Neurological risk of prolonged low dose exposure to imidacloprid in zebrafish

by

Megan Joy McCulloch

Thesis presented in partial fulfilment of the requirements for the degree of Master of Science in the Faculty of Medicine and Health Sciences (Pharmacology) at Stellenbosch University

Supervisor: Dr T Kellermann, Division of Clinical Pharmacology (Stellenbosch University)
Co-supervisor: Prof C Smith, Division of Clinical Pharmacology (Stellenbosch University)

Declaration of originality

I, the undersigned, hereby declare that the work contained in this thesis is my original work and that I have not previously submitted it, in its entirety or in part, at any other university for a degree.

Date: December 2022

Acknowledgements

I would like to express my deepest gratitude to my supervisor, Dr Tracy Kellermann. This endeavour would not have been possible without her. Thank you for always believing in me, even when I did not believe in myself. Most of all, thank you for granting me with various opportunities during my academic career to grow into the scientist you believed I could be.

I am also extremely grateful to my co-supervisor, Prof Carine Smith for her continual support and motivation. I appreciate and value all the knowledge and skills you have imparted to me. You are an incredible leader.

I am deeply indebted to my parents, Diane and Derek McCulloch. Words cannot express my gratitude for your endless love, support and the numerous sacrifices you have made for me during my academic career.

My fiancé, Thomas Campbell, thank you for being my number one supporter, motivator and for your endless encouragement during the completion of this work. Lastly, thank you for always being my sound board and dictionary. I appreciate you.

I am extremely grateful to my friends, Thomas and Annalise Marx for their endless support during my academic career and their amazing friendship. Side by side or miles apart, you are always close to my heart.

Thank you to all my peers who have been there for me, encouraged and supported me during the completion of this degree. I would like to make a special mention to Miss Janica Conradie and Miss Vanessa Louw for their willingness to help on countless occasions despite working towards the completion of their own degree.

Thanks should also go to the Department of Pharmacology and Faculty of Medicine and Health Sciences for funding me during the completion of this degree as well as Harry Crossley for funding this project.

Dr Mare' Vlok for always being up for a chat and for always listening when things were not going according to plan. Your infinite knowledge and good advice still finds me in awe. You are an inspiration.

Finally, I would like to give thanks to our Heavenly Father for giving me the strength and for blessing me with the opportunity to further my education. Your love and grace is what kept me going.

Abstract

Background

Imidacloprid (IMI) is a systemic neonicotinoid insecticide intended to replace the organophosphate pesticides in agriculture. Extensive use of these pesticides increases the risk to the environment and non-target organisms such as humans due to their potential bioaccumulation and toxicity. Researchers studying the effect of IMI on human nicotinic receptors (α4β2) have reported that IMI may have more substantial side effects on humans than originally anticipated. This research project aimed to assess the possibility of long-term neurological risks following prolonged, low dose IMI exposure within an *in vivo* zebrafish model.

Methods

A protein precipitation extraction from zebrafish brain, liver and gill homogenate was applied followed by LC-MS/MS detection of neurotransmitters and IMI and its primary metabolites. Protein precipitation was conducted using methanol:acetonitrile (1:1 v/v) as the precipitating solvent. Phenethylamine-d4 and IMI-d4 were used as internal standards for the neurotransmitter and IMI LC-MS/MS methods, respectively. For the neurotransmitters, chromatographic separation was achieved using a Poroshell column (3.0 x 100 mm, 2.7 µm) using a gradient elution mode at a flow rate of 0.45 mL/min and an analysis time of 7 min. Mobile phase A and B consisted of water with 0.1% formic acid and acetonitrile, respectively. For IMI and its metabolites, chromatographic separation was achieved using a biphenyl column (2.1 x 100 mm, 2.7 µm) with gradient elution at a flow rate of 0.4 mL/min. The total analysis time was 8.5 min. Mobile phase A and B consisted of water and methanol respectively, both with 5 mM ammonium formate and 0.1% formic acid. The two developed methods underwent a partial validation to certify that both methods were precise, accurate and reliable. Zebrafish larvae were exposed to IMI at four and five days post fertilisation to determine the no observed adverse effect level (NOAEL) of IMI. After this, adult zebrafish were exposed to the NOAEL concentration for 21 days. Key endpoints included behaviour indicative of neurocognitive decline and possible bioaccumulation in the adult zebrafish brain, liver and gills. Neurotransmitter concentrations were measured in the adult zebrafish brain tissue at the end of the treatment period to evaluate changes in neurotransmitter signalling and potential neurological risks using the developed LC-MS/MS method. Bioaccumulation of IMI and its metabolites in zebrafish brain, liver and gills was evaluated using LC-MS/MS.

Results

The calibration curve fits a quadratic (weighted 1/C) regression over the concentration range of 31.3 - 1000 ng/mL for acetylcholine, gamma-aminobutyric acid, serotonin and dopamine. The calibration curve for IMI and its metabolites fits a quadratic (weighted 1/C) regression over the concentration range of 1.95 - 125 ng/mL

for imidacloprid-urea and IMI, 0.244 - 125 ng/mL for desnitro-imidacloprid and 3.91 - 125 ng/mL for 5-hydro imidacloprid. The NOAEL of IMI in zebrafish larvae was determined to be 2.5 µg/L. No significant morphological changes were observed in the adult zebrafish during the treatment period. Behavioural changes observed during the pesticide exposure period included decrease in appetite of the treatment group. The treatment group was also observed swimming at the bottom of the tank in comparison to the control group. Although IMI, IMI-urea and desnitro-IMI could not be detected in any of the tissue specimens, 5-hydro IMI was detected at relatively high concentrations in the liver (0.793 ng/mg tissue) and gill epithelial tissue (117 ng/mg tissue). Only concentrations of gamma-aminobutyric acid and acetylcholine were detected and quantified in both the treated and control group. The treated group showed a 1.4-fold decrease and 1.9-fold increase in acetylcholine and gamma-aminobutyric acid, respectively in comparison to the control. Serotonin and dopamine could not be detected due to their levels being below the limit of quantitation of this method.

Conclusion

Robust LC-MS/MS methods were developed for the detection and quantitation of IMI, desnitro-imidacloprid, imidacloprid-urea and 5-hydro-imidacloprid, as well as serotonin, dopamine, acetylcholine and gamma-aminobutyric acid neurotransmitters in 200 µL zebrafish brain, liver and gill epithelial tissue homogenate. The behavioural changes observed in the adult zebrafish could be an indication of one of two things, anxiety or sedative effect. This is verified by the increase in gamma-aminobutyric acid neurotransmitter levels. Together with the evaluation of bioaccumulation of imidacloprid and its metabolites within the brain, liver and gill epithelial tissue of zebrafish, this study provides an indication of the potential risk to human health following chronic neonicotinoid exposure. Overall, our findings further contribute to existing literature and suggest that IMI does pose a threat to more than just insects and therefore requires further investigation.

Opsomming

Agtergrond

Imidacloprid (IMI) is ‘n sistemiese neonikotinoïed insekdoder wat geoormerk is om die organofosfaat plaagdoders in die landbou te vervang. Die wydverspreide gebruik van hierdie plaagdoders verhoog die risiko vir die omgewing en nie-teiken organismes soos mense, as gevolg van hul potensiële bioakkumluasie en toksisiteit. Narvorsers wat die effek van IMI op menslike nikotienreseptore (α4β2) bestudeer, het gerapprteer dat IMI meer aansienlike newe-effekte op mense kan hê as wat oorspronklik verwag is.

Metodes

‘n Proteïen presipitasie van gehomogeniseerde sebravisbrein, lewer en kieu monsters is toegepas, gevolg deur LC-MS/MS-meting van neuro-oordragstowwe, IMI en sy primêre metaboliete. Proteïen presipitasie is uitgevoer met die gebruik van metanol:asetonitriel (1:1, v/v) as die presipiterende oplosmiddel. Phenethylamine-d4 en IMI-d4 is gebruik as interne standaarde vir die neuro-oordragstowwe en IMI LC-MS/MS metodes, onderskeidelik. Vir die neuro-oordragstowwe is chromatografiese skeiding bereik met behulp van n Poroshell-kolom deur gebruik te maak van ‘n gradiënt-eleuringsmodus teen ‘n vloeispoed van 0.45 mL/min en ‘n ontledingstyd van 7 min. Mobiele fase A en B het bestaan uit water met 0.1% mieresuur en asetonitriel onderskeidelik. Vir IMI en sy metaboliete is chromatografiese skeiding bereik met behulp van n biphenyl kolom met gradiënt-eleuring teen ‘n vloeispoed van 0.4 mL/min. Die totale ontledingstyd was 8.5 min. Mobiele fase A en B het onderskeidelik uit water en metanol bestaan beide met 5 mM ammoniumformaat en 0.1% mieresuur. Die twee ontwikkelde metodes het 'n gedeeltelike validering ondergaan om te sertifiseer dat beide metodes presies, akkuraat en betroubaar was. Sebravislarwes is vier en vyf dae na bevrugting aan IMI blootgestel om die “geen waargenome nadelige effek”-vlak van IMI te bepaal. Hierna is volwasse sebravisse vir 21 dae aan die bepaalde konsentrasie blootgestel. Neuro-oordragstof konsentrasies is gemeet in die volwasse sebravis breinweefsel aan die einde van die behandelingsperiode om veranderinge in neuro-oordragstof seinoordrag en potensiële neurologiese risiko's te evalueer deur die ontwikkelde LC-MS/MS metode te gebruik. Bioakkumulasie van IMI en sy metaboliete in sebravisbrein, lewer en kieue is geëvalueer met behulp van LC-MS/MS.

Resultate

Die kalibrasiekurwe pas by 'n kwadratiese (geweegde 1/C) regressie oor die konsentrasiereeks van 31.3 - 1000 ng/mL vir asetielcholien, gamma-aminobottersuur, serotonien en dopamien. Die kalibrasiekurwe vir IMI en sy metaboliete pas by 'n kwadratiese (geweegde 1/C) regressie oor die konsentrasiereeks van 1.95 - 125 ng/mL

vir imidacloprid-ureum en IMI, 0.244 - 125 ng/mL vir desnitro-Imidacloprid en 3.91 - 125 ng/mL vir 5-hidro-imidakloprid. Die geen waargenome nadelige effekvlak van IMI in sebravislarwes is bepaal as 2.5 µg/L. Belangrike eindpunte het gedrag ingesluit, wat dui op neurokognitiewe agteruitgang. Voorlopige data dui ook op moontlike bioakkumulasie in die volwasse sebravisbrein, lewer en kieue. Geen betekenisvolle morfologiese veranderinge is in die volwasse sebravis tydens die behandelingsperiode waargeneem nie. Gedragsveranderinge wat tydens die blootstelling aan plaagdoders waargeneem is, sluit afname in eetlus van die behandelingsgroep in. Die behandelingsgroep is ook waargeneem wat aan die onderkant van die tenk geswem het in vergelyking met die kontrolegroep. Alhoewel IMI, IMI-ureum en desnitro-IMI nie in enige van die weefselmonsters opgespoor kon word nie, is 5-hidro IMI by relatief hoë konsentrasies in die lewer (0.793 ng/mg weefsel) en kieuepiteelweefsel (117 ng/mg weefsel) opgespoor. Slegs konsentrasies gamma-aminobottersuur en asetielcholien is in beide die behandelde en kontrolegroep opgespoor en gekwantifiseer. Die behandelde groep het 'n 1.6-voudige afname en 1.9-voudige toename in asetielcholien en gamma-aminobottersuur onderskeidelik in vergelyking met die kontrole getoon. Serotonien en dopamien kon nie opgespoor word nie omdat hul vlakke onder die drumpel van kwantifisering van hierdie metode was.

Gevolgtrekking

Robuuste LC-MS/MS-metodes is ontwikkel vir die opsporing en kwantifisering van IMI, desnitro-IMI, imidacloprid-ureum en 5-hidro imidakloprid, sowel as serotonien, dopamien, asetielcholien en gamma-aminobottersuur neuro-oordragstowwe in 200 µL sebravisse brein, lewer en kieuepiteelweefsel homogeniseer. Die gedragsveranderinge wat by die volwasse sebravis waargeneem word, dui ‘n toksisiteit/verdovend effek aan en word geverifieer deur die verhoging in gamma-aminobottersuur vlakke. Saam met die evaluering van bioakkumulasie van imidacloprid en sy metaboliete binne die brein, lewer en kieuepiteelweefsel van sebravis, verskaf hierdie studie 'n aanduiding van die impak op menslike gesondheid na chroniese neonikotinoïedblootstelling. Oor die algemeen dra ons bevindinge verder by tot bestaande literatuur en stel voor dat IMI wel 'n bedreiging vir meer as net insekte inhou en daarom verdere ondersoek verg.

Table of contents

List of Figures

List of Tables

List of abbreviations and symbols

°C	Degrees Celsius
%CV	Coefficient of variation
µM	Micromolar
cm	Centimetres
5-HT	Serotonin
α4β2	Alpha-4-Beta-2
ACh	Acetylcholine
AChE	Acetylcholinesterase
ACN	Acetonitrile
ADHD	Attention deficit hyperactivity disorder
ADME+T	Absorption, distribution, metabolism, excretion and toxicity
AOX	Aldehyde oxidase
BBB	Blood-brain barrier
ChAT	Choline acetyltransferase transporter
6-CNA	6-Chloronicotinic acid
CNS	Central nervous system
CYP	Cytochrome P450
DA	Dopamine
DCA	3, 4-Dichloroaniline
DCM	Dichloromethane
Dpf	Days post fertilization
EA	Ethyl acetate
EPA	US Environmental Protection Agency
EQS	Environmental Quality Standards
ERK	Extracellular signal-regulated kinase
ESI	Electrospray ionisation
EU	European Union
FAO	Food and Agriculture Organization of the United Nations
FDA	USA Food and Drug Administration
FET	Fish embryo acute toxicity test
GABA	Gamma–aminobutyric acid
GC-MS	Gas chromatography coupled to mass spectrometry

GFAP	Glial fibrillary acidic protein
Hex	Hexane
HFBA	Heptafluorobutyric acid
HILIC	Hydrophilic interaction liquid chromatography
Hpf	Hrs post fertilization
HPLC	High–performance liquid chromatography
HREC:BES	Health Research Ethics Committee: Bioethics and Safety Committee
6-HNA	6-Hydroxynicotinic acid
IMI	Imidacloprid
IMI-NH	Desnitro imidacloprid
IMI-NNO	Nitrosoguanidine-imidacloprid
ISTD	Internal standard
KCl	Potassium chloride
LC-MS/MS	Liquid–chromatography tandem mass spectrometry
LD_{50}	Lethal dose 50
LLE	Liquid-liquid extraction
LLOQ	Limit of quantitation
LOAEL	Lowest observed adverse effect level
MAPK	Mitogen-activated protein kinase
MCOD	Methoxycoumarin O–demethylase
MeOH	Methanol
$MgSO_4$	Magnesium sulphate
MOA	Mechanism of action
MRM	Multiple reaction monitoring
MS/MS	Tandem mass spectrometry
m/z	Mass-to-charge
nAChRs	Nicotinic acetylcholine receptors
NaCl	Sodium chloride
ng	Nanogram
NNO	Nitrosoguanidine imidacloprid
NOAEL	No observed adverse effect level
5-OH IMI	5-Hydroxy imidacloprid
OHDA	6-Hydroxydopamine
PPT	Protein precipitation

QC	Quality controls
RBC	Red blood cells
RPLC	Reversed phase liquid chromatography
SAVC	South African Veterinary Council
SL	Standard body length
SOPs	Standard Operating Procedures
SPE	Solid phase extraction
SS	Stock solution
TCA	Trichloroacetic acid
TMS	Tricaine methanesulfonate
UHPLC–MS/MS	Ultra-high performance liquid chromatography-tandem mass spectrometry
USA	United States of America
UV	Ultraviolet
VAChT	Vesicular acetylcholine transporter
WHO	World Health Organization
WS	Working solution

Chapter 1: Introduction and literature review

1.1 Background

According to the Food and Agriculture Organization of the United Nations (FAO), a pesticide is any compound or combination of compounds produced to distinctly hinder, harm or control any pest, including transmitters of human or animal disease. A pesticide is a general name that can be further subdivided into herbicides, insecticides, fungicides, antimicrobial agents, pool chemicals, and many more insect-and rodent-managing devices [(1)(2)(14)]. The classification of pesticides can be based on their target, their mechanism of action (MOA) or their chemical make-up [(3)]. While pesticide usage has economic benefits in large-scale agriculture, exposure can lead to detrimental effects on humans and other vertebrates [(4)(5)].

There are many health problems that may be related to pesticide exposure. In particular, the nervous system is extremely susceptible to many pesticides belonging to various chemical families [(6)(7)]. The development of the human brain takes place over an extended period, its maturation continuing through adolescence and young adulthood. Examining the course of brain development in distinct samples of the general population is crucial for understanding exposures as well as stressors in the child's and adults' physical and social environment that sculpts human brain development [(8)]. Ideally, a longitudinal follow-up in each individual would be required but this lacks feasibility therefore it would be good to develop experimental models for this.

This particular study focused on one specific pesticide, more particularly, a neonicotinoid insecticide. Despite neonicotinoids decreased affinity for the nicotinic receptors of vertebrates, negative effects have been documented in vertebrates [(9)]. More importantly, proof of human pesticide risk is predominantly from developed countries despite their use in developing countries, including South Africa, being excessive and largely uncontrolled [(7)(10)].

Additionally, there is a hypothesis stating that numerous untested neurotoxic substances may be behind the so called "silent pandemic", in which early life exposures are causing many neurodevelopmental disorders [(10)]. For the most part, genetic factors only account for approximately 30 - 40% of all the occurrences of neurodevelopmental dysfunction therefore non-genetic and environmental factors do play a role in the causation [(10)]. Currently, there is proof that the concentrations of possible neurotoxic substances has risen in our surroundings and that these substances may interrupt the maturation of the central nervous system (CNS) in humans [(11)]. To date, it has been widely accepted that the developing nervous system has increased vulnerability to neurotoxicants in comparison to adults. That means, in order to ensure the protection of

individuals, it is crucial to have definitive models to recognize and evaluate environmental pesticides that may cause harm [6][10][12].

1.2 Pesticide use and need in agriculture

As the human population continues to increase, additional crops are required to meet this growing need. The outcome of this is the increased use of pesticides to increase the crop yield [1][6][13][14]. The yearly application of pesticides to crops in the European Union (EU) surpasses 140 000 tonnes [15]. A minimal percentage of the applied pesticide reaches the intended biological target, while the majority of the pesticide is dispersed into the surroundings where it can have negative consequences [16]. The primary routes of entry of pesticides into water bodies include agricultural runoff, leaching and spray drift [17][18]. These chemicals significantly affect wildlife, water quality as well as air quality [14]. The extensive use of these pesticides also increases the risk to biodiversity of freshwater aquatic surroundings due to their potential bioaccumulation and toxicity [2][17]. Insecticide residues in the environment have a detrimental effect on human health as well as the environment [7][19]. It is therefore necessary to record and evaluate any possible impacts of these pesticides on biodiversity.

Additionally, bees, which are known to support the growth of trees, flowers and other plants have been largely affected by the increased utilization of pesticides. Bees also play an essential role in the pollination of the foods that we consume, therefore a decrease in the bee population may have a substantial effect on crop yield [14][20][21]. The EU provisionally forbade the use of imidacloprid and two other neonicotinoid pesticides in 2013 due to observable bee-poisoning incidents [3][22][23]. This puts a question mark over the safety of and potential undesired outcomes following the use of this particular pesticide.

In South Africa, impoverished communities are faced with poor housing, poor service delivery, unemployment and overpopulation which exacerbates persistent pest infestations associated with risks of disease. Informal businesses supply highly toxic, efficient, low cost and illicit street pesticides, either those which are legally registered for agricultural use but transferred into unlabeled containers or prohibited street pesticides. The former are not registered for household utilization due to their high acute toxicity, presenting a substantial health risk to informal vendors and consumers [24][25].

Turning attention to the specific group of insecticides relevant to this study, nicotine, an alkaloid present in the leaves of the genus *Nicotiana,* for some time was acknowledged for its insect-killing and pharmacological characteristics. However, due to its non-systemic, volatile and highly toxic characteristics to both insects and mammals, it was substituted for neonicotinoid insecticides [26][27]. These particular insecticides have been used for more than 20 years and globally, they are registered in more than 120 countries [28][29]. All

neonicotinoids have a similar structure to nicotine and their main target in insects is the cholinergic system [23][26]. One of the crucial differences between nicotine and the neonicotinoids is that nicotine is positively charged at physiological pH in contrast to neonicotinoids that are not but have an electronegative nitro- or cyano-functional group [23]. Nicotine can affect the development of the mammalian nervous system as ordinary developmental neurotoxicants and there has been increased scrutiny over the fact that human exposure to neonicotinoids may result in a rise in the likelihood of developmental neurotoxicity [30].

The neonicotinoid pesticides include a variety of different insecticides. The most widely used neonicotinoids include imidacloprid, thiacloprid, clothianidin and thiamethoxam [3]. The neonicotinoid insecticides especially have drawn significant attention globally as there has been an overwhelming rise in evidence over the past 10 years regarding possible detrimental risks to humans, non–target insects, aquatic invertebrates as well as an impact on the surrounding environment after use [31][32][33]. There have been various *in vivo* and *in vitro* studies which have debated the fact that neonicotinoids are less toxic to mammals. They revealed important toxicological potencies of imidacloprid on various mammalian organs such as the brain, liver, and their normal physiological functioning [33][34]. A systematic review on various neonicotinoid studies published between 2005 and 2015 discovered only eight studies that focused on the effects that this pesticide has on human health [35]. Despite the extensive research relating to neonicotinoid insecticides, many gaps remain. Firstly, researchers should focus on neonicotinoids as a class as well as individually instead of as a mixture with other pesticides. Secondly, include the sampling of food or drinking water utilizing validated biomarkers. Another crucial gap identified in the literature is the lack of an environmentally relevant idea of neonicotinoid exposure [35].

Prior to a pesticide being registered, there is a major focus on studying the effects of the parent entities although farmers and the public are frequently exposed to both the parent compounds as well as their breakdown products [26]. Therefore, in this current study, imidacloprid and three of its primary metabolites will be comprehensively assessed to determine the possibility of neurocognitive decline following prolonged exposure to low levels of IMI in adult zebrafish (*Danio rerio*).

1.3 Zebrafish as an animal model for toxicological studies

Up to the present time, many toxicological studies have been performed using mice, rats or rabbits as test organisms, but this is associated with high costs as well as well as lengthy experimental research periods [36][37]. Zebrafish reproduce within 30 min of morning light onset, hatch within 2 - 3 days post fertilization (dpf), start developing complex behavior within the first 7 days and are sexually mature within 3 - 4 months. At this time, they are considered as adults. For these reasons, after being exposed to a specific substance

they can easily be monitored over the complete lifespan of the zebrafish in comparison to the rodent models which take up to 6 months to reach maturity [38][39][40].

Additionally, between 48 and 72 hrs post fertilization (hpf), which is essentially during early development, the majority of organs are almost completely developed with the exception of the organs situated in the gastrointestinal tract, which become fully functional by 7 dpf. Adult zebrafish exhibit a varied collection of intricate behaviors such as memory and learning, social interactions as well as looking for prey [41][42]. Their capability to take up compounds through the water, also known as immersion administration, enables pharmacological and toxicological research, especially for compounds which have a high water solubility [39]. The adult zebrafish take up drugs via the gut in contrast to the larvae, where drugs are predominantly taken up via their gills and skin. As a result, zebrafish have become a helpful model to study chemical as well as developmental and neurobehavioural toxicology [38][43][44].

Zebrafish have been primarily utilised in developmental biology as well as molecular genetics but the advantage of their use in toxicology and drug discovery has been acknowledged. More morphological, biochemical and physiological details at all phases of development in juveniles and adults of both male and female are known about the zebrafish in comparison to any other species of fish. Therefore, zebrafish have become an optimal model for toxicology research [38].

Biological outcomes of chemicals have been thoroughly assessed in zebrafish and this is generally accomplished by merely dissolving a chemical in the water. This is followed by the monitoring of any phenotypic alterations and/or toxic endpoints [37][42]. The movement of zebrafish is an intricate behaviour that needs a coherent response of the brain functions, nervous system as well as visual pathway. Consequently, this behavioural pattern, together with automated tracking techniques, is gaining more and more attention for its utilisation in high-throughput screening of neurotoxic compounds. It is important to acknowledge that most toxicological studies using zebrafish as a model have concentrated on environmental pollutants, however a growing number are developing in the area of pharmaceutical toxicology [42]. This emphasises the utility of zebrafish as a model for toxicological studies.

There are many benefits of using zebrafish as a model for toxicological research such as their small embryos that allow for acceptable sample sizes to be assessed using one cell-culture plate to supply various experimental replicates at a single time. This allows for high-throughput analysis for toxicity testing as well as decreases the amount of test substances that would be required [42][45]. Additionally, what makes this species indispensable is their high fertility and transparency. A pair of adult zebrafish are able to lay

approximately 200 - 300 eggs per spawning and this yield can be supplied every 5 - 7 days on condition that the fish are maintained properly. Moreover, the larvae are transparent up until 9 - 14 dpf which allows for the non-invasive assessment of the development of organs as well as end points indicative of toxicity [38][41][42].

As a non-mammalian species, they do have specific disadvantages for simulating human disease such as lacking certain mammalian organs such as lungs and mammary glands. However, due to their versatility and distinctive characteristics, it is believed that zebrafish will play a progressively important role in identifying and studying disease as well as therapeutic discovery [41].

Additionally, despite being phylogenetically more distantly related to humans than rats, several similarities have been identified between zebrafish and humans. 71% of human proteins and 82% of disease-causing proteins in humans have a well characterized ortholog in zebrafish [41][46][47]. Their organ systems, some of which have adapted for life in an aquatic environment, accomplish much the same role as their human equivalent, suggesting a surprisingly well conserved physiology [42][48][49]. Moreover, the use of zebrafish as an analog to mammalian models has both economic and logistic benefits due to their small size and fairly simple maintenance [38][50].

Progress in the field of mass spectrometry will increase the use of zebrafish to study absorption, distribution, metabolism, excretion and toxicity (ADMET) [48]. The diversity of the zebrafish makes them extremely useful in various research fields. There is also increasing evidence that the majority of the overall signal transduction pathways are also conserved. In addition, because of our evolutionary past, several of the anatomical and physiological characteristics of fish are comparable to humans. An example of this is the multiple characteristics that zebrafish and mammalian brains have in common including the highly conserved neurotransmitter structures as well as systems [51]. The important shared neurotransmitter systems include glutamate, gamma-aminobutyric acid (GABA), dopamine (DA), adrenaline, noradrenaline, serotonin (5-HT) and acetylcholine (ACh) [42][47][52]. Additionally, zebrafish larvae exhibit behavioural patterns comparable to mammalian models after exposure to neuroactive compounds thereby further rendering zebrafish ideal for studies assessing neurotoxic effects [53].

More importantly, progress has been made by recent studies substantiating the existence of zebrafish basal ganglia, cortex, amygdala and hippocampus-like circuits [54][55][56][57]. The generation of the zebrafish CNS starts as early as 16 hpf, with neuronal development. By 17 hpf, the zebrafish brain forms its sub-regions, the midbrain-hindbrain boundary, cerebellum and thalamus together with the formation of the brain ventricles [58]. The blood-brain barrier (BBB) starts to emerge at 20 hpf, displaying the well-developed microglia and complete barrier structure at 3 dpf [52][59]. The primary vascular plan in the developing zebrafish

is similar to that of other vertebrates. Drug efflux transporters present at the BBB have been identified as the main determinant affecting the pharmacokinetics as well as the pharmacological behavior of various CNS drugs [60]. The ATP-binding cassette (ABC) efflux transporters, located in the BBB, can adequately remove the drug from the CNS and ultimately restrict the brain uptake when the drug is a substrate of these transporters. The ABC gene family has been annotated and analyzed phylogenetically in zebrafish. 77% of all human ABC genes have a zebrafish orthologue [60]. These specific efflux transporters are vital in the regulation of various xenobiotics including pesticides [61]. The fundamental neural signaling systems, which are ontogenetically similar to mammalian systems, form between 18 and 32 hpf and include the catecholaminergic, (GABA)ergic, serotonergic, and glutamatergic system and therefore can also be utilized as a pharmacological and toxicological target [62][63]. Figure 1.1 [47] below illustrates the dispersal of modulatory neurotransmitters in the zebrafish and human brain [47]. Important to note is that many behavioral studies have recognized the strong similarity between the functions of zebrafish and human brain regions [42].

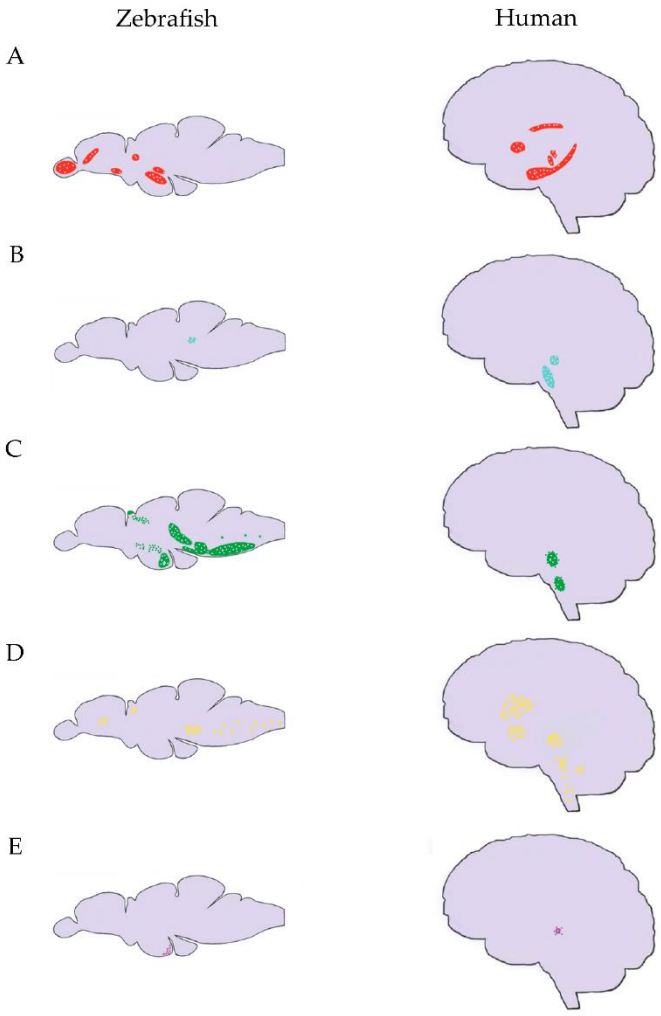


Figure 1.1: The dispersal of modulatory neurotransmitters in the zebrafish and human brain. (A) Location of dopamine (red) in the zebrafish (left) and human (right) brains; (B) Location of norepinephrine (blue) in the zebrafish (left) and human (right) brains; (C) Location of serotonin (green) in the zebrafish (left) and human (right) brains; (D) Location of acetylcholine (yellow) in the zebrafish (left) and human (right) brains; (E) Location of histamine (purple) in the zebrafish (left) and human (right) brains; (B) Location of norepinephrine in in the zebrafish (left) and human (right) brains. The brains are not to scale for representation purposes [47]

Cholinergic neurons are characterized by the appearance of choline acetyltransferase transporter (ChAT), which plays a role in ACh production as well as the vesicular ACh transporter (VAChT), which is responsible for loading ACh into vesicles. Presynaptic vesicular discharging of ACh into the synaptic cleft results in the stimulation of the ACh receptors by binding to them, before being broken down by acetylcholinesterase enzyme (AChE) into choline and acetate. These products are then transported into the presynaptic neurons by the high-affinity choline transporter (HACT) [64]. In the embryonic zebrafish, motorneurons begin expressing ChAT transcript from 16 hpf and the ChAT protein is detected in 2-day old embryos. Additionally,

AChE and several nicotinic acetylcholine receptor (nAChR) subunit transcripts appear in the spinal cord during early development. A spatiotemporal map published by Rima et al (2020) indicated that 1-day old embryos expressed the complete system for ACh release [64][65][66].

On the other hand, the use of zebrafish as a model of human liver endogenous and exogenous substance metabolism needs the entire range of CYP genes. The identification and annotation of these genes in zebrafish have been performed in terms of their phylogenetic relationships to human CYP enzymes. This crucial study was documented by Goldstone et al (2010) [67]. They specified a total of 94 CYP genes in the zebrafish genome. They also documented, on the basis of homologous amino-acid sequences, that these genes conformed into 18 CYP gene families that are also found in humans as well as other mammalian species. The CYP enzyme families 1 - 4 that are primarily responsible for the metabolism of exogenous compounds have a greater diversity in zebrafish in comparison to humans. Nonetheless, investigation of shared synteny displays an evolutionary association between human and zebrafish CYP genes. There is also a great level of conservation between human and zebrafish sequences with regards to the CYP families 5 - 51 [43][67].

Overall, the use of zebrafish is a compact but extremely robust model as it provides an economical balance between *in vitro* experiments and more intricate, mammalian systems. The numerous benefits of this model allow for simple adoption of novel methods, technologies and fields of study [68]. In fact, many behavioural assays have been performed for both neurological and/or neuropharmacological research in zebrafish due to their behaviour, also known as locomotor activity, being astonishingly indicative of their neurophysiology [69].

1.4 Imidacloprid, a neonicotinoid insecticide

Imidacloprid (IMI) [1-(6-chloro-3-pyridylmethyl)-N-nitroimidazolidin-2-ylideneamine] is a systemic neonicotinoid insecticide belonging to the chloronicotinyl nitroguanidine chemical family intended to replace the organophosphate pesticides in agriculture [39][70]. IMI's insect-killing activity is 12 times higher than that of nicotine and more systemic [26]. IMI is referred to as a "systemic" neonicotinoid as it can be dispersed throughout all plant tissues, where it can ultimately persist for long periods of time, due to its small size and high water solubility. This property of IMI makes it effective against sucking as well as some chewing insects. They are also dispersed into the plant's pollen and nectar, which exposes non-target organisms such as pollinating honeybees and bumblebees [22][29][71][72][73].

In addition, IMI is also considered as one of the most popular and extensively used neonicotinoids in the world and is commercially known as Confidor and Provado [34][74][75]. The various uses of IMI include the

protection of plants/crops against insects and mites, urban pest control as well as veterinary use, against ticks and fleas on dogs, cats and cattle [72][75]. To date, 90% of all corn and 50% of soybeans are grown from seeds that were treated with neonicotinoids. They are also extensively utilized on other cereal and oil crops [76][77]. The extensive use of neonicotinoids is also due to their flexibility of use. They can be utilized as foliar sprays, bait, granular formulations, soil drench or in irrigation water [77]. The frequent and abundant use of these insecticides increases the risks and hazards that could be associated with them [22].

IMI is a colourless crystal with a melting point of 144 °C and has a molecular mass of 255.6 g/mol [74]. The half-life of IMI in soil has been previously reported to be in the range from 28 - 1 250 days. In addition to this, it has been found to be more persistent in soils high in organic matter than in soils that contain a high percentage of water [77][78][79]. Long-term precipitates of IMI in the soil pose a significant risk to a variety of organisms residing in the soil as well as human health [79]. Repeated use of IMI can also result in a build–up in the soil [22]. IMI has a half-life of longer than 30 days in water at a pH between 5 and 9 [78]. IMI is broken down in sunlight thereby reducing its risk in surface bodies of water but it has the ability to run off into groundwater where it lasts for longer durations and this further contributes to the risk of IMI to the health of non-target organisms such as humans [80]. According to a review assessing surface water in a variety of different countries, neonicotinoids were often observed in surface water with concentrations ranging between 0.001 - 320 µg/L [32]. Given the scale of utilization of neonicotinoids, their persistent nature in soils, their leaching ability as well as their systemic nature within plant tissue, it is obvious that the majority of organisms living in arable surroundings will be exposed to them [77][81]. The main question is whether normal levels of exposure have a likelihood of giving rise to serious individual- or population-level effects.

People who do not come into direct contact with these insecticides can be exposed when consuming fruits and vegetables collected from agricultural land where neonicotinoids were used. A study reported that from 29 fruit and vegetable samples, neonicotinoids were observed in 72% of the fruits and 45% of the vegetables and due to their high water solubility, humans can also be exposed by drinking contaminated water [32][82][83].

Despite being moderately toxic (oral acute dose LD_{50} is 50 to 500 mg/kg; class II by World Health Organization (WHO) and toxicity category II; US. EPA, 1994), the understanding of human poisoning is not clear. Ingestions and inhalations have been associated with gastrointestinal irritation, respiratory failure, neuropsychiatric symptoms, rhabdomyolysis, ventricular fibrillation and death [70][84]. Acute exposure to IMI has been reported to be associated with symptoms similar to nicotinic signs such as fatigue, cramps, muscle weakness and twitching and it has been found that IMI does not irritate the eyes or skin [85][86]. To date, there is no antidote

available for IMI poisoning therefore patients are treated symptomatically [70][85]. The chemical structure of IMI is illustrated in Figure 1.2.

