Quantification of pyrethroid and organophosphate metabolites in hair and urine samples of rural children from the Western Cape, South Africa using the two-dimensional gas chromatography-mass spectrometry.

by

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Declaration

This thesis is presented in fulfilment of the requirements for the degree of Doctor of Philosophy (PhD) in Trichology and Cosmetics Science, Faculty of Health Sciences, University of Cape Town. I declare that the work on which this dissertation is based is my original work, that I am the author (unless explicitly stated otherwise), and that I have not previously submitted any part of it for any qualification.

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Abstract

Extensive use of organophosphates (OPs) and pyrethroids (PYRs) in the Western Cape's farming regions raises significant health concerns, particularly for children, whose developing bodies are more susceptible to the toxic effects of these chemicals. Regular monitoring of these insecticide concentrations in human biological matrices is essential to identify and minimize exposure risks. However, existing chromatography monitoring systems encounter challenges such as sensitivity and matrix effects when quantifying these compounds in complex biological samples. This study evaluated a two-dimensional gas chromatography coupled with a time-of-flight mass spectrometry (2-D GCxGC TOF/MS) based method, specifically designed for analysis of compounds in complex matrices such as urine and hair matrices. Furthermore, the 2D GCxGC TOF/MS was available in-house and proved to be less expensive than outsourcing an analysis using a triple quadrupole mass spectrometer.

The development and validation of the analysis method involved several key steps. First, the extraction techniques were optimized, utilizing QuEChERS for urine samples and solid-liquid extraction for hair samples. This was followed by the fine-tuning of metabolite derivatization using N-tert-butyldimethylsilyl-N-methyltrifluoroacetamide (MTBSTFA) to ensure efficient analysis. The choice of extraction methods was tailored to each sample type to maximize recovery and data reliability. Additionally, MetaboAnalyst 5.0 was used on non-targeted analysis data, offering insights into exposure variations and the effects of OP and PYR exposure on metabolic pathways. Both targeted and non-targeted analysis was performed using 2-D GCxGC TOF/MS. The study focused on children from farming areas in Grabouw and the Hex River Valley, monitored over two cycles (2017 and 2018). The developed and validated method was applied to urine samples (n = 122) collected from 61 participants and hair samples (n = 176) collected from 176 children. Alongside these biological samples, detailed demographic, geographic, and agricultural practice data were collected to provide a comprehensive context for the analysis.

The 2-D GCxGC TOF/MS technique demonstrated a limit of detection (LOD) ranging from 0.19 to 0.89 ng/mL and a limit of quantification (LOQ) ranging from 0.58 to 2.69 ng/mL for the targeted metabolites ((dimethylphosphate-DMP, diethylphosphate-DEP, dimethylthiophosphate-DMTP, diethylthiophosphate-DETP, and 3-phenoxybenzoic acid-3PBA). Additionally, the method detection

limit (MDL) for urine samples were between 0.16- 5.96 ng/mL and MDL for hair were between 2.27-99.45 pg/mg. Although the LOD and LOQ in this study were higher than those typically reported using liquid chromatography-mass spectrometry (<0.1 ng/mL), the method effectively detected OP and PYR exposure levels in children from farming areas in the Western Cape, where agricultural activities lead to elevated exposure. Although extraction recoveries in this study were generally above 50%, the results remained below the typical range of 70-130 %. This could be due to complexity of polar metabolites within the urine matrix likely contributed to inefficient interactions with derivatization agents, additionally, the derivatization process also altered the properties of the analytes and matrix components, further reducing recovery rates. Ethyl acetate proved to be the most effective solvent for enhancing extraction and detection rates. The method was applied to the samples and the analysis showed OP and PYR metabolites in 50% of urine samples and 38% of hair samples.

The method's robustness was demonstrated by its ability to detect and quantify metabolites at low levels. Universal exposure to OPs and PYRs among the children was indicated, likely driven by seasonal and agricultural practices. Notable findings included the detection of DMP, DEP, DETP, and 3-PBA at varying concentrations, suggesting episodic exposure events. The integration of MetaboAnalyst 5.0 with 2-D GCxGC TOF/MS provided a detailed examination of the metabolic pathways affected by insecticide exposure which revealed alterations in amino acid concentrations including glycine, serine, and threonine, suggesting potential disruptions in protein synthesis, energy metabolism and the nuanced biochemistry of human exposure to OPs and PYRs. Furthermore, non-targeted analysis revealed subtle shifts in the abundance of various metabolites involved in oxidative stress response and xenobiotic metabolism pathways, providing insights into the biochemical effects of OP and PYR exposure in children.

The study demonstrated the efficiency of 2-D GCxGC TOF/MS in detecting and quantifying OP and PYR metabolites in biological samples, highlighting its potential for broader environmental and health research. The findings suggest potential geographical and agricultural practice-based variations in exposure levels, warranting further investigation into the nature of OP and PYR exposure in the region. Furthermore, the integration of analytical tools like MetaboAnalyst 5.0 enhanced the understanding of the metabolic impacts of OP and PYR exposure thus prepares for further research groundwork in this crucial field. This supports the need for customized biomonitoring strategies to manage the health risks

of insecticide exposure in vulnerable groups, especially children. Continuous monitoring and advanced analytical methods are essential for safeguarding these at-risk populations.

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%	Percent
3PBA	3-phenoxybenzoic acid
4F3PBA	4-flouro-3-phenoxybenzoic
AChE	Acetylcholinesterase
BC	Before Christ
BCP	4-bromo-2-chlorophenol
Cis DBCA	Cis-(2,2-dibromovinyl)-2,2, -dimethylclopropane-1carboxylic acid
Cis DCCA	Cis-(2,2-dibromovinyl)-2,2, -dimethylcyclopropane-1carboxylic acid
CYP	Cytochrome P450
DAP	Dialkylphosphate
DEDTP	Diethyldithiophosphate
DEP	Diethylphosphate
DETP	Diethylthiophosphate
DMDTP	Dimethyldithiophosphate
DMP	Dimethylphosphate
DMTP	Dimethylthiophosphate
EDC	Endocrine Disrupting Compounds
EPA	Environmental Protection Agency
GC-MS/MS	Gas Chromatography - Tandem Mass Spectrometry
HSR	Hair and Skin research laboratory
IMPy	2- isopropyl- 6- methyl- 4-primidiol
IRAC	Insecticide Resistance Action Committee
LOD	Limit of detection
LOQ	Limit of Quantification
OPs	Organophosphates
QuEChERS	Quick Easy Cheap Effective Rugged and Safe
RSD	Relative Standard Deviation
SD	Standard Deviation
SDS	Sodium Dodecyl Sulphate
TCPy	3, 5, 6- trichloro-2-pyridinol

Trans DCCA	Trans-(2,2-dibromovinyl)-2,2, -dimethylcyclopropane-1carboxylic acid
WHO	World Health Organization
ng mL^{-1}	nanogram per mL
pg/mg^{-1}	picogram per milligram
µg g^{-1}	Micrograms per gram
µg l^{-1}	Micro grams per liter
µg	Microgram

Chapter 1

Introduction and literature review

1.1 Introduction

In modern agricultural practices, industrial processes, and public services the use of pesticides is considered indispensable in safeguarding against losses induced by pests and diseases[1-4]. Using pesticides in agriculture presents numerous benefits, including increased food production, better pest and disease control, improved labour efficiency, and protection of crops and livestock. As a result, pesticides have contributed to reduced hunger, a stronger agricultural economy, longer life expectancy, and lower infrastructure maintenance costs[1,2,4].

The term "pesticide" refers to chemicals like insecticides, herbicides, fungicides, and rodenticides used to eliminate pests that threaten crops, livestock, and humans. These chemicals can be naturally derived or synthetically produced5. Historically, pests have caused significant damage and diseases, leading to the development of various substances to manage them effectively[5-7].

1.1.1 Pesticide use

The historical use of sulfur compounds dates back to around 4500 BC when the Sumerians employed them as insecticides to repel insects and mites[8,9]. Elemental sulfur was also recognized for its fungicidal properties and is the oldest known fungicide, with formal recommendations for its use dating to 1802[10-13]. Ancient civilizations like the Egyptians, Greeks, and Chinese also employed compounds as poisons and insecticides9. In Europe, growing agricultural issues such as potato blight and powdery mildew led to a surge in pesticide use, especially insecticides, during the 1900s as illustrated in **Figure 1.1**[9].

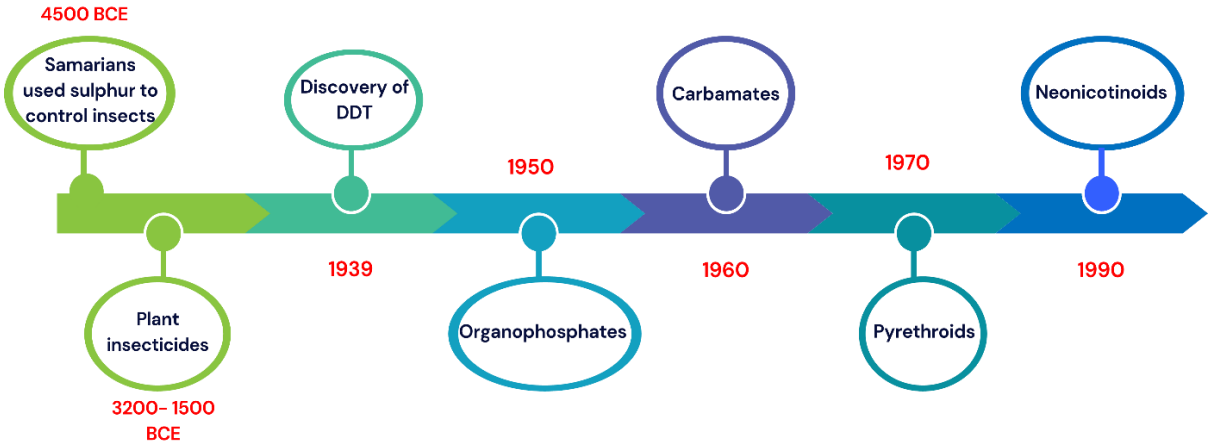
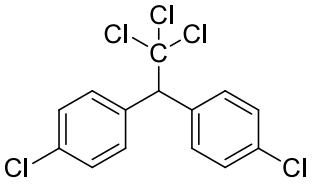


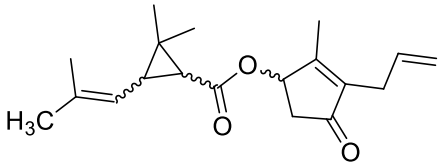
Figure 1. 1: Scaled schematic timeline of insecticide control evolution from 4500 BC to present day, adapted from 'Pesticides' Source[14]

In the 20th century, the agricultural sector witnessed a paradigm shift with the advent of synthetic insecticides as shown in **Figure 1.1**. Synthetic insecticides include organochlorine pesticides (OCPs) such as dichlorodiphenyltrichloroethane (DDT) and the organophosphates (OPs) class followed by carbamates, pyrethroids (PYRs), and lastly neonicotinoids[15]. The different structures of DDT, organophosphates, carbamates, pyrethroids and neonicotinoids are shown below in **Figure 1.2**.

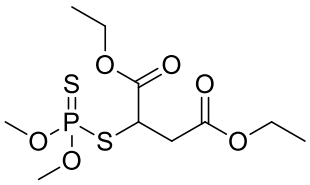
While DDT's initial success was overshadowed by its environmental persistence and health concerns, the ban on OCPs led to the rise of modern pesticides like organophosphates (OPs) and pyrethroids (PYRs) [16-30]. These newer options that are less persistent and toxic raise concerns due to their impact on the nervous system[16,18,19,22,31-35] . PYRs faced declining effectiveness as insects developed resistance[36-38]. The search for effective and environmentally responsible pest control solutions continues, with ongoing research and development of new insecticide approaches.



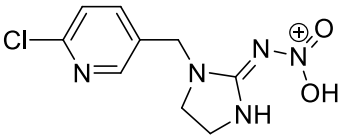
Dichlorodiphenyltrichloroethane (organochlorine)



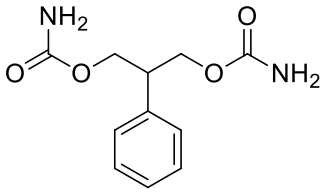
Allethrin (pyrethroid)



Malathion (organophosphate)



Imidacloprid (neonicotinoid)



Felbatol (carbamate)

Figure 1. 2: Structures of the dichlorodiphenyltrichloroethane (OCP), an OCP, Malathion (OP), Felbatol (carbamates), Allethrin (PYR) and imidacloprid (neonicotinoid)

1.1.2 Insecticide Classification.

Insecticides are categorized based on diverse attributes such as chemical composition, entry mechanism, toxicity levels, and mode of action (MOA)[39]. Established in 1984, the Insecticide Resistance Action Committee (IRAC) is an international consortium of crop protection companies that include ADAMA Agricultural Solutions Ltd, Agricultural Biotechnology (AgBiTech), Badische Anilin und Soda Fabrik (BASF), and Bayer[40]. IRAC is globally recognized as the global authority on insecticide classification by MOA, aiding in resistance management[41] and offering guidance to prevent resistance development[42]. In South Africa and across Africa, IRAC's classification system plays a vital role in combating insecticide resistance[43,44]. IRAC oversees over 25 MOA-classified insecticides, as noted by Sparks et al. (2015) with certain examples including OPs and PYRs presented in **Table 1.3**[40].

Table 1. 1: Insecticide classification by mode of action. Only 3 of 25 reported mode of action classes are shown in table[40,45].

IRAC group	Site of mode of action	Insecticide active examples
Nerve and muscle targets	Inhibit action of the nervous system's channel and enzyme.	OPs PYRs Carbamates
Growth and development targets	Mimic growth hormones and disrupt growth	Methoprene Hydroprene Pyriproxyfen Benzoylureas

Understanding class-specific properties of OPs and PYRs ensures accuracy in measuring exposure levels in biomonitoring and elucidate the potential health risks, ultimately contributing to a more informed and effective management of OP and PYR hazards

1.1.3 Utilizing Insecticides for Insect Pest Control Management

1.1.3.1 Current use pesticides (CUPs) for controlling insect pests.

The adoption of CUPs, including PYRs, has marked progress in pest control due to their sustainability and lower toxicity to fish[46,47]. CUPs that target insect-specific receptors are valued for their selective toxicity[48,49]. A key benefit of CUPs is their ability to efficiently eliminate pests while breaking down quickly in the environment, reducing the risk of harmful accumulation in humans and ecosystems[48]. However, widespread use has led to pest resistance through mutations and decreased insecticide effectiveness[48,50]. To address this, alternative strategies have been developed, as discussed in the following section.

1.1.3.2 Alternative methods for controlling insect pests.

Adopting alternative pest control methods is crucial for reducing environmental impacts and safeguarding non-target species, humans included, while maintaining effective pest management [51,52]. Alternative methods also serve to preserve biodiversity and promote sustainable agricultural practices[53,54]. The commonly used alternative pest control methods include:

i. **Biological control:** Biological control emerges as a pivotal alternative in pest management and leverages natural enemies such as predators, parasites, and pathogens to suppress pest populations[55,56]. A prime example is the use of ladybirds in controlling aphids and Colorado potato beetles, showcasing effective pest reduction through predation[57,58], as shown in **Figure 1.3**. This method has gained popularity globally, including in the United States, Russia, and parts of the Caribbean.

Figure 1. 3: Ladybug feeding on an amphid as a biological control technique[59]

ii. **Natural chemical control:** Natural chemical control uses substances derived from natural compounds, predominantly botanical sources such as Pyrethrin[60,61]. Pyrethrin, extracted from *chrysanthemum cinerariae folium* plants (see **Figure 1.4**), found in varied regions like Tasmania, Australia, and Kenya[60,62-64].

Figure 1. 4: *Chrysanthemum cineraria folium* plant[65]

iii. **Genetic control:** Genetic control in pest management involves integrating genetic elements into pest populations to reduce or eradicate them[66,67]. Techniques like pest sterilization are employed, targeting species such as the pink bollworm *Pectinophora gossypiella* and the Mediterranean fruit fly, which impact a wide range of crops[68-71]. Genetic control is preferred for

its ecological sustainability despite higher costs and it creates environments where pests cannot reproduce or transmit diseases[72].

iv. **Integrated pest control or integrated pest management (IPM):** Integrated Pest Management (IPM) represents a comprehensive, science-based pest control approach[73-75]. IPM combines biological control, cultural practices such as crop rotation or soil management, and selective pesticide use, effectively reducing dependence on chemical pesticides and controlling pest populations[76-80]. Originated in Brazil in the 1960s for soybean crops, IPM has been globally adopted for various crops, including maize[81,82]. IPM's benefits are manifold, reducing economic and health risks, preventing pest resistance to insecticides, and promoting sustainable management[79,83]. **Figure 1.5** depicts the iterative steps of the IPM cycle, emphasizing its adaptability and the need for ongoing research and adaptation to pest changes[78].

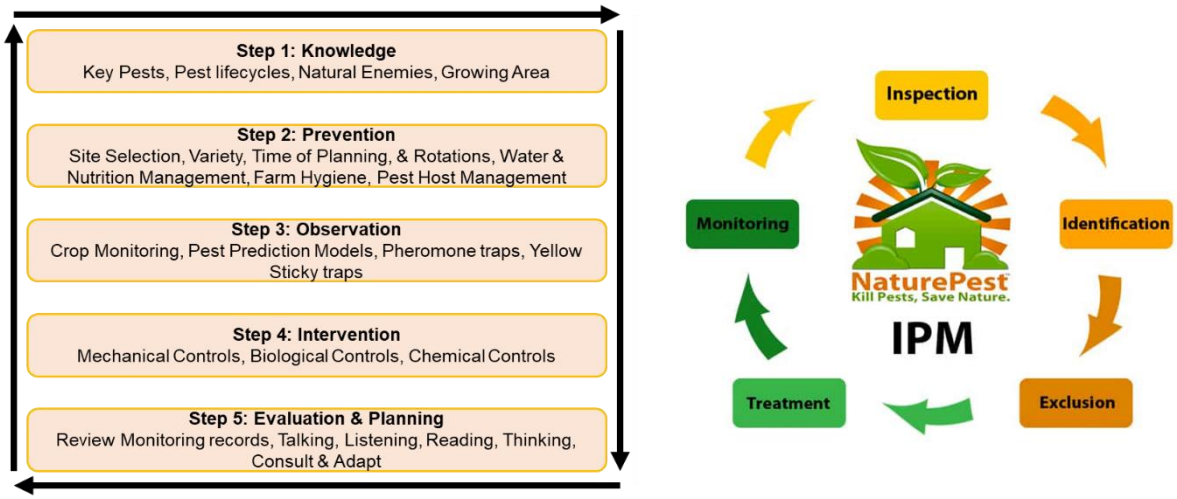


Figure 1. 5: Integrated Pest Management (IPM) steps/cycle[78].

The reliance on insecticides persists despite the adoption of alternative insect control methods, primarily due to the limitations in their efficacy. While these alternative approaches offer ecological and health benefits, their effectiveness often falls short compared to insecticides. For instance, the effectiveness of essential oil-based insecticides varies with concentration, oil type, and application method[84]. In Moroccan citrus orchards, biological controls are less effective against aphids compared to conventional insecticides [85,86]. Advanced techniques like RNA interference (RNAi) face challenges such as high labour, resource demands, repeated applications, and significant development costs [84,87,88]. Additionally, IPM requires extensive knowledge but struggles with regulatory and social acceptance[88,89].

The limited effectiveness and cost of alternative pest control methods, emphasizes the ongoing reliance on insecticides. Despite integrating alternative methods, the need for insecticides often increases to compensate for the gaps in effectiveness of alternative methods. Hence, the pest management landscape reflects not only the inherent limitations of alternative methods but also the ongoing, and sometimes increased, necessity for insecticide use to ensure adequate pest control.

1.1.3.3 OP and PYR uses in developing countries.

In developing countries, especially in Africa, the widespread use of OPs and PYRs is primarily due to their affordability and effectiveness compared to more costly alternatives[90,91]. OPs are commonly applied to staple crops like maize, rice, and vegetables, boosting yields and supporting food security[92-94]. Similarly, PYRs are valued for their stability and effectiveness, making them suitable for field crops and pest control in storage [95-97]. Moreover, PYRs are critical in public health efforts, particularly in vector control programs aimed at reducing mosquito populations and combating malaria[98]. Despite the availability of alternative methods, reliance on OPs and PYRs continues due to their accessibility and proven success.

Regulation of OPs and PYRs in Africa and other developing regions is guided by national laws and international conventions, such as the Stockholm Convention, which limits the use of highly toxic substances [94,99]. While countries like Kenya and South Africa have more structured regulatory frameworks, enforcement is inconsistent, often due to limited resources and gaps in farmer education [100-102]. The World Health Organization (WHO) also provides guidelines for using PYRs in vector control, including indoor residual spraying (IRS)[98,103]. However, weak enforcement, particularly in rural areas, continues to pose risks, as seen in South Africa[104].

Improving regulation requires stronger frameworks, harmonized laws, and better education on integrated pest management. Additionally, cost-effective monitoring systems are essential to reduce pesticide pollution and protect human and environmental health [104-113]. This research focuses on enhancing safety measures related to insecticide use in South African farming communities by identifying and measuring exposure levels.

1.1.3.3.1 OP and PYR Exposure in South Africa

The South African agricultural sector is a significant market in Sub-Saharan Africa for OP and PYR insecticides[104,110,114,115]. The country's role as a major agricultural exporter to Europe has catalysed diverse research efforts to scrutinize OP and PYR use and monitoring in its farming regions, especially the Western Cape [116-119]. Fascinatingly, research in the Western Cape farming region in South Africa sheds light on human insecticide exposure through various pathways, as illustrated below:

i. **Drinking portable and recreational water**- In South Africa, regulating water quality with a focus on insecticides is a pressing issue. London *et al*. (2005) reported widespread contamination of surface and groundwater with various insecticides, including OP and PYR[120]. Dalvie *et al*. (2003) highlighted similar contamination in the Western Cape's rural water systems [121]. Several studies have shown that OP and PYR pollution negatively impacts aquatic life, with contamination affecting fish in the region [122-124]110-112. This poses risks to humans through direct consumption of polluted water or indirectly via contaminated fish.

   Recent research by Curchod *et al*. (2019) and Chow *et al*. (2023) found significant OP and PYR contamination in the Hex River and Grabouw regions, worsened by drought conditions[125]. Chow *et al*. (2023) also noted increased pollution during peak spraying periods[126]. These studies underscore the need to address insecticide contamination in water sources for environmental and human health safety.

ii. **Diet**- Investigations into the diets of South African people revealed a concerning presence of insecticides. Dalvie & London et al. (2009) and Buah-Kwofie et al. (2019) detected insecticide residues in both local and imported raw wheat, a staple food in South Africa[106]. Similarly, high insecticide levels were found in vegetables and peanuts from rural Kwazulu Natal, emphasizing the urgent need for stricter monitoring and regulation to protect the health of those reliant on these foods[127].

iii. **Inhalation**- Studies have highlighted the link between air pollution, inhalation exposure, and related health effects in South Africa. Veludo *et al*. 2022 found insecticides in the air between July 2017 and July 2018 in agricultural areas of the Western Cape [128]. Concurrently, Degrendele *et al*. 2022 reported insecticide levels in both air and soil in the same Western Cape's rural settings[129]Additionally, Fuhrihmn *et al*. 2020 actively broadened this scope by

uncovering a widespread presence of insecticides in the air in 12 African countries. The 12 countries included South Africa's Western Cape farming areas, demonstrating the extensive environmental prevalence of insecticide in both agricultural and urban settings settings[130]. These studies collectively stress the critical importance of strategies to curb the risks of inhaling these insecticides.

iv. **Soil and dust**: Besides air insecticide pollution, Degrendele *et al*. 2022 also observed insecticide residues in dust and soil in Western Cape's agricultural areas. The findings indicate that dust in homes and schools in these regions contains insecticide residues, posing risks of inhalation or ingestion[129,131]. This discovery adds another layer to the complex challenge of managing environmental insecticide contamination.

Furthermore, studies conducted in the Western Cape of South Africa reported the presence of insecticides and their metabolites, such as OP and PYR, in urine samples from both adults and children in farming areas[132-134]. This evidence provides a direct link to environmental exposure and becomes significant, especially when compared with research focusing on the presence of insecticides in water, air, soil, and dust, underlining the widespread impact of these substances on human health.

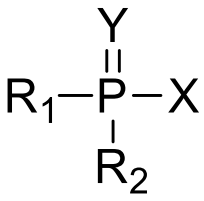
Despite progress in monitoring Op and PYR in environmental and biological samples, these efforts are sporadic and lack cohesion. There is an urgent need for continuous, comprehensive monitoring to safeguard farming communities and consumers from excessive exposure. Moreover, a critical research gap exists in systematically examining biological samples, especially in vulnerable populations like children. Detecting insecticides, such as OPs and PYRs, in various samples highlights their prevalence in the environment, necessitating further investigation into their effects on human health and the ecosystem. The subsequent section further explores the usage of OP and PYR insecticides to control pests.

1.1.4 Organophosphate and pyrethroid insecticides

1.1.4.1 Organophosphate and pyrethroid overview

1.1.4.1.1 Organophosphate

OPs are chemical entities usually synthesized by combining phosphorus compounds with organic counterparts like alcohols, amines, or carboxylic acids[135]. The composition of OPs generally encompasses a methyl or ethyl unit, oxygen (O) or sulphur (S), along with a distinct organic group (X), referred to as the leaving group, as depicted in **Scheme 1.1**[136]. The properties of OPs, that is, the physical, biological, and chemical aspects, vary depending on the specific R and X groups attached to the phosphorus atom (P)[136].



Scheme 1. 1: Schematic representation of an OP structure, denoted as P for phosphorus, R for ethyl or methyl group, Y representing oxygen or sulphur, and X signifying a distinct organic group. The latter is the leaving group, unique to specific OP, encompassing hydroxide, cyanide, phenoxy, or halogen[136,137].

OPs exhibit lower lipophilicity, leading to less accumulation in adipose tissue and making them more susceptible to degradation. Consequently, this increases their toxicity to living organisms, including humans, and non-target species, while reducing their persistence [138]. Despite these risks, the widespread availability and short environmental half-life of OPs continue to drive their common use as insecticides in agricultural and residential areas, particularly in developing nations. The populations most vulnerable to OP exposure include farmers, women, children, and farmworkers who often lack essential resources such as proper education, language skills, or protective gear[139]. Furthermore, the widespread presence of OPs in everyday environments such as homes, cosmetics, farms, hospitals, and foods lead to increased human and environmental exposure [140-144].

Certain OPs, such as chlorpyrifos are actively absorbed through the skin, respiratory system, and gastrointestinal tract into the blood for metabolism[145,146]. This rapid absorption and metabolism process

emphasizes the need for increased awareness and enhanced regulatory measures, highlighting the necessity to protect vulnerable groups and the environment from the harmful effects of OP exposure.

1.1.4.1.2 Pyrethroids

PYRs form a synthetic insecticide class structurally similar to natural pyrethrins from *Chrysanthemum cinerariaefolium* plants[147,148]. To combat the rapid degradation of pyrethrins under sunlight, PYRs were developed to enhance pest control effectiveness and environmental stability while offering a lower toxicity to mammals thus making PYRs more environmentally friendly alternatives to OPs[147,149]. Consequently, PYRs became the predominant insecticide in mosquito and lice control, accounting for 40% of indoor vector control products[221]. In contrast to OPs, PYRs exhibit a range of chemical structures, each derived from modifications to pyrethrin's composition, and are classified into Type I and Type II categories based on their specific interactions with insect and human nerve sodium channels, which influences their insecticidal effects differently[147,150,151].

i. **Type I PYRs** are the most toxic PYRs and bind to the closed nerve sodium channel in a way that causes an over-excitation of nerve cells, leading to the rapid advent of symptoms. Over-excitation results in an increased activity of the channel, which leads to paralysis and death. Examples of Type I PYRs include permethrin and cypermethrin[148].
ii. **Type II PYRs** bind to the open nerve sodium channel in a way that causes a slowing of the channel's activity, hence the slow onset of symptoms. This results in a decreased activity of the channel, which leads to paralysis and death. Examples of Type II PYRs include deltamethrin and lambda-cyhalothrin[148].

PYRs stand out from OPs due to their unique mode of action (MOA), characterized by rapid knockdown effects on insects and high selectivity for target species[147,152,153]. This selectivity is partly attributed to the smaller anatomical structures of insects and the enhanced sensitivity of their sodium channels, compared to those in humans, allowing PYRs to target pests without harming humans. Additionally, PYRs also possess hydrophobic properties, enhanced chemical stability, and resistance to environmental factors like light[47]. Consequently, PYRs have gained substantial favour across both residential and agricultural applications[154].

While PYRs are less hazardous than other insecticides, they are metabolized into toxic compounds, potentially resulting in bioaccumulation and associated health risks. The toxicity level of PYR varies based on factors such as the specific type of PYR involved, exposure levels, the unique metabolic responses of organisms, and prevailing environmental conditions[155-157]. This understanding lays the groundwork for the next section that discusses metabolism.

1.1.4.2 Organophosphate and pyrethroid insecticide mode of action and metabolism

1.1.4.2.1 Introduction to mode of action and metabolism of OP and PYR.

OPs and PYRs are widely used insecticides that exert their toxic effects primarily through neurotoxic mechanisms, with OPs inhibiting acetylcholinesterase (AChE) and causing an accumulation of acetylcholine in the synaptic cleft, and PYRs altering sodium channel function to induce prolonged nerve depolarization[158-160]. In humans, these insecticides are rapidly metabolized and excreted, typically within 24 to 48 hours, with relatively short half-lives ranging from a few hours to several days, effectively preventing significant bioaccumulation[161-165]. However, unlike OPs, PYRs can linger in adipose tissue with half-lives for up to 2-3 weeks[166], underscoring the importance of monitoring OP and PYR exposure. To comprehensively assess the health impacts of insecticide exposure, it is essential to understand how insecticides enter the human body, as illustrated in **Figure 1.6**,[167-172].

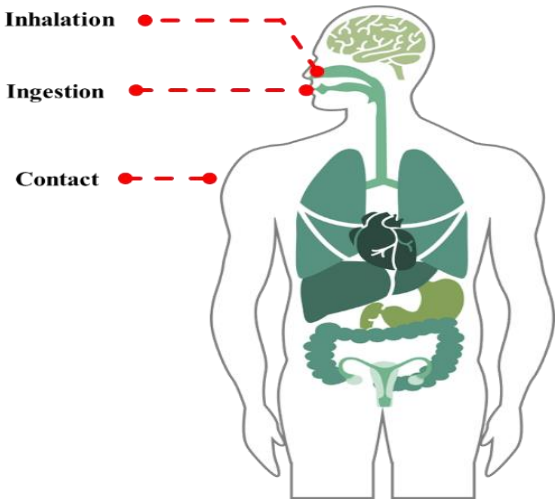


Figure 1. 6: Insecticide exposure routes include inhalation, ingestion, and direct contact[170].

Upon entering the bloodstream through various entry points like the throat, nasal passage, lungs, or in-utero, OPs and PYRs undergo metabolism. Metabolism transforms OPs and PYRs into active oxons

and more polar metabolites, which are partially charged and thus more water-soluble, aiding their elimination from the body through urination[173,174]. The blood circulates these insecticides throughout the body, leading to metabolism, excretion, storage, or bioaccumulation in body fat[170].

Human bodies actively metabolize substances such as waste, drugs, and foreign compounds, including insecticides, through processes that enhance their polarity. This increase in polarity is crucial for detoxifying and eliminating OPs and PYRs, ensuring the safe removal of these substances from the body[175,176]. The metabolic pathway encompasses phases (Phases I and II), as illustrated in **Figure 1.7**, where Phase I involves introducing functional groups into drug molecules through oxidation, reduction, or hydrolysis reactions. The resultant polar metabolites are distinguished by an asymmetric distribution of electrical charge, which is attributed to the incorporation of functional groups such as hydroxyl (-OH), carbonyl (C=O), or amino (-NH2). The presence of these functional groups significantly enhances the solubility of the metabolites in water, which is a polar solvent. However, this modification leads to a decrease in their solubility in nonpolar solvents[176,177].

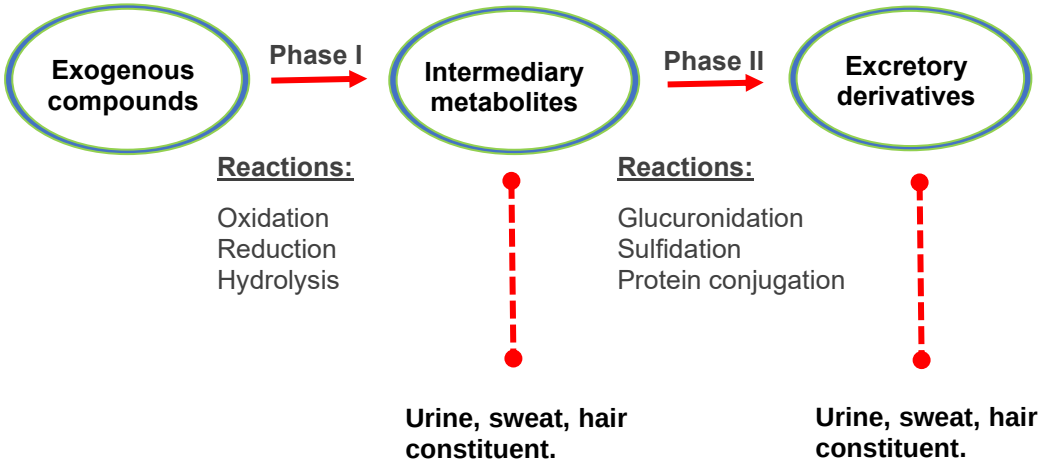


Figure 1. 7: Exogenous substances metabolism involves Phase I and Phase II processes[178,179].

Phase II metabolism involves conjugation reactions where endogenous hydrophilic substances attach to foreign substances or Phase I metabolites, increasing their polarity and inactivation of the parent foreign substance. This process increases their polarity and typically leads to the inactivation of the parent foreign substance through glucuronidation, sulfation, and glutathione conjugation reactions. As

a result, Phase II metabolism produces highly polar foreign substance metabolites, which the body can excrete more readily in urine or feces.[180].

The enzymes responsible for Phase I reactions are predominantly the cytochrome P450 family of oxidases (CYT). In contrast, Phase II reactions are facilitated by enzymes such as UDP-glucuronosyltransferases (UGTs), glutathione S-transferases (GSTs), sulfotransferases (SULTs), and N-acetyltransferases (NATs). Although the liver is the primary site for both Phase I and II metabolic processes, other organs like the skin, lungs, kidneys, and intestines also possess significant metabolic capacity, contributing to drug metabolism and elimination[181-184].

The excretion of metabolized substances occurs through various routes, including urinary excretion, faecal elimination, hair, and exhalation. In certain cases, substances can also be eliminated through sweat or breast milk. This diversity in excretory pathways ensures that the body can efficiently remove a wide range of substances, including those resulting from metabolism, thereby maintaining homeostasis, and preventing potential toxic accumulation.[185-187]. Some compounds become sufficiently water-soluble for elimination and are excreted after Phase I metabolism introduces a functional group. However, other compounds require further processing through Phase II metabolism when Phase I metabolites are not sufficiently polar for excretion, or their biological activity needs further reduction or elimination. The specific metabolic pathway a drug takes depends on its chemical structure and the presence of suitable functional groups for conjugation reactions[188,189].

The metabolism of OPs and PYRs into polar metabolites in the liver and kidneys is a biological marker for exposure to OPs and PYRs[190-194]. The following sections explore the mode of action and metabolism of OPs, and then PYRs, offering insight into their biological impacts and the mechanisms through which exposure can be assessed.

1.1.4.2.2 Mode of action of OPs

OPs oxon metabolites inhibit the acetylcholinesterase enzyme (AChE), which breaks down the neurotransmitter acetylcholine (ACh) in the human nervous system[195-197]. AChE's inhibition leads to the accumulation of ACh in synapses, disrupting normal neural function. Both AChE and ACh are present in target and non-target organisms, resulting in similar effects upon OP exposure[198,199].

AChE which is located at the synapse as illustrated in **Figure 1.9** (image edited from Ikonomopoulou *et al*. 2013[200]), plays a vital role in neuron functionality, primarily by terminating impulse transmission and returning neurons to a resting state[200,201]. Under normal circumstances, transmitting of a neural impulse trigger the release of ACh into the synapse, where AChE breaks it down into acetic acid and choline. Consequently, the breakdown maintains the equilibrium of neurotransmitter levels[195,200,202]. The body then assimilates the acetic acid, and choline gets repurposed for synthesizing new ACh [201,203]. However, this balance is disrupted when OP oxon metabolites inhibit AChE, leading to an excessive buildup of ACh. Such accumulation causes heightened health disorders that are discussed in the health impact section [196,201,202,204].

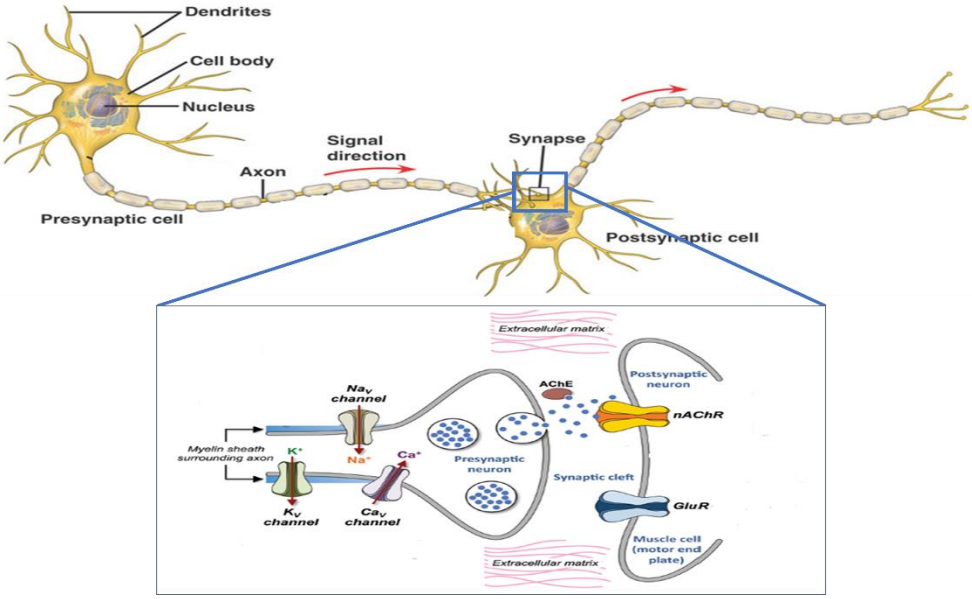


Figure 1. 8: Synapse between two neurons[200].

Additionally, the normal function of AChE is disrupted by the presence of unmetabolized OP, as the OP undergoes phosphorylation by binding to the hydroxyl group at the active site of AChE, resulting in a structural alteration of the enzyme[205-208]. This interaction impedes the hydrolysis of ACh, and if the OP remains within AChE beyond 48 hours, a permanent binding occurs, altering the enzyme's structure and rendering it resistant to reactivation[206,207,209]. Consequently, the inhibition of AChE disrupts neuron function, causing significant and often fatal damage to the nervous system, rendering treatment options ineffective[205,209]. Assessing OP exposure involves determining AChE levels in the blood and quantifying OP metabolites like DAPs in biological samples[210]. Given these risks, efforts have been made to substitute OPs with safer insecticide alternatives, including PYRs, to mitigate these impacts [162,211,212].

1.1.4.2.3 Metabolism of OPs

In the metabolic pathway of OPs, CYT enzymes primarily drive Phase I metabolism conducting oxidation reactions and hydrolysis processes crucial for converting OPs[213,214]. This phase transforms OPs into their active 'oxon' forms or hydrolyses them into more polar metabolites like dialkylphosphates (DAPs) [215] resulting in a combination of non-toxic DAPs and the more toxic oxon derivatives of OPs[216,217]. The specific oxons formed depend on the parent compound undergoing metabolism[218-221], and are known as potent cholinesterase inhibitors that induce acute toxicity and neurotoxic effects[218-220,222-224].

Despite their detrimental health impacts, Paraoxonases (PONs) enzymes step in to metabolize oxons into DAPs, facilitating their excretion from the body[170,225,226]. A 2019 study by Marscicllar and colleagues observed the significant involvement of both CYT and PON enzymes in the Phase I metabolism of OPs, particularly focusing on chlorpyrifos. CYT enzymes metabolise chlorpyrifos into DAPs and more toxic oxon compounds, which PONs hydrolyse into DAPs [39]. Among these enzymes, PON which is a plasma enzyme that plays a pivotal role in hydrolysing these toxic metabolites contributes substantially to the detoxification of OPs[213,214].

Although various OP metabolites are formed during Phase I, TenHoeve *et al*. in 1997 pointed out that conjugating OP metabolites in Phase II can be challenging due to their small molecular size. This difficulty is compounded by the observation that most OPs do not readily conjugate with appropriate carrier proteins [213,227,228]. Additionally, OP metabolites' highly lipophilic chemical structure allows them to dissolve in lipids or fats and distribute throughout the body without further modification through Phase II metabolism[229,230]. The metabolism of any compound, including OPs, can vary depending on various factors related to the compound's structure and properties. As a result of OPs generate a wide range of metabolites, including nonspecific DAPs common to several OPs, and specific metabolites unique to each type of OP [231-236], for instance, 3,5,6-trichloro-2-pyridinol (TCPY) is a, specific metabolite of chlorpyrifos, while 4-nitrophenol (PNP) is specific metabolite of parathion and malathion dicarboxylic acid (MDA) is a specific metabolite of malathion[237-241]. However, this study focuses on the nonspecific metabolites shown in **Figure 1.8**.

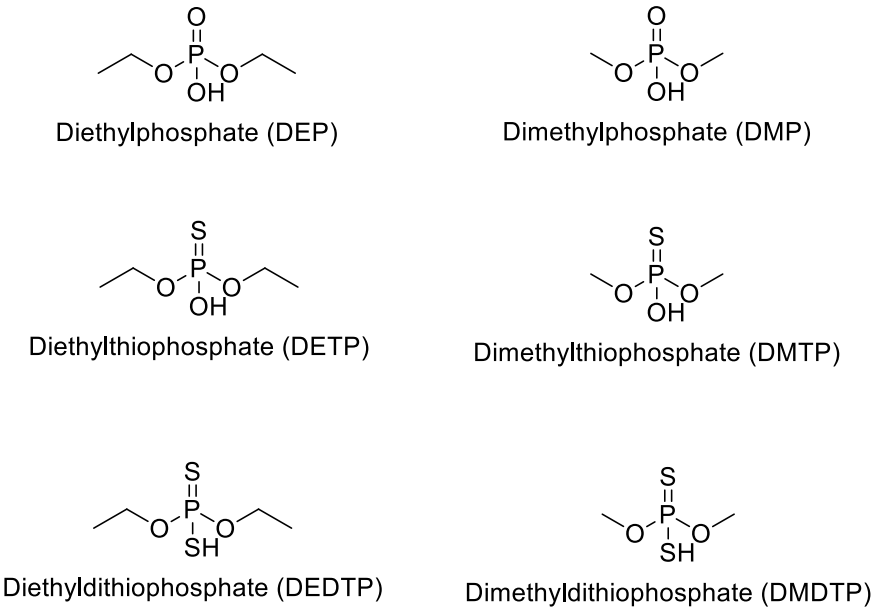


Figure 1. 9: Chemical structures and abbreviations of DAPs, OPs' metabolites commonly measured for OP exposure[241].

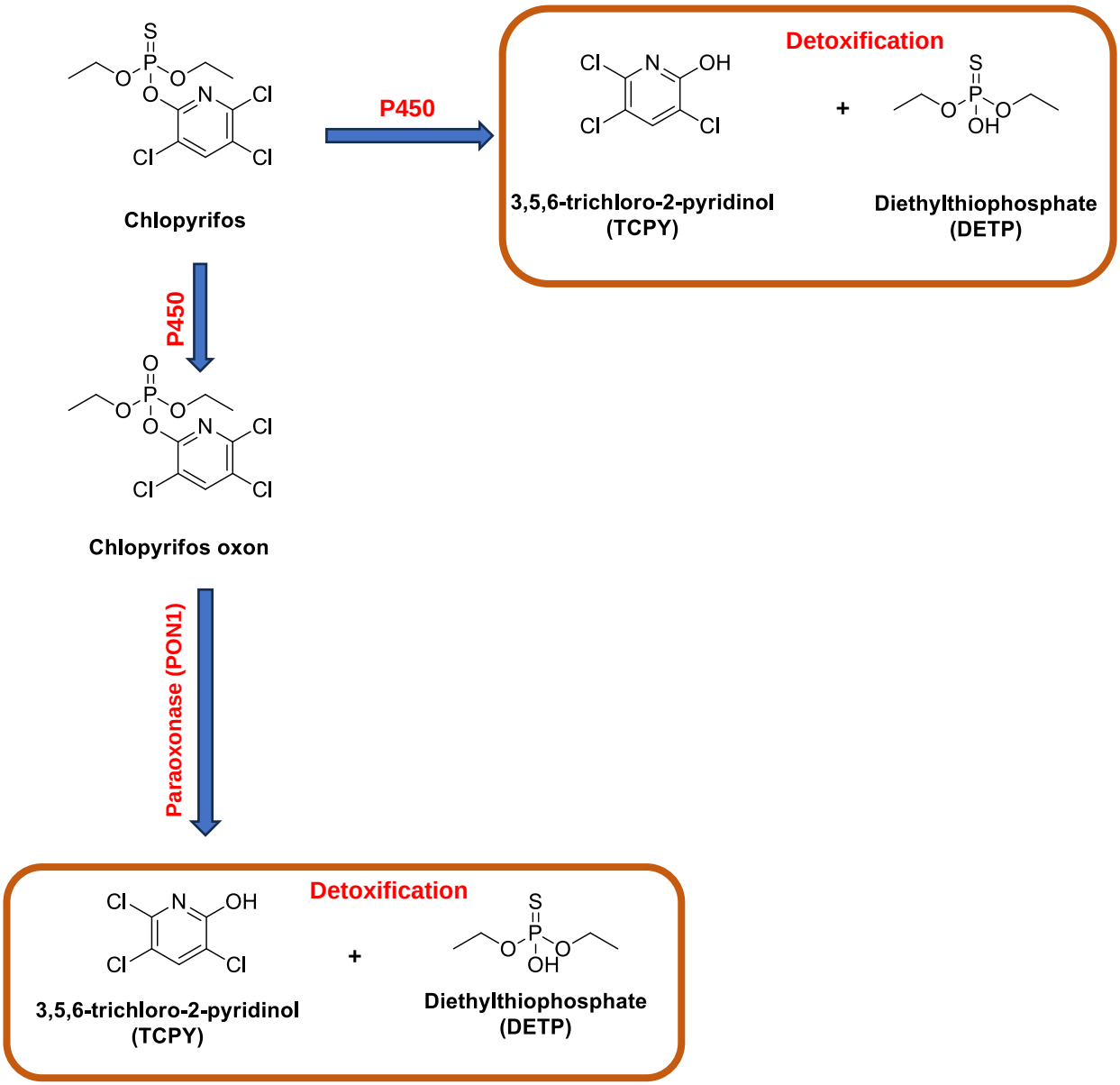
DAPs are essential markers for monitoring OP exposure in biomonitoring[231-236]. **Table 1.1** complements this by showcasing the respective parent OP insecticides that give rise to these DAPs. **Figure 1.8** and **Table 1.1** provide a comprehensive view essential for understanding OPs and their metabolites in this study.

Table 1. 2: Non-specific OP metabolites of common insecticides [242-244].

Classification	Parent Insecticide	Metabolites
OP	Azinphos methyl, chlorpyrifos-methyl, dichlorvos (DDVP), dicrotophos, dimethoate, fenitrothion, fenthion, methyl parathion, oxydemeton-methyl, phosmet, pirimiphos-methy, temephos, naled, tetrachlorviphos, trichlorfon	DMP
OP	chlorethoxyphos, chlorpyrifos, coumaphos, diazinon, disulfoton, ethion, malathion, parathion, phorate, sulfotepp, terbufos,	DEP
OP	Chlorethoxyphos, chlorpyrifos, coumaphos, diazinon, disulfoton, ethion, parathion, phorate, sulfotepp, terbufos,	DETP
OP	Azinphos methyl, chlorpyrifos-methyl, dimethoate, isazaphos-methyl, fenitrothion, fenthion, methyl parathion, oxydemeton-methyl, phosmet, pirimiphos-methy, temephos,	DMTP
OP	Disulfoton, Ethion, Phorate, Terbufos,	DEDTP
OP	Azinphos-methyl, Dimethoate, Malathion, Phosmet,	DMDTP

DAPs are detectable in biological samples such as human hair and urine and serve as reliable indicators of OP exposure across diverse populations, including those in occupational settings and the public. Therefore, DAPs provide insights into exposure to various parent OP chemicals and are recognized as non-toxic biomarkers in biomonitoring studies that can indicate minimal OP exposure without clinical symptoms [197,245]. However, DAPs not only indicate exposure to OPs but also to environmental sources like flame retardants and plasticizers, which, through similar metabolic pathways, can generate the same DAPs as OPs.[246-249]. This prevalence of DAPs in various chemicals that are metabolized upon exposure suggests that their presence should not be exclusively attributed to OP[250].

DMP, DEP, DMTP, and DETP are commonly measured as they are metabolites for a wide range of OP insecticides, whereas DMDTP and DEDTP are specific to certain OPs. These variations highlight the diversity in OP metabolism, which stems from the unique chemical structure of each compound and necessitates specific enzymes and pathways for processing pathways[251-255]. Chlorpyrifos which is a commonly used OP undergoes metabolism to become chlorpyrifos oxon and other metabolites as shown in **Scheme 1.2**, The oxon metabolite inhibits the acetylcholinesterase enzymes, a hydrolytic enzyme vital for nerve function, thereby affecting the nervous system. Furthermore, chlorpyrifos undergoes detoxification into DAPs (DETP and DEP), or it produces a unique metabolite, 3,5,6-trichloro-2-pyridinol (TCPY), specific to chlorpyrifos[256-258]. The structures within the yellow boxes represent DAP metabolites and a distinctive metabolite specific to chlorpyrifos; both excreted in urine. This highlights the complexity and specificity of OP metabolism and its implications for biomonitoring and health risk assessment.



Scheme 1. 2: Modified illustration depicting the metabolic pathway of chlorpyrifos[258].

OP oxon metabolites exhibit markedly higher potency than their original OP compounds due to their stronger affinity for the acetylcholinesterase enzyme.

Mode of toxic action of pyrethroids

PYRs disrupt nerve conduction by targeting voltage-gated sodium channels in neurons, a trait shared across insect and mammal nervous systems[150]. The sodium channels which are essential for transmitting impulses in neuron cells, are blocked, or bound by PYRs leading to impaired impulse transmission. The binding affinity of PYRs to sodium channel receptors is a fundamental aspect of their neurotoxic effect[150]. PYRs prevent nerve polarization, causing prolonged depolarization and paralysis[151]. This process is further elucidated in **Figure 1.10** (diagram Modified from Ikonomopoulou

et al. 2014[200]), which illustrates how PYRs influence the activation and inactivation of sodium channels, leading to hyperexcitability in the nervous system[259]. The consequent generation of abnormal, repetitive nerve impulses can cause various symptoms in humans that are discussed in the health impact section[260].

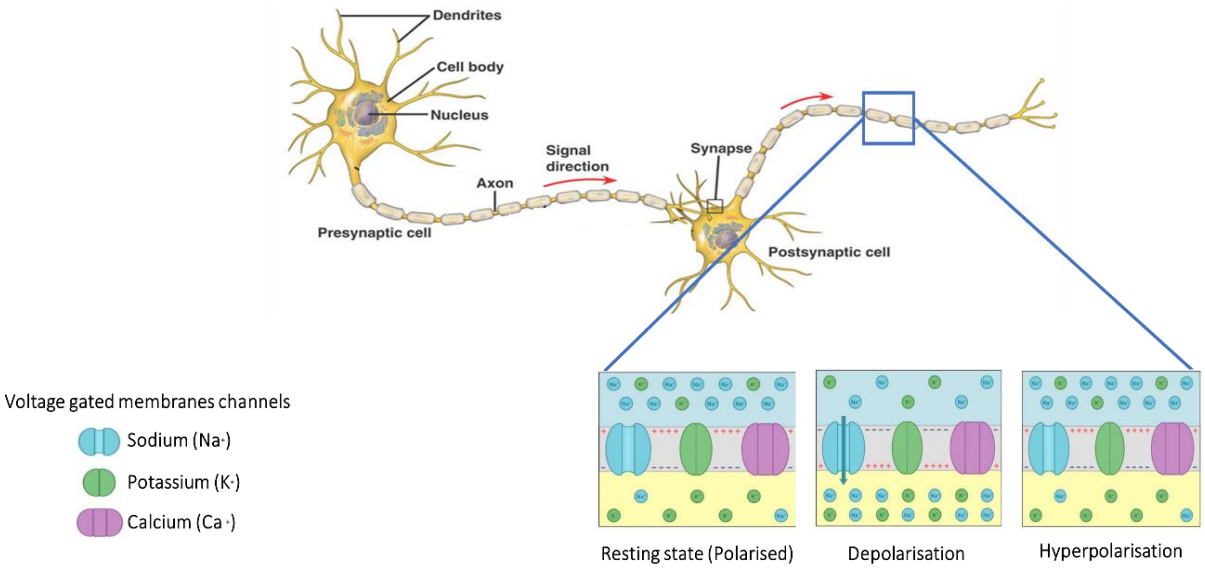


Figure 1. 10: PYRs mode of action[200].

