS mode was utilized to further develop the detection limit. EIM was also used to better resolve cyfluthrin and the remaining isomers of cypermethrin from background signals on both instruments. Ion chromatograms reconstructed from EIM filtration, close to the overall detection limits, can be seen in Figure 2.1a for the internal source and Figure 2.1b for the external source.

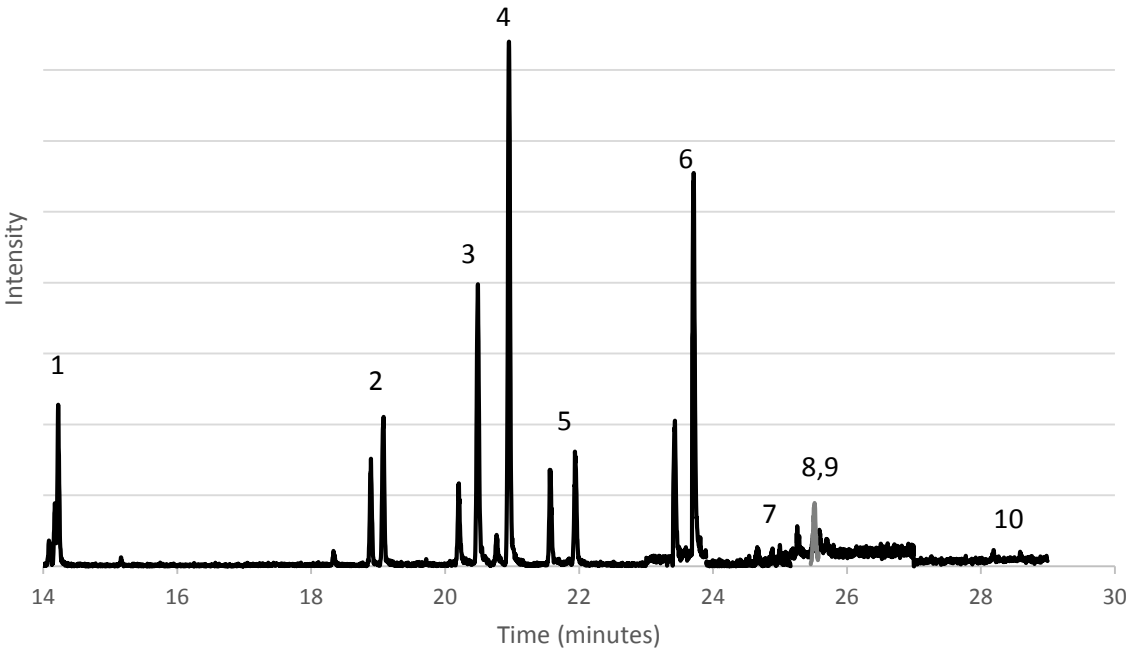
2.4.3 Comparison of internal and external ionization

While there were little to no visible differences in the peak shapes between internal and external ionization, there were differences in signal intensity. A greater signal improvement was seen in the internal source switching from general scan to SIS mode, 10-fold versus 3-fold in the external source. However, the original signal in the external source had a lower background and about 4-6 times higher signal intensity. The signal-to-noise, or S/N, ratios

Table 2.3 Characteristics and fragmentation patterns of the pyrethroid parents.

Pyrethroid	Molecular Weight	Quantitative Ion (% Abundance)		Qualitative Ion (% Abundance)		Additional Fragments (% Abundance)	
		Internal	External	Internal	External	Internal	External
Alle	302.4	123 (100%)	123 (100%)	136 (20%)	136 (25%)	81 (40-45%) 79 (15-45%) 91 (25-30%)	81 (40-50%) 79 (50%)
Res	338.4	123 (90-100%)	143 (100%)	143 (60%)	123 (100%)	170 (65-100%) 128 (30-40%) 81 (50%)	171 (75%) 128 (75%) 81 (45%)
Tetra	331.4	164 (100%)	164 (100%)	123 (30%)	123 (20%)	136 (10%) 81 (15%)	107 (10%) 81 (15%)
Pheno	350.5	123 (100%)	123 (100%)	183 (35-60%)	183 (95%)	81 (30-35%)	165 (15%) 81 (45%)
Cyhalo	449.9	197 (100%)	181 (100%)	181 (65-90%)	197 (50%)	141 (25-40%)	208 (20%) 161 (20%) 141 (20%)
Perm	391.3	183 (100%)	183 (100%)	163 (20-30%)	163 (15%)	127 (20%) 91 (20%)	165 (20%) 127 (15%) 91 (10%)
Cyflu	434.3	163 (30-100%)	206 (100%)	226 (40-75%) 206 (5-80%)	226 (40%)	127 (35-100%) 91 (70-100%)	163 (80%) 127 (60%) 91 (40%)
Cyper	416.3	165 (100%) 163 (65-100%)	181 (100%)	181 (50-100%)	163 (60-80%)	127 (30-100%) 91 (50-90%)	127 (50%) 91 (40%)
Fen	419.9	197 (100%)	ND	167 (20%)	ND	141 (15%)	ND
Delta	505.2	253 (15-100%)	181 (100%)	256 (50%)	253 (50%)	172 (40-100%)	255 (20%) 172 (40%) 93 (50%)

a) Internal source, 250 pg injection



b) External source, 50 pg injection

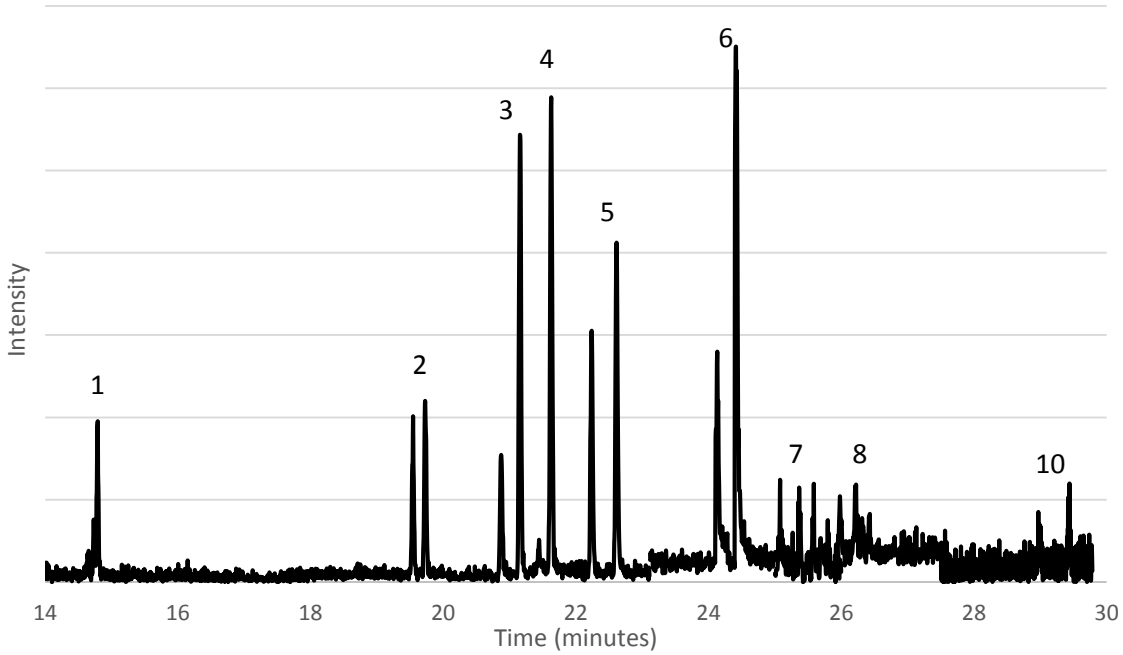


Figure 2.1 Reconstructed ion chromatogram from individual EIM filters for a) internal ionization, and b) external ionization. Peaks are as follows: (1) allethrin, (2) resmethrin, (3) tetramethrin, (4) phenothrin, (5) cyhalothrin, (6) permethrin, (7) cyfluthrin, (8) cypermethrin, (9) fenvalerate, (10) deltamethrin.

were between 10 – 30 on the external source at 50 ppb and 10 – 20 on the internal source at 100 ppb. The chromatographic signal counts for 10 – 50 ppb in the internal were indistinguishable, therefore signals below 50 ppb were assessed to be inaccurate on the internal source. The isomeric ratios were comparable between instruments and to the known literature values, Table 2.4. The detection limits and limits of quantification were lower overall, in the external source, typically half that of the internal source in the later eluting analytes, Table 2.5, making the external source at least 5 times more sensitive. At 50 ppb, the internal source produced 4-87% CV within days and interday variation of 33-103%. The external source produced 3-26% CV within days and interday variation of 9-17%. The analyte-specific % CV on both sources can be seen in Table 2.6. These variations indicate the external source is much more stable than the internal source and that it is more precise.