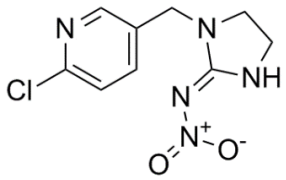


Figure 1.2: Chemical structure of imidacloprid. Image created using Biorender

1.5 Mechanism of action of imidacloprid in insects

Neonicotinoids are substances that act as agonists, similar to nicotine and nicotinoids in humans, on the insect's nicotinic acetylcholine receptors (nAChRs) [23][71][72][87][88]. These receptors are members of a superfamily of ligand-gated ion channels and are primarily responsible for quick excitatory cholinergic neurotransmission. To be more specific, neonicotinoids act on the α4β2 nAChR subtype opening cation channels. The nitromethylene, nitroimine or cyanoimine group which is rich in electrons allows the neonicotinoid to selectively target insects by binding more forcefully to insect nAChRs in comparison to mammalian nAChRs [23][71][88]. The identification of the high affinity IMI binding sites in insect nAChRs were observed in a wide range of insects such as green peach aphid, glassy-winged sharpshooter, cockroach as well as the fruit fly [3][89].

In insects, the nicotinic receptors can only be found in the CNS, and therefore it is essential for neonicotinoids to cross the CNS ion barrier for them to exert their effects [26][90]. Ultimately, the pesticide binds irreversibly to insect receptors during which, at low levels, nerve impulses are spontaneously conducted, followed by the inability of the neuron to generate any signal at all at higher levels. In contrast to ACh, AChE does not act on nicotine nor IMI, perhaps causing their continued action on the nAChRs ultimately leading to desensitization and blocking of the nicotinic receptor causing the death of the insect [3][26][72][90][91]. This mechanism of action is depicted in Figure 1.3 [27].

Neonicotinoids have also been reported to imitate the action of neurotransmitters and competitively inhibit the nAChR, by competing with the natural neurotransmitter ACh [72][85]. Neonicotinoids are typically toxic to insects in tiny quantities. For instance, the $LD_{50,}$ which is the quantity that kills half of the individuals, for ingestion of IMI in honeybees is only 5 nanogram (ng) per insect [77].

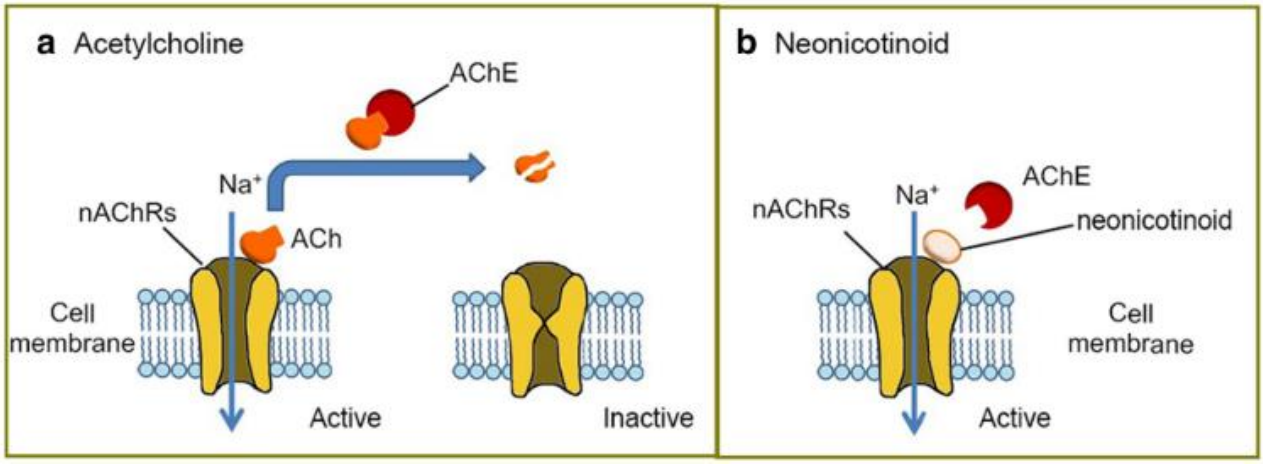


Figure 1.3: The action of nicotinic acetylcholine receptors in the presence of acetylcholine (a) and a neonicotinoid insecticide (b). These receptors are generally activated by binding ACh. Subsequently, they are deactivated when ACh is broken down by AChE resulting in the production of octane and choline (a).Similar to ACh, neonicotinoids can bind to and activate the nicotinic ACh receptors. In comparison to ACh, they cannot cause deactivation by AChE and thereby resulting in the overstimulation of the nervous system and ultimately cell death (b) [27]

1.6 Mechanism of action of imidacloprid in vertebrates

From a previously conducted study, IMI was documented to act as either an agonist or antagonist of nAChRs at 10 µM in rat pheochromocytoma cells [91][92]. Exposure to IMI *in utero* resulted in reduced sensorimotor performance and a rise in glial fibrillary acidic protein (GFAP) expression in the motor cortex and hippocampal region of the brain of neonatal rats. The nAChRs are crucial for the normal development of the brain. Another study reported that administration of IMI across the concentration range, 1 - 100 µM induced intracellular excitatory Ca^{2+} influxes in cerebellar neurons. It is likely that the interaction of ACh with nAChRs changes the morphology of the receptors, which may cause neonicotinoids to interact with the mammalian nAChRs [91]. Similarly, another study documented that IMI is a low-efficacy, high-affinity agonist of the human α4β2 receptor. This specific receptor subtype forms majority of the high-affinity nicotinic binding sites in the brain [93].

In addition, IMI has been shown to be a partial agonist by upregulating the α4β2 subtype in mouse M10 cell line and has been shown to desensitize the nAChR [3]. Cellularly, the direct outcome of the receptor activation is a rise in the amount of intracellular calcium and therefore membrane depolarization. This ultimately results in the activation of an intricate downstream signaling pathway [3][94]. A crucial constituent playing a role in the nAChR downstream signaling pathway is mitogen-activated protein kinase (MAPK), also known as the extracellular signal-regulated kinase (ERK). This ERK pathway is an imperative intermediate in the

signaling from the receptor to expression of particular genes [3][95]. Various human pathologies associated with the nervous system have been correlated to genetic changes of these receptor genes [3][96]. In addition, these receptors also play a role at various stages in many neurodegenerative disorders such as Parkinson's and Alzheimer's disease [3]. There is an increasing amount of information which associates long-term low dose pesticide exposure to several diseases such as cancers, reproductive health problems, as well as various neurological diseases [3][97]. IMI has shown partial agonistic activity with the recombinant chicken α4β2 as well as α7 receptors. IMI has also displayed agonistic activity on the nAChRs in BC3H1 muscles cells as well as mouse N1E-115 neuroblastoma cells [3].

In comparison to insects, these nAChRs are found in both the CNS as well as at the neuromuscular junction, spinal cord and various brain regions in vertebrates [73][90]. According to literature, the binding affinity of IMI at the mammalian nicotinic receptors is less than in insects and this is mainly due to fundamental pharmacophore differences between organisms. The key role-player in the neonicotinoids selectivity towards the insect's nAChR is the 2-nitroimine-imidazolidine group [3][26].

However, there are many inconsistencies in studies pertaining to the effect of neonicotinoids on the vertebrate nervous system. In spite of studies reporting the toxicity of neonicotinoids to mammals, essentially due to their action at the α4β2 receptor subtype in the brain, they are thought to be environmentally friendly insecticides [90][89]. However, the breakdown products of neonicotinoids may display marked toxic effects [23].

According to literature, the breakdown products of IMI that possess the nitroguanidine pharmacophore, such as IMI olefin, still show toxicity to honeybees. IMI olefin has also shown greater activity on nAChRs of honeybees in comparison to IMI itself [79][98]. Consequently, the main reason fewer studies focus on the possible impact of neonicotinoids on mammalian physiology is because of the common perception that the decreased affinity of these insecticides for the vertebrate nAChR's makes it unlikely to have any substantial health risks to mammals despite the fact that these receptors are largely dispersed throughout human and rodent brains during all stages of development [99][100][101].

In mammals, the nAChR possesses a conserved core of electron-rich aromatic amino acid residues within the extracellular loops inhibiting the electronegative tip of neonicotinoids from connecting with the receptor. However, neonicotinoid breakdown products that lack this electronegative tip, such as desnitro-IMI (IMI-NH), can obtain selectivity for the mammals nAChR, specifically the α4β2 subtype at the neuromuscular junction, as depicted in Figure 1.4 [23] below [26][102][103][104]. Researchers studying the effect of IMI on human nicotinic receptors (α4β2) have reported that IMI may have more substantial side effects on humans than originally anticipated [105].

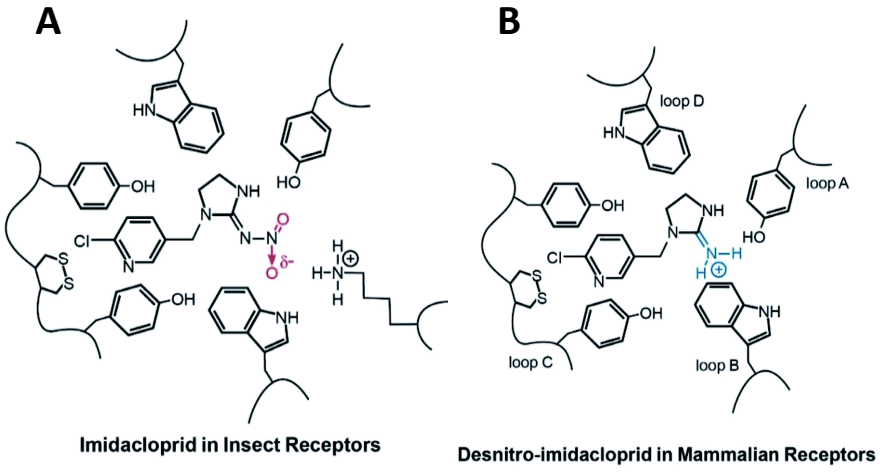


Figure 1.4: The electronegative pharmacophore of the neonicotinoid displays selective binding to the insect nAChR (A). Insecticidal target specificity is lost when the metabolite, desnitro-imidacloprid, without the nitro group, binds to the vertebrate nAChR (B) [23]

According to Rodrigues et al (2010), it has also been discovered that thiamethoxam, a neonicotinoid insecticide similar to IMI, or its metabolites, may affect the cholinergic system of rats. This effect on the cholinergic system illicits biochemical and behavioral outcomes that could be related to the development of neurodegenerative diseases such as Alzheimer's disease and involves neuronal cell loss, decline of ChAT, sodium-dependent choline transporter and AChE activities, as well as a decline in ACh in the hippocampus and cortical parts of the brain [88]. The ACh receptor has also been characterized to regulate dopaminergic neurotransmission, primarily by intensifying DA release in nigrostriatal and mesolimbic dopaminergic systems. Dopaminergic axons possess clear subtypes of heteromeric nAChRs, which permits striatal ACh as well as its agonists to function, by regulating DA release. Due to the high affinity of these various neonicotinoids to the α4β2 nAChR subtype, which is most abundantly found in the striatum, it could potentially mean they also play a role in striatal DA release and modulation [106].

It is important to acknowledge that the liver was found to be the main target organ following IMI exposure in rats and dogs. This was confirmed by the appearance of liver necrosis or hypertrophy, increased serum enzymes as well as changes in other clinical chemistry variables such as triglycerides, cholesterol as well as the blood clotting time [3][107]. Liver toxicity following low dose IMI exposure should be further evaluated. Neonicotinoid toxicity to insects has been thoroughly assessed, however, their exact effects on mammalian nAChRs remain to be clarified.

1.7 Metabolism of imidacloprid

It is crucial to understand the various metabolic pathways as well as the environmentally relevant breakdown products of neonicotinoids in order to evaluate the likelihood of any health risks. The metabolic processes of these insecticides can either result in an increase or a decrease in its potency, all dependent on the substrate and specificity of the nAChR. Many of these breakdown products are biologically active and often have increased toxicity to either insects or vertebrates. Most of the time, the change in bioactivity is usually due to the transformation of the pharmacophore, which is the electron-rich nitro- or cyano-functional group, particularly on the parent neonicotinoid. As has been noted, an example of this is IMI-NH, which is a breakdown product of IMI relative to insect nAChRs as well as insecticidal activity. However, it is also an activated derivative for mammalian nAChRs. Since it displays toxicity to mammals, it can be reviewed as a bioactivation. This can be explained by the dispersion of the positive charges at the guanidine thereby giving it its potential to bind to mammalian α4β2 nAChRs, which is illustrated in Figure 1.4 above [23][34][72][98][108]. Therefore, it is also vital to understand and recognize the metabolism as well as the different breakdown product forms of these insecticides *in vivo* to further evaluate their toxicological risks.

The CYP enzymes play a crucial role in various physiological processes but particularly in the transformation of various drugs and xenobiotics. A few of the CYPs such as CYP1, CYP2 and CYP3 have important roles specifically in speeding up oxidative biotransformation of chemical substances such as drugs and toxicants and therefore play a role in their persistence and actions. The majority of all drugs used clinically are metabolized by the CYP enzymes [109]. The CYP450 enzymes, which are mainly found in the liver, as well as cytosolic aldehyde oxidase (AOX) are predominantly responsible for the metabolism of neonicotinoids. A study conducted by Swenson et al (2013) documented that AOX plays a crucial role in mouse IMI hepatic nitroreductase *in vivo.* This was the first study to provide definitive proof that not only CYPs are involved in the metabolism of neonicotinoids but also AOX. It was also the first study to confirm the toxicological significance of AOX in *in vivo* metabolism of chemical compounds. The biotransformation of IMI to IMI-NH and nitrosoguanidine-IMI (IMI-NNO) is mainly due to AOX, not CYPs [23][43][26][98][110].

In zebrafish, the liver is the biggest organ in the abdominal region and plays a critical role in homeostasis as well as the protection against chemical substances. Despite the structural arrangement of the zebrafish liver being different from that of the livers of rats and humans, its drug-metabolizing characteristics are comparable in zebrafish, rats and humans. In addition, the zebrafish liver is very similar to the liver of mammals in terms of biological purpose which is the processing of fats, vitamins, proteins and starch as well as the production of serum proteins. At 5 dpf, the zebrafish larvae already have a fully functioning liver [43][109].

Figure 1.5 [111] below is a comparison of the liver structure in humans and zebrafish. The most obvious difference is that the zebrafish liver does not have normal portal triads. The liver cells are organized in tubules, and the intrahepatic bile ducts are positioned in the middle. Comparable to mammals, zebrafish liver cells also secrete bile salts into bile ducts though bile canaliculus on the apical side. Blood vessels are positioned on the basal side of the liver cells, mimicking the sinusoid capillaries in the mammalian liver [111].

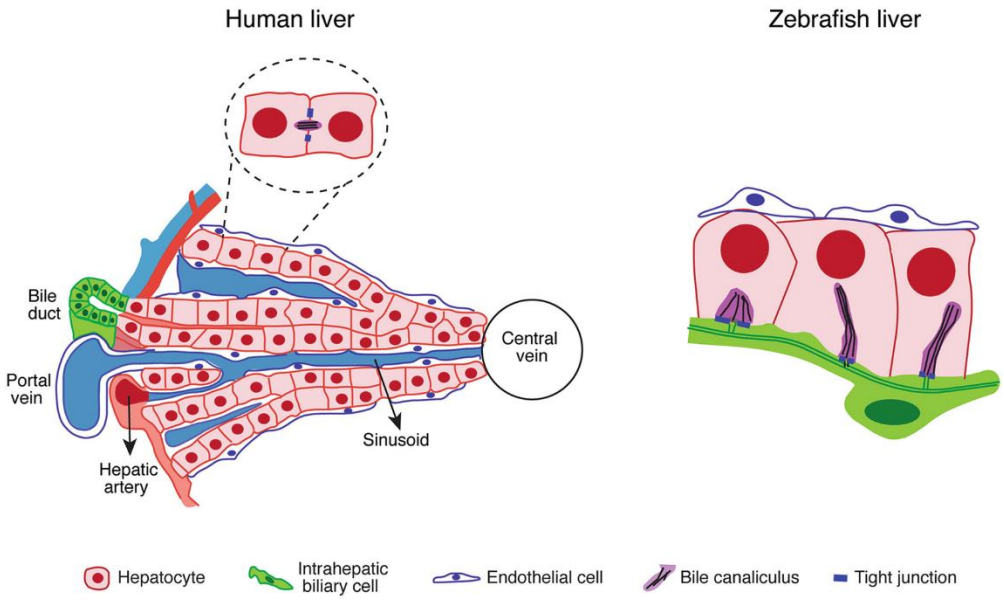


Figure 1.5: Liver architecture in humans and zebrafish [111]

Equally important, neonicotinoids go through both phase I and phase II metabolism in insects, mammals and plants. In summary, IMI is oxidized to the 5-hydroxy IMI (5-OH IMI) as well as IMI olefin breakdown products. It also undergoes reduction to IMI-NH, IMI urea as well as the nitrosoguanidine and aminoguanidine breakdown products. The most copious CYP enzyme in humans, CYP3A4, is also the most active CYP enzyme for oxidation of the imidazolidine moiety of IMI [26][110][112]. As depicted in Figure 1.6 [112] below, IMI is broken down by CYP enzymes via two vital pathways: the first one being imidazolidine hydroxylation and desaturation to produce 5-OH IMI and IMI olefin respectively, and second nitroimine reduction and cleavage to produce the nitrosoimine, guanidine and urea counterparts. Each of IMI's breakdown products seem to differ with regard to their effects, affinities or potencies towards the target receptor [34][113][114]. Neonicotinoids and their breakdown products have been observed in a variety of human biological fluids including urine, hair and serum [22].

Similarly, in a study conducted by Nimako et al (2021), various IMI breakdown products such as 5-OH IMI were found in the blood, kidney, lungs and testis of mice. In this same study, it was reported that IMI-NH displayed notable accumulation in the liver, brain, testis, lungs and kidney of mice. Toxicologically, this particular accumulation of IMI-NH in the brain is of concern as this metabolite is known to be more toxic to

mammals. It was also recommended that more studies should focus on the evaluation of the fate of IMI in various organs following prolonged, low-dose exposure [34].

Additionally, a human pharmacokinetic model for neonicotinoids was set up, *in vivo*, and it was observed that IMI was predominantly metabolized and excreted in urine as its various metabolites [110][115]. The predominant breakdown products of IMI in mammals has been reported to be 6-Chloronicotinic acid (6-CNA), its glycine conjugate and 5-OH IMI [116].

According to literature, higher levels of IMI's metabolites in relation to IMI itself was found in honey and together with the abovementioned, this is a clear indication that more focus should be allocated to the breakdown products when evaluating the risks of utilization of these insecticides [21].

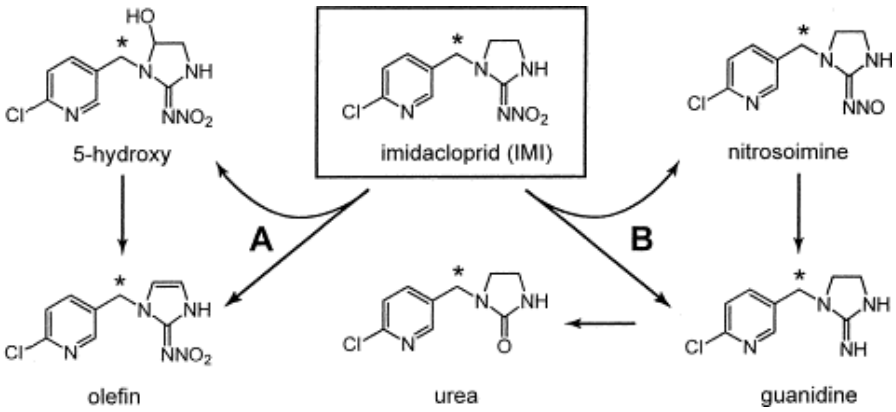


Figure 1.6: Imidacloprid metabolism by human CYP450 isozymes includes different pathways. Hydroxylation and desaturation of the imidazolidine moiety to produce the 5-hydroxy and olefin derivatives (A). Reduction and cleavage of the nitroimine substituent to produce the nitrosoimine, guanidine, and urea derivatives (B). Asterisks delegate positions of tritium labelling [112]

1.8 Nicotinic acetylcholine receptors

Neuronal nAChRs are a family of ligand gated ion channels. These receptors are pentameric and are composed of different sets of subunits and the varying subunits have different pharmacological and kinetic characteristics as well as in their cellular and subcellular positioning in the brain. The opening of these receptors results in the flow of cations such as sodium, potassium and calcium ions across the membrane [105][117][118]. A net influx of these cations through the channel pore results in depolarization and increased neuronal excitation [30][103]. The α7 oligomer is one of the main nicotinic receptors found in the brain and is distinguished by its rapid activation, low affinity and high calcium permeability [103]. This subtype has been reported to be involved in the pathophysiology of neurodegenerative diseases. This is partly due to their high density in the areas of the brain that play a role in cognitive processes. The α4β2 nicotinic receptors are

predominantly found in the thalamus. These receptors have been implicated in Alzheimer's disease, Parkinson's disease as well as schizophrenia. They are also involved in many functional processes such as cognition, learning and memory, arousal, cerebral blood flow as well as metabolism [103][117].

As has been noted, these receptors are also evident on dopaminergic neurons and therefore are involved in the release of dopamine [30][119]. The stimulation of these nicotinic receptors causes depolarization of the membrane potential as well as an increased firing rate in dopaminergic neurons. Overstimulation of these receptors may depolarize the neurons to a point beyond the firing limit and thereby cause the deactivation of sodium channels, consequently failing to give rise to an action potential [120].

Recent studies have reported that these receptors aren't only found in nerve cells but also in adipocytes, macrophages, lymphocytes, keratinocytes, hepatic, dendritic, lung and intestinal epithelial cells. The result of this is that the negative effects of IMI could occur in various organs and tissues including the nervous system [121][122].

Many studies have reported that chronic exposure of the receptor to an antagonist generally results in upregulation, or a rise in the amount of receptors. In contrast, chronic exposure of a receptor to an agonist results in downregulation, or a reduction in the amount of receptors. However, this is not seen with some of the other nAChRs, most distinctly the α4β2 nAChR subtype. When this subtype is exposed to an agonist for longer periods, α4β2 nAChR numbers are upregulated [103][123]. Desensitization of the α4β2 receptors in the brain are usually a result of chronic exposure of these receptors to partial agonists. This ultimately results in a long-term decrease in the reactivity to agonists. Another previously conducted study confirmed that under chronic nicotine exposure, α4β2 nAChRs sustain a nicotine–induced upregulation of the amount of receptors, subsequently resulting in an increased sensitivity to nicotine [120][123].

Additionally, compounds that change neurotransmitter signaling in the nervous system may have long–term effects in the developing nervous system. It is therefore important to consider any substance that interferes with adult neurotransmission as a possible developmental neurotoxicant. More conclusive information is needed regarding the ability of specific neonicotinoids to cause developmental neurotoxicity [30].

1.9 Degradation of imidacloprid

As has been noted, many studies have reported that IMI metabolites are more toxic in comparison to their parent entity, therefore degradation does not confer a decrease in toxicity [29][124]. IMI is a polar substance and has been reported to be relatively chemically stable at neutral and acidic pH but degrades slowly in alkaline conditions [125][126]. IMI is likely to sustain several physicochemical processes in soil-water

surroundings such as sorption, photodegradation as well as hydrolysis. This could affect how it behaves in its surroundings. Sorption is one of the most crucial processes as it subsequently has an effect on the translocation and transformation processes of pesticides as well as their bioavailability. Additionally, photodegradation and hydrolysis are also important transformation processes [(80)].

The quickest degradation pathway for neonicotinoids was shown to be photodegradation, but this process is often affected by various other considerations such as the depth of the water, light penetration and microbial activities. The resulting products of IMI photocatalytic degradation include: 6-CNA, IMI-NH, IMI olefin, IMI-urea and IMI hydroxyl. The nitroguanide group is generally the active part of the neonicotinoid undergoing photodegradation. It has also been reported that pH is another crucial parameter that is involved in the degradation of neonicotinoids in aquatic environments [(32)(127)].

1.10 Evaluation of previously conducted studies

To assess the toxicity of a compound, it is crucial to recognize the outcomes of toxicity as well as their dose-response associations, interpret the way in which this toxicity occurs as well as determine the toxicodynamics of a compound [(45)]. Sublethal effects of IMI have been observed in vertebrate wildlife - mammals, birds, fish, amphibians and reptiles. These effects involve genotoxicity, cytotoxicity, compromised immune system, decreased growth and reproductivity at levels much lower than expected to cause death. Most of the neonicotinoids have a moderate to high acute oral LD_{50} [(23)].

According to literature, the likelihood for neurotoxicity in humans due to developmental IMI exposure is unknown. A study conducted by Abou-Donia et al (2008) concluded that an *in utero*, large sublethal dose of IMI to pregnant rats resulted in substantial developmental, neurobehavioural as well as neuropsychiatric irregularities in the offspring of the rats. There was also a noteworthy rise in AChE activity in various parts of the brain. The abundant and frequent use of IMI as well as its increased affinity toward insect neuronal ACh receptors has led to the proposal that IMI may be safe for the mammalian nervous system, although this study demonstrated that maternal exposure to an increased sublethal dose of IMI during gestation resulted in serious sensorimotor impairments. The dose for this particular study was decided on in order to mimic an acute, high-level, sublethal exposure setting. This study also emphasized the need for further research in order to determine the prolonged effects following low dose IMI exposure [(122)].

Additionally, a study conducted by Kimura-Kuroda et al (2012), concluded that at concentrations larger than 1 µM, IMI and nicotine exert comparable excitatory effects on mammalian nAChRs. This ultimately suggests that neonicotinoids may detrimentally affect the health of humans, particularly the developing nervous system as seen with nicotine. The significance of neonicotinoid exposure in humans is specifically crucial in

children as the nAChRs are vital for normal brain development. During brain development the α4β2 and α7 nAChR subtypes have been involved in neuronal proliferation, cell-death, migration, differentiation, synapse formation as well as neural-circuit formation [91][128].

In support of measuring neurotransmitter levels following IMI exposure, a study performed by Kara et al (2015) showed that IMI at doses of 0.5, 2, and 8 mg/kg hindered cognition and learning behaviors in newborn and adult rats [121]. Another study reported a substantial decline of 38, 53, and 52% serotonin, dopamine, and GABA levels, respectively, in the IMI-treated rat's homogenized brain in comparison to the control group. The IMI-treated groups (adult and adolescent) were orally dosed with IMI (1 mg/kg) for 60 days successively [129].

Turning attention to the use of zebrafish as a model, a study was conducted by Krishnan et al (2018) to test the hypothesis that IMI exposure does in fact relate to a rise in mortality and decreased body length of zebrafish. In this study, the zebrafish were exposed to IMI for 5 dpf. Body length and the mortality of embryos were monitored daily in four different IMI concentrations ranging from 100 µg/L to 10 000 µg/L. The concentrations chosen were according to levels that could be attained in the environment, which seems to be vastly higher than documented in literature, therefore the limitation of this study is that it is not a true representation of environmental exposure of IMI [32]. The results from this study confirmed the hypothesis that IMI impedes body length development in the zebrafish embryos in a dose-dependent manner despite the high concentrations used. As an evaluation of growth, body length can portray both cellular and molecular alterations. This study also showed that IMI substantially causes a rise in deaths in zebrafish [130].

A study performed by Crosby et al (2015) was ultimately used to assess the neurobehavioral damage caused by developmental IMI exposure in zebrafish across their lifespan. All zebrafish, in different age groups (larval, adolescent and adult), were exposed to IMI for 0 - 5 dpf. The larval activity was evaluated at 6 dpf and included swimming behavior as well as response to light and dark surroundings. A different group was dosed and raised in the same manner and assessed at 1.5 months of age to observe sensorimotor reactivity and habituation, social behavior and novel tank swimming during adolescence. Another group was assessed at 3 months of age after being dosed and treated identically. A low and high dose of IMI was used, 45 and 60 µM respectively. The results indicated that exposure to IMI during early development does change the behavior of zebrafish both directly after being exposed (24 hrs later) and after 1.5 or 3 months residing in a condition-controlled environment. Similarly to nicotine, IMI substantially decreased swimming activity of the zebrafish larvae during the dark period of the larval swimming assay. The effects of these chemicals were evident as early as 6 dpf and continued through adolescence and into adulthood. IMI also had an impact on novel tank exploration resulting in adult and adolescent zebrafish to persistently stay near the tank floor for longer

periods of time in comparison to the control. This type of behavior could be an indication of exposure to chemicals that cause anxiety. Therefore it seems as though developmental exposure to IMI has long–term anxiogenic outcomes in zebrafish, but this has not been confirmed [(39)].

In addition, a study was conducted using zebrafish during early development to monitor alterations in neurotransmitter levels after IMI exposure. The proliferation and differentiation of the individual neuronal cell types have been thoroughly examined, however understanding about alterations in neurotransmitter levels during early development and in response to environmental pollutants is very limited. The zebrafish embryos were exposed to IMI the first 5 days of development. An interesting effect was noticed following the exposure to IMI. There was a reduction in ACh levels which is typically seen after exposure to familiar AChE–inhibiting pesticides. The consequences of IMI on neurotransmitter levels in this study validate the behavioral-induced effect of IMI in zebrafish reported before, displaying detrimental effects linked to IMI exposure in non-target organisms. The AChE inhibitors inhibit the enzyme activity to hydrolyze ACh to choline. Therefore, for this type of pesticide, a rise in ACh levels would be anticipated in the synaptic cleft. IMI replaces ACh binding site in the nAChRs, thereby resulting in a build-up of ACh in the neuronal cleft. This study's findings suggest a contrasting mechanism in which ACh is reduced due to a probable rise in AChE enzyme [(131)]. This contradicting information increases the urgency for further research regarding changes in ACh levels following prolonged, low dose IMI exposure.

1.11 Quantification of imidacloprid and its metabolites

Quantitation of pesticides and their breakdown products in biological fluids and tissues of laboratory animals is crucial for toxicological studies as it will correlate target tissue dose to the biological outcomes caused by such substances. Also, if we want to better our understanding of the effects of pesticides and their metabolites on human health, we need to clearly understand the distribution of these pesticides in the brain and various other tissues such as the liver. More importantly, details regarding the bioaccumulation of IMI and its related metabolites within the mammalian system is seriously lacking.

As has been noted, IMI does not penetrate the CNS, however many studies have reported on adverse effects relating to the nervous system following IMI exposure such as negatively affecting the memory and learning in rats [(26)(121)(132)]. Therefore, this further contributes to the urgency for more in depth studies of IMI distribution in the brain and the effects thereof. This brings us to another objective of this study of developing a sensitive, specific and robust analytical method for quantifying IMI and its metabolites within the liver, brain and gill epithelial tissue of adult zebrafish.

Chromatographic methods are needed for the definitive detection and quantification of pesticides and their metabolites in trace amounts [133]. To date, there are numerous toxicological screening methods used by toxicology laboratories such as gas chromatography coupled to mass spectrometry (GC-MS), which was previously considered as the gold standard for the detection of compounds. However, GC-MS involves laborious preparation of samples as well as the difficulty of detecting heat-sensitive and polar compounds, therefore these methods are rapidly being substituted for liquid chromatography tandem-mass spectrometry (LC-MS/MS) methods [134][135].

Unlike GC-MS, LC-MS/MS is not restricted to volatile or small compounds. Using LC-MS/MS makes it possible to detect compounds with a broad range of polarities without the need for derivatization before analysis [136]. LC-MS/MS is one of the most definitive modern analytical methods and by using LC-MS/MS, more polar compounds can be analyzed concomitantly in a single run [136][137].

More specifically, liquid chromatography is a separation technique used to isolate the individual constituents of a mixture. This technique involves mass transfer of a sample through a polar mobile phase and non-polar stationary phase, specific to reverse-phase chromatography [138]. Mass spectrometry utilizes methods of ionization that ultimately leads to the separation and detection of charged molecules. A method involving varying stages of mass analysis is known as tandem mass spectrometry (MS/MS). As illustrated by Figure 1.7 [139] below, the first analyzer (Q1) is involved in the isolation of the precursor ion. The second analyzer (Q2), also known as the collision cell, is where the precursor ion is fragmented. The collision cell usually contains neutral, static gas such as nitrogen or argon. The final analyzer (Q3) analyzes the product ions produced from the fragmentation that occurred in Q2 [134][139].

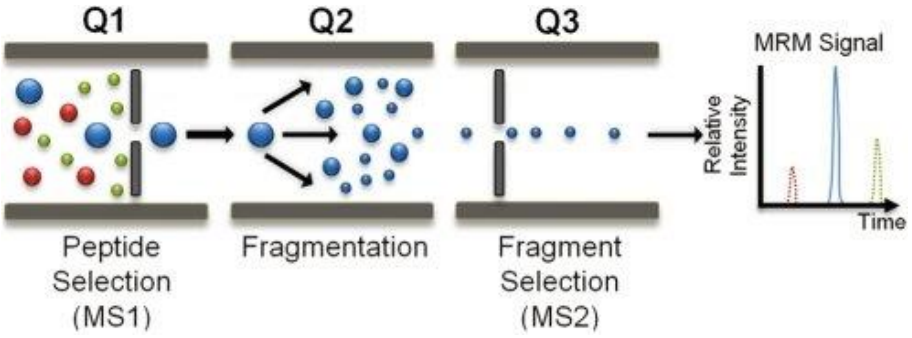


Figure 1.7: Multiple Reaction Monitoring Mass Spectrometry (MRM-MS). MRM-MS utilises the signal of selected MS2 fragment ions for quantification. It is commonly conducted on a triple-quadrupole instrument: Q1 selects a precursor ion, Q2 fragments the precursor ion and Q3 selects a specific fragment ion for the detector [139]

Table 1.1 below portrays IMI and its metabolites' precursor and product ions mass-to-charge ratios as reported in literature.

Table 1.1: Literature review summary of imidacloprid and its metabolites' precursor and product ions mass-to-charge (m/z)

Analyte	**Monoisoptopic Mass**	**Ionization mode**	**Precursor ion (m/z)**	**Production mass (m/z)**	**Product ion mass (m/z)**	**Reference**
Imidacloprid	255	Positive	256	209, 128	175, 239	Pubchem, [134], [140]
IMI urea	211	Positive	212	205, 126, 99.0, 195	171, 128	Pubchem, [140],[141]
Desnitro IMI	210	Positive	211	126	175, 90.0	Pubchem, [142]
5-OH IMI	271	Positive	272	225, 144, 191	226, 126, 228	Pubchem, [142]

An additional stage that is often neglected in the analysis of compounds is the preparation of samples. Sample preparation is often very labour-intensive and tedious. The efficiency of the sample preparation becomes crucial when it is necessary to quantify pesticides in biological fluids. The development of a multi-analyte technique is difficult as it is important to evaluate the several classes of varying compounds with varying polarities, solubilities as well as volatilities that need to be extracted and assessed concomitantly [134].

The main objectives of sample pre-treatment include the elimination of any interference, extraction of the compounds as well as increasing the final signal of the compound by increasing its concentration. By extracting

the target compound from somewhat large sample volumes into only a few microliters of eluent results in the preconcentration of the compound. This procedure also results in an improvement of accuracy and precision of the results by bringing the compound concentration into the dynamic range of the particular analytical instrumentation. It has been documented that the detection of neonicotinoid insecticides in several matrices generally undergo two critical stages. These stages include the extraction from sample matrices as well as the partitioning of analytes from ions or compounds that coexist in the matrix [19][143].

According to literature, many studies have focused on quantifying only IMI and one if its major metabolites, 6-CNA, using rat, human and rabbit biological fluids and tissues [116][140][144][145], however very few studies have focused on the other crucial metabolites of IMI [26][146]. This is once again another clear and critical limitation. As has been noted, imidacloprid's metabolites may be more toxic than IMI itself, in particular, IMI-NH. To accentuate the importance of analyzing all imidacloprid's major metabolites and also due to the impact of IMI on bees, a study was performed by Codling et al (2016) to assess whether neonicotinoids were detectable in bees as well as their hive products. The findings of this study reported higher concentrations of IMI metabolites, relative to IMI in honey, which suggests that the main focus should be on the metabolites when evaluating the risks of the utilization of IMI as an insecticide [21].

1.12 Quantification of neurotransmitters

Neurotransmitters are compounds produced in the presynaptic neuron, which exist in the presynaptic terminals, where they are discharged to exert a distinct effect on the postsynaptic neuron or effector organ. Once these neurotransmitters have been discharged into the synaptic cleft, they interact with receptors from the postsynaptic neurons by playing a crucial role in their regulation of membrane polarization as well as their ionic transport. The neurotransmitters play a very important role in neuronal communication which is ultimately involved in learning, memory and behavior [147].

Exposure to environmental pollutants can cause behavioral alterations via the changes of the neurotransmitter systems. The proliferation and differentiation of the individual neuronal cell types have been thoroughly examined, however understanding of alterations in neurotransmitter levels in response to environmental pollutants is limited [131]. A few of the crucial neurotransmitters present in the nervous system include DA, 5-HT, GABA and most importantly for this study due to the main target of IMI being the nAChRs, ACh [148].