Understanding the metabolism and mode of action of PYRs and other insecticides like OPs is crucial for accurately assessing human exposure OPs and PYRs have been detected in various consumer products such as hair products, beverages, skin products, foods, furniture, and cosmetics[261-263]. While these concentrations often comply with safety limits based on animal studies, the potential health risks to humans might not be fully represented [264-267]. Comprehensive exposure assessments are therefore vital to investigate the extent of OP and PYR exposure and their health impacts on human populations, as informed by epidemiological studies.

1.1.4.2.4 Pyrethroid metabolism

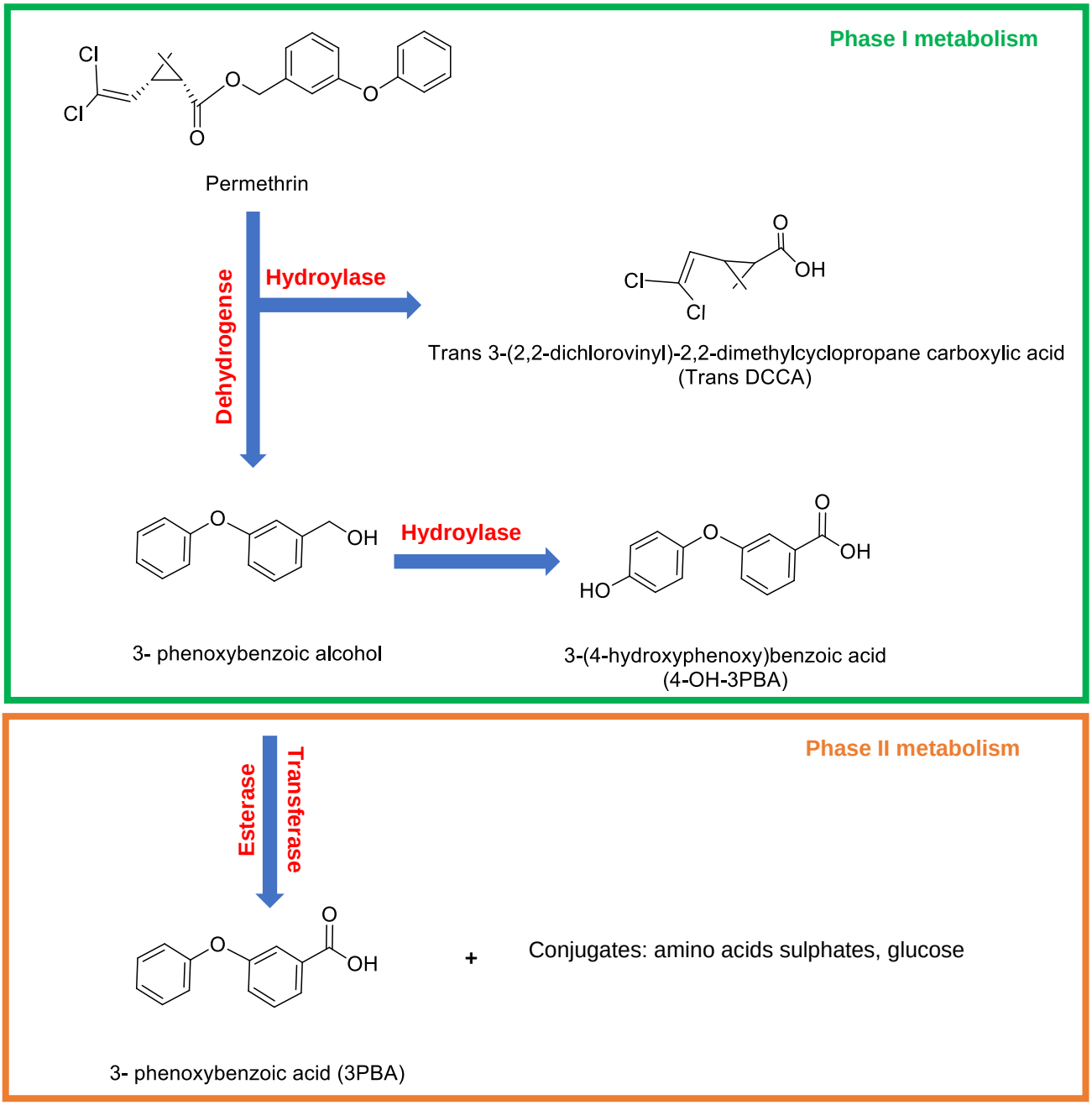
PYRs undergo rapid metabolic transformations, primarily through alcohol or acid moiety oxidation, conjugation, and ester cleavage reactions. CYT enzymes facilitate oxidation processes in phase I, while Glutathione S-transferases (GSTs), N-acetyltransferases (NAT), and other enzymes facilitate phase II by conjugating the PYR metabolites to more soluble moieties that can be excreted.[268]. Hydrophilic conjugates, including glucuronides, sulfates, and amino acid conjugates, constitute a significant portion of PYR metabolism in mammals[269]. These swift metabolic processes contribute to low toxicity and

generate hydrophilic and lipophilic conjugate metabolites[152,268]. A common PYR metabolite is 3-phenoxybenzoic acid (3-PBA), stemming from common PYRs, as shown in **Table 1.2** [237].

Table 1. 3: Non-specific PYR metabolites of common insecticides [242-244].

Classification	Parent Insecticide	Metabolites
PYR	Cyhalothrin, cypermethrin, deltamethrin, ethonfenprox, esfenvalerate, fenpropathrin, permethrin, Tralomethrin, phenothrin	3-PBA

Permethrin is a widely used PYR and is a prime example of PYR metabolism. Its broad application ranges from commercial insecticides to clothing treatments and head lice infestations, underscoring its importance in understanding PYR metabolism[270-277]. The metabolic pathway of permethrin, detailed in **Scheme 1.3** (image adopted from McCarthy *et al* 2005 [278]), includes Phase I and II reactions. In Phase I, hydroxylases and dehydrogenases increase permethrin's polarity, while in Phase II, enzymes like esterases transform it into water-soluble forms for excretion[164,273,279,280].



Scheme 1. 3: Metabolism of Permethrin[278].

3-PBA serves as a key biomarker for assessing human exposure to various PYRs, with most metabolites rapidly excreted as conjugates in urine[245,281]. However, the parent compounds are not excreted in urine due to their high lipophilicity and rapid metabolism[281,282]. Several studies suggest that urine and plasma are significant biological matrices for detecting and analysing conjugated PYR metabolites[283-285]. However, other matrices like hair require more focused research, as 3-PBA does not typically appear conjugated in hair samples.

Hydrochloric acid and enzyme hydrolysis are commonly used in analytical processes to transform conjugated PYR metabolites into their free forms, facilitating detection and quantification [282,286]. PYRs have been identified as endocrine disruptors, interfering with hormone signalling pathways in humans and wildlife, it is important to understand their mode of action and metabolism.

1.1.4.3 Health Impacts of OP and PYR Exposure.

Exposure to OPs and PYRs poses significant short-term and long-term health risks. Animal studies have reported that short-term exposure can cause acute symptoms such as dyspnoea, cough, bronchospasm, headaches, skin allergies, visual impairments, excessive bodily secretions, and muscle weakness[161,287-290]. Additionally, exposure can trigger the release of neurotransmitters like dopamine that lead to oxidative stress and neurotoxicity[147,196,197,291-298]. These immediate effects may progress to more severe conditions like delayed neuropathy, characterized by numbness and cramping[196]

Long-term exposure to OPs and PYRs has been linked to changes in brain function and cognitive impairments, notably in attention and memory[299 300]. Furthermore, prolonged exposure leads to neurotoxic, genotoxic, and immunotoxic effects. There is also a growing concern over the potential impact on reproductive immune, endocrine, and vital organs health[301-304]. Long-term exposure is reportedly alarming in children as they are more susceptible to neurological and developmental abnormalities and are at an increased risk of OP and PYR-related toxicity[305-307]. The observation that low doses of OP and PYR affect the mammalian nervous system emphasizes the necessity for cautious use and exposure assessment[153,198,308,309] these findings collectively underscore the serious and far-reaching risks associated with short- and long-term exposure to OPs and PYRs.

As such, the increasing awareness of the potential risks associated with OP's and PYR's exposure signifies the importance of establishing comprehensive insecticide exposure assessment initiatives.

1.2 Exposure Assessment

Exposure assessment is a critical process in environmental health, occupational health, and epidemiology. It involves estimating or measuring the magnitude, frequency, and duration of exposure to an agent including insecticide, along with the number and characteristics of the population exposed. The aim is to understand and quantify the levels of exposure to chemicals, pollutants, or other hazardous agents.

The exposure assessment of insecticides heavily relies on the classification of insecticides as it guides the selection of biomarkers and analytical methods, ensuring accurate monitoring of exposure levels[310,311]. Researchers and public health officials can better understand and manage the risks associated with these chemicals by linking specific insecticide classes with tailored biomonitoring approaches.

1.2.1 Biomonitoring

Monitoring is typically a recurring, systematic, and precautionary endeavour to guide potential corrective measures, distinct from diagnostic procedures[312]. Presently, human biomonitoring (HBM) is the primary means for measuring insecticide exposure and involves quantification of insecticide exposure biomarkers (insecticides and their metabolites) in biological samples[238,313]. In the context of this investigation, the focus was on urine and hair matrices for evaluating exposure to OPs and PYRs through HBM.

1.2.1.1 Biological matrices in OP and PYR biomonitoring.

Routine implementation of HBM is essential for setting OP and PYR safety regulations to accurately measure internal exposure levels[314]. Blood, serum, and plasma are commonly used to quantify persistent insecticides like OCPs [238,315], with blood being particularly effective due to its interaction with organs where chemicals accumulate[316]. For OPs and PYRs, urine is preferred, as these insecticides are rapidly metabolized, resulting in lower concentrations in blood[313,317]. While blood collection is invasive and may discourage participation, urine collection is non-invasive, offers larger sample

volumes, and is the primary route of insecticide excretion. However, it mainly reflects short-term exposure and can vary significantly in metabolite concentrations [318-320].

There is growing interest in non-invasive matrices like saliva, hair, breast milk, and mucus for HBM, especially for vulnerable groups like children and pregnant women[316,321]. Hair is valuable for assessing both short- and long-term insecticide exposure, revealing cumulative exposure patterns over time [321-324]. Hair samples are easy to collect, store, and transport, with the durable structure allowing long-term analysis[325-327]. However, differentiating between internal and external contamination in hair remains challenging[326,328].

Community-level exposure studies often rely on urine samples[329-332], but these are resource-intensive and costly[333]. To address this, innovative methods are needed to improve HBM efficiency and accuracy, providing essential data on the link between exposure and health outcomes[334]. Analytical procedures, including sample pre-treatment and detection techniques like mass spectrometry or chromatography, are critical to accurately identify and quantify insecticide metabolites[313,335,336]. The analysis of OP and PYR in urine and hair will be detailed in **Chapters 3** and **4**.

1.2.1.2 Sample analysis: Chromatographic techniques for detection and quantification.

Bioanalytical analysis or sample analysis is a procedure for identifying and quantifying specific chemicals within a biological matrix[337]. Various techniques have been developed and applied to scrutinize OPs and PYRs in urine, such as enzyme-linked immunosorbent assay (ELISA) and chromatography [338,339]. While ELISA can be a rapid and cost-effective method for screening OP and PYRs residues in food samples, it may not be as suitable for analysing metabolites in human biological samples[340]. ELISA assays typically require the availability of specific antibodies for each target analyte, which may not be readily available for all insecticide metabolites[341]. Additionally, ELISA assays may have limitations in terms of sensitivity and specificity compared to chromatography methods[341]. Compared to chromatography, ELISA and immunoassay methods often struggle with non-specificity and have difficulty distinguishing between similar chemicals and their metabolites, while mass spectrometry offers higher accuracy by directly identifying individual compounds

Chromatography is preferred for its versatility and wide detection range, including UV absorbance and mass spectrometry (MS), for detecting insecticide metabolites[342]. Coupling chromatography with MS enhances analytical capabilities, providing detailed molecular weight and fragmentation pattern data of analytes, thus ensuring high specificity and sensitivity suitable for diverse biomonitoring applications[238,343,344]. Techniques like liquid chromatography (LC) and gas chromatography (GC) are particularly effective for identifying and quantifying OP and PYR metabolites in complex biological samples [238,343]. These methods enable simultaneous separation and identification of multiple compounds, essential for insecticide mixture analysis and toxicological assessments[343].

Chromatography operates on differential compound distribution between stationary and mobile phases[345]. The stationary phase remains fixed, while the mobile phase, a gas in GC or liquid in LC, flows through the stationary phase. This interaction causes compounds to separate based on their affinity towards each phase, allowing for effective component[238,345,346]. **Chapter 2** comprehensively discusses chromatography and mass spectrometry.

The focal point of this study was the utilization of chromatography, specifically a GC technique for identifying and quantifying metabolites derived from OPs and PYRs extracted from hair and urine matrices.

1.2.1.2.1 Gas Chromatography instrument

Gas chromatography (GC) operates on the principles of separation and quantification by vaporizing analytes and efficiently separating them on a stationary phase. This technique excels in identifying and detecting complex mixtures of volatile compounds in the gaseous phase using a compatible detector [347-349]. The GC technique is constructed from key components: an injector, an oven-encased capillary column, and a detector. These elements collaborate to facilitate the separation and analysis of compounds. The choice of the GC column, the programming of temperature, and the selection of the detector type are all determined by the specific characteristics of the analytes under scrutiny[350-352]. The GC platform is renowned for its capacity to achieve high-resolution separations, rapid analysis, and exceptional sensitivity attributes that collectively render it indispensable across a spectrum of analytical applications[353,354].

The GC analytical process involves introducing a sample vaporized through heat application and propelled along the column by a carrier gas (typically helium, hydrogen or nitrogen) originating from the injector point. Inside the column (the stationary phase), the mixture undergoes separation predicated on the compounds' volatility and structural attributes. Throughout GC analysis, controlled temperature elevation or alteration prompts the elution of analytes. The resultant temperature gradient delineates analyte retention times and separation efficacy [355].

In this research study, a highly sensitive and structurally insightful approach was employed, which combined a mass spectrometer (MS) with GC, forming a GC/MS. The MS unit encompasses three integral components: an ion source, a mass analyser, and a detector, collectively discerning ions based on their respective size and charge, see **Figure 1.11**[355]. Within the GC/MS system there are two dominant ionization methods which are electron impact (EI) and chemical ionization (CI)[356,357]. Unlike electron impact (EI), CI is considered gentler, preserving molecular ions due to reduced fragmentation[357]. CI is advantageous for polar and non-volatile compound analysis and is employable with various reagent gases[358].

This study focused on EI ionization which pairs well with GC-MS and serves to analyse volatile and semi-volatile organic compounds[359-361]. It involves analyte molecules encountering high-energy electrons from an electron gun, forming a positively charged molecular ion (M+). This method is particularly useful for small molecules, large biomolecules, and complex mixtures[362-365]. Moreover, the process results in molecular fragmentation generating a distinctive pattern of ion fragments essential for substance identification and structural analysis[366-368]. Consequently, the mass spectrum from EI provides insights into the molecular weight and composition of compounds aiding in their identification and structural elucidation in GC-MS analysis[363,364,369]. EI's ability to produce consistent fragmentation patterns makes it crucial for organic chemistry analyses and serves as a foundational technique in environmental analysis forensic science and the examination of organic compounds[369].

These methodologies contribute to the generation of ions for subsequent analysis, thereby furnishing valuable insights into the composition and structure of compounds.

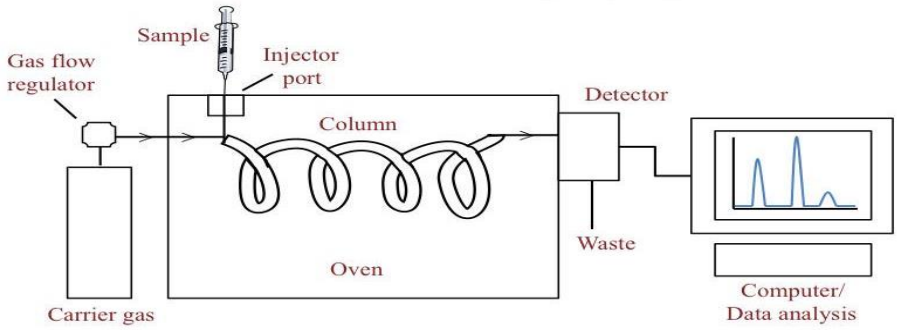


Figure 1. 11: Main components of GC[355].

1.2.1.2.1.1 Analysis of OP and PYR in biological samples using gas chromatography

Various human biological samples have been successfully analysed for detecting and quantifying OP and PYR metabolites using GC-MS methods, with a focus on meticulous sample preparation. Srivastava *et al*. (2017) reported a sensitive method for estimating OP and PYR residues in human plasma using GC-MS, emphasizing the effectiveness of the Quick, Easy, Cheap, Effective, Rugged, and Safe (QuEChERS) approach for sample preparation[370]. Hardy *et al*. (2015) demonstrated the versatility of GC-MS in detecting OP and PYR metabolites in urine and hair via Gas Chromatography-Tandem Mass Spectrometry with Negative Chemical Ionization (GC-MS/MS-NCI) [371].

Iqbal *et al*. (2020) modified the QuEChERS extraction method with GC-MS to detect OP and PYR in blood and urine, further highlighting GC-MS's adaptability[372]. Simaremare *et al*. (2019) correlated OP and PYR levels in blood and urine using GC-MS[197], while Pérez *et al*. (2010) demonstrated the precision of isotope dilution GC-high-resolution mass spectrometry for plasma analysis[373]. These studies confirm the efficiency of GC-MS in profiling OP and PYR metabolites across various human matrices, making it crucial for biomonitoring and toxicological assessments.

1.2.1.2.1.2 Advantages and disadvantages of using GC-MS.

GC-MS is used in OP and PYR metabolite analysis due to its sensitivity and selectivity, especially when paired with the MS. This combination effectively detects OP and PYR insecticides and their metabolites across various matrices. GC-MS's versatility allows for the screening of multiple insecticides, making it crucial for comprehensive residue monitoring [374]. Additionally, it enhances the separation of OP and

PYR metabolites from matrix co-extracts, improving detectability and reducing interference from other compounds [375]. However, GC-MS is complex and time-consuming, requiring meticulous sample preparation and skilled personnel [376]. Also, GC-MS is not designed to analyse polar, non-volatile, or thermolabile compounds, which can restrict its applicability in analysis of polar compounds. The need for derivatization of certain insecticides and metabolites due to their non-volatile nature further complicates the analysis [377,378].

This study used a GC technique (The 2-Dimensional Gas Chromatography coupled with Time-of-Flight Mass Spectrometry – 2D GCxGC ToF/MS) for the quantification of OP and PYR metabolites in urine and hair samples.

1.2.1.2.1.3 Justification for 2D GC-ToF/MS use in Quantifying PYR and OP Metabolites.

The LC technique is commonly used for analysing polar metabolites like those from OPs and PYRs due to its suitability for LC separation mechanisms[133,379-383]. However, this study selected 2D GCxGC-ToF/MS to overcome the limitations of traditional LC and GC-MS techniques. LC is less effective for volatile and semi-volatile analytes, and traditional GC-MS struggles with resolving complex mixtures, which can hinder the identification and quantification of trace-level compounds[384-386]. Therefore, 2D GCxGC-ToF/MS was chosen for its enhanced separation capabilities and improved compound detection, providing a more effective solution for these analytical challenges[387-390].

The decision to utilize 2D GCxGC-ToF/MS over UPLC-MS/MS is grounded in the distinct advantages offered by 2D GCxGC-ToF/MS in both targeted and non-targeted metabolite profiling in complex biological matrices like urine and hair[391]. While UPLC-MS/MS is well-established for non-volatile compounds due to its superior sensitivity and precision in quantification, 2D GCxGC-ToF/MS excels in scenarios where a combination of high-resolution separation and comprehensive metabolic profiling is required[392,393]. The coupling of the two-dimensional GC and the time-of-flight mass spectrometer (ToF/MS) allows for superior separation of complex mixtures, thereby reducing matrix interferences, which can be a limitation in LC and GC MS[390,394-399]..

The ToF/MS detector's capability to analyse diverse compounds within a single sample, ranging from small metabolites and chemicals to large biomolecules like proteins and peptides [400]. This enhances both the sensitivity and accuracy of quantification through advanced optimization techniques like isotope dilution for detecting low-abundance metabolites in urine and hair[401-404] . Moreover, 2D GCxGC-ToF/MS excels in non-targeted analysis, an essential component of this study's objectives, as it allows for the discovery of unknown metabolites potentially affected by OP and PYR exposure[405-408] .

Although ToF-MS has traditionally been considered less sensitive for quantification compared to quadrupole systems, recent improvements in detector performance allow it to provide robust quantitation when optimized for specific biomarkers such as DAP and 3PBA in environmental studies [408-411]. This dual capability (targeted and non-targeted analysis) enables the comprehensive metabolic pathway analysis planned in the study, offering a wider scope of analysis. As shown in **Table A2.13** in the Appendix and **Table 2.4** in **Chapter 2**, the LOD and LOQ of 2D GCxGC TOF/MS in this study are comparable to those reported in the literature for OP and PYR metabolites using LC and GC techniques. This data demonstrates that when the ToF/MS is optimized it can deliver comparable quantification results. Thus, the choice of this method is justified not only for its robust quantification capabilities but also for its ability to simultaneously perform comprehensive metabolic profiling.

Furthermore, the choice of 2D GCxGC-ToF/MS was also influenced by its accessibility at the time of the study. While UPLC-MS/MS might be the preferred method in some settings, resource limitations and instrument availability can play a significant role in methodological decisions, particularly in research conducted in developing countries or resource-constrained environments.

1.2.1.3 Sample data analysis.

Data analysis primarily utilises statistical methods to assess analytical precision and accuracy[412]. In this study, calibration curves, standard reference materials, and quality control samples were key components in quantifying analytes and evaluating method performance. This analysis provided insights into analyte concentrations, their distribution in sample matrices, and emerging trends.

The study used Excel's regression statistical and Stastica tools were used to shed light on potential health risks in the population studied using the HBM data. MetaboAnalyst 5.0, a web-based tool for statistical analysis and interpretation of metabolomic data, played a crucial role in the study[413]. This tool, known for its effectiveness in handling data from analytical techniques like GC-MS[414], enabled univariate and multivariate statistical analyses, pathway analysis, and enrichment analysis. It was a crucial resource for interpreting the metabolic impacts of insecticide exposure within the human body. Leveraging GC-MS data, the MetaboAnalyst 5.0 provided a comprehensive understanding of the complex bodily reactions to these insecticides, aiding in the assessment of potential health effects. The metabolomic data is presented in **Chapter 5**.

1.3 Study overview

1.3.1 Study Rationale

In Sub-Saharan Africa, children face a disproportionately high risk from environmental health hazards[415]. Children's heightened vulnerability to environmental toxins means that exposure during their crucial early developmental stages can lead to immediate and long-term health disorders. Despite this, there remains a significant gap in research on the impact of insecticide use on children's health, particularly in South Africa[210,416-418].

Children are particularly susceptible to accidental ingestion of insecticides, primarily due to their frequent hand-to-mouth activities and their higher relative intake of food and fluids. Furthermore, the commercial farming sector often prioritizes economic benefits over health and safety concerns. A significant hurdle in epidemiological studies investigating the health impacts of insecticide exposure in South African children is the lack of robust methods for exposure assessment, such as biomonitoring.

This study aims to tackle these challenges by developing a GC-MS analytical method 2-D GCxGC ToF/MS for the detection of OP and PYR metabolites in urine and hair matrices. The research includes an epidemiological study among children residing in the agricultural communities of Hex River and Grabouw areas of the rural Western Cape, South Africa. The study explores exposure levels across various farming systems, the proximity to agricultural areas, and gender-based differences in exposure.

1.3.2 Research aim and objectives.

1.3.2.1 Aim

To develop and validate a 2-D GCxGC TOF/MS analytical method for quantifying OP and PYR metabolites in urine and hair samples, and to compare metabolite levels in children living in the Grabouw and Hex River farm areas of the Western Cape, South Africa. Additionally, the study aimed to develop a method capable of performing both targeted analysis of specific metabolites and non-targeted analysis to explore metabolic profiles in urine simultaneously.

1.3.2.2 Objectives

The aim as mentioned above would be achieved by following objectives:

1. Develop a modified extraction method for the extraction of OPs/ PYRs non-specific exposure biomarkers (DAP and 3PBA) in urine and hair specimens of human populations.
2. Develop and validate a GCxGC ToF/MS method for the quantification of OPs/ PYRs biomarkers and identifying non-target metabolites in urine samples by:
    i. Optimizing the 2D GCxGC TOF/MS method for the accurate quantification of OP and PYR metabolites in complex matrices.
    ii. Addressing sensitivity and matrix effect challenges that affect the quantification of metabolites in urine and hair samples.
    iii. Leveraging the high sensitivity and broad detection capabilities of TOF/MS for both targeted and non-targeted metabolite analyses.
    iv. Ensuring the method is optimised for trace-level analysis, providing reliable data on OP and PYR exposure in complex biological samples.
3. To conduct a comparative assessment of the OPs/PYRs exposure biomarker concentrations as follows:
    i. Between children living on farms and those not living on farms in Grabouw and Hex River.

ii. At two time points (2017 (Cycle 1) and 2018 (Cycle 2)) in apple (Grabouw) and grape (Hex River) farms.

iii. Gender disparity in the two geographical areas

4. To use MetaboAnalyst 5.0 to identify metabolic pathways potentially affected by OP exposure, utilizing non-target metabolite data.

1.3.3 Dissertation outline

1.3.3.1 Chapter 1

This chapter first provides a background literature review on pesticides in general, specifically on OP and PYR, focusing on usage, risks, environmental occurrence and exposures, metabolism, and biomonitoring. Secondly, the chapter presents the thesis chapters' rationale, aims, objectives, and outline.

1.3.3.2 Chapter 2

Chapter 2 of the thesis details the extraction of DAPs and 3-PBA from biological samples (urine and hair) and the development and validation of an innovative 2-D GCxGC ToF method. Within this chapter, the validation parameters are thoroughly discussed, highlighting the calibration curve, limits of detection and their validation, and the recovery percentage and precision of the extraction techniques. The chapter also meticulously describes the analysis of quality control samples, such as spiked blank urine and hair samples.

1.3.3.3 Chapter 3

Chapter 3 describes the application of the validated method on urine samples collected from children living on farms and close to farms in the Grabouw and Hex River areas of the Western Cape in South Africa. QuEChERs extraction method was developed and used to extract the metabolites of interest from urine samples prior to hydrolysis of 3-PBA in urine samples. The validated method’s parameter was compared with other studies’ method validation parameters to determine if this study’s method is

suitable for biomonitoring. Further comparisons were made with other studies' urine metabolite concentrations (studies done in the Western Cape's farming communities) to our study's metabolite concentration (µg/g).

1.3.3.4 Chapter 4

This chapter presents the application of the developed and validated 2-D GCxGC-TOF/MS method on a hair sample matrix. The hair samples were collected from children living in the farming areas of Grabow and Hex River of the Western Cape. Solid liquid extraction was used to extract the metabolites of interest from hair samples before analysis. Comparison was done between other hair analysis studies on the metabolites of interest and our study in pg/mg.

1.3.3.5 Chapter 5

This chapter focuses on the use of the MetaboAnalyst software to determine other metabolic pathways affected by OP and PYR exposure. The non-target analysis urine data from cycle 1 was used as well as peak areas and names of the metabolites that were not the metabolites of interest' for the determination of the metabolic pathways.

1.3.3.6 Chapter 6

This chapter presents the overall conclusions and recommendations of the study.

Chapter 2

The Development of Extraction Methods and 2D GCxGC ToF/MS Methods for Quantifying Dialkylphosphates and 3-Phenoxybenzoic Acid in Urine and Hair samples.

2.1 Introduction

Developing and validating chromatography and mass spectrometry (MS) analytical methods is essential for identifying and quantifying compounds in biological samples during biomonitoring studies. During method development, the focus was to find optimal parameters for separating and enhancing the detection of DAPs and 3-PBA metabolites while validating and assessing the method's reliability. Customizing these methods is crucial to accurately identify and measure compounds and biological markers specific to the Western Cape's distinct environmental and biological diversity[316,419,420]. African laboratories face accessibility, affordability, and unique public health issues [421]. Tailoring analytical methods to local demands overcomes challenges and boosts scientific capacity and innovation.

In response to this approach, this study focused on developing and validating the Quick, Easy, Cheap, Effective, Rugged, and Safe (QuEChERS) extraction method and the solid-liquid extraction (SLE) method as well as a novel 2-D GCxGC ToF/MS technique. The methods validated were specifically for analysing biological samples from children living on farms in the Western Cape of South Africa. This innovative approach aims to explore the capabilities of 2-D GCxGC-ToF/MS in detecting OP and PYR metabolites in biological samples, a novel application in insecticide residue analysis. Therefore, the study highlights the method's potential in enhancing environmental and public health research by providing improved sensitivity and specificity for monitoring insecticide exposure.

2.1.1 Differences between comprehensive 2-dimensional gas chromatography and gas chromatography

The key components of a 2-D GCxGC ToF/MS include two capillary columns (one-dimensional (1-D) and two-dimensional (2-D)), along with a thermal modulator, which facilitates the separation of co-eluting compounds in complex matrix samples such as urine and hair [392,422]. The GC-MS comprises of a single column (1-D), a mass analyzer, and a detector for generating mass spectra from the samples, as shown in **Figure 2.1** below [392]. In both setups, the analysed samples pass through the sample inlet and the 1-D column. However, the 2-D GCxGC ToF/MS uses a modulator (hot and cold jets) and a 2-D column to separate compounds further and produce detailed chromatograms.

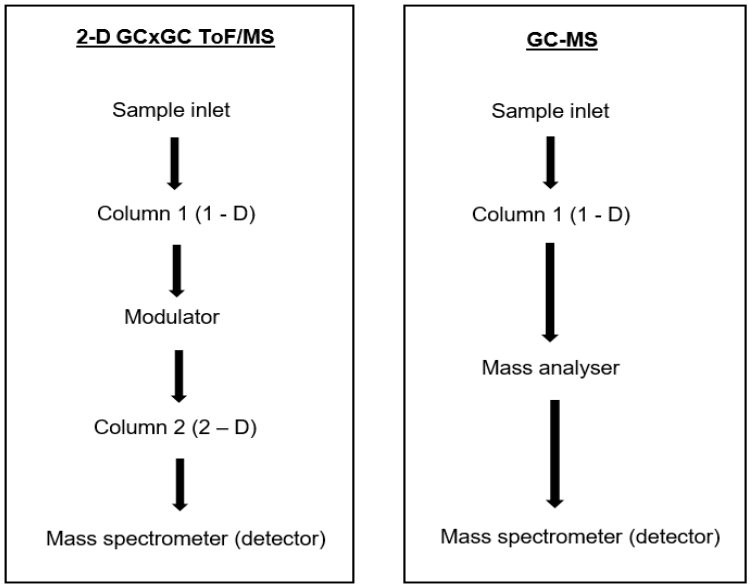


Figure 2. 1: Schematic representation of the differences between a 2- D GCxGC ToF/MS and a 1- D GCMS[392].

The two capillary columns in the 2-D GCxGC system connect seamlessly via micro-unions for a leak-free transfer of sample compounds from the 1-D column to the 2-D column. These columns enhance separation beyond traditional 1-D GC capabilities by featuring distinct stationary phases. Initially, sample injection into the 1-D column separates compounds by the compounds' boiling points [423-425]. The thermal modulator accumulates the analytes in pulses by first 'freezing' them with a cold jet to concentrate them into a narrow band from the 1-D column. A hot jet then 'unfreezes' analytes to vaporize them rapidly for injection into the 2-D column. The sequential process of trapping, concentrating, and

rapidly heating and releasing the analytes boosts compound separation and resolution, enabling detailed analysis of complex mixtures, as illustrated in **Figure 2.2**.

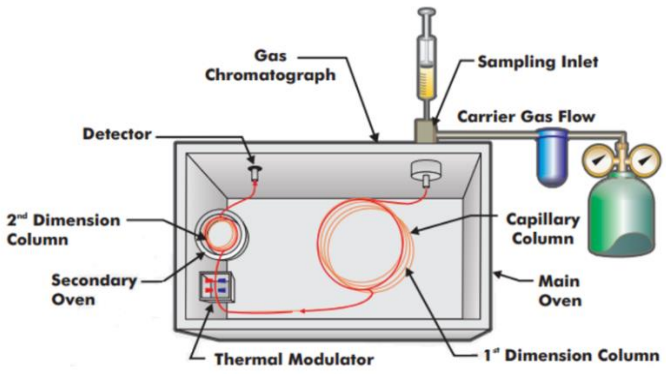


Figure 2. 2: GCxGC schematic diagram[388].

The difference in the column stationary phases and lengths of the two columns are critical to the 2-D GCxGC system's enhanced analytical performance. The 1-D column is usually about 30 meters long, which contrasts with the 2-D column, which is much shorter, typically ranging from 1 to 1.2 meters. This deliberate difference in column lengths, coupled with the modulating period, markedly improves compound separation and resolution[387,391,422,423].

After 2-D separation, an MS is used to measure the mass-to-charge of ions[426,427]. The separation achieved in the 1-D column is preserved and presented in a 1D chromatogram along with a 2-D chromatogram that visually represents the compound's distinct intensity (see **Figure 2.3**).

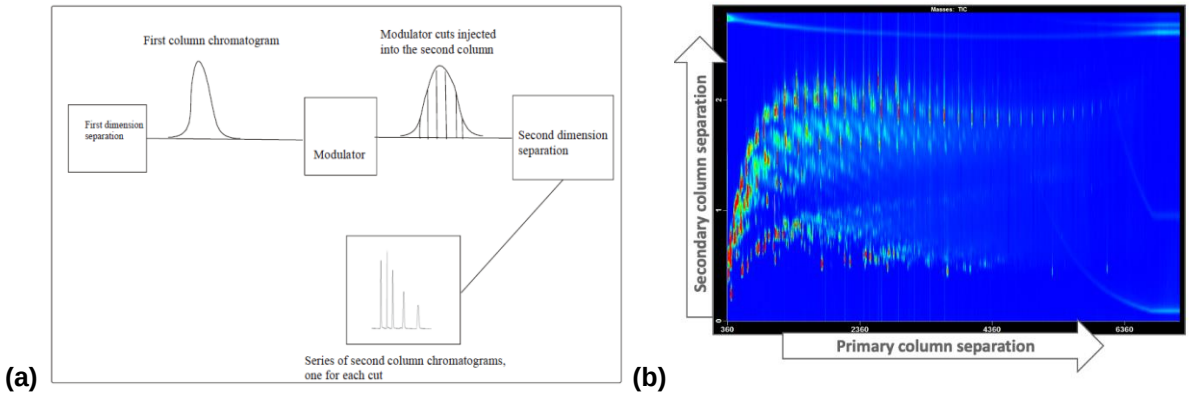


Figure 2. 3: (a) Representation of the steps involved in comprehensive 2-D GCxGC technology. (b) A 2-D chromatogram representing both 1-D column and 2-D column retention time axes.

2.1.1.1 Mass spectrometer - Time of Flight Mass Spectrometer

Various mass analysers, such as triple quadrupole (QqQ), Orbitrap MS, and Time-of-Flight (ToF) MS, are used post-ionization to accurately identify and quantify compounds, including detecting trace levels [406,428,429]. While each system has unique advantages, the ToF/MS has high accuracy, rapid data acquisition, and compatibility with chromatographic separations. Additionally, the ToF/MS is important as it can analyse diverse compounds within a single sample, ranging from small metabolites and chemicals to large biomolecules like proteins and peptides.

The broad mass range significantly boosts its versatility and usefulness in proteomics, metabolomics, environmental analysis, and pharmaceutical research that often analyses samples with complex mixtures of molecules of varying sizes and masses. Moreover, combining ToF/MS with GCxGC transforms the setup into an exceptionally powerful tool for quantifying trace-level analytes. This combination leverages retention times and m/z ratios, offering specific insights into compounds[430]. A heat transfer line connects the 2-D GCxGC to the ToF/MS to prevent the condensation of analytes and preserve the integrity and efficiency of analysing complex samples [407,429,431]; see **Figure 2.4**.

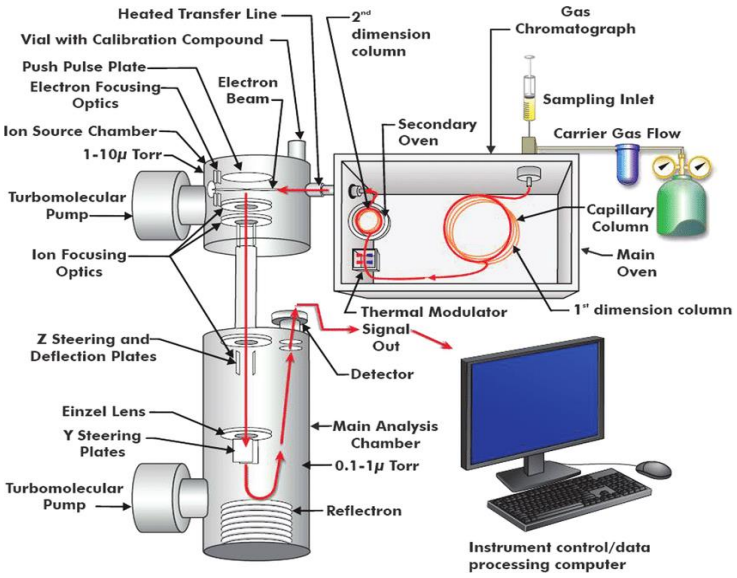


Figure 2. 4: 2- D GCxGC ToF/MS schematic diagram[393,432].

Within the framework of MS techniques, for comprehensive metabolite or compound analysis by the MS tool, targeted and non-targeted analysis are used as strategies, each tailored to meet specific research objectives in the field of metabolomics and beyond.

2.1.1.1.1 Targeted versus non-targeted compound analysis.

The targeted analysis strategy aligns with MS's ability to selectively detect predefined sets of compounds or metabolites with high sensitivity and specificity by utilizing retention times and mass-to-charge ratios (m/z) of commercially available reference standards [433]. Reference standards are typically provided with a certificate of analysis that details their purity, composition, and other relevant properties. These standards can be specific compounds or mixtures[434]. MS's precise quantification and structural identification capabilities are indispensable for targeted analysis as they allow for the detailed study of aspects of the metabolome under defined conditions [435,436].

Additionally, targeted analysis is important in comparing biomonitoring data to exposure guidelines and assessing regulatory compliance to ensure a focused and accurate examination of metabolites [437-441]. Moreover, the focused nature of targeted analysis ensures a focused and accurate examination of specific metabolites of interest, enhancing its utility in time-sensitive research. Consequently, this approach is particularly beneficial when the research goal is clear and focused, such as quantifying known biomarkers or investigating specific metabolic pathways [442-444].

In contrast, Bletsou *et al.* 2017 define “non-target strategy or screening” as identifying compounds for which no previous knowledge is available, which researchers typically conduct after target screening[445]. Non-targeted screening leverages MS's broad scanning ability to capture an extensive profile of compounds in a sample[446,447]. Consequently, researchers commonly use this approach for exploratory or discovery-based research to uncover novel compounds, identify unknown compounds, or reveal unexpected changes in metabolic profiles.

However, sample preparation is important for both non-targeted and targeted analysis in chromatography and MS studies as it is a step that converts complex samples into clear, interpretable data, thereby facilitating scientific breakthroughs[448,449]. Sample preparation removes interferences in the sample, boosting detection, sensitivity, and accuracy for non-targeted analysis. At the same time, sample preparation concentrates specific compounds to simplify the sample separation for targeted analysis to enhance quantification precision and reliable analysis[450,451]. Thus, effective sample preparation is indispensable for achieving high-quality results in MS.

2.1.2 Sample preparation prior to 2-D GCxGC ToF/MS analysis.

The sample preparation is crucial for obtaining reliable results in the chromatography and MS analysis of human biological samples. It significantly influences the metabolite profile patterns detected and quantified and the biological insights gained. Using a suitable sample preparation method ensures the accuracy and reliability of the metabolite analysis, highlighted by reference [452].

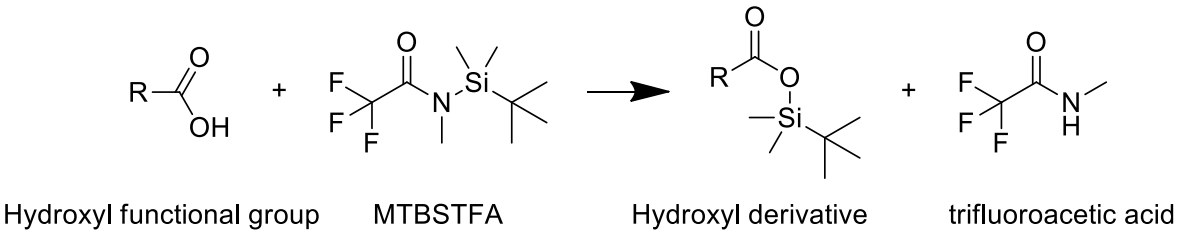
In GC analysis, especially with techniques like 2-D GCxGC ToF/MS, non-volatile compounds present challenges. The compounds form strong bonds with column stationary phases and face vaporization difficulties, leading to separation and elution inefficiencies[453]. To overcome these challenges, non-volatile compounds undergo pre-treatment with a derivatizing agent. This derivatization step enhances the reliability and accuracy of the metabolite analyses and significantly boosts the technique's sensitivity. Consequently, it allows for detecting trace metabolite amounts, substantiated by references [240,454,455].

2.1.2.1 Derivatisation of polar non-volatile compounds to volatile compounds

Derivatization involves coupling the non-volatile metabolites with a selected derivatizing agent, which can be easily separated and detected by analytical techniques such as 2-D GCxGC ToF/MS [455,456].

Consequently, this converts the metabolites to volatile, stable, and detectable derivatives [430,456-460]. Derivatisation is crucial not only to safeguard the 2-D GCxGC ToF/MS instrumentation but also to preserve the integrity of the analytical protocol[347,461-463]. Among various derivatizing agents employed, N-Methyl-N-(tert-butyldimethylsilyl) trifluoroacetamide (MTBSTFA) emerges as a popular derivatizing agent due to its effectiveness in silylating non-volatile compounds. This process involves adding a silyl group, specifically a trimethylsilyl (TMS) group, to functional groups such as hydroxyl (-OH), carboxyl (-COOH), thiol (-SH), and amines ($-NH_2$), making it suitable for diverse compounds[458,464-466].

MTBSTFA's popularity stems from its ability to produce reproducible and robust derivatives, resulting in sharp, well-defined peaks and the improvement of the sensitivity and specificity of analysis[458]. Moreover, MTBSTFA derivatives often exhibit enhanced thermal stability, which is advantageous for the high-temperature conditions typically employed in GC. The reliability and efficiency of MTBSTFA in derivatizing a broad range of compound classes, such as polar metabolites of insecticides[241,339,465,467], contribute to its widespread use in environmental and toxicological studies[458,465,468,469]. **Scheme 2.1** below represents a typical derivatization reaction of compounds containing a hydroxy group (-OH).



Scheme 2. 1: A general schematic representation of the derivatization reaction of hydroxyl functional groups with MTBSTFA.

Despite the advantages of sample preparation and derivatization techniques, it is important to acknowledge and address matrix effects. These effects can considerably influence the precision of metabolite quantification in complex biological matrices, particularly in targeted analysis.

2.1.2.2 Matrix effects

The International Union of Pure and Applied Chemistry (IUPAC) gold book defines matrix effects as the overall influence of all components within a sample, apart from the analyte of interest, on the accuracy of analytical quantification. This description highlights how components within a sample matrix can impact the accurate quantification of a target metabolite, leading to potential inaccuracies in the

analysis. Interference can enhance or suppress the analyte's signal for detection and quantification at various analytical stages, such as sample preparation, detection, and measurement [470-472].

The popular forms of interference reported are chemical and spectral interference [473-476]. Chemical interference occurs when sample components interact with the target analyte by altering the analyte's chemical form or causing competing reactions. Similarly, spectral interference occurs when sample components affect light absorption, emission, or scattering at wavelengths similar to the target analyte[477-479]. These interferences challenge analytes' precise quantification, leading to incorrect conclusions and potentially harmful decisions. Mitigating matrix effects, especially in insecticide biomonitoring, involves a comprehensive approach that spans the experimental design (preventative) and analytical (corrective) phases.

2.1.2.2.1 Use of internal standards to combat matrix effects.

Isotopically labelled internal standards (ISTDs) are chemically identical to the target analyte but incorporate stable isotopes into their structure, aiding in mimicking the analyte's behaviour during analysis. ISTD facilitates precise adjustments for variations in sample preparation, instrument responses, and other experimental factors, thereby ensuring reliable and precise results[480,481]. By differentiating the isotopically labelled standard from the analyte, even in complex matrices, they compensate for analyte loss or analytical response variations, significantly improving the quantification of trace metabolites and combating matrix effects [482,483].

Choosing the right ISTD is crucial for analysis success and reliability, offering advantages like enhanced accuracy, matrix effect correction, and result comparability across different instruments or laboratories [481,484,485].. Comparing the analyte's signal to the ISTD accurately determines the analyte's concentration, especially in complex matrices where matrix effects may skew analysis[484-486]. Furthermore, the ISTD method can also be used for baseline normalization and concentration correction in mass spectrometry and other analytical technologies [487-492].. The current study leverages the 2-D GCxGC ToF/MS technique for biomonitoring OP and PYR exposure in complex matrices like urine and hair by integrating isotopically labelled ISTD with this technique.

2.1.3 Research Methodology

Introducing the 2-D GCxGC ToF/MS method for quantifying DAPs and 3-PBA in children's biological samples (urine and hair) from Western Cape's agricultural regions marks a revolutionary step in biomonitoring research. This study pioneers the biomonitoring field by using sophisticated analytical technique in a specific population for the first time, establishing a novel approach in the biomonitoring domain. Furthermore, there is no current literature on using the 2-D GCxGC ToF/MS method for detecting or quantifying OP and PYR metabolites in urine and hair samples, positioning this research as a pioneering effort in Africa and possibly globally.

This Chapter outlines the comprehensive process of accurately quantifying metabolites in urine and hair samples using the 2-D GCxGC ToF/MS analytical method. This encompasses preparing reference standards and ISTD, choosing suitable solvents, fine-tuning the derivatization method, and evaluating the 2-D GCxGC ToF/MS's sensitivity and specificity regarding detection and quantification limits. Furthermore, it involves creating calibration curves for each metabolite and examining their linearity, which is integral to method development and validation.

During the method development and validation phase, an extraction method was selected based on recovery percentages (%) for urine samples, and a common extraction method was selected for hair samples. Subsequently, the chosen extraction method was assessed for % recoveries and precision as essential validation parameters that use quality control samples. This approach validates that the method is reliable and reproducible and establishes a robust foundation for the analytical process.

Following method development and validation, the method was systematically applied to the study samples, and the data was statistically analysed, as discussed in **Chapters 3** (urine samples) and **4** (hair samples).

2.1.3.1 Chemicals and reagents.

All the analytical grade chemicals and reagents utilized in this research were obtained from Sigma-Aldrich, South Africa. Analytical reference standards: DMP, DEP, DMTP, DEP, and 3-PBA, with purities higher than 99% from Toronto Research Chemicals Inc (TRC)Canada. Additionally, three ISTDs, namely DMP-d^6, DMTP-d^6 potassium salt, and 3PBA-$_{12}C^6$ were obtained from Toronto Research Chemicals Inc (TRC) Canada with purities higher than 99%. Sigmatrix Urine Diluent, 98.0% was

obtained from DLD scientific cc, Durban, South Africa. A solution of β-glucuronidase enzyme derived from Helix pomatia-2 (≥ 100,000 units/mL, IMCSzyme®3S, USA) and rapid hydrolysis buffer (IMCSzyme®3S, USA). Solid Phase Extraction (SPE) cartridges: Oasis HLB (3 mL, 60 mg) (Sigma-Aldrich, South Africa). N-tert-butyldimethylsilyl- N-methyltrifluoroacetamide (MTBSTFA) (Sigma-Aldrich, South Africa). HPLC-grade solvents such as ethyl acetate, hexane, acetonitrile, toluene, and methanol were purchased from Radchem (Johannesburg, South Africa) and Sigma-Aldrich, South Africa.

The subsequent sections and the following Chapters of the study detail the method development and validation parameters, and the comprehensive statistical analysis conducted on the obtained data.

2.2 Results and Discussion

2.2.1 Method development

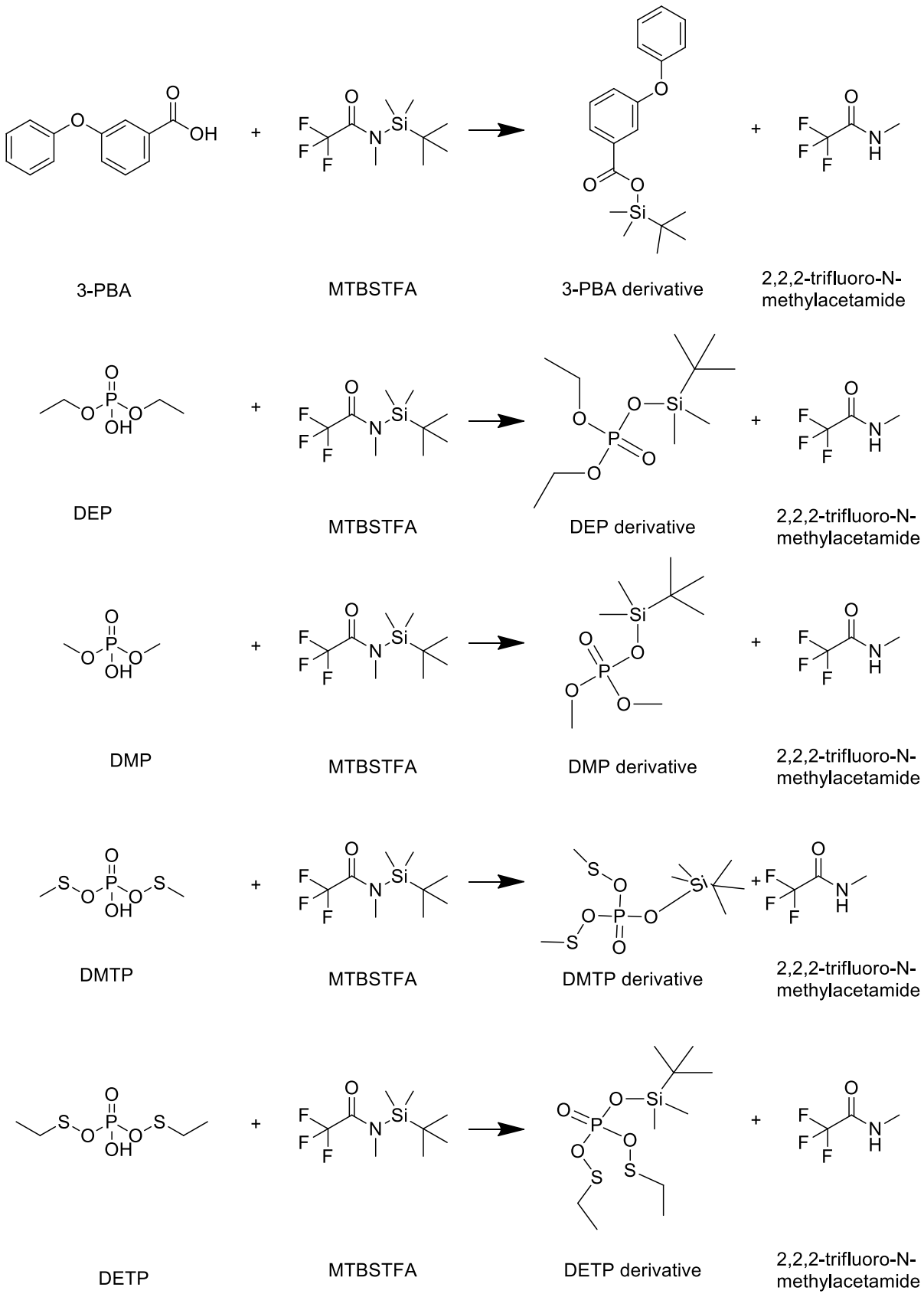
In this study, the metabolites of interest were derivatised for 2-D GCxGC ToF/MS analysis as they are non-volatile polar compounds[463,493-495]. Therefore, optimizing the derivatisation step is crucial as it ensures the efficient and reproducible conversion of non-volatile analytes into semi-volatile derivatized counterparts[460] This step, which is the first step of method development and validation, ensures ease of separation, resolution, and quantitative determination of the metabolites using the 2-D GCxGC ToF/MS. Optimization entailed systematically examining solvent choice, reaction time, and temperature to enhance derivatization efficiency, selectivity, and applicability [460].