2.4.4 Applicability to microsomal samples

In addition to neat standards, microsomal extracts were analyzed on both instruments as part of a metabolism study and compared for sensitivity. Samples with media reagent components were analyzed first, then samples with all components were analyzed.

On both instruments, permethrin was detected in the media component samples with typical matrix effects, roughly 20-30% signal suppression (fewer counts). However, when all components were present and the RLMs were not allowed to react, the matrix effects were substantial: on the internal source, there was 60-80% signal loss and on the external source, the signal loss was between 80-95%. Permethrin signal was detected using the

Table 2.4 Comparison of experimental pyrethroid parent isomeric ratios to known values.

Pyrethroid	Internal Source	External Source	Literature Isomeric Ratio
Alle	1 : 1.6 : 5.3	1 : 1.6 : 4.8	1 : 4
Res	1 : 1.1	1 : 1.3	1 : 7
Tetra	1 : 3.9	1 : 3.8	1 : 4.5
Pheno	1 : 29.8	1 : 22.9	1 : 19
Cyhalo	1 : 1.1	1 : 1.4	Unknown
Perm	1 : 3.6	1 : 3.5	1.3 : 1
Cyflu	1.5 : 1.1 : 1 : 1.3	1.5 : 1.5 : 1.3 : 1	2.5 : 1 : 1
Cyper	2.8 : 1 : 1.7 : 1.5	2.8 : 1 : 1.8 : 1.9	2 : 1 : 1.4
Fen	1	ND	1
Delta	1.4 : 1	1.2 : 1	1

Table 2.5 Comparison of pyrethroid parent sensitivity in SIS mode. DLs denoted by S/N ≥ 3 and LOQs denoted by 3 x DL.

Pyrethroid	Internal Source		External Source	
	DL (ppb)	**LOQ (ppb)**	**DL (ppb)**	**LOQ (ppb)**
Alle	50	150	10	30
Res	50	150	10	30
Tetra	50	150	10	30
Pheno	50	150	10	30
Cyhalo	50	150	10	30
Perm	75	150	25	75
Cyflu	100	300	50	150
Cyper	250	750	50	150
Fen	50	150	ND	ND
Delta	250	750	100	300

Table 2.6 Repeatability and reproducibility of combined pyrethroid parent isomers on both sources at 50 ppb. ND= not detected, NA= not applicable.

	Internal Source				External Source			
% CV	**Day One**	**Day Two**	**Day Three**	**Interday**	**Day One**	**Day Two**	**Day Three**	**Interday**
Alle	7.26	8.08	8.09	45.44	9.54	5.75	3.46	10.94
Res	14.53	7.60	7.18	49.67	6.30	7.70	5.27	9.35
Tetra	86.61	8.45	6.56	44.69	4.63	7.26	6.44	12.15
Pheno	8.71	4.32	4.48	37.26	7.24	8.90	8.52	10.52
Cyhalo	20.04	8.12	11.40	61.31	4.32	14.62	4.90	13.50
Perm	7.80	13.29	26.59	33.62	4.21	15.53	6.87	14.66
Cyflu	ND	ND	ND	NA	ND	ND	26.12	NA
Cyper	ND	ND	ND	NA	14.22	13.08	6.02	17.20
Fen	ND	27.17	45.63	102.56	ND	ND	ND	NA
Delta	ND	ND	ND	NA	ND	ND	ND	NA

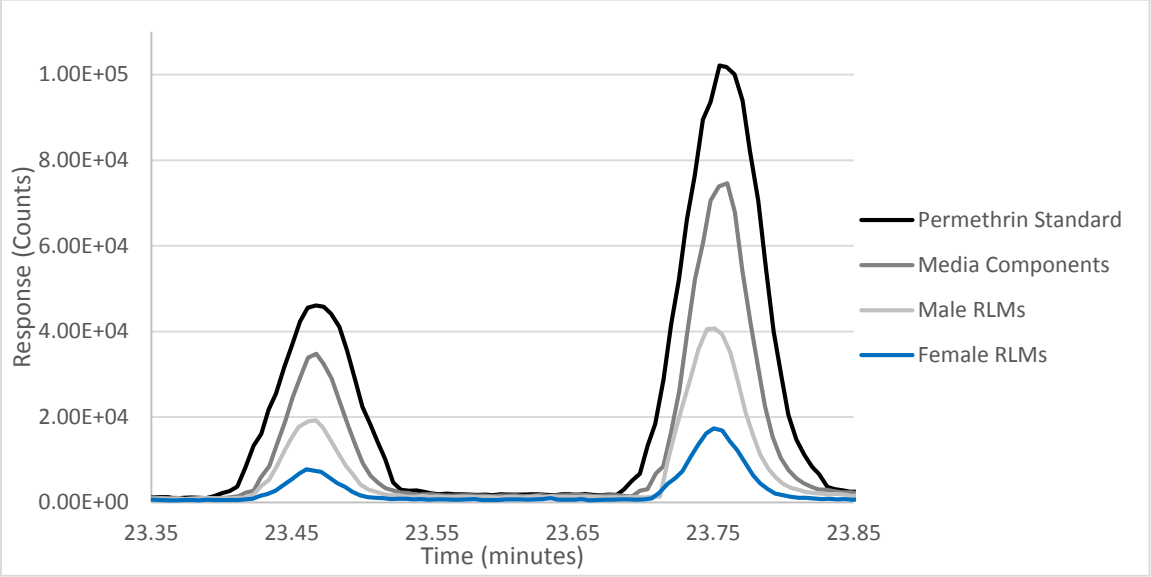
external ionization source in the $10^5 – 10^6$ range, while the internal source was 10-times less, again demonstrating the greater sensitivity of the external source (Figure 2.2).

2.5 Discussion

The method developed was intended to accurately quantify 10 pyrethroids in a single sample and determine any differences due to the ionization source. Baseline separation of eight pyrethroids was accomplished on both instruments, and the retention times were closely aligned across instruments, demonstrating the reproducible quality of identical columns. Fenvalerate was not seen on the external source and co-eluted with one isomer of cypermethrin on the internal source. The inability to be seen on the external source could indicate fenvalerate may not be ionizable with the external source or the ions are lost in transit to the trap. While it was identified on the internal source, albeit co-eluted with the second isomer of cypermethrin, extracted ion monitoring (EIM) was able to de-convolute the co-elution peak. EIM was also used to better resolve the remaining isomers of cypermethrin and cyfluthrin from background. The isomeric ratios were comparable across instruments and to the known literature values, as were the fragmentation patterns of each pyrethroid analyte.