DA and 5-HT are involved in many activities such as motor control, emotion, stress and cognition. The chemical structure of DA (A) and 5-HT (B) is illustrated in Figure 1.8 below. Since it has been noted that the target site of these insecticides are present on the dopaminergic neurons, it suggests, as previously documented, that

they are involved in the release of DA [148][149]. In addition, behavioral evidence previously documented suggests that nAChRs present in the hippocampus of the brain are present on serotonergic neurons and therefore have the ability to control 5-HT release [150]. This emphasizes the need for the analysis of changes in DA and 5-HT levels following IMI exposure. According to literature, any changes in dopamine's extracellular levels has been found to be associated with schizophrenia and Parkinson's disease [149][151]. Schizophrenia and Parkinson's disease has been associated with an increase and decrease in dopamine activity, respectively [151][152].

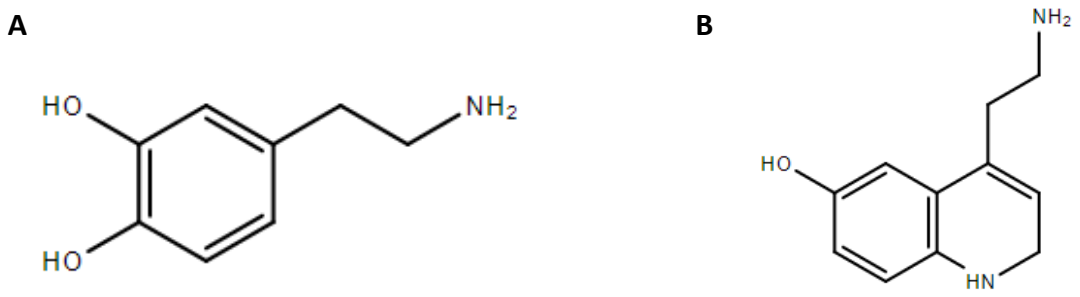


Figure 1.8: Chemical structures of dopamine (A) and serotonin (B). Images created using Biorender

The primary inhibitory neurotransmitter in the CNS has been reported to be GABA, which plays a vital role in the physiological activities of the brain. Therefore, the monitoring of GABA in bio-fluids following IMI exposure is crucial as alterations in its levels are related to several neurological disorders including epilepsy, seizures, anxiety as well as bipolar disorder [149]. The chemical structure of GABA is illustrated in Figure 1.9 below. Epilepsy and seizures have been associated with a decrease in GABA levels [153][154].

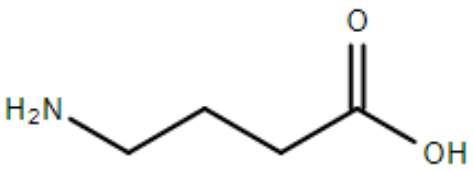


Figure 1.9: Chemical structure of GABA. Image created using Biorender

Even more important for this particular study is ACh, which is recognized as the main neurotransmitter of the autonomic nervous system. ACh forms part of the parasympathetic nervous system and is responsible for the contraction of smooth muscles, dilation of blood vessels as well as slowing the heart rate [155]. ACh also plays a crucial role in memory and learning. The significance of ACh is in its relationship with specific pathologies such as Parkinson's disease and Alzheimer's. Parkinson's and Alzheimer's disease has been reported to be

associated with an increase and decrease in ACh levels, respectively [156][157]. It is therefore also vital to monitor any alterations in ACh levels following IMI exposure. The chemical structure of ACh is illustrated in Figure 1.10 below.

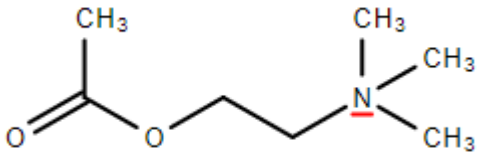


Figure 1.10: Chemical structure of ACh. Image created using Biorender

As has been noted, LC-MS/MS has become a vital practice to study compounds, specifically neurotransmitters in this case, in trace amounts in zebrafish. These neurotransmitters are highly polar therefore reversed phase liquid chromatography (RPLC) can often only be used if derivatization is involved as it is essential to allow for better retention as well as to improve the selectivity and sensitivity. However, certain derivatization agents can result in interference in the chromatography. On the other hand, separation could be improved via the addition of an ion-pair reagent. At the same time, when using mass spectrometry, an ion-pair reagent could result in ion suppression [147][149].

A study conducted by Santos–Fandila et al (2015) resulted in the development and validation of an ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC–MS/MS) method for the quantitation of a variety of different neurotransmitters within the ultra-trace range including GABA, 5-HT, DA and ACh. The overarching aim of this study was to apply this method to the analysis of neurotransmitters in zebrafish brain. The best separation was obtained utilizing mobile phase A that consisted of water, acetonitrile, heptafluorobutyric acid, an ion-pair reagent, as well as concentrated formic acid in a ratio of 900:100:1:1 (v/v/v/v) and mobile phase B as 0.1% (v/v) formic acid in acetonitrile (ACN). The samples were extracted using 1% formic acid in methanol and 5 µL was injected onto LC-MS/MS for analysis. This was the first study to utilize UHPLC-MS/MS to analyze a range of neurotransmitters, their metabolites and precursors in zebrafish. This method allows for the facilitation of this study and future research related with the development of the zebrafish brain [158].

Additionally, a study conducted by Aragon et al (2017) reported on the development of a sensitive analytical method for the detection of various neurotransmitters in zebrafish larvae, however this was done using GC-MS, which as has been noted, does require laborious sample preparation as well as decreased efficiency in detecting polar compounds and this ultimately is problematic as the monoamine neurotransmitters are highly polar. It was necessary for derivatization of the neurotransmitters to decrease the polarity thereby improving

the sensitivity. This is another pitfall of this developed method due to the possibility of the derivatising agent interfering with the chromatography [159].

In contrast, Tufi et al (2016) used an analytical method based on hydrophilic interaction liquid chromatography (HILIC) coupled to MS/MS to measure neurotransmitters in whole zebrafish embryos and larvae. The only downfall of this method is that it was performed in larvae (4 hpf - 5 dpf), which is not a true measure of chronic exposure effects of IMI on the changes in neurotransmitter levels [131].

In short, the measurement of these neurotransmitters in the CNS has great importance, particularly in the neuroscience discipline. This is mainly because of the integral part that these compounds play in normal physiological functioning. Any changes in their homeostasis could potentially lead to the occurrence of a neurological disorder/s [147][149].

1.13 Hypothesis statement

Despite the limited research regarding chronic exposure to lower concentrations of IMI, the perception exists that the lower levels do not pose a health or environmental risk. However due to the high solubility of IMI in an aqueous environment, we hypothesized that chronic exposure to the NOAEL of IMI would pose neurological risk.

1.14 Aim and objectives of this research

This research project aimed to assess the possibility of long-term neurological risks following prolonged, low dose IMI exposure within an *in vivo* zebrafish model. *In vivo* animal models and analytical studies were required to identify the magnitude of the effect of lower dose, prolonged IMI exposure. Moreover, for this study, more sensitive, selective, accurate and efficient analytical methods were essential to generate quantitative data in terms of bioaccumulation of IMI and its primary metabolites as well as the measurement of neurotransmitter levels following IMI exposure.

Specific objectives were formulated as follows:

- To employ a toxicity assay to determine the NOAEL within an *in vivo* zebrafish larval model of IMI exposure.
- To investigate the likelihood of general toxicity within an *in vivo* zebrafish larval model by using stereomicroscopy.
- To investigate the likelihood of neurotoxicity within an *in vivo* zebrafish larval model by monitoring behavior up to 5 dpf using Daniovision software.

- To measure acetylcholine, dopamine, serotonin and gamma-aminobutyric acid levels in the brain of zebrafish adults using LC-MS/MS as a measure of neuronal signaling after prolonged, low dose IMI exposure.
- To evaluate the potential accumulation of IMI and its primary metabolites in the brain, liver and gill epithelial tissue of the adult zebrafish using LC-MS/MS after prolonged, low dose IMI exposure.

We present the research performed to address these aims in the following two chapters. Chapter 2 will describe the analytical method development required. Chapter 3 describes experimental research conducted in zebrafish larvae and adults with which we determined neurological risk of IMI. Each of these chapters will contain a discussion of the relevant data. Final conclusions will be presented in chapter 4.

Chapter 2: Development of analytical assays for the determination of IMI, its metabolites and neurotransmitters in zebrafish tissue homogenate

2.1 Introduction

LC-MS/MS, as a multi-target screening technique, has become a key analytical tool in the clinical and forensic toxicological laboratory environment [160]. The ultimate aim of analytical toxicology is the detection, identification and quantification of chemical compounds as well as breakdown products in biological samples. Clinical toxicology encompasses both the detection and treatment of poisoned or drug-affected individuals, involving a broad range of chemical compounds such as pesticides or solvents [161].

In contrast to conventional methods, mass spectrometry has many benefits since structural information can be provided, and additionally, improved sensitivity and selectivity can be attained. Moreover, when a triple quadrupole analyser is utilised and MRM is selected, the signal-to-noise (S/N) ratio is considerably higher and selectivity can be enhanced. Nowadays, the development of rapid and reliable analytical methods is crucial, allowing for the quick, concomitant detection of multiple compounds in an unlimited number of samples [162].

Taking into consideration the mechanism of action of IMI and the importance of neurotransmitters in the nervous system, it is essential to assess for any changes in neurotransmitter levels following neonicotinoid exposure. In this study, the overarching aim was to develop a LC-MS/MS method with the capability of concomitantly detecting IMI, three of its metabolites as well as various neurotransmitters in adult zebrafish tissue homogenate. The identification of the target compounds using the developed LC-MS/MS method was based on mass accuracy, fragment ions as well as retention time.

2.2 Materials and methods

2.2.1 Reference standards

All the chemicals used in this study were of analytical grade.

Pesticide

IMI (98%), IMI-urea (98%), desnitro-IMI hydrochloride (98%), 5-hydro IMI (98%) and IMI-d4 internal standard (ISTD) reference standards were purchased from Toronto Research Chemicals (TRC) (Toronto, Canada).

Neurotransmitters

Acetylcholine chloride (99%), dopamine hydrochloride (97.7%), Y-aminobutyric acid (99.8%) and serotonin hydrochloride (99%) reference standards were purchased from Sigma Aldrich (Saint Louis, United States of America (USA)). The ISTD reference standard, phenethylamine-d4 (96%) was purchased from TRC (Toronto, Canada).

Buffers and solvents

The acetonitrile (ACN) and methanol (MeOH) were purchased from Romil Limited (Cambridge, United Kingdom). The ethyl acetate, dichloromethane and hexane were supplied by Kimix Chemical and Lab Supplies (Cape Town, South Africa). A Synergy® UV water purification system was used for all high purity water required in this study (Merck, Darmstadt, Germany). Formic acid, ammonium acetate and ammonium formate were supplied by Fisher Scientific Chemicals (New Hampshire, USA). Heptafluorobutyric acid (HFBA), an ion-pairing reagent, was supplied by Sigma Aldrich (Saint Louis, USA).

2.2.2 Matrix source

Individual zebrafish carcasses were supplied by the Zebrafish Research Unit located at Stellenbosch University, Faculty of Medicine and Health Sciences in the Division of Clinical Pharmacology. These carcasses were utilized as the test matrix for extraction method development purposes. The zebrafish were euthanised by an overdose of tricaine-methanesulfonate as part of normal husbandry procedures, usually due to an observed failure to thrive. This procedure was performed by a South African Veterinary Council (SAVC)-authorised staff member, under supervision of a SAVC-registered veterinarian.

Briefly, these zebrafish carcasses were cut into smaller portions, weighed and placed into 2 mL homogenizing tubes. This was followed by the addition of five times more volume of the homogenization solvent (0.2% formic acid) to the mass of the tissue. These samples were then homogenized using a Bead Ruptor Elite bead mill homogenizer (Georgia, USA) with the addition of four 2 - 4 mm metal beads to assist in this process. The homogenizer was set to operate at a speed of 6 milliseconds (ms) for 30 seconds.

2.2.3 Apparatus and Instrumentation

The Rediscan function on the Beckman DU 640 spectrophotometer (California, USA) was used to quantitatively measure the ultraviolet wavelength of the target analytes. A vortex and an ultrasonic cleaner was used to mix liquid samples rapidly and vigorously forcing all analytes into solution allowing for optimal solubility. The use of the Eins Sci centrifuge (Steeledale, South Africa) was to separate particles from a solution according to their size, shape, density, thickness of the medium as well as rotor speed. The Stuart sample concentrator (Staffordshire, United Kingdom) was used to accelerate the evaporation process of samples for analysis. The Bead Ruptor Elite bead mill homogenizer was used to homogenize biological samples by mechanical shearing prior to the extraction of the target analytes. Oasis PRiME HLB sorbent (60 mg) 3cc Vac extraction cartridges were supplied by Waters corporation (Massachusetts, USA) and were used to remove lipids and phospholipids. Quantitation was performed on the Shimadzu Prominence LC-20ADXR liquid chromatographer coupled to Shimadzu 8040 Mass Spectrometer with a SIL-20ACXR autosampler, CTO-20A column oven and an electrospray ionisation source (Kyoto, Japan). Data analysis was conducted using LabSolutions Insight. Method development was also performed on a Shimadzu HPLC instrument (Integrated Shimadzu LC-2050C 3D coupled with a binary pump, autosampler, column compartment and photodiode-array detector and ex-bioanalytics software).

2.2.4 Preparation of stock solutions and ISTD's

According to the certificate of analysis and the solubility of each analyte, an appropriate solvent was chosen in which to prepare stock solutions. As illustrated in Table 2.1 below, a stock solution (SS) of IMI, IMI-urea, desnitro-IMI hydrochloride and 5-hydro IMI were prepared such that the final concentration was 1 mg/mL, adjusted for purity. Additionally, Imidacloprid-d4 (IMI-d4) SS was prepared in the vial in which it was received to avoid loss such that the final concentration was 2.5 mg/mL. The stock solutions were stored at approximately - 80 °C prior to use.

Table 2.1: Preparation of pesticide stock solutions

Analyte	% purity	Solvent	Weighted mass (mg)	Adjusted mass (mg)	Solvent volume (µL)
Imidacloprid	98.0	MeOH	1.18	1.16	1156
Imidacloprid-urea	98.0	MeOH	0.910	0.892	892
Desnitro-imidacloprid	98.0	MeOH	1.18	0.986	986
5-Hydro imidacloprid	98.0	MeOH	0.570	0.559	559
Imidacloprid-d4	100	ACN	2.50	2.50	2500

Similarly, as depicted in Table 2.2 below, a SS of acetylcholine chloride, dopamine hydrochloride, gamma-aminobutyric acid and serotonin hydrochloride were prepared such that the final concentration was 1 mg/mL, adjusted for purity. The ISTD, phenethylamine-d4 was previously prepared at 1 mg/mL in MeOH. SS were stored at approximately – 80 °C prior to use.

Table 2.2: Preparation of neurotransmitter stock solutions

Analyte	% purity	Solvent	Weighted mass (mg)	Adjusted mass (mg)	Solvent volume (µL)
Acetylcholine chloride	100	Water	1.06	1.06	1060
Dopamine hydrochloride	97.7	Water	1.04	1.02	1020
Gamma-aminobutyric acid	99.8	Water	1.50	1.50	1500
Serotonin hydrochloride	99.0	Water	1.03	1.02	1020

2.2.5 Preparation of working solutions and calibration standards

2.2.5.1 Imidacloprid and metabolites

Calibration standards were prepared in zebrafish tissue homogenate (mass of tissue:volume, 1:5, m/v) at room temperature. This was performed by first preparing working solutions (WS) which were serially diluted in MeOH. A pooled IMI stock solution was prepared at a final concentration of 5 µg/mL (SS1). Each WS was used to spike the tissue homogenate. It is important to note that the spiking volume into the matrix must be less than or equal to 5% of the total volume. This mixture was vortexed and sonicated to ensure optimal solubilisation. The preparation of the working solutions and calibration standards are presented in detail in Table 2.3 below.

Table 2.3: Preparation of working solutions and calibration standards for IMI and its metabolites

Working Solutions (WS)	Blank solvent (MeOH) volume (µL)	Spiking solution	Spiking solution volume (µL)	WS Solution (µg/mL)	Vol WS into 0.190 mL tissue (µL)	STD	Final Concentration (ng/mL)
WS1	100	**SS1**	100	2.50	10.0	**STD1**	125
WS2	100	**WS1**	100	1.25	10.0	**STD2**	62.5
WS3	100	**WS2**	100	0.625	10.0	**STD3**	31.3
WS4	100	**WS3**	100	0.313	10.0	**STD4**	15.6
WS5	100	**WS4**	100	0.156	10.0	**STD5**	7.81
WS6	100	**WS5**	100	0.0780	10.0	**STD6**	3.91
WS7	100	**WS6**	100	0.0390	10.0	**STD7**	1.95
WS8	100	**WS7**	100	0.0200	10.0	**STD8**	0.977
WS9	100	**WS8**	100	0.0100	10.0	**STD9**	0.488
WS10	100	**WS9**	100	0.00500	10.0	**STD10**	0.244
WS11	100	**WS10**	100	0.00200	10.0	**STD11**	0.122
WS12	100	**WS11**	100	0.00100	10.0	**STD12**	0.0610

2.2.5.2 Neurotransmitters

As depicted in Table 2.4 below, the neurotransmitter calibration standards were prepared in the homogenization solvent (0.2% formic acid) at room temperature instead of in the zebrafish tissue homogenate. This was done by first preparing working solutions which were serially diluted in water. The preparation of these calibration standards in the homogenization solvent was primarily due to the presence of endogenous neurotransmitter levels. This posed a challenge when needing to compensate for these endogenous levels after spiking the tissue with the WS. Therefore, a pooled neurotransmitter stock solution was prepared at a final concentration of 40 µg/mL (SS1) and each working solution was used to spike the blank 0.2% formic acid. This mixture was vortexed and sonicated to ensure optimal solubilisation.

Table 2.4: Preparation of working solutions and calibration standards for neurotransmitters

Working Solutions (WS)	Blank solvent (0.2 % Formic acid) volume (µL)	Spiking solution	Spiking solution volume (µL)	WS Solution (µg/mL)	Vol WS into 0.190 mL tissue (µL)	STD	Final Concentration (ng/mL)
WS1	100	**SS1**	100	20.0	10.0	**STD 1**	1000
WS2	100	**WS1**	100	10.0	10.0	**STD 2**	500
WS3	100	**WS2**	100	5.00	10.0	**STD 3**	250
WS4	100	**WS3**	100	2.50	10.0	**STD 4**	125
WS5	100	**WS4**	100	1.25	10.0	**STD 5**	62.5
WS6	100	**WS5**	100	0.625	10.0	**STD 6**	31.3
WS7	100	**WS6**	100	0.313	10.0	**STD 7**	15.6
WS8	100	**WS7**	100	0.156	10.0	**STD 8**	7.81
WS9	100	**WS8**	100	0.0781	10.0	**STD 9**	3.91
WS10	100	**WS9**	100	0.0391	10.0	**STD 10**	1.95

2.2.6 Preparation of working solutions and quality controls

2.2.6.1 Imidacloprid and metabolites

Quality controls (QC) were prepared at room temperature using the same methodology that was used for the preparation of the calibration standards in Section 2.2.5 above. Firstly, WS were prepared, which were serially diluted in MeOH before being spiked into blank tissue homogenate. The preparation of the WS and QC's are presented in detail in Table 2.5 below. The inclusion of two QC lows was to ensure validation for all the various analytes lowest limit of quantitation.

Table 2.5: Preparation of working solutions and quality controls for IMI and its metabolites

Working Solutions (WS)	Blank solvent (water) volume (µL)	Spiking solution	Spiking solution volume (µL)	WS concentration (µg/mL)	Volume (µL) WS spiked into 0.190 mL tissue	QC	Final concentration (ng/mL)
WSQ2	150	**SS**	100	2.00	10.0	**QC H**	100
WSQ3	250	**WSQ2**	250	1.00	10.0	**QC M**	50.0
WSQ4	250	**WSQ3**	250	0.500	10.0	**SYS 1**	25.0
WSQ5	250	**WSQ4**	250	0.250	10.0	**SYS 2**	12.5
WSQ6	120	**WSQ5**	100	0.110	10.0	**QC L**	5.70
WSQ7	750	**WSQ6**	100	0.00500	10.0	**QC L**	0.700

2.2.6.2 Neurotransmitters

Similarly, as depicted in Table 2.6 below, QC's were prepared in 0.2% formic acid at room temperature by first preparing WS which were serially diluted in water.

Table 2.6: Preparation of working solutions and quality controls for neurotransmitters

Working Solutions (WS)	Blank solvent (water) volume (µL)	Spiking solution	Spiking solution volume (µL)	WS concentration (µg/mL)	Volume (µL) WS spiked into 0.190 mL 0.2 % formic acid	QC	Plasma concentration (ng/mL)
WSQ2	300	**SS1**	200	16.0	10.0	**QC H**	800
WSQ3	250	**WSQ2**	250	8.00	10.0	**QC M**	400
WSQ4	250	**WSQ3**	250	4.00	10.0	**SYS 1**	200
WSQ5	200	**WSQ4**	200	2.00	10.0	**SYS 2**	100
WSQ6	150	**WSQ5**	100	0.800	10.0	**QC L**	40.0

2.2.7 Determination of the wavelength range

Working solutions of IMI, IMI-urea, IMI-NH and 5-OH IMI at 10 µg/mL were scanned between 200 nm and 800 nm. This was conducted using an UV spectrophotometer against MeOH as a blank after baseline correction. From the scan, the wavelength of IMI, IMI-NH and 5-OH IMI was found to be 270 nm, 268 nm and 275 nm respectively. Additionally, from the scan, it was observed that IMI-urea did not have an ultraviolet absorbance.

2.2.8 LC-MS/MS conditions

LC-MS/MS was chosen as it is more specific and sensitive than conventional HPLC. Often conventional HPLC methods utilise mobile phases that consist of high concentrations of non-volatile buffer salts that are incompatible with LC-MS/MS methods. In this study, two distinct methods were developed:

2.2.8.1 Method 1: IMI and its metabolites

This first method was developed and optimised for the detection and quantitation of IMI, IMI-urea, desnitro-IMI, 5-hydro IMI and IMI-d4.

Chromatography

For LC-MS/MS, IMI and three of its metabolites were infused at a concentration of 1 µg/mL whereas the ISTD was infused at a concentration of 10 µg/mL onto the mass spectrometer to obtain precursor and product ion transitions, evaluating both the positive and negative (ESI +/-) ionisation modes. IMI, its metabolites and the

ISTD predominantly yielded singly charged protonated precursor $[M+H]^+$ ions with varying mass-to-charge ratio's (m/z) in Q1 full scan spectra. Furthermore, fragmentation of the precursor ions took place using argon as the collision gas and by applying 15, 20, 24, 15 and 17 eV collision energy for IMI, IMI-urea, IMI-NH, 5-OH IMI and IMI-d4 respectively. These m/z ratios as well as the most abundant product ions of IMI and its metabolites are depicted in Table 2.7 below.

Table 2.7: Mass-to-charge ratio's (m/z) obtained in Q1 full scan spectra for IMI and its metabolites

Analyte	Monoisoptopic mass	Ionization mode	Precursor ions (m/z)	Product ion mass (m/z)
Imidacloprid	255.1	Positive	256.0	209.1, 175.1, 210.0
IMI urea	211.1	Positive	211.9	128.0, 171.2, 126.1
Desnitro IMI	210.1	Positive	210.9	126.1, 175.2, 133.1
5-hydro IMI	271.1	Positive	272.1	225.2, 226.1
IMI-d4	259.1	Positive	260.1	213.2, 179.2, 214.1, 216.1

Following LC-MS/MS method development, the best chromatography and good retention was achieved with a Shimadzu, Shim-pack Velox biphenyl (2.1 x 100 mm, 2.7 µm) analytical column and mobile phase using a gradient elution. Mobile phase A and B consisted of 0.1% formic acid and 5 mM ammonium formate in water and MeOH, respectively. A gradient was run from 25 to 90% B over 8.5 min. The LC time program started at 25% B for 30 sec, followed by a gradual increase to 90% B over 3.5 min. It was held at 90% B for 2 min before reducing the %B to 25% over 30 sec. To equilibrate and attain a final analysis time of 8.5 min, %B was held at 25% for an additional 2 min. The flow rate was maintained at 0.4 mL/min. Finally, a volume of 20 µL of each sample containing the target analytes was injected onto the analytical column.

2.2.8.2 Method 2: neurotransmitters

Another LC-MS/MS method was developed and optimised for the detection and quantitation of acetylcholine, dopamine, gamma-aminobutyric acid and serotonin as well as the ISTD, phenethylamine-d4.

Chromatography

All four neurotransmitters were infused at a concentration of 1 µg/mL onto the mass spectrometer to obtain precursor and product ion transitions, evaluating both the positive and negative (ESI +/-) ionisation modes. Due to previous work done with phenethylamine-d4 in the department's analytical laboratory, it had already been infused onto the mass spectrometer. This made it possible to simply add its transitions to the final MRM method obtained for the neurotransmitters.

All the target compounds predominantly yielded singly charged protonated precursor $[M+H]^+$ ions with varying mass-to-charge ratio's (m/z) in Q1 full scan spectra. Moreover, fragmentation of the precursor ions

took place using argon as the collision gas and by applying 14, 25, 13, 13 and 15 eV collision energy for ACh, DA, GABA, 5-HT and phenethylamine-d4, respectively. Table 2.8 indicates the MRM transitions of all four neurotransmitters and phenethylamine-d4.

Table 2.8: Mass-to-charge ratio's (m/z) obtained in Q1 full scan spectra for the neurotransmitters

Analyte	Monoisoptopic mass	Ionization mode	Precursor ions (m/z)	Product ion mass (m/z)
Acetylcholine	146.1	Positive	147.2	87.1, 43.1
Dopamine	153.1	Positive	154.1	91.2, 65.2, 119.0
Gamma-aminobutyric acid	103.1	Positive	104.2	87.1, 69.1, 43.1
Serotonin	176.1	Positive	177.2	160.2, 115.1, 117.1
Phenethylamine-d4	125.1	Positive	126.2	109.1, 79.1, 51.9

Following LC-MS/MS method development, the best chromatography was achieved with an Agilent InfinityLab Poroshell 120 EC-C18 (3.0 x 100 mm, 2.7 µm) analytical column and mobile phase using a gradient elution. Mobile phase A and B consisted of 0.1% formic acid in water and ACN, respectively. A gradient was run from 10 to 60% B over 7 min. The LC time program started at 10% B for 1 min, followed by a gradual increase to 60% B over 2 min after which it was held at 60% B for 30 sec before reducing the %B back to 10% over 30 sec. To equilibrate and attain a final analysis time of 7 min, %B was held at 10% for an additional 3 min. The flow rate was maintained at 0.45 mL/min. A heating block temperature of 300 °C yielded optimal results for all four neurotransmitters. Finally, a volume of 20 µL of each sample containing the target analytes was injected onto the analytical column.

Final MS/MS settings for both the developed methods is illustrated in Table 2.9 below.

Table 2.9: Electrospray ionisation and MS/MS settings for method 1 and 2

	Method 1	Method 2
Mass Spectrometer	Shimadzu 8040	Shimadzu 8040
Parameter	Value	Value
Nebulising gas flow rate (L/min)	3	3
Drying gas flow rate (L/min)	15	15
Desolvation line temperature (°C)	250	250
Heating block temperature (°C)	400	300
Scan type	MRM	MRM
Dwell time (ms)	99	76
Column oven temperature (°C)	30	30

2.2.9 Extraction protocol

Prior to LC-MS/MS analysis, it was crucial to obtain as high as possible extraction recovery with negligible or low matrix effects to improve the sensitivity and reliability of LC-MS/MS analysis. Various approaches were undertaken to obtain the optimal extraction procedure for both methods, as stated above.

From the start, it was crucial to ensure that all the target analytes in this study could be extracted using a single extraction method. This was due to the final zebrafish tissue samples undergoing analysis for the detection and quantitation of the target analytes being limited in number and consecutively run on the two developed LC-MS/MS methods. Therefore, the final extraction method, protein precipitation (PPT) was chosen predominantly due to its capability to extract all the target analytes concomitantly.

In summary, the finalised PPT extraction method is depicted in Figure 2.1 below.

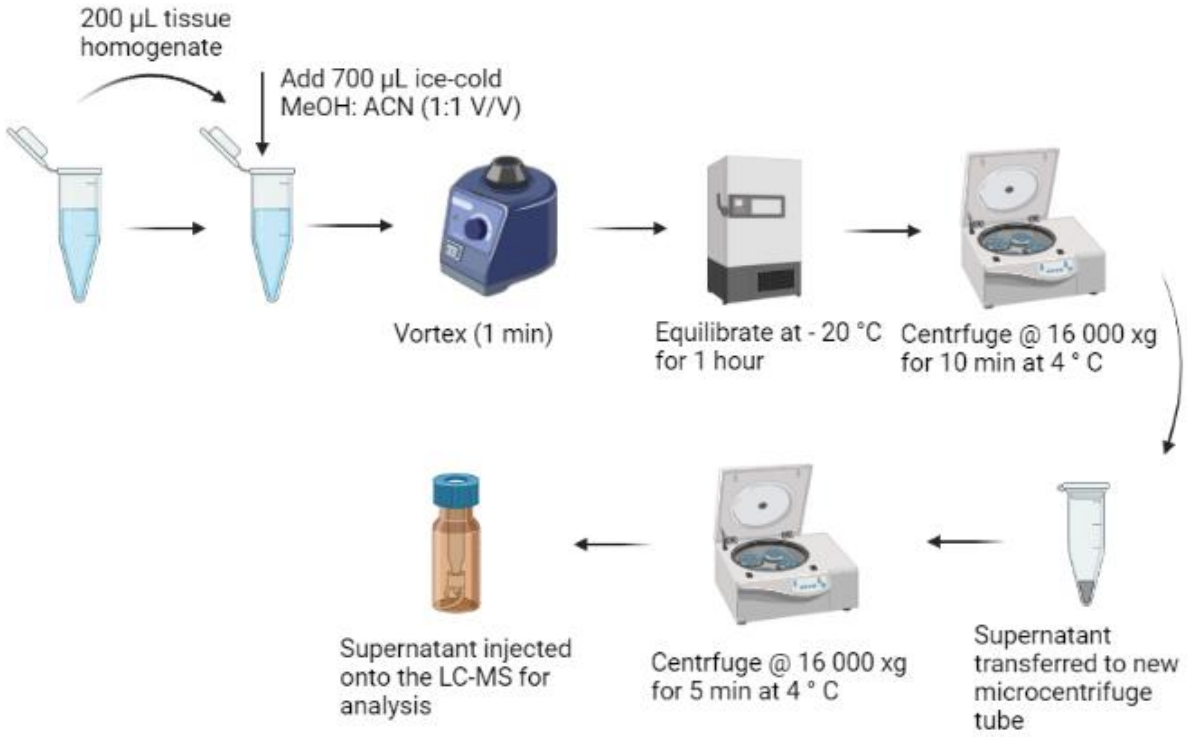


Figure 2.1: The final protein precipitation extraction method. Image created using Biorender

2.3 Pre-validation recovery assessment

Recovery pre-validation experiments were performed on the Shimadzu 8040 LC-MS. Recovery experiments are used in the evaluation of the actual recovery and loss of analyte during the extraction procedure. It is calculated by comparing the peak areas of the extracted QC samples in six-fold with the peak areas of blank matrix in six-fold, first extracted and then spiked with the target analytes at the theoretical concentrations (theoretical, represents 100% recovery).

2.3.1 IMI and its metabolites

Six QC's at each concentration (high, medium and low) were extracted as per the analytical method. Additionally, theoretical high, medium and low concentrations of IMI and its metabolites were used to reconstitute extracted, blank matrix.

2.3.2 Neurotransmitters

The neurotransmitters recovery assessment was done slightly differently. This was due to the standards and QC's being prepared in the homogenisation solvent instead of matrix as described in Section 2.2.5.2. Six QC's at each concentration (high, medium and low) were extracted as per the analytical method. In addition, theoretical phenetylamine-d4 ISTD was used to reconstitute extracted blank matrix.

2.4 Stock solution stability assessment – IMI and its metabolites

Stock solution stability for each individual analyte was evaluated in the suitable solvent and for the period that covers the intended time of use. This stock solution was used to assess the performance of the analytes under certain conditions. This experiment was performed on HPLC. The results from this experiment will give an indication of how these stock solutions should be handled.

2.4.1 Benchtop stability

Aliquots of SS prepared at 1 mg/mL on 19 May 2021 were left on bench on 12 January 2022 for approximately 5 hrs. Subsequently, 5 hrs later (08:00 am - 13:21 pm), these stock solutions were diluted to 10 µg/mL in MeOH. This was done in triplicate for each analyte. As an indication of benchtop stability, these thawed stock solutions were analysed against long-term storage stock solutions also prepared on 19 May 2021. These stock solutions were also diluted to 10 µg/mL in MeOH. These specific long-term storage samples stored at -80 °C were used for the comparison to ensure stock solution accuracy.

2.5 Solubility assessment – IMI and metabolites

In order to assess the solubility of IMI and its metabolites in the appropriate solvent, freshly prepared stock solutions at 1 mg/mL were diluted to 10 µg/mL in MeOH in six-fold. The first set of triplicate samples for each analyte was simply vortexed for a min. The second set of triplicate samples for each analyte was vortexed for 1 min and sonicated for an additional min. Furthermore, as an indication of solubility, these freshly prepared stock solutions were analysed against each other in terms of vortex vs vortex and sonication. This assay was also performed on HPLC. These results would provide an indication on how to ensure optimal solubilisation of IMI and its metabolites stock solutions.

2.6 Results

2.6.1 Optimisation of LC-MS/MS conditions

The analytical challenge of this study was accurately detecting and quantitating the target analytes in a single analysis. This was primarily due to the high sensitivity that was required for the detection of trace amounts of the target analytes. As has been noted previously, MS parameters were optimised for each compound as well as the internal standards.

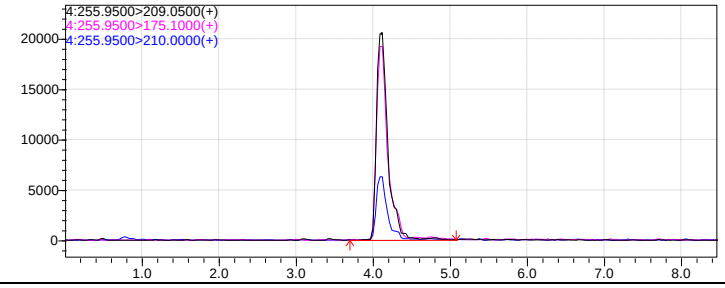
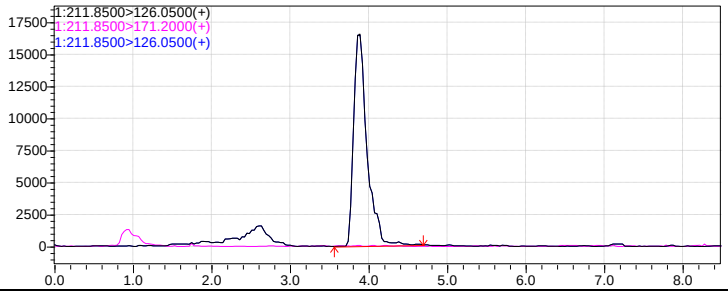
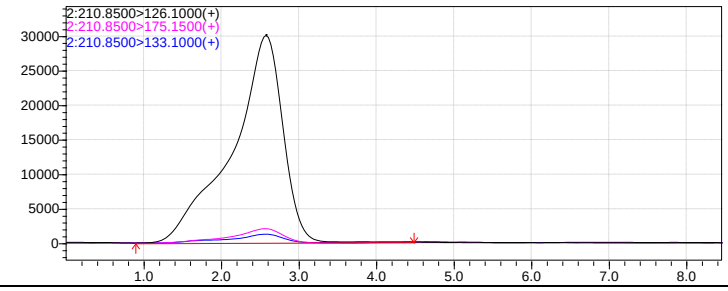
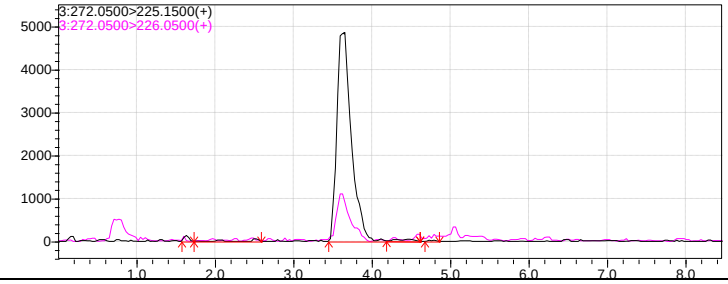
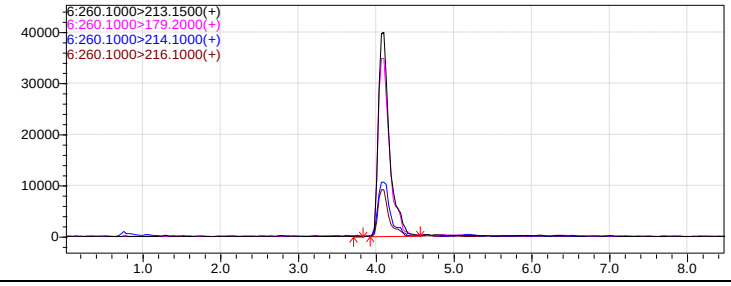
2.6.1.1 IMI and its metabolites

Based on the literature search for IMI and its metabolites, the majority of the mobile phases used for LC-MS/MS analysis consisted of low concentrations of formic acid and methanol as the organic solvent [21][34][140][141]. As previously discussed, IMI and its metabolites were infused separately at 1 µg/mL. The best ionisation response for all the precursor ions of the target analytes was achieved with a mixture of MeOH:water (1:1, v/v) with 0.1% formic acid. These m/z ratios as well as the most abundant product ions of IMI and its metabolites, are depicted in Table 2.7.