Subsequently, the preparation of stock, working reference, and internal standards was conducted to set benchmarks for the analytical protocol, facilitating the identification and quantification of target analytes in urine and hair samples. This strategic approach ensures the analytical method's reliability and precision, laying a solid foundation for the subsequent stages of method validation.

2.2.1.1 Optimisation of the derivatisation method through variation of solvent, temperature, and time.

Solutions (100 ng/mL) containing reference standards were prepared in different solvents. To each solution, 20 µL of the derivatizing agent was added.

The study conducted equivalent calculations and found that 2 uL of MTBSTFA would suffice to derivatize 1 mg/mL of metabolites. Nevertheless, the complexity of urine samples, with their competing compounds, necessitated a higher quantity of the derivatizing agent than initially estimated [496]. Following Guo *et al*.'s 2017 method, the study used 20uL of MTBSTFA for successfully derivatising DAPs in urine samples at 90°C for 30 minutes [241]. Subsequently, other parameters such as temperature and reaction time were optimised where the amount of the derivatising agent remained constant. The chemical structures of the derivatizing agent and the derivatized metabolite structures are illustrated in **Scheme 2.2**.



Scheme 2. 2: Reaction schemes of the derivatizing agent MTBSTFA with standards and internal standards.

2.2.1.1.1 Solvent optimisation

The choice of solvent considers factors such as compatibility with the derivatization reagent, solubility of the analyte and reagent, stability of the derivatization products, and compatibility with the analytical technique to achieve efficient and accurate analysis[497-500]. Five solvents, methanol, hexane, toluene, acetonitrile (ACN), and ethyl acetate, were used for solvent optimisation. During the derivatization process, solvents such as methanol, hexane, and toluene yielded no observable peaks, unlike ACN and ethyl acetate, indicating a clear difference in solvent effectiveness. Notably, ethyl acetate produced larger peak areas for metabolites at a concentration of 100ng/mL than ACN. The Appendix details this finding (**Figures A 2.1** to **A 2.8**). The smaller peak areas obtained with ACN may be due to the incomplete formation of metabolite derivatives during derivatization [501-503].

Ethyl acetate's success as a solvent in the derivatization process might be attributed to its unique chemical structure, which is polar aprotic and does not have hydrogen atoms directly connected to the electronegative oxygen atoms. These structural features enhance its chemical reactivity, its effectiveness in extraction processes, and its ability to act as an excellent solvent in derivatization reactions, as noted by Zhang *et al*. 2023 and Le *et al*. 2021 [504,505]. Therefore, the absence of hydroxyl groups means ethyl acetate does not compete with the metabolites of interest that often contain hydroxyl groups for the derivatizing agent (MTBSTFA). This non-competitive nature positions ethyl acetate as a superior solvent choice over methanol and other solvents for derivatization. This leads to its effectiveness in 2-D GCxGC-ToF/MS analysis of DAPs and 3-PBA.

Consequently, ethyl acetate was not only used as a analysis solvent but also an extraction solvent for this study due to its affinity with polar compounds, making it superior to methanol or ACN, which are commonly used to extract DAPs and 3-PBA from urine and hair samples for analysis using other analytical techniques. This selection was based on its proven efficiency in dissolving polar substances, thereby streamlining the metabolite extraction process[506]. Ethyl acetate's effectiveness in isolating various compounds, such as flavonoids, alkaloids, phenolic acids, and glycosides, has been well-documented, aligning with the research objectives to extract and analyse polar, non-volatile compounds effectively [507-511]. Kumar *et al*. 2023 further validated its use, noting high % recovery rates and low matrix effects in DAP extraction from urine samples using a LLE method[512].

2.2.1.1.2 Optimum derivatization conditions of OP and PYR metabolites

The temperature and time parameters were systematically varied to optimize the derivatization process. Optimization started with time variation to ensure complete derivatization of the analytes while keeping the derivatising agent concentration constant in ethyl acetate at 90 °C. The reaction time was adjusted in 30-minute intervals, ranging from 0 to 90 minutes. Upon determining the optimal time, temperature adjustments followed in 10ºC intervals, starting from room temperature (20 ºC) and increasing to 90 ºC.

The optimum conditions for complete derivatization of metabolites were identified at 60 ºC with a reaction time of 60 mins. At these conditions, the peak area no longer increased. These findings are in **Table 2.1** and detailed through chromatograms in Appendix **Figure A 2.9**, illustrating how optimised conditions facilitated the volatilization and stabilization of these metabolites for the 2-D GCxGC ToF/MS detection.

Table 2. 1: Optimum conditions were determined and then used to derivatize OP and PYR metabolites.

Derivatisation agent	Derivatising method	Diluent	Temperature (°C)	Time (mins)	Amount
MTBSTFA	Silylation	Ethyl acetate	60	60	20 µL

The development of the analytical method also involved fine-tuning the 2-D GCxGC ToF/MS conditions for detecting and quantifying the derivatised metabolites after optimizing the derivatisation step.

2.2.1.2 GCxGC-ToF/MS analysis method development

Optimizing the parameters and MS detection settings of the 2-D GCxGC ToF/MS is crucial for enhancing the method's analytical performance and reliability in identifying and quantifying derivatized metabolites in complex biological samples. This optimization boosts the capacity to detect and monitor specific metabolites, aiding in a thorough analysis of OP and PYR exposure. The use of ISTD, including DMP-d^6, DMTP-d^6, and 3PBA-$^{12}C_6$, improves the accuracy and dependability of quantitative measurements. The detailed parameters and MS detection settings are below:

A Leco® Pegasus 4DToF/MS system with an Agilent GC model 7890 and a GERSTEL MPS-2 auto-sampler was used for method development, validation, and sample quantification. The first column (1-

D), a 30m x 0.25mm x 1.0 µm coated with a 1.0 µm film Restek Rxi 5Sil MS stationary phase, was paired with a second, shorter column (2-D) of 1 meter in length and 250 µm in diameter coated with 0.25 µm of Restek Rxi 17Sil MS stationary phase. Both columns were operated at a constant flow rate of 1.0 mL/min. The ion acquisition delay was set at 4 minutes. Samples were injected with a volume of 1µL± 0.1 in split-less mode with the inlet temperature at 250°C using a Topaz liner/wool. Temperature programming for the 1-D column started at 80°C and held for 30 seconds before ramping up to 300°C at a rate of 20°C/min, where it was then maintained for 2 minutes. However, the 2-D column temperature was systematically set to be 5°C (+5) above the corresponding temperature gradient of the 1-D column. The modulator temperature was initially set 15°C above the 2-D column's temperature. The hot jet was adjusted to 250°C, the cold jet to -50°C, and the modulation cycle was established at 4 seconds. The transfer line temperature was 310 °C, while the ion source (EI) temperature was 250°C. The scan range was 35 amu to 550 amu at a scan rate of 100 spectra per second.

After setting the derivatising and analytical conditions, the total injection runtime was 21 minutes, which included a 7-minute cooling time. The ions (parent and daughter ions) for detecting and quantifying the reference standards and their respective internal standards are specified by their retention times, as shown in **Table 2.2**.

Table 2. 2: 2-D GCxGC–ToF/MS parameters used for the detection of parent and daughter ions (analyte) of metabolites.

Analyte	RT (min)	Parent ion	Daughter ions
DMP	5:06	183	153 105
DEP	6:03	211	155
DMP-d6	5:09	189	111
DMTP	5:30	199	105 169
DETP	6:09	227	171
DMTP-d6	5:27	205	111
3PBA	10:30	271	227 197
3PBA-12C6	10:36	277	203 233

Retention time = RT

For ion parameters, 2D precursor ion masses, and chromatograms (1D and 2D) for each metabolite, see the Appendix section, **Figures A 2.1** to **A 2.8**. Method development was followed by method validation.

2.2.1.3 Preparation of standard stock and working solutions.

Stock solutions (1 mg/mL) for reference and ISTD standards were prepared by dissolving approximately 1 mg of solid powder in 1 mL of ethyl acetate in a volumetric flask. These solutions were stored at -20°C in flasks sealed with paraffin film. The working reference standard mixtures from 0.001 to 100 µg/mL were made by diluting the ethyl acetate stock solutions. Stock solutions were renewed monthly to ensure stability, highlighting the approach's quantitative effectiveness.

2.2.2 Method validation

The developed analysis method was validated following internationally recognized guidelines for single laboratory validation of analytical techniques. Data quality relies on robust techniques, tailored validation criteria, and adherence to guidelines like the International Conference on Harmonization (ICH), which ensure data reliability[513]. Per international standards, as delineated in the guidelines of both the Food and Drug Administration (FDA) and the European Medicines Agency (EMA) for the validation of bioanalytical techniques, the extent and nature of method validation vary based on the specific application of the bioanalytical method[514,515].

The validation protocol evaluated key parameters including accuracy, precision, linearity, detection, and quantification limits, crucial for accurately quantifying analytes. These metrics were derived from the peak area ratio of the analyte to the internal standard [480,516,517]. Validation utilised reference standards and ISTD to establish an internal calibration curve, essential for determining linearity, limit of detection (LOD), and limit of quantification (LOQ). Typically, these values are ascertained through an external or matrix-based calibration method that generates a calibration curve from standard solutions with known concentrations. The internal standard calibration curve method is crucial for accurately quantifying analyte concentrations in samples by adding a consistent ISTD to both reference standards and samples. This method ensures the ratio of analyte to ISTD remains constant to overcome variations from instrument drift or sample loss. It proves especially effective in complex matrices, enhancing reliability by compensating for signal or procedural variations.

Internal standard calibration curves and matrix-matched calibration curves employ different methodologies. The former involves using standards dissolved in a solvent or matrix that differs from the sample's, whereas the latter ensures the matrix of the standards aligns with the sample's matrix[518]. However, employing an internal calibration curve is favoured in this study for its ability to effectively correct for variations caused by sample preparation and matrix effects across different matrices[519,520]. This method streamlines the analytical process by reducing the need for separate calibration curves for each matrix, saving time and decreasing the likelihood of errors.

ISTD proves advantageous in complex matrices such as urine and hair as it significantly compensates for matrix effects arising from varying compositions and endogenous compounds [521]. This technique ensures precise and reliable measurements across these diverse matrices and analytical techniques[520,522,523]. Linearity, LOD, and LOQ are calculated using an Excel regression tool on the calibration curve data for each metabolite.

2.2.2.1 Calibration curve standards

For internal standard calibration in this analytical method, a range of 17 concentrations was prepared, varying from a low of 0.007 ng/mL to a high of 100 ng/mL (0.007; 0.005; 0.003; 0.001; 0.07; 0.05; 0.03; 0.01; 0.5; 0.1; 1; 5; 10; 25; 50; 75; 100 ng/mL). These concentrations were achieved through precise dilution in ethyl acetate to ensure wide coverage for accurate equipment calibration. The calibration curve included more points at lower concentrations to enhance the method's precision in detecting the analytes at trace levels and determining the LOD, which is a critical aspect of the sensitivity of the analysis.

The internal standard calibration curves were prepared, derivatised, and analysed over two separate days to assess their reproducibility and reliability. Consequently, this approach enabled the calculation of standard deviations that provided a quantitative measure of the method's consistency over time. The results of this process are presented in the Appendix section, from Table A 2.1 - 2.12. The calibration curve's linearity, an important factor in the method's analytical performance, was evaluated by analysing key statistical metrics such as the correlation coefficient (R^2). This coefficient measures how closely the data points align with the linear regression line. A R^2 value of 1 signifies a strong linear relationship, and a value of 0 suggests no relationship or correlation[524,525].

Consequently, analysis of the internal standard calibration curve's linearity is foundational in determining the method's reliability for quantitative measurements.

2.2.2.1.1 Linearity

Linearity is defined by the direct proportionality between instrument response and known analyte concentrations to ensure that the response from an instrument is directly related to the concentration of an analyte. Additionally, the assessment involves the application of appropriate statistical methods, such as regression line calculation, which favours either unweighted or weighted to account for significant variance. Measuring linearity through the regression lines' average slope, 95% confidence intervals, and the R^2 value forms an important foundation for accurately interpreting test results from unknown samples and converting these responses into precise concentrations [526].

Consequently, this principle is used in different fields such as environmental analysis, pharmaceuticals, clinical diagnostics, and food safety because it confirms that the analytical method mirrors true changes in analyte concentration[527,528]. Additionally, determining linearity not only bolsters the reliability of the method but also the accuracy of the instrument's signal [129]. Normalization of quantitative measurements in metabolite analysis is achieved through the peak area ratio of the metabolite analyte to the internal standard, effectively addressing and correcting inconsistencies arising during the sample preparation process. It mitigates potential fluctuations attributed to sample handling, extraction efficiency, and other preparatory steps, thus enhancing the accuracy and reliability of quantitative measurements.

This study used Excel regression statistical tests to evaluate the internal standard calibration curve model's precision fit and subsequently used for routine analyses[529-531]. The results are shown in Table 2.5, and the regression calculations are shown in **Appendix Table A2.1 to A12**. Establishing linearity is important for accurately determining the LOD and LOQ, as it ensures a consistent and predictable analytical response across the relevant concentration range. Precision and accuracy at the LOD and LOQ levels may be compromised without a linear relationship, undermining the method's reliability in detecting and quantifying low-level analytes[532,533].

2.2.2.1.2 Limit of detection (LOD) and limit of quantification (LOQ)

LOD represents the lowest analyte concentration reliably detectable under the experimental conditions. In contrast, LOQ signifies the minimum quantifiable concentration with acceptable accuracy and

precision. The standard deviation of the signal was then multiplied by 3 for LOD and multiplied by 10 for LOQ to identify the threshold above which there was a 95% certainty that the signal differed from the blank value, assuming a blank value of zero[534,535].

The method's sensitivity (LOD and LOQ) was assessed by measuring the standard deviation of the calibration replicates [511]. The LOD and LOQ were calculated using the Excel linear regression tool and the equation for each metabolite calibration curve. This method, available in Excel, is known for its simplicity and efficiency in computing LOD and LOQ values from calibration curves. **Equations 2.1** and **2.2** show the calculation used to determine the sensitivities: the standard deviation of the response (δ) and the standard deviation of the slope in the linear relationship (S)[511]:

Equation 2.1

$$LOD = 3\delta/S$$

Equation 2.2

$$LOQ = 10\delta/S$$

Table 2.3 shows the calibration range, linearity, LOD, and LOQ of GCxGC ToF/MS; the LOD values were subsequently compared to other GC technologies' LOD and LOQ values. Comparison of the 2-D GCxGC ToF/MS LOD to other studies' LOD comparison helps ensure a method's quality and reliability, aiding decision-making.

Table 2. 3: Calibration data and method validation parameters for 2-D GCxGC- ToF/MS analysis of DMP, DEP, DMTP, DETP, and 3-PBA.

Metabolite	Cal range ng/mL	Linearity (R^2)	LOD ng/mL (±std)	LOQ ng/mL (±std)
DMP	0.0001- 100	0.995	0.50 (± 0.08)	1.50 (± 0.25)
DEP	0.007- 100	0.992	0.37 (± 0.04)	1.14 (± 0.13)
DMTP	0.007- 100	0.999	0.89 (± 0.17)	2.69 (± 0.52)
DETP	0.007- 100	0.998	0.74 (± 0.36)	2.23 (± 1.10)
3PBA	0.007- 100	0.999	0.19 (± 0.08)	0.58 (± 0.25)

In this study, 2-D GCxGC ToF/MS LOD and LOQ values were compared with those obtained using GC-MS, GC-MS/MS by other studies, and an LC-MS/MS technique published by Fiserova *et al*. 2021 [133], as outlined in **Table 2.4**. This comparative analysis was chosen because the referenced studies employed GC techniques and derivatization methods for analysing identical metabolites. In contrast, the LC method was particularly relevant as it was developed and validated for quantifying the same metabolites in urine samples of children in the same geographic locations (Grabouw and Hex River in the Western Cape) as those sampled in this study.

The comparison aimed to evaluate the GCxGC-ToF/MS technique's effectiveness in analysing OP and PYR metabolites by comparing its sensitivity (LOD and LOQ) to established methods and to verify the technique's capacity for detecting metabolites at low concentrations within complex matrices. The objective centred on determining if the 2-D GCxGC ToF/MS LOD values were comparable to other GC-MS technologies and LC-MS/MS. The 15 published studies that used the GC technique were identified and listed in Appendix **Table A 2.13**, all concerning urine sample analysis.

Table 2. 4: presents LOD and LOQ concentrations obtained by GCxGC MS and other techniques (GCMS and LCMS).

2-D GCXGC ToF/MS			Literature comparison		
Name	**LOD ng/mL (±std)**	**LOQ ng/mL (±std)**	**GC/MS LOD ranges ng/mL *a****	**Fiserova (LC) LOD ng/mL *b****	**Fiserova (LC) LOQ ng/mL**
DMP	0.50 (± 0.08)	1.50 (± 0.25)	0.06 – 22.50	0.63	2.15
DEP	0.37 (± 0.04)	1.14 (± 0.13)	0.02 – 22.50	0.24	0.80
DMTP	0.89 (± 0.17)	2.69 (± 0.52)	0.05 – 22.50	0.04	0.15
DETP	0.74 (± 0.36)	2.23 (± 1.10)	0.03 - 22.50	0.03	0.07
3PBA	0.19 (± 0.08)	0.58 (± 0.25)	0.01 -200	0.02	0.06

LOD- Limit of Detection, LOQ- Limit of quantification, Cal- Calibration
LC- liquid chromatography
*a**- 15 other studies
*b**- Fiserova *et al* 2021

The LOD values for DAPs and 3-PBA obtained through 2-D GCxGC ToF/MS analysis closely match the reported GC/MS ranges. For instance, this study's LOD for DMP at 0.50 ng/mL falls within the broad GC/MS range of 0.06 – 22.50 ng/mL. Similarly, DEP, DMTP, DETP, and 3-PBA LODs align with their respective GC/MS ranges, underscoring the 2-D GCxGC ToF/MS technique's accuracy in detecting low metabolite concentrations in urine and hair samples. This accuracy mirrors the efficacy of established GC/MS methods. Comparing these findings with Fiserova *et al*. 2021, the LC technique in that study achieved lower LOD and LOQ values, underscoring LC's enhanced sensitivity. LC's increased sensitivity comes from its simplified sample preparation process, which eliminates the need for derivatization, which is essential in GC to boost the volatility of polar compounds. [536-538]. LC uses non-polar C18 columns, facilitating easier elution of polar analytes, in contrast to GC's reliance on polar columns that enhance interaction and complicate elution, thus raising LODs[539].

Despite the method's LOQ being 0.1 ng/mL, the calibration lower range was set as low as 0.007 ng/mL by the instrument's software to ensure the full linear response of the instrument could be evaluated. The 0.007 ng/mL point was included because it is an experimentally determined value that helps establish the line of best fit and assess the method's linearity(R2) which was 0.999. While concentrations below 0.1 ng/mL cannot be reliably quantified due to background noise and uncertainty, the inclusion of these lower points supports a broader understanding of the instrument's sensitivity and provides more data for calibration, improving the accuracy of quantification near the LOQ

Ethyl acetate was selected as the solvent to enhance metabolite interaction and derivatization efficiency for 2-D GCxGC ToF/MS analysis, a decision differing from Fiserova *et al*.'s preference for methanol, which effectively dissolves polar compounds, thereby improving detection[133,540,541]. This solvent choice and the distinct MS technologies employed (ToF in this study versus MS/MS in Fiserova *et al*. 2021) play a pivotal role in the variability observed in LOD and LOQ values. ToF is commended for its high mass accuracy, resolution, and exceptional high-throughput capabilities. It enables broad-spectrum analysis that captures an extensive range of chemicals within samples and is beneficial for rapid compound screening and complex mixture analysis[542]. Despite ToF's broad capabilities, MS/MS is celebrated for its precision in targeting and analysing specific compounds within complex mixtures, significantly reducing background noise, and enhancing measurement accuracy, especially vital for detecting trace levels of compounds[543-547].

Technological advancements in ToF/MS have broadened its analytical applications, including challenging analyses of non-volatile or highly polar analytes, although this requires careful consideration of LOD and LOQ values[548,549]. The integration of 2-D GCxGC with ToF/MS has strengthened its analytical role, markedly improving the resolution and separation of sample components, making the 2-D GCxGC-ToF/MS setup a powerful tool for dissecting complex mixtures into distinct, analysable elements[407,550-552].

2.2.2.2 Development of metabolite extraction method for urine and hair samples

Efficiently extracting DAPs and 3-PBA from urine and hair is essential due to their roles as biomarkers for environmental exposure for biomonitoring studies, as discussed in Chapter 1. These biomarkers indicate exposure to OPs and PYRs, which require sophisticated extraction methods for accurate quantification due to their low concentrations in biological matrices[553,554]. Techniques such as lyophilization, solid-phase extraction, liquid-liquid extraction (LLE), solid-liquid extraction, and derivatization enhance % recovery rates and reduce matrix effects[316,555-560], highlighting the importance of selecting the appropriate method based on sample type and analyte for accurate, sensitive analysis.

The current study adapted extraction methods for metabolites from urine and hair samples based on established protocols from Fiserova *et al*. 2021 and Knipe *et al*. 2016, which focused on metabolites of interest[133,243]. These methods were used to extract metabolites effectively and to validate the analysis method by determining % recovery and precision measures. This validation confirmed the methods' efficiency and reliability in isolating metabolites from samples collected from children in the Western Cape of South Africa, affirming the analytical approach's robustness.

The study chose enzymatic hydrolysis over acidic hydrolysis with HCL for deconjugation of 3-PBA for urine extraction and analysis. This preference was due to enzymatic hydrolysis's ability to selectively break down specific bonds using naturally occurring enzymes, ensuring precision and selectivity without harming other molecules. Additionally, enzymatic hydrolysis was favoured for its environmental friendliness, avoiding strong acids, reducing waste, and preventing the side reactions or degradation of analytes that the harsher, faster acidic hydrolysis could cause [133,561-563]. However, hair samples underwent washing to eliminate external contaminants, followed by pulverization to enhance metabolite extraction, omitting hydrolysis for 3-PBA based on existing hair analysis methods[564]. The extracted urine

and hair samples were then subjected to derivatization for detection and quantification by the 2-D GCxGC ToF/MS technique. These steps are comprehensively described in the following sections.

2.2.2.2.1 Quality control samples

Quality control (QC), typically categorized into blanks and spikes, is important for ensuring the accuracy and reliability of analytical methods. These samples serve as benchmarks for analysis method validation and performance monitoring. Blanks detect contamination and establish background levels, spiked samples that add analytes, test method accuracy, and matrix effects. Regular QC analysis allows laboratories to identify errors, ensure method precision, and maintain data integrity[527,565].

This study used QC samples to develop a reliable protocol for analysing trace metabolites in complex biological specimens like urine and hair. These QC samples were spiked with known concentration standards and ISTD levels relative to the calibration range. QC samples were prepared and analysed alongside the internal standard curve and study samples. This approach facilitated the evaluation of precision and accuracy in the method, particularly addressing matrix effects, which is a significant challenge as co-eluting substances can impair ionization efficiency, leading to inaccuracies in quantification [566,567]. The study effectively countered matrix effects by adopting specific sample preparation techniques and utilizing ISTD, ensuring reliable quantification despite potential variability and losses during analysis [568,569].

Commercially sourced synthetic urine and milli Q water samples functioned as QC blanks for developing the urine extraction method. In contrast, the hair extraction protocol employed human samples free from OP and 3-PBA metabolites. These blanks helped identify and rectify any contamination or analytical system interference[570,571]. QC samples were spiked at concentrations of 5 ng/mL (Lower concentration level- LCL), 50 ng/mL (medium concentration level- MCL), and 100 ng/mL (high concentration level- HCL), with ISTD added at 20 ng/mL which was crucial for validating the internal standard calibration curve and ensuring accurate quantification. Following preparation, QC samples underwent extraction and subsequent analysis using the designated extraction method for each biological matrix, significantly improving the method's precision and reliability.

Additionally, the study leveraged QC samples to ascertain the % recovery and precision (inter and intra-day) of the urine and hair extraction methods. This analysis verified the methods' robustness and

effectiveness in quantifying trace metabolites within complex matrices via 2-D GCxGC ToF/MS. The development of these extraction methods used urine and hair QC samples and is elaborated in subsequent sections.

2.2.2.2.2 Extraction method for urine samples

The current research explored and adapted a combined SPE and QuEChERS extraction technique originally developed for LC analysis by Fiserova *et al*. 2021 to effectively extract metabolites of varying polarities [133]. This adaptation involved employing QC samples to assess the extraction method's effectiveness and reliability within the new analytical context by focusing on the % recoveries; three extraction methods solid phase extraction (SPE), QuEChERS, and a hybrid of the two) were evaluated, which is discussed comprehensibly in the urine extraction method **section 2.2.2.2.3**. The extraction method that demonstrated the highest % recovery rates was subsequently chosen to ensure the most accurate and reliable 2-D GCxGC ToF/MS analysis.

For this study, the Oasis HLB cartridge was used due to its prior successful use in a study involving urine samples from the same participants residing in Grabouw and Hex River farms. Also, the Oasis HLB cartridge was chosen for its hydrophilic-lipophilic balanced sorbent, which retains both polar and non-polar analytes thus making it ideal for extracting a wide range of metabolites from complex matrices like urine[572]. Alternatives, such as C18 and Weak Cation Exchange (WCX), were not considered due to financial constraints, despite their specific advantages for non-polar or basic analytes, respectively[573,574]. With additional funding, future studies will explore alternative SPE sorbents to further optimize recovery rates.

Selecting an extraction method establishes a benchmark for performance in clean matrices and evaluates the method's resilience and reliability within complex biological matrices. Moreover, the hydrolysis of conjugated 3-PBA in urine samples is important in ensuring the accuracy of PYR exposure analysis. This dual approach highlights the method's sensitivity, accuracy, and suitability for real-world urine sample analysis.

2.2.2.2.2.1 Urine hydrolysis of 3-PBA metabolite in the urine sample

The study applied *β*-glucuronidase enzyme to urine samples for deconjugation in the initial sample preparation phase. Consequently, the enzyme facilitated the release of 3-PBA from its conjugate for extraction and subsequent derivatization before GC-MS analysis, as illustrated in **Scheme 2.3**.

Urine sample

+ β- glucuronidase enzyme ⟶ 3-phenoxy benzoic acid (3PBA) + Glucose Sulphur proteins

Scheme 2. 3: urine sample hydrolysis.

The deconjugation method was based on the Fiserova *et al*. 2021 study, where the same enzyme was employed for the hydrolysis. Enzyme hydrolysis involved the addition of 127.5 µL of β-glucuronidase solution (2000 units/mL in 1 mM ammonium acetate with 20 ng/mL internal standard) to 500 µL of urine. This was followed by incubation at 37°C for 90 minutes with continuous agitation to facilitate deconjugation before extraction.

2.2.2.2.3 Urine extraction method selection

The study developed the extraction method using QC samples (water and synthetic urine) with the MCL (50 ng/mL).

a) Solid Phase Extraction

HLB Oasis SPE cartridges were used to separate target analytes from QC samples. Initially, the cartridges were activated and equilibrated with 1 mL of methanol followed by 1 mL of 1% acetic acid in Milli-Q water before introducing the sample, as shown in **Figure 2.5**. Post-loading, the cartridges were washed with 1 mL of 5% methanol in 1% acetic acid to purge impurities. Subsequently, 1 mL of ethyl acetate eluted the target analytes, and the eluant was then reduced to 500 µL at room temperature and transferred to a glass vial for derivatisation and analysis.

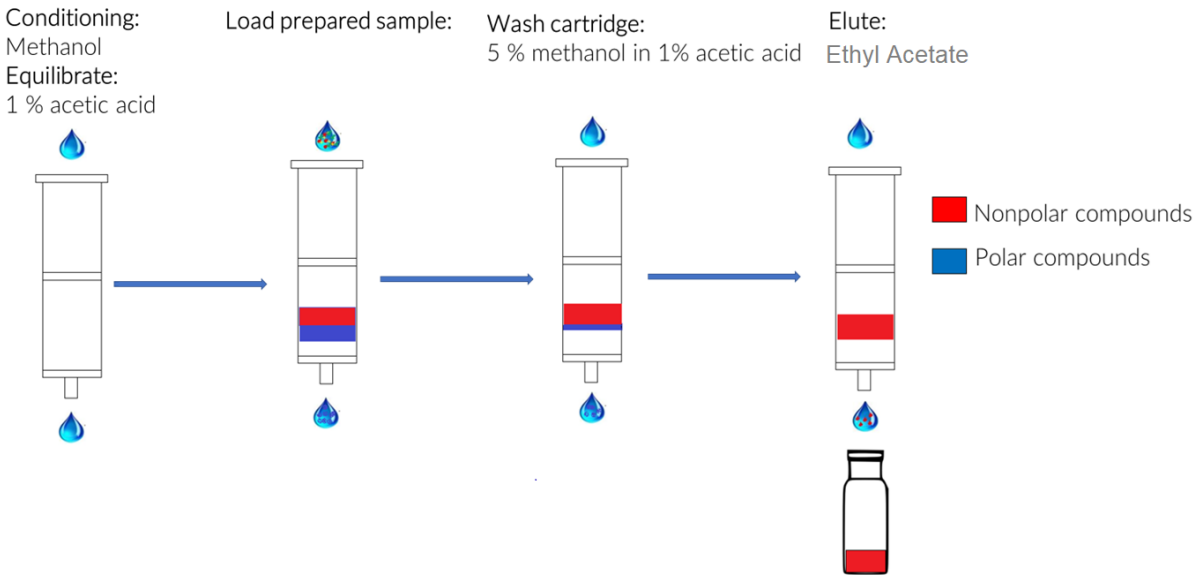


Figure 2. 5: SPE extraction method.

b) QuEChERs extraction methodology

The QuEChERs method streamlines the extraction and purification of analytes from QC samples. This technique began with mixing QC samples with 500 µL ethyl acetate (methanol replacement) and salts (200 mg MgSO4 and 100 mg NaCl), followed by vigorous shaking for one minute and centrifugation at 11,000 rpm for 5 minutes to separate into two layers. The upper layer was transferred to a clean centrifuge tube containing 10 mg C18 sorbent and 60 mg $MgSO_4$, which was shaken for one minute and centrifuged again. From this, 400 µL of the supernatant was moved to a glass vial for derivatization and analysis, see **Figure 2.6**.

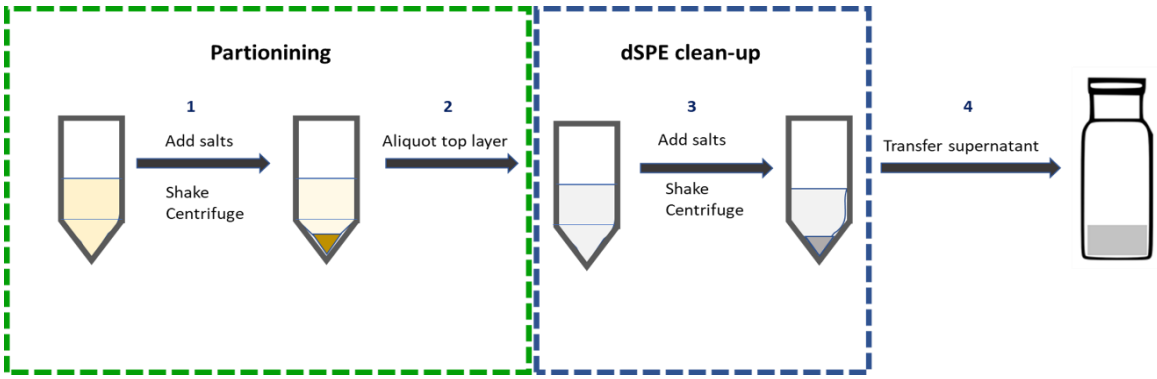


Figure 2. 6: QuEChERs extraction method.

c) The combined SPE and QuEChERs extraction method

A QC sample was loaded onto an HLB Oasis SPE cartridge, previously activated and equilibrated with 1 mL of methanol and 1 mL of 1% acetic acid in Milli-Q water. The unretained fraction was collected for QuEChERs extraction, and the retained matrix was washed with 5% methanol and 1% acetic acid and eluted with 1 mL of ethyl acetate, as shown in **Figure 2.7**.

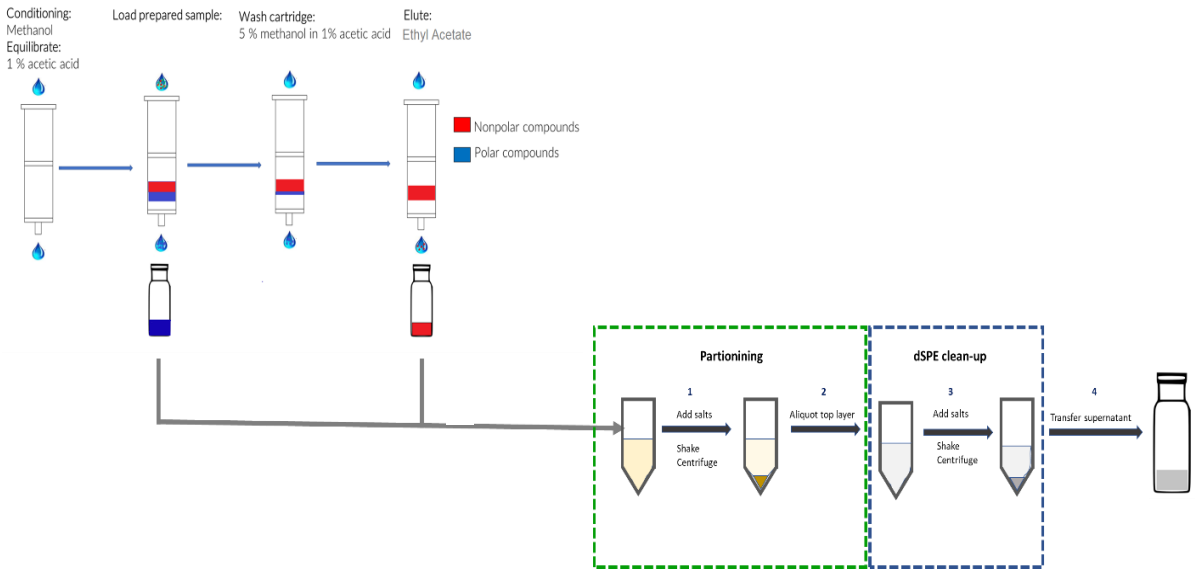


Figure 2. 7: The combined SPE and QuEChERs extraction protocol.

In a comparative assessment of extraction techniques, the QuEChERS method exhibited higher % recovery rates of metabolite derivatives in both water and synthetic urine samples, as demonstrated in **Figure 2.8**. Moreover, the analysis highlighted significant variations in recovery efficiency across different matrices, with water consistently yielding higher recovery rates than synthetic urine. This discrepancy stems from the complex composition of synthetic urine, which more accurately simulates human urine and introduces potential matrix interferences that impact extraction efficiency.

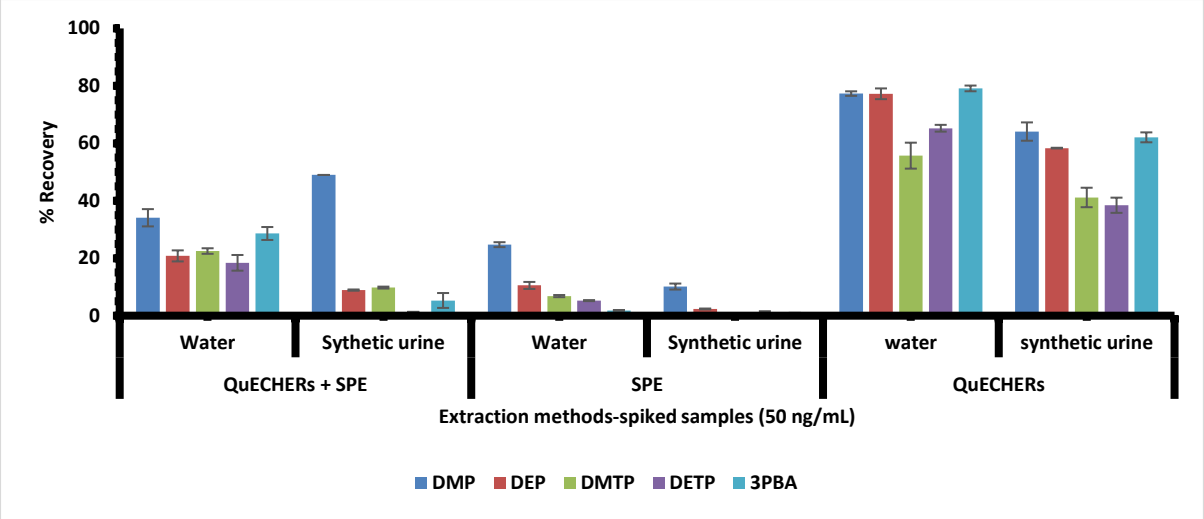


Figure 2. 8 : Percentage recoveries from different extraction methods.

The study determined that the QuEChERS method surpassed the SPE method and the combination of QuEChERs and SPE in both matrices. This indicated QuEChERs' sufficiency for metabolite extraction without the necessity for additional SPE cleanup procedures. This result was partially due to the hydrophilic nature of SPE cartridges, which restricted the efficient extraction of polar metabolites with ethyl acetate. Furthermore, the investigation revealed that the derivatization process could affect recovery rates with all three methods, showing % recoveries below 80%. This finding led to further exploration into whether the derivatisation process influences % recovery, considering that derivatization is an additional step needed to analyse the metabolites of interest using the 2-D GCxGC ToF/MS.

2.2.2.2.4 Effect of derivatisation on the percentage recovery

The study explored how the derivatisation step influences the % recoveries of the metabolites from urine samples when employing the QuEChERs extraction method. The QuEChERs extraction method previously demonstrated high efficacy in achieving higher % recovery rates than other extraction methods (as observed and discussed in the above section). To assess the derivatisation step's impact, the study employed two methods:

- **Pre-spike derivatization** (derivatised before addition to the matrix): In this approach, reference standards at a concentration of 50 ng/mL and ISTD at 20 ng/mL are derivatized prior to their addition to the blank sample matrix (water and synthetic urine sample) before % recovery analysis. % recovery analysis is discussed in detail in **section 2.2.2.3**. Water matrix was chosen

because it is free from matrix compounds, and synthetic urine was chosen as it simulates actual urine samples with matrix compounds. The matrices were used to assess the effectiveness of separate metabolite derivatization and their % recovery during the QuEChERs extraction process. The Pre-spike derivatisation method evaluates the effect of derivatising metabolites without matrix interference and the extraction method's efficacy with pre-derivatized analytes. Consequently, the method provides insight into the pre-derivatization impact on metabolite % recovery and the efficiency of the extraction and analysis process.

- **Post-spike derivatization** (derivatised within the matrix): Contrarily, in this method, the standards and ISTD are added to the sample matrix and then extracted using the QuEChERs method. Derivatization was performed after the extraction step. This sequence evaluates the % recovery of the metabolites when they are derivatized within the matrix after the extraction process, highlighting the impact of the matrix on the derivatization effectiveness and the subsequent recovery rate.

The bar graph (**Figure 2.9**) presents a comparison of the % recovery for the DAPs and 3PBA, pre-spike derivatisation, and post-spike derivatisation. All metabolites exhibit a high % recovery in water for pre-spike, with DMP showing the highest % recovery. However, when the metabolites are derivatized within the water matrix, a slight reduction in % recoveries are observed. In synthetic urine, pre-spike derivatization led to lower % recovery rates than water, with 3PBA experiencing a notable decrease. The % recovery rates are further reduced when derivatization occurs within the synthetic urine matrix. This pattern indicates that both the timing of derivatization and the type of matrix can significantly influence the effectiveness of the recovery process, with water being a more favourable matrix than synthetic urine for the pre-derivatization of these compounds.

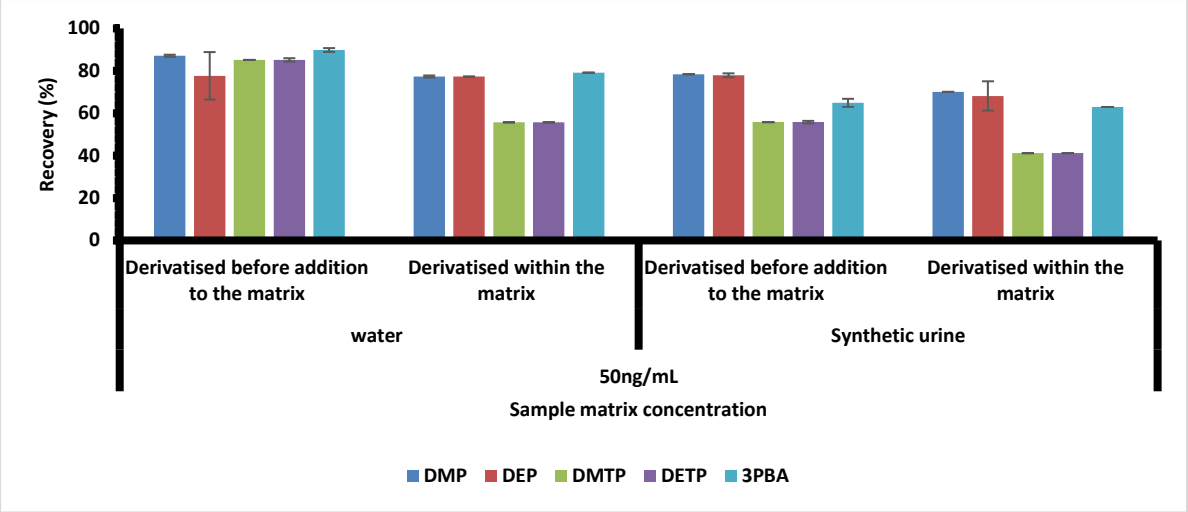


Figure 2. 9: % Recoveries of derivatised metabolites from pre-spike and post-spike methods.

Derivatization enhances the detection of metabolites; however, any inefficiency in this process, such as incomplete derivatization, analyte decomposition, or unintended side reactions, can significantly reduce analyte concentrations[575,576], severely impacting analysis accuracy and precision. Additionally, Pawliszyn *et al*. 2012 described how compounds in a sample matrix can disrupt the derivatization process as they compete with the derivatizing agent. This reduces the agent's availability for the target analytes, potentially leading to incomplete derivatization[577]. Such interference can adversely affect the analytes' recovery and quantification in the analytical process. These findings are important for environmental, biomonitoring, pharmaceutical, and clinical research, guiding the selection of appropriate derivatization agents and methods to improve accuracy and reliability in analytical data.

Therefore, it is important to delve into the selection of derivatizing agents capable of minimizing matrix effects while maintaining analyte integrity to overcome the challenges in metabolite recovery. Concurrently, investigating alternative extraction techniques and solvents opens new pathways to refine extraction methods to discover approaches yield higher % recoveries alongside dependable results. Within this research framework, the QuEChERS method was validated for extracting metabolites from urine samples, which marked a significant phase in affirming its suitability and efficiency for 2-D GCxGC ToF/MS analysis needs. This validation process involved the evaluation of the method's % recovery and precision capabilities that ensure its compliance with the demands for precise and reliable metabolite analysis.

The initial evaluation focused on the QuEChERS method's efficacy in recovering metabolites and ensuring precision from urine samples to lay the groundwork for further research. The study progressed to developing an extraction technique for hair samples, which was characterized by high % recovery rates and is further detailed in subsequent sections of the study.

2.2.2.2.5 Extraction for hair samples

Solid-liquid extraction (SLE) is a predominant method used for metabolite extraction from hair in human biomonitoring studies for insecticide exposure. Researchers have successfully employed SLE using solvent variants to isolate OP and PYR metabolites, demonstrating its widespread use, reliability, and effectiveness [243,247,578,579]. SLE efficiently processes hair's complex biological matrix, ideally suited for polar and stable metabolites like DAPs and 3-PBA, ensuring accurate quantification[580-582].

The absence of % recovery data in similar studies does not diminish the inferred efficacy of the SLE, evidenced by the consistent detection of metabolites in a significant portion of hair samples. For instance, Hardy *et al*. 2021 observed detectable concentrations of the metabolites of interest in over 93% of the 93 women's hair samples examined, while Peng *et al*. 2020 found metabolites in more than 98% of the 204 hair samples analysed[578,583]. These high detection rates are consistent with findings from other studies where more than half of the samples showed detectable levels of metabolites, suggesting that SLE is highly effective in extracting significant quantities of analytes. Such robust detection rates serve as indirect evidence of the SLE's efficiency and validate its suitability for the study's objectives.

The current study tailored the SLE method by selecting ethyl acetate as the extraction solvent over the more commonly used methanol or acetonitrile in response to specific research needs. Similar to previous research, this study employed a GC technique for analysing and quantifying DAP and 3-PBA metabolites in hair samples. However, it diverged by using MTBSTFA as the derivatising agent instead of Pentafluorobenzyl Bromide (PFBBr) to reflect a methodological adaptation based on the enhanced sensitivity for the target compounds, chemical compatibility with the sample matrix, suitability for the analytical instruments used, or the ability to achieve the desired reaction efficiently in this investigation[243,247,578,579]. Despite these modifications, the continued use of GC-MS for analysing and quantifying metabolites aligns with established practices in the field.

2.2.2.2.5.1 Hair sample preparation and DAP and 3-PBA extraction

The preparation of hair samples for DAP and 3-PBA extraction began with thorough washing using a 1% SDS solution and methanol, which removes external contaminants. This step is critical to ensure the purity of the samples. Subsequently, the samples undergo drying and pulverization to enhance their surface area, thereby increasing the efficiency of the extraction process. A mechanical grinder typically performs this pulverization, achieving a uniform consistency. The pulverized samples are extracted using ethyl acetate as the solvent. In this process, the finely ground hair mixes with ethyl acetate and undergoes overnight agitation to maximize the interaction between the solvent and the hair particles, facilitating an efficient transfer of target analytes into the solvent. The extracted solution is then derivatized and analysed using 2-D GCxGC ToF/MS, as discussed in Chapter 4.

Following the development of urine and hair sample extraction methods, namely QuEChERs and SLE, the performance of these methods was quantitatively assessed by determining the % recoveries and precision parameters. These aspects are pivotal for validating the reliability and accuracy of this study's extraction and analysis methods. This evaluation not only justifies the methodological choices made but also confirms the robustness and reproducibility of the experimental outcomes.

2.2.2.3 % recoveries and precision of QuEChERS and SLE extraction methods

Evaluating the efficacy of an analytical method in accurately extracting and quantifying target analytes from samples hinges on "% recovery," a key metric that not only assesses the method's ability to retrieve these analytes accurately but also signifies the precision of their quantification[584]. Conversely, precision reflects the consistency of measurement reproducibility, determined by calculating the relative standard deviation (RSD) of replicate measurements where an ideal outcome would show variability within 20% of a nominal or reference value, thus ensuring measurement consistency and dependability [585].

Precision presents intra-day and inter-day precision, where the former assesses the uniformity of measurements within a single day, and the latter assesses the consistency of results across multiple days. This dual assessment is crucial in extensive studies involving many participants, underscoring the necessity for a method that exhibits steadfast reliability within and across days[585].

Central to determining % recovery, spiked QC samples are used to evaluate the precision and trustworthiness of the QuEChERs and SLE extraction methods[586,587]. Through the employment of QC

samples that are spiked at LCL, MCL, and HCL concentration levels, the analysis encompasses both pre-spike scenarios (where analytes are introduced into blank matrices before extraction) and post-spike scenarios (where analytes are added post-extraction). This comprehensive approach allows for the evaluation of metabolite recoveries against the backdrop of standard concentrations, as outlined in **Equation 2.3**, where "A" and "B" represent the average peak area of post-spikes (n ≥ 3) and the peak area for pre-spike samples, respectively[588,589].

Equation 2.3

$$\%\, Recovery = \left(\frac{\boldsymbol{B}}{\boldsymbol{A}}\right) * 100$$

Precision quantifies the variability or dispersion of a set of values (concentration or % recovery values). In this study, % RSD is represented as a percentage of the mean of % recovery values, indicating how much individual values in a set of repeated measurements or analyses deviate from their average (mean). The lower the % RSD, the more consistent and reliable the data set is, indicating higher precision in the measurements or analyses[590,591]. This research study determined intra-day precision by analysing 9 blank synthetic urine samples for each spiked three QC concentration levels on the same morning (9 am). On the other hand, inter-day precision was determined by analysing QC samples spiked with three concentration levels 9 times in every analytical batch over a 2-day cycle, usually in the morning (from 9 am). Subsequently, the RSD or variance obtained at each concentration level during intra-day and inter-day analysis was evaluated to facilitate the estimation of the coefficient of variation (CV) for the entire analytical value chain.

The CV is a statistical metric that quantifies the variability within the analytical value chain. It is defined as the ratio of the standard deviation to the mean and is often expressed as a percentage. This approach was important in assessing and confirming the accuracy, reliability, and validity of the analytical findings. The accuracy was estimated by dividing the theoretically expected metabolite level by the experimentally determined value and multiplying the quotient by 100, as shown in **Equation 2.4** below. Commonly, acceptable accuracy criteria define a range of ±15% from the reference value. Various sources have established these criteria, including references [529,530,592-594]. In this study, the reference values for accuracy criteria are 5, 50, and 100 ng/mL, and % recovery values are deemed accurate if they fall within ±15% of reference values. Similarly, for precision, it is required that the

percent % RSD does not exceed 15%, ensuring that the measurements are consistent and reliable around the mean. **Equation 2.4**

$$\%RSD = \frac{\delta}{\bar{x}} * 100\%$$

Where δ is the relative standard deviation whilst $\bar{x}$ is the experimentally derived mean[595].

Following this foundational exposition, the subsequent sections will detail the specific % recoveries and precision metrics for the QuEChERs extraction method applied to urine samples and the SLE method for hair samples, encapsulating the essence of method validation in analytical chemistry % recoveries and precision determination for the QuEChERs extraction method for urine samples.

Table 2.5 revealed that % recoveries for urine samples ranged from 44% to 70%. DMP and 3-PBA showed the highest recovery percentages, with averages of 66.45% and 60.29% across QC concentrations, respectively. DEP followed with an average % recovery of 57.13%. DMTP and DETP, however, exhibited the lowest average % recoveries at 43.67% and 49.61%, respectively, for the three QC concentration samples. The uniformity across the three QC concentrations suggested that % recoveries for each were nearly identical, indicating consistency. The interaction between the derivatizing agent and matrix components likely reduced the % recovery, a subject that has been discussed earlier. This recovery falls below the anticipated benchmark of 80% or higher that analytical method validation commonly aims for. Using a synthetic urine matrix for recoveries served as a solid foundation for applying the QuEChERS extraction method to the study's quantification analysis of urine samples.

The table also highlighted that precision (%RSD) showed higher values for samples processed and analysed within a 24-hour cycle than those assessed after 24 hours. Intra-day precision remained within the recommended % RSD range, showcasing a high degree of consistency with %RSD values from 1.20% to 11.05% for same-day analyses. On the other hand, inter-day precision varied more significantly, with %RSD values from 0.56% to 37.53%, indicating less consistency for measurements taken on different days. Factors such as instrument variations, changes in sample preparation, and environmental impacts were cited as possible reasons for the variability in inter-day precision[596-598]. Thus, analyses conducted within 24 hours demonstrated preferable precision, reducing the likelihood of data variance. Nevertheless, adopting stringent quality control measures and meticulous calibration

was essential for ensuring data reliability and comparability across different days for extended analysis periods.

Table 2. 5: % recovery and precision method validation results for urine sample extraction.

Name	Recovery % (std)			Precision					
				Intraday (%RSD)			Inter-day (%RSD)		
Metabolites	**5 ng/mL**	**50 ng/mL**	**100 ng/mL**	**5 ng/mL**	**50 ng/mL**	**100 ng/mL**	**5 ng/mL**	**50 ng/mL**	**100 ng/mL**
DMP	61.95 (0.79)	70.07 (10.31)	67.33 (2.56)	8.98	9.01	1.51	13.50	20.86	3.26
DEP	50.55 (10.64)	59.03 (1.47)	61.80 (2.28)	5.22	2.95	3.16	1.66	20.35	0.56
DMTP	44.71 (1.83)	41.15 (8.24)	45.17 (5.75)	3.99	6.61	2.59	38.71	18.13	3.09
DETP	61.90 (8.47)	46.54 (12.48)	40.40 (1.07)	3.04	1.99	1.20	5.77	20.36	1.60
3-PBA	56.29 (1.64)	62.94 (3.40)	61.65 (10.77)	11.05	3.30	1.13	37.53	27.67	1.34

2.2.2.3.1 Comparison of % recoveries in the current study with other studies utilizing GC techniques

The % recoveries achieved in this study aligned with those documented in other research analysing the same metabolites with GC techniques over the past decade (2012–2022), see **Appendix Table A 2.14** which shows the extraction techniques, solvents, derivatising agents, % recovery and GC column used by the different studies. These studies, incorporating various extraction methods and derivatizing agents, reported 48% to 94% recoveries. Significantly, **Table 2.6** showed that two studies using the same derivatizing agent (MTBSTFA) utilized in the current investigation found recoveries within the observed range. Specifically, these studies yielded recoveries from 54.08% to 82.49% for GC-MS/MS analysis and 48% to 91% for GC-MS analysis. For 2-D GCxGC ToF/MS analysis, recoveries varied between 40% and 70%. This uniformity in recovery rates across different studies highlights the reliability and comparability of the methodology applied in our research to those reported in scientific literature throughout the last decade.