While both instruments are ion trap mass spectrometers, their advantages are different. In this study, the internal source achieved greater sensitivity in changing the ion preparation to selective ion storage. The chromatograms produced on the internal source instrument between 10 – 50 ppb looked similar, indicating a nonlinear relationship between concentration and signal in this concentration range. The external source had higher signal

a) Internal source



b) External source

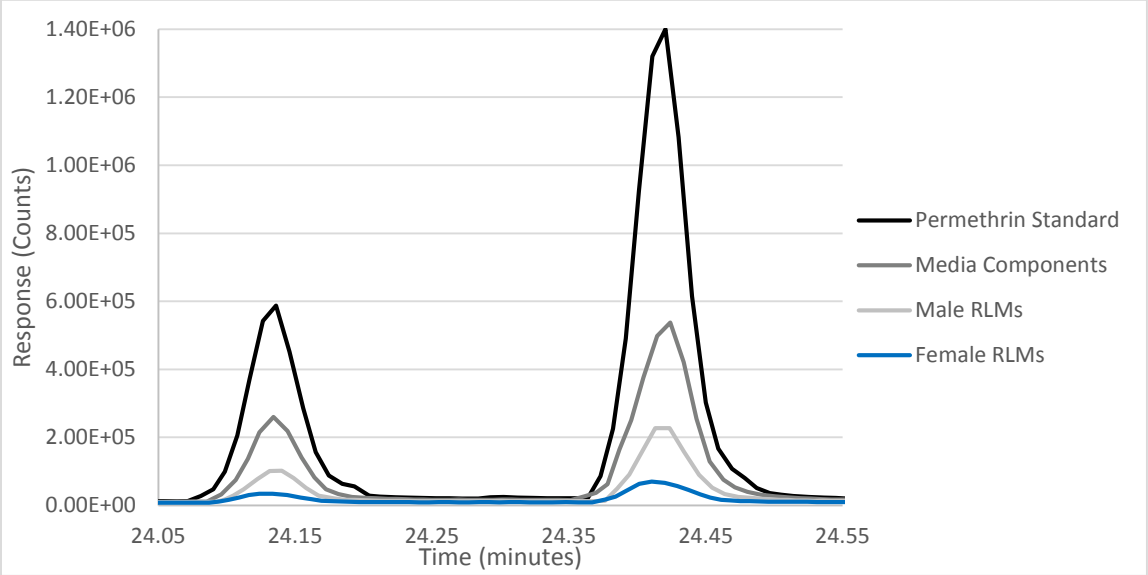


Figure 2.2. Demonstration of matrix suppression effects on the measurement of permethrin as analyzed by the a) internal and b) external ionization sources. The standard (black line) is at the concentration all samples were tested at. The media component samples (dark grey) experience typical media effects, while the samples from male rat liver microsomes (light grey) and female RLMs (blue), stopped before any reactions could occur, sustained substantial media effects.

intensities, even in general scan mode, and had an overall quieter background also present in SIS mode. The greater signal intensity and lower background helped produce lower DLs and LOQs, as well as lower coefficients of variance for both inter- and intraday using external ionization.

Another difference between the instruments was age. The internal source instrument is about 20 years old, while the external source is about 5 years old. Both had good maintenance records, with their sensitivities closely monitored over time with a performance standard, which has led to changing the electron multiplier within a year prior to the conduction of the study. Lastly, the Agilent instrument is capable of internal and external ionization, though changing between sources is quite difficult and not recommended by the manufacture service personnel.

In order to determine the sensitivity of the method for real world samples, microsome enzyme samples were analyzed on both instruments. These samples showed a large matrix effect on both instruments, making it difficult to study the kinetics. The greater signal suppression with the female RLMs could be due to difference in lipid content between genders. However, there was a higher signal on the external source, despite matrix suppression.

2.6 Conclusions

A GC-ITMS method was used to separate and detect a suite of pyrethroids within a single sample at relatively low ppb levels using ion trap mass spectrometers. Differences in the ionization source alone were distinguished, providing evidence that the external source is

more sensitive and detection limits were lower than those on the internal source. CVs were typically lower on the external source as well, even at lower concentrations. However, the external source was unable to detect fenvalerate, while the internal source could. If fenvalerate is a target analyte, the internal source would be necessary.

While the extraction method was not effective at removing matrix effects from liver microsomes on permethrin signal intensity, it proved permethrin can be extracted from one type of *in vitro* sample and produced the same result shown in standards. The external source had better detection limits, lower background, and greater sensitivity than the internal source.

Based on my results, future studies should investigate extraction protocols that reduce signal suppression creating a method sensitive enough to monitor metabolism. Given the semi-volatile nature of these compounds and the aqueous components of this *in vitro* system, it may be worth determining whether liquid chromatography – mass spectrometry is better suited for analysis.

3.0 AIM TWO Effect of pyrethroids on cytochrome P450 activity using a fluorescence-based technique

3.1 Abstract

The metabolic reactions of 7-ethoxyresorufin (7-ER) in untreated Wistar rat liver microsomes (RLMs) were monitored to assess the inhibitory capabilities of two pyrethroids singly and in combination. Results show that 7-ER alone provided Michaelis-Menten kinetics: male RLMs produced a K_m of 9.83 ± 5.56 μM and a V_{max} of 0.44 ± 0.14 nmol/min/mg microsomal protein and the female RLMs produced a K_m of 7.84 ± 0.79 μM and a V_{max} of 0.46 ± 0.02 nmol/min/mg microsomal protein. Both permethrin and cypermethrin were able to generate inhibition in the system, at different rates. When combined, they produced minimal inhibition but not an additive effect as hypothesized.

3.2 Introduction

Metabolism is generally broken down into Phase I and Phase II pathways, where Phase I includes hydrolysis and oxidation, and Phase II consists of conjugation. Typically, oxidation includes adding a hydroxyl group to a compound as a means of detoxification. This type of metabolism can be performed by cytochrome P450 (CYP) enzymes, which are a superfamily of enzymes with many different isozymes that vary in specificity as a result of the size of their active site (Johnson and Stout, 2005).

Pyrethroid insecticides target voltage-gated sodium channels in neurons of the central and peripheral nervous system. Permethrin, the most prevalent type I pyrethroid, is used in the

treatment of lice and ticks, making the World Health Organization's List of Essential Medicines (Organization, 2017). Cypermethrin, a commonly used type II pyrethroid, is used in household pesticides, usually ant and cockroach killers.

The toxicity of pyrethroids is not of concern in mammals because of their rapid metabolism (Casida and Quistad, 1998, DeMicco, et al., 2010, Du, et al., 2013, Nasuti, et al., 2003, Scollon, et al., 2009, Tange, et al., 2014). One relevant metabolic pathway is oxidation by CYPs, of which there are many isozymes that can act on pyrethroids. In rodent species, the 1A, 2B, 2C, 3A, and 2D families are active in pyrethroid oxidation (Scollon, et al., 2009). As with any species, gender differences are present in CYP profiles between male and female rats (Ryan and Levin, 2013). While pyrethroids can be rapidly metabolized by CYPs, a wide variety of compounds are metabolized by CYPs. Therefore, inhibiting the metabolism of pyrethroids or the other xenobiotics present could lead to increased risk of toxicity. Studies have investigated single and combinations of pesticides (Abu-Qare and Abou-Donia, 2008, Choi, et al., 2004, Khan, et al., 2013), but the effect of pyrethroid mixtures has not been well characterized.

In this acute *in vitro* study, 7-ethoxyresorufin (7-ER), a CYP1A-specific substrate, is used as a marker of CYP1A activity and used to assess the impact permethrin and cypermethrin, two of the most commonly used pyrethroids, have singly and in combination on the metabolism of 7-ER in both male and female Wistar rat liver microsomes (RLMs).

3.3 Experimental

3.3.1 Materials

7-ethoxyresorufin and resorufin were purchased from Sigma-Aldrich (St. Louis, MO). Individual native pyrethroid standards of permethrin and cypermethrin were from ChemService (West Chester, PA). Untreated liver microsomes pooled from 200 male or 100 female Wistar rats were obtained from Sekisui XenoTech (Lenexa, KS). NADPH from Acros Organics (Fair Lawn, NJ) was used as the energetic co-factor. Phosphate buffer was made from potassium monobasic and dibasic phosphates, both of which were purchased from Sigma-Aldrich.

3.3.2 Determination of protein content

Protein content was assessed using the Pierce BCA assay kit (ThermoScientific, Bridgewater, NJ), which uses bovine serum albumin (BSA) as the standard protein. BSA was prepared at concentrations between 0 – 2000 μg/mL in phosphate buffer. Untreated male and female rat liver microsome (RLM) samples were tested at three dilutions: 1:10, 1:50, and 1:100. 25 μL of each standard protein and RLM protein sample were loaded into wells of a clear, flat-bottomed 96-well plate (ThermoFisher, Waltham, MA). 200 μL of the working reagent, which is a mixture of bicinchoninic acid and cupric sulfate, was added to each well and after 30 minutes at 37°C, absorbance was measured at 562 nm by the SpectraMax M3 Multimode Microplate Reader (Molecular Devices, Sunnyvale, CA) controlled by SoftMax Pro 6.3 software.