Method development started with the main aim of attaining efficient retention and resolution of the peak of interest by assessing various chromatographic parameters. These parameters involved the selection of mobile phase, flow rate, analytical column and injection volume. Various volume ratios (5:95, 20:70, 25:75, v/v) of water:MeOH and water:ACN combinations with formic acid (0.05, 0.1, and 0.2%) and ammonium formate (2 mM) and acetate (2 mM, 5 mM) buffer were investigated as a mobile phase. Two different analytical columns were evaluated. This included a Poroshell (3.0 x 100 mm, 2.7 µm) and biphenyl (2.1 x 100 mm, 2.7 µm) analytical column. Another crucial condition that was assessed to achieve optimal chromatographic peak shapes was the outcome of flow rate from 0.3 mL/min to 0.45 mL/min as well as injection volumes of 5, 10 and 20 µL.

As depicted in Table 2.10 below, the retention times for IMI, IMI-urea, IMI-NH, 5-OH IMI and IMI-d4 were 3.90, 3.70, 2.50, 3.50 and 4.00 min respectively. The final LC-MS/MS conditions are described in Section 2.2.8.1 above. Chromatograms for each target analyte are also illustrated in Table 2.10 below.

Table 2.10: Retention times and chromatograms of IMI, its metabolites and internal standard at the highest calibration standard concentration

Analyte	Retention time (min)	Chromatograms at 125 ng/mL
Imidacloprid	3.90	
Imidaclorprid-urea	3.70	
Desnitro-imidacloprid	2.50	
5-hydro imidacloprid	3.50	
Imidacloprid-d4 at 2.50 ng/mL	4.00	

2.6.1.2 Neurotransmitters

Based on literature for the neurotransmitters, the majority of the mobile phases used for LC-MS/MS analysis consisted of varying concentrations of formic acid and ACN as the organic solvent [131](163)(164)(165). The mixture of ACN:water (50:50, v/v) with 0.1% formic acid achieved the best ionisation during infusion which allowed the creation of MRMs. These m/z ratios, as well as the most abundant product ions of all the neurotransmitters, are depicted in Table 2.8.

Similarly to method 1, the main aim of the chromatographic parameters was to attain efficient retention and resolution of the peaks of interest. These parameters involved the selection of mobile phase, flow rate, analytical column and injection volume. Various volume ratios (20:80, 10:90, 5:95, v/v) of water:ACN combinations were investigated as a mobile phase with formic acid (0.1%) as well as ammonium formate (25 mM, 2 mM) and ammonium acetate (5 mM) buffer on a Poroshell (3.0 x 100 mm, 2.7 µm) analytical column. Additionally, other crucial conditions that were assessed to achieve optimal chromatographic peak shapes was the flow rate (from 0.3 mL/min to 0.45 mL/min) and injection volumes of 5, 10 and 20 µL. The outcome of the utilisation of an ion-pair reagent, HFBA, as an additive in mobile phase A as previously documented by Santos-Fandila et al (2015) was also assessed [158].

Additionally, following an injection of a standard curve, it was observed that further optimisation was required to compensate for serotonin on-instrument. The heating block temperature was assessed from 250 - 450 °C, as shown below in Figure 2.2.

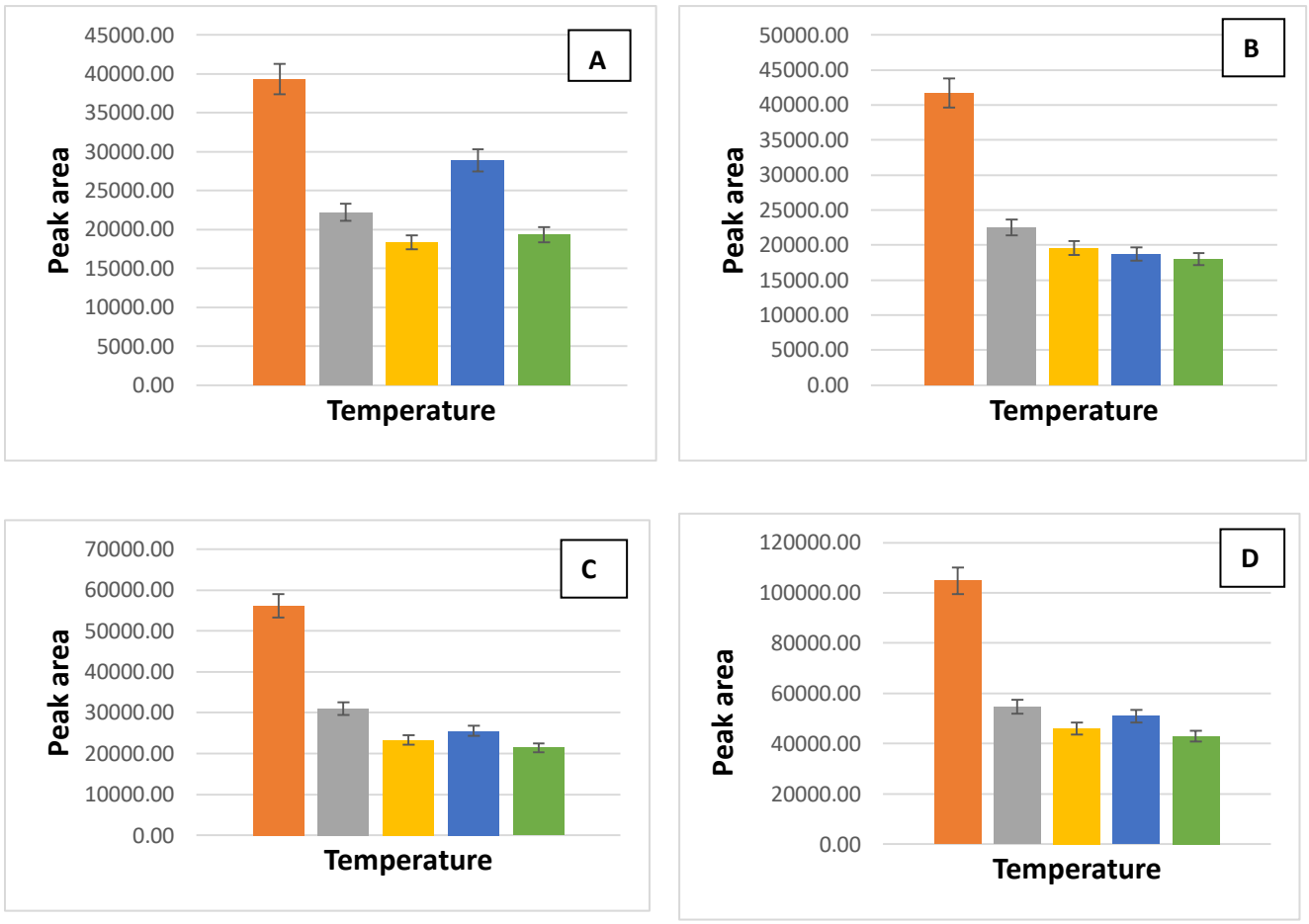
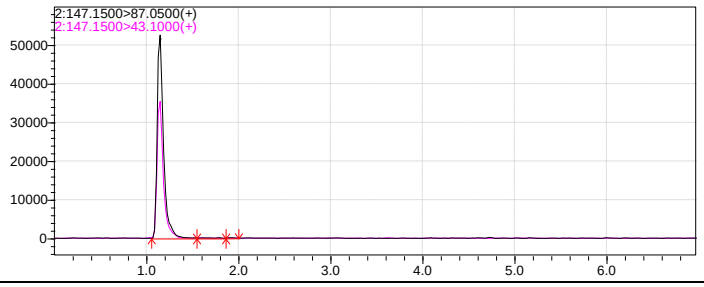
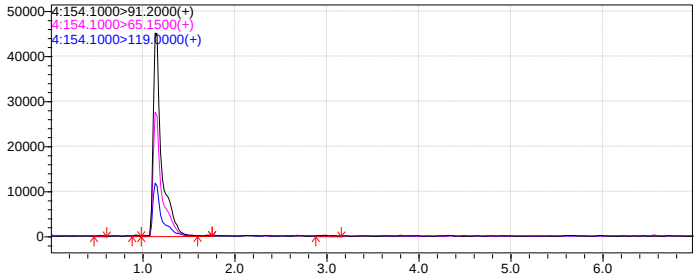
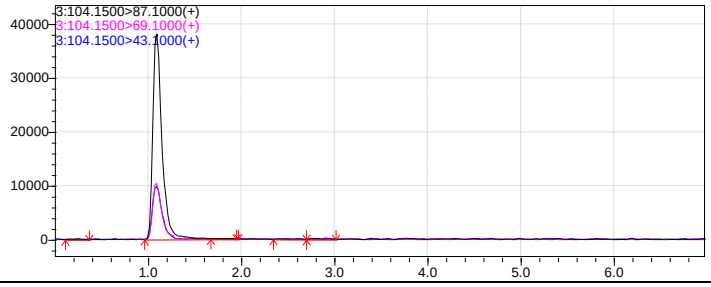
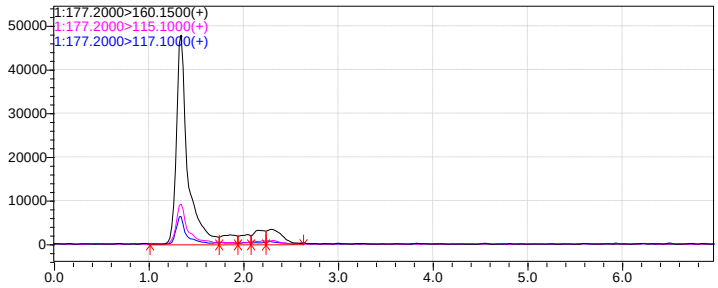
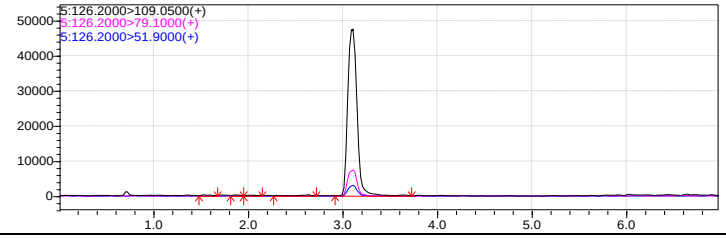


Figure 2.2: The relationship between peak area and heating block temperature (°C) for acetylcholine (A), dopamine (B), gamma-aminobutyric acid (C) and serotonin (D). (Orange - 250 °C; grey - 300 °C; yellow - 350 °C; blue - 400 °C and green - 450 °C)

Subsequently, from the results it was concluded that all the analytes yielded optimal results with a heating block temperature of 300 °C. This conclusion was based on the variability (% CV) between each sample as well as the peak area.

As depicted in Table 2.11 below, the retention times for ACh, DA, GABA, 5-HT and phenetylamine-d4 were 1.15, 1.15, 1.10, 1.40 and 3.07 min respectively. The final LC-MS/MS conditions are described in Section 2.2.8.2 above. Chromatograms for each target analyte are also illustrated in Table 2.11 below.

Table 2.11: Retention times and chromatograms of the neurotransmitters and internal standard at the highest calibration standard concentration

Analyte	Retention time (min)	Chromatograms at 1 000 ng/mL
Acetylcholine (ACh)	1.15	
Dopamine (DA)	1.15	
Gamma-aminobutyric acid (GABA)	1.10	
Serotonin (5-HT)	1.40	
Phenethylamine-d4 at 100 ng/mL	3.07	

2.6.2 Optimisation of the extraction procedure

Two extraction approaches were undertaken, LLE and PPT. At the same time, it was equally important to ensure optimal extraction results for IMI-d4 and phenetylamine-d4 internal standards using the same developed and optimised extraction method. IMI-d4 and phenetylamine-d4 were added to the precipitating solvent [(MeOH:ACN (1:1, v/v)] at 2.50 ng/mL and 100 ng/mL, respectively, with the ultimate aim of yielding a peak area of between 300 000 – 500 000 arbitrary units.

2.6.2.1 IMI and metabolites

The LLE process was conducted using dichloromethane (DCM), ethyl acetate (EA) and ethyl acetate:hexane (1:1 v/v) (EA:Hex). IMI and its metabolites were prepared at a final concentration of 12.5 ng/mL in zebrafish tissue homogenate. A small volume (100 µL) of 1% trichloroacetic acid (TCA) prepared in water was added to the sample. The purpose of the addition of TCA to the samples was to remove any proteins in the matrix that could cause interference, ultimately contributing to the sample clean-up. A volume of 300 µL of each of the investigated LLE solvents was added to 100 µL of tissue homogenate, after which the samples were vortexed for 1 min and centrifuged at 16 000 xg for 5 min at 4 °C.

Subsequently, for the samples containing EA and EA:Hex (1:1, v/v), the top layer was removed and transferred to a new microcentrifuge tube and dried under a gentle stream of nitrogen at room temperature. For the samples containing DCM, the top layer was discarded and the bottom later was dried under a gentle stream of nitrogen at room temperature. The dried samples were reconstituted in 200 µL water:MeOH (1:1, v/v), vortex-mixed and centrifuged. All the samples were injected onto the LC-MS/MS to observe whether the target analytes remained in the aqueous layer or migrated into the organic solvent. This LLE procedure is depicted in Figure 2.3 below.

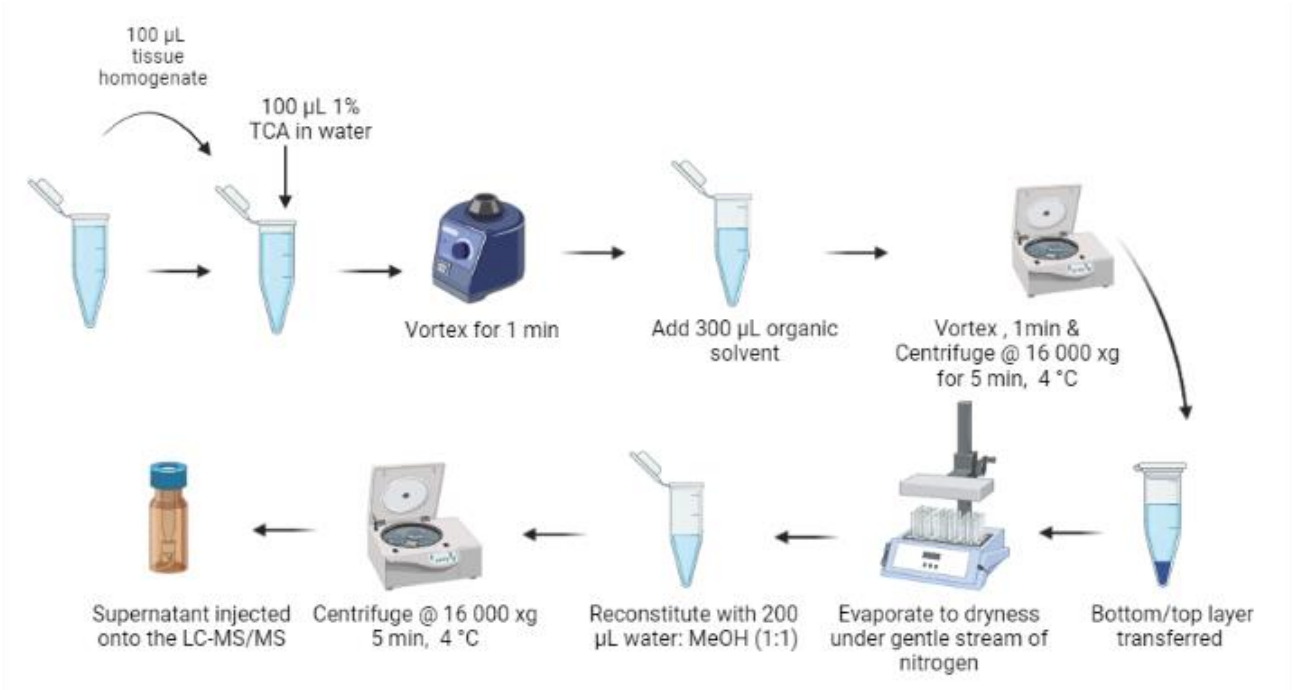


Figure 2.3: The liquid-liquid extraction method development. Image created using Biorender

In conjunction with the above LLE experiment, a PPT extraction method was performed using ice-cold 10% TCA, ACN, MeOH and ACN:MeOH (1:1, v/v). Similarly, IMI and its metabolites were prepared at 12.5 ng/mL in zebrafish tissue homogenate. A volume of 300 µL of each of the investigated PPT solvents was added to 100 µL of tissue homogenate.

Subsequently, the samples were vortex-mixed and sonicated in an ice-bath for 10 min and allowed to equilibrate at -20 °C for 1 hr. This was followed by centrifugation at 16 000 xg for 10 min at 4 °C. The supernatant was transferred to a new microcentrifuge tube and evaporated to dryness under a gentle stream of nitrogen at room temperature. The dried samples were reconstituted in 200 µL water:MeOH (1:1, v/v) and centrifuged again. A volume of 20 µL was injected onto the LC-MS for analysis. This PPT procedure is depicted in Figure 2.4 below.

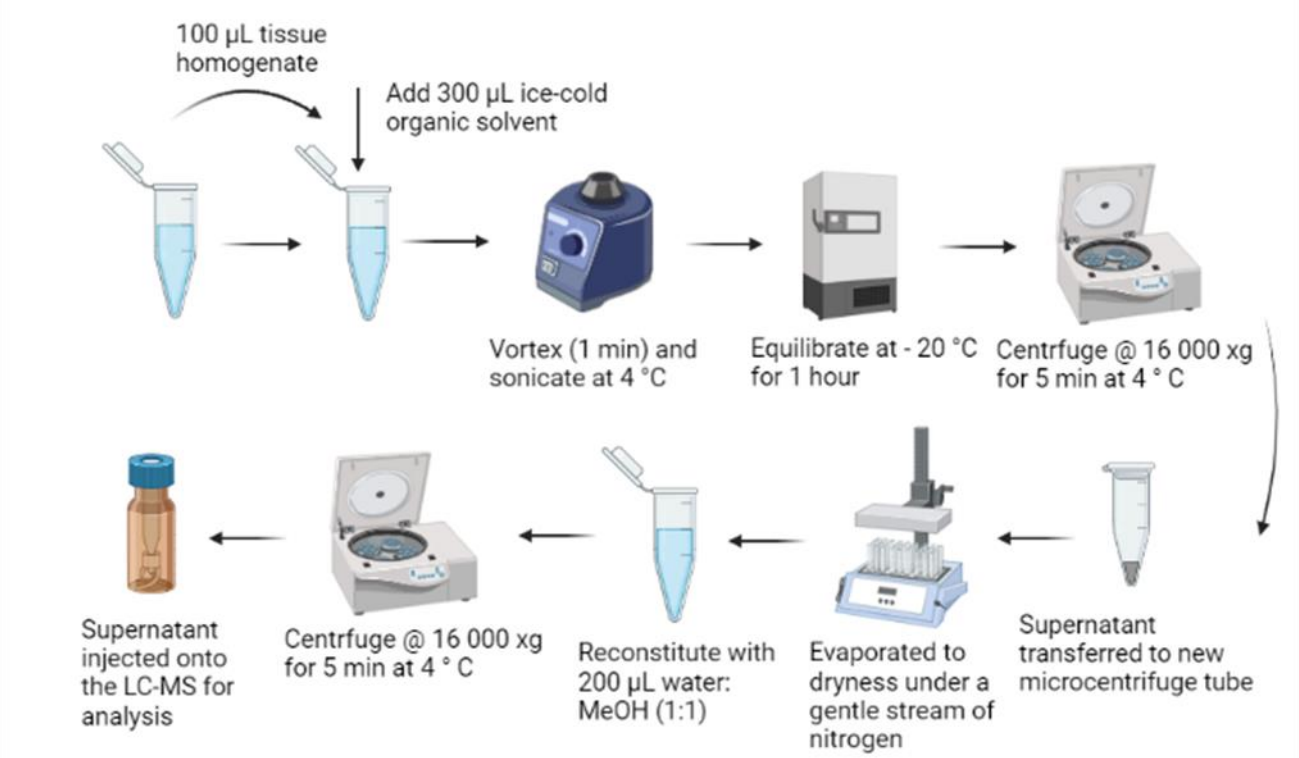


Figure 2.4: The protein precipitation extraction method development. Image created using Biorender

From the results obtained, it was concluded that all the target analytes yielded optimal extraction results with the MeOH:ACN (1:1, v/v) as the precipitating solvent. However, extraction of calibration standards yielded an unsatisfactory calibration curve. Additionally, there was some carry-over present in the blank samples. The method therefore required optimisation. This optimisation involved the assessment of various conditions to eliminate any confounding factors. A few of these conditions involved using HLB extraction cartridges to remove any unwanted phospholipids from the samples before LC-MS/MS analysis and assessing the outcome of the concentration step by comparing samples that were dried down and samples that did not undergo the drying down step. Another optimisation assessment involved assessing various reconstitution solutions.

The results obtained following these experiments for each individual analyte is depicted in Figure 2.5 below.

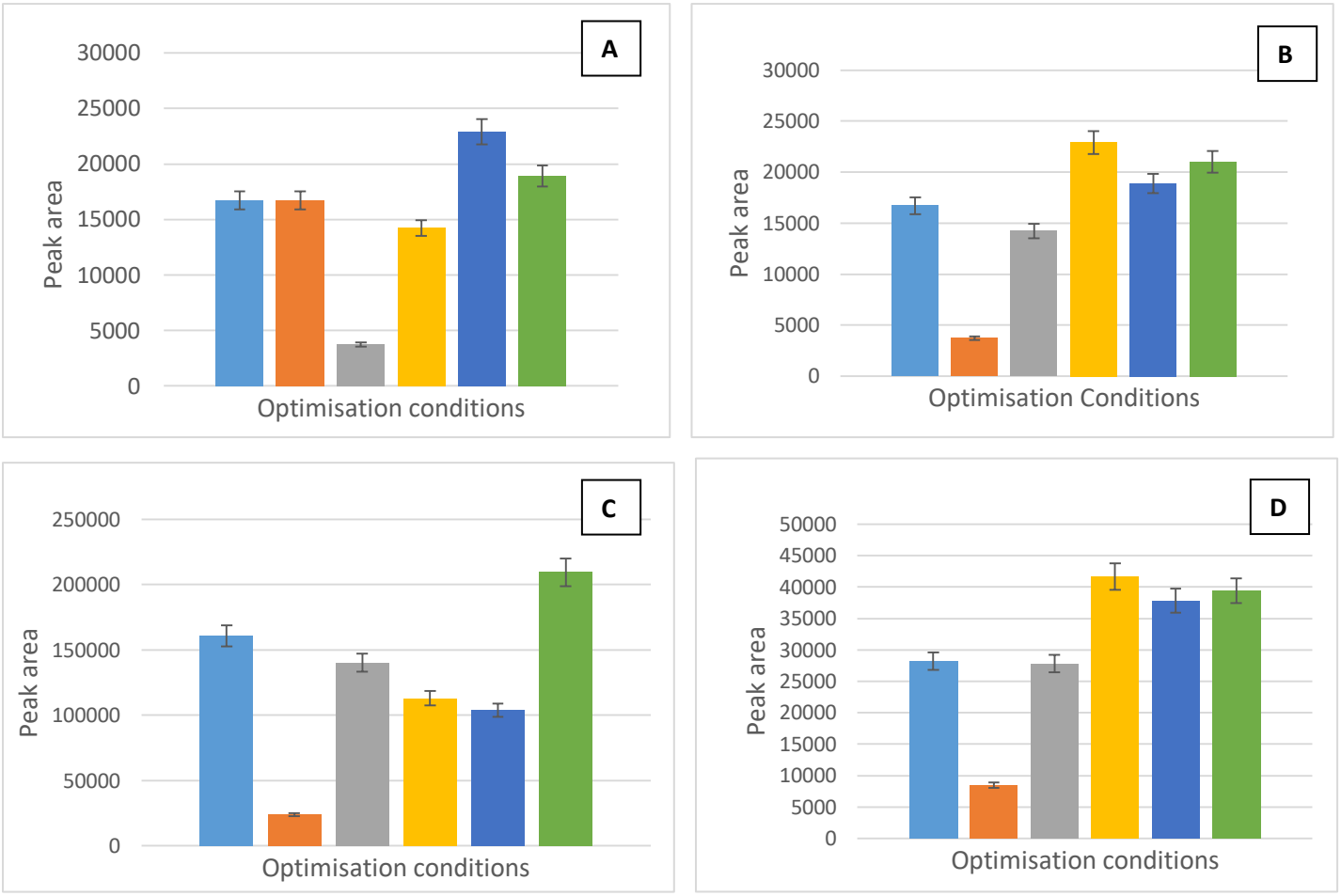


Figure 2.5: The relationship between the average peak area of IMI-urea (A), 5-hydro IMI (B), IMI-NH (C) and IMI (D) and various extraction optimisation conditions. (Light blue - supernatant injected directly onto LC-MS; orange - supernatant eluted through HLB cartridges; grey - supernatant concentrated under nitrogen and reconstituted in MeOH:water (1:1, v/v); yellow - supernatant eluted through HLB cartridges, concentrated under nitrogen and reconstituted in MeOH:water (1:1, v/v); dark blue - supernatant eluted through HLB cartridges, concentrated under nitrogen and reconstituted in MeOH:water (9:1, v/v); green - supernatant dried down and reconstituted in MeOH:water (9:1, v/v))

It was important to note that during the extraction method development and optimisation, challenges would be encountered in the attempt to concomitantly extract all the target analytes. Subsequently, from the results, it was concluded that all the analytes yielded optimal results with three optimisation conditions listed below. This conclusion was based on the precision (%CV) as an indicator of variability between each sample, as well as the peak areas.

1. Supernatant directly injected onto LC-MS for analysis.

2. Supernatant eluted through HLB prime extraction cartridges, concentrated under nitrogen and reconstituted in MeOH:water (1:1, v/v).

3. Supernatant eluted through HLB prime extraction cartridges, concentrated under nitrogen and reconstituted in MeOH:water (9:1, v/v).

2.6.2.2 Neurotransmitters

The neurotransmitters underwent the same extraction method development procedures as IMI and its metabolites described above. In addition, to finalise the PPT extraction method, the neurotransmitters were exposed to the three conditions stated above, which yielded optimal results for IMI and its metabolites.

The results obtained for this experiment were analysed by considering the variability (%CV) between each sample as well as the peak area. The results for each individual analyte are illustrated in Figure 2.6 below.

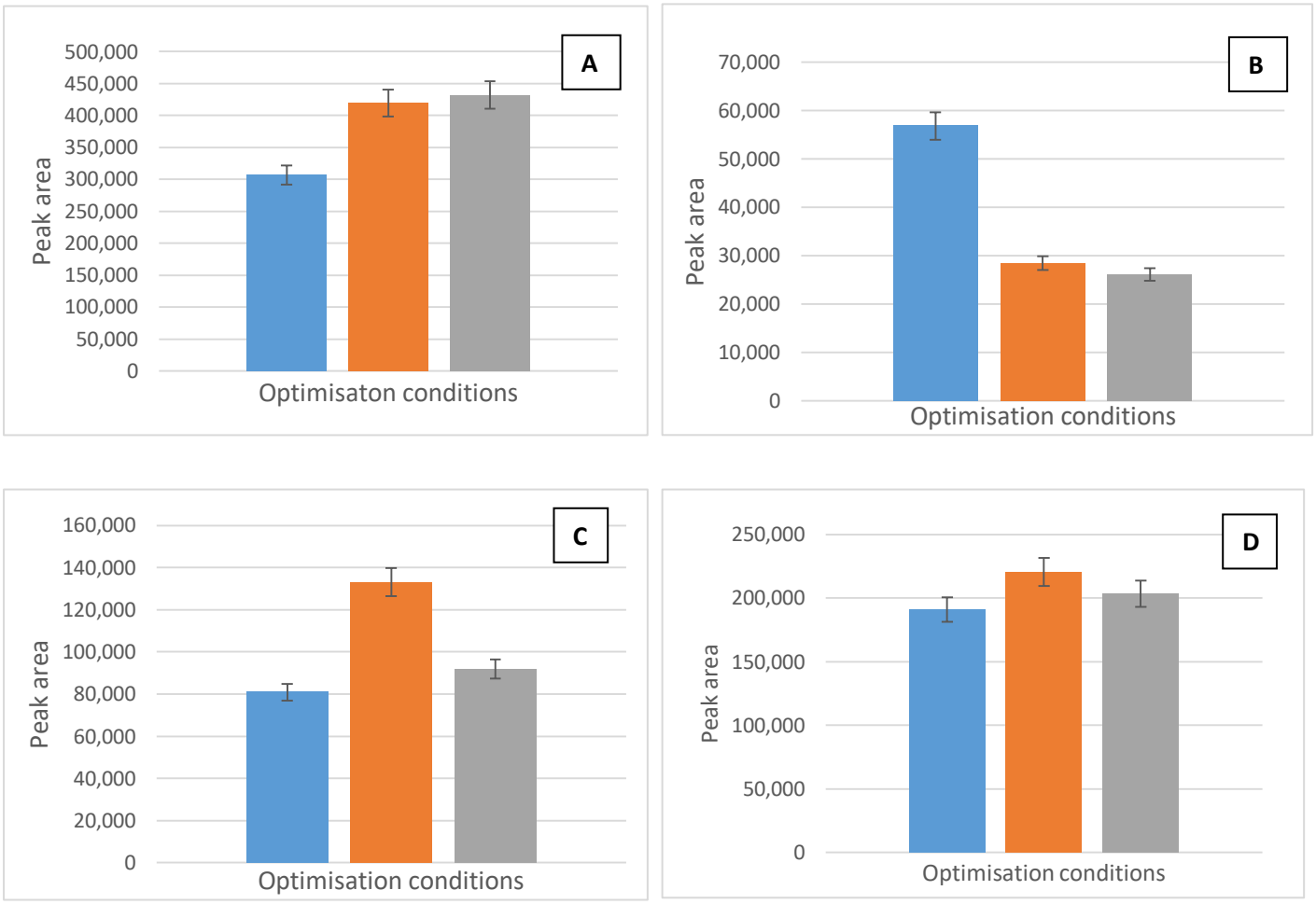


Figure 2.6: The relationship between the average peak area of serotonin (A), acetylcholine (B), dopamine (C) and gamma-aminobutyric acid (D) and various extraction optimisation conditions. (Blue - supernatant injected directly onto LC-MS; orange - supernatant eluted through HLB cartridges, concentrated under nitrogen and reconstituted in MeOH:water (1:1, v/v) and grey - supernatant concentrated under nitrogen and reconstituted in MeOH:water (9:1, v/v))

Overall, following all the method development and optimisation of the extraction procedure, the greatest yield and sensitivity was obtained using a standard PPT extraction method for all the target analytes in this study, without the concentration step. This final extraction method is illustrated in Figure 2.1.

2.6.3 Calibration curve

A calibration curve refers to the relationship between a known concentration of an analyte and the response from an instrument. This enables the estimation of the concentration of the analyte in an unknown sample [166]. The calibration range for IMI and its metabolites was determined by using a reference range previously published in literature by Yang et al (2021) [146]. The calibration range for the neurotransmitters was determined by using published neurotransmitter levels during early zebrafish development [131]. The

overarching aim for both methods was to quantitate levels as low as possible as the concentrations in the final tissue samples could not be predicted.

The data best fits a quadratic regression with 1/C (c is concentration) weighting for all the target analytes. Table 2.12 below indicates the calibration ranges for each specific analyte.

Table 2.12: Calibration ranges for imidacloprid and its metabolites, as well as for the neurotransmitters

Target analyte	Calibration range (ng/mL)
Imidacloprid	1.95 - 125
Imidacloprid-urea	
Desnitro-Imidacloprid	0.244 - 125
5-hydro Imidacloprid	3.91 - 125
Acetylcholine	31.3 – 1 000
Gamma-aminobutyric acid	
Dopamine	
Serotonin	

The precision of the calibration standards and quality controls for each of the target analytes is summarised below. The in-house acceptance criteria for standards and QC's pertaining to pre-clinical work specify that they must have been between 80 and 120% accuracy and precision between replicates of less than or equal to 20%.

The calibration curves of the various target analytes in this study are presented in appendix 2.

2.6.3.1 IMI and metabolites standards accuracy and precision

IMI, IMI-urea, desnitro-IMI and 5-OH IMI calibrations standards and quality controls yielded accuracies between 86.8 - 101%, 82.1 - 97.4%, 86.5 - 120% and 88.3 - 105%, respectively. The tables depicting this information are shown in Tables 2.13 - 2.16 below.

Table 2.13: The accuracy and precision of IMI calibration standards and quality controls

Sample ID	Nominal concentration (ng/mL)	Mean Observed Concentration (ng/mL)	Standard deviation	% CV	% Accuracy	n
S1	125	125	19.2	15.4	100.0	2 of 2
S2	62.5	63.0	7.10	11.3	100.8	2 of 2
S3	31.3	30.1	0.200	0.6	96.2	2 of 2
S4	15.6	16.9	1.10	6.2	92.7	2 of 2
S5	7.81	7.05	0.300	3.8	90.7	2 of 2
S6	3.91	4.38	0.000	0.4	89.2	2 of 2
S7	1.95	1.82	0.000	0.2	93.3	2 of 2
QC H	111	119	12.1	10.2	93.6	6 of 6
QC M	61.0	70.3	3.50	5.0	86.8	4 of 6
QC L	5.57	5.79	1.00	16.6	96.2	4 of 6

IMI had an observed overall accuracy and precision for calibration standards and QC's that were within the acceptable limits stated in Section 2.7.3 above.

Table 2.14: The accuracy and precision of IMI-urea calibration standards and quality controls

Sample ID	Nominal concentration (ng/mL)	Mean Observed Concentration (ng/mL)	Standard deviation	% CV	% Accuracy	n
S1	125	132	15.9	12.1	95.0	2 of 2
S2	62.5	57.7	2.70	4.8	92.3	2 of 2
S3	31.3	28.3	n/a	n/a	90.4	1 of 2
S4	15.6	15.2	0.400	2.6	97.4	2 of 2
S5	7.81	7.19	0.800	11.7	92.1	2 of 2
S6	3.91	4.51	0.200	4.5	86.7	2 of 2
S7	1.95	1.85	0.000	0.1	94.9	2 of 2
QC H	111	122	16.1	13.1	90.9	4 of 6
QC M	61.0	74.3	3.60	4.8	82.1	3 of 6
QC L	5.57	5.06	0.500	9.4	90.8	6 of 6

IMI-urea had an observed overall accuracy and precision for calibration standards and QC's that were within the acceptable limits. One replicate of calibration standard 3, was excluded from the curve as it was not within acceptable limits. However, 50% of calibration standard 3 passed, therefore making it acceptable.

Table 2.15: The accuracy and precision of desnitro-IMI calibration standards and quality controls

Sample ID	Nominal concentration (ng/mL)	Mean Observed Concentration (ng/mL)	Standard deviation	% CV	% Accuracy	n
S1	125	125	9.81	7.8	99. 7	2 of 2
S2	62.5	61.9	2.27	3.7	101.0	2 of 2
S3	31.25	31.7	0.864	2.7	98.7	2 of 2
S4	15.63	15.8	0.335	2.1	98.6	2 of 2
S5	7.81	7.20	0.247	3.4	108.6	2 of 2
S6	3.91	3.99	0.526	13.2	97.9	2 of 2
S7	1.95	1.87	0.0425	2.3	104.4	2 of 2
S8	0.977	0.95	n/a	n/a	97.2	1 of 2
S9	0.488	0.540	n/a	n/a	110.7	1 of 2
S10	0.244	0.211	n/a	n/a	86.5	1 of 2
QC H	111	99.59	2.94	3.0	111.5	4 of 6
QC M	61.0	50.91	12.2	24.0	119.8	3 of 6
QC L	5.57	5.43	0.800	14.7	102.6	3 of 6

IMI-NH had an observed overall accuracy and precision for calibration standards and QC's that were within the acceptable limits. One replicate of calibration standard 8, 9 and 10, was excluded from the curve as it was not within acceptable limits. However, 50% of these calibration standards passed, therefore making it acceptable

Table 2.16: The accuracy and precision of 5-OH IMI calibration standards and quality controls

Sample ID	Nominal concentration (ng/mL)	Mean Observed Concentration (ng/mL)	Standard deviation	% CV	% Accuracy	n
S1	125	125	0.923	0.7	100.0	2 of 2
S2	62.5	63.1	6.97	11.0	99.0	2 of 2
S3	31.3	30.2	2.73	9.0	96.5	2 of 2
S4	15.6	17.3	0.343	2.0	90.4	2 of 2
S5	7.81	8.07	n/a	n/a	103.3	1 of 2
S6	3.91	4.21	0.129	3.1	92.9	2 of 2
QC H	111	120	9.30	7.8	92.9	4 of 6
QC M	61.0	69.1	5.90	8.6	88.3	5 of 6
QC L	5.57	5.32	5.90	12.3	105	4 of 6

5-OH IMI had an observed overall accuracy and precision for calibration standards and QC's that were within the acceptable limits. One replicate of calibration standard 5, was excluded from the curve as it was not within acceptable limits. However, 50% of calibration standard 5 passed therefore making it acceptable.