Table 2. 6: Comparison of GC techniques' urine extraction methods, derivatizing agents, solvents, and recoveries between study data and literature.

GC technique	Extraction technique	Extraction solvent	Derivatizing agent	Recovery (%)
GC-MS	LLE[197,244]	Hexane [197,244]	MTBSTFA[197,244]	48-90 [197] 89-94[244]
GC- MS/MS	LLE and SPE[241,381]	Methanol or acetonitrile containing formic acid [241] Hexane[381]	MTBSTFA [241,381]	100[241] 54.08-82.49[381]
GCxGC ToF/MS	QuEChERs	Ethyl acetate	MTBSTFA	40-70

2.2.2.4 % recoveries and precision determination for the SLE extraction method for hair samples

Hair QC samples were prepared as detailed in the hair preparation section 2.2.2.2.5, and this was followed by both pre-spiking and post-spiking procedures with LCL, MCL, and HCL. Pre-spiking and post-spiking procedures were crucial for assessing the SLE method's precision and % recoveries. As shown in **Table 2.7**, DMP showed high recovery rates across all concentrations, achieving 95% at 5 and 100 ng/mL and 88% at 50 ng/mL. This indicates an efficient extraction process for DMP while intra-day precision is consistent, with %RSD values ranging from 1.86 to 3.02 for all the metabolites.

The inter-day precision also maintains this consistency where %RSD values range between 2.63 and 3.67. DEP also exhibits high recovery rates, particularly 94 % at 50 ng/mL and 87 % at 100 ng/mL. However, its intra-day precision shows higher variability, with % RSD values between 6.41 and 7.30, but it demonstrates better consistency across different days, particularly at higher concentrations (1.76 %RSD). DMTP records lower recovery rates at 50 ng/mL (73 %) than DMP and DEP. Its intra-day precision is good, with low %RSD values from 0.82 to 3.10.

DETP presents consistent recovery rates of around 85% across concentrations. It shows moderate variability in intra-day precision (2.50 to 3.38 %RSD) but maintains consistent inter-day precision, especially at higher concentrations. 3PBA has varying recovery rates, with an exceptionally high rate at 100 ng/mL (98%). Its intra-day precision maintains remarkable consistency across intra-day and inter-day, as low %RSD values indicate, proving that hair samples can be prepared and analysed on the same day or on different days.

Table 2. 7: % recovery and precision method validation results for hair sample extraction.

Name	Recovery % (std)			Precision					
				Intraday (%RSD)			Inter-day (%RSD)		
Metabolites	5 ng/mL	50 ng/mL	100 ng/mL	5 ng/mL	50 ng/mL	100 ng/mL	5 ng/mL	50 ng/mL	100 ng/mL
DMP	95 (1.40)	88 (1.26)	95 (0.57)	1.86	3.02	2.81	3.67	2.63	2.63
DEP	95 (3.77)	94 (1.53)	87 (5.14)	6.41	6.96	7.30	4.70	1.76	1.76
DMTP	86 (1.27)	73 (1.47)	79 (0.55)	2.14	3.10	0.82	11.86	0.86	0.86
DETP	86 (2.92)	85 (3.60)	82 (0.75)	2.50	3.38	3.30	7.62	1.68	1.68
3PBA	80 (0.86)	81 (1.13)	98 (0.55)	1.87	2.38	2.03	1.73	0.81	0.81

2.2.2.5 Method detection limit for urine samples

The Method Detection Limit (MDL) is the lowest concentration of an analyte that can be reliably detected under specific analytical conditions[599]. It reflects the sensitivity of the method in complex biological samples such as urine and hair. Unlike the LOD, which is often determined using solvent-based standards, the MDL accounts for matrix effects that influence analyte detection in biological samples. In this study, blank urine and hair samples were spiked with metabolite standards at concentrations of 5, 50, and 100 ng/mL, and percent recoveries were calculated. Urine samples showed significant matrix interference, resulting in inconsistent recoveries, while hair samples provided reliable and consistent results. The MDL was calculated based on the 5 ng/mL spiked samples, with three replicates for urine and six for hair. Using the standard formula[599],

$$\mathrm{MDL} = \mathrm{t}\,(\mathrm{n}-1, 0.99) \times \mathrm{S},$$

where S is the standard deviation of replicates and t is the appropriate t-value for a 99% confidence level. The MDLs for urine were reported in ng/mL, while those for hair were reported in pg/mg, reflecting the concentrations present after pre-treatment and extraction see **Table 2.8** below. The MDL calculations are comprehensively described in Appendix section **Table A2.15** and Table **A2.16**.

Table 2. 8: MDL values for urine and hair samples

	MDL Urine (ng/mL)	MDL Hair (pg/mg)
DMP	5.96	2.27
DEP	3.04	7.59
DMTP	0.16	5.48
DETP	5.08	5.04
3-PBA	0.17	99.45

MDLs for biological matrices are often higher than LODs because matrix components and derivatization processes influence both analyte recovery and signal detection. Biological samples, such as urine and hair, contain endogenous compounds that interfere with analyte detection, particularly when derivatization agents are involved[600]. In addition, derivatization can be challenging due to incomplete reactions or variability in reaction conditions which leads to inconsistent results and unreliable MDL

values[502]. Moreover, instrument sensitivity limitations play a role in increasing MDLs, especially when detecting low concentrations of analytes in complex biological matrices[601]. The lack of standardized reference materials for urine and hair adds to the difficulty, as calibration and validation procedures become more complex without well-defined matrix standards[602]. Furthermore, inconsistencies introduced during sample preparation, such as extraction and purification steps, affect the precision needed for accurate MDL determination[603-605]

2.3 Conclusion

The study developed and validated a novel analytical method using 2D GCxGC-TOF/MS to quantify DAPs and 3-PBA in urine and hair samples, marking the first application of this technique for insecticide biomonitoring in human biological samples. The method represents an advancement as a new application in human health studies and a milestone in South Africa, where the instrument's use now extends beyond environmental sample analysis. Selecting MTBSTFA as the derivatizing agent and fine-tuning factors such as solvent choice, reaction time, and temperature, significantly boosted the detection and analysis of the target compounds. Ethyl acetate emerged as the most effective solvent for enhancing the extraction and detection rates of the compounds.

Further, the study refined extraction processes for urine and hair samples, applying SLE for hair and the QuEChERS method for urine. The extraction methods led to high recovery rates and consistent precision. This refinement demonstrated marked improvements in percent recovery and precision over previous methods and suggested its applicability across different biological matrices. The methods proved effective and robust in evaluating % recovery and precision for urine and hair samples. Although recovery rates for urine samples varied among metabolites, these results aligned with those of other studies employing different GC techniques. High recovery rates for DMP and DEP in hair samples, alongside consistent precision across metabolites, affirmed the SLE method's efficiency which is a finding supported by its successful detection of compounds in comparable research.

2.3.1 Limitation

The current study successfully demonstrated the quantitative ability of a 2D GCxGC-TOF/MS method in the analysis of DAPs and 3-PBA in biological matrices. However, the method performance, as indicated by low % recovery, suggests that the QuEChERS extraction protocol for urine may need further optimization to reduce matrix effects for optimal method accuracy. Due to the complexity of the urine matrix and the presence of competing compounds, the derivatization process encountered challenges that could have led to incomplete derivatization or analyte degradation and significantly affected the method's accuracy and precision. In addition, comparing the method performance with the latest available literature, can be biased due to differences in specific analytical conditions for each study.

Chapter 3

The Assessment of Exposure to Insecticide Residues in Farm Children from the Western Cape: Evaluation Using Advanced 2-D GCxGC ToF/MS

3.1 Introduction

Biomonitoring plays an important role in environmental health research by providing a direct measure of human exposure to chemicals and helping to assess the associated health risks[238,606]. The current study used the developed and validated 2-D GCxGC ToF/MS analysis method to quantify DAPs and 3-PBA in children living in apple (Grabouw) and grape (Hex River) farming areas in the Western Cape, South Africa [607-609]. The novel analysis method was used to detect the metabolites in urine and hair samples, enhancing exposure assessment in South Africa. Analysis of hair samples is comprehensively discussed in **Chapter 4**.

Not only did the study focus on targeted analysis but it also explored non-targeted analysis in urine samples to identify other possible health risks due to insecticide exposure in these regions and the results are presented in **Chapter 5**. The primary methodology centred on urine and hair sample analysis; however, other research frameworks have also been employed to understand the health impacts of these chemicals. These frameworks include genotoxicity monitoring, occupational exposure studies, and age-specific investigations, which collectively provide a broader perspective on the environmental and health risks of agricultural chemicals[610-614]. While the study in question specifically exploited targeted and non-targeted analyses to gauge the exposure and effects of OP and PYR, acknowledging these alternate methodologies highlights the multidimensional nature of research in this area and the importance of employing various strategies to fully assess the potential health risks.

The current research study qualifies as a cohort study as it assesses OP and PYR exposure in farm children, dividing them into two distinct groups: farm, comprising children living on farms, and nonfarm,

children residing within a 5 km radius of farms. The study collects and analyses biological samples from the children over two annual cycles. The study aims to assess OP and PYR exposure comprehensively by including children from diverse demographic backgrounds. The methodology, discussed in the following sections, encompasses detailed sample collection, preparation, and analysis, ensuring reliable and accurate results, characteristics essential for a successful cohort study.

The current study screened and quantified five metabolites (DMP, DEP, DMTP, DETP, and 3-PBA) in biological samples from school children in Grabouw and Hex River farming areas of the Western Cape to assess OP and PYR exposure levels. This chapter introduces the innovative application of the 2-D GCxGC ToF/M.S method in this study area. Unlike prior research that often relied on different analytical techniques, such as GC-MS and LC-MS/MS, this novel application contributes significantly to biomonitoring. By using this advanced method for biological samples obtained from the Western Cape region of South Africa, the study enhances the understanding of OP and PYR metabolite analysis in various biological matrices, offering essential insights into children's exposure levels in these farming communities.

3.2 Metabolite extraction and analysis: from study sample collection to data interpretation

This research shows a specific order of metabolites analysis, which includes sample collection, sample preparation, extraction methods, analysis techniques, and finally, data analysis. These interdependent stages collectively form a comprehensive approach crucial for ensuring the accuracy and dependability of the analytical process[615]. Numerous methodologies have been documented for extracting metabolites of OPs and PYRs from various biological matrices, encompassing urine, hair, breast milk, placenta, and other matrices[334]. The details of metabolite extraction and analysis are outlined below, providing a step-by-step elucidation of the methodology employed in this comprehensive examination.

The study is a sub-study of a longitudinal investigation of the "Child Health Agricultural Pesticide Study in South Africa," which aimed to investigate the potential adverse effects of agricultural pesticide exposure on the reproductive development and neuro-behaviour of farm children living in rural Western Cape, South Africa[616]. The longitudinal study was conducted in schools in three pesticide-intensive farming areas of the Western Cape (Piketburg, Hex River, and Grabouw) regions where insecticides

exposure had been found in various environmental media and among farm workers[109,121,617,618]. The core purpose of this research was to evaluate the impact of insecticide exposure on adolescent boys and girls over a period of three years, from 2017 to 2019.

A cohort of 1000 children was enlisted from schools in the Hex River Valley, known for grape farming, the Grabouw area, where apple farming is the main agricultural activity, and the Piketberg region, recognized for its wheat and fruit cultivation. The recruitment strategy ensured a balanced representation of participants regarding area, gender, and living conditions, whether on farms or off-farm. This was achieved through a random systematic sampling method from seven schools across the specified areas. Over the years, urine samples were collected at five intervals, starting with a baseline in the initial year, then three rounds in the subsequent year, and finally, during the third year for follow-up purposes. Hair samples were also collected at only two rounds (the baseline and once more in the second year) due to the participants' reluctance to consent to their hair being collected.

3.2.1.1 Sample Size Calculations

Sample size is important for research validity and reliability because it enhances true effect detection and reduces errors, thus sharpening precision [619]. Statistical power, influenced by sample size, effect size, significance level, and data variability, should reach 80% to ensure detection of true effects; increasing sample size boosts power but necessitates balancing costs and diversity [620]. Precise sample size and adequate power are essential for meaningful results, such as evaluating insecticide impacts on health[621]. High statistical power signifies reliable findings, but low power increases the risk of overlooking real effects, leading to false negatives [622,623].

The significance level often stands at an alpha of 0.05, and effect size directly affects statistical power [624]. Enhancing sample or effect size strengthens statistical power, whereas lowering the significance level may improve power but raises the chance of false positives. Power analysis, facilitated by tools like G*-power software, is crucial for determining the right sample size to detect significant effects efficiently [625]. Conducting power analysis before the study helps ascertain the required sample size (a priori power analysis), and evaluating power afterwards is vital to gauge the study's strength and reliability (post hoc power analysis) [626-629].

*Power software conducts T-statistical tests to analyse differences in means between two distinct groups (farm and non-farm) in the current study's category of interest. An independent samples t-test determines the presence of a significant difference between the groups' means. Additionally, the test assesses if the average values (mean) of the groups differ statistically, considering their variance and sample size. The categories are gender, residential, geographical area, and timeline (cycles). A post hoc determination of the necessary sample size, with an alpha (α) error probability set at 0.08 and an effect size of 2, indicated a requirement for 6 samples (n = 6) in each group category; see **Figure 3.1**. The study exceeded the initial sample size requirements, collecting more than six samples per category, which is evident in **Figure 3.2**. The sample size curves in this figure demonstrate the influence of increasing sample sizes on the statistical power for the specified effect size of 2.

Consequently, the actual sample size ensured a robust statistical power above the 80% threshold commonly sought and reduced the probability of Type II errors, which enhanced the confidence in the detected effects. The study set the alpha error probability at 0.08, which exceeded the standard 0.05 and thus required a larger sample to sustain the study's power[625]; the condition was fulfilled. The ample sample size strengthened the validity of the study's conclusions and provided a more dependable foundation for interpreting the effects of insecticide exposure on the populations under investigation.

***t* tests** – Means: Difference between two independent means

(Two groups)

Analysis:	Post hoc: Compute achieved power		
Input:	Tail(s)	=	Two
	Effect size d	=	2
	α err prob	=	0.08
	Sample size group 1	=	6
	Sample size group 2	=	6
Output:	No centrality parameter δ	=	3.46410
	Critical t	=	1.94809
	Df	=	10
	Power (1−β err probability)	=	0.92425

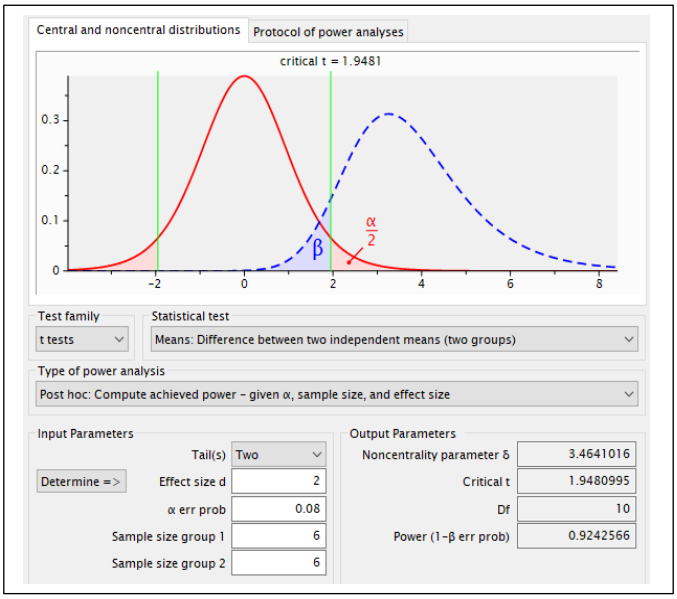


Figure 3. 1: Curves showing sample size for two independent groups using input characters in the review.

Figure 3.4 power curve shows that the probability of accurately pinpointing true effects rises as both sample size and effect size escalate. Such an increase enhances the capacity to detect genuine differences as sample size expands and heightens sensitivity for discerning variations with effect size growth. The analysis required a two-tailed input and necessitated 12 samples while accounting for a degree of freedom of 10 and a critical-t value of 1.948, leading to an actual power of 0.92 (92 %). By incorporating more than 6 participants for each category, as detailed in **Table 3.2**, the study ensured robust statistical power for the hair sample participant group, reinforcing the investigation's credibility.

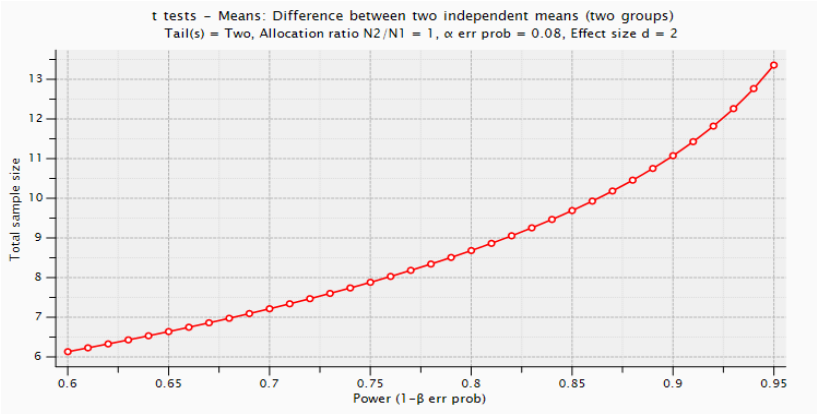


Figure 3. 2: A power curve showcasing g how statistical power varies as sample size or effect size changes.

3.2.2 Study ethics.

The cohort study received ethical approval from the University of Cape Town's Research Ethics Committee (HREC), with the reference number HREC 234/2009, for urine collection and analysis (see Appendix **Figure A 3.1**), while the ethics approval (HREC 181/2019) for hair collection was issued separately for this study, see Appendix **Figure A 3.2**. The University of Cape Town conducted the sample collection in collaboration with the Swiss Tropical and Public Health Institute (Swiss TPH) in Switzerland. Other research collaborators are the University of Witwatersrand in Johannesburg, South Africa, and the Research Centre for Toxic Compounds in the Environment (RECETOX) in Burno, Czech Republic.

3.2.3 Sub-study sample collection.

In this study, hair samples were collected in addition to the urine samples during the baseline study conducted in May-August 2017 (Cycle 1) preceding the spraying season in October-November 2018 (Cycle 2) in the spraying season of these insecticides in these two areas. These urine and hair samples were obtained from boys and girls residing in farm and nonfarm areas in the Hex River Valley and Grabouw farming areas, as illustrated in **Figure 3.3** below.

Figure 3. 3: Geographical locations of Grabow and Hex River study sites for this sub-study.

3.2.3.1 Sociodemographic characteristics of urine sample participants

Recent investigations revealed exposure of Grabouw and Hex River residents, including women, farm workers, and children, to OPs and PYRs, with higher concentrations found in children's wristbands compared to adults[121,131,132,616,630-633]. The socio-demographic analysis of the children who provided urine samples (See **Table 3.1**) reveals significant differences in age, household size, income, and geographical location between farm and non-farm children. Farm children were generally younger, with a median age of 10 years compared to 11 for non-farm children ($p = 0.01$), and more likely to live in larger households, with 57% of farm families having 5-6 members.

Household income between the two groups were also significant ($p = 0.05$), with less non-farm families, with incomes above R3201.00. Less than two-thirds (62%) of mothers attained a secondary education and less than half (40%) of parents were married. These findings align with studies by Molomo *et al*. (2021) and Chetty-Mhlanga *et al*. (2018), which showed that children in rural settings are of low socio-economic status [616,634].These socio-demographic factors further emphasize the need for targeted interventions to address the environmental and economic vulnerabilities faced by farm children, who are disproportionately affected by these risks.

Piketburg was excluded from the study due to participants' reluctance to provide biological specimens, specifically hair samples. The study population consisted of girls and boys between 9-16 attending primary or secondary schools in the Grabouw and Hex River farming areas, living on farms and in neighbouring communities within a 5 km radius. Exclusions included children who did not meet age criteria, did not consent to sample collection, parents who did not provide consent, and lived more than 5 km from the farms.

Table 3. 1: Urine sample participant and guardian sociodemographic characteristics by farm and non-farm residents.

	Total	Farm	Non-farm	
	N=61	N=43	N=18	*p*-value
Participant sociodemographic characteristics				
Age in years	10 (9, 11)	10 (9, 11)	11 (10, 12)	***0.01***
Age categories				
9-11 years	50 (82)	40 (93)	10 (56)	***0.001***
12-14 years	11 (18)	3 (7)	8 (44)	
Sex				
Female	42 (69)	33 (77)	9 (50)	***0.04***
Male	19 (31)	10 (23)	9 (50)	
Grade categories				
2^{nd}-3^{rd} (foundation phase)	11 (18)	8 (19)	3 (17)	0.40
4^{th}-6^{th} (intermediate phase)	49 (80)	35(81)	14 (78)	
7^{th}-9^{th} (senior phase)	1 (2)	0	1 (5)	
Guardian sociodemographic characteristics				
Mother education level attained				
n_1=50 n_2=35 n_3=15				
No schooling	3 (6)	2 (6)	1 (7)	0.31
Primary education	16 (32)	9 (26)	7 (47)	
Secondary education	31 (62)	24 (69)	7 (47)	
Marital status				
n_1=50 n_2=35 n_3=15				
Married/Cohabiting	20 (40)	16 (46)	4 (27)	0.47
Widowed	3 (6)	2 (6)	1 (7)	
Divorced/Separated	8 (16)	6 (17)	2 (13)	
Never married	19 (38)	11 (31)	8 (53)	
Mother employed in past year	28 (56)	18 (51)	10 (67)	0.56
n_1=50 n_2=35 n_3=15				
Size of Household				
n_1=50 n_2=35 n_3=15				
≤4 people	9 (18)	2 (6)	7 (47)	***0.001***
5-6 people	27 (54)	20 (57)	7 (47)	
>6 people	14 (28)	13 (37)	1 (7)	
Household income				
n_1=50 n_2=35 n_3=15				
R0-R3200	12 (24)	7 (20)	5 (33)	***0.05***
R3201or more	23 (46)	20 (57)	3 (20)	
Refused/Don't know	15 (30)	8 (23)	7 (47)	
Geographical location				
Grabouw	41 (67)	39 (91)	2 (11)	<***0.001***
Hex river valley	20 (33)	4 (9)	16 (89)	

- p-value of ≤0.05 considered statistically significant.
- continuous data reported as median (Interquartile range - IQR) and p-values derived using Mann Whitney U-test.
- proportions (%) reported as n/N (except if data is missing - denominator added in the variable name column) and p-values derived using Chi-squared/Fishers Exact tests.

3.2.3.2 Sample collection.

There are three methods for urine collection: 24-hour collection, First-Morning Void (FMV), and Spot Sample, each offering distinct advantages and limitations within the biomonitoring framework. The 24-hour urine collection is regarded as the gold standard, and it entails accumulating all urine excreted over a 24-hour period thus providing an exhaustive depiction of the daily exposure[635-637]. However, this method is prone to sampling errors such as under or oversampling and imposes a significant logistical burden on participants, necessitating continuous collection[329,636,638,639]. Although the FMV method captures the first urine sample of the day, it offers a less burdensome alternative and yields a concentrated snapshot of overnight metabolite accumulation, it correlates well with 24-hour samples and is especially valuable for biomarkers with diurnal variations[640-644]. Nevertheless, FMV is susceptible to exposure misclassification, raising concerns about its accuracy in certain contexts[645].

Spot urine sample collection requires only a single sample collected at a specified time, consequently, this streamlines the collection process and reduces participant burden [51]. This method offers practical benefits for large-scale studies, as it requires just a single sample and avoids the logistical challenges of 24-hour monitoring[645-648]. This approach is valuable for detecting insecticide metabolites efficiently, making it suitable for extensive epidemiological researchl[649-651]. However, the reliability of spot samples is occasionally questioned due to potential variability in urine volume and concentration[652,653].

Considering these aspects, the current research opted for spot urine collection to biomonitoring OPs and PYRs metabolites, representing a strategic compromise that enhances both the accuracy and feasibility of the study. The method balances practicality for large-scale application with the informative value essential for assessing environmental chemical exposures. Spot urine samples were collected in individual 50 ml plastic cups for each participant in private area in a classroom at the school. A trained research assistant separated each participant's urine sample into four 4-ml cryovials, color-coded, and stored on dry ice for delivery to the Hair and Skin Laboratory at Groote Schuur Hospital, University of Cape Town. The samples were refrigerated and kept at –20°C in a light-proof fridge. Special precautions were taken to prevent contamination throughout sample collection and all participants were fully informed of the appropriate procedures to be followed. The total urine samples collected in the two cycles are shown as a Venn diagram in **Figure 3.2**.

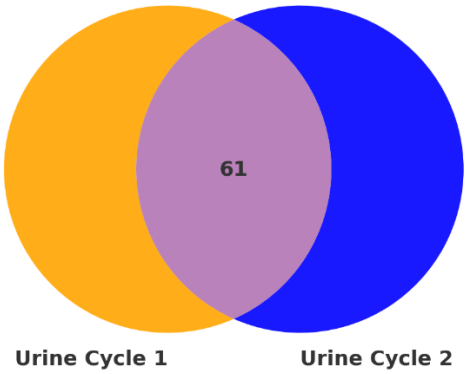


Figure 3. 4: Number of participants with urine samples collected from cycle 1 and 2.

Figure 3.4 depicted a cohort's consistency across two urine collection cycles, with 61 participants appearing in both instances, which ensured methodical analysis through an unvarying sample size. In **Table 3.2**, data analysis revealed a higher female participation in the study, with 41 females compared to 20 males. Grabouw's farm-specific data highlighted a pronounced female majority of 31 against 8 males. Both genders showed equally minimal representation in non-farming sectors, summing to a mere two individuals.

Table 3. 2: Urine population information on gender and residence of participants from Grabouw and Hex river.

Gender	Hex river		Grabouw		
	Farm	Nonfarm	Farm	Nonfarm	Total
Female	4	5	31	1	41
Male	3	8	8	1	20
Total	7	13	39	2	61

3.2.4 Laboratory analysis

The novel 2-D GCxGC ToF/MS technique was applied to analyse children's urine samples for exposure levels. Statistical analyses were conducted to compare exposure between children living close to and distant from farms and to investigate how exposure affects health. The study highlighted the 2-D GCxGC ToF/MS technique's adaptability by employing it for both targeted and non-targeted analyses in urine samples. This versatility underscores the technique's broad relevance and pivotal role in evaluating insecticide exposure's environmental and health impacts on children in farming communities.

Furthermore, the research seamlessly incorporated analytical method development and validation into the protocol. The protocol included detailed sample preparation and the application of the analytical method for urine sample analysis. This process encompasses several tasks, such as the hydrolysis of study urine samples using an enzyme, metabolite extraction using the QuEChERs method, derivatization, and subsequent analysis. Subsequently, statistical data analysis was conducted on the analysed study samples to extract meaningful insights that show the extent of exposure to OP and PYR in children living on farms and those living 5km close to the farms.

3.2.4.1 Chemicals and reagents

Analytical reference standards: DMP, DEP, DMTP, DEP, and 3-PBA; internal standards: DMP-d_6, DMTP-d_6, and 3-PBA $^{12}C_6$, with purities higher than 99% were obtained from Toronto Research Chemicals Inc (TRC) Canada. Sigmatrix Urine Diluent was utilized as the blank urine (B.U.) for Q.C. samples was obtained from DLD scientific cc, Durban, South Africa. A solution of β-glucuronidase enzyme derived from Helix pomatia-2 (≥ 100,000 units/mL, IMCSzyme®3S, USA) and rapid hydrolysis buffer (IMCSzyme®3S, USA). N-tert-butyldimethylsilyl- N-methyl trifluoroacetamide (MTBSTFA) (Sigma-Aldrich, South Africa) and ethyl acetate. HPLC grade solvents were purchased from Sigma-Aldrich, South Africa and Radchem (Johannesburg, South Africa).

3.2.5 Data analysis

Statistical data analysis was done using Stata v16 computer software. Descriptive statistics such as frequencies, percentages, mean, and standard deviations were performed to summarize the characteristics of the study population, and results are presented as (Mean ± Standard Deviation). Associations between the insecticide concentrations detected in the cycles (cycle 1 and cycle 2)

analysed as continuous data and gender (Male and Female), area of residence (Grabouw and Hex River) and location (farm and non-farm) analysed as dichotomous variable were tested using Mann Whitney test because the data was not normally distributed. A P-value ≤0.05 was considered as significant.

A methodological approach undertaken to address the challenge of detecting low-level toxins. The square root transformation of the LOD is employed to manage non-detects and values below the LOD in a statistically robust manner[654,655]. This transformation is essential in reducing the skewness of the distribution of toxin concentrations. Moreover, it stabilizes the variance across the range of measured values which is critical for the application of many statistical tests and models that assume homogeneity of variance. This approach is especially useful in datasets with a high proportion of values below the LOD, ensuring that these non-detects do not disproportionately affect the analysis. This sensitivity is crucial for accurately representing and assessing the health risks associated with low-level toxin exposure[656-658].

3.3 Results and Discussion

3.3.1 Metabolite concentrations in urine samples

A 2-D GCxGC ToF/M.S. analytical method served as a tool for accurately measuring the concentrations of these metabolites in urine samples (absolute concentration (ng/mL)), offering invaluable insights into human exposure to OPs and PYRs.

An overview of the quantified absolute concentrations of metabolites (ng/mL) in urine samples collected from school children living in the rural areas of the Western Cape is shown in **Table 3.3**. The study analysed 122 urine samples over the two cycles and found varying detection frequencies (DF) for DAPs metabolites with DMP and DMTP present in 62 % and 67 % of the total number of samples analysed respectively while 3-PBA had a detection frequency of 61 %. At least one metabolite was detected in each urine sample indicating that every child in the study had been exposed to OPs or PYRs.

Cycle 1 analysis showed median DEP and DMTP concentrations significantly exceeded Cycle 2 levels with p-values under 0.05 highlighting time-based exposure variation. Further analysis comparing children from farming and non-farming backgrounds revealed a significant difference as the median concentration of DETP in the urine of farm children was 6.50 ng/mL, surpassing that in non-farm

children (3.64 ng/mL). This underscores the impact of residing near farming areas on OP and PYR exposure. In the broader dataset, DEP showed the highest median concentration at 9.67 ng/mL, while 3PBA had the lowest at 2.86 ng/mL. During the cycle 1, DEP peaked at 16.21 ng/mL, while non-farm children had the lowest median concentration of 3-PBA at 2.22 ng/mL. These variations could be attributed to different levels of OP and PYR use in farming areas compared to non-farming regions, leading to higher exposure in agricultural settings.

Table 3. 3: Summary of concentrations of OP and PYR metabolites detected in urine samples collected from children, expressed as median and interquartile range (IQR) values in ng/mL.

Variable(total)	DF Total (%)	DMP ng/mL Median (IQR)	DF Total (%)	DEP ng/mL Median (IQR)	DF Total (%)	DMTP ng/mL Median (IQR)	DF Total (%)	DETP ng/mL Median (IQR)	DF Total (%)	3-PBA ng/mL Median (IQR)
Total (122)	76 (62)	7.83 (0.71-19.40)	80 (66)	9.67 (4.14-19.17)	82 (67)	4.86 (1.25-9.65)	81 (66)	6.37 (1.05-13.34)	74 (61)	2.86(0.93-5.30)
Cycle 1 (61)	35 (57)	9.72 (0.71-20.42)	37 (61)	16.21 (3.81-36.40)	43 (71)	6.91 (1.25-14.76)	41 (67)	4.87 (1.05-13.78)	41 (67)	2.83(0.93-4.40)
Cycle 2 (61)	41 (67)	7.05 (0.71-19.38)	43 (71)	7.71 (4.16-13.36)	39 (64)	4.02 (1.25-6.01)	40 (66)	6.75 (3.43-12.27)	33 (54)	2.88(1.11-6.40)
p-value		0.56		***0.02***		***0.01***		0.74		0.78
Farm (82)	55 (76)	9.13 (0.71-19.39)	50 (69)	10.33 (3.38-18.91)	62 (86)	5.38 (1.25-11.15)	61 (85)	6.50 (3.50-15.13)	49 (68)	2.88(0.93-5.30)
Non-farm (40)	21 (70)	6.25 (2.31-19.42)	30 (100)	9.46 (7.14-19.42)	20 (67)	1.25 (1.25-6.31)	20 (67)	3.64 (1.05-8.86)	25 (83)	2.22(1.05-4.79)
p-value		0.72		0.30		0.07		***0.05***		0.86
Grabouw (82)	53 (65)	7.05 (0.71-17.95)	51 (62)	7.89 (3.38-14.98)	60 (73)	5.38 (1.25-10.84)	59 (72)	6.50 (1.05-15.24)	50 (61)	2.78(0.27-4.40)
Hex river (40)	23 (58)	8.60 (2.31-19.47)	29 (73)	14.15 (7.68-26.06)	22 (55)	1.25 (1.25-7.54)	22 (55)	4.02 (1.05-9.12)	24 (60)	3.11(1.29-6.98)
p-value		0.68		***0.04***		0.10		0.22		0.40
Female (84)	56 (68)	7.92 (0.71-19.43)	59 (72)	9.87 (4.16-22.37)	53 (64)	5.48 (1.25-9.57)	52 (63)	5.32 (1.05-14.14)	51 (62)	2.73(0.27-6.13)
Male (38)	20 (50)	7.47 (3.56-18.79)	21 (53)	9.47 (4.12-18.71)	29 (73)	4.06 (1.25-9.65)	29 (73)	6.99 (3.44-12.71)	23 (58)	3.29(1.37-4.28)
p-value		0.83		0.77		0.46		0.45		0.58
<LOD (% RSD) corrected		46 (38%)		42 (34%)		40 (33%)		41 (34%)		48 (39%)
<LOQ (% RSD)		16 (13%)		3 (2%)		27 (22%)		13 (11%)		18 (15%)

Data reported as median (IQR that is 25 % and 75 % of the data) for continuous data.
Associations between continuous data were tested using the Mann-Whitney test.
P-value ≤0.05 considered as significant; % RSD= % relative standard deviation; DF= Detection Frequency
*Samples below limit of detection (LOD) were assigned a value calculated by dividing the LOD value (**Table 2. 3**)of each metabolite by square root of 2 to give: DMP=0.71; DEP=0.52; DMTP=1.25; DETP=1.05; 3PBA=0.27*

Additionally, **Table 3.3** revealed children in the Hex River Valley region had notably higher median DEP levels at 14.15 ng/mL compared to 7.89 ng/mL in Grabouw indicating regional variances in OP and PYR utilization or exposure risks. Gender analysis reveals that girls have higher median DMTP levels at 5.48 ng/mL while boys show elevated median DETP levels at 6.99 ng/mL. This finding aligns with studies suggesting gender-specific differences in exposure or metabolism of OPs. Research indicates that such disparities may influence reproductive health and hormone levels in a gender-dependent manner[234,659,660].

An average of 43% of the metabolites were non-detects and had values below the LOD, while an average of 13 % of the metabolites were below LOQ, as shown in **Table 3.3**. The LOD and not detects were assigned a value obtained by dividing the LOD for each metabolite by the square root of 2[634]. This correction addresses the potential underestimations of exposure levels caused by the detection and quantification of low-level metabolites. It facilitated a nuanced analysis and ensured the health implications of minor OP and PYR exposures were accurately represented. The study aimed to provide a more accurate depiction of exposure distribution across the population by incorporating these corrected values.

3.3.1.1 Comparison of metabolite concentrations between studies done in children of Grabouw and Hex River

Concurrent studies in the Western Cape's Grabouw and Hex River regions by Fiserova *et al*. 2021 and Molomo *et al*. 2021 documented children's insecticide exposure. Fiserova *et al* .2021 gathered data in the spraying season (October–November in 2018) revealing particular insecticide concentrations while Molomo et al. 2021 collected pre-spraying season data between April 2007 and March 2008[133,634], and this study collected pre-spraying season data between May and August 2017 (cycle1) and in spraying season between October - November 2018 (cycle 2).The timelines are essential to grasp the fluctuating insecticide exposure levels among children in these regions. The disparity in sampling periods, as shown in **Table 3.4** which outlines DAPs and 3-PBA detection via LC-MS/MS, offers a detailed perspective on exposure which that exposure to OPs and PYRs is continuous through the year. Moreover, this persistent presence suggests a need to reconsider exposure risk assessments and monitoring strategies[133,634].

Table 3. 4: Table shows the median metabolites concentrations (ng/mL) in urine samples from previous studies done in the Western Cape.

Study	Area	S.P. and AM used	DMP ng/mL	DEP ng/mL	DMTP ng/mL	DETP ng/mL	3-PBA ng/mL
Current study	Grabouw and Hex River	61 Children 2-D GCxGC ToF/MS	7.83	9.67	4.86	6.37	2.86
Fiserova *et al* 2021[133]	Hex River	20 Children LC-MS/MS	10.94	< LOQ	1.68	0.63	0.53
Molomo et al 2021[634]	Grabouw, Hex River and Piketburg	Boys LC-MS/MS	32.60	5.50	16.70	X	X

Associations between continuous data were tested using the Mann-Whitney test
S.P.- study population
AM- an analytical method used
<LOQ- concentrations below the limit of quantification
X- not analysed

In comparing metabolite concentrations across three studies, Molomo *et al*. 2021 recorded the highest median values for DMP and DMTP at 32.60 ng/mL and 16.70 ng/mL, respectively, as shown in Table 3.3. In contrast, the Fiserova *et al*. 2021 study reported the lowest median values for other metabolites, averaging at 0.95 ng/mL. The current study, however, noted higher median values for 3-PBA (2.86 ng/mL), DETP (6.37 ng/mL), and DEP (9.67 ng/mL), showcasing the 2D GCxGC ToF/MS method's effectiveness in identifying OP and PYR metabolites. The observed variations in metabolite concentrations during both spraying and non-spraying seasons suggest that children's exposure to OP and PYR is influenced by factors like area, location, and gender, not solely by OP and PYR application schedules. These findings highlight the importance of incorporating such variables into risk assessments to fully comprehend the changes of OP and PYR exposure in children.

The study's analytical approach was instrumental in detecting these fluctuations, which offered insights into the effects of insecticide use and underscored the need to consider seasonal variations when assessing children and farm communities' exposure to OPs and PYRs. Moreover, adjusting urine sample concentrations for creatinine levels post-quantification was essential for consistent metabolite concentration expression (e.g., µg/g of creatinine). This standardization allows more precise exposure level comparisons and interpretations across individuals, times, and populations. Compensating for urinary flow rate differences through this correction technique ensures that observed concentration variations accurately represent changes in exposure levels to OPs and PYRs rather than mere differences in fluid intake. This approach guarantees a dependable measure of exposure. The following discussion on creatinine and creatinine-corrected data delves deeper into the methodology employed for assessing exposure to environmental contaminants, highlighting the precision and reliability of the study's analytical processes.

3.3.2 Creatinine quantification

Creatinine is a critical biomarker in forensic and biomonitoring studies to monitor and quantify compounds such as xenobiotics and insecticide metabolites in urine samples because it is a byproduct from creatine breakdown in muscles and excreted through urine[661,662]. Creatinine's constant excretion rate underscores its utility in evaluating sample completeness, serving as an integrity marker for samples, [663]. Additionally, creatinine measurement is essential for kidney function assessment and compound quantification in urine and serves as a dependable urine volume indicator influenced by

muscle mass, age, gender, and hydration status[664]. Integrating creatinine quantification into insecticide metabolite monitoring proved crucial because it enabled dilution correction by normalizing drug or metabolite concentrations to creatinine levels, thus ensuring accurate and comparable outcomes. It also played a key role in compliance assessment by identifying adulterated samples, which suggested tampering and helped monitor hydration status as abnormal creatinine levels could affect interpretations of drug concentrations or metabolite excretion rates[665-670].

In epidemiological studies, normalizing chemicals in human urine samples to creatinine or specific gravity measurements improves comparability. Although specific gravity may provide reliable corrections, it is best measured in fresh samples due to its volume requirements and analysis time considerations [671]. According to WHO guidelines, the acceptable range for urine creatinine is 30 – 300 mg/dL, with deviations indicating possible health concerns [672,673]. The descriptive analysis of urine samples before creatinine correction, to be discussed, will offer detailed insights into exposure assessments.

3.3.2.1 Measurement of creatinine in study samples

In our current study, quantification of creatinine was outsourced to the esteemed Bio Analytical Research Corporation South Africa (BARC-SA) BARC global central laboratory. BARC SA employed a Kinetic colorimetric assay based on the Jaffé method, specifically the Creatinine Jaffé Gen.2 – Urine, with an analyser measuring range spanning from 0.027 to 32.5 mmol/L (equivalent to 0.31 to 367 mg/dL). This approach used a conversion factor of mmol/L × 11.3 = mg/dL, ensuring urine data standardization and enhancing the accuracy and reliability of our OP and PYR metabolite biomonitoring results.

Creatinine calculation analysis of the study samples was done in Microsoft Excel using the data analysis tool (descriptive analysis) and the pivot table. Creatinine-corrected urine samples were obtained using the calculation below (**Equation 3.1**) [671,674,675]. The quantified metabolite urine concentrations (ng/mL) by the 2-D GCxGC ToF/MS were subsequently corrected by creatinine concentrations (mg/dL) to give a metabolite concentration of ug/g.

Equation 3.1

$$\left(\frac{metabolites\ concentration\ in\ urine\ \left(\frac{ng}{mL}\right)}{creatinine\ concentration\ \left(\frac{mg}{dL}\right)}\right) * 100$$

A total of 24 urine samples had creatinine concentrations outside the WHO recommended range (30 – 300 mg/dL) and were not included in the urine-corrected data, see Appendix **Table A 3.1**. The 24 urine samples excluded belong to participants who could have hydration issues affected by diet, medication use, or underlying health conditions. Hydration significantly affects urine concentration. Dehydration leads to concentrated urine, raising compound concentrations, while increased fluid intake dilutes urine, lowering compound concentrations[676,677]. Circadian rhythms influence urine compound concentrations, with renal functions displaying ultradian patterns[678]. Variations in the daily metabolism of elements like sodium, potassium, and creatinine lead to fluctuating urine compound levels throughout the day[679,680].

Circadian rhythms impact urine compound concentrations, as renal functions may follow an ultradian pattern. Different circadian patterns exist for sodium, potassium, and creatinine metabolism, implying varying daily influences on urine compound levels. These conditions can be checked and verified by a medical practitioner.

3.3.2.2 Comparison of absolute concentrations and creatinine corrected concentrations.

The absolute (ng/mL) and creatinine corrected (ug/g) concentrations' DFs mean, and median values shown in **Table 3.5** show that the DF of each metabolite in the different concentrations were in the same magnitude in percentages (about 60%). The table also shows that DMP and 3-PBA had the highest and lowest mean concentrations in both ng/mL and µg/g, showing that the participants are more exposed to OPs than PYRs. Also, the median values for both ng/mL and µg/g showed that DEP and 3-PBA had the highest and lowest concentrations, still supporting that the children are exposed to OPs more than the PYRs.

Table 3. 5: Summary of absolute creatinine corrected metabolite concentrations in urine samples.

Metabolites	Detection frequency		Mean		Median (Minimum – Maximum)	
	ng/mL (%)	µg/g (%)	ng/mL	µg/g	ng/mL	µg/g
DMP	76/122 (62)	65/98 (66)	12.53	19.82	7.83(1.37 - 135.87)	10.61 (1.59 - 141.29)
DEP	80/122 (66)	67/98 (68)	15.61	30.50	9.67(0.52 - 83.06)	16.31 (0.52 - 281.33)
DMTP	82/122 (67)	65/98 (66)	8.58	18.31	4.86(1.00 - 46.34)	8.35 (1.64 - 173.20)
DETP	81/122 (66)	64/98 (65)	9.73	19.04	6.37(2.4 - 66.20)	10.90 (2.65 - 147.84)
3-PBA	74/122 (61)	60/98 (61)	5.21	14.16	2.86(0.27 - 52.70)	4.02 (0.30 - 298.64)

Table 3.6 summarized creatinine-corrected concentrations of metabolites in children's urine samples, highlighting patterns across cycle locations and between farm and nonfarm environments. The first cycle exhibited higher median DMP and DEP concentrations, hinting at seasonal exposure impacts. Yet, p-values like 0.50 for DMP and 0.08 for DEP showed no significant differences across cycles, suggesting steady exposure. Farm children had elevated median metabolite levels compared to nonfarm children, with DEP at 11.43 µg/g for farm versus 18.11 µg/g for nonfarm children. However, these differences lacked statistical significance (p-value for DEP = 0.40), suggesting agricultural activities might influence exposure without a clear distinction based on residence.

Comparing results from Grabouw and Hex River revealed regional differences in OP and PYR use or exposure risks, with Hex River showing a significantly higher median value for DEP at 22.66 µg/g (p-value = 0.05) than Grabouw. The gender-based analysis found no significant differences in metabolite concentrations between females and males, with p-values of 0.81 for DMP and 0.60 for DEP, indicating similar exposure levels across genders. Although many comparisons lack statistically significant differences, the data establishes a basis for grasping exposure changes and highlights the need for more research to clarify OP and PYR exposure complexities among children in agricultural environments.

Table 3. 6: Summary of creatinine corrected concentrations of OP and PYR metabolites detected in urine samples collected from children, expressed as median and interquartile range (IQR) values in µg/g.

Variable (total)	DF Total (%)	DMP µg/g Median (IQR)	DF Total (%)	DEP µg/g Median (IQR)	DF Total (%)	DMTP µg/g Median (IQR)	DF Total (%)	DETP µg/g Median (IQR)	DF Total (%)	3-PBA µg/g (IQR %)
Total (98)	65 (66)	10.61 (3.86 - 26.94)	67 (68)	16.31 (6.07 - 34.19)	65 (66)	8.35 (5.25 - 21.25)	64 (65)	10.90 (6.21 - 19.44)	60 (61)	4.02 (1.95 - 7.66)
Cycle 1 (50)	30 (60)	12.91 (3.62 - 31.04)	32 (64)	23.03 (6.17 - 38.62)	36 (72)	9.44 (5.38 - 31.72)	34 (68)	9.30 (4.35 - 23.44)	34 (68)	4.18 (2.01 - 6.93)
Cycle 2 (48)	35 (73)	9.06 (3.86 - 24.54)	35 (73)	9.46 (5.96 - 22.83)	29 (60)	7.62 (4.62 - 18.08)	30 (63)	11.29 (7.68 - 17.70)	26 (54)	3.49 (1.48 - 14.61)
p-value		0.50		0.08		0.08		0.69		0.76
Farm (66)	48 (73)	13.14 (3.76 - 31.14)	41 (62)	11.43 (3.53 - 36.38)	50 (76)	8.65 (5.31 - 25.15)	49 (74)	12.63 (6.21 - 22.97)	40 (61)	4.78 (1.95 - 10.80)
Non-farm (32)	17 (53)	10.53 (4.16 - 18.18)	26 (81)	18.11 (8.06 - 32.69)	15 (47)	7.37 (4.62 - 15.20)	15 (47)	9.02 (5.77 - 13.50)	20 (63)	3.04 (1.97 - 6.78)
p-value		0.64		0.40		0.23		0.19		0.44
Grabouw (65)	41 (63)	10.72 (3.86 - 30.49)	58 (89)	9.46 (3.26 - 24.17)	44 (68)	8.93 (5.29 - 24.76)	51 (79)	13.32 (6.21 - 22.97)	47 (75)	4.87 (1.74 - 10.66)
Hex river (33)	20 (61)	10.62 (3.46 - 22.47)	25 (76)	22.66 (10.02 - 34.28)	18 (55)	7.95 (4.64 - 18.58)	23 (70)	9.02 (4.72 - 12.60)	19 (58)	3.01 (2.24 - 6.68)
p-value		0.88		***0.05***		0.33		0.15		0.76
Female (64)	(70)	10.62 (3.62 - 31.04)	(72)	16.40 (5.76 - 35.45)	(61)	9.06 (5.70 - 20.83)	(84)	11.76 (5.65 - 19.91)	(89)	3.86 (1.73 - 10.66)
Male (34)	(59)	11.72 (5.72 - 21.06)	(62)	15.70 (7.51 - 23.29)	(77)	6.66 (4.59 - 25.22)	(68)	10.84 (6.39 - 18.19)	(79)	4.18 (1.97 - 6.78)
p-value		0.81		0.60		0.28		0.78		0.81

Data reported as median (IQR that is 25 % and 75 % of the data) for continuous data.
Associations between continuous data were tested using the Mann-Whitney test.
P-value ≤0.05 considered as significant.
% RSD= % relative standard deviation; DF= Detection Frequency.

3.3.2.3 Comparison of Cycles for farm and nonfarm residents

The two cycles' urinary metabolite levels of OP and PYR were compared by farm/nonfarm, gender, and study area. Detailed statistical analysis of the three factors according to cycles is shown in **Tables A 3.3 to 3.5**.

Figure 3.6 shows no significant difference in farm and nonfarm metabolite concentrations, as the *p*-values for each metabolite in the two cycles were greater than 0.05. This observation shows that the participants residing in the farm and nonfarm areas of both Grabouw and Hex River farming areas are similarly exposed to OPS and PYRs at the same magnitude.

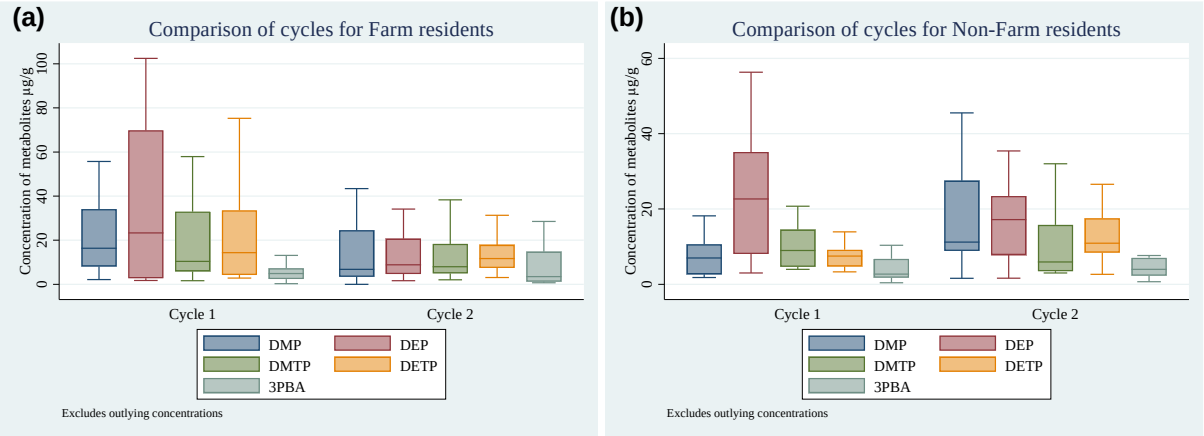


Figure 3. 5: Distribution boxplots outlining the metabolite concentrations from urine samples of farm and non-farm residents obtained during cycle 1 and 2.

3.3.2.4 Comparison of metabolite levels across cycles for gender

Figure 3.7 shows no significant gender differences in exposure to OPs and PYRs before or during spraying seasons across both cycles. Hence, female, and male participants face similar exposure levels to OP and PYR, irrespective of spraying times.

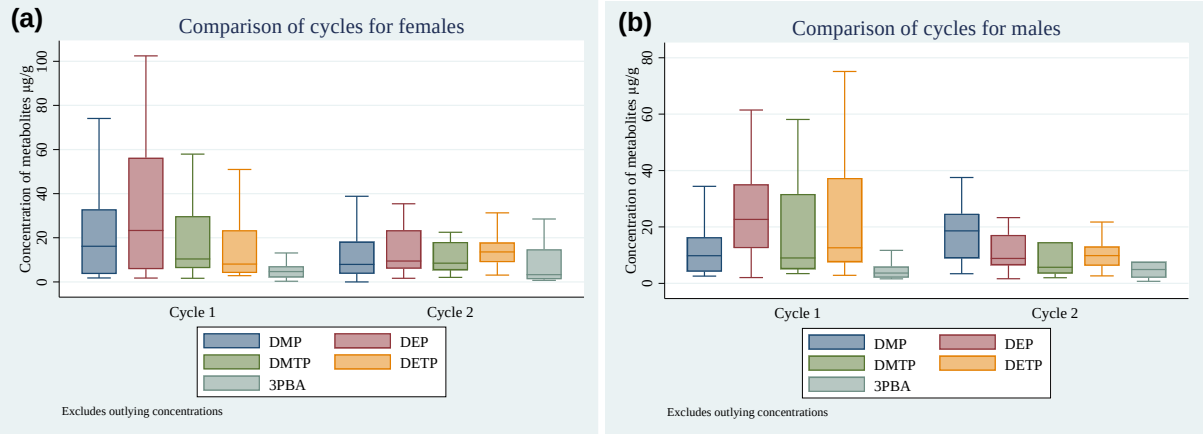


Figure 3. 6: Distribution boxplots outlining the metabolite concentrations from urine samples of females and males' residents obtained during cycle 1 and 2.