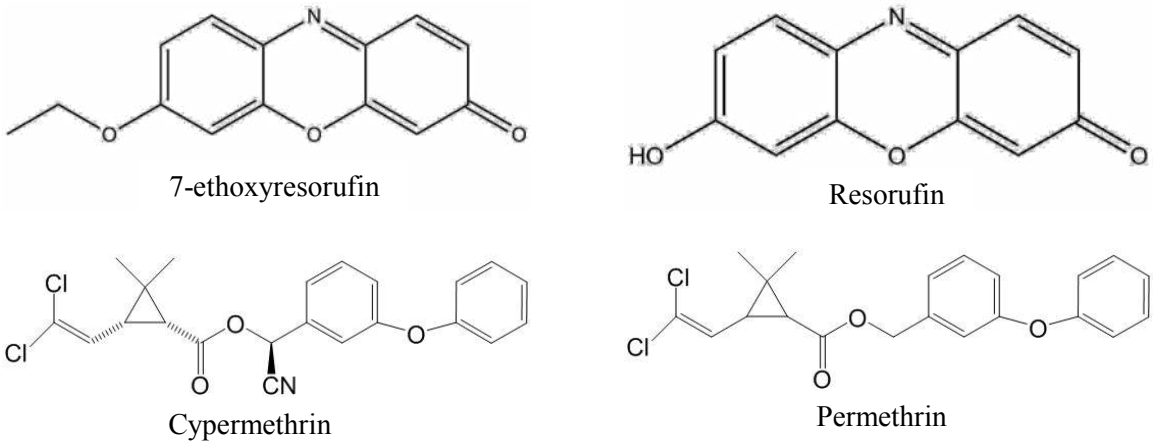


Figure 3.1. Chemical structures of 7-ethoxyresorufin, resorufin, and two pyrethroids.

3.3.3 Determination of CYP1A activity

Production of resorufin, the metabolite of 7-ethoxyresorufin (7-ER), was used as a metric of CYP1A activity. Reactions were performed in black 96-well plates with clear bottoms (Greiner Bio-One, Monroe, SC) and held at a constant 37°C. The fluorescence signal of resorufin was monitored by the SpectraMax using 535 nm and 580 nm for the excitation and emission wavelengths, respectively. A standard curve of resorufin was made by incubating heat-inactivated male and female RLMs with phosphate buffer and the range of resorufin concentrations. This curve was measured in a single capture and used to quantify results from enzymatic reactions.

Kinetic assays were measured in 30 second intervals over the course of 30 minutes. Reaction mixtures contained 10 mM phosphate buffer (pH 7.4) and 450 μM NADPH, and were initiated by addition of male or female RLMs to produce a concentration of 0.1 mg/mL and a final total volume of 200 μL. For kinetic assays, reaction mixtures contained 0.25 – 12.5 μM 7-ER.

3.3.4 Inhibition assays

The inhibitory effects of permethrin and cypermethrin on CYP1A activity were examined, using α – naphthoflavone as a positive control. Reaction mixtures were similar to the description above, with 7-ER held constant at 10 μM for all inhibition assays. Permethrin and cypermethrin stock solutions were in acetonitrile and diluted in phosphate buffer to produce <2% organic solvent within the reaction wells to prevent enzyme inhibition (Chauret, et al., 1998, Easterbrook, et al., 2001, Li, et al., 2010). The diluted stocks were

added to the reaction mixtures as described above in a volume of 80 μL/compound for individual inhibitions or 50 μL/compound for combination inhibitions. The final concentrations ranged from 0 – 1 μM within the reaction mixture. Assays monitored production of resorufin in 30 second intervals over the course of 30 minutes.

3.3.5 Blank controls

For the kinetic assays, the blank controls contained untreated male or female RLMs, NADPH, and buffer. For the inhibition assays, the blank controls contained male or female RLMs, the pyrethroid(s) of interest in the same proportion as the assay, NADPH, and buffer. The average fluorescence measured over the course of 30 minutes was subtracted from the resorufin standard curve as a means of correction. Each set of experiments had its own corrected standard curve, which was applied to quantify the results of the given assay.

3.3.6 Data processing and statistical analysis

Data were analyzed using GraphPad Prism 7.04 (La Jolla, CA). Reactions were run in biological triplicate, with 3 – 5 technical replicates per concentration per biological replicate.

Kinetic assays utilized 0.25 – 12.5 μM of 7-ER to determine K_m and V_{max} values for each CYP. Individual inhibition assays were analyzed to first determine inhibitory concentration values, then combination inhibition assays assessed a potential additive effect. The RLMs treated with 7-ER were used as the control, and were compared to the RLMs treated with

7-ER and varying concentrations of permethrin, cypermethrin, or a combination of the two pyrethroids.

The amount of resorufin produced was converted into a percent of control, while the reaction rate of the production was also calculated for all concentrations of each condition, as an additional means of determining inhibitory capability. These data were used to perform two-way ANOVAs with Tukey post-hoc tests to determine the statistical differences between genders and concentrations. Significance was accepted at $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$.

3.4 Results

3.4.1 Protein Content

Although multiple dilutions of both untreated male and female Wistar rat liver microsomal (RLM) proteins were analyzed, only the 1:100 dilution fell within the range of the assay. Based on comparing the results of the microsome samples to the albumin standard curve, the male RLMs had 13.41 ± 0.64 mg protein/mL and the female RLMs had 15.66 ± 0.44 mg protein/mL. These values are relatively comparable to the Certificates of Analysis, which state both the male and female RLMs have 20 mg protein/mL. While the specific CYP1A protein content was not assessed, according to Ryan and Levin (Ryan and Levin, 2013), male liver microsomes of Long-Evans and Sprague-Dawley rats possess 0.01 nmol each of CYP1A1 and CYP1A2/mg microsomal protein, while the female counterparts contain 0.01 nmol CYP1A1/mg microsomal protein and 0.05 nmol CYP1A2/mg microsomal protein (Ryan and Levin, 2013).

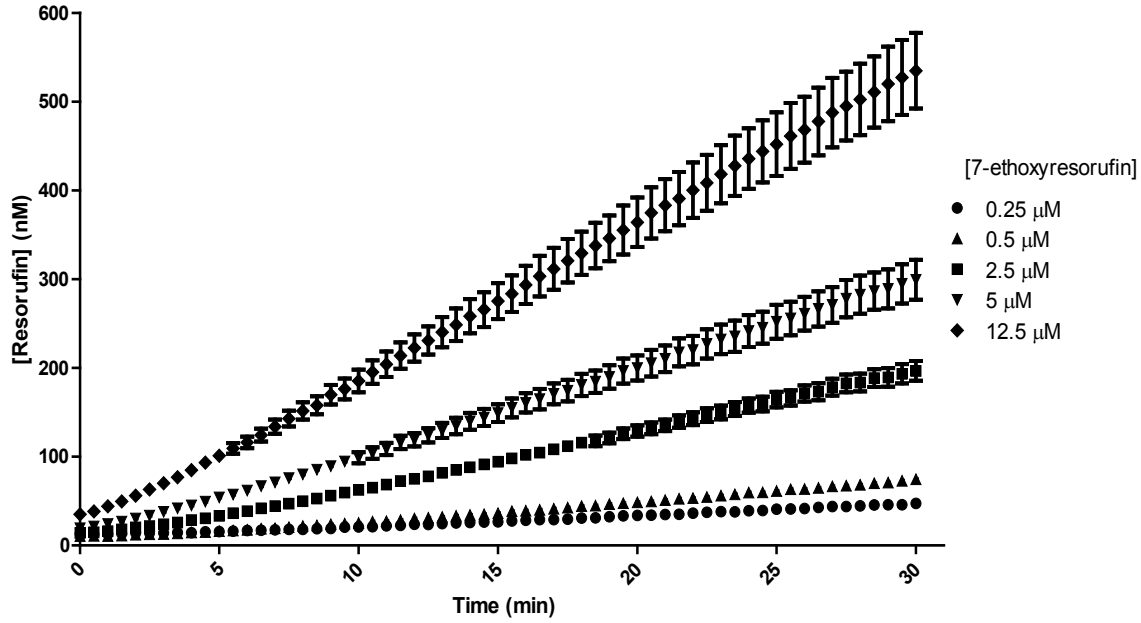
3.4.2 CYP1A Activity

The metabolism of 7-ethoxyresorufin (7-ER) to resorufin by untreated male or female Wistar rat liver microsomes (RLMs) was examined for CYP1A activity. At every concentration of 7-ER, female RLMs produced more resorufin (Figure 3.2). Untreated male RLMs produced a K_m of 9.83 ± 5.56 μM and a V_{max} of 0.44 ± 0.14 nmol/min/mg protein, while in untreated female RLMs, they produced a K_m of 7.84 ± 0.79 μM and a V_{max} of 0.46 ± 0.02 nmol/min/mg protein (Figure 3.3). This activity was greatly inhibited in the positive control using the CYP1A inhibitor α – naphthoflavone.