2.6.3.2 Neurotransmitters standards accuracy and precision

ACh, GABA, DA and 5-HT calibrations standards and quality controls yielded accuracies between 85.6 - 119%, 87.8 - 99%, 90.4 - 107% and 94.8 - 99.7 %, respectively, as depicted in Tables 2.17 - 2.20 below.

Table 2.17: The accuracy and precision of ACh calibration standards and quality controls

Sample ID	Nominal concentration (ng/mL)	Mean Observed Concentration (ng/mL)	Standard deviation	% CV	% Accuracy	n
S1	1000	1193	n/a	n/a	119.3	1 of 2
S2	500	470	3.80	0.8	94.0	2 of 2
S3	250	250	10.2	4.1	99.6	2 of 2
S4	125	134	8.70	6.5	93.4	2 of 2
S5	62.5	68.2	0.200	0.2	91.6	2 of 2
S6	31.3	26.8	0.900	3.4	85.6	2 of 2
QC H	800	755	46.7	6.2	94.4	6 of 6
QC M	400	410	20.8	5.1	97.6	6 of 6
QC L	40.0	44.0	2.60	5.9	91.7	5 of 6

ACh had an observed overall accuracy and precision for calibration standards and QC's that were within the acceptable limits. One replicate of calibration standard 1, was excluded from the curve as it was not within acceptable limits. However, 50% of calibration standard 1 passed therefore making it acceptable.

Table 2.18: The accuracy and precision of GABA calibration standards and quality controls

Sample ID	Nominal concentration (ng/mL)	Mean Observed Concentration (ng/mL)	Standard deviation	% CV	% Accuracy	n
S1	1000	1010	53.6	5.3	99.0	2 of 2
S2	500	473	15.0	3.2	94.6	2 of 2
S3	250	257	6.40	2.5	97.3	2 of 2
S4	125	139	5.40	3.9	90.3	2 of 2
S5	62.5	54.9	n/a	n/a	87.8	1 of 2
S6	31.3	28.7	4.90	17.2	91.7	2 of 2
QC H	800	854	46.7	5.5	93.6	6 of 6
QC M	400	427	13.8	3.2	93.7	6 of 6
QC L	40.0	39.2	3.10	7.9	98.0	5 of 6

GABA had an observed overall accuracy and precision for calibration standards and QC's that were within the acceptable limits. One replicate of calibration standard 5, was excluded from the curve as it was not within acceptable limits. However, 50% of calibration standard 5 passed therefore making it acceptable.

Table 2.19: The accuracy and precision of DA calibration standards and quality controls

Sample ID	Nominal concentration (ng/mL)	Mean Observed Concentration (ng/mL)	Standard deviation	% CV	% Accuracy	n
S1	1000	1004	100	10.0	99.6	2 of 2
S2	500	492	n/a	n/a	98.4	1 of 2
S3	250	267	n/a	n/a	106.8	1 of 2
S4	125	113	12.5	11.1	90.4	2 of 2
S5	62.5	64.3	n/a	n/a	102.9	1 of 2
S6	31.3	31.8	1.21	3.8	98.3	2 of 2
QC H	800	819	91.7	11.2	97.7	5 of 6
QC M	400	385	27.0	7.0	96.3	6 of 6
QC L	40.0	41.6	5.43	13.1	96.2	5 of 6

DA had an observed overall accuracy and precision for calibration standards and QC's that was within the acceptable limits stated. One replicate of calibration standard 2, 3 and 5 was excluded from the curve as it was not within acceptable limits. However, 50% of calibration standard 5 passed therefore making it acceptable

Table 2.20: The accuracy and precision of 5-HT calibration standards and quality controls

Sample ID	Nominal concentration (ng/mL)	Mean Observed Concentration (ng/mL)	Standard deviation	% CV	% Accuracy	n
S1	1000	995	184	18.5	99.5	2 of 2
S2	500	498	39.3	7.9	99.6	2 of 2
S3	250	251	12.3	4.9	99.7	2 of 2
S4	125	126	13.1	10.3	98.9	2 of 2
S5	62.5	61.3	5.07	8.3	98.1	2 of 2
S6	31.3	31.5	0.665	2.1	99.3	2 of 2
QC H	800	758	73.8	9.7	94.8	6 of 6
QC M	400	407	30.7	7.5	98.2	6 of 6
QC L	40.0	40.3	4.20	10.3	99.2	3 of 6

5-HT had an observed overall accuracy and precision for calibration standards and QC's that were within the acceptable limits.

Overall, the results from the assays above indicate an acceptable calibration range for each target analyte in this study. The lowest limit of quantitation (LLOQ) was set at the concentration of the lowest validated standard for each target analyte. Additionally, the results indicate that the method provides sufficient accuracy and precision over the entire range based on analyte peak area with a quadratic calibration curve (weighted by 1/C concentration).

2.6.4 Pre-validation recovery assessment

2.6.4.1 Recovery

Recovery refers to the efficiency of the extraction process documented as a percentage of the known quantity of an analyte taken through the sample extraction as well as the processing steps of the method [(167)]. According to Sonawane et al (2014), recoveries do not need to be 100%. However, the recoveries should be consistent, precise and reproducible [(168)]. The final % recovery and overall %CV for all the target analytes in this study is demonstrated in Tables 2.21 - 2.25 below. The average recovery of a quantitative analytical method must be consistent and precise. The measured recovery should not be greater than 20% for any specific concentration of the analyte at which it is determined. Recovery reproducibility between the different concentrations must not be greater than 20%.

2.6.4.1.1 IMI and metabolites

The blank space left in the tables below at the high concentration at sample 6 was due to the loss of sample during the experimental procedure.

Table 2.21: Recovery of IMI from zebrafish tissue homogenate

	High Concentration (111 ng/mL)		Medium Concentration (61 ng/mL)		Low Concentration (5.57 ng/mL)	
	Extraction: Analyte Peak Area	**Spiked Solution: Analyte Peak Area**	**Extraction: Analyte Peak Area**	**Spiked Solution: Analyte Peak Area**	**Extraction: Analyte Peak Area**	**Spiked Solution: Analyte Peak Area**
Sample 1	123 210	133 042	69 304	96 620	6 932	9 263
Sample 2	110 023	171 738	93 335	99 307	6 193	9 391
Sample 3	164 779	151 888	83 350	105 174	10 198	9 287
Sample 4	150 332	143 169	86 979	99 930	8 045	6 735
Sample 5	137 246	191 106	96 785	92 729	10 915	11 158
Sample 6		138 361	102 206	93 455	9 417	[1 246 306]*
Average	137 118	154 884	88 660	97 869	8 617	9 167
STDEV	21 606	22 327	11 637	4 629	1 869	1 577
% CV	15.8	14.4	13.1	4.7	21.7	17.2
% Recovery		88.5		90.6		94.0
				Average % Recovery		91.0
				Average % CV		3.0

*Outliers omitted from data analysis

Table 2.22: Recovery of IMI-urea from zebrafish tissue homogenate

	High Concentration (111 ng/mL)		Medium Concentration (61 ng/mL)		Low Concentration (5.57 ng/mL)	
	Extraction: Analyte Peak Area	Spiked Solution: Analyte Peak Area	Extraction: Analyte Peak Area	Spiked Solution: Analyte Peak Area	Extraction: Analyte Peak Area	Spiked Solution: Analyte Peak Area
Sample 1	98 328	161 283	68 820	88 250	7 937	6 033
Sample 2	99 585	190 784	92 374	96 088	7 480	11 267
Sample 3	146 503	169 125	91 196	96 683	7 204	9 342
Sample 4	124 428	173 589	90 056	70 787	8 729	6 762
Sample 5	124 837	188 218	90 511	98 152	8 685	10 544
Sample 6		151 467	104 750	83 592	7 955	11 445
Average	118 736	172 411	89 618	88 925	7 998	9 232
STDEV	20 149	15 245	11 598	10 531	618	2 328
% CV	17.0	8.8	12.9	11.8	7.7	25.2
% Recovery		68.9		101.0		86.6
				Average % Recovery		85.4
				Average % CV		18.7

Table 2.23: Recovery of desnitro-IMI from zebrafish tissue homogenate

	High Concentration (111 ng/mL)		Medium Concentration (61 ng/mL)		Low Concentration (5.57 ng/mL)	
	Extraction: Analyte Peak Area	Spiked Solution: Analyte Peak Area	Extraction: Analyte Peak Area	Spiked Solution: Analyte Peak Area	Extraction: Analyte Peak Area	Spiked Solution: Analyte Peak Area
Sample 1	610 426	1 180 025	725 072	568 117	6 729	12 349
Sample 2	733 054	1 528 906	914 204	961 694	6 521	12 460
Sample 3	867 656	1 734 415	923 128	816 951	7 791	11 441
Sample 4	736 970	1 398 169	839 925	688 879	9 553	9 975
Sample 5	[1 031 653]*	1 692 175	974 293	776 280	[21 587]*	[4 747]*
Sample 6		1 111 907	[1 110 985]*	777 475	6 631	16 099
Average	737 027	1 440 933	875 324	764 899	7 445	12 465
STDEV	105 052	258 903	96 722	131 322	1 284	2 262
% CV	14.3	18.0	11.0	17.2	17.2	18.1
% Recovery		51.1		114.0		59.7
				Average % Recovery		75.1
				Average % CV		45.7

*Outliers omitted from data analysis

Table 2.24: Recovery of 5-OH IMI from zebrafish tissue homogenate

	High Concentration (111 ng/mL)		Medium Concentration (61 ng/mL)		Low Concentration (5.57 ng/mL)	
	Extraction: Analyte Peak Area	Spiked Solution: Analyte Peak Area	Extraction: Analyte Peak Area	Spiked Solution: Analyte Peak Area	Extraction: Analyte Peak Area	Spiked Solution: Analyte Peak Area
Sample 1	37 476	42 943	33 880	31 768	3 812	[5 302]*
Sample 2	46 558	58 764	39 910	35 196	2 975	3 976
Sample 3	51 394	65 804	44 083	34 495	3 743	3 444
Sample 4	48 448	46 922	26 522	36 792	2 904	[1 890]*
Sample 5	56 814	61 019	34 924	32 216	[1 957]*	2 431
Sample 6		46 784	34 805	36 865	2 582	2 333
Average	48 138	53 706	35 687	34 555	3 203	3 046
STDEV	7 109	9 330	5 949	2 191	545	798
% CV	14.8	17.4	16.7	6.3	17.0	26.2
% Recovery		89.6		103.0		105.0
				Average % Recovery		99.4
				Average % CV		8.5

*Outliers omitted from data analysis

All the analytes displayed an average recovery ranging from 75.1 - 99.4% across the high, medium and low concentrations. None of the analytes exceeded a %CV of 20% except for IMI-NH with a %CV of 45.7. As it stands, for a pre-clinical method, this was deemed acceptable by a senior scientist. This analyte, IMI-NH, was challenging and this was a compromise that had to be made for the other analytes.

2.6.4.1.2 Neurotransmitters

As previously discussed, the recovery of the neurotransmitters was predominantly based on the extraction recovery of the ISTD due to the presence of endogenous neurotransmitter concentrations in matrix. A similar approach was used by Aragon et al (2017) [(159)].

Table 2.25: Recovery of phenetylamine-d4 in zebrafish tissue homogenate

	Concentration (100 ng/mL)	
	Extraction: Analyte Peak Area	Spiked Solution: Analyte Peak Area
Sample 1	288 422	353 154
Sample 2	299 123	330 990
Sample 3	334 836	373 573
Average	307 460	352 572
STDEV	24 304	15 672
% CV	**7.9**	**4.4**
% Recovery		**87.2**

2.6.4.2 Benchtop stability of IMI and its metabolites stock solutions

Benchtop stability is conducted to assess the stability of the analytes at room temperature, as would occur during sample processing prior to analysis. These results provide an indication as to whether the stock solutions should be kept on ice during processing or not. Additionally, the results indicate that assays could be completed within the time-frame for which the analyte is identified as stable. This assay was conducted using HPLC for all the analytes except for IMI-urea which did not have an UV absorbance. Therefore, IMI-urea's stock solution stability assessment was done on LC-MS. Both the %CV and the % difference have to be below 15%. A % difference that is greater than 15% in comparison to the long-term samples peak area is an indication of on-bench stock solution instability.

Tables 2.26 below represents the results obtained from the on-bench stock solution stability assay as described in Section 2.4.1. The table also portrays the average peak area of the benchtop stability stock solutions, the precision (%CV) as well as the calculated difference after being left on bench for approximately 5 hrs. It is indicated from the results that the %CV and % difference for the various target analytes is within 15%. Therefore, on-bench stock solution stability for IMI and its metabolites is indicated for at least 5 hrs.

Table 2.26: Benchtop stock solution stability results for IMI and its metabolites

Analyte	Solvent	Average peak area	% CV	% difference
IMI-urea	MeOH	1 417 373	2.4	2.9
IMI	MeOH	1 105 964	6.0	12.0
IMI-NH	MeOH	45 303	0.4	0.0
5-OH IMI	MeOH	1 133 725	6.5	0.2

2.6.4.3 Solubility assessment– IMI and metabolites

A solubility assessment was performed to determine the effects of only vortexing and vortexing and sonicating of stock solutions during procedures. Table 2.27 below represents the results obtained from the solubility assay as described in Section 2.5. The table also portrays the average peak areas of the freshly prepared stock solutions that were vortexed and sonicated, the precision (%CV) as well as the calculated difference between the two conditions. Similarly, this assay was also conducted using HPLC for all the analytes except for IMI-urea. Both the %CV and the % difference have to be below 15%. A % difference that is greater than 15% in comparison to the freshly prepared (vortexed only) stock solutions is an indication of a solubility issue.

From the results depicted in Table 2.27, it is depicted that all the analytes had a %CV and % difference within 15% which does suggest that solubility is not an issue.

Table 2.27: Solubility assessment results for IMI and its metabolites

Analyte	Solvent	Average peak area	% CV	% difference
IMI-urea	MeOH	1 883 461	12.5	3.5
IMI	MeOH	1 376 872	4.2	10.7
IMI-NH	MeOH	45 452	1.0	1.3
5-OH IMI	MeOH	809 896	3.3	9.4

2.7 Discussion

In this chapter, we were able to develop LC-MS/MS methods for the quantitation and detection of IMI, three of its primary metabolites and four neurotransmitters in adult zebrafish brain, liver and gill epithelial tissue. Due to the pre-clinical nature of this study, it was not necessary to do a full validation according to FDA guidelines [(167)]. However, despite this, all the measures and precautions were taken to ensure that the method has reliability, accuracy, precision as well as repeatability.

2.7.1 Optimisation of LC-MS/MS conditions

Initially, the plan was to develop a method for IMI and five of its metabolites. However, due to challenges faced with the infusion, two metabolites, namely IMI-olefin and 6-CNA, were excluded from the method. The difficulty of including these two analytes in this multiplex method was due to it predominantly yielding singly charged deprotonated precursor $[M-H]^-$ ions with varying m/z in Q1 full scan spectra as has been reported in literature [(34)(146)]. This would result in more challenging ionisation. An additional LC-MS/MS method with different mobile phases would be required to facilitate the negative ionisation mode as it would still be crucial to ensure that all the target analytes could be extracted using a single method.

To initiate the chromatography method development for IMI and its metabolites, a biphenyl analytical column was chosen despite the extensive use of a C18 analytical column in previous studies [(21)(140)(141)(169)]. This choice of analytical column was based on the difference between a C18 and biphenyl column. According to literature, the separation given by a C18 column is less effective in comparison to a phenyl column. In addition, the resolution given by a phenyl column has been reported to be better than a C18 column [(170)].

A method compatible for IMI, IMI-urea and 5-OH IMI was successfully developed and optimised. However, a few crucial observations were made with regards to IMI-NH. Firstly, as illustrated in Table 2.10, the peaks are wider in the front half than they are in the back resulting in an asymmetrical effect. This is also known as fronting. It has been reported that the main cause of this is poor solubility of the sample [(171)]. Despite the solubility assay results indicating no solubility issues with IMI-NH, there was a limitation to this assay. It was performed assessing the solubility of the analyte in a solvent prepared as a stock solution, however IMI-NH may not have optimal solubility in the actual tissue matrix. As previously discussed, due to the complexity of

the development of a multiplex method, it was necessary to compromise to ensure that all the target analytes could be detected utilising a single LC-MS/MS method. However, this observation does deem it necessary for further chromatography development for IMI-NH. Secondly, after analysing the summary of the calibration standards and quality controls precision and accuracy, it was observed that the %CV between the QC medium samples was above 20%. This observation suggests increased variability between the individual samples. The inclusion of an isotopically labelled internal standard for IMI-NH could improve these results by compensating for instrumental drift during an analytical run.

When considering chromatography development of method two, the majority of previously conducted studies focusing on the quantitation of neurotransmitters utilised analytical columns which are excellent for the retention of polar analytes [147][163][172][173]. Therefore, a Poroshell analytical column was chosen for this method as it has been reported to be the most versatile providing increased selectivity [174]. The choice of column for both methods yielded good peak shapes, separation, and short analytical runs. A deuterated internal standard of a neurotransmitter precursor was also included to compensate for early elution of these polar compounds.

2.7.2 Optimisation of extraction procedure

With regards to the extraction of IMI and its metabolites from various tissues and biological fluids, the majority of previously conducted studies utilised a SPE method [34][115][145][175]. When considering extraction method development for the neurotransmitters, the majority of previously conducted studies also utilised a protein precipitation extraction [131][159].

Various studies have been developed for the determination of IMI and a few of its primary metabolites in mice, human and rabbit biological fluids and tissues as well as in water and agricultural food sources [16][34][140][176][175][177][178]. A previously conducted study performed the detection of IMI in various human biological fluids following a case of fatal insecticide ingestion. This published method also used a protein precipitation method but used HPLC for detection [116]. Another study utilized a QuEChERS-HPLC-MS/MS method on crayfish tissue and generated various calibration ranges for IMI and a few of its metabolites. However, these ranges were above the ranges used in this present study [146].

Subsequent to the extensive extraction method development described in Section 2.6.2, this study made use of a protein precipitation extraction method. This choice of extraction method was made not only because of its simplicity and low cost but also due its ability to concomitantly extract all the target analytes in this study. Based on the high recovery range detected across all the compounds, it can be deduced that the extraction method is effective and reliable at extracting all the target compounds from zebrafish tissue homogenate.

2.7.3 Calibration curve and pre-validation assessments

These methods underwent a partial validation and presented accuracy and robustness using a single intra-day validation batch. Since these methods were used for pre-clinical assessments, a single batch was deemed sufficient. A duplicate of the standards at each level and six replicates of each quality control (QC high, medium and low) underwent analysis in this intra-day validation batch. Additional pre-validation experiments that were performed included recovery for both methods and benchtop stability for IMI and its metabolites. As illustrated in Section 2.7.4, all the analytes displayed an average recovery ranging from 75.1 - 99.4%.

Despite the extensive research on the analysis and quantification of various neurotransmitters in the brain of zebrafish following exposure to various exogenous agents, there are very few studies that have focused on the analysis and quantification of these neurotransmitters in adult zebrafish brains following IMI exposure [158][179][180]. The majority of the studies that have analyzed changes in neurotransmitter levels following acute IMI exposure have utilized zebrafish larvae until 5 dpf as a test organism, which is a critical limitation as this does not confer a true representation of the prolonged neurotoxicity effects of IMI exposure on zebrafish [131][159].

To the best of our knowledge, this is one of the first developed LC-MS/MS methods to report the quantitation of IMI, IMI urea, 5-OH IMI, IMI-NH, 5-HT, DA, GABA and ACh in various adult zebrafish tissues.

Chapter 3: In *vivo* (zebrafish) investigations following imidacloprid exposure

3.1 Introduction

IMI has been reported to contaminate multiple surface waters around the globe at levels that pose serious risk to various biodiversity's. According to a review published by Morrissey et al (2015), approximately half of the accessible water monitoring studies documented observable IMI levels given its extensive use as well as its various applications. The reported observable levels of IMI ranged from 0.001 to 320 µg/L. Estimated values of some accidental leakages might reach up to mg/L [181][182][183].

Several studies' main aim is to determine the effect of IMI at high concentrations or during the development of species [144][184][185]. These are clear and very crucial limitations. Firstly, this means that the longer term effects of low level IMI exposure is still unclear. Secondly, it means that the effect on adult forms have been neglected in research. It would be of much greater use if concentrations that can be attained in the surroundings are used to characterize pesticide exposures as well as to determine the effect of IMI in post-development stages. This is especially important when evaluating long-term toxicity effects of IMI, which is limited in research.

Therefore, the overarching aim of this study was firstly to use zebrafish larvae as an *in vivo* model to determine NOAEL of IMI exposure and secondly to determine longer-term neurotoxicity risk in adult zebrafish chronically exposed to NOAEL IMI.

3.2 Materials and methods

3.2.1 Chemicals and reagents

Imidacloprid (IMI, CAS: 138261-41-3, LOT: 6-MIC-12-1, purity: 98%) was supplied by Toronto Research Chemicals (Canada). Once received, it was stored as directed at -20°C. A SS of IMI was prepared at 1 mg/mL in MeOH purchased from Romil Ltd (Cambridge, United Kingdom). Additionally, aliquots of smaller volumes of this SS was prepared and stored in a sealed cryobox at -80 °C to avoid photodegradation. The positive control, 3, 4-dicholoroaniline, purchased from Sigma Aldrich (Saint Louis, USA) was prepared in ethanol, at a stock solution concentration of 1 mg/mL. The same procedure as above was followed for this SS. The compound, tricaine-methanesulfonate was supplied by Sigma Aldrich. Tricaine-methanesulfonate was utilised as an anaesthetic/euthanisation agent in this study.

3.2.2 Animal model: ethical considerations

Longfin *Danio rerio* (zebrafish) eggs were obtained from the Zebrafish Research Unit located at Stellenbosch University, Faculty of Medicine and Health Sciences, Division of Clinical Pharmacology. All experimental assays were performed in accordance with ethical guidelines. Research protocols used in this study were reviewed and approved by the Research Ethics Committee: Animal Care and Use (protocol # ACU-2021-21865). Early-life stages of zebrafish are not protected by South African (and global) research ethics bodies as they are not recognised as sentient animals before 5 dpf [186]. In the Zebrafish Research Unit, all fish were maintained at standard laboratory conditions in accordance with standard zebrafish husbandry protocols. The water was maintained at 28 °C, pH 6.8 - 7.3 and the fish were exposed to a 14/10 light/dark cycle. This well-maintained water was used as dilution water in the preparation of all test solutions in all assays conducted during this study. The adult zebrafish were fed twice daily at approximately 2.5% body mass with commercially available semi-floating Hikari micropellets.

3.2.3 Maintenance of the eggs and larvae

Adult fish were spawned following standard husbandry procedures and eggs obtained at 4 hpf. Eggs and larvae were maintained in E3 embryo media. A 50 x concentrated stock solution of E3 medium was prepared as follows, 14.6 g sodium chloride (NaCl), 0.650 g potassium chloride (KCl), 2.20 g calcium chloride ($CaCl_2$) and 4.05 g magnesium sulphate ($MgSO_4$). A WS was prepared fresh by diluting 50x stocks in autoclaved, distilled water. The pH of the media was adjusted to 7.4 using 0.5 M sodium bicarbonate. The media of the eggs were refreshed every morning. The refreshing of the media involves the removal of approximately ¾ of the media in the petri dish and replacing it with fresh media. The hatching of the larvae takes place between 2 - 3 dpf and the empty chorions should be removed during the refreshing of the media. Additionally, during the refreshing of the eggs media, it is crucial to observe the eggs under a stereomicroscope (World Precision Instruments, USA) to remove any dead or abnormally shaped eggs/embryos. Once the eggs have hatched, only half the media needs to be replaced daily. Eggs and larvae were maintained in a climate chamber (Axiology, model: BJP-M150) at a constant temperature of 28.5 °C and 14/10 light/dark cycle (lights on at 8am).

3.3 Experimental design and pesticide exposure concentrations

3.3.1 Larvae assay

The reported observable levels of IMI mentioned in Section 3.1 was used as a reference in this study to determine the NOAEL in zebrafish larvae to which the adult zebrafish would be exposed to for 21 days. As has been noted, the zebrafish BBB is fully developed at 3 dpf. Therefore the larvae used for this study were

exposed to varying IMI concentrations for a period of 24 hrs, starting at 4 dpf. This was crucial to ensure that any abnormalities detected during or following the IMI exposure could be associated with the pesticide crossing the BBB.

The larvae were plated into a 96-well microtiter plate and treated accordingly. The larvae were pipetted up with 200 µL E3 medium. The well of a 96-well plate can take a maximum volume of 250 µL therefore 50 µL of the various IMI concentrations were introduced to the plate. Due to the five times dilution of the concentration of IMI and the positive control once introduced to the well, the original concentrations needed to be prepared at a concentration five times that of the intended concentration in the well.

The zebrafish larvae were exposed to three different IMI concentrations (0.001, 5 and 25 µg/L), a positive (tox) control (3, 4-dichloroaninline) as well as a negative control (E3 media). The tox control, 3, 4-dichloroaniline (DCA), is an aniline pesticide and is the recommended compound in the Fish Embryo Acute Toxicity (FET) test as outlined in the OECD guidelines for the testing of chemicals. The toxicity of the positive control, DCA, to fish species at early-life stages is well reported [(187)(188)].

The IMI treatment concentrations were prepared in E3 media as serial dilutions from the SS which was prepared in MeOH as stated on IMI's certificate of analysis. DCA was prepared in ethanol and serially diluted using E3 media such that the final concentration to which the larvae would be exposed in the well was 4 mg/mL. It was crucial to ensure that the final MeOH and ethanol content to which the larvae were exposed was less than 2.5% and 0.5% respectively, to eliminate any confounding factors [(189)]. This experiment involved a 96-well microtiter plate where n=12 for each IMI concentration. Once dosing was complete, the plate was incubated at 28.5 °C for 1 hr.

One hrs post-exposure, the plate was viewed under a stereomicroscope to monitor survival as well as any clearly visible abnormalities such as oedema, deformed body shape and swim bladder. Following this observation, the plate was incubated for another 24 hrs. 24 hrs post-acute exposure, tracking was performed to observe any behavioural alterations using the Noldus Ethovision instrument (Noldus Information Technology, Wageningen, Netherlands). The plate was transferred to the Ethovision device for tracking visualisation for 30 min. The behavioural activity that was monitored using the Ethovision software included distance moved (mm) over time (min).

Once the tracking visualisation was complete, the plate was visualised under the stereomicroscope again to monitor the same factors mentioned above, this time after being exposed to IMI for 24 hrs. For this specific 24-hrs visualisation, to allow for more accurate and optimal microscopy, the zebrafish larvae were sedated using tricaine. The objective of this was to ensure that the larvae fell onto their side for images to be taken,

thereby allowing clear visualisation of any abnormalities. Tricaine-methanesulfonate (TMS), commonly known as MS-222, is a white odourless powder with a high water-solubility. It is predominantly used as an anaesthetic to sedate fish during handling by depressing their central and peripheral nervous systems thereby also reducing their sensory perception during any procedure. Despite the controversial topic of fish being able to feel pain, there is substantial evidence that indicates that the utilisation of anaesthetics promotes the wellbeing of the fish [(190)].

See Figure 3.1 below for a more detailed representation of how this dose-response experiment was conducted. This was to ensure accurate finalisation of the concentration to which the adult zebrafish would be exposed to for 21 days. Upon completion of the larval experiment, the larvae were transferred to a 50 mL falcon tube using a micropipette and euthanised accordingly using tricaine by a staff member authorised for this procedure by the SAVC.

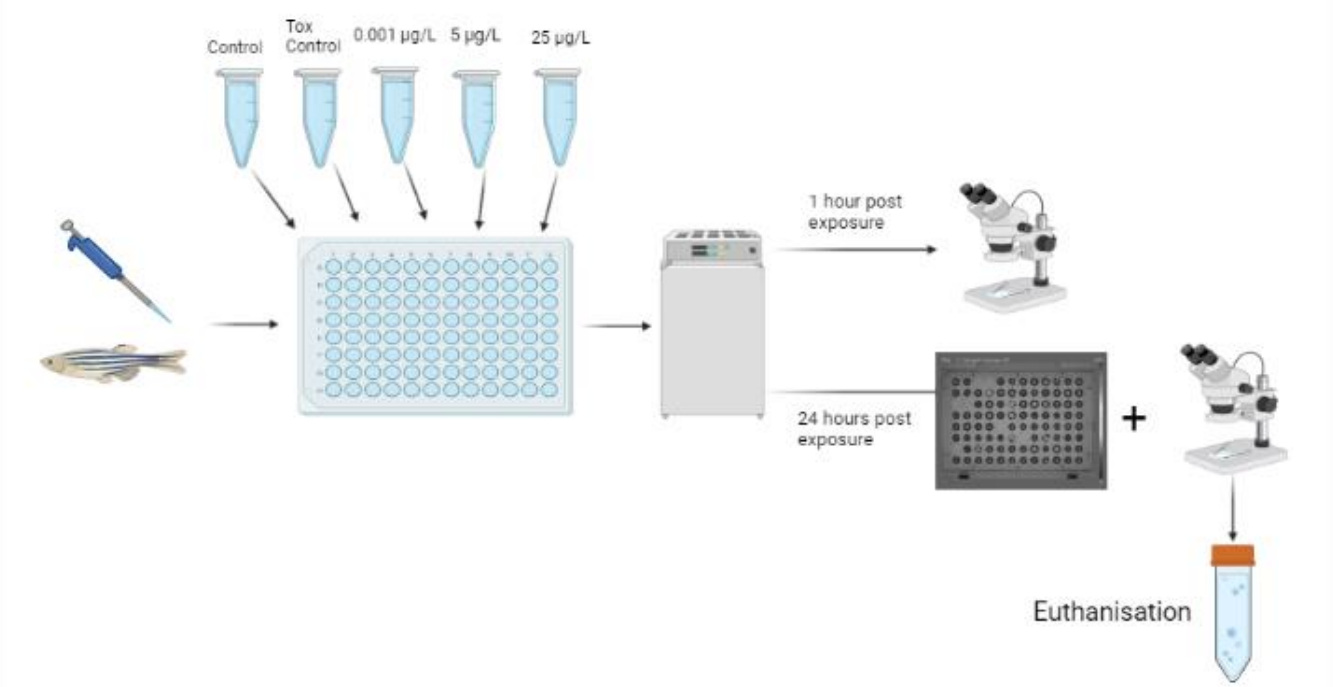


Figure 3.1: A diagram illustrating the procedure of exposing the zebrafish larvae to various IMI concentrations in a 96-well microtiter plate. Image created using Biorender

3.3.1.1 Statistical analysis

One-way ANOVA was utilised in the determination of the main effects of treatment followed by a Bonferroni post hoc test. Biological repeats of at least n=12 per treatment condition was used. A p-value of <0.05 was considered statistically significant.

3.3.2 Adult fish assay

3.3.2.1 Experimental fish husbandry

Healthy adult zebrafish were used in this 21-day pesticide exposure experiment. Adult zebrafish of similar length and age (average length: 3.2 cm, 7 months old) were chosen for the study. There were 10 and 11 fish allocated to the treatment and control group respectively. Following the toxicity assay experiment conducted in the zebrafish larvae, it was concluded that the NOAEL of IMI was 2.5 µg/L. The adult zebrafish were treated with this NOAEL concentration for 21 days. The set-up of the 21-day study is demonstrated in Figure 3.2 below.

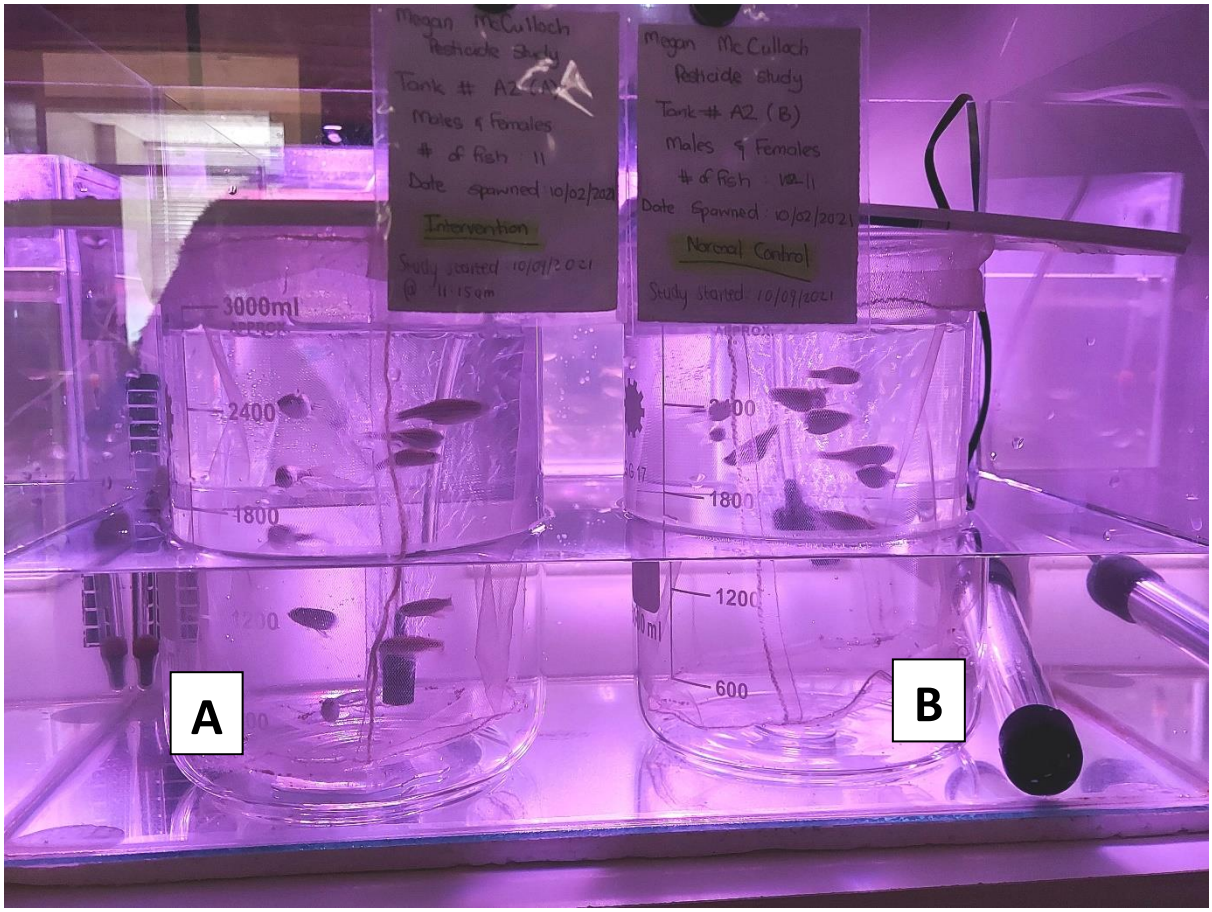


Figure 3.2: An image representing the set-up of the 21-day pesticide exposure experiment in adult zebrafish. A-Treatment group (n=10) and B-Control group (n=11)

As illustrated in Figure 3.2 above, there were two glass beakers each filled with 3 L of water. These beakers were placed in a tank filled halfway with water that was maintained at a temperature of 28 °C using a submersible glass aquarium heater. Additionally, an air stone was placed into each beaker to allow for the circulation of air by producing tiny bubbles filled with oxygen. As has been noted, IMI undergoes photodegradation, therefore to prevent this from occurring, the zebrafish were exposed to low light during this 21-day study.

Furthermore, the beakers of water were refreshed daily to prevent accumulation of IMI in the treatment group's water. This was done by allowing two beakers to acclimatise overnight at 28 °C in a pre-heated laboratory oven (Ecotherm, Labotech). Prior to the addition of the zebrafish, treatment group (A) and control group (B) to the beakers, the pH was checked daily to ensure it was stable at a pH of ± 7. In order to cause minimal distress to fish, they were housed within purpose-made nets in the beakers so that they did not have to be netted for transfer each day. The fish were fed as per normal, twice daily during this 21-day exposure period. The test solution of 2.5 µg/L was prepared by diluting an IMI stock solution in conditioned water. Moreover, the experimental group was treated at the same time daily. See appendix 1 for the detailed description of how this test solution of IMI was prepared.

During the 21-day exposure period, two water samples of 1 mL each were obtained from the treatment group for 5 consecutive days. One sample was taken immediately after dosing the treatment group with the test solution and another sample was taken 24 hrs after dosing. These samples were stored at -80 °C until LC-MS/MS analysis so that the extent of degradation of IMI during a 24-hrs period could be determined.