3.3.2.5 Comparison of metabolite levels for cycles between Grabouw and Hex River

Figure 3.8 shows that there is a significant difference (p-value of 0.04) in DMP concentrations only (an OP metabolite) in the Grabouw area between cycle 1 and cycle 2, where it is observed that cycle 1 has the highest median value than cycle 2 for DMP, see **Table A 3.5** in the supplementary section. In contrast, the Hex River area showed no significant differences in DMP concentrations across both cycles.

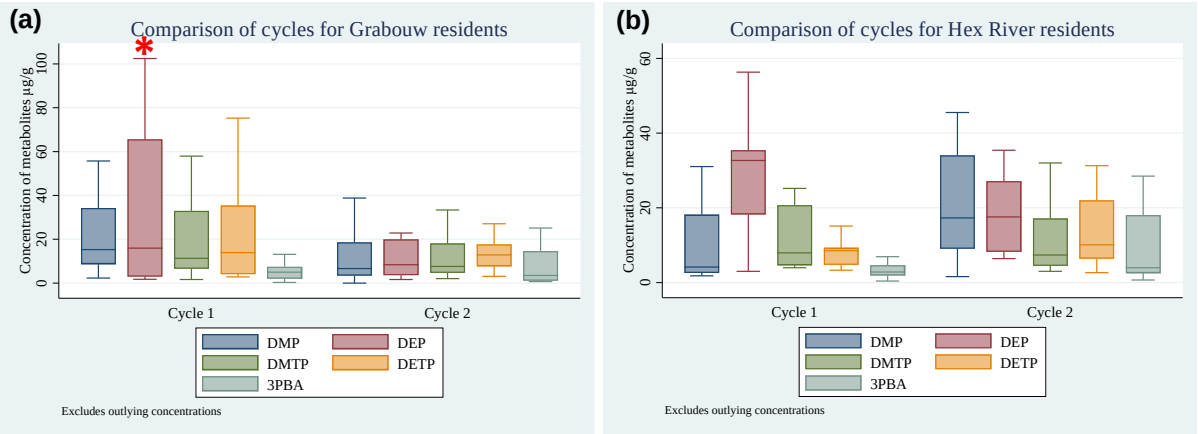


Figure 3. 7: Distribution boxplots outlining the metabolite concentrations from samples of and Hex River and Grabouw residents obtained during cycle 1 and 2.

The variation observed in the concentrations of DMP, an OP metabolite, across different cycles in Grabouw and between the geographical areas can be directly linked to the study's findings by Curchod *et al*. 2020.

During the drought period from 2017 to 2018, the study identified a notable increase in chlorpyrifos and other insecticides within the river waters of Western Cape. Grabouw had more diverse and concentrated insecticides in its surface water than the Hex River Valley because it focuses on apple and pear farming, while the Hex River Valley, known for table grape production, detected only two insecticide types. The study also linked Grabouw's variable chlorpyrifos application and consequent DMP level fluctuations to seasonal agricultural demands and drought conditions, which likely reduced water's dilution capacity, raising insecticide concentrations and affecting DMP measurements outcome[125].

The contrast in insecticide application between Grabouw and the Hex River valley is dictated by the primary crops grown and explains the geographical variation in DMP levels. Grabouw's extensive use of a wide range of insecticides, chlorpyrifos included, led to greater and more diverse exposure to OP metabolites such as DMP. Curchod *et al*. 2020 noted a mismatch in insecticide usage reporting, with the discrepancy between environmental insecticide detection and farmers' records indicating incomplete use documentation. This gap challenges accurate exposure risk assessment and complicates correlating reported insecticide use with environmental and metabolite detections, thus hindering understanding of environmental exposure[125]. Moreover, Fuhrimann *et al*. 2020 showed ongoing exposure to CUPs like OPs in Grabouw and Hex River residents through air samples from 2010 to 2018, implying farm spraying air pollution leads to metabolite presence across two cycles. Although exposed, the current study found no significant gender differences in metabolite concentrations, showing OP and PYR exposure was uniform across genders[130].

However, uniform exposure levels do not rule out other influences on individual metabolite concentrations like behaviours, lifestyles, and environmental factors affecting hydration and urine concentration. These elements can alter how the body absorbs and processes insecticides, causing metabolite concentration variations not evident in simple gender comparisons. The lack of statistical gender differences in metabolite concentrations underlines the complexity of exposure risk assessment and stresses considering a wider array of factors beyond gender[634].

3.3.3 Comparison of metabolite concentration in similar studies in the Western Cape

In comparison to similar studies done in the farming areas of the Western Cape, particularly Grabouw and Hex River, **Table 3.7** outlines the use of different analytical methods on different populations (SP and AM) and their findings across various studies focusing on the presence of specific chemicals (DMP, DEP, DMTP, DETP, 3-PBA) in different populations.

Table 3. 7: Summary of creatinine corrected concentrations of OP and PYR metabolites detected in urine samples of the current study and previous studies done in the Western Cape, expressed as median (µg/g).

Study	S.P. and AM used	DMP µg/g	DEP µg/g	DMTP µg/g	DETP µg/g	3-PBA µg/g
Current study	Children 2-D GCxGC ToF/MS	10.61	16.31	8.35	10.90	4.02
Fiserova *et al.* 2021[133]	Children LC-MS/MS	22.36	< LOQ	1.96	1.10	1.16
Motsoeneng *et al* 2015[681]	Adult female workers And LC-MS/MS	29.63	4.27	21.87	3.87	3.40

SP- study population
AM- an analytical method used.
<LOQ- concentrations below the limit of quantification
Associations between continuous data were tested using Mann Whitney test.

Table 3.7 shows the variation in OP and PYRs exposure levels among different groups (children, adults, and adult female workers) and highlights how analytical methods like 2-D GCxGC ToF/M.S. and LC-MS/MS, along with factors like occupation and daily activities, affect exposure outcomes. Using 2-D GCxGC ToF/M.S., the current study detected higher DMP, DEP, and DETP levels in children than Fiserova *et al*. 2021 reported using LC-MS/MS. In contrast, Motsoeneng *et al*. 2015 found the highest DMP levels in adult female workers, hinting at occupational or environmental exposure differences. Adults had higher median DMP and DMTP values than children, likely because adults spend more time working in fields, whereas children are usually at school or home[133,681].

3.4 Conclusion

This study successfully utilized non-invasive spot urine samples to evaluate insecticide exposure among children in farming communities, demonstrating the method's efficiency and accuracy. Immediate metabolite snapshots proved highly suitable for children's biomonitoring research, with the study exceeding its sample power calculations. The research exceeded the recommended sample sizes and included a diverse pool of participants from various demographics, thereby significantly enhancing its findings' statistical power and reliability. By employing advanced 2-D GCxGC ToF/MS techniques, the study achieved detailed profiling of OP and PYR metabolites, enabling a more precise assessment of exposure levels. Consequently, the study uncovered widespread OP and PYR exposure among participants, attributing the exposure patterns to seasonal changes in insecticide application. This finding provides a detailed view of how agricultural practices affect health and the environment.

The study's robustness was further supported by employing creatinine correction, allowing for more accurate comparisons of metabolite concentrations. This comprehensive analysis considered factors like hydration levels and circadian rhythms, thus offering a nuanced understanding of OPs and PYRs exposure risks in agricultural settings. Consequently, the findings revealed universal exposure to OPs and PYRs among children in both farm and nonfarm environments and significant differences in exposure levels influenced by geographical location and farming practices. This research contributes valuable insights into biomonitoring, emphasizing the need for continued vigilance in protecting vulnerable populations, especially children, from insecticide exposure.

By advancing our knowledge of biomonitoring in agricultural communities and underscoring the importance of methodological precision and comprehensive analysis, this study makes a significant contribution to the domain. It informs future research directions and supports the development of more effective strategies for minimizing insecticide exposure among children in farming regions. Future research studies can explore alternative exposure assessment mediums and further examine the long-term health implications of these exposures. The forthcoming chapter will delve into the methodology's application to hair samples to expand the understanding of the various mediums through which insecticide exposure can be assessed.

3.4.1 Limitations

The study quantified OP and PYR metabolites in children from agricultural areas using spot urine samples which is practical for large-scale studies. However, spot urine samples may have impacted metabolite detection accuracy due to variable urine concentrations and volumes per individual. Additionally, handling logistics for urine collection and storage needs expensive professional management to prevent contamination and degradation.

The study collected more samples from farm areas than non-farm areas, which could bias the results. This imbalance might not accurately reflect the range of OP and PYR exposure experienced across various agricultural settings. Although the study had enough participants to ensure the statistical reliability of its findings (statistical power), it may not accurately represent the diverse and complex exposure to organophosphates and pyrethroids in broader rural agricultural communities. These areas have varied insecticide use and exposure scenarios that the study's sample might not fully capture. The study design's focus on specific intervals and locations might not capture the full range of exposure scenarios across various agricultural practices and seasons affecting the findings' applicability. Therefore, despite providing valuable data on children's exposure to OP and PYR in selected areas, the study underscores the need for a comprehensive approach encompassing year-round and multi-regional research to fully understand the extent and impact of OP and PYR exposure.

Chapter 4

Analysis of Diakylphosphates and 3-phenoxybenzoic acid in Hair Using 2-D GCXGC TOF/MS

4.1 Introduction

In biomonitoring studies for insecticide exposure body fluids such as blood, urine, or saliva have traditionally been utilized to assess short-term exposure [682,683]. However, hair samples have become a superior matrix for long-term exposure assessment due to their ability to accumulate exposure data over months [316,317,606,683]. Hair's average growth rate of 1 cm per month allows for a more comprehensive analysis of chronic exposure patterns, rather than transient spikes due to recent diet or behaviour [684,685]. Hair analysis offers a unique capability for long-term insecticide exposure assessment, providing a stable, long-term record beyond the initial exposure period, complementing insights from other matrices, and making hair samples a practical choice for monitoring insecticide variations [310,320,684,686].

Hair analysis is increasingly used in forensic and clinical toxicology for drug detection and diagnostic applications. However, the field lacks standardized methodologies for extraction and analysis. The Society of Hair Testing (SoHT) has introduced guidelines (that are discussed in the following sections) to streamline hair analyses, but to accurately interpret the results a deep understanding of the hair anatomy and physiology is required [687,688]. This knowledge is crucial for accounting for variables affecting compound absorption, retention, and degradation in hair samples [687].

4.1.1 Hair anatomy

Hair anatomy involves the structure and composition of hair and incorporates key components with distinct functions. Human hair consists of lipids and proteins and fulfils various roles such as skin protection along with sexual and social communication [689]. The anatomy splits into two primary segments: the hair shaft and the root.

The hair shaft is structured in three layers: the core medulla, the melanin-enriched cortex in the middle, and the protective cuticle on the outside [690]. Meanwhile, the hair root is embedded in the follicle beneath the skin and includes the hair bulb at the base, where cellular activity promotes the formation of the hair shaft [691,692] as depicted in **Figure 4.1**.

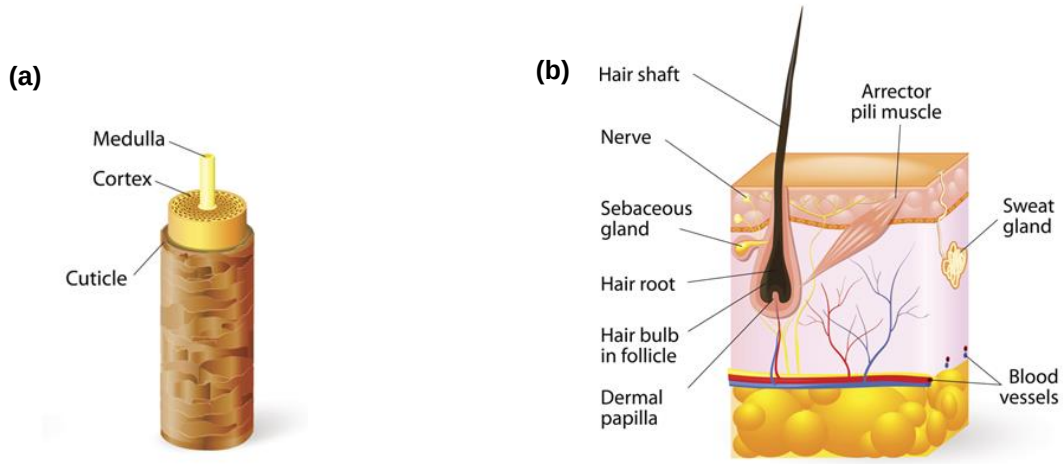


Figure 4. 1: illustrates a) a hair shaft and b) a hair root along with the associated glands and the dense capillary networks in the surrounding tissue[693].

The hair shaft's structure is important for the hair's physiology and integrity as depicted in **Figure 4.1 a)** and it includes the medulla, cortex and cuticle which play crucial roles. The medulla enhances thermal insulation and hair strength through its compound-retaining loosely arranged cells and air spaces. Additionally, the medulla is found in thicker, terminal hairs on the scalp and beard [694]. The cortex comprises of keratin filaments, matrix proteins, and melanin pigments and is vital for the hair's structural diversity and strength. Consequently, the cortex provides elasticity, texture, and enhances compound retention [695-699].

The cuticle envelopes the cortex to serve as the hair shaft's protective layer. Cuticle scales protect against chemical, thermal, and light damage and enable substance interaction. Therefore the cuticle is essential for retaining external elements like insecticides or drugs, thereby maintaining the hair shaft's integrity and improving hair health and appearance [700].

As depicted in **Figure 4.2 b)**, the hair root's anatomy underpins growth and nourishment, with the root anchoring the hair and the bulb at its base facilitating elongation. Moreover, the follicle encapsulating the root and bulb, undergoes cycles of growth, regression, and rest, essential for hair development.

Additionally, the dermal papilla located at the bulb's base supplies nutrients and growth signals while the isthmus and infundibulum near the follicle strengthen its connection to the skin surface [701-703]. Furthermore, sebaceous glands adjacent to the follicle secrete sebum, moisturizing and safeguarding hair and skin, while sweat glands play a pivotal role in hair growth [704,705]. Concurrently, the contraction of the arrector pili muscle elevates the hair, aiding in the distribution of sebum along the shaft and enhancing the health of both hair and skin. Substances entering the hair through the blood vessels in the dermal papilla, including nutrients, drugs, and toxins, in the hair root's intricate anatomy and its significance in assessing health and environmental exposures[700,706,707].

4.1.2 Hair physiology

Hair physiology involves various components and structures that contribute to its health and biological functions. Hair acts as a protective barrier, regulates temperature, senses environmental changes, and conditions the skin through sebum production thus, playing a crucial role in biomarker discovery and environmental monitoring due to its growth cycle and structure [708-712]. Melanin, produced by follicular melanocytes, determines hair colour, providing a range from black and brown (eumelanin) to red, yellow, and blonde (pheomelanin), and protects against UV damage[713].

Hair's capacity to retain substances during its growth phases makes it an effective indicator of chronic exposure and long-term substance use, essential for understanding substance distribution over time and for biomarker and environmental analysis [320,714-717]. The hair growth cycle, consisting of the anagen, catagen, and telogen phases, is a dynamic process essential for interpreting these substance retention patterns, as depicted in **Figure 4.2**.

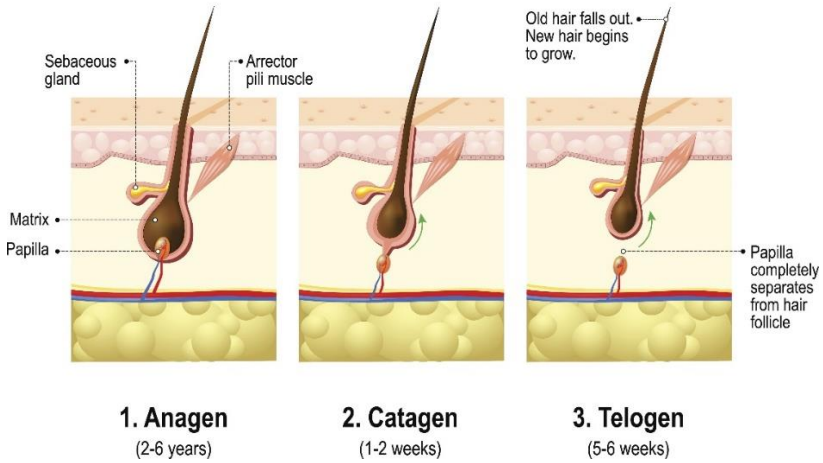


Figure 4. 2: Phases of hair growth[693].

Active hair growth characterizes the anagen phase, lasting 2-6 years and setting the hair shaft's length [706]. The subsequent catagen phase marks a transitional period of follicle regression and shrinkage, leading to the telogen phase where follicles enter a dormant state. Hair shedding signals the cycle's completion, transitioning back into the anagen phase. The catagen and telogen phases last for 1-2 and 5-6 weeks, respectively, with human head hair typically growing about one centimetre monthly [706,715-718]. Determining precise hair growth rates within each phase is complex, as individual differences in growth rates are influenced by genetics and health conditions [719-722].

Moreover, the anagen phase facilitates the incorporation and deposition of compounds along the shaft as hair grows. Therefore, the anagen phase allows for the accumulation of trace elements and substances within the hair, creating a long-term record of exposure[723].

4.1.2.1 Mechanism of compound incorporation in hair

The process through which compounds and chemicals are incorporated into hair is multifaceted and not entirely understood yet. Nevertheless, it is widely acknowledged that this process is affected by several factors, including the structure of human hair fibres, properties of the drugs or compounds, and the physiological characteristics of individuals[704]. The incorporation of compounds into the hair can occur via sweat or sebum and external contamination[705,724], as illustrated in **Figure 4.3**.

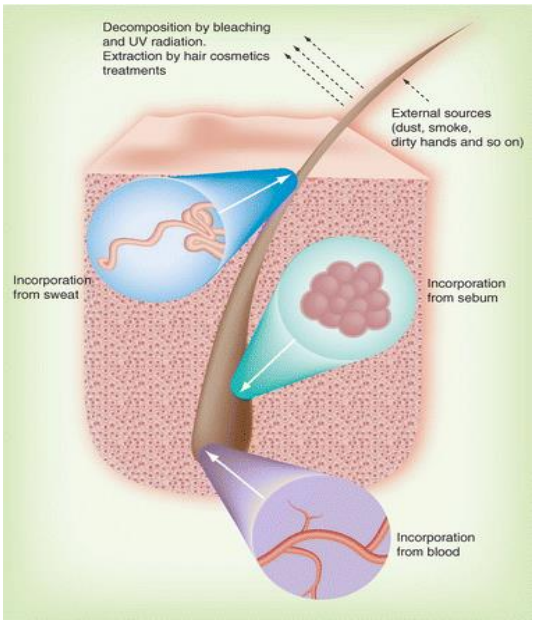


Figure 4. 3: Mechanisms of compounds incorporation into hair[704].

Melanin concentration in hair affects the absorption and detection of various compounds; higher melanin levels result in increased substance absorption. Furthermore, the pigmentation which is determined by melanin content results in varied concentration profiles within the hair[725,726]

Concurrently, Oberg *et al*. 2007 highlighted the dual functionality of hair follicles in compound absorption, acting as both a reservoir and a portal for topically applied substances [727]. Moreover, the hair's structure, enhanced by sebum from sebaceous glands which facilitates this absorption process, particularly aiding in the integration of lipophilic substances such as insecticides. These substances dissolve in the sebum that coats the hair shaft, thereby optimizing the absorption process [700,725,728].

Over time, various research groups have developed diverse methods for sampling, extraction, and analysis in hair research. Yet, comparing results across these differing methodologies has been challenging, primarily due to inconsistencies in interpreting and comparing findings. To tackle these issues, the Society of Hair Testing (SoHT) has established guidelines to standardize the protocols for sampling, extraction, and analysis in hair research [688].

The hair analysis process is multi-step and is discussed in more detail in the subsequent sections. These steps are crucial for understanding the complexities and nuances involved in preparing, analysing, and interpreting hair analysis results, especially within the context of target compound identification and biomonitoring.

4.2 Collection and storage of hair samples

The integrity and reliability of hair analyses in forensic, medical, and environmental research hinge on meticulous sample collection and storage [687,729,730]. Initially, proper labelling and collection are crucial, followed by storage under optimal conditions to preserve sample stability and integrity. Specifically, collecting head hair from the posterior vertex is recommended due to its consistent growth rate [706]. Moreover, recording details like the collection site, length, colour, and treatments enhances the interpretability of the results.

Additionally, storing a pencil-thick strand of hair wrapped in aluminium foil and placed in Ziplock bags is advised for maintaining sample condition[688,730]. Furthermore, in the absence of head hair, alternative sources like pubic, axillary, or beard hair are considered viable [687]. Therefore, ensuring hair samples are stored in a dry, dark environment at room temperature is paramount for preserving their integrity, underscoring the critical role of careful storage practices in achieving accurate analytical outcomes. In addition to the technical considerations for sample integrity, socio-demographic factors also play a crucial role in understanding the exposure risks associated with different populations.

4.2.1 Segmentation

In hair sample analysis and segmentation, the process of cutting a lock or multiple strands at consistent intervals, develops a detailed compound exposure profile [731]. Typically, segments, aligned by their proximal ends, are cut into regular lengths, often around 1 cm or more, based on the study's required time resolution. This method relies on the standard hair growth rate of approximately 1 cm per month, thus allowing estimations of substance intake over specified periods. Segmental analysis mainly uses head hair, where accurately identifying the hair's root end is crucial for reliable results [688].

4.2.2 Decontamination of hair by washing.

Decontamination through washing hair samples is important for ensuring the accuracy and reliability of analyses in forensic toxicology, drug testing, and environmental monitoring [732]. Washing removes surface materials like hair products, sweat, sebum, and environmental contaminants, which can interfere with the analysis or affect the extraction recovery [732]. This step is especially important in drug testing, where distinguishing between internal and external contamination is fundamental [733,734].

Decontamination is crucial for analysing environmental pollutants in hair samples, as it selectively removes external contaminants to differentiate between internal and external exposures [564]. However, there is no universally accepted standard for wash procedures, but SoHT recommends using a combination of organic solvents and aqueous solutions for an effective wash process that effectively removes surface contaminants while preserving hair compounds[322,688].

4.2.3 Cutting and pulverising hair

Hair analysis relies on the important steps of cutting and pulverizing hair samples to extract and analyse compounds present. Pulverizing has proven to speed up the extraction process, showcasing improved efficiency and faster analysis rates[735]. Meier *et al*.'s 2017 study has demonstrated that pulverization of hair leads to higher extraction efficiencies of compounds than only cutting the hair [736,737]. However, the choice between cutting and pulverizing depends on the studied substances, as various analyses demand specific approaches[738-740]. This highlights the need for tailored sample preparation methods based on the compounds under investigation.

4.2.4 Extraction of compounds from hair matrices

After decontamination and pulverisation, compound extraction from hair samples is critical in various analytical procedures. The extraction of compounds from hair samples employs diverse methods tailored for efficient isolation for analysis[741]. Releasing drugs from within the hair matrix is crucial for detection and quantification.

Various extraction methods, including incubation in non-damaging solvents like methanol or buffered solutions with ultrasonication or pulverization assistance[742], are employed to isolate analytes from hair, as discussed in Chapter 2 under hair extraction method development. Notably, SLE is a prevalent choice for extracting insecticides and their metabolites from hair samples[315,319,684,743-745], highlighting its significance in the current study.

Adhering to the Society of Toxicological Hair (SoTH) guidelines has significantly enhanced the standardization of extracting compounds from hair, leading to the development of reliable metabolite extraction methods[688]. Techniques such as chromatographic separation and mass spectrometry, particularly LC-MS and GC-MS, are essential for efficiently extracting and identifying insecticide

metabolites. These methods are chosen for their sensitivity and selectivity and are crucial for analyzing a wide range of compounds in biological specimens[746].

LC-MS and GC-MS have broadened their application in hair analysis, identifying substances ranging from drugs to environmental pollutants. LC-MS/MS excels in analysing polar and non-volatile compounds, making it ideal for insecticide metabolite analysis. On the other hand, GC-MS complements LC-MS by analysing volatile and semi-volatile compounds, proving critical for assessing human exposure to insecticides[743,747]. Researchers such as Margariti & Tsatsakis *et al*. 2009 and others have demonstrated the effectiveness of GC-MS in measuring insecticide metabolites in hair, employing methods such as decontamination and derivatization[315,580,743]. Chapter 4 explores the application of a novel in-house developed and validated 2-D GCxGC ToF/MS for identifying and quantifying insecticide metabolites in hair samples from children in the agricultural communities of Grabouw and Hex River in the Western Cape, South Africa.

The introduction of sensitive analytical technologies, like chromatography, has enhanced the detection of low concentrations of insecticide metabolites in hair. Building on this foundation, a 2-D GCxGC ToF/MS method was developed and validated to quantify OP and PYR non-specific metabolites in hair samples.

4.3 Experimental method

As a starting point, the hair sample preparation phase involved pre-washing followed by hair pulverization then the hair was extracted using the developed SLE method, as discussed in subsequent sections.

As outlined in the study design section of **Chapter 3**, this investigation is part of the Child Health Agricultural Pesticide Study in South Africa, a longitudinal study conducted from 2017 to 2019 in the Western Cape's agricultural regions of Grabouw and Hex River. Children who consented to the study provided hair samples, with only 184 participants providing hair during two out of five cycles. These participants were distinct from the 61 in the urine study, highlighted in **Chapter 3**. Samples weighing less than 20 mg were deemed inadequate for reliable analysis and thus excluded, leading to the analysis of 176 hair samples. The sociodemographic information pf the 176 participants is outlined in the section below.

4.3.1.1 Sociodemographic characteristics of hair sample participants

Table 4.1 shows significant differences between farm and non-farm children in sex, household size, and income. These findings were compared to international studies which analysed insecticide metabolites in hair samples of mostly adults. Despite limited research on children, the data highlighted exposure patterns and socio-demographic disparities between farm and non-farm populations.

Table 4. 1: Hair sample participant and guardian sociodemographic characteristics by farm and non-farm residents.

	Total	Farm	Non-farm	
	N=176	N=132	N=44	*p*-value
Participant sociodemographic characteristics				
Age in years	11 (10, 12)	11 (10, 12)	11 (11, 13)	***0.04***
Age categories				
9-11 years	105 (60)	82 (62)	23 (52)	0.13
12-14 years	66 (37)	48 (36)	18 (41)	
15-16 years	5 (3)	2 (2)	3 (7)	
Sex				
Female	152 (86)	118 (89)	34 (77)	***0.04***
Male	24 (14)	14 (11)	10 (23)	
Grade categories				
2nd-3rd (foundation phase)	19 (11)	16 (16)	3 (17)	0.24
4th-6th (intermediate phase)	130 (74)	99 (78)	31 (74)	
7th-9th (senior phase)	27 (15)	17 (6)	10 (9)	
Guardian sociodemographic characteristics				
Mother education level attained				
n_1=139 n_2=102 n_3=37				
No schooling	8 (6)	7 (7)	1 (3)	0.38
Primary education	38 (27)	25 (25)	13 (35)	
Secondary education	93 (67)	70 (69)	23 (62)	
Marital status				
n_1=139 n_2=102 n_3=37				
Married/Cohabiting	63 (45)	48 (47)	15 (41)	0.69
Widowed	11 (8)	7 (7)	4 (11)	
Divorced/Separated	19 (14)	15 (15)	4 (11)	
Never married	46 (33)	32 (31)	14 (38)	
Mother employed in past year	88 (63)	67 (66)	21 (57)	0.33
n_1=139 n_2=102 n_3=37				
Size of Household				
n_1=139 n_2=102 n_3=37				
≤4 people	34 (25)	21 (21)	13 (35)	0.16
5-6 people	67 (48)	50 (49)	17 (46)	
>6 people	38 (27)	31 (30)	7 (19)	
Household income				
n_1=139 n_2=102 n_3=37				
R0-R3200	53 (38)	35 (34)	18 (49)	0.29
R3201or more	53 (38)	42 (41)	11 (30)	
Refused/Don't know	33 (24)	25 (25)	8 (22)	
Geographical location				
Grabouw	80 (45)	57 (43)	23 (52)	0.29
Hex river valley	96 (55)	75 (57)	21 (48)	

- p-value of ≤0.05 considered statistically significant.
- continuous data reported as median (Interquartile range - IQR) and p-values derived using Mann Whitney U-test.

The current study included 176 children with 132 from farms and 44 from non-farm areas. Farm children had a median age of 11 years (IQR 10-12), while non-farm children were slightly older at a median age of 11 years (IQR 11-13), a statistically significant difference (p = 0.04). Gender differences were notable, with 89% of farm children being female compared to 77% of non-farm children (p = 0.04). Larger households were more common among farm families (49% had 5-6 members), though this was not significantly different from non-farm households (p = 0.16). There were no significant differences in maternal education (most mothers completed secondary education), marital status, or maternal employment between the two groups. Income patterns showed 41% of farm households earned above R3201, while 49% of non-farm households earned R0-R3200, though these differences were also not statistically significant (p = 0.29). Geographically, 57% of farm children lived in the Hex River Valley, while 48% of non-farm children were from Grabouw, though this difference was not significant (p = 0.29).

When comparing these findings with international research, the results align with studies that have highlighted socio-economic disparities between rural and non-rural populations. For instance, Béranger *et al*. (2020) observed that pregnant women from rural agricultural regions in France, particularly those with lower socio-economic status faced higher levels of insecticide exposure due to their rural residence and limited education[748]. This aligns with the higher vulnerability observed in rural farm settings, such as the larger household sizes and lower income levels found in our study, although education levels did not show significant disparity. Similarly, Peng *et al.* 2020 emphasized the role of socio-demographic factors like income in determining exposure levels in urban Chinese populations with rural populations often experiencing greater risks, a trend mirrored in the income disparities seen in this study[578]. In addition, Hardy *et al*. 2021 and Iglesias-González *et al*. 2020 highlighted the increased environmental health risks faced by rural populations in proximity to agricultural activity[321,583]. This is consistent with the findings in the present study, where geographical and socio-economic factors in rural areas, such as larger household sizes and income variability, could amplify vulnerability to environmental hazards, including insecticide exposure.

The socio-demographic differences in age, gender, household size, and location observed in this study suggest increased environmental risk for children in agricultural areas. While some differences, like household income and size, were not statistically significant, the overall trends align with international findings that rural, low-income, farm communities face higher environmental health risks. These results

highlight the need for targeted interventions in agricultural regions, especially for children and other vulnerable populations. Further research is needed to understand how socio-economic factors contribute to environmental exposures and health outcomes in rural communities.

4.3.2 Sample collection and storage.

The hair samples were collected from 176 participants (girls and boys in two time points (August 2017 and November 2018)) from Grabouw and Hex River. 124 participants submitted hair samples in cycle 1 (before the spraying season), 52 participants provided hair samples in cycle 2 (during the spraying season), while only 4 participants contributed hair samples in both cycles, as shown in **Figure 4.4**. Informed consent was obtained from all participants.

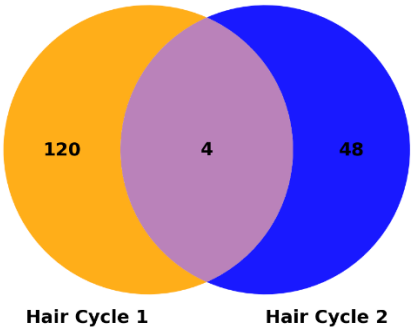


Figure 4. 4: The number of participants from whom hair samples were collected in cycle 1 (August 2017) and cycle 2 (November 2018).

While **Figure 4.4** shows the participation by cycle, **Table 4.3** displays the gender distribution of participants from farm and non-farm areas, highlighting fewer participants from non-farm areas compared to those from farm areas and a predominance of female over male participants.

Table 4. 2: Distribution of gender and farm residence among all child participants from whom hair samples were collected during both cycles in the study.

	Number of participants Study area				
	Hex river		Grabouw		
Gender	**Farm**	**Non-Farm**	**Farm**	**Non-Farm**	**Total**
Female	67	18	51	16	152
Male	8	3	6	7	24
Total	75	21	57	23	176

Hair samples were collected over two weeks based on participant availability and consent by a team trained in hair and skin sample collection. This team included students, a professional male barber for male participants, and a qualified female nurse for female participants, working during school hours (10 am to 1 pm) in school classrooms. To prevent cross-contamination, hair collection for each participant occurred in a sterile environment, which was cleaned and sterilized after each session. Upon collection, the hair was immediately labelled with details such as participant number, place of collection, date, and time, then wrapped in foil paper, placed in ziplock bags, and stored in a dark cupboard.

4.3.3 Materials and methods

4.3.3.1 Materials and chemicals

An incubator facilitated the extraction phase, while a centrifuge and 1.5 mL centrifuge tubes from Sigma International were employed for both hair collection and extraction activities during incubation. Additionally, omni microtubes and beads were used alongside the Omni Bead Ruptor for the pulverization of samples and were provided by Omni International, Georgia, USA.

Analytical reference standards: DMP, DEP, DMTP, DEP, and 3-PBA; internal standards: DMP-d6, DMTP-d6, and 3-PBA 12C6, with purities higher than 99% were obtained from Toronto Research Chemicals Inc (TRC) Canada. N-tert-butyldimethylsilyl- N-methyl trifluoroacetamide (MTBSTFA) (Sigma-Aldrich, South Africa) and ethyl acetate.

4.3.3.2 Preparation of standards

Internal Standards were prepared at a concentration of 1 mg/ml in ethyl acetate. Working solutions were prepared by diluting standards and internal standards of interest in ethyl acetate to achieve 5, 50, and 100 ng/ml concentrations. Meanwhile, 20 ng/ml internal standards solutions were used for QC purposes to monitor the calibration graph linearity. All reference and working standard solutions were stored below -20°C.

4.3.4 Hair sample preparation

4.3.4.1 Washing of hair samples

About 30 mg of hair from each participant was weighed into separate Petri dishes and washed using 300 µL of the 5 % sodium dodecyl sulfate (SDS) solution following the procedure developed by Duca *et al.* 2014 [564]. After that, the hair was agitated for 2 mins, followed by the addition of 400 µL of de-ionized water. The mixture was further agitated for 1 minute, after which 300 µL of 100% methanol was added and the agitation was continued for another 1 minute. The hair was then removed from the solution and air-dried on aluminium paper at room temperature for at least 4 hours.

4.3.4.2 Pulverising of hair samples

The air-dried hair was placed in 2 ml microtubes and 6 steel balls (2.4 mm) from Omni International, Georgia, USA, for pulverization into fine powder. Using the Omni Bead Ruptor 24 from Omni International, Georgia, USA, the hair was pulverized in 4 cycles of 30 seconds each at a speed of 10 m.s-1. After this, 20 mg of pulverized hair was weighed and transferred to 1.5 mL Eppendorf tubes for metabolite extraction.

4.3.4.3 Extraction

Solid-liquid extraction (SLE) was utilized to extract metabolites of interest from hair samples. The procedure involved the addition of 300 µL of ethyl acetate containing an internal standard (20 ng/ml) into a 1.5 centrifuge tube containing 20 mg of pulverised hair. The samples were then vortexed, mixed for 10 seconds, and incubated overnight (12 hrs) at 40 °C on a shaker set at 350 rpm. After overnight extraction, the samples were vortexed again and centrifuged at 13000 rpm for 10 minutes. The supernatant (~280 µL) was subsequently collected and transferred into glass vials for derivatization. Finally, 2 µL of the derivatized sample extract was injected into the 2-D GCxGC ToF/MS for metabolite separation and quantitative analysis.

4.4 Results and discussion

4.4.1 Hair sample analysis

In this study, a comprehensive analysis of 176 hair samples was conducted, and the concentrations (ng/mL) obtained from the 2-D GCxGC ToF/MS analysis were subsequently converted to pg/mg using **Equation 4.1**. The Equation essentially calculates the concentration of the compounds in picograms per milligram of hair (pg/mg), starting from its concentration in the solvent (ng/mL) and considering the specific volumes (0.3 mL) and masses (20 mg of hair) used in the extraction process, while the 1000 is a multiplication factor for converting nanograms (ng) to picograms (pg). This conversion is important for standardizing measurements and making them comparable, regardless of the amount of hair or solvent used in different experiments. The conversion was done to improve the order of magnitude and level of significance of the trace concentrations of metabolites detected in the hair samples under study using the internal standard calibration curve (ng/mL) as a baseline reference.

Equation 4.1

$$\boldsymbol{metabolite\ concentration\ in\ hair}\ \left(\frac{\boldsymbol{pg}}{\boldsymbol{mg}}\right) = \left(\frac{\boldsymbol{absolute\ concentration}\ \left(\frac{\boldsymbol{ng}}{\boldsymbol{mL}}\right) * \mathbf{0.3}\ \boldsymbol{mL}\ (\boldsymbol{solvent\ volume\ used})}{\mathbf{20}\ \boldsymbol{mg}\ (\boldsymbol{hair\ extracted})}\right) * \mathbf{1000}$$

In the study, hair samples with concentrations below the LOD were transformed from ng/mL to pg/mg, aligning with the approach for urine samples. This transformation enhances the accuracy of detecting low-level toxins, a situation frequently encountered in hair analysis. Literature acknowledges that hair typically shows low concentrations of toxins, reflecting its selective absorption and incorporation of substances. Moreover, factors like hair growth variations and external influences affect these concentrations[322,749,750]. Therefore, this statistical method is essential for precise exposure assessment and bias minimization in hair sample analysis, especially given the hair's variable compound distribution.

4.4.1.1 Diakylphosphate and 3 phenoxy benzoic acid concentrations (pg/mg) in hair samples

The OP and PYR metabolite levels, as well as detection frequencies (DF), mean, and median values for calculated concentrations (pg/mg), are shown in **Table 4.3**. As shown in **Table 4.3**, DETP exhibited the highest detection frequency at 47 %, while DMTP was the lowest at 31 %. The observation suggests

that the DETP metabolite is the most prevalent, while DMTP is the least prevalent in the hair samples. On the other hand, DEP presented the highest mean concentration at 15.61 pg/mg, pointing towards a more consistent or prevalent exposure to OP sources among the population studied. In contrast, 3-PBA metabolite had the lowest median concentration at 5.21 pg/mg, suggesting the variability in exposure levels or the metabolism of OP compounds compared to PYR compounds.

Table 4. 3: Summary of metabolite concentrations in hair samples (pg/mg).

Metabolites	Detection frequency (DF/total (%))	Mean pg/mg	Median (Minimum-Maximum) pg/mg
DMP	59/176 (34)	12.53	10.61 (10.61 – 797.25)
DEP	65/176 (37)	15.61	7.85 (7.85 – 637.05)
DMTP	54/176 (31)	8.58	18.88 (18.88 – 562.05)
DETP	82/176 (47)	9.73	15.70 (15.70 – 567.45)
3-PBA	75/176 (43)	5.21	4.03 (4.03 – 882.30)

*Samples below limit of detection (LOD) were assigned a value calculated by dividing the LOD value (**Table 2. 3**)of each metabolite by square root of 2 to give: DMP=10.61; DEP=7.85; DMTP=18.88; DETP=15.70; 3PBA=4.03 (pg/mg)*

Further analysis of the table showed that DMP was detected in 34 % of samples with a median concentration of 12.53 pg/mg. Although the median concentration for DMP is 10.61 pg/mg, the wide range from 10.61 to 797.25 pg/mg signifies substantial variability in individual exposure levels. Similarly, for DEP and DETP, the broad concentration ranges (7.85 – 637.05 pg/mg for DEP and 15.70 – 567.45 pg/mg for DETP) reflect diverse exposure within the population, despite a considerable portion of detections being lower in concentration.

The data revealed that although many individuals have exposure levels around the median, instances of much higher exposures occur, as shown by the maximum values. This pattern indicates that most of the participants might experience low to moderate exposure to OP and PYR, yet a smaller group faces significantly higher exposure levels. Moreover, the spread in exposure levels for all metabolites, especially with some values being markedly higher, underscores the importance of understanding the range and factors contributing to OP and PYR exposure. The study thus highlights the necessity of examining environmental and occupational factors, such as insecticide use practices, proximity to treated areas, and personal behaviours, to fully comprehend the exposure and the potential health risks associated with OP and PYR insecticides.

Moreover, the variability in metabolite levels may be attributed to individual metabolic differences, the episodic nature of contact with OP and PYR, and factors such as compound localization within hair,

environmental contamination, and hair treatments[751]. These factors, combined with the sensitivity of the analytical methods employed, contribute to the observed variability in metabolite levels detected in hair samples[710,725,752,753]. This underscores the need for meticulous methodological considerations in monitoring and analysing insecticide exposure to accurately assess its potential health risks.

4.4.1.2 Comparative analysis of OP and PYR metabolites concentrations in hair samples of the current study with other studies,

The comparative analysis of OP and PYR metabolite concentrations in hair samples showed a complex landscape of exposure levels across varied demographics and geographies, with significant emphasis on the influence of residential settings, see **Table 4.4**. The current study analysed 176 South African children from the rural farm and non-farm backgrounds. It revealed that median concentrations of the tested metabolites (DMP, DEP, DMTP, DETP, and 3-PBA) in 1 cm long hair samples weighing 20 mg were reported as 10.61, 7.85, 18.88, 15.70, and 4.03 pg/mg, respectively. These values represent transformed data due to the original concentrations being below the LOD or undetectable. Despite this transformation, the detection frequencies (DF%) were relatively low (33% for DMP, 37% for DEP, 31% for DMTP, 47% for DETP, and 43% for 3-PBA), indicating minimal to no OP and PYR exposure among the cohort. Contrastingly, other studies shown in the table did not report using such corrections for their data. The absence of corrected values in these studies could be due to different analytical approaches or detection capabilities, where concentrations of OP and PYR metabolites were above the LOD and thus directly measurable.

This finding contrasts with those from other regions. For instance, Knipe *et al*. 2016 detected DMP and DEP in Sri Lankan cohorts using 1 cm hair samples weighing 100 mg but did not specify DF, suggesting potential differences in methodological rigor or reporting standards. Moreover, Hardy *et al*. 2021 reported significant DEP exposure in French women with 3 cm long hair samples weighing 50 mg and achieved a 100% detection frequency, suggesting either higher exposure or a more sensitive detection method[243,583]. The comparison reveals how hair length and sample weight can influence detection sensitivity. Additionally, the variation in insecticide levels detected across studies implies that agricultural practices and environmental conditions significantly affect exposure. Therefore, establishing standardized biomonitoring methodologies is crucial to accurately assess and compare insecticide exposure in different populations and regions.

Iglesias-González *et al.* 2020 analysed 142 French children from both farm and urban settings, utilizing 6 cm, 50 mg hair samples and reported a 100% detection frequency for 3-PBA, underscoring the influence of sample size and living environments on exposure assessment [321]. Similarly, Beranger *et al*.2020 and Peng *et al*. 2020 identified OP and PYR metabolites in French pregnant women and Chinese women, respectively, with Peng et al. employing 12 cm, 50 mg hair samples to uncover a wide range of DMP concentrations [748] [578]. These findings highlight the ubiquitous nature of OP and PYR exposure, impacting individuals in urban areas and revealing exposure disparities among children in varied environments and adults across different nations. Notably, during this analysis, there was a lack of data on OP and PYR metabolites in hair samples from African farming communities.

Examining DF % for OP and PYR metabolites revealed a broad spectrum of exposure levels worldwide. Amongst South African children significantly lower DF% (less than 50 %) for all metabolites were observed indicating lesser exposure in this group. Conversely, Hardy et al. 2021 in France and Peng *et al*. 2021 in China found much higher DF%, with some metabolites present in all women samples, highlighting significant exposure, particularly in agricultural settings [578,583]. The contrast between the higher DF% in adult populations in France and China and the lower rate in South African children might be influenced by hair length and the quantity of hair collected, along with geographical and lifestyle factors affecting OP and PYR exposure levels. This variation highlights the importance of considering physical factors like hair characteristics in public health initiatives aimed at monitoring and mitigating OP and PYR exposure across diverse populations and environments.

Table 4. 4: Median OP and pyrethroid metabolite concentrations (pg/mg) in hair samples in the current and previous studies done by other researchers.

Researchers	SP and AM were used.	Country (residence)	Hair length - cm (amount of hair used for analysis- mg)	DMP (pg/mg) Median DF (%)	DEP (pg/mg) Median DF (%)	DMTP (pg/mg) Median DF (%)	DETP (pg/mg) Median DF (%)	3-PBA (pg/mg) Median DF (%)
Current study	176 Children 2-D GCxGC ToF/MS	South Africa (rural farm and non-farm setting)	1 (20)	10.61 (33)	7.85 (37)	18.88 (31)	15.70 (47)	4.03 (43)
Knipe *et al.* 2016[243]	50 women and men GC-MS/MS	Sri-Lanka (farm)	1 (100)	1.00 (-)	6.88 (-)	<LOD (-)	1.17 (-)	1.05 (-)
Hardy *et al.* 2021[583]	93 women GC-MS/MS	France (farm)	3 (50)	1.16 (87)	51.10 (100)	(-) (4)	- 0.85 (100)	1.10 (100)
Iglesias-González *et al.* 2020[321]	142 children GC-MS/MS	France (farm and urban)	6 (50)	0.89 (43)	1.66 (86)	<LOD (22)	0.14 (68)	2.36 (100)
Beranger *et al.* 2020[748]	311 pregnant women GC-MS/MS	France (Urban)	9 (-)	0.86 (84)	7.46 (98)	0.03 (56)	0.88 (97)	1.69 (100)
Peng *et al.* 2020[578]	204 women GC-MS/MS	China (Urban)	12 (50)	3.61 (100)	1.68 (100)	0.15 (95)	0.89 (100)	0.56 (99)

<LOD: Less than the level of detection
SP- study population
DF- detection frequency.
AM- the analytical method used.
(-)- not mentioned.

The variability in detection, as illustrated by the use of 50 mg (6 cm) of hair in studies like that of Iglesias-González *et al*. 2020, compared to 20 mg (1 cm) in the current study, highlights how methodological factors, specifically hair sample size, can influence metabolite detectability [321]. This underpins the challenges in directly comparing studies without accounting for these differences. Additionally, in the current study, the decision to use 20 mg of hair (1cm) was partly due to the hesitancy of children to provide larger hair samples. This additional context emphasizes the complexities of conducting research with child participants and further complicates direct comparisons between studies without considering these nuanced methodological and practical differences. Furthermore, accurate assessment of OP and PYR metabolite concentrations necessitates careful consideration of the length and weight of hair samples, as these factors jointly determine the detection capacity for chronic exposure assessment.

Iglesias-Gonzales study analysed 6 cm hair length and reported metabolites values of DMP: 0.89, DEP: 1.66, DETP: 0.14, 3-PBA: 2.36 pg/mg. Dividing these concentrations by the hair length (6 cm), representing six months of exposure, potentially brings the calculated monthly values for each metabolite below the LOD for a 1 cm hair sample. This finding suggests that shorter hair lengths may not accumulate enough metabolites to surpass the LOD, affecting these substances' detectability. The study emphasizes that longer hair samples provide a more detailed exposure record, improving metabolite detection, particularly for low or seasonal exposure. Differences in exposure between children and adults and across geographic locations highlight how lifestyle, environmental factors, and insecticide use contribute to varying exposure levels [754-756]. Additionally, non-agricultural PYR sources, like lice treatments, could also affect metabolite concentrations, adding to the complexity of assessing exposure accurately.

As of this current study, existing literature barely explored the impact of hair length and analysis mass on detecting insecticide metabolites, presenting an opportunity for future research. The difference in metabolite concentration due to hair length highlights the need for standardized biomonitoring protocols for reliable exposure assessments. Furthermore, the lack of data concerning OP and PYR metabolite concentrations in the hair of individuals from African farming communities highlights a significant gap in research. Filling this gap is crucial for creating public health policies and interventions that meet the specific needs of these populations.

The following sections detail and discuss the current study's comprehensive Analysis of OP and PYR metabolite concentrations found in the hair samples of children from farms in the Western Cape.

4.4.1.3 Statistical summary of metabolite concentrations (pg/mg) found in the hair samples collected from children.

Table 4.5 shows the analysis of metabolite concentrations in children's hair samples, revealing detailed insights into exposure levels to five metabolites. The study segmented the data into categories, including overall totals, cycles of data collection, comparisons between farm and non-farm locations, specific areas such as Grabouw and Hex River, and gender differences. The detection frequencies (DF) of metabolites DMP, DEP, DMTP, DETP, and 3-PBA were 34 %, 37 %, 31 %, 47 %, and 43 %, respectively. Despite the DF, median concentrations were predominantly below the LOD, signifying that most of the samples had undetectable metabolite concentrations. The interquartile range (IQR), representing the spread from the 25th to the 75th percentiles, illustrates the range of detectable concentrations. Notably, concentrations at the 75th percentile were above the LOD for all metabolites, indicating detectable levels in over 25% of the samples. 3-PBA stood out with the highest 75th percentile concentration at 32.63 pg/mg.

Table 4. 5: Summary of concentrations of metabolites found in hair samples from children, expressed as median and interquartile range (IQR) values in pg/mg.

Variable	DF Total (%)	DMP pg/mg median (IQR)	DF Total (%)	DEP pg/mg median (IQR)	DF Total (%)	DMTP pg/mg median (IQR)	DF Total (%)	DETP pg/mg median (IQR)	DF Total (%)	3-PBA pg/mg median (IQR)
Total (176)	59 (34)	10.61 (10.61 - 21.08)	65 (37)	7.88 (7.88 - 104.03)	54 (31)	18.88 (18.88 - 18.88)	82 (47)	15.70 (15.70- 15.70)	75 (43)	4.03(4.03- 32.63)
Cycle 1 (124)	38 (31)	10.61 (10.61 - 40.73)	42 (34)	7.88 (7.88 - 115.28)	31 (25)	18.88 (18.88 - 18.88)	54 (44)	15.70(15.70- 15.70)	49 (40)	4.03(4.03- 37.12)
Cycle 2 (52)	21 (41)	10.61 (10.61 - 10.61).	23 (44)	7.88 (7.88 - 13.2)	23 (44)	18.88 (18.88 - 1.90)	28 (54)	15.70(15.70- 15.70)	26 (50)	4.03(4.03- 4.03)
p-value		***0.04***		***0.03***		0.47		***0.01***		***0.002***
Farm (132)	38 (29)	10.61 (10.61 - 10.61)	43 (33)	7.88 (7.88 - 96.08))	18 (14)	18.88 (18.88 - 18.88)	32 (24)	15.70(15.70- 15.70)	26 (20)	4.03(4.03- 38.10)
Non-farm (44)	21 (48)	10.61 (10.61 - 32.15)	22 (50)	7.88 (7.88 - 111.68)	18 (41)	18.88 (18.88 - 18.88)	28 (64)	15.70(15.70- 15.70)	26 (59)	4.03(4.03- 20.25)
p-value		0.69		0.98		0.91		0.69		0.79
Grabouw (80)	43 (54)	10.61 (10.61 - 29.25)	48 (60)	7.88 (7.88 - 102.60)	39 (49)	18.88 (18.88 - 18.88)	60 (75)	15.70(15.70- 15.70)	54 (68)	4.03(4.03- 37.50)
Hex river (96)	16 (17)	10.61 (10.61 - 7.50)	22 (18)	7.88 (7.88 - 108.15)	15 (16)	18.88 (18.88 - 18.88)	22 (23)	15.70(15.70- 15.70)	21 (22)	4.03(4.03- 19.95)
p-value		0.67		0.73		0.87		0.51		0.58
Female (152)	43 (29)	10.61 (10.61 - 20.85)	48 (32)	7.88 (7.88 - 110.10)	42 (28)	18.88 (18.88 - 18.88)	51 (33)	15.70(15.70- 15.70)	44 (29)	4.03(4.03- 35.25)
Male (24)	16 (67)	10.61 (10.61 - 21.30)	17 (67)	7.88 (7.88 - 89.70)	14 (58)	18.88 (18.88 - 18.88)	18 (75)	15.70(15.70- 15.70)	16 (67)	4.03(4.03- 27.45)
p-value		0.87		0.47		0.97		0.85		0.97
<LOD (%RSD) (corrected)		120 (65 %)		114 (62 %)		125 (68 %)		97 (53 %)		104 (57 %)
<LOQ (%RSD)		8 (4 %)		1 (0.5 %)		38 (21 %)		46 (25 %)		12 (7 %)

Data reported as median (IQR that is 25 % and 75 % of the data) for continuous data.
Associations between continuous data were tested using the Mann-Whitney test.
P-value ≤0.05 considered as significant; % RSD= % relative standard deviation; DF= Detection Frequency
*Samples below limit of detection (LOD) were assigned a value calculated by dividing the LOD value (***Table 2. 3***)of each metabolite by square root of 2 to give: DMP=10.61; DEP=7.88; DMTP=18.88; DETP=15.70; 3PBA=4.03.*

A significant finding from the comparative analysis between cycles 1 and 2 was the decrease in 75th percentile concentrations of metabolites from the pre-spraying season (cycle 1) to the spraying season (cycle 2). Specifically, the 75th percentile DEP concentration showed a pronounced decrease from 115.28 pg/mg in cycle 1 to 13.20 pg/mg in cycle 2. This trend of varying concentrations across cycles was consistent for other metabolites, though to varying extents. Statistical analysis further substantiates these observations. A p-value below 0.05 generally indicates statistical significance, suggesting a less than 5% chance that the observed differences occurred by chance[757]. In this context, significant differences in metabolite concentrations between cycles were confirmed for DMP (p=0.04), DEP (p=0.03), DETP (p=0.01), and 3-PBA (p=0.002), while DMTP showed no significant change (p=0.47). No significant differences were found when comparing farm versus non-farm locations (p-values from 0.69 to 0.98), nor between the specific regions of Grabouw and Hex River (p-values from 0.51 to 0.87). The gender-based comparison also revealed no significant differences in metabolite exposure (p-values from 0.47 to 0.97).