3.4.3 Inhibition by Permethrin

In the control samples, male RLMs produced greater amounts of resorufin than their female counterparts. While it was not significant when any concentration of permethrin was added, the trend persisted. When comparing within gender, there were no significant reductions, though both genders showed greater reductions with increasing permethrin concentration. Using the male RLMs, there was 17% inhibition of resorufin production with 0.01 and 1 μM permethrin and 8% with 0.1 μM permethrin, while the female RLMs experienced about 25% inhibition at 0.01 and 0.1 μM, and 32% at 1 μM (Figure 3.4, Table 3.1a). Similar to the resorufin production reduction, both male and female RLMs show an almost dose-response trend with decreased reaction velocity correlated to increasing permethrin concentrations (Table 3.2a).

a)



b)

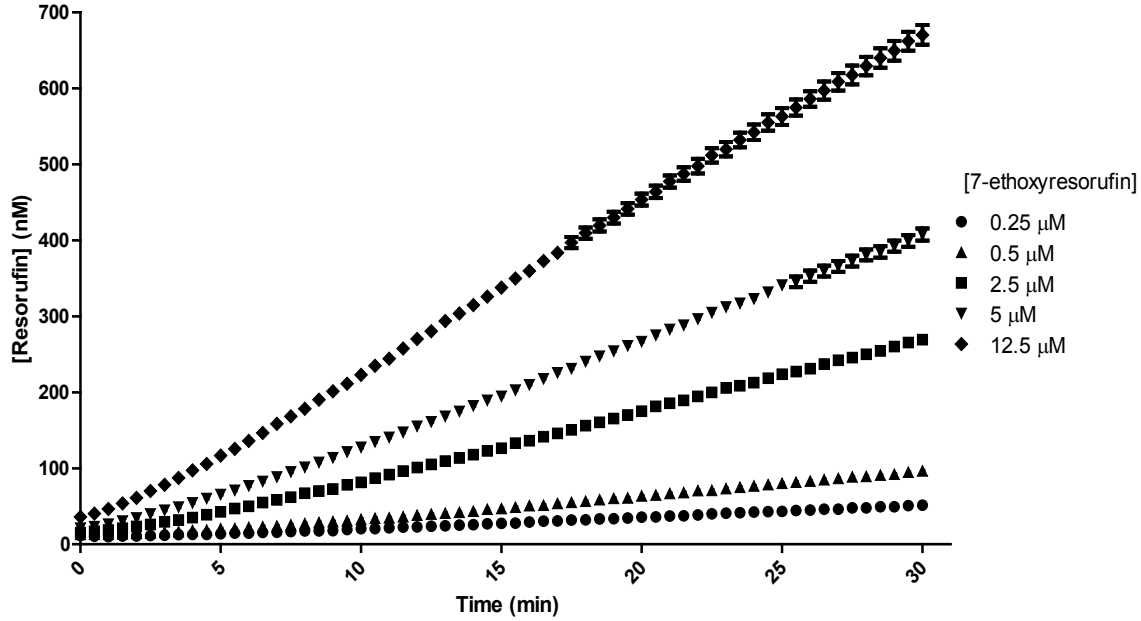


Figure 3.2. Metabolism of 0.25 – 12.5 μM 7-ethoxyresorufin to resorufin by untreated male (a) and female (b) Wistar rat liver microsomes (biological triplicate, n = 3 – 5/ replicate/concentration).

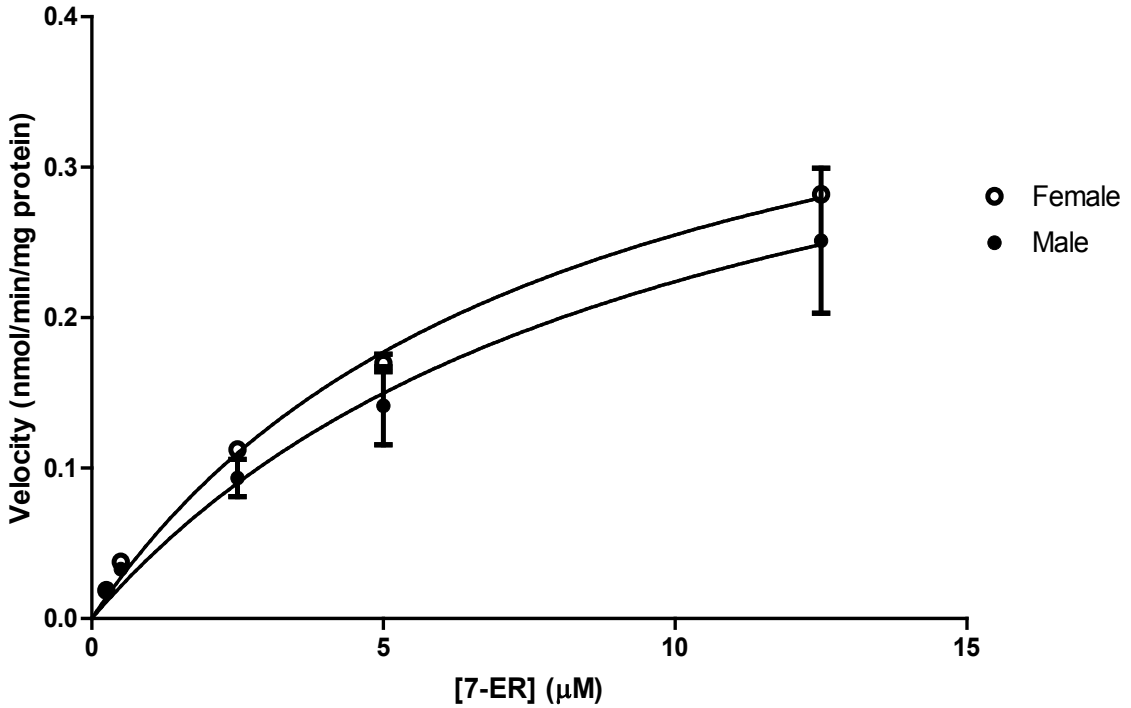
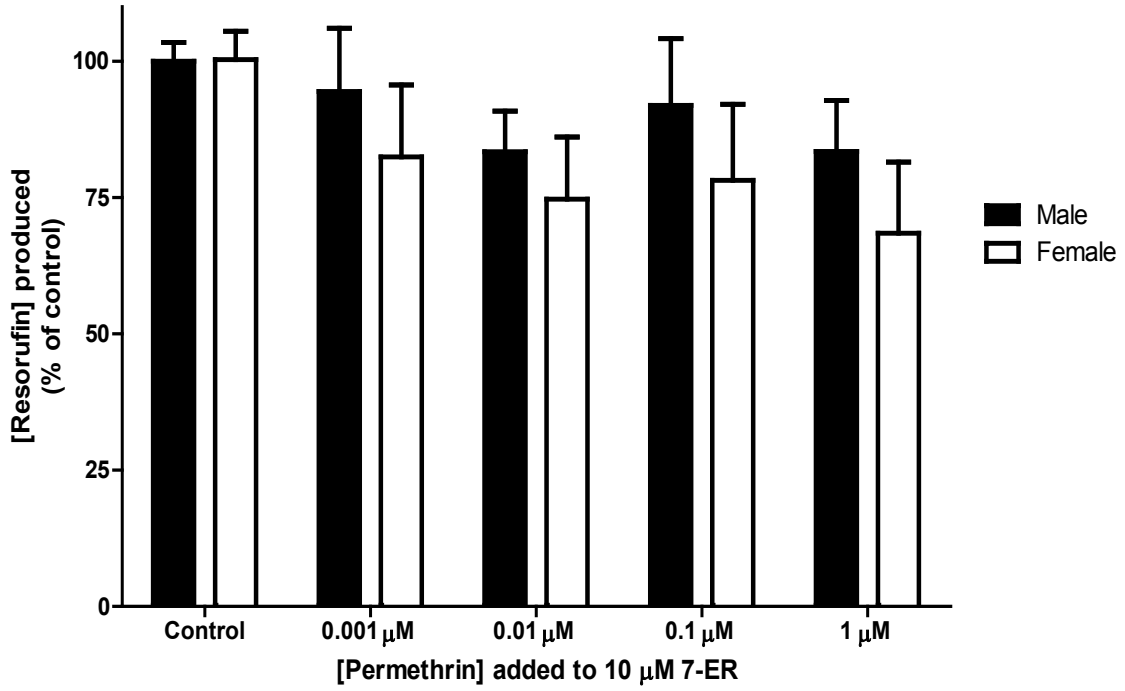