3.3.2.2 Body length

After 21 days of IMI exposure, all the zebrafish were sacrificed using 300 mg/L tricaine by prolonged immersion. The fish were kept in the solution for approximately 3 min following the termination of opercular movement. A ruler was used to measure the standard body length (SL) in centimetres (cm), from the tip of the mouth to the tip of the tail. See appendix 1 for reference photos taken during this procedure. Following this measurement, the zebrafish were sacrificed as soon as possible to prevent the degradation of the neurotransmitters. The zebrafish were removed from the tricaine solution, lightly patted dry with paper towel and pinned to a dissecting mat through the fleshy part of the tail. The brain, liver and gills were dissected out and snap-frozen using liquid nitrogen in 0.5 mL microcentrifuge tubes. These samples were stored at -80 °C until LC-MS/MS analysis. Statistical analysis was performed to determine if the difference observed between the treated and control group was statistically significant.

3.4 Application of the developed and optimised LC-MS/MS method

Subsequent to the 21-day IMI exposure period, the amount of IMI and its metabolites in the various tissue was determined using the developed and optimised LC-MS/MS method in this study. A calibration curve was generated using a general matrix (zebrafish whole body homogenate) in order to quantify the target analytes in the various tissue specimens. Additionally, this method was also used to detect and quantify various neurotransmitters in the adult zebrafish brain tissue. Finally, this method was also used to detect and quantify IMI and its metabolites in water samples taken immediately after dosing (± 2 min post-dose) and water

samples taken 24 hrs post-dose during the IMI exposure period. Due to the small size of the brain, liver and gill epithelial tissue, it was deemed necessary to pool the samples resulting in a small sample size.

Statistical analysis was performed on the data obtained for this study using GraphPad Prism 8.0.2. The data from all the experimental groups underwent a normality test using Shapiro-Wilk test. GraphPad Prism was also utilised to plot the appropriate graphs.

3.5 Results

3.5.1 Larvae morphology and behaviour

The following results were obtained from the toxicity assay experiment conducted in larvae (4 - 5 dpf).

3.5.1.1 One–hrs post IMI exposure

There was no significant difference in morphology between the negative control and the IMI-treated larvae. The survival rate was 100% in all groups.

3.5.2 Larvae 24-hrs post exposure – tracking and microscopy results

After 24 hrs exposure to IMI, there were no mortalities nor any obvious malformations. However, the more sensitive activity tracking revealed effects of prolonged exposure to IMI. These effects are illustrated in Figure 3.3 and 3.4 below.

From this graph (Figure 3.3), it is clear that the tox control had a decrease in total distance moved in comparison to the control. Therefore this observation regarding the tox control validates the results of this assay.

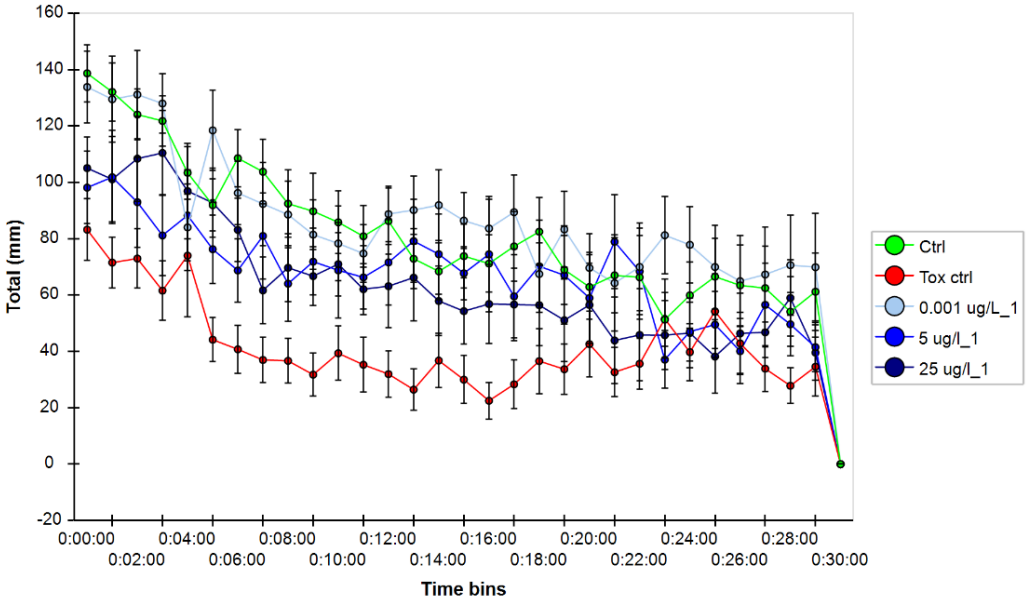


Figure 3.3: A line graph obtained directly from the EthoVision software representing the total distance moved and time (min) following 24-hrs IMI exposure

Figure 3.4 below illustrates a dose-dependent decrease in total distance moved following 24 hrs IMI exposure.

The Bonferroni ($p<0.05$) post-hoc test revealed that the tox control and the 25 µg/L treatment condition yielded a statistically significant decrease in total distance moved in comparison to the control. In contrast, despite not quite reaching statistical significance, there was a clear decrease in total distance moved in the 5 µg/L treatment condition in comparison to the control. This does suggest toxicity, and therefore the 5 µg/L treatment condition seemed to be the LOAEL dose. However, the primary objective of this toxicity assay was to determine the NOAEL of IMI.

Finally, with the postulation of the 5 µg/L dose as the LOAEL of IMI following 24-hrs exposure, it was decided to take 50% (2.5 µg/L) of the LOAEL dose as the NOAEL of IMI.

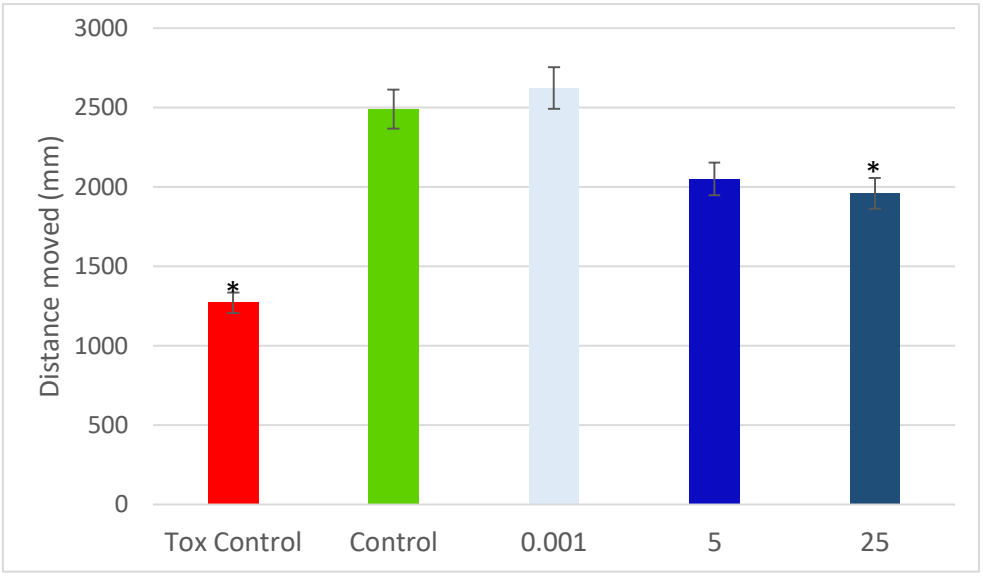


Figure 3.4: The relationship between the distance moved following 24 hrs imidacloprid exposure at various treatment conditions. ANOVA main effect of treatment, $p<0.05$. Asterisks (*) refers to the treatment conditions significantly different from the negative control group

Upon completion of the larval activity tracking using the EthoVision software, the larvae were sedated using 164 mg/L TMS by immersion. The fish were kept in the solution for approximately 2 - 3 min after which they would fall onto their side. This would allow for accurate microscopy following the 24 hrs IMI exposure. After allowing some time for the larvae to fall onto their side, the larvae were observed under the stereomicroscope to determine the survival rate as well as to identify any obvious morphological changes. The images that were taken following the 24 hrs exposure was compared to a healthy zebrafish larvae at the same stage of life that was not exposed to the pesticide at all.

As depicted in Figure 3.5 below, there were no morphological changes consistently present in all larvae following the 24 hrs IMI exposure. However, there were some minor morphological changes observed in some of the treated larvae such as a deflated swim bladder as well as oedema.

It was also noted that oedema was present at IMI concentrations as low as 5 µg/L, which suggests toxicity. However, during this study, it was particularly crucial to find the NOAEL of IMI, to which the adult zebrafish would be exposed to for 21 days. Therefore these results further support the assignment of 5 µg/L as the LOAEL of IMI in this study.

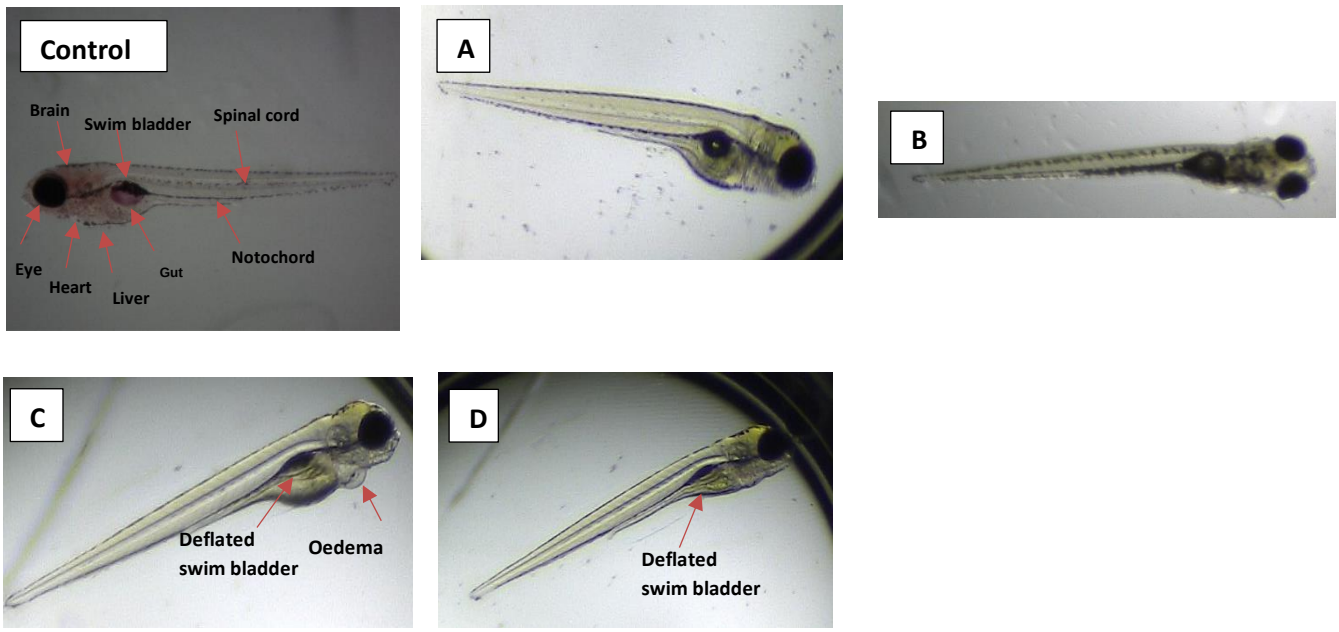


Figure 3.5: Morphological changes observed in larvae following 24-hrs IMI exposure at various concentrations. (A - 0.001 µg/L; B - 2.5 µg/L; C - 5 µg/L; D - 25 µg/L)

Following the analysis of both phenotypic and ethovision data, it was concluded that the NOAEL for IMI was 2.5 µg/L, 50% of the LOAEL of IMI. The determination of this NOAEL allowed for the start of the 21-day IMI exposure period in adult zebrafish.

3.5.3 21-day pesticide exposure period in adult zebrafish

During the 21-day IMI exposure period, a few crucial observations were made. On day four of the study, a decrease in appetite of the treatment group was observed. In addition, on numerous occasions, it was clear that the treatment group was swimming at a lower level in comparison to the control group. This was confirmed by a senior scientist in the department. This behaviour could be an indication of the fish experiencing anxiety on perceiving the presence of IMI in their water.

3.5.3.1 Body length

From the SL measurements, the average standard body length for the treated and control group was found to be 3.25 and 3.21 cm, respectively. Following statistical analysis, a t test revealed that this difference in the average SL was not significant (p=0.716).

3.5.3.2 Tissue bioaccumulation of IMI and its metabolites

We applied the developed method one in the current study to evaluate the accumulation of IMI and its metabolites in the liver, brain and gill epithelial tissue of adult zebrafish subsequent to a 21-day exposure period to low dose (NOAEL dose) IMI.

Following the LC-MS/MS analysis, it was observed that IMI was not detected in any of the tissues. The metabolites, IMI-urea and IMI-NH were also not detected in any of the tissues. However, 5-OH IMI was detected in the liver and the gill epithelial tissue. The concentration of 5-OH IMI detected in the liver and gill epithelial tissue was obtained from the LC-MS in ng/mL. This concentration was used to calculate the final concentration of 5-OH IMI in ng/mg liver and gill epithelial tissue. Refer to appendix 3 to see the detailed calculations of how this was executed. This was done for both the treated and control group.

Figure 3.6 below portrays the concentration of 5-OH IMI detected in the IMI treated group in comparison to the control group for the liver tissue. Due to the small sample size (n=1) for each group, statistical analysis could not be conducted to determine the significance. However, the fact that the treated group showed an 8-fold increase in 5-OH IMI in comparison to the control, should not be ignored and warrants more research in a larger sample size.

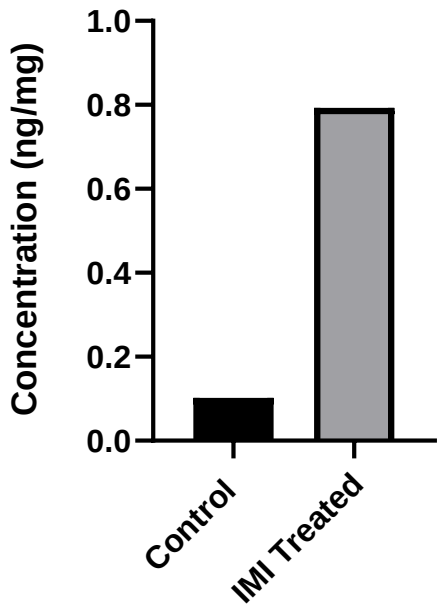


Figure 3.6: A bar graph illustrating the relationship between the IMI treated (n=1) and control (n=1) group and the concentration of 5-hydro imidacloprid detected in the liver tissue

Figure 3.7 below portrays the concentration of 5-OH IMI detected in the treated group in comparison to the control group for the gill epithelial tissue. The observed concentration in ng/mL obtained from the LC-MS is an estimate which was calculated using the peak area to ISTD peak area ratio. This estimation was necessary due to the concentration that was detected on the LC-MS being above the upper limit of quantification. In other words, it falls out of the calibration range utilised in this study.

Similarly, due to the small sample size (n=1) for each group, statistical analysis could not be conducted to determine the significance. However, the treated group showed a 14-fold increase in 5-OH IMI in comparison to the control. This observation suggests that 5-OH IMI also accumulated in the gill epithelial tissue of the adult zebrafish following IMI exposure period.

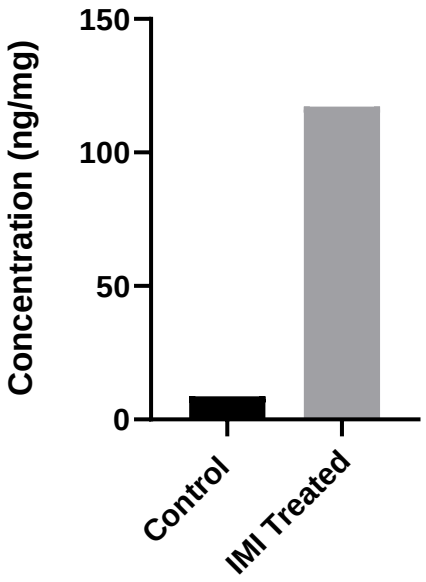


Figure 3.7: A bar graph illustrating the relationship between the IMI treated (n=1) and control (n=1) group and the concentration of 5-hydro imidacloprid in the gill epithelial tissue

3.5.3.3 Changes in neurotransmitter levels

The same brain samples that were used to assess the bioaccumulation of IMI and its metabolites were analysed on the developed method 2 to assess the likelihood of neurological risk following prolonged, low dose IMI exposure. There were three and four biological replicates for the control (n=3) and treated group (n=4) respectively.

Following the LC-MS/MS analysis, only concentrations of GABA and ACh were detected and quantified in both the treated and control group. In contrast, 5-HT and DA could not be detected due to their levels being below

the limit of quantitation of this method. Similarly, the concentrations of GABA and ACh detected in the brain tissue was obtained from the LC-MS/MS in ng/mL. This concentration was used to calculate the final concentration of GABA and ACh in ng/mg brain tissue. A concentration in ng/mg tissue for each pooled sample for the treated (n=4) and control (n=3) group was calculated. Subsequently, an average was taken to get a final concentration in ng/mg tissue for each group. Refer to appendix 4 to see the detailed calculations of how this was performed as well as the specific masses for each brain, liver and gills. Theses masses illustrate the complexity of working in such small samples.

During data analysis, an experimental error was detected. For some of the samples, a much smaller volume of zebrafish tissue homogenate was obtained, however it was not accounted for when performing the extraction as per the analytical method. This resulted in a greater dilution of the sample in comparison to the 4.5-fold dilution in the actual extraction method. This error has been accounted for in all the calculations as demonstrated in appendix 4.

Statistical analysis was performed using GraphPad Prism 8.0.2. A Shapiro-Wilk test was conducted to determine the distribution of the data. The data was found to be normally distributed. Subsequently, a parametric t-test was performed to determine statistical significance.

Figure 3.8 below portrays the concentration of ACh detected in the treated group in comparison to the control group in the brain tissue. The treated group showed a 1.4-fold decrease in ACh in comparison to the control. Despite this clear decrease in ACh in the treated group, it was found to be insignificant (p=0.47).

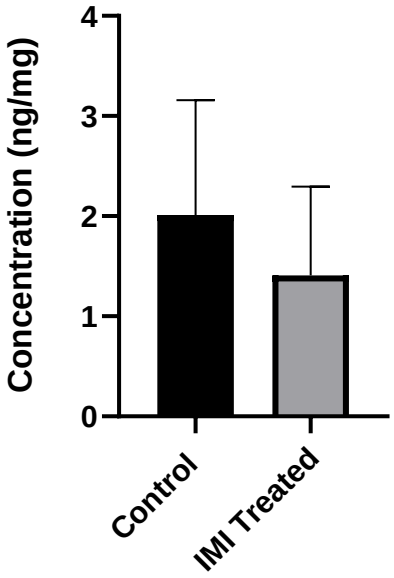


Figure 3.8: A bar graph illustrating the relationship between the IMI treated (n=4) and control group (n=3) and the concentration of acetylcholine in the brain tissue

Figure 3.9 below portrays the concentration of GABA detected in the treated group in comparison to the control group in the brain tissue. The treated group showed a 1.9-fold increase in comparison to the control. This observed increase in the treated group was found to be statistically significant following an unpaired T test (p=0.05).

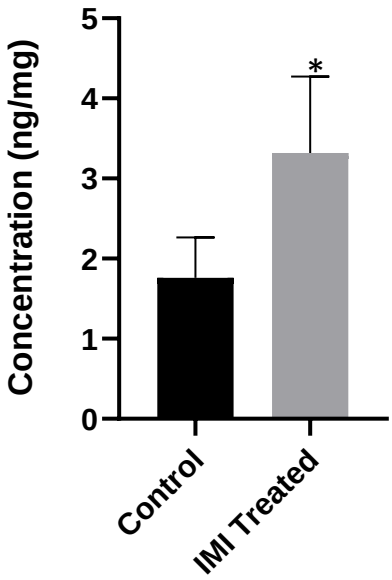


Figure 3.9: A bar graph illustrating the relationship between the IMI treated (n=4) and control (n=3) group and the concentration of GABA in the brain tissue. Asterisks (*) refers to the treatment group being significantly different from the negative control group

3.5.3.4 Detection and quantitation of IMI and its metabolites in water samples taken during the IMI exposure period

Finally, we applied the developed method 1 to detect and quantify IMI and its metabolites in water samples taken ± 2 min post-dose and 24 hrs after dosing during the IMI exposure period. A calibration curve was generated using water as a matrix in order to quantify the target analytes in the water samples. There were 5 replicates for each test group (n=5).

Statistical analysis was performed using GraphPad Prism 8.0.2. A Shapiro-Wilk test confirmed that the data is normally distributed. Subsequently, a parametric t-test was performed to determine statistical significance. In addition, descriptive statistics were performed to determine the coefficient of variation (%CV) between the individual samples in each group.

Following the LC-MS/MS analysis, only concentrations of IMI was detected and quantified in both groups. None of the metabolites of IMI were detected in the water samples. Figure 3.10 below portrays the concentration of IMI detected in the water samples obtained at two different time points during the pesticide exposure period. Based on the averages of the two groups, the water samples obtained 24 hrs post-dose

underwent a 1.1-fold decrease in comparison to the ± 2 min post-dose group. Despite this clear decrease in IMI in the 24 hrs post-dose group, it was found to be insignificant (p=0.248). It is also clear from the graph below that there is great variability between the samples in each test group. The %CV between the samples in the ± 2 min post-dose and 24 hrs post-dose group was calculated to be 25.7% and 59.6% respectively.

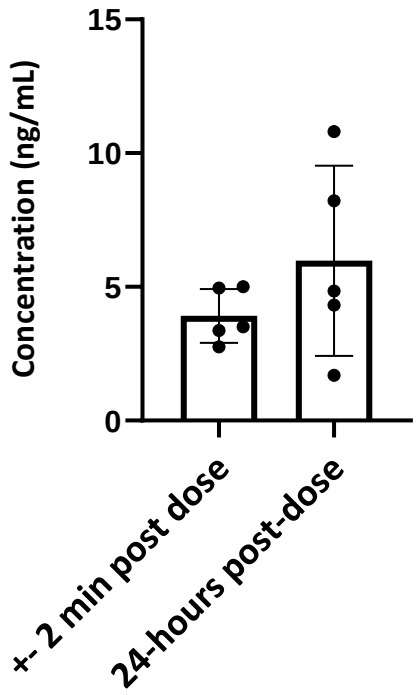


Figure 3.10: A bar graph illustrating the relationship between the concentrations of IMI in water samples obtained at two different time points during the pesticide exposure period (n=5)

3.6 Discussion and conclusion

In this chapter, we were able to determine the NOAEL of IMI by utilising an *in vivo* zebrafish larval model to investigate the possibility of general and neuro-toxicity utilising stereomicroscopy and Daniovision software, respectively. Following this determination, adult zebrafish were exposed to the NOAEL for 21 days to assess the chronic effects of low dose IMI.

Several studies have used zebrafish embryos, larvae, adolescents and adults to investigate various effects of IMI, ranging from morphological to neurological effects. These studies have reported that IMI has an effect on embryo length and mortality as well as neurobehavioral defects following developmental IMI exposure that persists into adolescence and adulthood [(130)(131)(191)]. Despite these studies' useful findings, it is critical to note that the levels of IMI to which the zebrafish were being exposed exceeded the levels that are environmentally relevant.

3.6.1 Larvae assay

In this study, successful toxicity assays were performed by exposing the zebrafish larvae at 4 - 5 dpf to IMI concentrations that could be attained in the environment [181].The behavioural outcomes of early life exposures to IMI are not well interpreted in vertebrates, regardless of its extensive use in agriculture and structural similarity to nicotine, which is generally considered as neurotoxic [23][26]. Swimming activity of the zebrafish larvae was quantified as an indication of the behavioural outcomes following 24 hrs IMI exposure.

An increase in total distance moved was observed at 0.001 µg/L IMI during the toxicity assay. This observed hyperlocomotion has been reported to be as a result of excessive stimulation of the nervous system [192]. This increased activity is also interpreted as avoidance behaviour, i.e. they detect the presence of a noxious substance in their water and try to get away from it. In contrast, similarly to nicotine, the higher concentration (25 µg/L) and the tox control resulted in a statistically significant decrease in the total distance moved [39]. According to literature, this decreased movement could serve as a stress response allowing a better chance of survival [191]. These results, together with the phenotypic observations, justified the selection of the 2.5 µg/L as the NOAEL of IMI in the zebrafish larvae

3.6.2 21-day pesticide exposure period in adult zebrafish

During this period, two crucial observations were made. Firstly, it was noted that the treatment group had a reduced appetite. Although prior studies have not focused on decreased appetite following IMI exposure, studies have reported the negative effects of IMI on the growth of various species. This cannot be directly correlated to decreased appetite, however it could be associated with a decline in feeding rate [184][193].

On the other hand, nicotine has been reported to decrease appetite and change feeding patterns [194][195]. As previously discussed, the binding of nicotine to the nAChRs opens cation channels, allowing for the entry of sodium and calcium, which sequentially results in the release of various neurotransmitters. This release by the CNS ultimately influences chemicals that suppress appetite and increase the rate of metabolism [196]. The exact mechanism through which this occurs is not completely understood. Therefore, the observation in this study regarding decreased appetite does suggest that IMI exerts similar effects at the nAChRs in adult zebrafish.

Secondly, on observation, it was clear that the treatment group would typically swim near to the bottom of the tank in comparison to the control group. This observation has also been reported by Crosby et al (2015) following IMI exposure. This response has been reported to be similar to the response following nicotine exposure [39]. According to literature, this type of swimming behaviour is related to anxiety, which is often assessed as a novel tank diving test [197][198][199][200]. This suggests that IMI can induce anxiety-like behaviours.

Generally, anxiety-like behaviours caused by chemical compounds are primarily due to disturbances of neurotransmitter systems [197][199]. As IMI is regularly detected in surface water, IMI-induced anxiety-like behaviours could affect the survival of fish in the wild resulting in increased ecological effects.

Previous studies have demonstrated that acute exposure to nicotine has anxiolytic effects, whereas chronic exposure causes anxiety-like behaviour in adult zebrafish in the novel tank diving test. The anxiolytic effects caused by nicotine have been linked to the initial stimulation of the α7 and α4β2 nicotinic receptor subtypes, although the mechanism behind the anxiogenic effects following chronic nicotine exposure remains to be clarified [200][201][202].

3.6.3 Tissue distribution/accumulation of IMI and its metabolites

To date, data regarding tissue-specific build-up of IMI and its metabolites within the mammalian system is minimal [34]. Despite the lack of research regarding bioaccumulation of IMI in organisms, it has been predominantly reported to accumulate in detectable levels in the muscle tissue in birds [203][204]. The highest level of IMI was observed in the kidney, thyroid gland and adrenals suggesting that the main route of excretion is via the renal system [26]. According to a pharmacokinetic study, IMI has a rapid excretion rate in mammals. It raises the question of whether the IMI-related toxicological outputs noticed in mammalian species are definitely caused by IMI itself or by its various breakdown products [34].

We applied the developed method 1 in the current study to evaluate the accumulation of IMI and its metabolites in the liver, brain and gill epithelial tissue of adult zebrafish subsequent to a 21-day exposure period to low dose IMI.

Prior to LC-MS/MS analysis, the various tissue specimens were weighed, homogenized and extracted as per the analytical method. During this process, a few challenges were encountered. Firstly, due to the small size of the brain, liver and gill epithelial tissue, it was deemed necessary to pool the samples resulting in a small sample size. This does result in reduced statistical power. Secondly, despite the samples being placed on ice during this process, the samples seemed to thaw rapidly thereby making it very difficult to weigh out accurately.

The parent compound, IMI, was not detected in any of the assessed tissue specimens. This finding is in line with a previously conducted study by Nimako et al (2021), who reported that IMI was not detected in the liver of exposed mice as a result of IMI's rapid metabolic breakdown within the liver. This was following a 0.6 mg/kg body weight/day of IMI exposure for 24 weeks [34]. Additionally, another study also detected very low levels of IMI in the liver of lizards following a single oral-exposure to IMI [205]. However, Nimako et al (2021), did report

the detection of IMI in the brain of the exposed mice, indicating its ability to cross the BBB [34][205]. Another study reported the detection of IMI in amphibian brain tissue following exposure to varied IMI concentrations (0, 0.1, 1, 5, and 10 µg/L) for 21 days thereby also supporting its ability to cross the BBB [206]. Despite these findings, this present study suggests that either IMI cannot cross the BBB of adult zebrafish or was detected at levels below our LLOQ and therefore could not be quantitated. Therefore, our study is in accordance with other studies which have reported on the inability of IMI to cross the BBB [90][104][207]. However, further evaluation is required to confirm this statement due to the limitations and challenges experienced in this study.

Although IMI could not be detected in any of the tissue specimens, 5-OH IMI was detected in the liver (0.793 ng/mg tissue) and gill epithelial tissue (117 ng/mg tissue). It is important to note that it has been reported that metabolites often display increased toxicity and persistence than the parent compound itself [104][208].

The liver is the organ primarily affected by contaminants as it is involved with detoxification as well as metabolism. As has been noted, the main target organ following IMI exposure in rats and dogs were reported to be the liver [3][107]. This present study's results are therefore in line with this finding as the liver in adult zebrafish also seemed to be one of the main target organs following the IMI exposure period. This detection of 5-OH IMI in the liver in this study supports the CYP450-mediated imidazolidine hydroxylation pathway as a major route of IMI breakdown, as is seen in mammalian species [26]. In addition, this observation also supports what has been reported in a study conducted by Nimako et al (2021), where low levels of 5-OH IMI were detected in the liver. This finding could be explained by the spontaneous non-metabolic transformation of 5-OH IMI into the IMI olefin breakdown product [34].

The gills are vital organs for fish and is also the main target organ to be exposed to pollutants. It has been reported that pesticides do accumulate in the gills and their concentrations indicate the pesticide level in water where fish species live [17]. Therefore this high detection of 5-OH IMI in the gill epithelial tissue does give rise to serious concerns in that 5-OH IMI has been reported to be one of the predominant breakdown products detected in humans [116]. The detection of 5-OH IMI in the control group was unexpected. At this point, we can speculate, based on the average level of IMI detected in the water samples being greater than the amount administered, that the aged tap water used as per standard husbandry procedures, may have contained IMI as contaminant (which was then metabolised to 5-OH IMI by control animals as well). This does not only validate the necessity to research the effects of pesticides but also to test the various water sources used prior to a study being conducted. Future studies should rather house the zebrafish in prepared purified water. Nevertheless, due to the concentration of 5-OH IMI in the treated group being so much higher than in the control group, the presence of this metabolite in the control group is deemed not to be significant.

Toxicologically, the inability to detect IMI-NH in any of the tissue specimens in this present study does shed some light on the concerns regarding IMI-NH. This is due to its known ability to evoke nicotinic-type effects with an increased toxicity to mammals in comparison to IMI itself. However, according to literature, IMI is transformed to IMI-NH by AOX enzymes in mice [34][72][76][98][104][108]. Therefore, the inability of detecting IMI-NH in this study could be explained by a lack of AOX enzymes in zebrafish. However, there is no literature to support this. This indicates that further research is required.

Regardless of the extensive utilisation of IMI, its detection in many human studies is fairly low in comparison to the levels of other neonicotinoids outlined in biomonitoring studies [209][210][211]. In addition, only a small amount of IMI was detected in human urine samples following a pharmacokinetic study. These findings do propose that an increased amount of IMI undergoes transformation into various metabolite forms in the human body. The results from this study are therefore in line with the hypothesis despite only being able to detect a single metabolite. Following the analysis of the concentrations detected in the tissues in the present study, it can deduced that the liver and gills have increased likelihood of cumulative effects of IMI and its metabolites following a prolonged, low-dose IMI exposure in mammalian species.

3.6.4 Changes in neurotransmitter levels

Alterations in neurotransmitter levels during development may cause improper development of the CNS and harm typical neurological functions. Neurotransmitters are well-known to be changed in various neurological disorders such as Alzheimer's disease and attention deficit hyperactivity disorder (ADHD). Behavioral alterations, via changes in the neurotransmitter system, can be caused following exposure to environmental pollutants [131].

Despite the inability to detect 5-HT and dopamine, it has been reported that IMI does play a role in the release of both these neurotransmitters [148][149][150]. However, ACh and GABA were detected and quantified in the zebrafish brain tissue homogenate. GABA was detected and quantitated in both the treated and control group at 3.32 ng/mg tissue and 1.76 ng/mg tissue, respectively. In addition, ACh was detected and quantitated in both the treated and control group at 1.47 ng/mg tissue and 2.04 ng/mg tissue, respectively.

The increase in GABA levels in the treated group in this present study contradicts what was previously reported by Abd-Elhakim et al (2018) that showed a 52% decrease in GABA in rat brain tissue following IMI exposure [129]. However, the GABA-relevant literature on this topic is controversial, as a previously conducted study reported that the activation of hippocampal nAChR subtypes by endogenous ACh may result in the up- or down-regulation of GABA release [149][212]. More research is required to clarify this issue.

The significant increase in GABA in comparison to the control obtained in this present study does raise concerns. This is due to the known fact that GABA is associated with anxiety disorders, anxiety-like behaviours, schizophrenia as well as bipolar disorders [149][213]. The decreased activity and relative unresponsiveness during the larvae toxicity assay, as well as the observation made in the treatment group during the adult pesticide exposure period, demonstrates a sedative effect. This has been reported to result from a GABA agonist [214].

Therefore this increase in GABA preliminarily suggests that the behavioral changes observed in the larvae as well as the adult zebrafish during the IMI exposure period could be due to IMI resulting in a toxicity/numbing effect.

In contrast, a decrease in ACh was detected in the treated group. This result was surprising when considering IMI's mechanism of action. An increase in ACh following IMI exposure was expected as it replaces the ACh binding sites in the nAChRs. This would ultimately result in an accumulation of IMI in the neuronal cleft. However, this decrease in ACh following IMI exposure in zebrafish has also been reported by Tufi et al (2016). Similarly, this study suggests an opposite mechanism during which ACh levels decline due to a potential rise in AChE [131]. In addition, Abou-Donia et al (2008) reported a significant increase in AChE activity in response to IMI exposure in parts of rat brains which supports the abovementioned statement [122].

Therefore the findings in this present study, despite not being significant, contributes to literature regarding the probable rise in AChE thereby resulting in a decline in ACh levels following IMI exposure. As has been noted, a decrease in ACh levels has been associated with Alzheimer's disease [88][149][155]. Moreover, compounds that change neurotransmitter signaling in the nervous system may have long–term effects in the CNS [147]. Hence, the finding in this present study does raise concerns regarding the effect of IMI on non-target nAChRs.

3.6.5 Detection and quantitation of IMI and its metabolites in water samples taken during the IMI exposure period

The results from this assay did not yield conclusive results in terms of the true concentration to which the zebrafish were being exposed during the 21-day study. To improve these results, it may be preferable to obtain water samples more regularly during the pesticide exposure period, thereby increasing the sample size.

In contrast, with regards to the degradation of IMI, these results are in line with previously conducted studies. No degradation products, such as IMI-NH and IMI-urea, could be quantified during LC-MS/MS analysis, which substantiates that IMI is relatively chemically stable at neutral conditions [125][126]. In addition, it has been reported that IMI has a half-life of longer than 30 days in water at a pH between 5 and 9 [79]. Therefore, the

result of only detecting IMI in the water samples corresponds with the statement mentioned above as the pH was kept constant between 6 and 7 during the pesticide exposure period.

Chapter 4: Concluding remarks

Robust LC-MS/MS methods were developed for the detection and quantitation of IMI, desnitro-Imidacloprid, Imidacloprid-urea and 5-hydro Imidacloprid, as well as serotonin, dopamine, acetylcholine and gamma-aminobutyric acid neurotransmitters in 200 µL zebrafish brain, liver and gill epithelial tissue homogenate.

This study was a proof of concept using adult zebrafish as an animal model to test the effects of prolonged exposure to low levels of IMI. Despite a few limitations, this study has shown that zebrafish larvae and adults are a suitable model for toxicological research.

There remains an urgent need to identify the underlying mechanisms that link the effects of prolonged IMI exposure to the effects of nicotine. However, this present study has demonstrated that zebrafish, as seen with nicotine, also display anxiogenic behaviour following prolonged IMI exposure. Overall, the behavioral effects observed during prolonged IMI exposure suggests toxicity. The strong similarity in terms of behaviour, physiology and genetics between humans and zebrafish and the global rise in neonicotinoid use increases the ecological, environmental and human health concerns following prolonged exposure to low-dose IMI-containing surface water. These health concerns are further exacerbated by the high water solubility of IMI.

It has been proposed that the extensive utilisation of IMI and its specificity towards the insect's nAChRs might make it safe for the mammalian nervous system. Nevertheless, our results display that prolonged exposure to a low dose (NOAEL dose) of IMI does have increased neurological risk in adult zebrafish.

The biggest limitation of this current study was the small sample size utilised for the final sample analysis. This resulted in decreased statistical power and reproducibility. Future work should investigate ways of increasing the sample size, while keeping the three R's as a distinct mechanism for improving the welfare of animals utilised in research and thereby helping to improve the scientific outcomes.