The comprehensive analysis of metabolites in children's hair reveals complex dynamics, with variations in metabolite levels across different collection cycles suggesting environmental exposure influences. Interestingly, levels appear to be higher during the non-spraying season, indicating a variation by cycle, though the reasons for this remain unclear. The non-significant differences related to location, gender, and area might be attributed to the sample size, adding another layer of complexity to understanding the environmental impact on metabolite fluctuations. Indeed, insecticide spraying seasons emerge as a critical determinant, as reported by a study that documented the effects of environmental and occupational exposures to lead (Pb) on Pb concentrations in children's scalp hair [758].

The analysis reveals a pervasive exposure to OP and PYR insecticides across both farm and non-farm settings and various geographical locations, demonstrating a widespread environmental presence of these substances. This consistent exposure pattern, unaffected by farming status, location, or gender, highlights the critical need for ongoing surveillance and in-depth investigation into the health effects of such environmental contaminants. It underscores the importance of employing precise and sensitive detection techniques to accurately capture the complex changess of insecticide exposure, thereby emphasizing the necessity for comprehensive environmental health measures to safeguard public well-being.

4.4.1.4 Comparison of OP and PYR metabolites in hair during the two cycles among farm and non-farm residents

Figures 4.5 to **4.7** show only the concentrations of metabolites detected in hair samples, categorized by residential area, gender, and geographical location across two cycles. Detailed statistical analysis of the three factors according to cycles is shown in **Tables A 4.1** to **4.3**. The figures include corrected/ transformed values for less than LOD and non-detected metabolites. The figures are box plots that visually summarise a dataset's median, variability, and skewness, with the median shown as a line inside the box. Therefore, they allow for easy comparison of data distributions and trend spotting. Also, a red star in the figures indicates a statistically significant change (statistical difference), with a p-value below 0.05.

Figure 4.5 (a) notably shows a significant difference in farm metabolite concentrations between cycles 1 and 2 for DMP, DMTP, DETP, and 3-PBA, with higher values observed in cycle 1. This disparity might result from the larger participant size in cycle 1, but it highlights participants' exposure to OPs and PYRs. Additionally, the data for DEP display a notable spread, characterized by a higher median and a wider IQR compared to other metabolites, indicating greater variability among participants. Also, DEP and DETP concentrations decrease from cycle 1 (before spraying season) to cycle 2 (during spraying season), as shown by the downward shift in median values. Interestingly, non-farm participants (**Figure 4.5 (b)**) shows metabolite concentrations indicative of exposure to OPs and PYRs at similar magnitudes.

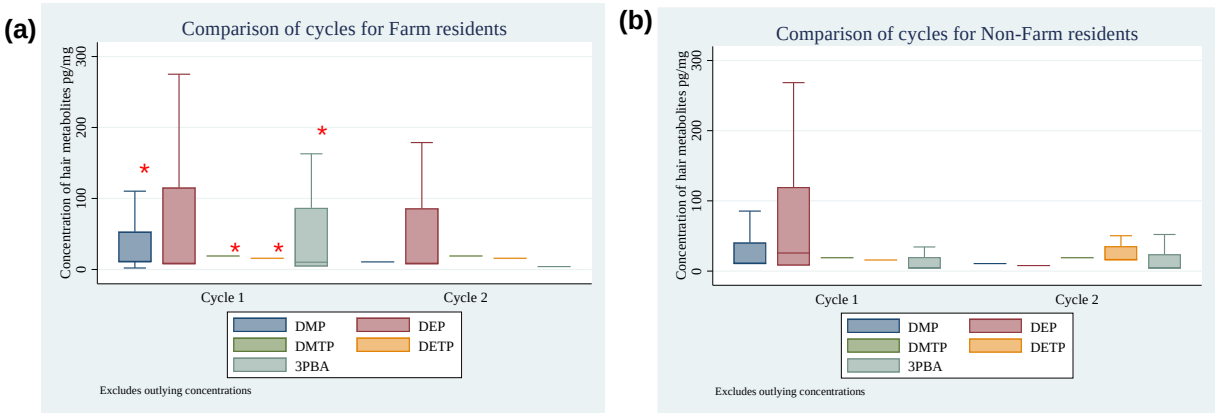


Figure 4. 5: Distribution boxplots outlining the metabolite concentrations from hair samples of farm and non-farm residents obtained during cycle 1 and 2.

The fluctuation in metabolite concentrations across seasons indicates variable exposure, but the exact reasons for this variability are not clear. It is uncertain whether these changes are due to differences in

insecticide application or other factors. In the context of occupational and environmental exposure, studies by Kimata *et al*. 2009 and Dalvie *et al*. 2011 further elucidate the persistence and variability of insecticide exposure. Kimata *et al*. highlighted that even after abstaining from spraying activities for a week, pest control workers exhibited significantly higher urinary concentrations of 3-PBA compared to the general population [759]. This indicates the lasting impact of occupational exposure and the potential for sustained PYR levels in the body. Similarly, Dalvie *et al*. 2011 found consistent levels of DAP in farm workers across the spraying season, suggesting a constant exposure risk regardless of the spraying cycle's phase [132].

The research discussed above reveals the persistent exposure to OP and PYR in both occupational and environmental contexts, underscoring the need for continuous monitoring and effective interventions to mitigate health risks associated with insecticide use, particularly in agricultural settings like the Western Cape of South Africa. Their findings highlight how insecticide exposure, extending beyond application sites, impacts communities and vulnerable groups such as children, reinforcing the importance of comprehensive strategies to reduce exposure. Studies by Chetty-Mhlanga *et al*. 2020 and Ochieng *et al*. 2013 linking insecticide exposure to adverse health outcomes further stress the urgency for robust environmental monitoring and public health interventions to protect communities in agricultural regions from the effects of insecticides[760,761].

4.4.1.5 Comparison of OP and PYR metabolites in hair during the two cycles between the Grabouw and Hex River Valley areas

Figure 4.6 reveals distinct patterns of metabolite concentrations in the hair samples of residents from Grabouw and Hex River across two cycles. Specifically, **Figure 4.6(a)** indicates a marked increase in both the median and IQR DEP concentrations in cycle 2 for Grabouw residents, alongside a significant variation in 3-PBA concentrations, suggesting an increased exposure in the initial cycle. Conversely, **Figure 4.6(b)** shows higher DEP concentrations in cycle 1 than in cycle 2 for Hex River residents, indicating a significant reduction in exposure.

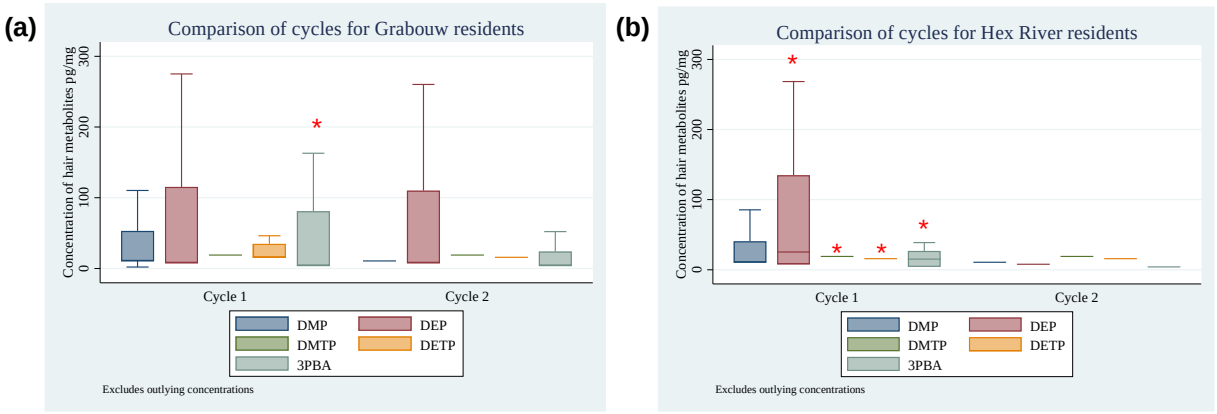


Figure 4. 6: Distribution boxplots outlining the metabolite concentrations from hair samples of Grabouw and Hex River residents obtained during cycle 1 and 2.

Figure 4.6 illustrates variations in OP and PYR metabolite levels in hair samples, demonstrating the impact of temporal and geographical factors on insecticide exposure. Specifically, in Grabouw, there was an increase in DEP levels during cycle 2, which resonates with the findings of Stout *et al*. 2009 who reported that the timing of agricultural activities, such as insecticide application, directly influences the levels of insecticide residues in residential areas, both urban (non-farm) and rural (farm)[762]. This correlation between agricultural insecticide application and residual levels in homes highlights the connection between environmental and biological indicators of exposure. Furthermore, the variation observed in 3-PBA levels in Grabouw is consistent with the ones reported by Barr *et al*. 2010 who highlighted widespread PYR exposure which was notably higher among children across various populations[563]. In contrast, the reduction in DEP levels in Hex River during the spraying season suggests an unexpected trend, possibly reflecting changes in local agricultural practices. This observation aligns with the findings of Bradman *et al*. 2007, who reported variations in insecticide exposure in agricultural areas, indicating that such exposures can fluctuate due to factors beyond the immediate application of insecticides. These factors could include changes in insecticide usage, application methods, or other environmental practices that influence the presence of DEP in the environment during the spraying season[763]. Previous research have shown that OP and PYR metabolite concentrations like DEP and 3-PBA are influenced by factors such as agricultural application patterns, proximity to farming activities, and seasonal changes in insecticide use, reflecting the complex variations of exposure[634].

The evidence of insecticide exposure in agricultural communities, shown by research in South Africa and internationally, highlights a pervasive concern. Biomonitoring studies of insecticides near Western Cape schools indicate significant exposure risks for school children, suggesting that exposure fluctuates

with seasonal insecticide applications, changes in farming practices, or the effectiveness of mitigation efforts[616,760,764]. Similarly, studies from the United States reveal seasonal fluctuations in insecticide metabolite levels in urine samples from children and adults, matching the periods of insecticide use [631,765]. Moreover, the distinct exposure patterns observed in Grabouw and Hex River underscore the influence of localized environmental and agricultural factors on insecticide exposure risks. Similar studies conducted in diverse geographic areas have consistently found that local practices, such as the timing of insecticide application, types of insecticide used, and proximity to spraying sites, are factors that significantly influence exposure levels among residents [766,767].

4.4.1.6 Comparison of metabolite levels across cycles for gender

Figure 4.7 uncovers gender-specific variations in metabolite concentrations across two cycles, with female participants (**Figure 4.7(a)**) exhibiting significantly higher concentrations of DMP, DEP, DETP, and 3-PBA in cycle 1 than in cycle 2, unlike their male counterparts (**Figure 4.7(b)**) who show no such difference. This observation hints at potentially higher exposure to OPs and PYRs among females, a disparity that might stem from distinct behavioural and occupational roles within agricultural settings. Women's engagement in tasks such as hand weeding or washing insecticide-contaminated clothing could elevate their exposure risk[768,769].

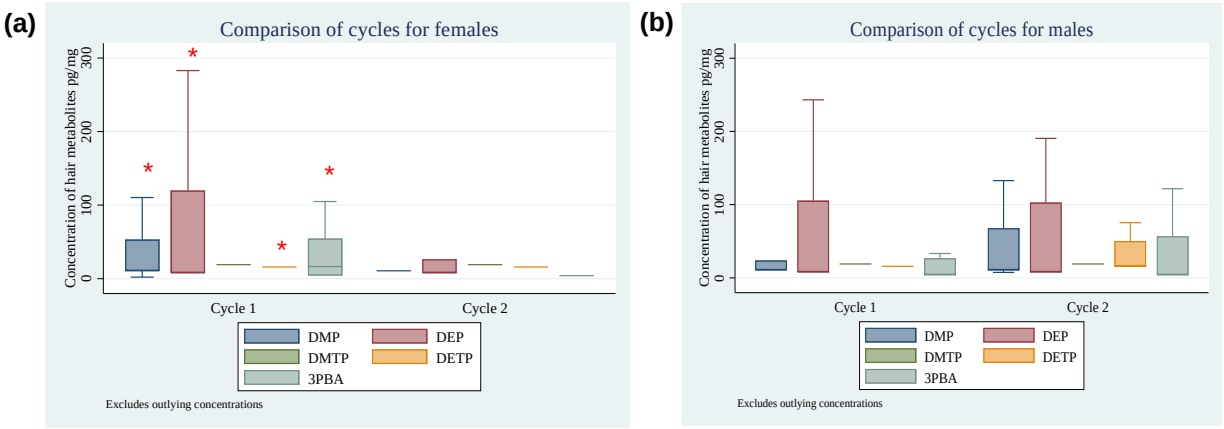


Figure 4. 7: Distribution boxplots outlining the metabolite concentrations from hair samples of females and male residents obtained during cycle 1 and 2.

The study's participant composition, with a significantly larger number of females (152) than males (24), introduces a crucial factor in interpreting these findings. The ample female sample size enhances the statistical power, possibly elucidating differences not as readily detected in the smaller male cohort. The

disparity in sample sizes necessitates meticulous analysis, as it significantly impacts the understanding of gender-specific insecticide exposure. Notably, the heightened exposure variability observed among females may imply that a particular subgroup of females experienced higher levels of exposure. Furthermore, this variability is likely intensified by inherent biological differences between genders in the absorption, metabolism, and elimination of OP and PYRs, thereby emphasizing the layered complexity of the risks associated with insecticide exposure [770,771].

4.5 Conclusion

Analysing hair sample metabolites in children illuminated complex exposure patterns to OPs and PYRs insecticides. The study has uncovered variable detection frequencies and concentration levels across different cycles and regions, revealing that exposure to OPs and PYRs is not static but fluctuates over time. Observations show gender-specific differences but do not lead to a significant disparity between males and females. The median concentrations and IQR values have indicated transformed low or non-detectable levels of OP and PYR metabolites, reflecting a general trend of low exposure within the studied population. Nonetheless, the study's findings reveal significant variations in the concentrations of certain metabolites across different cycles, indicating fluctuating exposure risks. Specifically, DMP, DEP, DETP, and 3-PBA concentrations showed significant differences, with p-values of 0.04, 0.03, 0.01, and 0.002, respectively, which suggested periods of heightened exposure.

In contrast, DMTP concentrations remained consistent across cycles ($p=0.47$), indicating stable exposure levels in both cycles. The results emphasize the need to understand diverse exposure patterns, from significant fluctuations to steadiness, highlighting the complexity of environmental exposures. Variability in metabolite concentrations in farm children's hair and regional differences between Grabouw and Hex River residents underscores the impact of local agricultural practices and environmental factors on exposure. These findings call for region-specific, temporally adjusted biomonitoring strategies to effectively manage OP and PYR exposure risks, underscoring the importance of tailored approaches in environmental health monitoring.

The findings highlight the importance of considering hair length and sample volume in future biomonitoring studies. Moreover, longer hair strands encapsulate a comprehensive exposure history that may exhibit metabolite concentrations above the LOD. However, the metabolite levels might have been significantly higher if the present study had utilized longer hair samples, similar to those in other

research studies. Additionally, this realization underscores the need for a deeper investigation into the effect of hair length on metabolite quantification, thus highlighting a promising direction for future research in environmental exposure and public health. Although this critical observation reveals a gap in the current literature, it also opens a rich avenue for future inquiry. Investigating the relationship between hair length and metabolite detectability could greatly enhance the methodologies for assessing environmental contaminant exposure. Furthermore, understanding this relationship is crucial for developing more accurate and reliable biomonitoring practices to gain more precise environmental health assessments.

While urine is a well-established medium for biomonitoring due to its ability to reflect recent insecticide exposure and its extensive validation for various metabolites, hair sampling is emerging as a complementary tool with distinct advantages. The capacity to document long-term exposure characteristics of hair to act as a historical record offers a unique opportunity to assess chronic exposure, which is particularly relevant for insecticides that accumulate over time or have delayed health effects. Advancements in analytical techniques are needed to establish hair analysis as a robust method in environmental health. The development of sensitive methods, such as those utilizing sophisticated mass spectrometry, could allow for the detection of lower levels of insecticides and their metabolites in hair samples. Furthermore, the standardization of hair collection and preparation methods would address current variability and improve the consistency of findings across different studies. As such, hair analysis holds the potential to become an equally established and informative biomonitoring approach, offering a longer-term perspective on insecticide exposure when combined with the more immediate reflections provided by urine analysis.

4.5.1 Limitations

The study's limitations included an uneven distribution of participants across sampling cycles, a gender imbalance, a lack of representation from non-farm areas, and a geographical restriction to two regions, which limits the generalizability of the study’s conclusions. Consequently, the study's ability to provide a comprehensive and representative analysis of OP and PYR exposure in the broader child population is restricted. Moreover, the use of short 1 cm, 20 mg hair samples may underestimate long-term OP and PYR exposure compared to longer hair samples and larger sample masses used in other studies. In addition, hair as a biological matrix presents challenges such as external contamination and variability

caused by environmental exposure, hair color, racial differences, and cosmetic treatments, all of which affect the accuracy of metabolite detection. As a result, cautious interpretation of the study's hair analysis results is necessary to ensure an accurate reflection of exposure levels.

Chapter 5

Impact of Insecticide Exposure on the Metabolomic Profiles of Children: Insights from MetaboAnalyst 5.0 and 2-D GCxGC ToF/MS Analyses

5.1 Introduction

Chapters 3 and **4** present biomonitoring data (DAPs and 3-PBA concentrations in urine and hair samples) that demonstrates children's exposure to OP and PYR in South Africa's Western Cape farming communities. The current chapter evaluates the impact of insecticide exposure on children's metabolomic profiles using non-targeted metabolomics data generated through using both 2-D GCxGC ToF/MS and MetaboAnalyst 5.0 tool. The non-targeted analysis approach broadens the biomonitoring investigation by revealing metabolic pathways affected by insecticide exposure thus providing a detailed view of the biochemical effects in the children's bodies.

Metabolomics is the study and analysis of small molecules or metabolites within a biological sample thereby uncovering crucial insights into cellular functions in the human body. Moreover, it assesses how these activities vary with conditions such as disease or exposure to chemicals, offering a detailed picture of metabolic processes. MetaboAnalyst 5.0 is a comprehensive web-based tool for data pre-processing, statistical analysis, and metabolite interpretation. Moreover, MetaboAnalyst 5.0 plays a significant role in advancing metabolomics by simplifying the analysis of complex metabolite data from analytical tools such as GC/MS and LC/MS and enhances understanding of the body's metabolic pathways. By facilitating efficient exploration and visualization of biological processes, MetaboAnalyst 5.0 aids in interpreting how these processes respond to different stimuli[772,773]. The tool improves data interpretation by facilitating a detailed examination of the disruption in metabolic pathways caused by insecticide exposures, thereby advancing from mere exposure measurements to a comprehensive understanding of their broader health implications.

Integrating targeted (**Chapter 3** and **4** data) and non-targeted approaches deepens insights into metabolic disruptions caused by insecticides and highlights complex biochemical reactions in children exposed to environmental toxins. This research fills a crucial gap in South African metabolomics offering valuable contributions to personalized healthcare and expanding our knowledge of health and disease processes.

The following sections discuss in depth how MetaboAnalyst 5.0 tool was used for the metabolomics study using urine sample non-targeted analysis data.

5.1.1 Metabolomics

Metabolomics is an important field for exploring metabolites within biological systems and contributes to understanding metabolic pathways affected by external and environmental factors like insecticides [773,774]. Techniques like mass spectrometry (MS) advance disease diagnosis, drug development, and environmental health analysis by accurately identifying and quantifying biomolecules while the integration of metabolomics with genomics and proteomics data from MetaboAnalyst 5.0 data base provides deeper insights into biological mechanisms and diseases, uncovering the effects of genetic variations on metabolic pathways and protein functions[773,775-794]. This comprehensive approach uncovers novel biomarkers and therapeutic targets leading to a more in-depth understanding of diseases such as diabetes. Furthermore, it propels cancer medical research and environmental science[793,795-797]. Examining environmental compounds, for instance, insecticides associated with neurotoxicity and developmental challenges in children, highlights the significance of metabolomics in revealing the impacts on health [798,799].

For this study, MetaboAnalyst 5.0 was used for urine-based non-targeted metabolomics analysis. Urine samples were optimal for this metabolomics study as the samples had a higher detection rate of metabolites like DAPs and 3-PBA and a wider compound spectrum.

5.1.1.1 Non-targeted analysis

Non-targeted metabolomics can comprehensively analyse the entire metabolome of an organism, tissue, or cell thereby capturing all metabolites to uncover metabolic changes and interactions without focusing on specific metabolites[773,774,780]. This approach does not rely on a priori knowledge and can identify unexpected metabolic alterations, potentially overlooked in targeted analyses[776]. Additionally,

non-targeted metabolomics plays a crucial role in assessing the metabolic responses of various organisms[800,801]. However the non-targeted metabolomics broad applicability, the analysis has lower sensitivity and selectivity than targeted approaches [774].There are several tools and software platforms available to analyse and interpret metabolomics data such as MetaboAnalyst 5.0 which will be the focus of this research.

5.1.2 MetaboAnalyst 5.0

The web-based tool is widely used to facilitate metabolite taxonomic and functional interpretation, and offers a user-friendly interface and batch processing, as it is a comprehensive resource for metabolomics and pathway analysis [802,803]. It is extensively used in various studies to reveal crucial metabolic pathways and to understand the mechanisms underlying different diseases and interventions [803-810]. Consequently, this platform enhances research efficiency in metabolic pathway analysis by simplifying the handling of large datasets [803]. Enhanced features of MetaboAnalyst 5.0 include improved pathway interpretation from non-targeted MS data, support for meta-analysis, and multi-omics analysis [803].

MetaboAnalyst 5.0's pathway analysis feature utilized 2-D GCxGC ToF/MS non-targeted data to support metabolic pathway analysis, metabolite set enrichment, and biomarker selection [806,811-817]. It has been instrumental in exploring metabolic pathways affected by diverse exposures and conditions in humans, aiding in understanding disease mechanisms and intervention strategies [800,818-820]. The tool leverages statistical methods and algorithms for significant pathway identification and visualization, integrating with key databases like KEGG and Human Metabolome Database (HMDB) for in-depth analysis [803,813,815,821,822]. The KEGG database contains data on genes, proteins, and metabolic pathways, detailing molecular interactions and reaction networks. It generates output in the form of pathway maps and modules that elucidate the roles of these components in cellular processes.

In contrast, HMDB includes detailed information on human metabolites, such as their structures, concentrations, and roles in metabolism. HMDB produces outputs like metabolite profiles and metabolic pathway maps, helping researchers understand the metabolic processes in the human body. The databases offer tools for analysing and visualising the intricate relationships among their the systematically documented and organized molecules entities (proteins, amino acids, genes, etc), proving vital for genomics, biochemistry, and systems biology research. Consequently, they furnish

insights into the molecular mechanisms driving biological functions and diseases, highlighting their significance in scientific exploration.

MetaboAnalyst 5.0 is a key tool for metabolic pathway analysis in various research fields which has facilitated significant studies on insecticide exposure. Through it, studies by Gu *et al*. 2020, Al-Natour *et al*. 2022, and Pu *et al*. 2019 have clarified metabolic disruptions from OP in rats, pinpointed pathway changes due to nanoparticles, and assessed heat stress in quail, highlighting its versatility in diverse biological studies[823-825]. These findings underscore the tool's applicability in revealing intricate metabolic and liver function responses across varied biological contexts.

As of this writing, MetaboAnalyst 5.0 has not been used to study the metabolic effects of insecticide exposure, specifically with biomonitoring data derived from advanced chromatographic techniques like GC/MS or LC/MS. However, this study highlights the novelty of applying this the 2-D GCxGC ToF/MS analytical technique in combination with MetaboAnalyst 5.0, which together offer a new approach to understanding the metabolic disturbances caused by insecticides in children living in the farming areas of the Western Cape. Therefore, this integration of advanced analytical technology with MetaboAnalyst 5.0 sets the research apart from previous studies that have not utilized this combination to explore the metabolic effects of insecticide exposure.

5.2 Experimental method

5.2.1 Extraction of compounds

The extraction methods and 2-D GCxGC ToF/MS analytical methods used to quantify targeted and non-targeted targeted metabolites in urine samples are reported in Chapter 2. The non-targeted data set comprised of [full scan (35-450 m/z) MS data], all detectable metabolite’s (m/z values and peak areas) in the analysis of 61 urine samples (farm and non-farm) from cycle 1 only. The term "[full scan (35-450 m/z) MS data]" refers to a method in MS where the instrument scans and records metabolite or compound ions across a wide range of mass-to-charge ratios, specifically from 35 to 450. This comprehensive scanning captures a broad spectrum of molecules present in a sample, allowing for an extensive analysis of its chemical composition.

The study focused on analysing urine samples from cycle 1 only to maintain data consistency and manage resources efficiently. Urine was chosen for this study because it offers a clear view of recent

insecticide exposure, unlike hair, where metabolite reliability varies with physical traits, complicating interpretation [826,827]. This approach, supported by MetaboAnalyst 5.0, optimized the detection of metabolic changes. The non-targeted data was processed using the MetaboAnalyst 5.0 web-based software and the steps are described in detail in the following section.

5.2.2 Statistical Analysis

MetaboAnalyst 5.0, evaluates the impact on metabolic pathways by processing and analysing metabolomics data. Initially, raw MS data from urine samples under non-targeted analysis are compiled into an Excel sheet. In the excel sheet, metabolites are listed in the first column, while the first row contains urine sample IDs alongside their corresponding peak areas (m/z). This setup ensures that each compound's data is organized and accessible for analysis The processed non-targeted data from 2-D GCxGC ToF/MS was used to perform statistical analysis using the 'MetaboAnalyst 5.0' www.metaboanalyst.ca (accessed on 16 December 2023)[802,817]. Through these structured steps listed below, MetaboAnalyst 5.0 systematically identifies and quantifies the variations in metabolite peak areas, linking them to specific metabolic pathways and thus elucidating the biochemical effects under study.

Step 1: Analyses included data processing, that is, checking if the compounds are labelled, and flagging and removing non-numeric values, derivatives and missing values from the CSV excel sheet that will be used for MetaboAnalyst analysis. The repeating or reoccurring metabolic names in a sample were removed to have only one metabolite name in the column from the Excel sheet with 61 participants compiled data. Also, derivatised compound names that the MetaboAnalyst 5.0 did not recognise were removed from the Excel sheet.

The data was further cleaned up and the most prevalent compounds in the 61 participants' urine samples were selected-. The compounds that were derivatised had the derivatised moiety removed using ChemDraw - see **Figure 5.1** below which is a snapshot of an Excel sheet containing some of the compounds that are derivatised (highlighted compounds) and had to be converted to underivatized compounds using ChemDraw.

Sample number	8	15	23	24	28	29	30	31	32	35	39
Compound name	Female	Male	Female	Female	Female	Female	Female	Female	Female	Female	Female
[1,4,5]Oxadithiepane	15	10281630	17547803	0	0	7374945	0	14013261	8270076	17295517	7243932
1-(2-Ethyl-[1,3]dithian-2-yl)-3-methyl-butan-1-ol	18	1026379	1117063	0	233487	0	0	0	0	3171929	4829770
1,7-Dimethylxanthine	8	259145	0	0	0	506016	0	0	6388563	0	0
1-Butanamine	19	2999775	0	23963080	0	1050714	15337220	25211282	0	235519	0
1-Iodo-2-methylundecane	18	42540262	44706356	49128666	0	47533768	47605617	0	54437441	41683585	25552309
1-Methyl-3-propyl-1H-pyrazole-5-carboxylic acid, TBDMS derivative	10	1673826	1948072	0	0	0	0	0	1053415	0	0
1-Trimethylsilyoxylundec-2-ene	10	9408061	0	0	0	0	0	0	3810812	0	0
2-((Prop-2-ynyloxy)carbonyl)benzoic acid	22	10708631	7564894	0	0	0	0	0	0	9006871	0
2-Octenoic acid, TMS derivative	12	4374662	6163758	0	2060946	3964959	1278021	37388722	2354121	1895018	3319580
2-Propanone, 1-(ethylthio)-	10	56434117	0	0	0	44472964	36122667	0	0	0	0
3-(Methylthio)benzoic acid, TBDMS derivative	13	46128085	0	0	0	0	0	2,81E+08	0	365223	20648560
3-Indoleacetic acid, TBDMS derivative	44	2,53E+08	96862028	0	508887	4,31E+08	24732567	7,5E+08	39439794	882231	0
4-(Methoxycarbonyl)phenol, TBDMS derivative	15	3896757	0	0	0	0	362121	1427335	12015130	751438	2122595
4-Hydroxybenzoic acid, 2TBDMS derivative	32	41897438	43211757	0	980638	8126415	22115304	10159924	23071059	48342526	35345670
5,8,11-Eicosatriynoic acid, tert-butyldimethylsilyl ester	19	3064706	0	0	0	0	5947196	729617	0	0	122792
à-D-Glucopyranoside, methyl 2-(acetylamino)-2-deoxy-3-O-(trimethylsilyl)-, cyclic methylboronate	13	964634	0	0	0	0	0	0	0	822358	1995928
Adipic acid, 2TBDMS derivative	11	1024223	195453	0	174771	0	239456	12328030	0	194911	0
Azelaic acid, 2TBDMS derivative	19	85239443	0	0	10699595	0	21133267	1,45E+09	0	1,06E+08	36603503
Benzamide, N-(4-cyanomethylphenyl)-	20	486665	0	0	0	0	248754	686264	0	0	74710
Benzeneacetic acid, TBDMS derivative	21	3177562	6313453	0	7504300	1791370	1668480	35700648	2441987	1842352	12527594
Benzoic Acid, TMS derivative	16	25819743	0	0	0	0	0	0	0	0	0
Bifenthrin	11	1614220	2250550	7766439	1234260	0	0	0	257780	2892801	1340150

Figure 5. 1: Example of derivatised compounds that were converted to non-derivatised compounds.

Step 2: Excel sheet (see **Figure 5.2 below)** which is a snapshot of an Excel with the details of compounds and samples) with selected metabolites was converted to a CSV file and was submitted to the MetaboAnalyst tool for Name/ID standardisation, where enrichment analysis occurs, that is, only well-annotated HMDB and KEGG ID compounds (i.e. those in the MetaboAnalyst 5.0 pathway libraries & metabolite sets) were mapped. The compounds that are not recognised such as derivatives are not considered for analysis.

Sample	Label	Oxadithie	2-Ethyl-3-	Dimethyl	1-Butanan	iodoalkan	1-Methyl-	undec-2-e	Prop-2-yn	2-Octenoi	Propanon	3- Methyl	3-Indolea	4- Methox	4-Hydroxy	icosa-5.8.	7-acetami
8	Farm	10281630	1026379	259145	2999775	42540262	1673826	9408061	10708631	4374662	56434117	46128085	2,53E+08	3896757	41897438	3064706	964634
15	Farm	17547803	1117063	0	0	44706356	1948072	0	7564894	6163758	0	0	96862028	0	43211757	0	0
23	Farm	0	0	0	23963080	49128666	0	0	0	0	0	0	0	0	0	0	0
24	Nonfarm	0	233487	0	0	0	0	0	0	2060946	0	0	508887	0	980638	0	0
28	Farm	7374945	0	506016	1050714	47533768	0	0	0	3964959	44472964	0	4,31E+08	0	8126415	0	0
29	Farm	0	0	0	15337220	47605617	0	0	0	1278021	36122667	0	24732567	362121	22115304	5947196	0
30	Farm	14013261	0	0	25211282	0	0	0	0	37388722	0	2,81E+08	7,5E+08	1427335	10159924	729617	0
31	Farm	8270076	0	6388563	0	54437441	1053415	3810812	0	2354121	0	0	39439794	12015130	23071059	0	0
32	Farm	17295517	3171929	0	235519	41683585	0	0	9006871	1895018	0	365223	882231	751438	48342526	0	822358
35	Farm	7243932	4829770	0	0	25552309	0	0	0	3319580	0	20648560	0	2122595	35345670	122792	1995928
39	Farm	0	0	0	1404596	0	0	0	0	0	0	0	0	0	0	0	0
43	Farm	0	0	1585339	352527	46633411	174786	0	32740017	0	12811626	0	0	0	1679456	0	0
45	Farm	0	2004988	0	4048559	47700013	638295	0	11139309	0	0	0	46415773	0	1168606	0	0
49	Farm	17285894	0	0	0	0	885651	0	26476489	9307890	0	0	227103	0	1117525	3251030	0
50	Farm	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	778284
51	Farm	0	0	7920417	0	0	1908415	0	21135878	0	0	0	7522378	1734420	4002572	0	0
53	Farm	0	0	0	2223339	0	0	0	0	0	0	1,1E+08	7,44E+08	0	35437656	26702414	1630025
75	Farm	10602854	0	0	0	0	0	0	31740448	0	34964839	54480412	1820700	0	1,76E+08	4176091	0
77	Farm	0	21108552	0	17250560	0	0	1,04E+08	63372389	5994739	0	1,35E+08	99654784	0	0	9491734	53018191

Figure 5. 2: Part of the excel sheet that was then converted to a csv file before loading onto the MetaboAnalyst 5.0 software.

enrichment analysis was followed by data normalisation which involved the normalisation of compounds by a row of the CSV sheet, followed by a global test and auto-scaling of the data. The Global Test is used to determine if metabolites in a pathway are related to a particular outcome, like disease status. It considers the fact that metabolites are interconnected in pathways, which can help address issues in testing at the individual metabolite level[828]. Finally, the results were presented in an overview pathway analysis image and a table. The analysis steps of the data are shown in **Figure 5.3** which were adapted from Xia *et al* 2009[413].

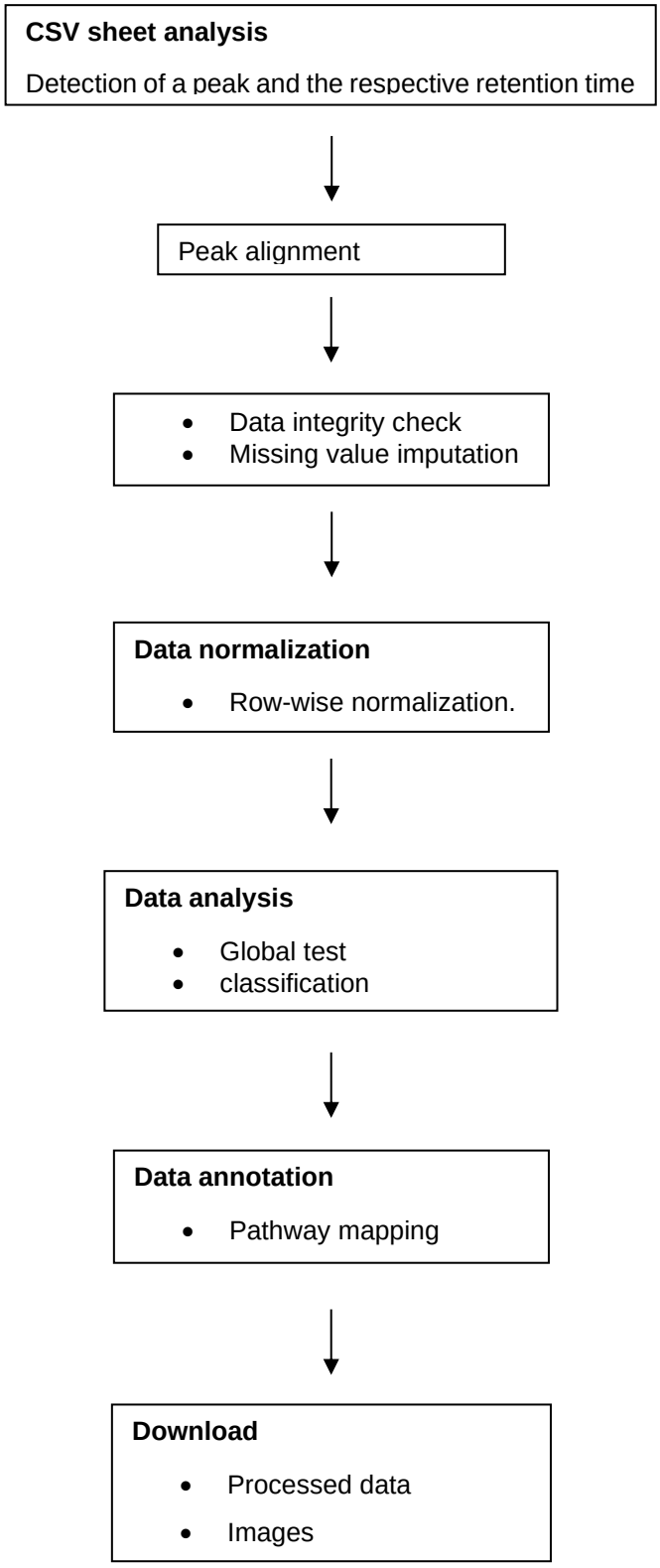


Figure 5. 3: A diagram illustrating MetaboAnalyst 5.0 workflow and data processing steps.

Step 3: Upon uploading excel CSV sheet into the MetaboAnalyst 5.0 pathway analysis tool, some metabolites were not recognised in most of the samples by the tool (the metabolites not recognised by MetaboAnalyst 5.0 are in yellow, see **Table 5.1** and these metabolites were not considered for further analysis.

Table 5. 1: Table showing some of the compounds not identified and considered for analysis by the MetaboAnalyst 5.0.

Query	Hit	HMDB	PubChem	KEGG	Details
Oxadithiepane		-	-	-	View
2-Ethyl-3-methyl-1-butanol		-	-	-	View
Dimethylxanthine		-	-	-	View
1-Butanamine	1-Butylamine	HDMB0031321	8007	-	View
Iodoalkane		-	-	-	View
1-Methyl-3-propyl-1H-pyrazole-5carboxylic acid		-	-	-	View
Undec-2-en-1-ol		-	-	-	View
Prop-2-ynyloxy carbonyl benzoic acid		-	-	-	View
2-Octenoic acid	2-Octenoic acid	HDMB0000392	5282713	-	View
Propanone. 1ethylthio		-	-	-	View
3- Methylthio benzoic acid		-	-	-	View
3-Indoleacetic acid	Indoleacetic acid	HMDB0000197	802	C00954	View

Step 4: After the pathways were determined by the MetaboAnalyst, the pathways with the highest impact factor and pathways with a metabolite that was prevalent in most of the pathways picked up are discussed in the results section. Impact factor refers to the pathway impact score in pathway analysis. This score is a measure of the significance and relevance of a particular metabolic pathway in the study, based on the experimental data.

5.3 Results and discussion

5.3.1 Matching csv files to the MetaboAnalyst 5.0 metabolic pathways

The MetaboAnalyst 5.0 identified 20 out of 106 compounds that are linked to various metabolic pathways. **Table 5.2** presents both the total number of compounds (Total Tmpd) associated with each pathway and the hits, which denote the compounds in the csv excel sheet that matched with the compounds in Total (Cmpd). The assessment of these hits relies on raw p-values, with a lower p-value (≤ 0.05) and an impact value near 1 highlighting significant pathway alterations between two groups, Farm and Non-Farm[829,830].

For instance, row 22 of **Table 5.2** shows the biosynthesis pathway for phenylalanine, tyrosine, and tryptophan, where two compounds (L-Phenylalanine and L-Tyrosine) have an impact value of 1.00 but a raw p-value of 0.78. This suggests that the pathway is not significantly affected by exposure to insecticide. Despite this, the impact factor underscores the significant role of the pathway in shaping the metabolomic profile. L-Phenylalanine and L-Tyrosine are key aromatic amino acids that play pivotal roles in human physiology. As precursors in the synthesis of neurotransmitters, they are fundamental to the production of proteins and melanin influencing skin and hair colour. Moreover, their involvement in maintaining neurotransmitter balance is vital for mood regulation and cognitive functions[831-834]. These amino acids are also of considerable interest in biotechnology, given their wide-ranging implications in health and disease management [835-837]. Additionally, Karen *et al*. 2001 noted that exposure to insecticides like permethrin (a PYR) and chlorpyrifos (a OP) disrupts dopaminergic pathways, potentially causing physiological and developmental issues in insects and mammals[838].

However, the analysis of compiled raw data from 61 participants showed that only 8 participants had detectable levels of at least one of the essential amino acids (L-Phenylalanine, L-Tyrosine, or

Tryptophan) in their urine samples. Such a discrepancy in amino acid levels warrants clinical evaluation and further diagnostic testing to investigate the underlying causes and implications of these metabolic differences in the 8 participants.

Table 5. 2: An overview of the impact of various compounds, in the urine samples, on different metabolic pathways.

Row	Pathway	Total Cmpd	Hits	Raw p	LOG10(p)	Holm adjust	FDR	Impact
1	Fatty acid biosynthesis	47	Hexadecanoic acid, Decanoic acid, Tetradecanoic acid, Octanoic acid	0.003505	2.4553	0.080614	0.080614	0.01473
2	Fatty acid elongation	39	Hexadecanoic acid	0.030856	1.5107	0.67883	0.30186	0
3	Fatty acid degradation	39	Hexadecanoic acid, omega-Hydroxy fatty acid	0.054552	1.2632	1	0.30186	0
4	Biosynthesis of unsaturated fatty acids	36	Hexadecanoic acid; Octadecanoic acid	0.078694	1.1041	1	0.30186	0
5	Glycine, serine and threonine metabolism	33	Glycine, Methylglyoxal	0.094111	1.0264	1	0.30186	0.25981
6	Nitrogen metabolism	6	HCO3-	0.14067	0.85181	1	0.30186	0
7	Pyruvate metabolism	23	(S)-Lactate, Methylglyoxal, ethanol	0.14468	0.8396	1	0.30186	0.0283
8	Glyoxylate and dicarboxylate metabolism	32	Glycine	0.15749	0.80274	1	0.30186	0.10582
9	Glutathione metabolism	28	Glycine	0.15749	0.80274	1	0.30186	0.08873
10	Primary bile acid biosynthesis	46	Glycine	0.15749	0.80274	1	0.30186	0.00758
11	Lipoic acid metabolism	28	Glycine	0.15749	0.80274	1	0.30186	0.0017
12	Porphyrin metabolism	31	Glycine	0.15749	0.80274	1	0.30186	0
13	Phenylalanine metabolism	8	L-Phenylalanine, Phenylacetic acid, L-Tyrosine	0.21855	0.66045	1	0.36542	0.35714
14	Tryptophan metabolism	41	L-Tryptophan, Anthranilate, Indole-3-acetate	0.23358	0.63156	1	0.36542	0.14507
15	Glycolysis / Gluconeogenesis	26	Ethanol, (S)-Lactate	0.26294	0.58014	1	0.36542	0

Table 5.2: Continuation.

Row	Pathway	Total Cmpd	Hits	Raw p	LOG10(p)	Holm adjust	FDR	Impact
16	Citrate cycle (TCA cycle)	20	Succinate	0.30187	0.52018	1	0.36542	0.03273
17	Alanine, aspartate and glutamate metabolism	28	Succinate	0.30187	0.52018	1	0.36542	0
18	Propanoate metabolism	22	Succinate	0.30187	0.52018	1	0.36542	0
19	Butanoate metabolism	15	Succinate	0.30187	0.52018	1	0.36542	0
20	Caffeine metabolism	10	Caffeine	0.42373	0.37292	1	0.48728	0
21	Ubiquinone and other terpenoid-quinone biosynthesis	18	4-Hydroxybenzoate, L-Tyrosine	0.54664	0.2623	1	0.5987	0
22	Phenylalanine, tyrosine and tryptophan biosynthesis	4	L-Phenylalanine; L-Tyrosine	0.78241	0.10657	1	0.81797	1
23	Tyrosine metabolism	42	L-Tyrosine	0.94454	0.02478	1	0.94454	0.13972

TotalCmpd= total compounds
Raw p= p-value
Holm adjust= procedure for adjusting p-values
LOG10(P)= logarithm (base 10)
FDR= False Discovery Rate

Table 5.2 and **Figure 5.4** showed that while some metabolic pathways showed an impact from insecticide exposure, their impact values were relatively low (below 0.5). **Figure 5.4** uses dotted circles to highlight pathways significantly impacted according to pathway impact analysis. Despite some pathways having an impact factor above 0.0, indicated by larger dots, the pathways were not considered significant. Consequently, the significance of a pathway's impact from insecticide exposure relies on statistical tests, like p-values which assess if effects are likely not by chance. Additionally, **Table 5.2** revealed that some pathways included significant hit compounds with raw p-values less than 0.05, indicating the influence of insecticide exposure on these pathways. However, the potential for these pathways to undergo substantial alterations appears to be limited. One possible explanation for this observation could be the relatively small number of compounds investigated as most of compounds were removed as they were derivatised compounds within the two population groups. These tests consider experiment variability and sample size. Therefore, a pathway showing an impact doesn't guarantee significance unless it meets statistical criteria, adjusted to reduce false positives through methods like the Bonferroni correction[839,840].

Additionally, the significance of a pathway's impact also weighs on its biological relevance[841]. If a pathway with a high impact factor doesn't align with the study's goals or its role in the organism's response to the insecticide is unclear, it won't be deemed significant. Only pathways that exhibit statistically solid and biologically meaningful changes are marked as significant, ensuring that the analysis of insecticide exposure's effects is accurate and dependable[841]. This information provided valuable insights into potential metabolic pathways influenced by insecticides exposure, aiding in a deeper understanding of the associated biological mechanisms.

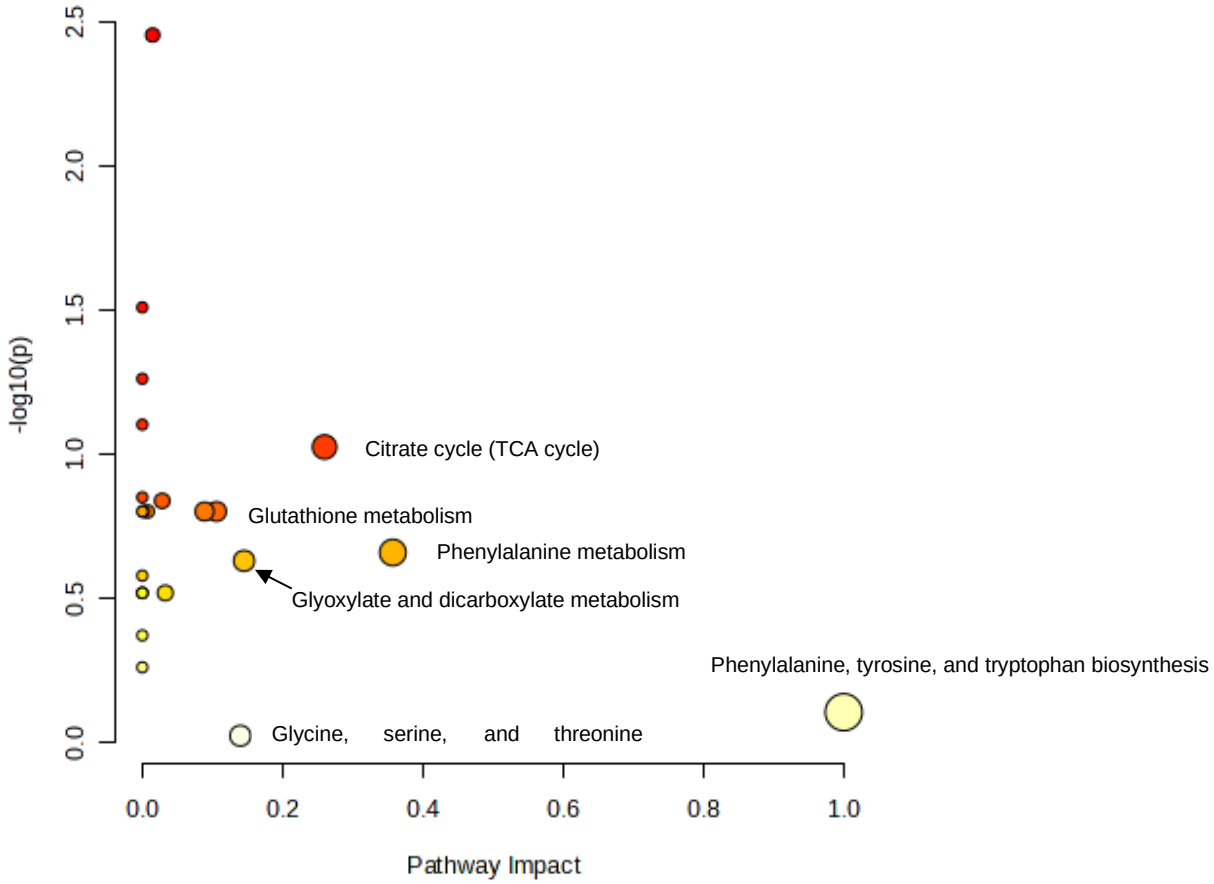


Figure 5. 4: Pathway impact analysis

Table 5.2 shows alterations in the prevalence of several amino acids in the Hit column and that includes glycine, serine, and threonine, indicating potential disruptions in protein synthesis and energy metabolism. Notably, glycine which is a nonessential amino acid, is highlighted as the most prevalent compound which suggest its involvement in multiple metabolic pathways. As noted by Alves *et al*. 2019, glycine plays a critical role in human physiology, participating in protein synthesis, glutathione production for antioxidant defence, and creatine formation[842]. Moreover, glycine plays a role in regulating blood sugar and has potential therapeutic uses in treating metabolic disorders such as obesity. Additionally, glycine's benefits include reducing oxidative stress and inflammation in metabolic syndrome [843,844]. This comprehensive role of glycine underscores its significance in overall metabolic health and disease management.

5.3.2 Detection of glycine non-essential amino acid

Detection of glycine by MetaboAnalyst indicates alterations in various metabolic pathways. Glycine's role in the synthesis of glutathione, a major antioxidant, and may influence metabolic disorders and mitochondrial function that affect various pathways [845]. Disorders in glycine metabolism can manifest in conditions like non-ketotic hyperglycinaemia, affecting neurological functions [842,843,846,847]. In this study, Glycine was detected in 23 out of 61 participants (2 from non-farm areas and 21 from farms) highlighting that farm-dwelling children's metabolic pathways could be influenced by insecticide exposure, though no significant pathway alterations were detected. **Figure 5.5** below show the participants who had glycine detected in their urine sample in non-targeted analysis, the Figure also shows what other amino acids were detected in each of the 23 participants. However, no significant effects on metabolic pathways were found related to the urinary levels of OP and PYR metabolites, despite increased glycine detection in farm-dwelling children.