Figure 3.3. Michaelis-Menten kinetic plot of ethoxyresorufin O-deethylation in untreated male (closed circles) and female (open circles) Wistar rat liver microsomes (biological triplicate, n = 3 – 5/ replicate/concentration). When incubated with male RLMs, K_m = 9.83 ± 5.56 μM and V_{max} = 0.44 ± 0.14 nmol/min/mg protein, and with the female RLMs, K_m = 7.84 ± 0.79 μM and V_{max} = 0.46 ± 0.02 nmol/min/mg protein.

a) Between genders



b) Within genders

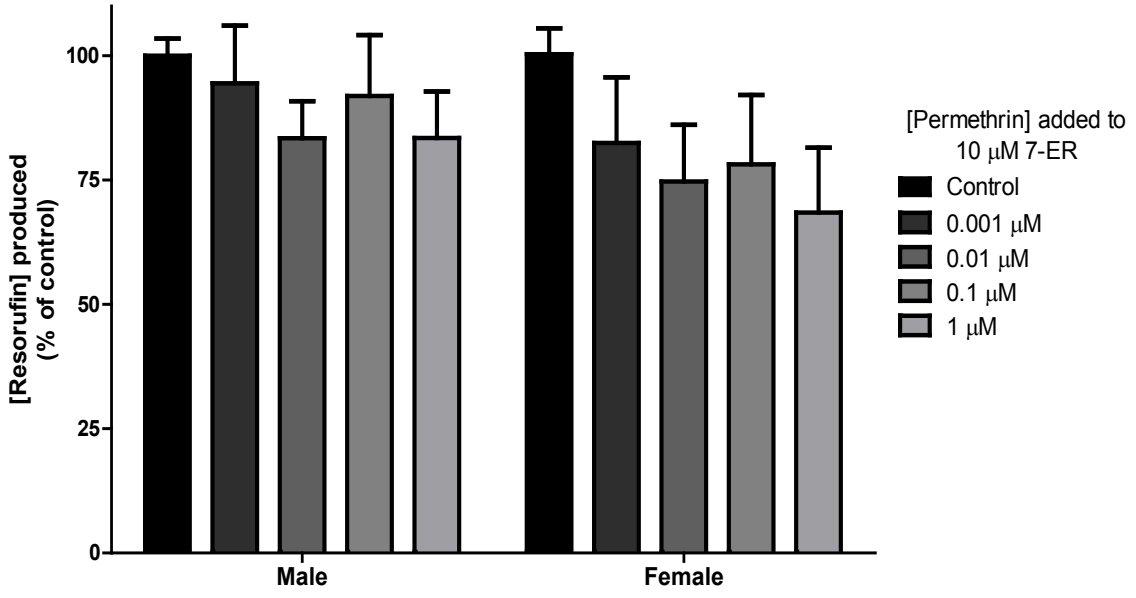


Figure 3.4. Comparing effect of permethrin on the metabolism of 7-ethoxyresorufin between genders (a) and within genders (b); untreated male (black bars) and female (white bars) Wistar rat liver microsomes (biological triplicate, n = 3 – 5/ replicate/concentration). Data were converted to percent of control and presented as mean ± SEM.

Table 3.1. Inhibition percentages for individual and combined incubations with 7-ethoxyresorufin. Comparison of inhibitory percentages of individual incubations of permethrin and cypermethrin (a), and comparison of estimated percentages and observed percentages for the pyrethroid combinations (b). Significant differences between concentrations within one gender are designated by differing letters.

a) Individual incubations

% inhibition	**Permethrin**		**Cypermethrin**	
[Pyrethroid] (μM)	**Male RLMs**	**Female RLMs**	**Male RLMs**	**Female RLMs**
0.001	6 ± 5^a	18 ± 6^a	6 ± 2^a	5 ± 1^a
0.01	17 ± 4^a	25 ± 4^b	6 ± 2^a	14 ± 3^a
0.1	8 ± 5^a	22 ± 6^a	6 ± 2^a	15 ± 2^a
1	17 ± 5^a	32 ± 5^c	8 ± 2^a	21 ± 3^b

b) Combination incubations

% inhibition	**Estimated**		**Observed**	
[Permethrin] + [Cypermethrin] (μM)	**Male RLMs**	**Female RLMs**	**Male RLMs**	**Female RLMs**
0.01 + 0.1	23	40	2 ± 2^a	4 ± 1^a
0.01 + 1	25	46	4 ± 2^a	6 ± 2^a
0.1 + 0.1	14	37	3 ± 1^a	9 ± 1^a
0.1 + 1	16	43	9 ± 2^a	8 ± 1^a
1 + 0.01	23	46	8 ± 4^a	8 ± 2^a
1 + 0.1	23	47	14 ± 5^a	10 ± 2^a
1 + 1	25	53	10 ± 6^a	4 ± 2^a

Table 3.2. Velocities of resorufin production (nmol resorufin/min/mg microsomal protein) for individual and combination incubations with 7-ethoxyresorufin. Comparison of rates for individual incubations with permethrin and cypermethrin (a), and comparison for combination incubations with permethrin and cypermethrin (b). Significant differences between concentrations within one gender are designated by differing letters.

a) Individual incubations

	Permethrin		Cypermethrin	
Concentration (μM)	**Male RLMs**	**Female RLMs**	**Male RLMs**	**Female RLMs**
0	0.254 ± 0.023^{a}	0.281 ± 0.001^{a}	0.115 ± 0.005^{a}	0.109 ± 0.002^{a}
0.001	0.229 ± 0.007^{a}	0.234 ± 0.017^{a}	0.109 ± 0.005^{a}	0.103 ± 0.002^{a}
0.01	0.205 ± 0.011^{a}	0.212 ± 0.014^{b}	0.109 ± 0.005^{a}	0.093 ± 0.001^{a}
0.1	0.221 ± 0.003^{a}	0.222 ± 0.018^{a}	0.108 ± 0.005^{a}	0.092 ± 0.001^{a}
1	0.201 ± 0.005^{a}	0.194 ± 0.016^{c}	0.106 ± 0.006^{a}	0.085 ± 0.001^{b}

b) Combination incubations

	Permethrin + Cypermethrin	
Concentration (μM)	**Male RLMs**	**Female RLMs**
0	0.128 ± 0.008^{a}	0.125 ± 0.001^{a}
0.01 + 0.1	0.125 ± 0.010^{a}	0.119 ± 0.001^{a}
0.1 + 1	0.122 ± 0.006^{a}	0.117 ± 0.001^{a}
0.1 + 0.1	0.124 ± 0.008^{a}	0.112 ± 0.000^{a}
0.1 + 1	0.116 ± 0.010^{a}	0.114 ± 0.001^{a}
1 + 0.01	0.121 ± 0.008^{a}	0.115 ± 0.001^{a}
1 + 0.1	0.118 ± 0.010^{a}	0.114 ± 0.001^{a}
1 + 1	0.121 ± 0.010^{a}	0.119 ± 0.001^{a}

3.4.4 Inhibition by Cypermethrin

The male RLMs produced greater amounts of resorufin than the female RLMs across all concentrations of cypermethrin. The only significant reduction in resorufin production from control was at 1 μM cypermethrin within the female RLMs, generating 21% inhibition, while 0.01 μM and 0.1 μM produced about 15% inhibition. Within the male RLMs, there was minimal reduction, 6 – 8%, in resorufin production regardless of cypermethrin concentration (Figure 3.5, Table 3.1a). The reaction velocities show a similar pattern to the resorufin production; the male RLMs have a slightly but non-significant reduction in velocity with increasing concentrations of cypermethrin, while the female RLMs show a dose-response trend with greater impact to velocity (Table 3.2a).