The reference standards of IMI and its metabolites were of high purity. However, due to the high cost of these high purity reference standards, only IMI had a deuterated ISTD (IMI-d4). The incorporation of a stable isotopically labelled analogue for IMI's metabolites in this study, especially IMI-NH, could have resulted in an improvement with regards to the robustness and sensitivity of the analytical method.

In addition to the optimisation of IMI-NH chromatography, further pre-validation experiments should be conducted for all the target analytes such as matrix effects, process efficiency as well as the stability of the compounds in the matrix. Future work should also involve further investigation of IMI's other metabolites such as 6-CNA and IMI-olefin.

The potential increase in AChE should be investigated to further contribute to the observed decrease in ACh detected in this study. Overall, our findings further contribute to existing literature and suggest that IMI does pose a threat to more than just insects and therefore requires further investigation.

References

1. Saeedi Saravi SS, Shokrzadeh M. Pesticides in the modern world - risks and benefits. InTechopen. 2011. DOI: 3–12. 10.5772/949

2. Zhang W, Jiang F. Global pesticide consumption and pollution: with China as a focus. Proc Int Acad Ecol Environ Sci. 2011; 1(2):125–44.

3. Malev O. Toxic effects of selected neonicotinoids through different organisational levels: in Vitro and in Vivo studies. Dissertation. 2012. https://www.bib.irb.hr/749544/download/749544.28Malev.pdf.

4. Damalas CA, Eleftherohorinos IG. Pesticide exposure, safety issues, and risk assessment indicators. Int J Environ Res Public Health. 2011;8(5):1402–19.

5. Adekunle, Akinbode SO, Oyekale TO, Koyi OV. Effects of agricultural pesticide utilization on farmers health in Egbeda local government area, Oyo State, Nigeria. Niger J Agric Econ. 2017;7(1):73–88.

6. Bjørling-Poulsen M, Andersen HR, Grandjean P. Potential developmental neurotoxicity of pesticides used in Europe. Environ Heal A Glob Access Sci Source. 2008; 50 (7). https://doi.org/10.1186/1476-069X-7-50

7. Blair A, Ritz B, Wesseling C, Freeman LB. Pesticides and human health. Occup Environ Med. 2015;72(2):81–2.

8. Tierney AL, Nelson CA. Brain Development and the Role of Experience in the Early Years. 2009;30(2):9–13. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23894221%0Ahttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC3722610.

9. Poliserpi MB, Cristos D, Pérez-Iglesias JM, Brodeur JC. Tissue distribution and sublethal effects of imidacloprid in the South American grayish baywing (*Agelaioides badius*). Chemosphere. 2021;284(June).

10. Grandjean P, Landrigan PJ. Neurobehavioural effects of developmental toxicity. Lancet Neurol. 2014;13(3):330–8.

11. Júlvez J, Paus T, Bellinger D, Eskenazi B, Tiemeier H, Pearce N, et al. Environment and brain development: Challenges in the global context. Neuroepidemiology. 2016;46(2):79–82.

12. Hogberg HT, Kinsner-Ovaskainen A, Hartung T, Coecke S, Bal-Price AK. Gene expression as a sensitive endpoint to evaluate cell differentiation and maturation of the developing central nervous system in primary cultures of rat cerebellar granule cells (CGCs) exposed to pesticides. Toxicol Appl Pharmacol. 2009;235(3):268–86. Available from: http://dx.doi.org/10.1016/j.taap.2008.12.014

13. Sharma A, Kumar V, Shahzad B, Tanveer M, Sidhu GPS, Handa N, et al. Worldwide pesticide usage and its impacts on ecosystem. SN Appl Sci. 2019;1(11):1–16. Available from: https://doi.org/10.1007/s42452-019-1485-1

14. Environmental Protection Agency. What is the difference between pesticides, insecticides and herbicides? Pesticide Effects on Food Production. 2010; 1–4. http://www.mendeley.com/research/1b0cceac-9e47-3a51-a91b-6044fba1732a/?utm_source=desktop&utm_medium=1.19.8&utm_campaign=open_catalog&userDocumentId=%7B0fcfc026-cc3b-447b-9296-bd539b8ca15b%7D

15. The use of plant protection products in the European Union Data 1992-2003. Eurostat statistical books. (2007) https://ec.europa.eu/eurostat/documents/3217494/5611788/KS-76-06-669-EN.PDF.

16. Tišler T, Jemec A, Mozetič B, Trebše P. Hazard identification of imidacloprid to aquatic environment. Chemosphere. 2009;76(7):907–14.

17. Muazzam B, Munawar K, Khan IA, Jahan S, Iqbal M, Asi MR, et al. Stress response and toxicity studies on zebrafish exposed to endosulfan and imidacloprid present in water. J Water Supply Res Technol- AQUA. 2019;68(8):718–30.

18. Zhang D, Zheng G, Zheng S, Guan W, Zhao W, Jia X. Assessing water quality of Nen River, the neighboring section of three provinces, using multivariate statistical analysis. J Water Supply Res Technol - AQUA. 2018;67(8):779–89.

19. Selahle SK, Mpupa A, Nomngongo PN. A review of extraction, analytical, and advanced methods for the determination of neonicotinoid insecticides in environmental water matrices. Reviews in Analytical Chemistry. 2021; 187–203. https://doi.org/10.1515/revac-2021-0134

20. Raymann K, Motta EVS, Girard C, Riddington IM, Dinser JA, Moran NA. Imidacloprid decreases honey bee survival rates but does not affect the gut microbiome. Appl Environ Microbiol. 2018;84(13).

21. Codling G, Al Naggar Y, Giesy JP, Robertson AJ. Concentrations of neonicotinoid insecticides in honey, pollen and honey bees (Apis mellifera L.) in central Saskatchewan, Canada. Chemosphere. 2016; 144:2321–8. Available from: http://dx.doi.org/10.1016/j.chemosphere.2015.10.135

22. Han W, Tian Y, Shen X. Chemosphere Human exposure to neonicotinoid insecticides and the evaluation of their potential toxicity: An overview. Chemosphere. 2018; 192:59–65. Available from: https://doi.org/10.1016/j.chemosphere.2017.10.149

23. Thompson DA, Lehmler HJ, Kolpin DW, Hladik ML, Vargo JD, Schilling KE, et al. A critical review on the potential impacts of neonicotinoid insecticide use: Current knowledge of environmental fate, toxicity, and implications for human health. Environ Sci Process Impacts. 2020;22(6):1315–46.

24. Rother HA. Pesticide vendors in the informal sector: Trading health for income. New Solut. 2016;26(2):241–52.

25. WHO. Guidelines on Public Health Pesticide Management Policy. 2010;60. Available from: http://www.who.int/whopes/resources/SEA_CD_214.pdf

26. Swenson TL. Neonicotinoid insecticide metabolism and mechanisms of toxicity in mammals. UC Berkeley Electron Theses Diss Title. 2013. https://escholarship.org/uc/item/8xw6m1kg.

27. Buszewski B, Bukowska M, Ligor M, Staneczko-Baranowska I. A holistic study of neonicotinoids neuroactive insecticides—properties, applications, occurrence, and analysis. Environ Sci Pollut Res. 2019;26(34):34723–40.

28. Bass C, Denholm I, Williamson MS, Nauen R. The global status of insect resistance to neonicotinoid insecticides. Pestic Biochem Physiol. 2015; 121:78–87. Available from: http://dx.doi.org/10.1016/j.pestbp.2015.04.004

29. Hladik ML, Main AR, Goulson D. Environmental risks and challenges associated with neonicotinoid insecticides. Environ Sci Technol. 2018; 52:3329–35.

30. Loser D, Hinoj, Osa MG, Blum J, Schaefer J, Brüll M, et al. Functional alterations by a subgroup of neonicotinoid pesticides in human dopaminergic neurons. Arch Toxicol. 2021;95(6):2081–107. Available from: https://doi.org/10.1007/s00204-021-03031-1

31. Sano K, Isobe T, Yang J, Win-Shwe TT, Yoshikane M, Nakayama SF, et al. In utero and lactational exposure to acetamiprid induces abnormalities in socio-sexual and anxiety-related behaviors of male mice. Front Neurosci. 2016;10(JUN):1–12.

32. Borsuah JF, Messer TL, Snow DD, Comfort SD, Mittelstet AR. Literature review: Global neonicotinoid insecticide occurrence in aquatic environments. Water (Switzerland). 2020;12(12):1–17.

33. Radolinski J, Wu J, Xia K, Hession WC, Stewart RD. Plants mediate precipitation-driven transport of a neonicotinoid pesticide. Chemosphere. 2019; 222:445–52. Available from: https://doi.org/10.1016/j.chemosphere.2019.01.150

34. Nimako C, Ikenaka Y, Akoto O, Fujioka K, Taira K, Arizono K, et al. Simultaneous quantification of imidacloprid and its metabolites in tissues of mice upon chronic low-dose administration of imidacloprid. J Chromatogr A. 2021; DOI: 1652. 10.1016/j.chroma.2021.462350

35. Cimino AM, Boyles AL, Thayer KA, Perry MJ. Review effects of neonicotinoid pesticide exposure on human health: A systematic review. Environmental Health Perspectives. 2017;125(2):155–62. http://dx.doi.org/10.1289/EHP515

36. Gonçalves ÍFS, Souza TM, Vieira LR, Marchi FC, Nascimento AP, Farias DF. Toxicity testing of pesticides in zebrafish—a systematic review on chemicals and associated toxicological endpoints. Environ Sci Pollut Res. 2020;27(10):10185–204.

37. Zon LI, Peterson RT. In vivo drug discovery in the zebrafish. Nat Rev Drug Discov. 2005;4(1):35–44.

38. Bailey J, Oliveri A, Levin ED. Zebraish model systems for developmental neurobehavioural toxicology. Embryo Today Rev. 2014;99(1):14–23.

39. Crosby EB, Bailey JM, Oliveri AN, Levin ED. Neurobehavioral impairments caused by developmental imidacloprid exposure in zebrafish. Neurotoxicol Teratol. 2015; 49:81–90. Available from: http://dx.doi.org/10.1016/j.ntt.2015.04.006

40. Singleman C, Holtzman NG. Growth and maturation in the zebrafish, Danio Rerio: A staging tool for teaching and research. Zebrafish. 2014;11(4):396–406.

41. Santoriello C, Zon LI, Santoriello C, Zon LI. Modeling human disease in zebrafish. J Clin Invest. 2012;122(7):2337–43.

42. Cassar S, Adatto I, Freeman JL, Gamse JT, Iturria I, Lawrence C, et al. Use of zebrafish in drug discovery toxicology. Chem Res Toxicol. 2020;33(1):95–118.

43. Vliegenthart ADB, Tucker CS, Pozo J Del, Dear JW. Zebrafish as model organisms for studying drug-induced liver injury. Br J Clin Pharmacol. 2014;78(6):1217–27.

44. Flores EM, Nguyen AT, Odem MA, Eisenhoffer GT, Krachler AM. The zebrafish as a model for gastrointestinal tract–microbe interactions. Cell Microbiol. 2020;22(3).

45. Hill AJ, Teraoka H, Heideman W, Peterson RE. Zebrafish as a model vertebrate for investigating chemical toxicity. Toxicol Sci. 2005;86(1):6–19.

46. Richardson R, Webster A, Moosajee M. The zebrafish eye - a paradigm for investigating human ocular genetics. Nat Publ Gr. 2017; 31:68–86.

47. Horzmann KA, Freeman JL. Zebrafish Get Connected: Investigating neurotransmission targets and alterations in chemical toxicity. Toxics. 2016;4(3):19.

48. MacRae CA, Peterson RT. Zebrafish as tools for drug discovery. Nat Rev Drug Discov. 2015;14(10):721–31. Available from: http://dx.doi.org/10.1038/nrd4627

49. Levin ED, Limpuangthip J. Timing of nicotine effects on learning in zebrafish. Psychopharmacology (Berl). 2006; 184:547–52.

50. Loerracher AK, Grethlein M, Braunbeck T. In vivo fluorescence-based characterization of cytochrome P450 activity during embryonic development of zebrafish (*Danio rerio*). Ecotoxicol Environ Saf. 2020;192 (November 2019):110330. Available from: https://doi.org/10.1016/j.ecoenv.2020.110330

51. Garcia GR, Noyes PD, Tanguay RL. Advancements in zebrafish applications for 21st century toxicology. Pharmacol Ther. 2016; 161:11–21.

52. Panula P, Chen Y, Priyadarshini M, Kudo H, Semenova S, Sundvik M, et al. The comparative neuroanatomy and neurochemistry of zebrafish CNS systems of relevance to human neuropsychiatric diseases. Neurobiol Dis. 2010;40(1):46–57. Available from: http://dx.doi.org/10.1016/j.nbd.2010.05.010

53. Christou M, Kavaliauskis A, Ropstad E, Fraser TWK. DMSO effects larval zebrafish (*Danio rerio*) behavior, with additive and interaction effects when combined with positive controls. Sci Total Environ. 2020; 709:134490. Available from: https://doi.org/10.1016/j.scitotenv.2019.134490

54. Kozol RA, Abrams AJ, James DM, Buglo E, Yan Q. Function Over Form: Modeling groups of inherited neurological conditions in function over form: Modeling groups of inherited neurological conditions in Zebrafish. Frontier. 2016;9(55).

55. Ganz J, Kroehne V, Freudenreich D, Machate A, Geffarth M, Braasch I, et al. Subdivisions of the adult zebrafish pallium based on molecular marker analysis. F1000 Res. 2015;308(3):1–20.

56. Filippi A, Mueller T, Driever W. vglut2 and gad expression reveal distinct patterns of dual GABAergic versus glutamatergic cotransmitter phenotypes of dopaminergic and noradrenergic neurons in the zebrafish brain. 2019;2037(2014):2019–37.

57. Maximino C, Gomes M, Renata K, Oliveira M, Jesus E De, Batista O, et al. ‘“Limbic associative”’ and ‘“autonomic”’ amygdala in teleosts: A review of the evidence. J Chem Neuroanat. 2013;48–49:1–13. Available from: http://dx.doi.org/10.1016/j.jchemneu.2012.10.001

58. Gutzman JH, Graeden EG, Anne L, Holley HS, Sive H. Formation of the zebrafish midbrain – hindbrain boundary constriction requires laminin-dependent basal constriction. Mech Dev. 2008;125(11–12):974–83. Available from: http://dx.doi.org/10.1016/j.mod.2008.07.004

59. Jeong J, Kwon H, Ahn J, Kang D, Kwon S, Ae J, et al. Functional and developmental analysis of the blood–brain barrier in zebrafish. Brain Res Bull. 2008; 75:619–28.

60. Li Y, Chen T, Miao X, Yi X, Wang X, Zhao H, et al. Zebrafish: A promising in vivo model for assessing the delivery of natural products, fluorescence dyes and drugs across the blood-brain barrier. Pharmacol Res. 2017; 125:246–57.

61. Buss DS, Callaghan A. Interaction of pesticides with p-glycoprotein and other ABC proteins: A survey of the possible importance to insecticide, herbicide and fungicide resistance. Pestic Biochem Physiol. 2008;90(3):141–53.

62. de Abreu MS, Genario R, Giacomini ACVV, Demin KA, Lakstygal AM, Amstislavskaya TG, et al. Zebrafish as a model of neurodevelopmental disorders. Neuroscience. 2020; 445:3–11. Available from: https://doi.org/10.1016/j.neuroscience.2019.08.034

63. Rico EP, Rosemberg DB, Seibt KJ, Capiotti KM, Silva RS Da, Bonan CD. Neurotoxicology and teratology zebrafish neurotransmitter systems as potential pharmacological and toxicological targets. Neurotoxicol Teratol. 2011;33(6):608–17. Available from: http://dx.doi.org/10.1016/j.ntt.2011.07.007

64. Rima M, Lattouf Y, Abi Younes M, Bullier E, Legendre P, Mangin JM, et al. Dynamic regulation of the cholinergic system in the spinal central nervous system. Sci Rep. 2020;10(1):1–13. Available from: https://doi.org/10.1038/s41598-020-72524-3

65. Seredick SD, Ryswyk L Van, Hutchinson SA, Eisen JS. Zebrafish Mnx proteins specify one motoneuron subtype and suppress acquisition of interneuron characteristics. Neural Dev. 2012;7(35):1–14.

66. Arenzana FJ, Clemente D, Rosario S. Development of the cholinergic system in the brain and retina of the zebrafish. Brain Res Bull. 2005; 66:421–5.

67. Goldstone J V, Mcarthur AG, Kubota A, Zanette J, Parente T, Jönsson ME, et al. Identification and developmental expression of the full complement of Cytochrome P450 genes in Zebrafish. BMC Genomics. 2010;11(643):1471–2164.

68. Horzmann KA, Freeman JL. Making waves: New developments in toxicology with the zebrafish. Soc Toxicol. 2018;163(1):5–12.

69. Basnet RM, Zizioli D, Taweedet S, Finazzi D, Memo M. Zebrafish larvae as a behavioral model in neuropharmacology. Biomedicines. 2019;7(1).

70. Kumar A, Verma A, Kumar A. Accidental human poisoning with a neonicotinoid insecticide, imidacloprid: A rare case report from rural India with a brief review of literature. Egypt J Forensic Sci. 2013;3(4):123–6. Available from: http://dx.doi.org/10.1016/j.ejfs.2013.05.002

71. Tomizawa M, Casida JE. Neonicotinoid insecticide toxicology: Mechanisms of selective action. Annu Rev Pharmacol Toxicol. 2005; 45:247–68.

72. Simon-Delso N, Amaral-Rogers V, Belzunces LP, Bonmatin JM, Chagnon M, Downs C, et al. Systemic insecticides (Neonicotinoids and fipronil): Trends, uses, mode of action and metabolites. Environ Sci Pollut Res. 2015;22(1):5–34.

73. Rogers KH, Mcmillin S, Olstad KJ, Poppenga RH. Imidacloprid poisoning of Songbirds following a drench application of trees in a residential neighborhood in California, USA. Environ Toxicol Chem. 2019; 38(8):1724–7. Available from: http://dx.doi.org/10.1002/etc.4473

74. PubChem. Compound summary - Imidacloprid. 2021. 1–55. 10.5517/cc84zhv. https://pubchem.ncbi.nlm.nih.gov/compound/Imidacloprid

75. Sun Q, Xiao X, Kim Y, Kim D, Yoon KS, Clark JM, et al. Imidacloprid promotes high fat diet-induced adiposity and insulin resistance in male C57BL/6J Mice. J Agric Food Chem. 2016;64(49):9293–306.

76. Cimino AM, Boyles AL, Thayer KA, Perry MJ. Effects of neonicotinoid pesticide exposure on human health: A systematic review. Environ Health Perspect. 2017;125(2):155–62.

77. Goulson D. An overview of the environmental risks posed by neonicotinoid insecticides. J Appl Ecol. 2013;50(4):977–87.

78. Junquera, P. Imidacloprid toxicological sheet for veterinary use in dogs, cats and livestock: poisoning, overdose, symptoms, tolerance, safety margin, antidote. Parasitipedia. 2018. IMIDACLOPRID TOXICITY, POISONING, INTOXICATION, ANTIDOTE, TOXICITY. Safety Summary for VETERINARY USE on Dogs and Cats (parasitipedia.net)

79. Wang Y, Han Y, Xu P, Guo B, Li W, Wang X. The metabolism, distribution and effect of imidacloprid in chinese lizards (*Eremias argus*) following oral exposure. Ecotoxicol Environ Saf. 2018;165(September):476–83. Available from: https://doi.org/10.1016/j.ecoenv.2018.09.036

80. Liu W, Zheng W, Ma Y, Liu K. Sorption and degradation of imidacloprid in soil and water. J Environ Sci Heal - Part B Pestic Food Contam Agric Wastes. 2006;41(5):623–34.

81. Akoijam R, Singh B. Metabolic degradation of imidacloprid in paddy field soil. Environ Monit Assess. 2014;186(10):5977–84.

82. Zhang Q, Li Z, Chang CH, Lou JL, Zhao MR, Lu C. Potential human exposures to neonicotinoid insecticides: A review. Environmental Pollution. 2018; 236: 71-81. https://doi.org/10.1016/j.envpol.2017.12.101

83. Chen M, Tao L, McLean J, Lu C. Quantitative analysis of neonicotinoid insecticide residues in foods: Implication for dietary exposures. J Agric Food Chem. 2014;62(26):6082–90.

84. Iyyadurai R, George IA, Peter JV. Imidacloprid poisoning-newer insecticide and fatal toxicity. J Med Toxicol. 2010;6(1):77–8.

85. Viradiya K, Mishra A. Imidacloprid poisoning. J Assoc Physicians India. 2011;59(9):594–5.

86. Chemical Watch Factsheet. 1994; 20003(202):200–2. https://www.beyondpesticides.org/assets/media/documents/pesticides/factsheets/Imidacloprid.pdf

87. Rogers KH, McMillin S, Olstad KJ, Poppenga RH. Imidacloprid poisoning of songbirds following a drench application of trees in a residential neighborhood in california, USA. Environ Toxicol Chem. 2019;38(8):1724–7. Available from: http://dx.doi.org/10.1002/etc.4473

88. Rodrigues KJA, Santana MB, Do Nascimento JLM, Picanço-Diniz DLW, Maués LAL, Santos SN, et al. Behavioral and biochemical effects of neonicotinoid thiamethoxam on the cholinergic system in rats. Ecotoxicol Environ Saf. 2010;73(1):101–7.

89. Tomizawa M, Casida JE. Selective toxicity of neonicotinoids attributable to specificity of insect and mammalian nicotinic receptors. Annu Rev Entomol. 2003; 48:339–64.

90. Sheets LP. Imidacloprid: A Neonicotinoid Insecticide. Hayes' Handbook of Pesticide Toxicology. Chapter 95. 2010; 2055–64.

91. Kimura-Kuroda J, Komuta Y, Kuroda Y, Hayashi M, Kawano H. Nicotine-like effects of the neonicotinoid insecticides acetamiprid and imidacloprid on cerebellar neurons from neonatal rats. PLoS One. 2012;7(2).

92. Nagata K, Iwanaga Y, Shono T, Narahashi T. Modulation of the neuronal nicotinic acetylcholine receptor channel by imidacloprid and cartap. Pestic Biochem Physiol. 1997;59(2):119–28.

93. Li P, Ann J, Akk G. Activation and modulation of human α4β2 nicotinic acetylcholine receptors by the neonicotinoids clothianidin and imidacloprid. J Neurosci Res. 2014;23(1):1–7. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3624763/pdf/nihms412728.pdf

94. Berg DK, Conroy WG. Nicotinic alpha-7 Receptors: Synaptic options and downstream signaling in neurons. J Neurobiol. 2002;53(4):512–23.

95. Chang KT, Berg DK. Voltage-gated channels block nicotinic regulation of CREB phosphorylation and gene expression in neurons. Neuron. 2001; 32:855–65.

96. Lindstrom J. Autoimmune diseases involving nicotinic receptors. J Neurobiol. 2002;53(4):656–65.

97. Parrón T, Requena M, Hernández AF, Alarcón R. Association between environmental exposure to pesticides and neurodegenerative diseases. Toxicol Appl Pharmacol. 2011;256(3):379–85. Available from: http://dx.doi.org/10.1016/j.taap.2011.05.006

98. Honda H, Tomizawa M, Casida JE. Neonicotinoid metabolic activation and inactivation established with coupled nicotinic receptor-CYP3A4 and -aldehyde oxidase systems. Toxicol Lett. 2006; 161:108–14.

99. Sheets LP, Li AA, Minnema DJ, Collier RH, Creek MR, Peffer RC. A critical review of neonicotinoid insecticides for developmental neurotoxicity. Crit Rev Toxicol. 2016;46(2):153–90.

100. Mesnage R, Biserni M, Genkova D, Wesolowski L, Antoniou MN. Evaluation of neonicotinoid insecticides for oestrogenic, thyroidogenic and adipogenic activity reveals imidacloprid causes lipid accumulation. J Appl Toxicol. 2018;38(12):1483–91.

101. Nakayama A. The neonicotinoids, acetamiprid and imidacloprid impair neurogenesis and alter the microglial profile in the hippocampal dentate gyrus of mouse neonates. J Appl Toxicol. 2019; 39:877–87.

102. Kanne DB, Dick RA, Tomizawa M, Casida JE. Neonicotinoid nitroguanidine insecticide metabolites: Synthesis and nicotinic receptor potency of guanidines, aminoguanidines, and their derivatives. Chem Res Toxicol. 2005;18(9):1479–84.

103. Houchat JN, Cartereau A, Le Mauff A, Taillebois E, Thany SH. An overview on the effect of neonicotinoid insecticides on mammalian cholinergic functions through the activation of neuronal nicotinic acetylcholine receptors. Int J Environ Res Public Health. 2020;17(9).

104. Thompson DA, Lehmler H, Kolpin DW, Hladik ML, Vargo JD, Schilling KE, et al. Environmental Science Processes & Impacts. A critical review on the potential impacts of neonicotinoid insecticide use : current knowledge of environmental fate, toxicity, and implications for human health. R Soc Chem. 2020; 22:1315–46.

105. Li P, Ann J, Akk G. Activation and modulation of human α4β2 nicotinic acetylcholine receptors by the neonicotinoids clothianidin and imidacloprid. J Neurosci Res. 2011;89(8):1295–301.

106. de Oliveira IM, Nunes BVF, Barbosa DR, Pallares AM, Faro LRF. Effects of the neonicotinoids thiametoxam and clothianidin on in vivo dopamine release in rat striatum. Toxicol Lett. 2010;192(3):294–7.

107. Svetlana E. Risk Characterisation document: Dietary and drinking water exposure. California Environmental Protection Agency. Department of Pesticide Regulation. 2006; (2). https://www.cdpr.ca.gov/docs/risk/rcd/imidacloprid.pdf

108. Parkinson RH, Gray JR. Neural conduction, visual motion detection, and insect flight behaviour are disrupted by low doses of imidacloprid and its metabolites. Neurotoxicology. 2019;72(September 2018):107–13. Available from: https://doi.org/10.1016/j.neuro.2019.02.012

109. Kubota A, Kawai YK, Yamashita N, Lee JS, Kondoh D. Transcriptional profiling of cytochrome P450 genes in the liver of adult zebrafish, *Danio rerio*. J Toxicol Sci. 2019;44(5):347–56.

110. Khidkhan K, Ikenaka Y, Ichise T, Nakayama SMM, Mizukawa H, Nomiyama K, et al. Comparative Biochemistry and Physiology, Part C Interspecies differences in cytochrome P450-mediated metabolism of neonicotinoids among cats, dogs, rats, and humans. Comp Biochem Physiol Part C. 2021;239(October 2020):108898. Available from: https://doi.org/10.1016/j.cbpc.2020.108898

111. Sousa T, Castro RE, Coutinho A, Rodrigues CMP, Prieto M, Fernandes F. Experimental cholestasis research. Methods Mol Biol. 2019; 1981:273–89. Available from: http://link.springer.com/10.1007/978-1-4939-9420-5

112. Schulz-jander DA, Leimkuehler WM, Casida JE. Chemical Research in Toxicology. Neonicotinoid Insecticides: Reduction and cleavage of Imidacloprid nitroimine substituent by liver microsomal and cytosolic enzymes. Toxicol Lett. 2002; 450:1158–65.

113. Cardone A. Imidacloprid induces morphological and molecular damages on testis of lizard (*Podarcis sicula*). Ecotoxicology. 2015;94–105.

114. Schulz-jander DA, Casida JE. Imidacloprid insecticide metabolism: human cytochrome P450 isozymes differ in selectivity for imidazolidine oxidation versus nitroimine reduction. Toxicol Lett. 2002; 132:65–70.

115. Harada KH, Tanaka K, Sakamoto H, Imanaka M, Niisoe T. Biological monitoring of human exposure to neonicotinoids using urine samples, and neonicotinoid excretion kinetics. PLoS One. 2016;1–16.

116. Fuke C, Nagai T, Ninomiya K, Fukasawa M, Ihama Y, Miyazaki T. Detection of imidacloprid in biological fluids in a case of fatal insecticide intoxication. Leg Med. 2014;16(1):40–3. Available from: http://dx.doi.org/10.1016/j.legalmed.2013.10.007

117. Cartereau A, Martin C, Thany SH. Neonicotinoid insecticides differently modulate acetycholine-induced currents on mammalian α7 nicotinic acetylcholine receptors. Br J Pharmacol. 2018;175(11):1987–98.

118. Gotti C, Clementi F, Fornari A, Gaimarri A, Guiducci S, Manfredi I, et al. Structural and functional diversity of native brain neuronal nicotinic receptors. Biochem Pharmacol. 2009; 78:703–11.

119. De Kloet SF, Mansvelder HD, De Vries TJ. Cholinergic modulation of dopamine pathways through nicotinic acetylcholine receptors. Biochem Pharmacol. 2015;97(4):425–38. Available from: http://dx.doi.org/10.1016/j.bcp.2015.07.014

120. Chen Y, Broad LM, Phillips KG, Zwart R. Partial agonists for α4β2 nicotinic receptors stimulate dopaminergic neuron firing with relatively enhanced maximal effects. Br J Pharmacol. 2012;165(4):1006–16.

121. Kara M, Yumrutas O, Demir CF, Ozdemir HH, Bozgeyik I, Coskun S, et al. Insecticide imidacloprid influences cognitive functions and alters learning performance and related gene expression in a rat model. Int J Exp Pathol. 2015;96(5):332–7.

122. Abou-Donia MB, Goldstein LB, Bullman S, Tu T, Khan WA, Dechkovskaia AM, et al. Imidacloprid induces neurobehavioral deficits and increases expression of glial fibrillary acidic protein in the motor cortex and hippocampus in offspring rats following in utero exposure. J Toxicol Environ Heal - Part A Curr Issues. 2008;71(2):119–30.

123. Melroy-Greif E, Stitzel A, Ehringer A. Nicotinic acetylcholine receptors: upregulation, age-related effects, and associations with drug use. Physiol Behav. 2016;176(1):100–106.

124. Casida JE. Neonicotinoid metabolism: Compounds, substituents, pathways, enzymes, organisms, and relevance. J Agric Food Chem. 2011;59(7):2923–31.

125. Pang S, Lin Z, Zhang Y, Zhang W, Alansary N, Mishra S, et al. Insights into the toxicity and degradation mechanisms of Imidacloprid via physicochemical and microbial approaches. Toxics. 2020;8(3):65.

126. Chen S, Deng J, Deng Y, Gao N. Influencing factors and kinetic studies of imidacloprid degradation by ozonation. Environ Technol (United Kingdom). 2019;40(16):2127–34. Available from: https://doi.org/10.1080/09593330.2018.1439105

127. Bonmatin JM, Giorio C, Girolami V, Goulson D, Kreutzweiser DP, Krupke C, et al. Environmental fate and exposure; neonicotinoids and fipronil. Environ Sci Pollut Res. 2015;22(1):35–67.

128. Dwyer JB, Mcquown SC, Leslie FM. The dynamic effects of nicotine on the developing brain. Pharmacol Ther. 2010;122(2):125–39.

129. Abd-Elhakim YM, Mohammed HH, Mohamed WAM. Imidacloprid impacts on neurobehavioral performance, oxidative stress, and apoptotic events in the brain of adolescent and adult rats. J Agric Food Chem. 2018;66(51):13513–24.

130. Krishnan A, Clyburn C, and Newcombe P. Developmental effects of the pesticide Imidacloprid on zebrafish body length and mortality, Washington DC. Europe PMC. 2018; 1–15. 10.1101/395327

131. Tufi S, Leonards P, Lamoree M, De Boer J, Legler J, Legradi J. Changes in neurotransmitter profiles during early zebrafish (Danio rerio) development and after pesticide exposure. Environ Sci Technol. 2016;50(6):3222–30.

132. Duzguner V, Erdogan S. Acute oxidant and inflammatory effects of imidacloprid on the mammalian central nervous system and liver in rats. Pestic Biochem Physiol. 2010;97(1):13–8. Available from: http://dx.doi.org/10.1016/j.pestbp.2009.11.008

133. Brandhonneur N, De Sousa Mendes M, Lepvrier E, Flejou Esseiva E, Chevanne F, Le Corre P. A micro-QuEChERS method coupled to GC-MS for the quantification of pesticides in specific maternal and fetal tissues. J Pharm Biomed Anal. 2015; 104:90–6. Available from: http://dx.doi.org/10.1016/j.jpba.2014.10.035

134. Tiya LL. Development of toxicology screening methods for common poisoning agents in South Africa. Dissertation. https://scholar.sun.ac.za/handle/10019.1/109098

135. Li W, Tse FLS. Special issue: Review dried blood spot sampling in combination with LC-MS/MS for quantitative analysis of small molecules. Biomed Chromatogr. 2010;24(October 2009):49–65.

136. Aga DS. Analytical Methods analysis of hormones and pesticides in surface water. R Soc Chem. 2019; 11:1436–48.

137. Sveshnikova N, Yuan T, Warren JM, Piercey-Normore MD. Development and validation of a reliable LC-MS/MS method for quantitative analysis of usnic acid in *Cladonia uncialis*. BMC Res Notes. 2019;12(1):1–6. Available from: https://doi.org/10.1186/s13104-019-4580-x

138. Pang B, Zhu Y, Lu L, Gu F, Chen H. The applications and features of liquid chromatography-mass spectrometry in the analysis of traditional Chinese medicine. Hindawi. (2016) http://dx.doi.org/10.1155/2016/3837270.

139. Boja ES, Rodriguez H. The path to clinical proteomics research: Integration of proteomics, genomics, clinical laboratory and regulatory science. Korean J Lab Med. 2011;31(2):61–71.

140. Kavvalakis MP, Tzatzarakis MN, Theodoropoulou EP, Barbounis EG, Tsakalof AK, Tsatsakis AM. Development and application of LC-APCI-MS method for biomonitoring of animal and human exposure to

imidacloprid. Chemosphere. 2013;93(10):2612–20. Available from: http://dx.doi.org/10.1016/j.chemosphere.2013.09.087

141. Seifrtova M, Halesova T, Sulcova K, Riddellova K, Erban T. Distributions of imidacloprid, imidacloprid-olefin and imidacloprid-urea in green plant tissues and roots of rapeseed (Brassica napus) from artificially contaminated potting soil. Pest Manag Sci. 2017;73(5):1010–6.

142. Raina-Fulton R, Behdarvandan A. Liquid chromatography-mass spectrometry for the determination of neonicotinoid insecticides and their metabolites in biological, environmental and food commodity matrices. Trends Chromatogr. 2006; 2:1–29.

143. Gruyter DE. Analytical techniques for trace element determination. Phys Sci Rev. 2017;1–14.

144. Kapoor U, Srivastava MK, Trivedi P, Garg V, Srivastava LP. Disposition and acute toxicity of imidacloprid in female rats after single exposure. Food Chem Toxicol. 2014; 68:190–5. Available from: http://dx.doi.org/10.1016/j.fct.2014.03.019

145. Gao B, Poma G, Malarvannan G, Dumitrascu C, Bastiaensen M, Wang M, et al. Development of an analytical method based on solid-phase extraction and LC-MS/MS for the monitoring of current-use pesticides and their metabolites in human urine. J Environ Sci. 2022; 111:153–63. Available from: https://doi.org/10.1016/j.jes.2021.03.029

146. Yang Q, Ai X, Dong J, Liu Y, Zhou S, et al. A QuEChERS-HPLC-MS/MS method with matrix matching calibration strategy for determination of Imidacloprid and Its metabolites in *Procambarus clarkii* (Crayfish) tissues. Molecules. 2021;26(274).

147. Tufi S, Lamoree M, de Boer J, Leonards P. Simultaneous analysis of multiple neurotransmitters by hydrophilic interaction liquid chromatography coupled to tandem mass spectrometry. J Chromatogr A. 2015; 1395:79–87. Available from: http://dx.doi.org/10.1016/j.chroma.2015.03.056

148. Plenis A, Olędzka I, Kowalski P, Miękus N, Bączek T. Recent trends in the quantification of biogenic amines in biofluids as biomarkers of various disorders: A Review. J Clin Med. 2019;8(5):640.

149. Castro V, Valenzuela C, Sanchez J, Pena K, Perez S, Ibarra J, et al. An update of the classical and novel methods used for measuring fast neurotransmitters during normal and brain altered function. Curr Neuropharmacol. 2014;12(6):490–508.

150. Kenny PJ, File SE, Neal MJ. Evidence for a complex influence of nicotinic acetylcholine receptors on hippocampal serotonin release. Journal of Neurochemistry. 2000;2–7.

151. Benazzouz A, Mamad O, Abedi P, Bouali-benazzouz R. Involvement of dopamine loss in extrastriatal basal ganglia nuclei in the pathophysiology of Parkinson's disease. Frontiers in Aging Neuroscience. 2014; 6(May):1–5.