Only the glycine pathway was drawn and discussed in relation to how the insecticides affect the pathway as it was found in more participants that any other amino acid and/ compounds. There is limited and inconclusive evidence to suggest that exposure to OPs and PYRs directly causes levels of glycine to be detected in urine. However, there are a few potential indirect mechanisms that warrant further research that are mentioned in the next section. Additional results will be explored when the samples are run on a LC for a non-targeted analysis to avoid derivatization which alters compounds to give more clarity in Metabolomics studies

	Participant ID	23	28	29	30	31	32	35	45	51	53	78	80	86	114	122	147	149	158	159	176	180	240	399
	Residence	Farm	Farm	Farm	Farm	Farm	Farm	Farm	Farm	Farm	Farm	Farm	Farm	Non-farm	Farm	Farm	Farm	Farm	Farm	Farm	Farm	Farm	Farm	Non_farm
Compound name		Peak areas																						
Glycine		9210102	6617638	13270646	7809693	19701687	2352078	3233673	4306571	68755	19704971	28410426	7584554	11746915	10461598	18390665	3974140	5128898	2363412	4322840	352828	42978860	632810	19347629
L-Phenylalanine, N-benzoyl-		760588	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Phenylalanine, 2TBDMS derivative		361165	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
L-Phenylalanine, 2TMS derivative		X	X	X	X	5466221	4459482	X	3889224	4490640	X	X	X	4650246	X	X	X	X	X	X	X	X	X	X
L-Serine, 3TBDMS derivative		643163	X	X	26054354	X	12195578	X	X	X	21006428	X	X	X	X	X	34796671	X	X	X	X	X	X	X
L-Serine, N-glycyl-		X	X	X	1582943	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
L-Tyrosine, 3TMS derivative		379068	X	X	X	X	X	X	X	X	X	X	4306616	X	2297193	X	X	X	X	X	X	X	X	X
L-3-Methoxytyrosine, 3TMS derivative		263458	X	X	33203	X	X	18334	X	X	X	X	X	X	356170	X	X	29790	X	X	X	X	X	X
L-Tryptophan, ethyl ester		X	520972	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Tryptophan		X	X	X	X	X	X	X	X	357512	X	X	X	X	X	X	X	X	X	X	X	X	X	X
L-Tryptophan		X	X	81884	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
l-Tryptophan, N-formyl-		X	X	X	X	X	X	X	639850	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
l-Tryptophan, TMS derivative		X	X	X	X	X	X	X	259898	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
L-Tryptophan, N-acetyl-		X	X	60544	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
L-Threonine, N-(trifluoroacetyl)-O-(trimethylsilyl)-, trim		X	X	161998	X	X	X	X	X	X	X	X	X	X	X	X	2580034	X	X	X	X	X	X	X
DL-Tryptophan, 5-methoxy-		X	X	X	X	X	156463	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	591453
L-Tryptophan, N(1)-(trimethylsilyl)-, trimethylsilyl ester		X	X	X	X	X	X	X	X	X	X	1373128	X	X	X	228530	X	X	X	X	X	X	X	X
L-Tryptophan, N-methyl-, methyl ester		X	X	X	X	X	X	X	X	X	X	X	X	233991	X	X	X	X	X	X	X	X	X	X
dl-Tryptophan ethyl ester		X	X	X	X	X	X	X	X	X	X	X	X	205911	X	X	X	X	X	X	X	X	X	X
d-Tyrosine		X	X	X	X	X	X	X	X	X	X	X	X	X	562073	X	X	X	X	X	X	X	X	X
tyrosine, N-(2-methyl-1-oxo-2-propen-1-yl)-		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	1446653	X	X	X	X	X	X	X

Figure 5. 5: Image showing the participants who had glycine and other amino acids detected in their urine samples.

5.3.2.1 Hypothesized implications of OP and PYR Exposure on the Glycine Metabolic Pathway

The non-targeted screening results suggest that insecticide exposure, particularly OP and PYR exposure, may disrupt crucial metabolic pathways, particularly those related to amino acid metabolism, oxidative stress, and mitochondrial function. Upon entering the body, insecticides, undergo biotransformation (metabolism), potentially interfering with enzymes involved in amino acid biosynthesis and degradation[848,849].

OPs and PYR exposure may lead to elevated levels of glycine in the human body through several mechanisms related to both neurotransmission and glycine pathway metabolism.

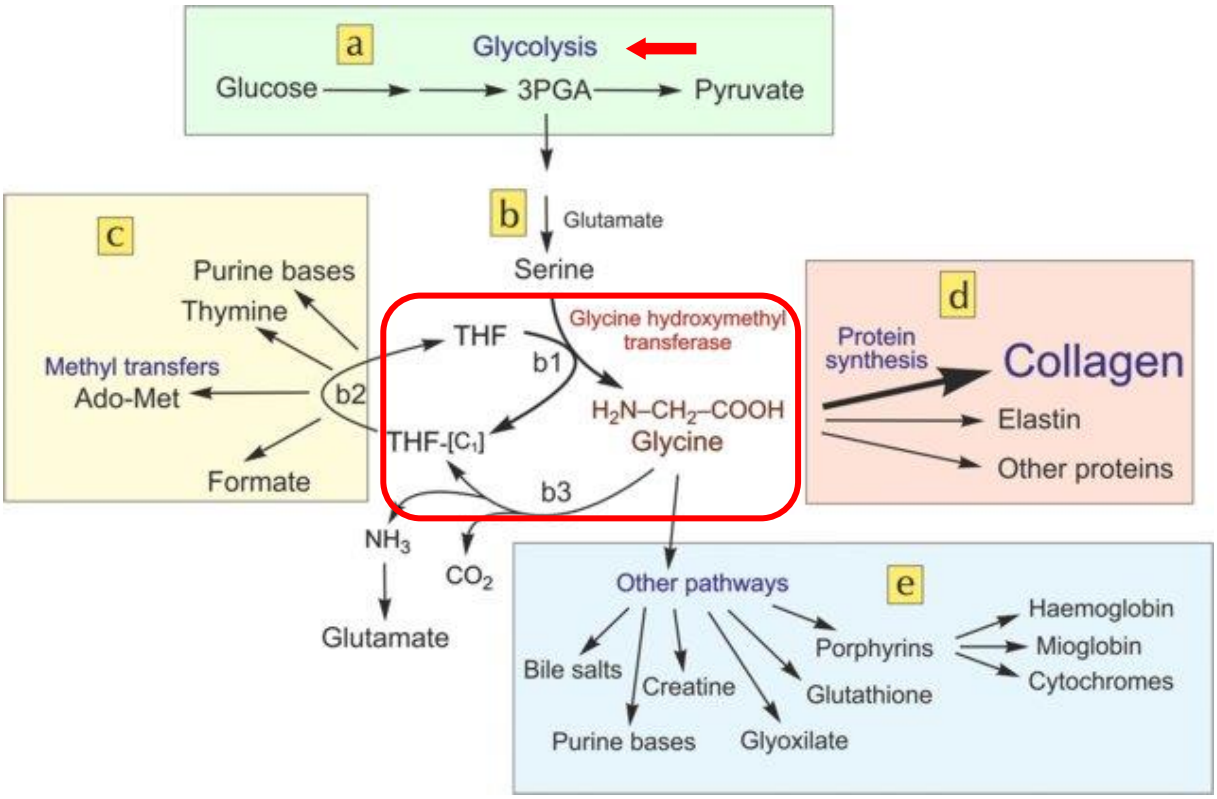


Figure 5. 6: Figure illustrates the glycine metabolism pathway: Interconnected pathways showcasing glycine's synthesis, degradation, and contribution to diverse cellular processes, with potential disruption points by OP/PYR metabolites highlighted by the red arrow on glycolysis pathway (a) and the red box (b), is where the study is assuming the OP and PYR insecticides affect. Image was adapted from de Paz *et al* 2018[850].

Figure 5.6 illustrates the glycine metabolism pathway from (a) to (e), starting from glycolysis (a), where glucose is converted into 3-phosphoglycerate (3PGA), an intermediate that leads to serine production. Section b shows the dynamic interplay between serine and glycine which is crucial for neurotransmitter synthesis. Serine is converted into glycine through the enzyme glycine hydroxymethyltransferase, highlighting the pathway's direct contribution to the production of glycine. From there, glycine enters various critical processes: in section c, glycine is involved in purine and thymine synthesis and supports methylation reactions through S-adenosyl methionine (AdoMet). In section d, glycine contributes to protein synthesis, particularly the formation of collagen and other structural proteins like elastin. Finally, section e shows glycine's involvement in other pathways, where it plays a role in the synthesis of important molecules such as creatine, glutathione, porphyrins, bile salts, and haemoproteins like hemoglobin[850-852] . This figure demonstrates the interconnected nature of glycine metabolism across both anabolic and catabolic processes.

The red arrow and box highlight potential areas of disruption in glycine metabolism due to OP and PYR exposure, leading to detectable levels of glycine in urine. This study hypothesizes that OP and PYR metabolites primarily affect pathway a (glycolysis) and pathway b (Serine-Glycine interconversion & one-carbon metabolism), contributing to the observed glycine elevation in the 23 participants. The OPs and PYRs disrupt glycine metabolism (pathway and b) by interfering with key processes that regulate glycine levels in the body. Glycine acts as an inhibitory neurotransmitter in the CNS, helping to control synaptic signals. However, OPs and PYRs block the function of the glycine cleavage system (pathway b), a critical pathway that breaks down excess glycine[853,854]. Additionally, these insecticides inhibit enzymes that facilitate glycine degradation, leading to its accumulation. This metabolic imbalance affects neurotransmission, which can result in glycine receptor dysfunction and trigger neurological effects like depression and anxiety[855-858]. The broader impact of OP and PYR exposure may goes beyond neurotransmission since it has been reported that reactive oxygen species (ROS) generated through insecticide exposure cause oxidative stress. Oxidative stress causes damage to biochemical pathways, including amino acid metabolism, where proteins and enzymes involved in regulating glycine, L-phenylalanine, and L-tyrosine are affected. Amino acids are essential for neurotransmitter synthesis, energy production, and the formation of antioxidants. Consequently, oxidative stress and metabolic dysfunction together create neurological and systemic imbalances that affect overall health[859-865].

The body's response to disruptions in glycine metabolism acts as a compensatory mechanism. Muscle breakdown releases glycine into the bloodstream, and the neuroprotective role of glycine triggers the body to increase glycine synthesis. This process aims to counteract neurotoxic effects from insecticide exposure, yet it also raises overall glycine levels[866-868]. Mitochondrial dysfunction, often caused by insecticide exposure, plays a key role since mitochondria are crucial for energy metabolism and glycine breakdown. When mitochondrial function declines, enzymes responsible for glycine degradation become less effective[869-871], worsening glycine accumulation and creating a cycle of metabolic imbalance.

The link between glycine and glutathione, a key antioxidant, adds further complexity. Glycine serves as a precursor to glutathione, and oxidative stress increases the body's need for this antioxidant[872-875]. Higher glycine levels may indicate the body's effort to produce more glutathione to fight reactive oxygen species. However, prolonged OP and PYR exposure can overwhelm this protective mechanism, especially when renal dysfunction limits glycine excretion[859-861]. The combined effect of reduced excretion and higher demand keeps glycine levels elevated, which can have both protective and harmful effects. The metabolic disruptions caused by insecticides, especially in children, raise concerns. Imbalances in amino acids, particularly glycine, can affect growth, development, and long-term health[876,877]. Reduced glycine levels also hinder glutathione production, leaving cells more prone to oxidative damage[878]. Understanding the relationships between glycine metabolism, oxidative stress, and mitochondrial function is crucial for evaluating the impact of environmental toxins on human health.

The detection of glycine in urine samples from 23 participants confirms its significance as a critical biomarker in metabolic health research. This finding aligns with evidence linking glycine to insulin resistance and type 2 diabetes. Moreover, the detection of glycine as an affected metabolic pathway in farm-dwelling children may be attributed to insecticide-induced oxidative stress, which increases glycine utilization for glutathione synthesis[879-881]. Alternatively, insecticides could directly disrupt glycine metabolism, affecting various physiological processes. Although dietary variations and underlying health conditions cannot be entirely ruled out, the association between glycine and metabolic disorders suggests its potential as a biomarker of insecticide exposure and its broader role in metabolic health.

The study emphasizes glycine's potential for non-invasive diagnosis and monitoring, showcasing the value of metabolic profiling in personalized healthcare. Advanced techniques like urine analyses and

NMR spectroscopy have further highlighted glycine's importance in diagnosing metabolic disorders. Profiling glycine conjugates, such as acylglycines, has linked glycine metabolism to conditions like coccidioidomycosis and diabetes[843,882-886]. These findings underscore glycine's potential as both a metabolic regulator and a biomarker for diverse health conditions.

To further enhance our understanding of the metabolic pathways affected by OPs and PYRs, the use of the mass spectrometer Time-of-Flight Mass Spectrometry (ToF-MS) combined with the 2-D GCxGC offers a powerful tool (non-targeted analysis technique). The technique enables the comprehensive detection of metabolic changes across various biological samples which allows researchers to identify and quantify a wide range of metabolites. By providing a detailed metabolic fingerprint, ToF/MS allows for the identification of disruptions in pathways, including those affected by environmental toxins like OPs and PYRs.

5.3.2.2 Toxicological Implications of OP/PYR Exposure: Metabolic Pathway Disruptions Revealed by ToF/MS

The use of ToF-MS in metabolomics is indispensable for understanding the metabolic disruptions caused by environmental pollutants, such as insecticides. ToF-MS offers exceptional mass accuracy and resolution, which are crucial for identifying low-abundance metabolites and subtle metabolic changes that other techniques might overlook. This capability is particularly significant in detecting metabolic pathways affected by insecticide exposure, such as amino acid metabolism, oxidative stress, and mitochondrial dysfunction[887,888]. Furthermore, ToF-MS enables researchers to capture transient metabolites, which are essential for analysing change processes in metabolism, especially in the context of oxidative stress where reactive oxygen species can alter metabolite profiles quickly[888]. For instance, studies have demonstrated how ToF-MS's high sensitivity has been critical in distinguishing biomarkers related to exposure and metabolic disruption in complex biological samples like urine. This technology's rapid data acquisition and high resolving power allow for a comprehensive metabolomic profile, advancing our understanding of how environmental toxins impact human health at the molecular level, making it a powerful tool in toxicology and environmental health studies [888,889].

By utilizing ToF-MS in metabolomic studies, researchers can uncover novel biomarkers and pathways that are critical for early detection of exposure-related health risks, offering significant insights into the

long-term health consequences of insecticide exposure, particularly in vulnerable populations such as children.

5.4 Conclusion

The study revealed that metabolite derivatization influenced their identification by the MetaboAnalyst, highlighting a critical aspect for future metabolomic research. Derivatization modifies metabolites to enhance detectability but may lead to derivatives unrecognized by MetaboAnalyst, which prefers non-derivatized compounds. Future research could leverage LC for untargeted analysis in urine and hair, circumventing derivatization issues encountered with the 2-D GCxGC ToF/MS. LC, not requiring derivatization, aligns more closely with MetaboAnalyst's database. Enriching MetaboAnalyst's database to encompass derivatized compounds and refining algorithms for their identification could improve the utility of 2-D GCxGC ToF/MS data in metabolomics. Integrating LC and GC data promises a broader metabolomic profile, enhancing our understanding of the metabolome.

The MetaboAnalyst 5.0 analysis pinpointed 20 out of 106 compounds related to metabolic pathways, thus providing significant insights into the metabolic consequences of insecticide exposure. In doing so, it notably identified essential amino acids like L-Phenylalanine and L-Tyrosine, alongside the non-essential amino acid glycine in urine samples. Despite the lack of significant disturbances in their pathways, the detection of these amino acids underlines their crucial roles in human physiology, particularly in neurotransmitter synthesis. Hence, the presence of these amino acids, even without marked pathway disruptions, hints at the subtle and complex influence of insecticide exposure on metabolic functions. Therefore, this finding stresses the importance of conducting in-depth clinical and epidemiological research to comprehend the extensive effects of insecticide exposure on public health. This is especially crucial for understanding its impact on the metabolic processes of susceptible individuals, like children in agricultural regions. The research should focus on clarifying how insecticides affect these metabolic processes and overall health, which will support the creation of specific public health strategies and policies. Additionally, investigating genetic susceptibility could open avenues for personalized medicine in addressing or forestalling the adverse effects of insecticide exposure.

Ultimately, such an integrated approach will enrich our understanding of the impacts of insecticide exposure and encourage the development of effective risk mitigation strategies.

The detection of glycine in 23 out of 61 participants highlights the need for further research to understand its clinical significance. While it's possible that these children fall within the upper range of normal glycine excretion, factors such as diet, metabolic variations, and minor differences in laboratory measurements could also influence the results. To clarify whether these glycine levels indicate any health concerns, detailed medical histories, clinical evaluations, and additional lab tests are required. Moreover, exploring environmental factors or exposures that may affect glycine metabolism is crucial. Overall, while this finding underscores the potential importance of glycine as a biomarker, a more comprehensive investigation is needed to determine its relevance in paediatric health.

Glycine plays a key role in metabolic pathways and mitochondrial function, impacting various metabolic processes. This underscores the importance of examining glycine in urine samples to probe its metabolic pathway links under varying conditions. Future research should explore how glycine levels change with diet and environmental factors such as insecticide exposure. This work is vital in understanding how diet and environment affect metabolic pathways. It could also lead to identifying biomarkers for the effects of environmental and dietary factors on health, particularly from harmful exposures. Delving into amino acids' roles in metabolism will broaden our understanding of metabolic diseases and support the development of better diagnostic and treatment methods.

5.4.1 Limitations

In evaluating the study's limitations, it is crucial to acknowledge that employing non-targeted metabolomics data through 2-D GCxGC ToF/MS and analyzed with MetaboAnalyst 5.0 broadens biomonitoring scope but faces constraints. The non-targeted analysis though comprehensive may suffer from reduced sensitivity and selectivity compared to targeted methods which could impair subtle metabolic alteration detection. In this study, the reliance on urine samples for non-targeted metabolomics highlights short-term exposure and overlooks the broader spectrum of compounds indicative of long-term exposure that hair samples could provide. Unfortunately, hair did not offer an extensive range of detectable compounds such as urine, leading to its exclusion from detailed analysis. Therefore, integrating both urine and hair samples in future research is essential to capture a full

spectrum of immediate and extended exposure to insecticides to ensure a thorough evaluation of their health impacts.

While MetaboAnalyst 5.0 offers advanced analysis capabilities it might not fully elucidate the complex interplay of metabolite interactions and pathways affected by external variables like diet or environment. Consequently, the study's primary focus on urine analysis may not completely convey the metabolic impact of insecticide exposure stressing the necessity for an integrated approach that combines various biological matrices and longitudinal data collection to thoroughly assess the health implications of insecticide exposure in these communities.

Chapter 6

Conclusion

A novel 2-D GCxGC ToF/MS analytical method was successfully developed and subsequently applied to the analysis of urine and hair samples of children in the Western Cape to detect and quantify DAPs and 3PBA metabolites. The study optimised the SLE process for hair samples and the QuEChERS method for urine sample extraction. The high recovery rates and precision in the analysis served as indicators that these extraction methods are both robust and reliable. The optimisation of the derivatization reaction (MTBSTFA derivatizing agent) through the selection of ethyl acetate as the solvent, timing the reaction, and controlling the temperature, significantly improved the reliability and accuracy of detection and quantification of DAPs and 3PBA. These methodological advancements are not just incremental but represent significant leaps in analytical chemistry.

Chapters 3 and **4** addressed the primary aim of the research study which was to ascertain exposure levels among children in farm areas of the Western Cape, by employing modified extraction methods for OPs/PYRs in urine and hair samples. The study not only achieved this through the application of the 2-D GCxGC ToF/MS quantification method but also extended its findings to offer a comparative exposure analysis across different demographics and timeframes. The study found that the children were exposed to OPs and PYRs in the same manner regardless of their sex, where they lived and the different farming systems they resided in. Therefore, these chapters underscore the precision of the developed method in monitoring OP and PYR exposure levels, thereby revealing critical public health insights. Such data is vital as it can inform policy and foster interventions aimed at safeguarding vulnerable groups, especially children in agricultural regions.

Furthermore, **Chapter 5** introduced the application of the 2-D GCxGC ToF/MS for the non-targeted analysis of urine samples. This approach facilitated a detailed examination of exposure changes by using the MetaboAnalyst 5.0 tool to investigate the potential impacts on metabolic pathways from insecticide exposure. Consequently, the application of the MetaboAnalyst 5.0 tool not only highlighted

its potential for broader research applications but also led to the observation of changes in amino acid concentrations, indicating altered metabolic pathways. This indicates that future studies could benefit from integrating advanced data analysis tools and bioinformatics.

This integration would enhance the interpretation of complex datasets, offering a more nuanced understanding of the metabolites' impact on the biochemistry of humans.

Overall, the study highlighted the method's robustness demonstrated through high recovery rates and consistent precision in both urine and hair samples. This robustness not only affirms the method's utility for broad applications in environmental and occupational health research but also marks a significant advancement in human biomonitoring techniques, particularly within the South African context.

Future studies

Future studies in non-targeted metabolomics should utilize the LC technique and focus on amino acids. The LC can analyse a broad range of metabolites including non-volatile, polar, and large molecules without needing derivatization. This capability makes LC essential for comprehensive metabolite profiling. While improving LC's approach, the importance of GC should not be overlooked as it is important for analysing volatile and semi-volatile compounds with high accuracy. However, in insecticide biomonitoring integrating extensive metabolite data from LC with bioinformatics tools like MetaboAnalyst 5.0 will enable advanced pathway analysis and biomarker discovery, thus deepening insight into the effects of insecticide exposure to biological processes in humans. The study of amino acid profiles in urine samples using LC techniques should take precedence to uncover the metabolic pathways influenced by environmental and physiological factors. This effort will lead to the identification of biological pathways disrupted by factors like insecticide exposure and assist in the early identification of biomarkers for intervention.

Furthermore, the transformative potential of tools like MetaboAnalyst 5.0 in insecticide biomonitoring should be highlighted. These tools facilitate comprehensive metabolic profiling and pathway analysis, providing a holistic perspective on the metabolic disruptions caused by insecticide exposure. Future research should employ these technologies to discover novel biomarkers and to unravel the systemic effects of insecticides on biological pathways. By integrating metabolomics with genomics and proteomics, researchers can achieve a multi-omics view, uncovering the mechanisms that link

insecticide exposure to diseases and improving our capacity to assess and predict health risks. The advanced application of these tools in biomonitoring will markedly enhance disease prevention and management strategies in environmental health research.

Recommendations

Recommendations for future research include focusing on analytical and epidemiological studies to clarify the health effects of insecticide exposure. Analytical studies should investigate dose-response relationships, which examine how varying amounts of insecticide exposure relate to health impacts, and assess the cumulative effects, looking at how combined exposures to different insecticides influence health over time. Epidemiological research should evaluate the long-term health consequences for diverse demographic groups, with a special focus on susceptible individuals like children in agricultural areas. To enhance public health, initiatives are needed to lower exposure through better insecticide application practices and mandatory use of protective gear. Updating policies with the latest scientific findings will lead to stronger public health safeguards, requiring more stringent insecticide regulation. Furthermore, increasing public and professional awareness and education on insecticide hazards and preventive practices, and providing targeted training for healthcare and agricultural workers, are essential steps in reducing exposure risks and protecting community health.

Appendix

Method development with reference standards and internal standards

Derivatised metabolite identification in ethyl acetate.

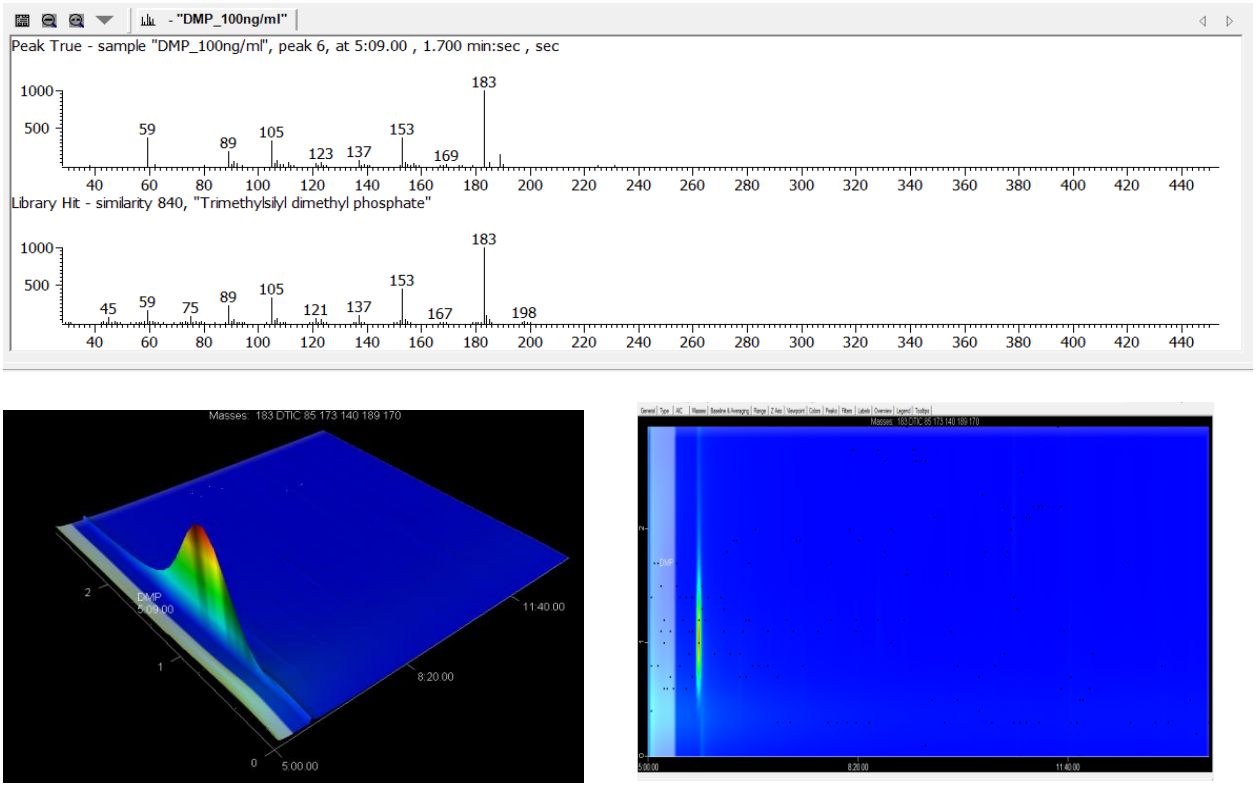


Figure A 2.1: ion chromatogram of DMP and the respective 2D chromatogram.

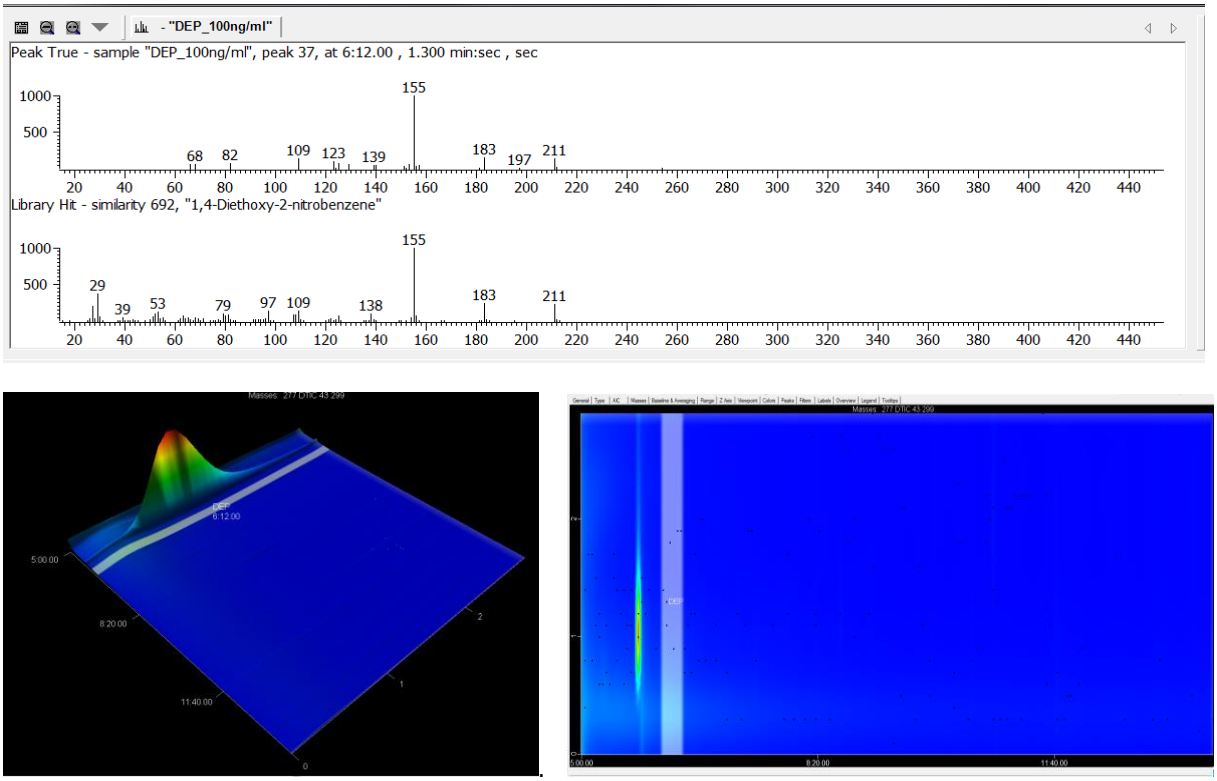


Figure A 2.2: ion chromatogram of DEP and the respective 2D chromatogram

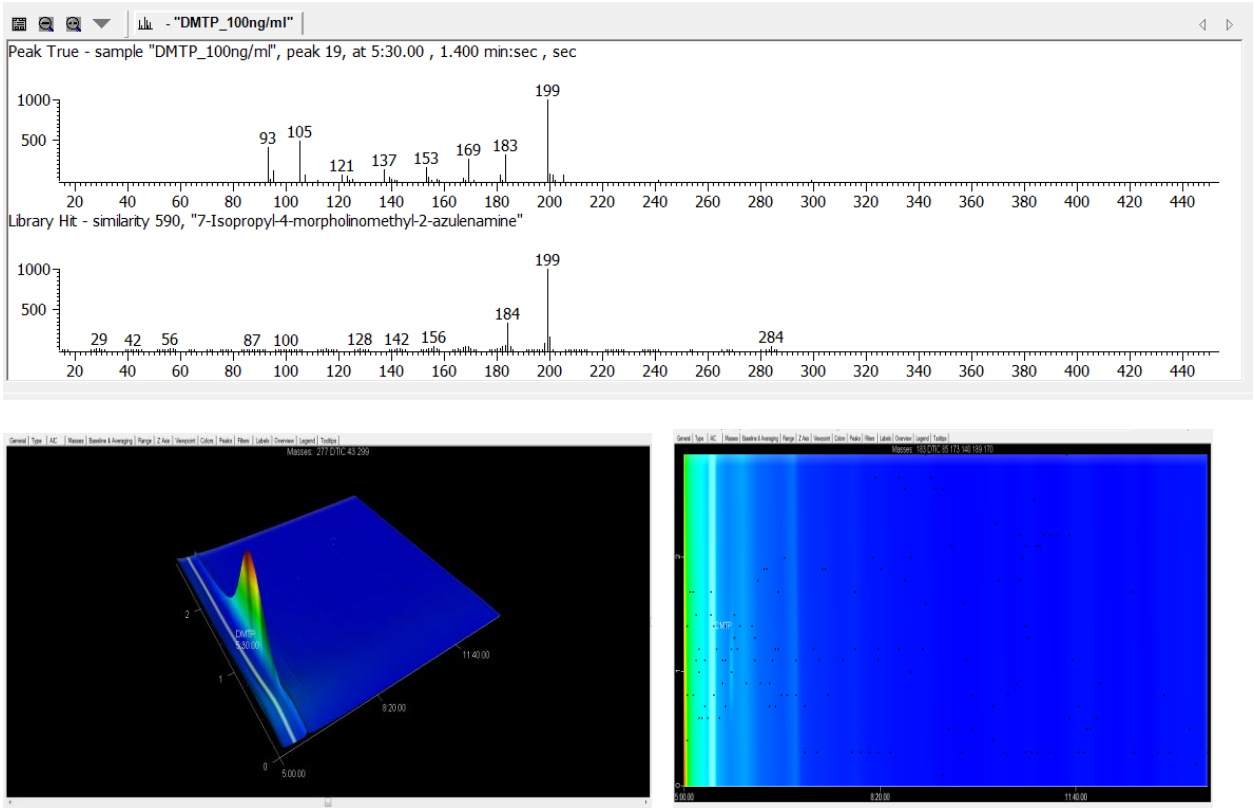


Figure A 2.3: ion chromatogram of DTMP and the respective 2D chromatogram

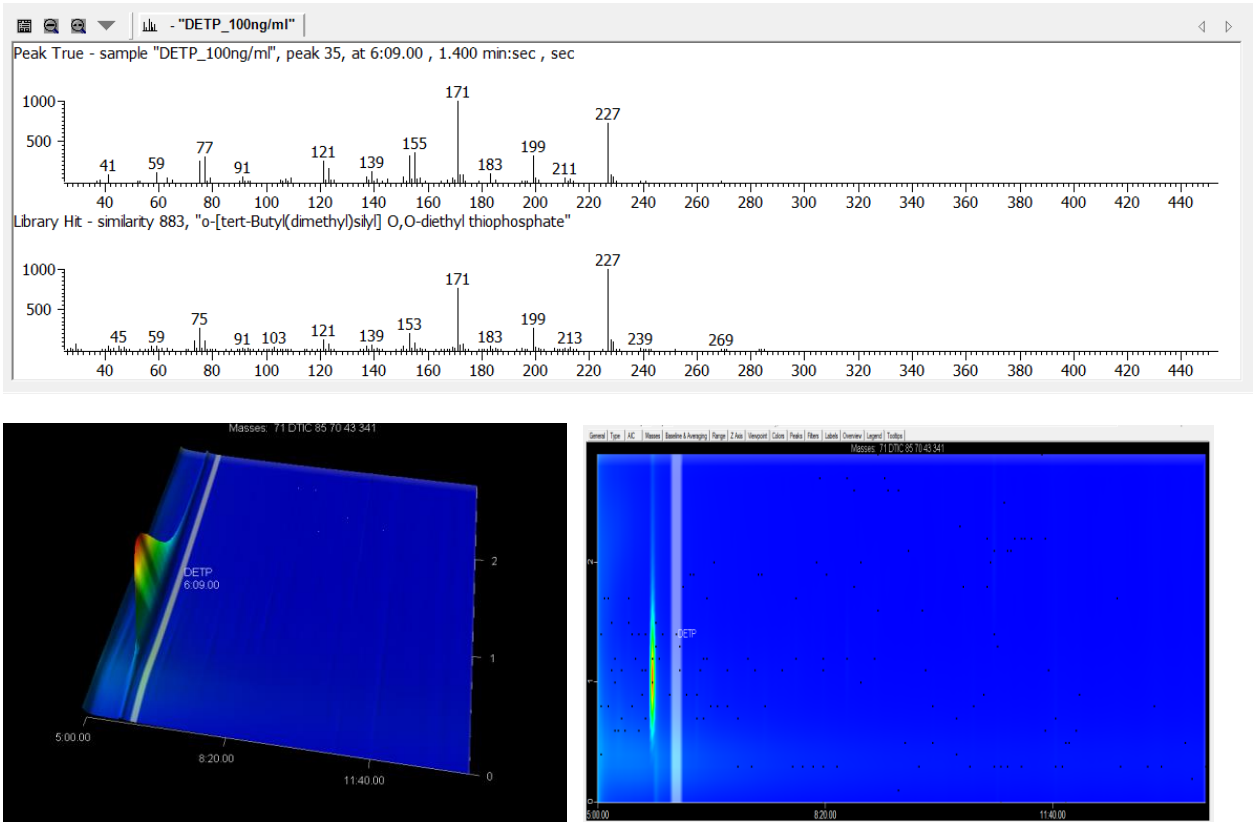


Figure A 2.4: ion chromatogram of DETP and the respective 2D chromatogram

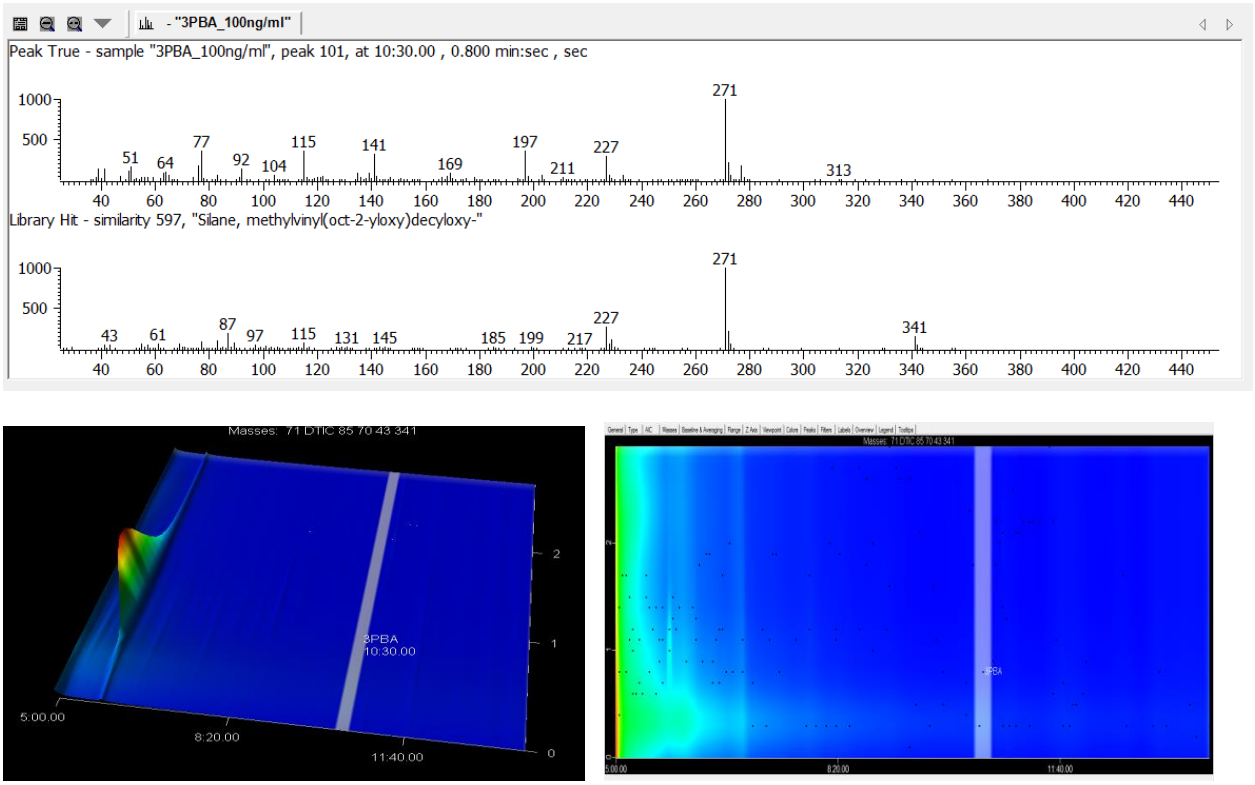


Figure A 2.5: ion chromatogram of 3-PBA and the respective 2D chromatogram

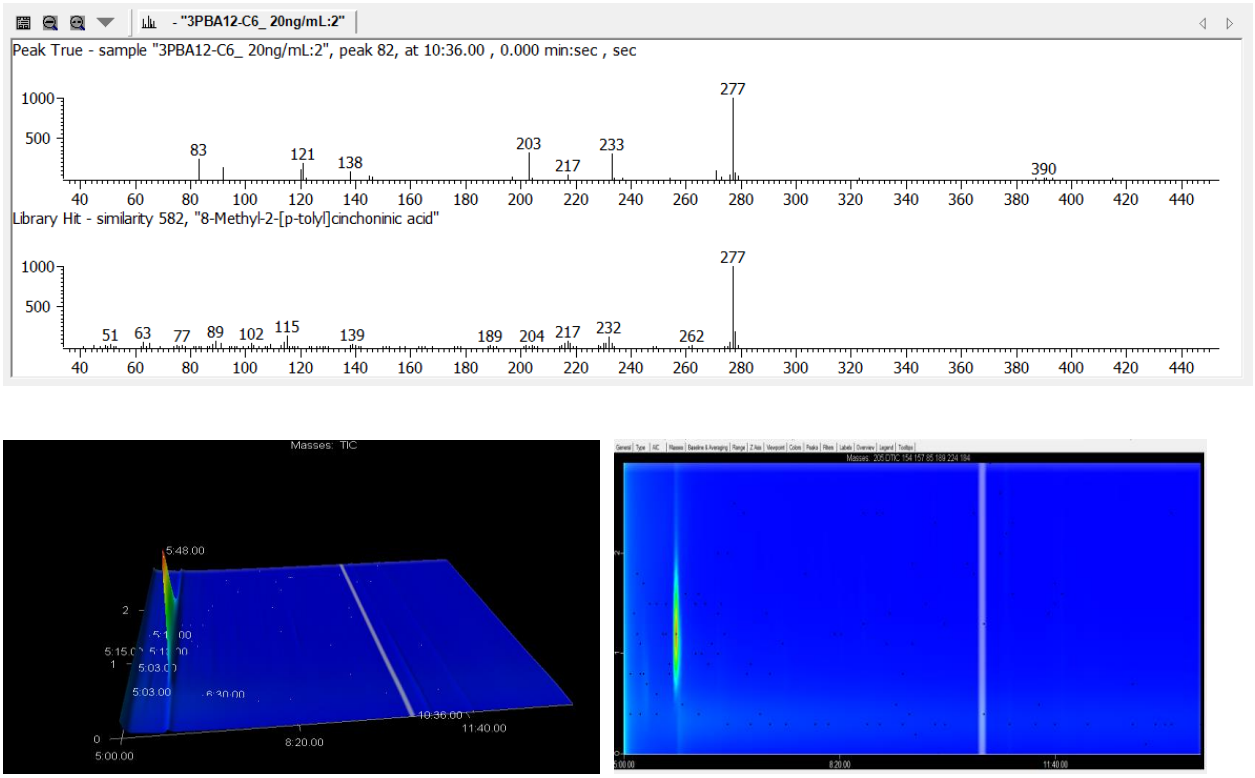


Figure A 2.6: ion chromatogram of 3PBA $^{6}C_{12}$ and the respective 2D chromatogram

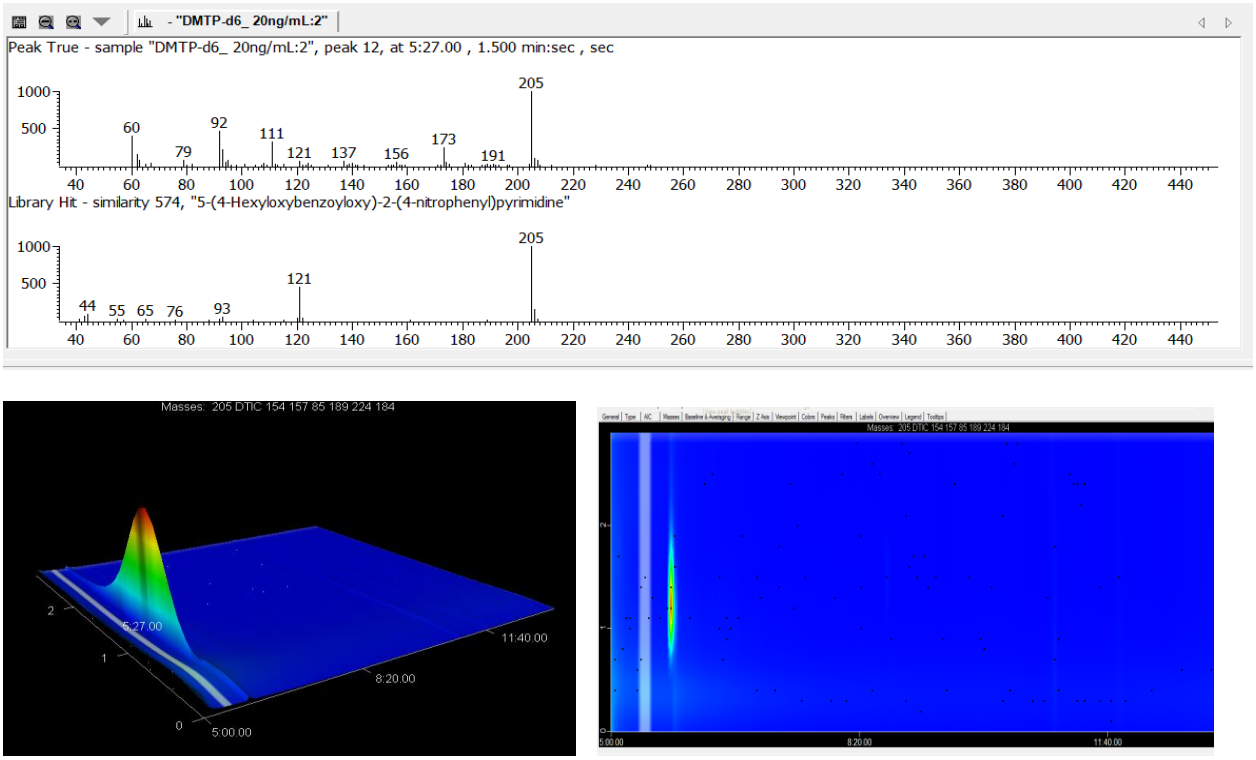


Figure A 2.7: ion chromatogram of DMTP-d^{6} and the respective 2D chromatogram

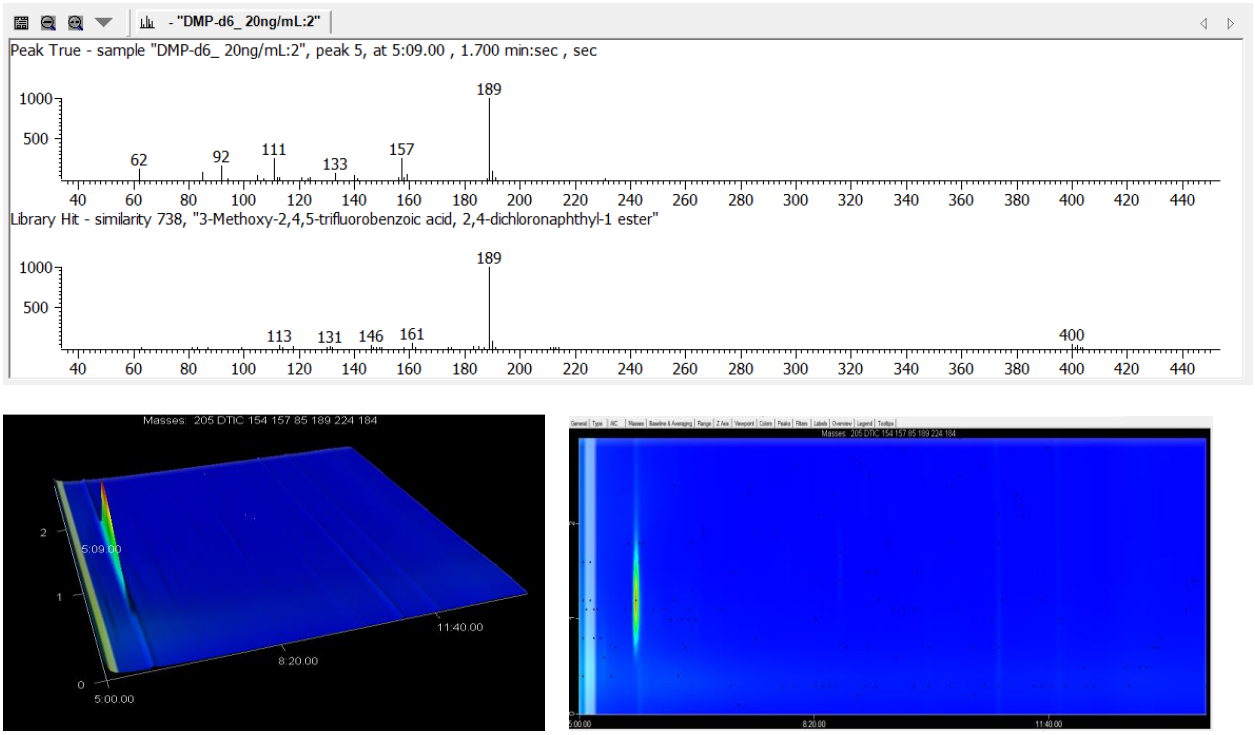


Figure A 2.8: ion chromatogram of DMP-d ^{6} and the respective 2D chromatogram

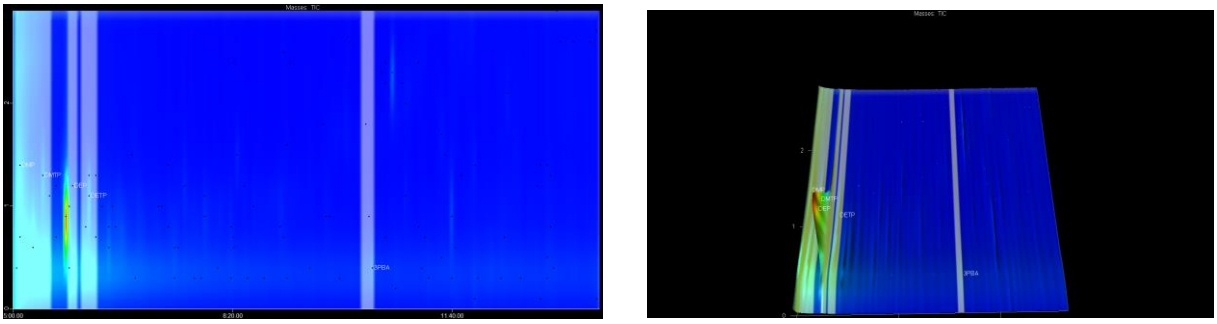
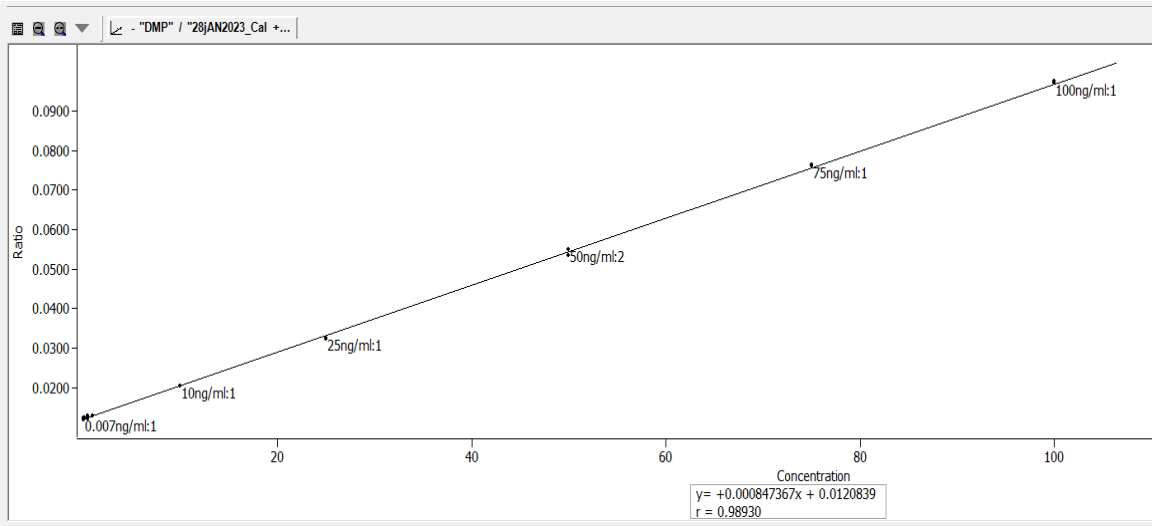


Figure A. 2.10: Ion chromatogram of a sample containing all the metabolites and the respective 2D chromatogram.