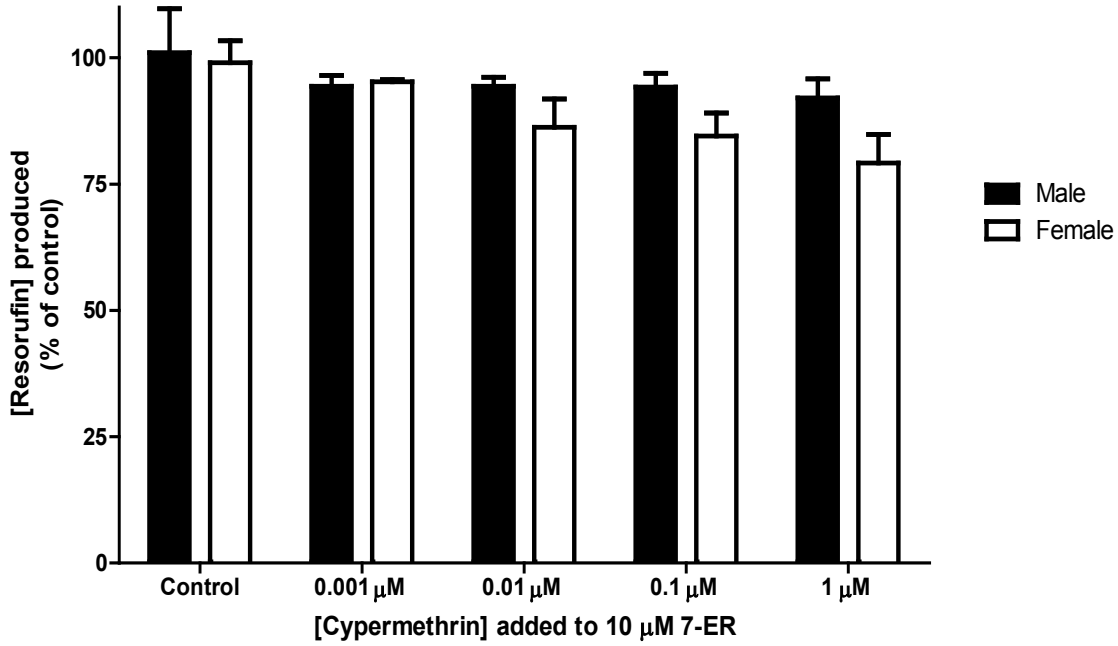
3.4.5 Inhibition by combinations of permethrin and cypermethrin

At lower permethrin concentrations, the male RLMs produced greater amounts of resorufin, but this trend reversed with higher permethrin concentrations, though none were significantly different. Between both sets of RLMs, the greatest reduction was 14% (Figure 3.6, Table 3.1b). The reaction velocities corroborate this; all velocities in both male and female RLMs hover around 0.12 nmol resorufin/min/mg microsomal protein despite pyrethroid concentrations (Table 3.2b).

3.5 Discussion

This acute study investigated the inhibition of the metabolism of 7-ethoxyresorufin (7-ER), a CYP1A specific substrate, by pyrethroids in Wistar rat liver microsomes. The Michaelis-Menten fit in GraphPad was used to calculate CYP1A kinetics, where the male rat liver microsomes (RLMs) had a higher K_m but the female RLMs had a higher V_{max}. With every

a) Between genders



b) Within genders

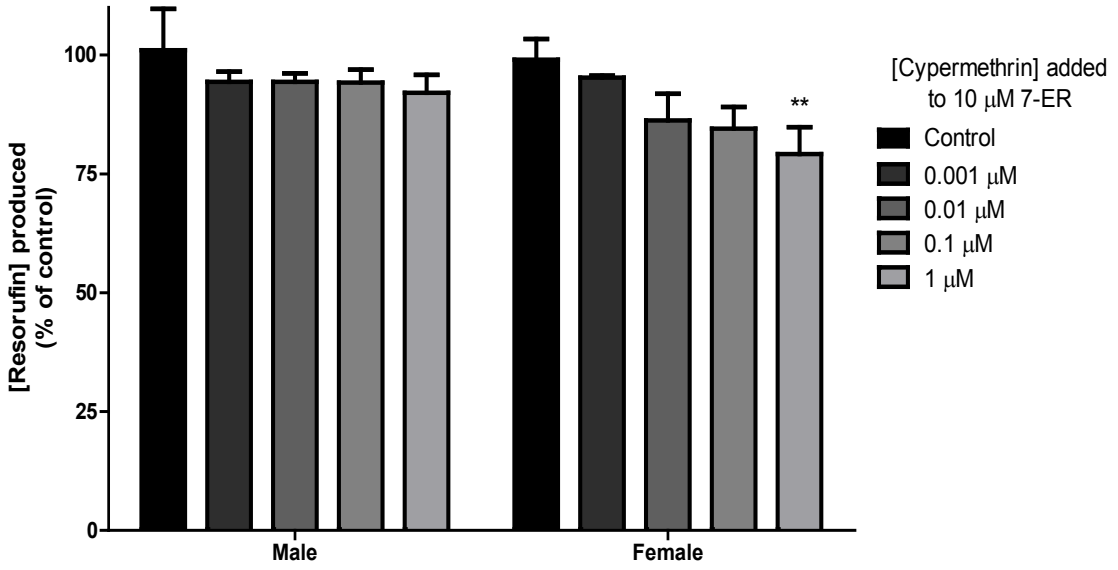
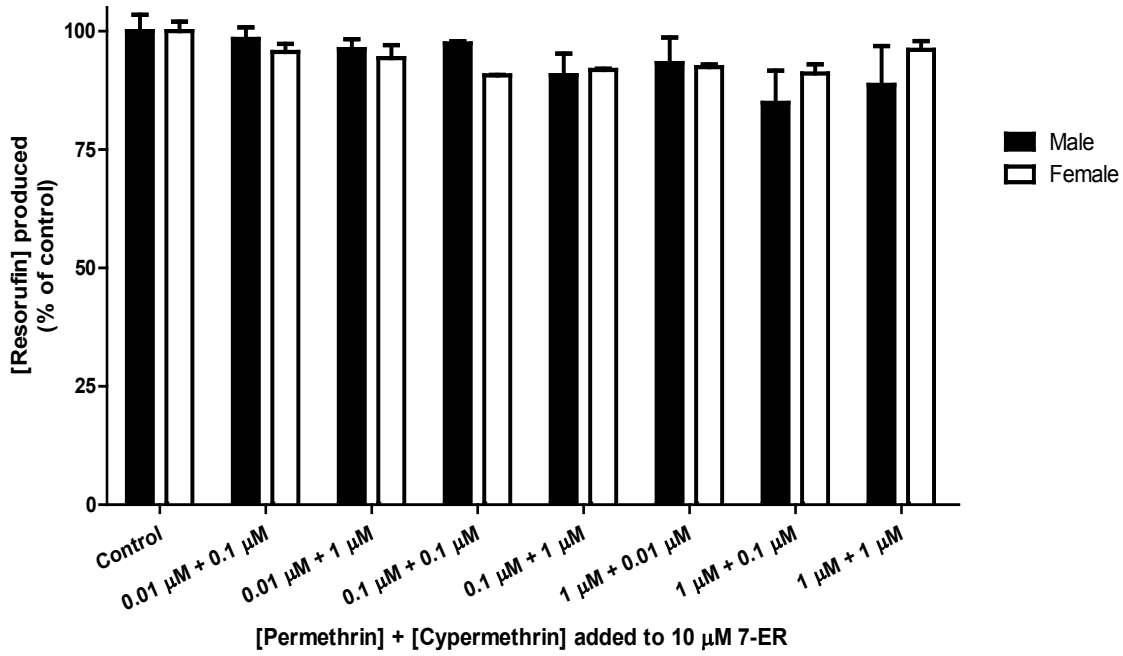