152. Kesby JP, Eyles DW, McGrath JJ, Scott JG. Dopamine, psychosis and schizophrenia: The widening gap between basic and clinical neuroscience. Transl Psychiatry. 2018;8(1). Available from: http://dx.doi.org/10.1038/s41398-017-0071-9

153. Sarlo GL, Holton KF. Brain concentrations of glutamate and GABA in human epilepsy: A review. Seizure. 2021;91(June):213–27. Available from: https://doi.org/10.1016/j.seizure.2021.06.028

154. Stuart A, Reviewed M, Ratini M. GABA (Gamma-Aminobutyric Acid). WebMD. 2021;22–5. https://www.webmd.com/vitamins-and-supplements/gaba-uses-and-risks. (Date accessed: 24 October 2022)

155. Rogers, K. Acetycholine. Britannica Online Encyclopedia. 2021. Available from: https://www.britannica.com/science/acetylcholine. (Date accessed: 29 April 2021)

156. McIntosh J. What to know about eczema. MedicalNewsToday. 2021;1–10. Available from: https://www.medicalnewstoday.com/articles/14417. (Date accessed: 24 October 2022)

157. Mashour GA. Acetylcholine: Working on working memory. Sci Transl Med. 2011;3(114):1510–2.

158. Santos-Fandila A, Vázquez E, Barranco A, Zafra-Gómez A, Navalón A, Rueda R, et al. Analysis of 17 neurotransmitters, metabolites and precursors in zebrafish through the life cycle using ultrahigh performance liquid chromatography-tandem mass spectrometry. J Chromatogr B Anal Technol Biomed Life Sci. 2015; 1001:191–201. Available from: http://dx.doi.org/10.1016/j.jchromb.2015.07.040

159. Aragon A, Legradi J, Ballesteros-Gómez A, Legler J, van Velzen M, de Boer J, et al. Determination of monoamine neurotransmitters in zebrafish (Danio rerio) by gas chromatography coupled to mass spectrometry with a two-step derivatization. Anal Bioanal Chem. 2017;409(11):2931–9.

160. Rauh M. LC–MS/MS for protein and peptide quantification in clinical chemistry. J Chromatogr B. 2012;883–884:59–67. Available from: http://dx.doi.org/10.1016/j.jchromb.2011.09.030

161. Kohler I. Multi-target screening of biological samples using LC–MS/MS: focus on chromatographic innovations. Bioanalysis. 2014; 6:1255–73.

162. González RR, Fernández RF, Luis J, Vidal M, Frenich AG, Luz M, et al. Development and validation of an ultra-high performance liquid chromatography–tandem mass-spectrometry (UHPLC–MS/MS) method for the simultaneous determination of neurotransmitters in rat brain samples. J Neurosci Methods. 2011; 198:187–94.

163. Li XS, Li S, Kellermann G. Simultaneous extraction and determination of monoamine neurotransmitters in human urine for clinical routine testing based on a dual functional solid phase extraction assisted by phenylboronic acid coupled with liquid chromatography-tandem mass spectromet. Anal Bioanal Chem. 2017;409(11):2859–71.

164. Eckstein JA, Ammerman GM, Reveles JM, Ackermann BL. Analysis of glutamine, glutamate, pyroglutamate, and GABA in cerebrospinal fluid using ion pairing HPLC with positive electrospray LC/MS/MS. J Neurosci Methods. 2008;171(2):190–6.

165. Han XM, Qin YJ, Zhu Y, Zhang XL, Wang NX, and Rang Y, et al. Development of an underivatized LC-MS/MS method for quantitation of 14 neurotransmitters in rat hippocampus, plasma and urine: Application to CUMS induced depression rats. J Pharm Biomed Anal. 2019; 174:683–95. Available from: https://doi.org/10.1016/j.jpba.2019.06.043

166. B. Subia JK and SCK. Linearity of calibration curves for analytical methods: A review of criteria for assessment of method reliability. InTechopen. 2010; 524:141–57.

167. U.S. Department of Health and Human Services Food and Drug Administration. Bioanalytical Method Validation Guidance for Industry Bioanalytical Method Validation Guidance for Industry. Biopharmaceutics. 2018; (May). https://www.fda.gov/files/drugs/published/Bioanalytical-Method-Validation-Guidance-for-Industry.pdf.

168. Sonawane L V, Poul BN, Usnale S V, Waghmare P V, Surwase LH. Pharmaceutica Bioanalytical Method Validation and Its Pharmaceutical Application- A Review. Pharm Anal Acta. 2014;5(3).

169. Abdallah O, Abdel Ghani S, Hrouzková S. Development of validated LC-MS/MS method for imidacloprid and acetamiprid in parsley and rocket and evaluation of their dissipation dynamics. J Liq Chromatogr Relat Technol. 2017;40(8):392–9. Available from: http://dx.doi.org/10.1080/10826076.2017.1310112

170. Madhu, K. What is the difference between C18 and phenyl column. 2022;1–7. (https://www.differencebetween.com/author/madhus). (Date accessed: 8 August 2022)

171. Kits A. Understanding chromatogram peaks – fronting, tailing, ghosting & rounded. Chemtech International. 2020; 1–6. https://chemtech-us.com/articles/understanding-chromatogram-peaks-fronting-tailing-ghosting-rounded-explained/.

172. Wojnicz A, Avendaño J, Casas AI, Freitas AE, López MG, Ruiz-nuño A. Simultaneous determination of 8 neurotransmitters and their metabolite levels in rat brain using liquid chromatography in tandem with mass spectrometry: Application to the murine Nrf2 model of depression. Clin Chim Acta. 2016; 453:174–81. Available from: http://dx.doi.org/10.1016/j.cca.2015.12.023

173. Kim T, Choi J, Kim H, Kim HR. Quantification of neurotransmitters in mouse brain tissue by using liquid chromatography coupled electrospray tandem mass spectrometry. Hindawi. 2014. http://dx.doi.org/10.1155/2014/506870

174. Powell, M. A Look at Column Choices. Engineer. Agilent Technologies. 2015 https://www.agilent.com/cs/library/eseminars/public/A_Look_at_Column_Choices_April_2015.pdf

175. Taira K, Fujioka K, Aoyama Y. Qualitative profiling and quantification of neonicotinoid metabolites in human urine by liquid chromatography coupled with mass spectrometry. PLoS One. 2013;8(11):1–12.

176. Mohamed F, Gawarammana I, Robertson TA, Roberts MS, Zawahir S, Jayamanne S, et al. Acute human self-poisoning with Imidacloprid compound: A neonicotinoid insecticide. PLoS One. 2009;4(4).

177. Wang A, Mahai G, Wan Y, Yang Z, He Z, Xu S, et al. Assessment of imidacloprid related exposure using imidacloprid-olefin and desnitro-imidacloprid: Neonicotinoid insecticides in human urine in Wuhan, China. Environ Int. 2020;141(May). Available from: https://doi.org/10.1016/j.envint.2020.105785

178. Curchod L, Oltramare C, Junghans M, Stamm C. Temporal variation of pesticide mixtures in rivers of three agricultural watersheds during a major drought in the Western Cape, South Africa. Water Res X. 2020; 6:1–12.

179. Mayol-Cabré M, Prats E, Raldúa D, Gómez-Canela C. Science of the Total Environment Characterization of monoaminergic neurochemicals in the different brain regions of adult zebrafish. Sci Total Environ. 2020; 745:141205. Available from: https://doi.org/10.1016/j.scitotenv.2020.141205

180. Dong G, Li X, Han G, Du L, Li M. Zebrafish neuro-behavioral profiles altered by acesulfame (ACE) within the range of “no observed effect concentrations (NOECs).” Chemosphere. 2020; 243:125431. Available from: https://doi.org/10.1016/j.chemosphere.2019.125431

181. Morrissey CA, Mineau P, Devries JH, Sanchez-Bayo F, Liess M, Cavallaro MC, et al. Neonicotinoid contamination of global surface waters and associated risk to aquatic invertebrates: A review. Environ Int. 2015; 74:291–303. Available from: http://dx.doi.org/10.1016/j.envint.2014.10.024

182. Moschet C, Wittmer I, Simovic J, Junghans M, Piazzoli A, Singer H, et al. How a complete pesticide screening changes the assessment of surface water quality. Environ Sci Technol. 2014; 48:5423–32.

183. Van Dijk TC, Van Staalduinen MA, Van der Sluijs JP (2013) Macro-Invertebrate Decline in Surface Water Polluted with Imidacloprid. PLoS ONE 8(5): e62374. doi:10.1371/journal.pone.0062374

184. Krishnan A. Developmental Effects of the pesticide Imidacloprid on zebrafish body length and mortality. 2018; 1–15. https://doi.org/10.1101/395327.

185. Islam MA, Hossen MS, Sumon KA, Rahman MM. Acute toxicity of Imidacloprid on the developmental stages of common carp (Cyprinus carpio). Toxicol Environ Health Sci. 2019;11(3):244–51.

186. Protocol Approval Letter. 2021;(May 2021):33136.

187. Klaus-Peter, W, Howard, I. OECD guidelines for testing of chemicals. Dermatotoxicology. 2013;(July):509–11. DOI: 10.3109/9781841848570-66.

188. Vieira LR, Hissa DC, de Souza TM, Chayenne A. S, Evaristo JAM, Nogueira FCS, et al. Proteomics analysis of zebrafish larvae exposed to 3, 4-dichloroaniline using the fish embryo acute toxicity test. Environ Toxicol. 2020;35(8):849–60.

189. Maes J, Verlooy L, Buenafe OE, de Witte PAM, Esguerra C V, Crawford AD. Evaluation of 14 organic solvents and carriers for screening applications in zebrafish embryos and larvae. PLoS One. 2012;7(10):1–9.

190. Carter KM, Woodley CM, Brown RS. A review of tricaine methanesulfonate for anesthesia of fish. Rev Fish Biol Fish. 2011;21(1):51–9.

191. Crosby EB, Bailey JM, Oliveri AN, Levin ED. Neurotoxicology and teratology neurobehavioral impairments caused by developmental imidacloprid exposure in zebrafish. Neurotoxicol Teratol. 2021;49(2015):81–90. Available from: http://dx.doi.org/10.1016/j.ntt.2015.04.006

192. Guerra LJ, Monique A, Andreatta V, Tiago DQ, Broock D, Osmar R, et al. Biochemical and behavioral responses in Zebrafish exposed to Imidacloprid oxidative damage and antioxidant responses. Arch Environ Contam Toxicol. 2021;81(2):255–64. Available from: https://doi.org/10.1007/s00244-021-00865-9

193. Agatz A, Cole TA, Preuss TG, Zimmer E, Brown CD. Feeding inhibition explains effects of Imidacloprid on the growth, maturation, reproduction, and survival of Daphnia magna. Environ Sci Technol. 2013; 47:2909–17.

194. Young-Hwan, Jo, Talmage, DA and Role, LW. Nicotinic receptor-mediated effects on appetite and food intake. J Neurobiol. 2014;23(1):1–7. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3624763/pdf/nihms412728.pdf

195. Mishra A, Chaturvedi P, Datta S, Sinukumar S, Joshi P, Garg A. Harmful effects of nicotine. Indian J Med Paediatr Oncol. 2015;36(1):24–31.

196. Audrain-Mcgovern J, Benowitz NL. Cigarette smoking, nicotine, and body weight. Clin Pharmacol Ther. 2011;90(1):164–8.

197. Liu S, Yu M, Xie X, Ru Y, Ru S. Carbofuran induces increased anxiety-like behaviors in female zebrafish (Danio rerio) through disturbing dopaminergic/norepinephrinergic system. Chemosphere. 2020; 253:126635. Available from: https://doi.org/10.1016/j.chemosphere.2020.126635

198. Ortiz-Severin J, Osorio F, Quiroz G, Reyes-parada M, Varas R, Moya PR. Effects of selective α4β2 nicotinic acetycholine receptor ligands on the behaviour of adult zebrafish in the novel tank diving test. Revewi Famacol Chile. 2015;8(October):35–40.

199. Nonnis S, Angiulli E, Maffioli E, Frabetti F, Negri A, Cioni C, et al. Acute environmental temperature variation affects brain protein expression, anxiety and explorative behaviour in adult zebrafish. Sci Rep. 2021;11(1):1–21. Available from: https://doi.org/10.1038/s41598-021-81804-5

200. Bencan Z, Levin ED. The role of α7 and α4β2 nicotinic receptors in the nicotine-induced anxiolytic effect in zebrafish. Physiol Behav. 2008;95(3):408–12.

201. Stewart AM, Grossman L, Collier AD, Echevarria DJ, Kalueff A V. Anxiogenic-like effects of chronic nicotine exposure in zebrafish. Pharmacol Biochem Behav. 2015; 139:112–20. Available from: http://dx.doi.org/10.1016/j.pbb.2015.01.016

202. Dean R, Duperreault E, Newton D, Krook J, Ingraham E, Gallup J, et al. Opposing effects of acute and repeated nicotine exposure on boldness in zebrafish. Sci Rep. 2020;1–11. Available from: http://dx.doi.org/10.1038/s41598-020-65382-6

203. Abu EH, Alam RTM, Ali SA, Hendawi MY. Ecotoxicology and environmental safety dose-related impacts of imidacloprid oral intoxication on brain and liver of rock pigeon (Columba livia domestica), residues analysis in different organs. Ecotoxicol Environ Saf. 2019;167(October 2018):60–8. Available from: https://doi.org/10.1016/j.ecoenv.2018.09.121

204. Naiel MAE, Shehata AM, Negm SS, Abd El-Hack ME, Amer MS, Khafaga AF, et al. The new aspects of using some safe feed additives on alleviated imidacloprid toxicity in farmed fish: a review. Rev Aquac. 2020;12(4):2250–67.

205. Wang Y, Zhang Y, Li W, Han Y, Guo B. Study on neurotoxicity of dinotefuran, thiamethoxam and imidacloprid against Chinese lizards (*Eremias argus*). Chemosphere. 2019; 217:150–7. Available from: https://doi.org/10.1016/j.chemosphere.2018.11.016

206. Campbell KS, Keller PG, Heinzel LM, Golovko SA, Seeger DR, Golovko MY, et al. Detection of imidacloprid and metabolites in Northern Leopard frog (Rana pipiens) brains. Sci Total Environ. 2022; 813:152424. Available from: https://doi.org/10.1016/j.scitotenv.2021.152424

207. Berheim EH, Jenks JA, Lundgren JG, Michel ES, Grove D, Jensen WF. Effects of neonicotinoid insecticides on physiology and reproductive characteristics of captive female and fawn white-tailed deer. Sci Rep. 2019;(September 2018):1–10. Available from: http://dx.doi.org/10.1038/s41598-019-40994-9

208. Hussain S, Hartley CJ, Shettigar M, Pandey G. Bacterial biodegradation of neonicotinoid pesticides in soil and water systems. FEMS Microbiol Lett. 2016;363(23):1–13.

209. Ospina M, Wong L, Baker SE, Serafim AB, Morales-agudelo P, Calafat AM. Exposure to neonicotinoid insecticides in the US general population: Data from the 2015–2016 national health and nutrition examination survey. Environ Res. 2019;176(June):108555. Available from: https://doi.org/10.1016/j.envres.2019.108555

210. Ueyama J, Aoi A, Ueda Y, Oya N, Sugiura Y, Ito Y, et al. Food Additives & Contaminants: Part A Biomonitoring method for neonicotinoid insecticides in urine of non-toilet-trained children using LC-MS/MS. Food Addit Contam Part A. 2020;37(2):304–15. Available from: https://doi.org/10.1080/19440049.2019.1696020

211. Zhang T, Song S, Bai X, He Y, Zhang B, Gui M. A nationwide survey of urinary concentrations of neonicotinoid insecticides in China. Environ Int. 2019;132(August):105114. Available from: https://doi.org/10.1016/j.envint.2019.105114

212. Maggi L, Sher E, Cherubini E. Regulation of GABA release by nicotinic acetylcholine receptors in the neonatal rat hippocampus. The Physiological Society. 2001; 89–100. https://doi.org/10.1111/j.1469-7793.2001.00089.x

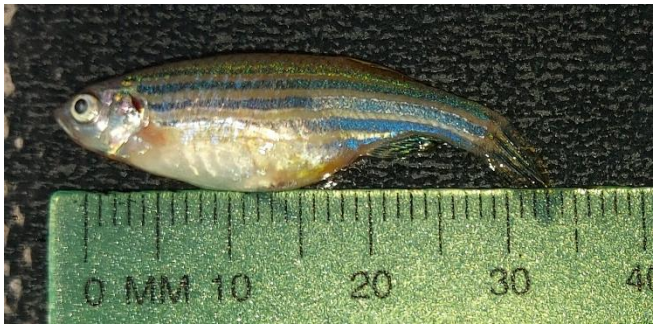
213. Zhang JG, Ma DD, Xiong Q, Qiu SQ, Huang GY, Shi WJ, et al. Imidacloprid and thiamethoxam affect synaptic transmission in zebrafish. Ecotoxicol Environ Saf. 2021;227(October):112917. Available from: https://doi.org/10.1016/j.ecoenv.2021.112917

214. Powrie Y, Strydom M, Aucamp M, Schellack N, Steenkamp V, Smith C. Zebrafish behavioral response to ivermectin: insights into potential neurological risk. Medicine in Drug Discovery. 2022;16(July):100141. Available from: https://doi.org/10.1016/j.medidd.2022.100141

Appendix 1

Preparation of the spiking IMI test solution concentration for the 21-day IMI exposure study in adult zebrafish

A stock solution of 1 mg/mL IMI was prepared in MeOH. The stock solution was diluted down to 7.5 µg/mL in environmentally-controlled water. Subsequently, a total volume of 1 mL of the test solution at 7.5 µg/mL was used to spike the treatment group containing a final volume of 3 L of water which resulted in a final exposure concentration of 2.5 µg/mL. This test solution of IMI was prepared daily to avoid any confounding factors.



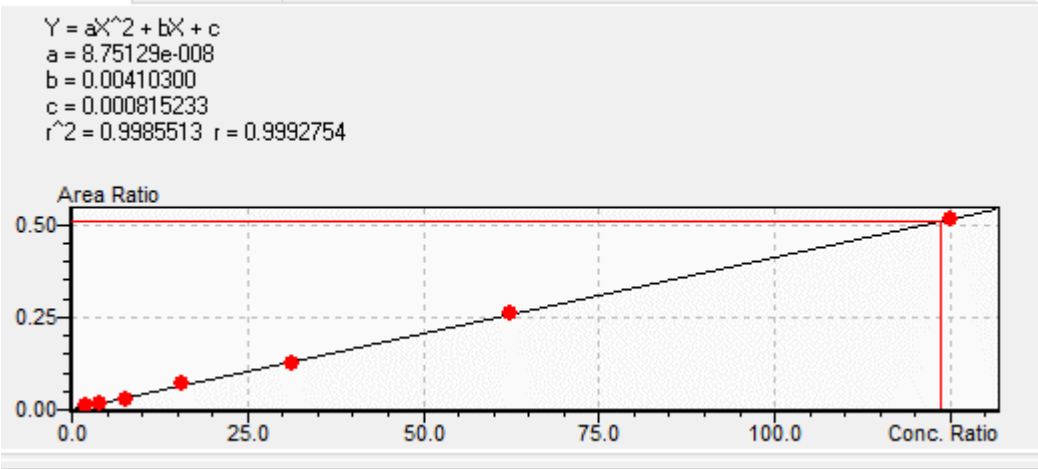
Body length detection in control group

Body length detection in treatment group

Appendix 2

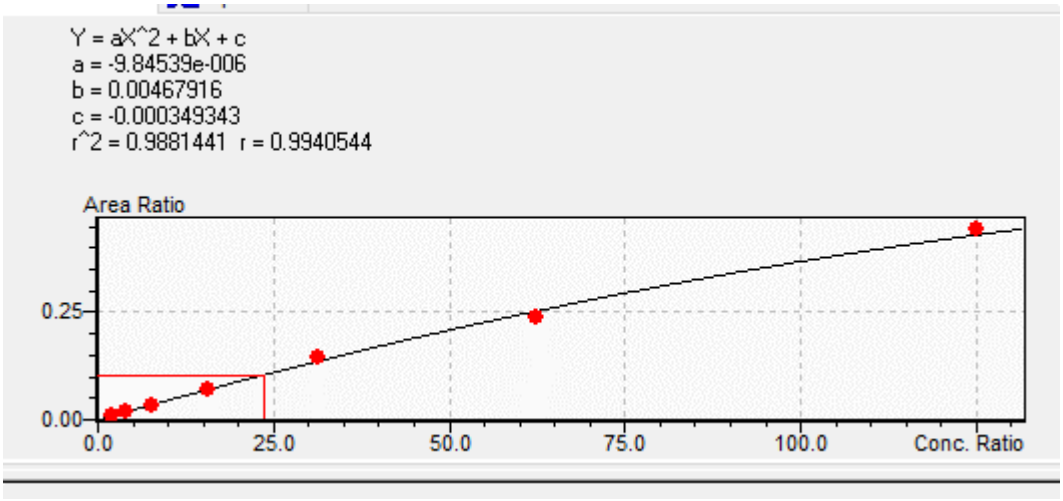
The calibration curves of the various target analytes in this study is presented in the Figures below.

- Imidacloprid



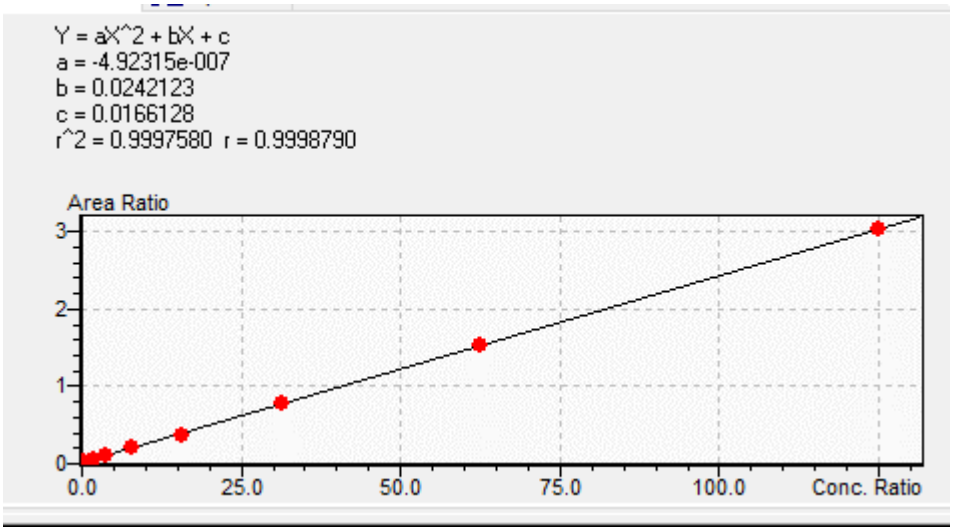
Calibration curve of imidacloprid from 0.488 - 125 ng/mL with a quadratic regression and 1/C weighting

- Imidacloprid Urea



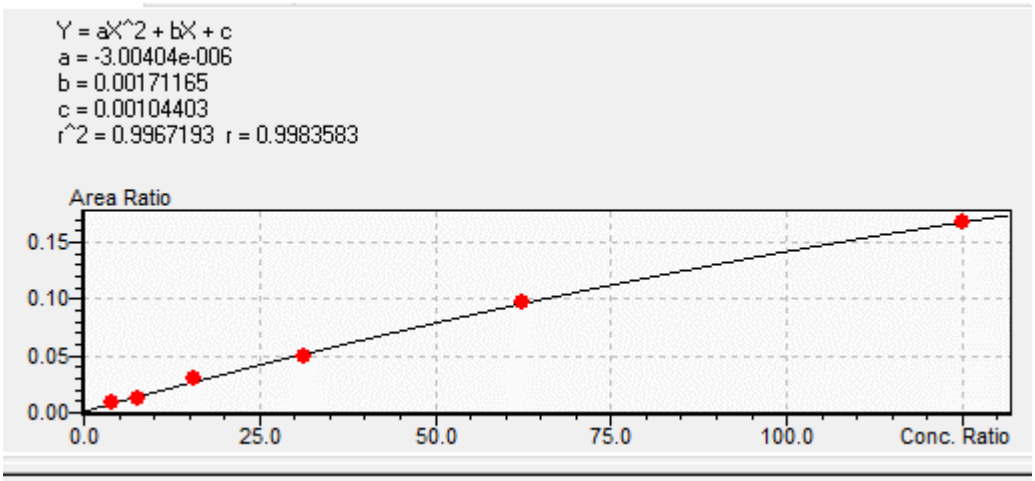
Calibration curve of imidacloprid-urea from 1.95 - 125 ng/mL with a quadratic regression and 1/C weighting

- Desnitro-imidacloprid



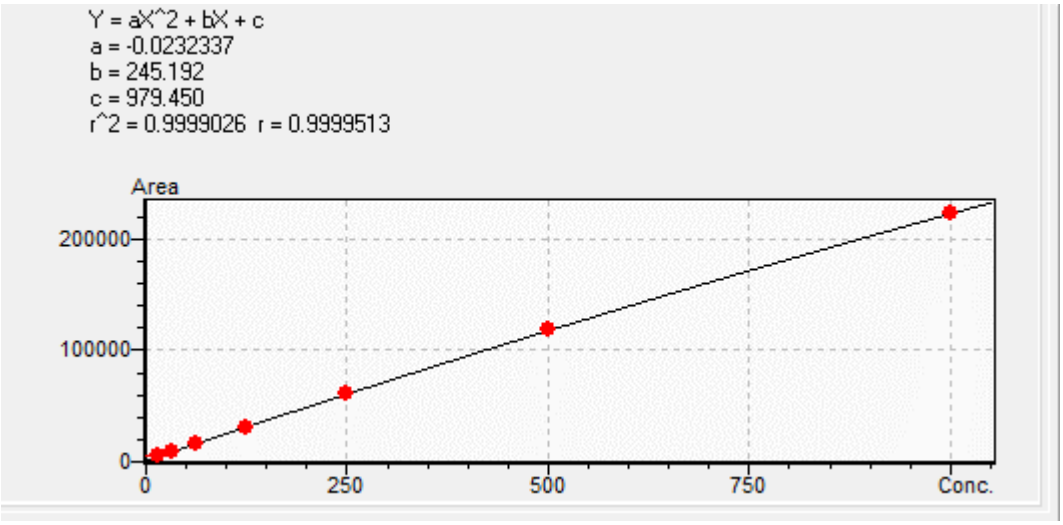
Calibration curve of desnitro-imidacloprid from 0.244 - 125 ng/mL with a quadratic regression and 1/C weighting

- 5-hydro imidacloprid



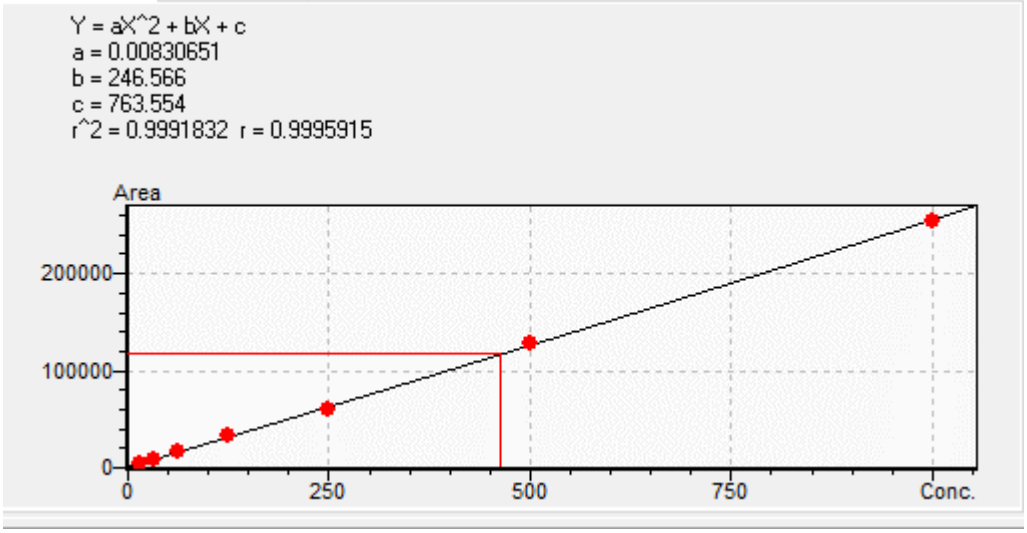
Calibration curve of 5-hydro imidacloprid from 3.91 - 125 ng/mLwith a quadratic regression and 1/C weighting

- Acetylcholine



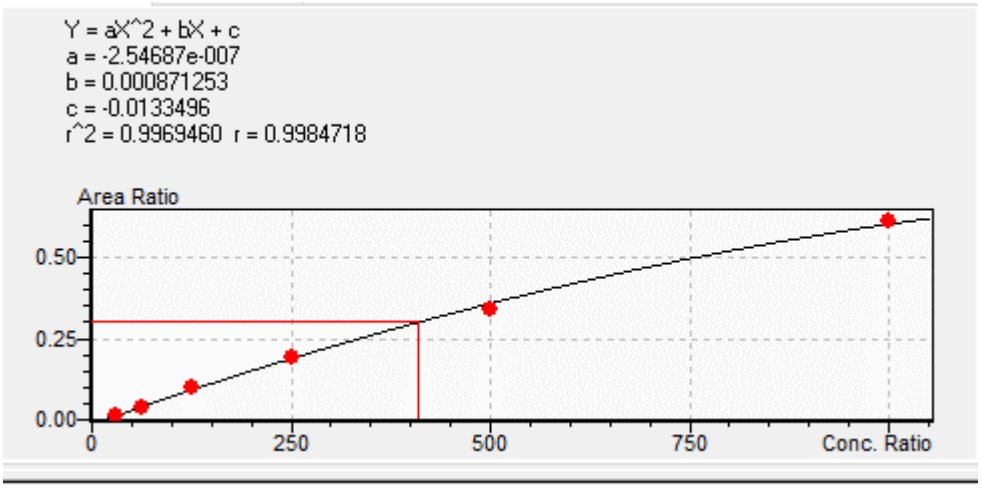
Calibration curve of acetylcholine from 31.3 - 1000 ng/mL with a quadratic regression and 1/C weighting

- Gamma-aminobutyric acid



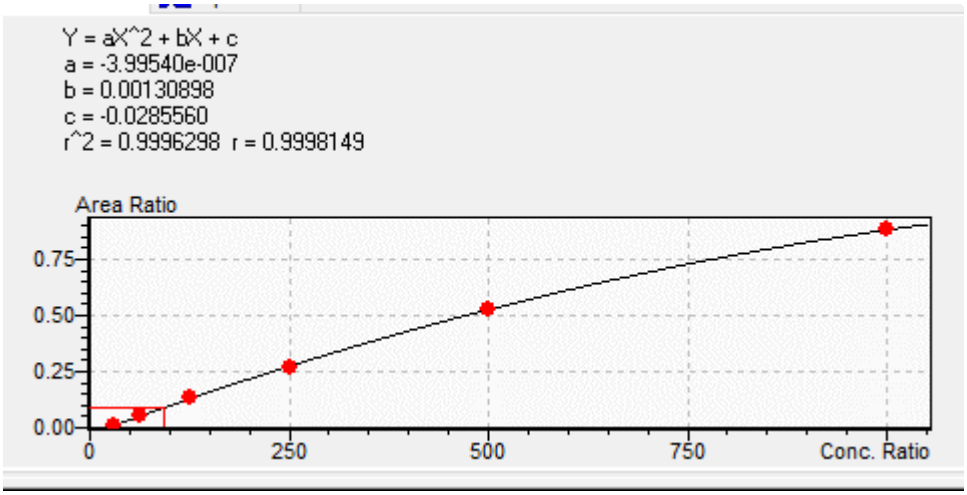
Calibration curve of GABA from 15.63 - 1000 ng/mL with a quadratic regression and 1/C weighting

- Dopamine



Calibration curve of dopamine from 31.3 - 1000 ng/mL with a quadratic regression and 1/C weighting

- Serotonin



Calibration curve of serotonin from 31.3 - 1000 ng/mL with a quadratic regression and 1/C weighting

Appendix 3

Calculating the final concentration of 5-OH IMI in the liver and gill epithelial tissue. The following formula was used to perform the calculations:

Concentration obtained from LC-MS/MS analysis (ng/mL)

_____ ng/mL X volume of homogenisation solution added (mL)

_____ ng/mg of tissue = _____ ng/mg tissue

Treated

A table illustrating the calculations to determine the final concentration of 5-OH IMI in the treated liver and gill epithelial tissue in ng/mg

Liver	Gills
Tube 1	
177.76 ng/mL x 0.441 mL 78.392 ng/ 98.9 mg tissue = 0.793 ng/mg tissue	20023.37 ng/mL x 0.274 mL 5486.403 ng/ 46.84 mg = 117 ng/mg tissue

Control

A table illustrating the calculations to determine the final concentration of 5-OH IMI in the control liver and gill epithelial tissue in ng/mg

Liver	Gills
Tube 1	
17.31 ng/mL x 0.355 mL 6.145 ng/ 60.37 mg = 0.102 ng/mg tissue	1474 ng/mL x 0.238 mL 350.812 ng/ 40.65 mg = 8.63 ng/mg tissue

Appendix 4

Calculating the final concentration of ACh and GABA in the brain tissue. The following formula was used to perform the calculations:

Concentration obtained from LC-MS/MS analysis (ng/mL)

_____ ng/mL X volume of homogenisation solution added (mL)

_____ ng/mg of tissue = _____ ng/mg tissue

TREATED

A table illustrating the calculations to determine the final concentration of ACh and GABA in the treated brain tissue in ng/mg

GABA	ACh
Tube 1	
821.49 ng/mL x 0.263 mL 216.052 ng/ 52.60 mg = 4.107 ng/mg tissue	139.54 ng/mL x 0.263 mL 36.699 ng/ 52.6 mg = 0.698 ng/mg tissue
Tube 2	
419.06 ng/mL x 0.312 mL 130.915 ng/ 62.29 mg = 2.099 ng/mg tissue	117.962 ng/mL x 0.312 mL 36.804 ng/ 62.29 mg = 0.591 ng/mg tissue
Tube 3	
398.08 ng/mL x 2 (due to experimental error) x 0.230 mL 183.117 ng/ 45.32 mg = 4.05 ng/mg tissue	216.12 ng/mL x 2 (due to experimental error) x 0.230 mL 99.415 ng/ 45.32 mg = 2.19 ng/mg tissue
Tube 4	
292.40 ng/mL x 2 (due to experimental error) x 0.240 mL 140.52 ng/ 46.50 mg = 3.02 ng/mg tissue	208.76 ng/mL x 2 (due to experimental error) x 0.240 mL 100.205 ng/ 46.50 mg = 2.15 ng/mg tissue

Control

A table illustrating the calculations to determine the final concentration of ACh and GABA in the treated brain tissue in ng/mg

GABA	ACh
Tube 1	
297.06 ng/mL x 0.284 mL 84.365 ng/ 56.81 mg = 1.49 ng/mg tissue	369.45 ng/mL x 0.284 mL 104.924 ng/ 46.81 mg = 1.85 ng/mg tissue
Tube 2	
468.41 ng/mL x 0.242 mL 113.355 ng/ 48.42 mg = 2.341 ng/mg tissue	190.75 ng/mL x 0.242 mL 46.162 ng/ 48.42 mg = 0.953 ng/mg tissue
Tube 3	
141.91 ng/mL x 2 (due to experimental error) x 0.210 mL 59.602 ng/ 41.03 mg = 1.45 ng/mg tissue	321.58 ng/mL x 2 (due to experimental error) x 0.210 mL 135.064 ng/ 41.03 mg = 3.29 ng/mg tissue

Therefore to determine the final concentration in ng/mg tissue for each group, an average was taken between the tubes/pooled samples. This result is depicted below.

- **GABA**

Control group = 1.76 ng/mg tissue

Treated group = 3.32 ng/mg tissue

- **ACh**

Control group = 2.04 ng/mg tissue

Treated group = 1.47 ng/m6g tissue

A table illustrating the masses in mg for the specific tissue for both the treated and control group

Control tissue	**Mass of tissue in each tube (pooled) in mg**
Brain (n=11)	Tube 1: 12.39 + 20.03 + 8.09 + 16.2 = 56.7 mg brain tissue Tube 2: 11.47 + 13.66 + 16.49 + 6.80 = 48.4 mg brain tissue Tube 3: 10.00 + 17.45 + 14.23 = 41.7 mg brain tissue
Liver (n=11)	Tube 1: 5.10 + 8.31 + 7.66 + 4.73 + 6.40 + 7.40 + 2.50 + 10.6 + 4.60 +4.70 + 8.70 = 70.7 mg liver tissue
Gills (n=11)	Tube 1: 2.35 + 6.70 + 5.40 + 3.00 + 2.5 + 7.00 + 5.60 + 5.00 + 2.20 + 5.00 + 2.70 = 47.6 mg gill epithelial tissue
Treated tissue	**Mass of tissue in each tube (pooled) in mg**
Brain (n=10)	Tube 1: 22.84 + 29.76 = 52.6 mg brain tissue Tube 2 = 12.62 + 23.87 + 25.80 = 62.3 mg brain tissue Tube 3 = 28.12 + 17.20 = 45.3 mg brain tissue Tube 4 = 7.50 + 7.33 + 31.67 = 46.5 mg brain tissue
Liver (n=10)	Tube 1: 5.00 + 5.30 + 20.3 +14.7 + 5.20 + 4.40 + 5.10 + 12.0 + 6.20 + 10.0 = 88.2 mg tissue
Gills (n=10)	Tube 1: 5.54 + 4.00 + 8.10 + 4.00 + 2.80 + 4.50 + 3.00 + 5.60 + 4.40 + 9.50 = 51.44 mg gill epithelial tissue