Internal standard calibration curve

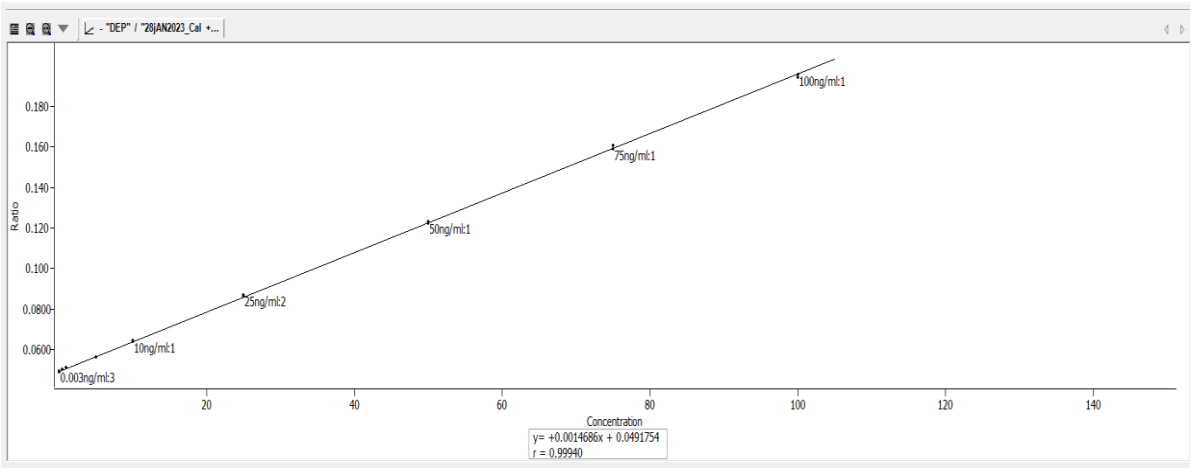
Calibration day 1

Table A 2.1: Regression analysis of DMP and calibration curve



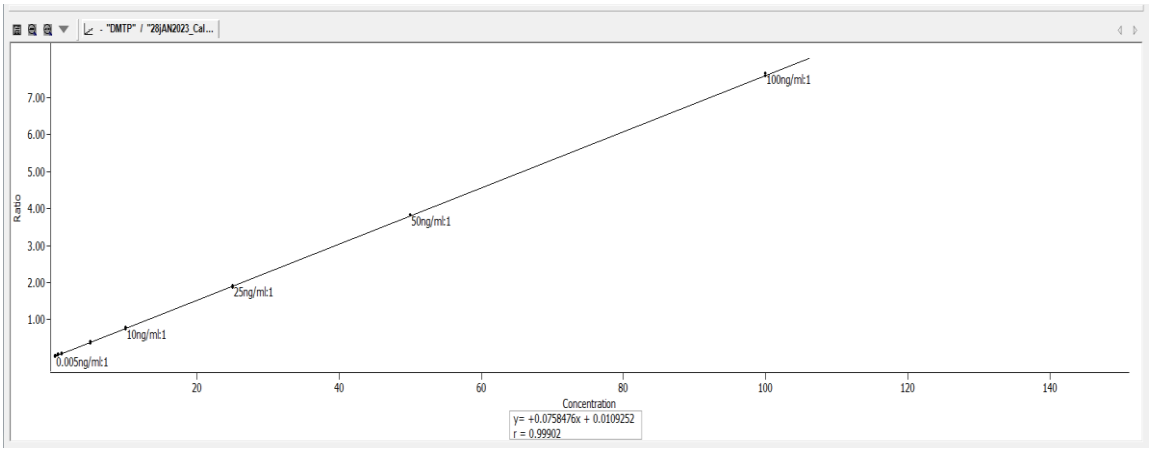
Summary output (DMP)	
Regression Stats	
Multiple R	0.99986328
R Square / Adj. R Square	0.99972658 / 0.99971681
Standard Error / Observations	380.678 / 30
ANOVA (df, SS, MS, F, Sig F)	
Regression	1, 1.48E+10, 1.48E+10, 102378.15, 1.95E-51
Residual	28, 4057645.214, 144915.90
Total	29, 1.48E+10
Coefficients	
Intercept	3096.75, 85.22, 36.34, 4.17E-25, [2922.20, 3271.31]
0.007	644.75, 2.02, 319.97, 1.95E-51, [640.62, 648.88]
Detection Limits	
LOD / LOQ	0.44 / 1.32

Table A 2.2: Regression analysis of DEP and calibration curve



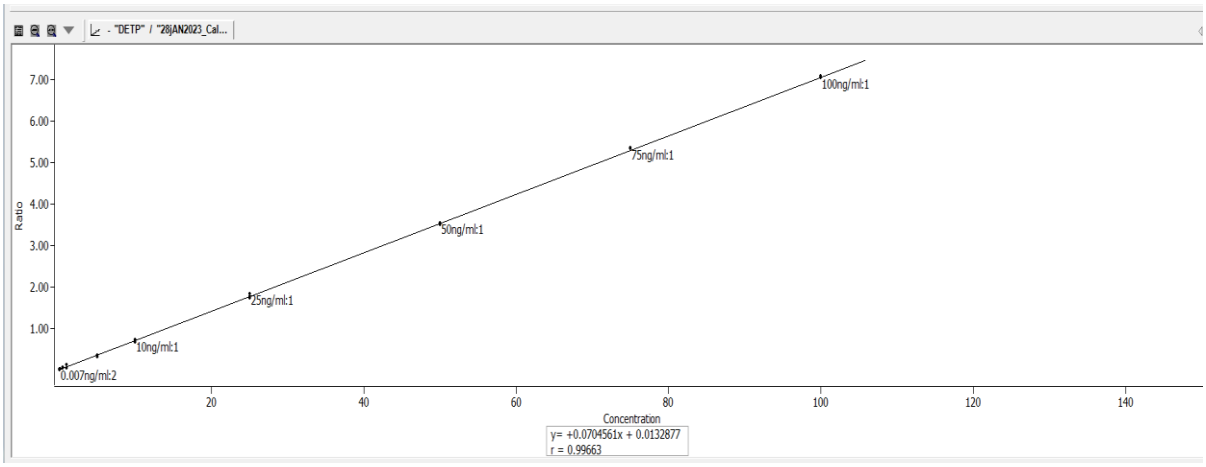
Summary output (DEP)	
Regression Stats	
Multiple R	0.99972700
R Square / Adj. R Square	0.99945500 / 0.99940900
Standard Error / Observations	3337.091 / 24
ANOVA (df, SS, MS, F, Sig F)	
Regression	1, 2.45E+11, 2.45E+11, 21992.06, 5.94E-21
Residual	12, 1.34E+08, 11136179
Total	13, 2.45E+11
Coefficients	
Intercept	804.3703, 1213.211, 0.66301, 0.519857, [-1838.99, 3447.73]
0.003	4044.618, 27.27373, 148.2972, 5.94E-21, [3985.194, 4104.042]
Detection Limits	
LOD / LOQ	0.99 / 3.00

Table A 2.*3*: Regression analysis of DMTP and calibration curve



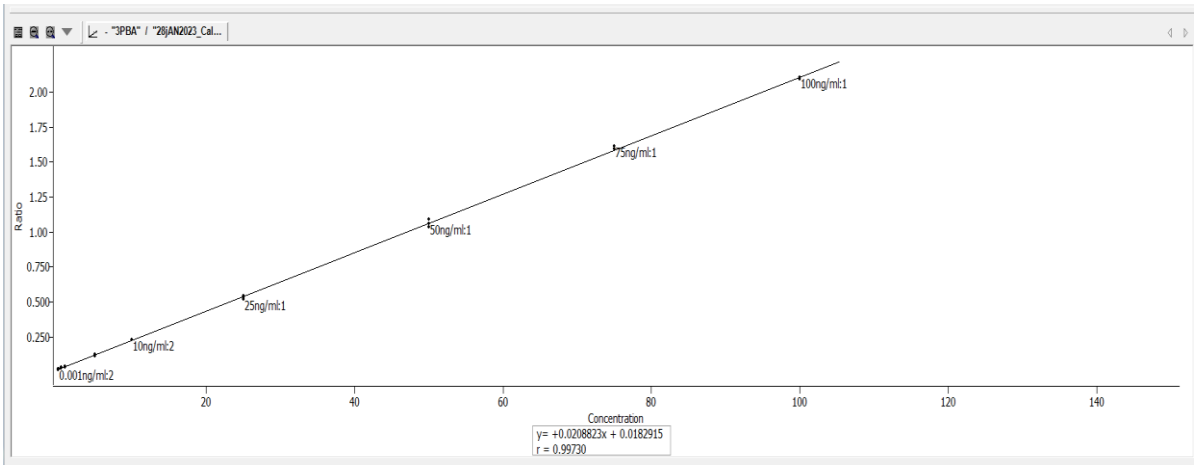
Summary output (DMTP)	
Regression Stats	
Multiple R	0.99946100
R Square / Adj. R Square	0.99892300 / 0.99887200
Standard Error / Observations	10942.47 / 23
ANOVA (df, SS, MS, F, Sig F)	
Regression	1, 2.33E+12, 2.33E+12, 19482.47, 1.18E-32
Residual	21, 2.51E+09, 1.2E+08
Total	22, 2.34E+12
Coefficients	
Intercept	-3321.78, 2862.822, -1.16032, 0.258941, [-9275.35, 2631.783]
0.007	12318.85, 88.25683, 139.5796, 1.18E-32, [12135.31, 12502.4]
Detection Limits	
LOD / LOQ	0.77 / 2.32

Table A 2.4: Regression analysis of DETP and calibration curve



Summary output (DETP)	
Regression Stats	
Multiple R	0.99972700
R Square / Adj. R Square	0.99945500 / 0.99940900
Standard Error / Observations	3337.091 / 27
ANOVA (df, SS, MS, F, Sig F)	
Regression	1, 2.45E+11, 2.45E+11, 21992.06, 5.94E-21
Residual	12, 1.34E+08, 11136179
Total	13, 2.45E+11
Coefficients	
Intercept	804.3703, 1213.211, 0.66301, 0.519857, [-1838.99, 3447.73]
0.007	4044.618, 27.27373, 148.2972, 5.94E-21, [3985.194, 4104.042]
Detection Limits	
LOD / LOQ	0.99 / 3.00

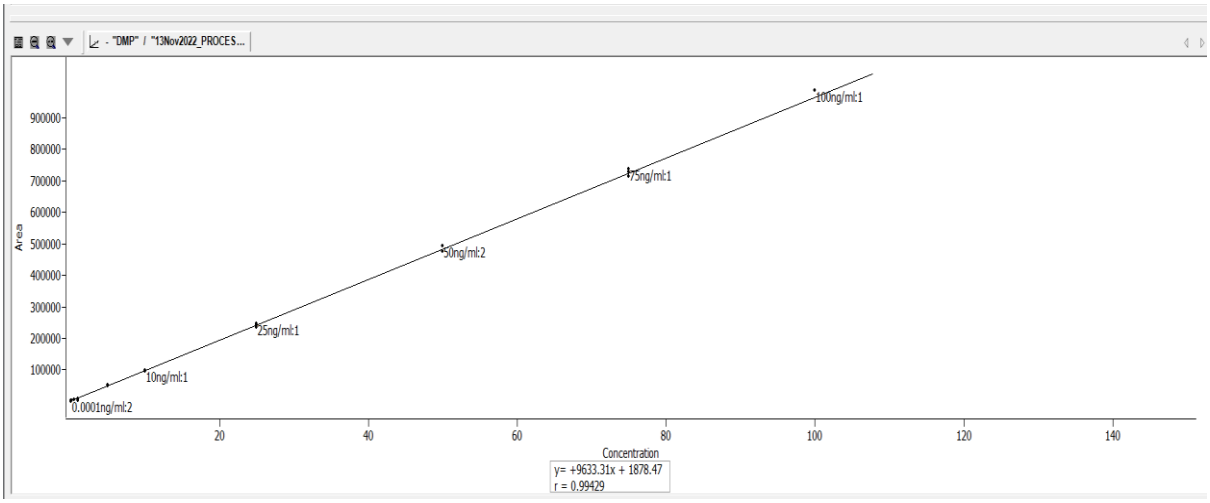
Table A 2.5: Regression analysis of 3PBA and calibration curve



Summary output (3PBA)	
Regression Stats	
Multiple R	0.99997196
R Square / Adj. R Square	0.99994391 / 0.99994240
Standard Error / Observations	584.11793 / 39
ANOVA (df, SS, MS, F, Sig F)	
Regression	1, 2.25E+11, 2.25E+11, 659703.8, 2.94E-80
Residual	37, 12624169.13, 341193.8
Total	38, 2.25E+11
Coefficients	
Intercept	3492.347389, 106.2471769, 32.87003, 5.51E-29, [3277.07, 3707.625]
0.007	2637.38504, 3.247125337, 812.2215, 2.94E-80, [2630.806, 2643.964]
Detection Limits	
LOD / LOQ	0.13 / 0.40

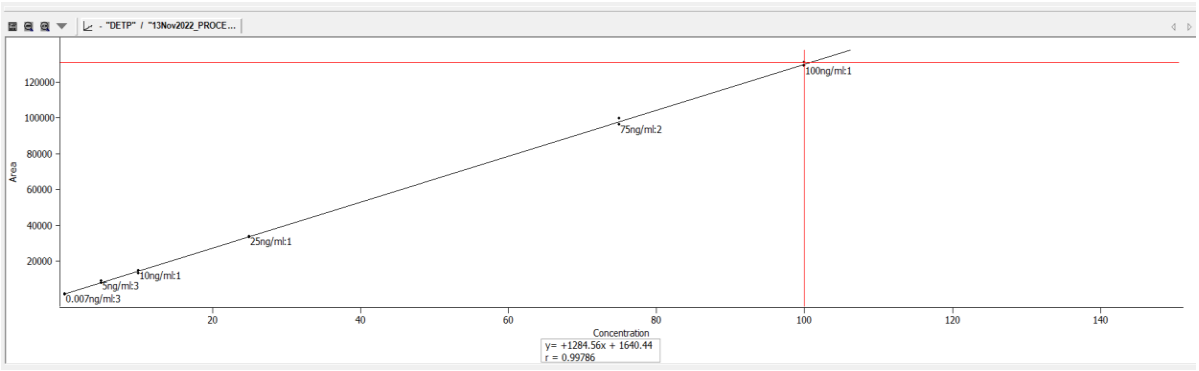
Calibration curve day 2

Table A 2.6: Regression analysis of DMP and calibration curve



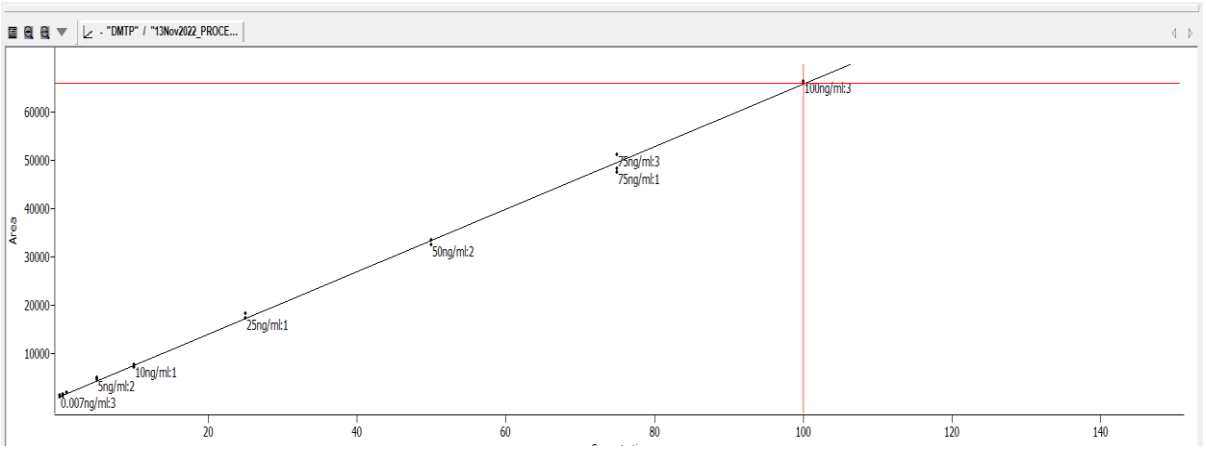
Summary output (DMP)	
Regression Stats	
Multiple R	0.99991057
R Square / Adj. R Square	0.99982116 / 0.99980327
Standard Error / Observations	4716.6502 / 23
ANOVA (df, SS, MS, F, Sig F)	
Regression	1, 1.2437E+12, 1.2437E+12, 55904.67007, 4.50302E-20
Residual	10, 222467892.3, 22246789.23
Total	11, 1.24392E+12
Coefficients	
Intercept	-545.920935, 1643.7685, -0.3321154, 0.74665842, [-4208.4654, 3116.6236]
0.0001	9799.3516, 41.445110, 236.44168, 4.50302E-20, [9707.0061, 9891.6970]
Detection Limits	
LOD / LOQ	0.55 / 1.68

Table A 2.7: Regression analysis of DEP and calibration curve



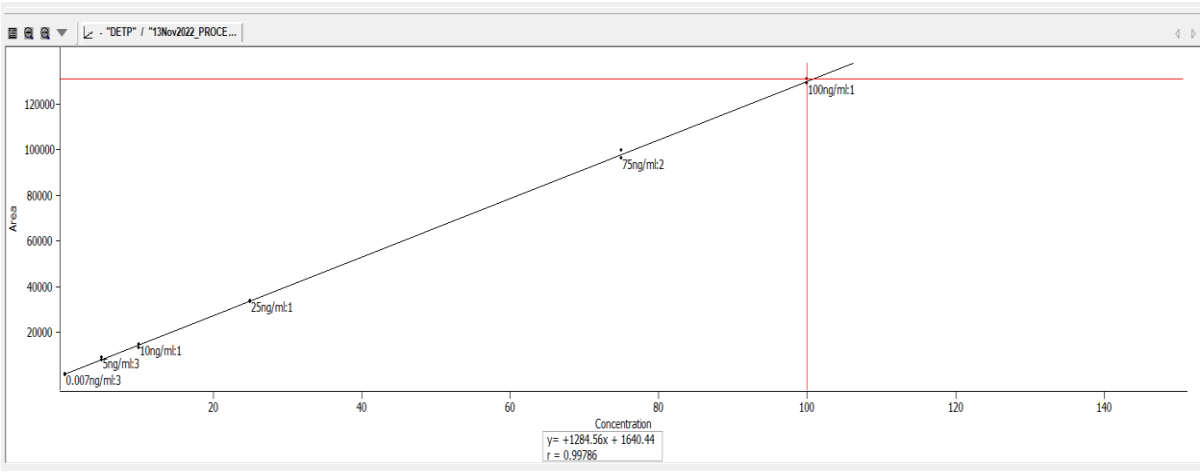
Summary output (DEP)	
Regression Stats	
Multiple R	0.99996200
R Square / Adj. R Square	0.99992300 / 0.99991200
Standard Error / Observations	98.8587 / 19
ANOVA (df, SS, MS, F, Sig F)	
Regression	1, 8.89E+08, 8.89E+08, 90980.41, 1.16E-15
Residual	7, 68411.3, 9773.043
Total	8, 8.89E+08
Coefficients	
Intercept	2000.928, 40.78896, 49.05562, 3.83E-10, [1904.477, 2097.378]
0.007	391.7626, 1.29882, 301.6296, 1.16E-15, [388.6914, 394.8338]
Detection Limits	
LOD / LOQ	0.34 / 1.04

Table A 2.8: Regression analysis of DMTP and calibration curve



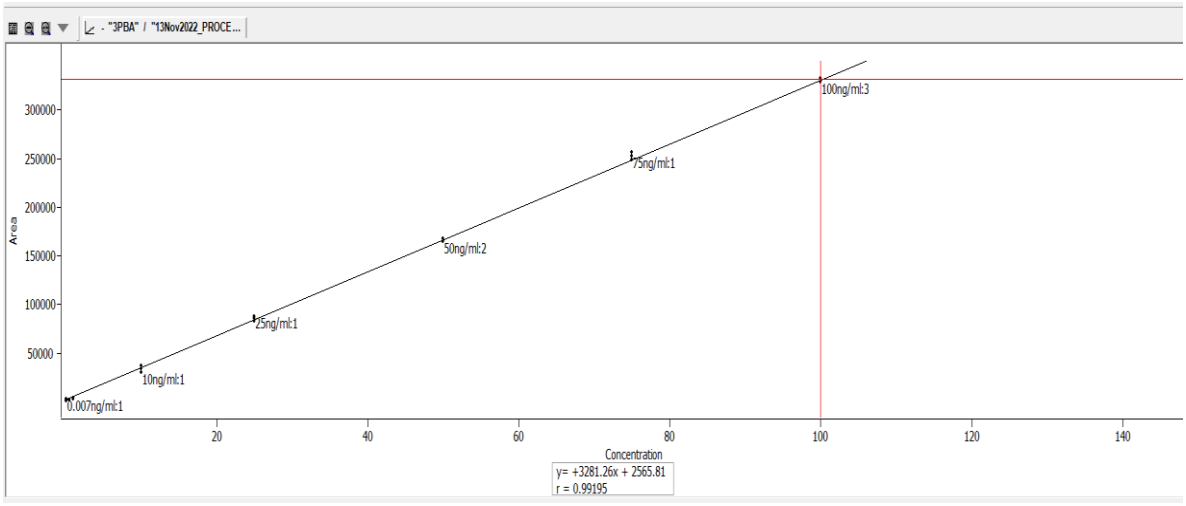
Summary output (DMTP)	
Regression Stats	
Multiple R	0.99970200
R Square / Adj. R Square	0.99940500 / 0.99934500
Standard Error / Observations	5553.597 / 22
ANOVA (df, SS, MS, F, Sig F)	
Regression	1, 5.18E+11, 5.18E+11, 16794.32, 1.84E-17
Residual	10, 3.08E+08, 30842440
Total	11, 5.18E+11
Coefficients	
Intercept	5065.198, 1934.111, 2.618877, 0.025647, [755.7314, 9374.665]
0.003	6319.722, 48.76596, 129.5929, 1.84E-17, [6211.064, 6428.379]
Detection Limits	
LOD / LOQ	1.01 / 3.06

Table A 2.9: Regression analysis of DETP and calibration curve



Summary output (DETP)	
Regression Stats	
Multiple R	0.99998500
R Square / Adj. R Square	0.99996900 / 0.99996100
Standard Error / Observations	333.9262 / 26
ANOVA (df, SS, MS, F, Sig F)	
Regression	1, 1.44E+10, 1.44E+10, 129090.1, 3.6E-10
Residual	4, 446026.7, 111506.7
Total	5, 1.44E+10
Coefficients	
Intercept	1481.631, 187.3497, 7.908371, 0.001383, [961.4647, 2001.797]
0.007	1288.5, 3.586229, 359.2911, 3.6E-10, [1278.543, 1298.457]
Detection Limits	
LOD / LOQ	0.48 / 1.45

Table A 2.10: Regression analysis of 3PBA and calibration curve



Summary output (3PBA)	
Regression Stats	
Multiple R	0.99933200
R Square / Adj. R Square	0.99866500 / 0.99833100
Standard Error / Observations	524.707 / 29
ANOVA (df, SS, MS, F, Sig F)	
Regression	1, 8.24E+08, 8.24E+08, 2992.623, 6.68E-07
Residual	4, 1101270, 275317.4
Total	5, 8.25E+08
Coefficients	
Intercept	1277.97, 243.4945, 5.248458, 0.006304, [601.9214, 1954.02]
0.007	3242.32, 59.26932, 54.70487, 6.68E-07, [3077.762, 3406.878]
Detection Limits	
LOD / LOQ	0.25 / 0.75

Calibration curve averages and standard deviations

Table A 2.11: Day 1 and Day 2 LOD and LOQ values

		LOD	**LOQ**
DMP	Day 1	0,44	1,32
	Day 2	0,55	1,67
DEP	Day 1	0,4	1,23
	Day 2	0,34	1,04
DMTP	Day 1	0,77	2,32
	Day 2	1,01	3,06
DETP	Day 1	0,99	3
	Day 2	0,48	1,45
3PBA	Day 1	0,13	0,4
	Day 2	0,25	0,75

Table A 2.12: Day 1 and Day 2 LOD and LOQ average and standard deviation values

	LOD		LOQ	
	average	stdev	average	stdev
DMP	0,50	0,08	1,50	0,25
DEP	0,37	0,04	1,14	0,13
DMTP	0,89	0,17	2,69	0,52
DETP	0,74	0,36	2,23	1,10
3PBA	0,19	0,08	0,58	0,25

Method validation other GC techniques LOD values

Table A 2.13: GC techniques'LOD parameters for metabolite analysis in urine samples [ng/mL] available in literature (10-year range 2012- 2022).

	DMP	DEP	DMTP	DETP	3PBA
GCMS	22.5 µg/L[890] 0.6 ng/mL[891] 0.06 ng/mL[892] 5.00 ng/mL[253,893] 0.15 µg L^{-1} [894] 2.0 µg L^{-1} [895]	22.5 µg/L[890] 0.2 ng/mL[891] 0.02 ng/mL[892] 0.034 ng/mL[253,893] 0.07 µg L^{-1} [894]	22.5 µg/L[890] 0.2 ng/mL[891] 0.05 µg L^{-1} [894]	22.5 µg/L[890] 0.1 ng/mL[891] 0.03 ng/mL[892] 0.028 ng/mL[253,893] 3.0 µg/L^{-1} [896] 0.05 µg L^{-1} [894]	0.0047 ug/L[897] 0.2- 200 ng/mL[898] 0.06 ng/mL[898] 0.1 µg/L[899] 0.1 ng/mL[900] 0.03 µg/L[901] 1 µg/L[902] 0.06 ng/mL[898] 0.02ng/mL[561] 0.1 ng/mL[903] 0.10 µg L^{-1} [904] 0.09 µg/L[905]
GCMS/MS	0.26 µg/l [906]	0.667 ug/L[241] 0.50 µg/l [906]	0.667 ug/L[241] 0.40 µg/l [906]	0.083 ug/L[241] 0.12 µg/l [906]	0.008 µg/L[241] 0.050 µg L[907] 0.010 µg L[908]

Method validation (recoveries)

Table A 2.14: Extraction techniques, solvents, derivatising agents, % recovery and GC column used for the quantification of the metabolites of interest in urine samples available in literature (10-year range 2012- 2022).

Technique	Extraction technique	Solvent	Derivatizing agent	Recovery (%)	column
GCMS	LLE (diethylether–acetonitrile (1:1, v/v)[890]	Toluene [890]	Pentafluorobenzyl bromide (PFBBr)[890]	56-90[890]	DB 5MS[890]
		Toluene[909]		63.8 – 86.1[909]	BP-10[909]
		Toluene[891]	PFBBr[909]	94–119[891]	HP-5[891]
		Toluene[892]	PFBBr[891]	83-101[892]	BPX5[892]
	SLE acetonitrile and 1 g of Na2SO4[909]	acetonitrile[253]	PFBBr[892]	93.64- 99.92[253]	DB-5MS[253]
		Toluene[893]	PFBBr[253]	93.64 to 99.92[893]	Rtx-65[894]
		Toluene[894]	PFBBr[893]	29- 31[896]	RTx®-5MS[896]
	LLE (diethylether–acetonitrile (1:1, v/v)[891]	tetrahydrofuran[896]	PFBBr[894]	88 [898]	
		Hexane[897]	PFBBr[896]	96.2- 105.8[899]	DB-XLB[897]
		Iso octane[898]	hexafluoro-2-propanol plus 20 µL of diisopropylcarbodiimide (DIC and HFIP)[897]	74–123 [901]	DB-5MS[898]
		toluene [899]		46[902]	DB-5MS[899]
	LLE (diethylether–acetonitrile (1:1, v/v)[892]	hexane [900]		96.2- 105.8[904]	VF-5 ms[900]
		iso octane[901]		91[905]	Rtx-65[901]
		Toluene[902]		90.48[197]	BP-5[902]
		Iso- octane[561]	DIC and HFIP[898]		Rtx 65[561]
	SPE (Bond elut-acetonitrile elution)[253]	Hexane[903]	H^2SO^4 in methanol[899]		VF-5 ms[903]
		Toluene[904]			DB-5 MS[904]
		Toluene[905]	DIC and HIFP[900]		DB-5 ms[905]
		Toluene[910]	DIC and HIFP[901]		BPX5[910]
	Automated SPE	acetonitrile[197,244]	PFBBr[902]		RTX-35[197,244]
			DIC and HIFP[561]		

	(acetonitrile elution)[893] LLE (diethylether–acetonitrile (1:1 v/v)) followed by water and hexane (3ml and 3 ml) followed by clean-up column[894] molecularly imprinted solid phase extraction (MISPE) (acetonitrile elution)[896] acetate buffer at pH 5 and extracted with HCl/hexane[897] SPE- 0.2 M sodium acetate buffer (pH 4.7), SPE-C18cartridges	DIC and HIFP[903] H2SO4 in methanol[904] TMSI and MTBSTFA[905] PFBBr[910] MTBSTFA[197,244]

	(200 mg, 3 mL), elution - acetonitrile[898] LLE- Hexane[899] LLE- hexane and methyl tert-butyl ether[900] SPE and LLE- tert-butyl methyl ether[901] LLE- sulphuric acid [902] LLE- tert-butyl-methyl ether [561] LLE - Hexane[903] LLE- Hexane[904] LLE- Toluene[905] LLE- diethyl ether- acetonitrile[910] LLE- hexane[197,244]

Technique	Extraction technique	Solvent	Derivatizing agent	Recovery (%)	column
GCMS/MS	LLE and SPE-hexane:isopropanol and HLB cartridges [241] LLE- MTBE [907] LLE- tert-butyl-methyl ether[908] Freeze drying and LLE-diethylether and acetonitrile[659] LLE-Hexane[381] Freeze drying and LLE-acetonitrile[911]	Toluene [241] Toluene[907] Iso octane[908] Hexane[659] Hexane[381] Hexane and acetone[911]	MTBSTFA[241] BSTFA[907] BSTFA-TMCS and DIC and HFIP[908] PFBBr[659] MTBSTFA[381] potassium carbonate and of 1-Chloro3-iodopropane (CIP)[911]	100[241] 103.5[907] 40[908] 91- 115[659] 100[381]	DB-5MS[241] DB-5 ms and Rtx-65[908] HP-5-MS[381]

Steps for calculating Method detection limits of biological samples

Step 1: Prepare the Samples

Collect multiple replicates (at least 3, but ideally 7) of the spiked biological sample (urine or hair) at a known low concentration near the expected detection limit (5 ng/mL). Analyse samples with GCxGC to obtain the measured concentrations for each replicate.

Step 2: Calculate the Mean

Find the mean of the replicates

Step 3: Calculate the Standard Deviation (S)

Calculate the standard deviation (S) of the replicates

Step 4: Determine the t-Value

Choose the appropriate t-value from the student's t-distribution for a 99% confidence level. The t-value depends on the number of replicates: For urine there was 3 replicates: t=4.303 and for hair there was 7 replicates: t=3.143

Step 5: Calculate the MDL

Calculate the MDL using the formula: $MDL = t(n-1, 0.99) \times S$

Step 6: Interpret the Results

The resulting MDL represents the lowest concentration (ng/mL) that can be reliably detected in your sample matrix (urine or hair).

MDL Calculation Results for Urine Samples

Table A 2.15: Method detection limits (MDLs) for OP and PYR metabolites in urine samples based on replicate measurements.

Metabolite	Replicate 1	Replicate 2	Replicate 3	Mean	Standard Deviation	MDL (ng/mL)
DMP	1.64	3.96	4.11	3.24	1.384	5.96
DEP	3.72	2.72	2.34	2.93	0.706	3.04
DMTP	1.95	2.00	2.02	1.99	0.037	0.16
DETP	2.09	2.16	4.12	2.79	1.18	5.08
3PBA	2.43	2.40	2.35	2.39	0.040	0.17

MDL Calculation Results for Hair Samples

The MDL in ng/mL were converted to pg/mg following the equation below:

Metabolite concentration in pg/mg = (Metabolite concentration in ng/mL×0.3mL/20mg)×1000

Metabolite concentration in ng/mL: MDL value obtained.

• 0.3 mL: Extraction volume used for hair sample preparation.

• 20 mg: Weight of hair used in the analysis.

• 1000: Conversion factor from ng to pg (1 ng = 1000 pg).

Table A 2.16: Method detection limits (MDLs) for OP and PYR metabolites in hair samples based on replicate measurements.

Metabolite	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5	Replicate 6	Replicate 7	Mean	Standard Deviation	MDL (ng/mL)	MDL (pg/mg)
DMP	4.65	4.67	4.72	4.62	4.58	4.70	4.68	4.66	0.048	0.151	2.27
DEP	4.74	4.89	4.57	4.52	4.41	4.70	4.71	4.65	0.161	0.506	7.59
DMTP	4.39	4.45	4.48	4.36	4.67	4.42	4.32	4.44	0.116	0.365	5.48
DETP	4.71	4.84	4.76	4.54	4.60	4.72	4.55	4.67	0.107	0.336	5.04
3PBA	5.17	4.98	4.70	0	0	4.76	4.77	3.48	2.11	6.63	99.45

UNIVERSITY OF CAPE TOWN

Health Sciences Faculty
Research Ethics Committee
Room E52-24 Groote Schuur Hospital Old Main Building
Observatory 7925
Telephone [021] 406 6338 • **Facsimile** [021] 406 6411
e-mail: sumayah.ariefdien@uct.ac.za

01 June 2009

REC REF: 234/2009

Dr MA Dalvie
Public Health & Family Medicine

Dear Dr Dalvie

PROJECT TITLE: FOLLOW-UP STUDY OF REPRODUCTIVE HEALTH EFFECTS DUE TO ENVIRONMENTAL PESTICIDE EXPOSURE AMONG BOYS IN THE WESTERN CAPE, SOUTH AFRICA.

Thank you for submitting your study to the Research Ethics Committee for review.

It is a pleasure to inform you that the Ethics Committee has **formally approved** the above-mentioned study.

Approval is granted for one year till the 05th June 2010.

Please submit an annual progress report if the research continues beyond the expiry date. Please submit a brief summary of findings if you complete the study within the approval period so that we can close our file.

Please note that the ongoing ethical conduct of the study remains the responsibility of the principal investigator.

Please quote the REC. REF in all your correspondence.

Yours sincerely

PROFESSOR M BLOCKMAN
CHAIRPERSON, HSF HUMAN ETHICS

Federal Wide Assurance Number: FWA00001637.
Institutional Review Board (IRB) number: IRB00001938

sariefdien

Figure A 3.1: Ethical approval for the longitudinal and cohort urine study.

UNIVERSITY OF CAPE TOWN
Faculty of Health Sciences
Human Research Ethics Committee

Room E53-46 Old Main Building
Groote Schuur Hospital
Observatory 7925
Telephone [021] 406 6626
Email: shuretta.thomas@uct.ac.za
Website: www.health.uct.ac.za/fhs/research/humanethics/forms

19 July 2019

HREC REF: 181/2019

Prof Aqiel Dalvie
Public Health and Family Medicine
Room 4.31, 4th floor
Falmouth Building

Dear Prof Dalvie

PROJECT TITLE: BIOMARKERS OF PESTICIDES UPTAKE IN HAIR AND URINE OF RURAL CHILDREN OF THE WESTERN CAPE, SOUTH AFRICA (SUB-STUDY LINKED TO 234/2009) (PHD CANDIDATE: MS M MUGARI)

Thank you for submitting your response to the Faculty of Health Sciences Human Research Ethics Committee.

It is a pleasure to inform you that the HREC has **formally approved** the above-mentioned study.

Approval is granted for one year until 30 July 2020.

Please submit a progress form, using the standardised Annual Report Form if the study continues beyond the approval period. Please submit a Standard Closure form if the study is completed within the approval period.
(Forms can be found on our website: www.health.uct.ac.za/fhs/research/humanethics/forms)

Please note that the ongoing ethical conduct of the study remains the responsibility of the principal investigator.

Please note that for all studies approved by the HREC, the principal investigator **must** obtain appropriate institutional approval, where necessary, before the research may occur.

The HREC acknowledge that the student, Mufaro Buhlebenkosi Mugari will also be involved in this study.

Please quote the HREC REF in all your correspondence.

Yours sincerely

PROFESSOR M BLOCKMAN
CHAIRPERSON, FHS HUMAN RESEARCH ETHICS COMMITTEE

Figure A 3.2: Ethical approval for the hair study.

Creatinine analysis

Sample preparation and creatinine analysis were done using the Jaffe method at BARC laboratories under lancet laboratories. Creatinine concentrations in urine samples are shown in. Table 13 According to WHO, the acceptable range of creatinine in urine samples is 30 – 300 mg/dL. In our study, twelve samples were out of this range, samples are highlighted in yellow. Creatinine calculation analysis of the study samples was done in Microsoft Excel using the data analysis tool (descriptive analysis) and the pivot table.[671,674,675].

Table A 3.1: Creatinine values in mg/dL

Sample Name	**Creatinine (mg/dL)**	**Sample Name**	**Creatinine (mg/dL)**
	Cycle 1		Cycle 1
8	16,40	50	47,06
15	110,86	51	30,54
23	72,51	53	269,68
24	46,27	75	84,05
28	91,97	77	140,61
29	81,90	78	98,75
30	154,18	80	84,73
31	46,38	86	60,18
32	65,04	90	70,59
35	97,28	96	20,81
39	70,70	99	26,58
43	15,61	114	104,18
45	11,54	122	8,60
49	88,12	129	40,16
147	109,16		
149	37,44		
150	29,52		
151	26,24		
153	72,85		
158	79,30		

Sample Name	Creatinine (mg/dL)	Sample Name	Creatinine (mg/dL)
159	81,90		
160	27,83		
176	21,83		
180	22,06		
240	111,65		
277	145,13		
291	247,39		
293	127,15		
299	98,08		
300	74,32		
303	62,78		
305	20,14		
306	164,48		
310	81,67		
331	47,28		
334	82,01		
340	68,32		
346	40,16		
350	121,38		
356	200,22		
368	45,25		
378	68,32		
387	117,31		
399	102,03		
416	82,58		
440	115,16		
494	95,59		

Validated 2-DGCxGC ToF/MS. method application on study samples.

Table A 3.1: Concentrations of metabolites in urine expressed in ng/mL before creatinine correction and in µg/g after correction. (concentrations falling outside the recommended range are labelled as Out-of-range (OR) according to WHO guidelines for creatinine concentrations)

Sample	DMP		DEP		DMTP		DETP		3PBA	
	ng/mL	µg/g	ng/mL	µg/g	ng/mL	µg/g	ng/mL	µg/g	ng/mL	µg/g
8	12,15	OR	12,82	OR	23,08	OR	15,13	OR	1,07	OR
15	18,15	16,37	2,24	2,02	<LOQ	<LOQ	<LOQ	<LOQ	1,78	1,61
23	29,84	41,15	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	14,88	20,52
24	<LOD	<LOD	3,73	8,06	6,72	14,52	<LOQ	<LOQ	4,79	10,35
28	<LOQ	<LOQ	2,39	2,60	6,91	7,51	4,32	4,70	4,40	4,78
29	<LOD	<LOD	2,24	2,74	4,81	5,87	<LOQ	<LOQ	3,82	4,66
30	20,77	13,47	36,40	23,61	31,59	20,49	4,87	3,16	16,44	10,66
31	1,68	3,62	32,39	69,84	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
32	20,67	31,78	14,98	23,03	5,72	8,79	15,24	23,43	3,26	5,01
35	9,72	9,99	1,70	1,75	<LOQ	<LOQ	<LOQ	<LOQ	<LOD	<LOD
39	<LOD	<LOD	11,47	16,22	<LOQ	<LOQ	<LOD	<LOD	5,30	7,50
43	4,76	OR	1,62	OR	<LOQ	<LOQ	2,82	OR	<LOD	<LOD
45	<LOD	<LOD	26,17	OR	<LOQ	<LOQ	5,88	OR	<LOQ	<LOQ
49	11,38	12,91	54,17	61,47	27,95	31,72	66,20	75,12	10,28	11,67
50	4,34	9,22	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOQ	<LOQ
51	1,41	4,62	3,81	12,47	12,57	41,16	13,78	45,12	3,30	10,80
53	6,46	2,40	65,18	24,17	4,43	1,64	13,34	4,95	<LOQ	<LOQ
75	28,91	34,40	<LOD	<LOD	4,29	5,10	31,42	37,38	4,28	5,09
77	<LOD	<LOD	<LOD	<LOD	46,34	32,96	<LOD	<LOD	2,83	2,01
78	26,60	26,94	<LOD	<LOD	7,46	7,55	19,20	19,44	<LOD	0,00
80	<LOQ	<LOQ	<LOQ	<LOQ	9,57	11,30	2,40	2,83	17,86	21,08
86	6,34	10,54	9,45	15,70	5,90	9,80	8,37	13,91	4,08	6,78
90	<LOD	<LOD	<LOD	0,00	18,89	OR	24,99	OR	<LOQ	<LOQ
96	<LOD	<LOD	39,81	191,26	<LOD	<LOD	<LOD	<LOD	2,73	13,12

Sample	DMP		DEP		DMTP		DETP		3PBA	
	ng/mL	µg/g	ng/mL	µg/g	ng/mL	µg/g	ng/mL	µg/g	ng/mL	µg/g
99	<LOD	<LOD	<LOD	<LOD	10,53	OR	3,61	OR	0,93	OR
114	<LOD	<LOD	<LOD	<LOD	26,20	25,15	33,06	31,73	<LOD	<LOD
122	<LOD	<LOD	<LOD	<LOD	14,89	OR	12,71	OR	<LOD	<LOD
129	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
147	<LOD	<LOD	<LOD	<LOD	7,27	6,66	19,86	18,19	2,13	1,95
149	<LOD	<LOD	<LOD	<LOD	14,76	39,42	9,74	26,01	1,00	2,67
150	<LOD	<LOD	83,06	OR	<LOD	<LOD	<LOD	<LOD	<LOQ	<LOQ
151	<LOQ	<LOQ	<LOD	<LOD	15,20	OR	3,11	OR	<LOD	<LOD
153	15,87	21,78	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
158	11,23	14,16	<LOD	<LOD	5,19	6,55	2,68	3,38	<LOD	<LOD
159	45,64	55,73	<LOD	<LOD	7,73	9,44	3,30	4,03	4,06	4,96
160	<LOD	0,00	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
176	9,82	OR	22,37	OR	9,29	OR	16,44	OR	<LOD	<LOD
180	20,42	OR	<LOD	<LOD	<LOQ	<LOQ	4,05	OR	1,52	OR
240	38,10	34,12	43,12	38,62	<LOQ	<LOQ	4,04	3,62	4,31	3,86
277	<LOD	<LOD	8,36	5,76	<LOD	<LOD	<LOD	<LOD	<LOQ	<LOQ
291	<LOD	<LOD	7,77	3,14	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
293	<LOD	<LOD	46,25	36,38	32,06	25,21	19,23	15,12	6,55	5,15
299	<LOQ	<LOQ	<LOD	<LOD	7,79	7,94	9,12	9,30	2,93	2,99
300	14,61	19,66	<LOD	<LOD	3,77	5,07	6,39	8,60	<LOD	<LOD
303	0,00	<LOD	33,51	53,38	<LOD	<LOD	<LOD	<LOD	1,67	2,66
305	6,25	OR	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	17,82	OR
306	<LOD	<LOD	42,72	25,97	<LOD	<LOD	<LOD	<LOD	6,88	4,18
310	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
331	<LOD	<LOD	16,21	34,28	<LOD	<LOD	<LOD	<LOD	<LOQ	<LOQ
334	2,84	3,46	<LOD	<LOD	<LOQ	<LOQ	3,87	4,72	1,05	1,28
340	<LOD	<LOD	2,05	3,00	14,16	20,72	8,61	12,60	<LOD	<LOD
346	1,67	4,16	14,11	35,14	23,34	58,12	3,39	8,44	<LOQ	<LOQ
350	3,21	2,64	<LOD	<LOD	9,65	7,95	9,10	7,50	<LOD	<LOD

Sample	DMP		DEP		DMTP		DETP		3PBA	
	ng/mL	µg/g	ng/mL	µg/g	ng/mL	µg/g	ng/mL	µg/g	ng/mL	µg/g
356	21,26	10,62	35,38	17,67	<LOD	<LOD	<LOD	<LOD	<LOQ	<LOQ
368	4,43	9,79	<LOD	<LOD	4,06	8,97	4,16	9,19	<LOD	<LOD
378	12,42	18,18	38,48	56,32	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
387	<LOQ	<LOQ	7,24	6,17	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
399	<LOD	<LOD	36,16	35,44	<LOD	<LOD	<LOD	<LOD	7,07	6,93
416	<LOQ	<LOQ	18,71	22,66	<LOQ	<LOQ	<LOQ	<LOQ	2,22	2,69
440	<LOD	<LOD	37,65	32,69	<LOD	<LOD	<LOD	<LOD	3,59	3,12
494	<LOD	<LOD	17,45	18,26	<LOQ	<LOQ	<LOQ	<LOQ	<LOD	<LOD

Table A 3.3: Farm and nonfarm metabolite concentrations (µg/g) found in the urine samples collected in cycle 1 and cycle 2 from children (median (IQR)

Variable	Farm				Nonfarm			
	Total µg/g Median (IQR))	**Cycle 1 µg/g Median (IQR)**	**Cycle 2 µg/g Median (IQR)**	**p-value**	**Total µg/g Median (IQR)**	**Cycle 1 µg/g Median (IQR)**	**Cycle 2 µg/g Median (IQR)**	**p-value**
DMP	13.14 (3.76 - 31.14)	16.37 (8.08 - 34.12)	6.76 (3.41 - 24.54)	**0.10**	10.54 (4.16 -18.18)	6.97 (2.64 - 10.62)	11.21 (8.87 - 27.52)	**0.12**
DEP	11.43 (3.53 - 36.38)	23.32 (2.74 - 69.84)	8.82 (4.64 - 20.67)	**0.22**	18.11 (8.06 -32.69)	22.66 (8.06 - 35.14)	17.18 (7.78 - 23.46)	**0.37**
DMTP	8.65 (5.31 - 25.15)	10.37 (5.87 - 32.96)	8.65 (5.31 -25.15)	**0.13**	7.37 (4.62 - 15.20)	8.97 (4.64 - 14.52)	5.93 (3.47 - 15.78)	**0.52**
DETP	12.63 (6.21 - 22.97)	11.68 (7.39 - 17.87)	14.35(4.19- 33.57)	**0.73**	9.02 (5.77 - 13.50)	7.50 (4.72 - 9.19)	10.90 (8.40 - 17.53)	**0.10**
3-PBA	4.78 (1.95 - 10.80)	4.87 (2.34 - 7.30)	3.49 (1.18 - 14.79)	**0.98**	3.04 (1.97 - 6.78)	2.69 (1.73 -6.78)	3.96 (2.29 - 7.05)	**0.51**

Table A 3.4: Grabouw and Hex River metabolite concentrations (µg/g) found in the urine samples collected in cycle 1 and cycle 2 from children. (median (IQR)

Variable	Grabouw				Hex River			
	Total µg/g Median (IQR)	**Cycle 1 µg/g Median (IQR)**	**Cycle 2 µg/g Median (IQR)**	**p-value**	**Total µg/g Median (IQR)**	**Cycle 1 µg/g Median (IQR)**	**Cycle 2 µg/g Median (IQR)**	**p-value**
DMP	10.72(3.86-30.49)	15.2(8.65 - 34.26)	6.65 (3.41 - 18.59)	**0.04**	10.62 (3.46 - 22.47)	4.16 (2.57 - 18.18)	17.30(9.06-34.06)	**0.06**
DEP	9.46 (3.26 - 24.17)	15.96(2.94 - 65.66)	8.36 (3.53 - 19.97)	**0.22**	22.66(10.02- 34.28)	32.69(18.26- 35.44)	17.57(8.23-27.15)	**0.11**
DMTP	8.93 (5.29 - 24.76)	11.30(6.55 - 32.96)	7.62 (4.62 - 18.08)	**0.07**	7.95 (4.64 - 18.56)	7.95 (4.64 - 20.72)	7.38 (4.46 - 17.18)	**0.74**
DETP	13.32(6.21-22.97)	13.91 (4.03 -35.40)	12.86(7.68- 17.67)	**0.80**	9.02 (4.78 - 12.60)	8.52 (4.72 - 9.30)	10.08(6.39-22.04)	**0.32**
3-PBA	4.87 (1.74 - 10.66)	4.96 (2.01 - 7.50)	3.49 (1.08 - 14.61)	**0.27**	3.01 (2.24 - 6.68)	2.84 (1.89 - 4.67)	3.96 (2.52 - 18.08)	**0.33**

Table A 3.5: Female and male metabolite concentrations (µg/g) found in the urine samples collected in cycle 1 and cycle 2 from children (median (IQR).

Variable	Female				Male			
	Total µg/g Median (IQR)	**Cycle 1 µg/g Median (IQR)**	**Cycle 2 µg/g Median (IQR)**	**p-value**	**Total µg/g Median (IQR)**	**Cycle 1 µg/g Median (IQR)**	**Cycle 2 µg/g Median (IQR)**	**p-value**
DMP	10.62(3.62-31.04)	16.17 (3.54-32.95)	7.93 (3.66-18.27)	**0.21**	11.72 (5.72-21.06)	9.79 (4.16-16.37)	18.59(8.87-24.69)	**0.16**
DEP	16.40(5.76-35.44)	23.32(5.76- 56.32)	9.46 (5.96 - 23.46)	**0.27**	15.70(7.51 - 23.29)	22.66(12.47-35.14)	8.82 (6.42 - 17.18)	**0.06**
DMTP	9.06 (5.70 - 20.83)	8.50 (5.31 - 18.08)	10.37(6.21- 29.86)	**0.23**	6.66 (4.59 - 25.21)	8.97 (5.07 - 31.72)	5.70 (3.44 - 14.62)	**0.13**
DETP	11.77(5.65-19.91)	8.06 (4.03 - 23.43)	13.56(8.88- 17.87)	**0.31**	10.84(6.39 - 18.19)	12.60(7.50 - 37.38)	9.84 (6.21 - 13.10)	**0.41**
3-PBA	3.86 (1.73 - 10.66)	3.26 (1.18 - 14.79)	4.66 (2.01 - 7.11)	**0.78**	4.18 (1.972 -6.78)	3.65 (2.00 - 5.97)	4.88 (1.97 - 7.66)	**0.81**

Table A 4.1: Farm and non-farm metabolite concentrations (pg/mg) found in the hair samples collected in cycle 1 and cycle 2 from children (median(IQR)

Variable	Farm				Non-farm			
	Total pg/mg Median (IQR)	**Cycle 1 pg/mg Median (IQR)**	**Cycle 2 pg/mg Median (IQR)**	**p-value**	**Total pg/mg Median (IQR)**	**Cycle 1 pg/mg Median (IQR)**	**Cycle 2 pg/mg Median (IQR)**	**p-value**
DMP	10.61 (10.61 - 20.33)	10.61(10.61– 40.80)	10.61(10.61-10.61)	**0.03**	10.61(10.61– 32.18)	10.61(10.61– 40.65)	10.61(10.61– 7.50)	**0.66**
DEP	7.88(7.88– 96.08)	7.88(7.88– 26.40)	7.88(7.88– 102.60)	**0.10**	7.88(7.88–111.68)	7.88(7.88–119.85)	7.88(7.88–7.88)	**0.12**
DMTP	18.88(18.88– 18.88)	18.88(18.88– 18.88)	18.88(18.88– 18.88)	**0.10**	18.88(18.88– 18.88)	18.88(18.88– 18.88)	18.88(18.88– 18.88)	**0.18**
DETP	15.70(15.70– 15.70)	15.70(15.70– 15.70)	15.70(15.70– 15.70)	**0.04**	15.70(15.70– 15.70)	15.70(15.70– 35.55)	15.70(15.70– 15.70)	**0.14**
3-PBA	4.03(4.03– 38.10)	4.03(4.03– 81.00)	4.03(4.03– 4.03)	**0.0034**	4.03(4.03– 20.25)	4.03(4.03– 19.95)	4.03(4.03– 24.00)	**0.25**

Table A 4.2: Grabouw and Hex River metabolite concentrations (pg/mg) found in the hair samples collected in cycle 1 and cycle 2 from children. (median (IQR)

Variable	Grabouw				Hex River			
	Total pg/mg Median (IQR)	**Cycle 1 pg/mg Median (IQR)**	**Cycle 2 pg/mg Median (IQR)**	**p-value**	**Total pg/mg Median (IQR)**	**Cycle 1 pg/mg Median (IQR)**	**Cycle 2 pg/mg Median (IQR)**	**p-value**
DMP	10.61(10.61- 29.25)	10.61(10.61– 40.80)	10.61(10.61- 10.61)	**0.16**	10.61(10.61– 7.50)	10.61(10.61– 40.6)	10.61(10.61– 10.61)	**0.10**
DEP	7.88(7.88–106.60)	7.88(7.88– 102.60)	7.88(7.88– 102.75)	**0.60**	7.88(7.88– 108.15)	7.88(7.88– 134.85)	7.88(7.88– 7.88	**0.04**
DMTP	18.88(18.88– 18.88)	18.88(18.88– 18.88)	18.88(18.88– 18.88)	**0.56**	18.88(18.88– 0.0148)	18.88(18.88– 0.0148)	18.88(18.88– 18.88)	**0.02**
DETP	15.70(15.70– 15.70)	15.70(15.70– 15.70)	15.70(15.70– 15.70)	**0.13**	15.70(15.70– 15.70)	15.70(15.70– 15.70)	15.70(15.70– 15.70)	**0.01**
3-PBA	4.03(4.03– 37.50)	4.03(4.03– 77.40)	4.03(4.03– 4.03)	**0.03**	4.03(4.03– 19.95)	4.03(4.03– 26.85)	4.03(4.03– 4.03)	**0.011**

Table A4.3: Female and male metabolite concentrations (pg/mg) found in the hair samples collected in cycle 1 and cycle 2 from children (median (IQR).

Variable	**Female**				**Male**			
	Total pg/mg Median (IQR)	**Cycle 1 pg/mg Median (IQR)**	**Cycle 2 pg/mg Median (IQR)**	**p-value**	**Total pg/mg Median (IQR)**	**Cycle 1 pg/mg Median (IQR)**	**Cycle 2 pg/mg Median (IQR)**	**p-value**
DMP	10.61(10.61- 20.85)	10.61(10.61- 53.10)	10.61(10.61-10.61)	**0.01**	10.61(10.61-21.30)	10.61(10.61–21.30)	10.61(10.61–7.50)	**0.84**
DEP	7.88(7.88–110.10)	7.88(7.88– 119.85)	7.88(7.88– 26.40)	**0.03**	7.88(7.88– 89.70)	7.88(7.88– 89.70)	7.88(7.88– 7.88	**0.43**
DMTP	18.88(18.88–18.88)	18.88(18.88 18.88)	18.88(18.88–18.88)	**0.60**	18.88(18.88–18.88)	18.88 (18.88–18.88)	18.88(18.88–18.88)	**0.64**
DETP	15.70(15.70–15.70)	15.70(15.70– 15.70)	15.70(15.70–15.70)	**0.01**	15.70(15.70–15.70)	15.70(15.70–15.70)	15.70(15.70–15.70)	**0.364**
3-PBA	4.03(4.03– 35.25)	4.03(4.03– 54.45)	4.03(4.03– 4.03)	**0.001**	4.03(4.03– 27.45)	4.03(4.03– 20.55)	4.03(4.03– 52.05)	**0.674**

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