Figure 3.5. Comparing effect of cypermethrin on the metabolism of 7-ethoxyresorufin between genders (a) and within genders (b); untreated male (black bars) and female (white bars) Wistar rat liver microsomes (biological triplicate, n = 3 – 5/ replicate/concentration). Data were converted to percent of control and presented as mean ± SEM. Asterisks (**) represent a statistical difference using a two-way ANOVA with a Tukey post-hoc test ($p \leq 0.01$).

a) Between genders



b) Within genders

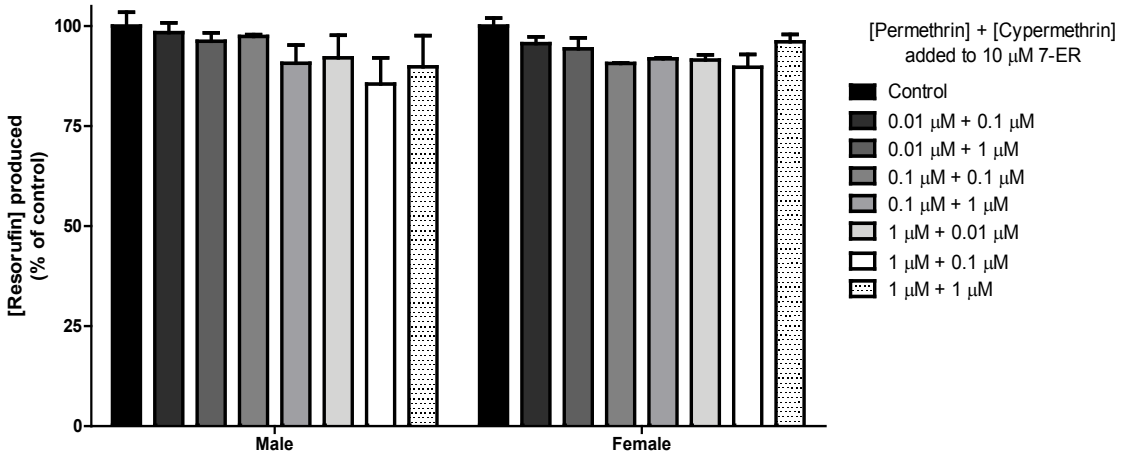


Figure 3.6. Comparing effect of permethrin and cypermethrin combinations on the metabolism of 7-ethoxyresorufin between genders (a) and within genders (b); untreated male (black bars) and female (white bars) Wistar rat liver microsomes (biological triplicate, n = 3 – 5/ replicate/concentration). Data were converted to percent of control and presented as mean ± SEM.

concentration of 7-ER, the female RLMs were able to produce greater amounts of resorufin. Given that female RLMs are supposed to have greater CYP1A content than their male counterpart (Ryan and Levin, 2013), as mentioned previously, this ability was understandable.

On its own, permethrin produced inhibition in both male and female RLMs. However, a 30% *cis*: 70% *trans* mixture was used in this study, and because c*is* permethrin has been shown to be a more potent inhibitor of CYP1A activity than *trans* permethrin (Abdou, et al., 2010), it is reasonable that higher concentrations of permethrin were required to inhibit 7-ER metabolism.

Cypermethrin is thought to be a more potent pyrethroid than permethrin due to its cyano group (DeMicco, et al., 2010, Sheets, 2000, Yang, et al., 2009). However, in this study, the male RLMs did not experience a large impact to their capability in metabolizing 7-ER, and the female RLMs exhibited less reduction with the addition of cypermethrin than they did with permethrin.

The greater inhibition of metabolism using both individual pyrethroids with the female RLMs could be a result of having fewer CYP classes to compensate with (Ryan and Levin, 2013), leaving CYP1A to bear most of the burden.

Combining permethrin and cypermethrin was thought to provide an additive effect, yet produced less inhibition than either produced singularly. The combinations used in this

study only produced a third of the expected inhibition in male RLMs, and in female RLMs a fifth of the expected inhibition was produced. This could be due to a preferential metabolism of 7-ER over the pyrethroids by CYP1A, compensation by other enzyme classes or due to the ratio of 7-ER to the pyrethroids.

The reaction velocities generated in both permethrin and cypermethrin individual incubations corroborate the reductions in resorufin, but show greater significance in both male and female RLMs. When combinations of permethrin and cypermethrin are incubated with the RLMs, the velocities are not significantly different from control.

This is an acute study due to the use of microsomes which are not viable for chronic experiments. However, chronic studies indicate in rats, mice, and dogs that low level chronic exposures do not produce severe symptoms and any symptoms seen were at levels well above the NOAEL/LOAEL (ATSDR, 2003).

3.6 Conclusions

Pyrethroids are thought to be non-toxic to mammals due to a plethora of metabolizing enzymes generating rapid detoxification. However, inhibition of another compound in a co-exposure could suggest a potential toxic mechanism for pyrethroid pesticides. This study showed the ability of permethrin and cypermethrin to inhibit the metabolism of a CYP1A substrate. While the combinations of these pyrethroids used here did not produce an additive effect, it suggests a need to further study the inhibitory potential of these pyrethroids and pyrethroid mixtures on other CYP enzymes.

4.0 GENERAL DISCUSSION

The work presented here showed that a gas chromatography – mass spectrometry method could be developed for pyrethroid pesticide measurement in suitable matrices. This method utilized an ion preparation mode that produced detection limits between 10 – 100 ppb. The extraction protocol, however, was not effective enough at controlling matrix suppression to study metabolism in a microsomal culture system. It was however used to differentiate the sensitivity differences between an internal and external ionization source. Matrix signal suppression using GC-MS measurement demonstrated the need for alternative methods of quantifying CYP activity. A spectrophotometer was utilized as a high-throughput, highly sensitive approach to monitor the metabolism of 7-ethoxyresorufin (7-ER), a CYP1A-specific, fluorogenic substrate, and how addition of pyrethroids, permethrin and cypermethrin, singly and in combination affected this metabolism.

In Chapter 3, the metabolism of 7-ER by means of ethoxyresorufin O-deethylation was monitored. The Michaelis-Menten kinetics of 7-ER were calculated, providing a baseline to compare the impact by pyrethroids against. On their own, permethrin was able to inhibit metabolism of 7-ER in both male and female rat liver microsomes, while cypermethrin really only inhibited metabolism of 7-ER in female rat liver microsomes. Additionally, combinations of permethrin and cypermethrin were tested based on the hypothesis that they would produce an additive inhibitory effect. This hypothesis was disproven by the combination experiments, which showed minimal impact by any combination, even at the highest concentrations of both pesticides.

Microsomes were used due to their concentration of CYP enzymes, though esterases are also present in the matrix because the centrifugation process does not completely remove them. While the hydrolytic pathway of metabolism is the more common route for pyrethroids, it has been thoroughly researched, leaving the oxidative pathway in need of further investigation. This study used rat liver microsomes as a more cost effective matrix that has utility in correlating to human processes as Cyp1a1 and Cyp1a2 in the rat are orthologs to the human CYP1A1 and CYP1A2.

Many questions regarding oxidative metabolism were brought up as a result of this study because the pyrethroid combination experiments disproved the hypothesis that these pyrethroids would produce an additive inhibition. As such, it is important to determine why singly permethrin and cypermethrin produced inhibition but not in combination. Additionally, because only one CYP family was studied here, other families should be evaluated, especially given the efficiency of some of the other CYP enzymes, as well as the difference in CYP profiles, in both rats and humans. Most commercial formulations contain piperonyl butoxide, which is a known CYP inhibitor. While the positive control used in this study was α – naphthoflavone, a well characterized CYP1A inhibitor, it would be worth assessing how well piperonyl butoxide correlates.

This study provided a good starting point to investigate pyrethroid pesticides and their potential for xenobiotic interaction via oxidative metabolism, and more work should be performed to assess the current pyrethroid formulations and known pyrethroid mixtures to

which people are exposed. Higher concentration exposures, as is seen with pesticide applicators, should also be assessed to help with occupational regulations.

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