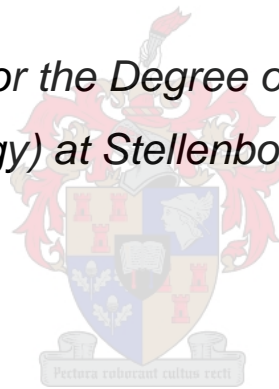# DEVELOPMENT OF TOXICOLOGY SCREENING METHODS FOR COMMON POISONING AGENTS IN SOUTH AFRICA

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Thesis presented for the Degree of Master of Science (Pharmacology) at Stellenbosch University

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Declaration

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.

Signature:………………….. Date:………………….

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Abstract

Background

Hospitals admit many patients suffering from acute poisoning on a regular basis. Over a single year period the Tygerberg Poison Information Centre (TPIC) dealt with 4 771 consultations related to human exposure to various poisonous substances. Of these consultations, exposures to medicines and pesticides accounted for the majority of cases.   As a response to this medical burden, this project aims to develop effective screening tools for both medicines and pesticides in human plasma. It is believed that such a tool could enhance how medical practitioners accurately treat and respond to serious poisonings. There are many toxicological screening methods employed today. In the past immunoassays (IAs) and gas chromatography coupled to mass spectrometry (GC-MS) were seen to be the gold standard for the identification of compounds. Due to false positive results associated with immunoassays and the labour intensive sample preparation associated with GC-MS, it is clear that there is a need for faster, more robust and more sensitive bioanalytical methods. This study's objective therefore is to develop a cost effective, fast and robust liquid chromatography tandem mass spectrometry (LC-MS/MS) screening tools for the identification of drugs and pesticides. Relevant drug and pesticide selection was done by consulting with the TPIC, a centre which deals with poisoning cases on a daily basis, as well as clinicians in the Division of Clinical Pharmacology who deal with patients admitted at Tygerberg Hospital daily and CropLife SA, an organisation that has the knowledge of which pesticides are currently in use in South Africa. A total of 37 drugs and 10 pesticides were identified as those often suspected to be involved in poisoning/overdose cases. Two methods were developed: An untargeted liquid chromatography quadrupole time-of-flight mass spectrometry (LC-TOFMS) method for the determination of therapeutic drugs; and a targeted LC-MS/MS method for the determination of pesticides.

Method

Analytes were extracted from CPD plasma using protein precipitation (PP). For the untargeted method, a Waters Synapt G2 Quadrupole Time-of-flight (QTOF) mass spectrometer (MS) fitted with an ultra-high performance liquid chromatography (UHPLC) was used. The separation was done using a Waters HSS T3 (1.8 μm, 2.1 ×150 mm) column. A gradient consisting of a mobile phase

of 0.1% formic acid in both water (A) and in acetonitrile (B) at a flow rate of 0.25 mL/min. Ionisation mode was set for both positive and negative (ESI +/-); Nitrogen as the desolvation gas was set at a flow rate of 650L/h; Desolvation temperature was set at 275˚C. The instrument was operated in the MS^E mode. The total run time was 37 min. Four concentration levels (10, 50, 100, 1000 ng/mL) were determined to cover the pharmacokinetic peak concentrations of all drugs. This range was determined according to sub-therapeutic, therapeutic and overdose type concentrations. Furthermore, the data generated from the untargeted method development process was used to accurately identify analytes using online search libraries.

For the targeted method, two liquid chromatographic separation methods were developed on a Shimadzu 8040 LC-MS/MS instrument. One gradient method using the Agilent Poroshell 120 EC-C18 (2.7 μm, 4.6 ×100 mm) column was developed for atrazine, MCPA, fipronil, methomyl and aldicarb, while a second isocratic method utilizing of the Restek Raptor Biphenyl (2.7 μm, 2.1 x 100 mm) column was also developed for imidacloprid. Both methods used mobile phase consisting of 0.1% formic acid in water (A) and in acetonitrile (B) with a flow rate of 0.5 mL/min. The LC program on the Poroshell method starts at 30% (B) for 2 min followed by a 0.5 minute ramp to 90% (B); 2.5 min at 90% (B); 0.5 min to 30% (B), equilibrate at 30% (B) for 2.5 min. The total run time is 9 min. The biphenyl method isocratic run comprises of (65%:35%, v/v) for a run time of 1.75 min. A semi-quantitative method with a quadratic curve fit with 1/x regression over the range of 20 to 600 ng/mL was chosen. The two targeted methods were subjected to a partial validation to ensure that the methods were accurate, robust and reliable.

Results

The untargeted method was able to detect and identify 92% of the therapeutic drugs included in the screen. Due to matrix effects and differences in compound ionisation some could not be detected at lower concentrations. Polarity and thus lack of retention on the columns, difficulty in ionisation, and the inability to produce stable fragments made the detection of valproate, levetiracetam and metformin not possible on the method.

A screening tool for simultaneous determination of pesticides was optimized. Due to several challenges associated with the LC-MS/MS determination of glyphosate, paraquat and deltamethrin, the study resorted to excluding these

three pesticides from the current methods. The targeted method was developed for the determination of seven pesticides. The USA Food and Drug Administration (FDA) and the European Medicines Agency (EMA) guidelines were followed for a partial validation of the semi-quantitative method. Three independent runs were assessed to measure accuracy and precision. The calibration curve was linear over the ranges 20 to 600 ng/mL. Matrix effects, recovery, process efficiency, on-bench stability, freeze/thaw stability, storage stability, and whole blood stability for the methods was successfully determined for the seven pesticides.

Conclusion

To respond to the medical burden of acute poisoning cases faced by our hospitals, this study has developed efficient screening tools for both relevant medicines and relevant pesticides in human plasma. The latter method was subjected to a partial validation, ensuring accuracy and reliability of results that will be applied to the clinical management of poisoning patients.

Abstrak

Agtergrond

Hospitale sien baie gevalle van pasiente wat vergiftig is. Tygerberg Gifstof Inligtingsentrum (TPIC) het 4771 gevalle in een jaar gehad waar mense aan verskillende gifstowwe blootgestel is. Die meeste van hierdie gevalle was die medisyne en pestisiede.

Hierdie projek het gepoog om metodes te ontwikkel waarmee daar vir hierdie stowwe getoets kan word in menslike plasma. So 'n metode kan 'n hulpmiddel vir dokters wees om die beste behandeling vinnig aan pasiente met vergiftiging te kan gee. Daar bestaan heelwat toksikologiese toetsmetodes waar daar vir meervuldige stowwe in een analise getoets kan word. In die verlede is immuumgebaseerde metodes en gaschromatografie tesame met massaspetrometrie (GC-MS) gesien as die goue standaarde vir die identifisering van gifstowwe. Daar is egter vals-positiewe resultate as die immuungebaseerde metodes gebruik word en die GC-MS metodes is lank en die monstervoorbereiding omslagtig. Daar is 'n behoefte aan vinniger, meer betroubare en sensiewe bio-analitiese metodes. Die doel van hierdie studie is om 'n koste-effektiewe, vinnige en robuuste metode te ontwikkel vir die identifisering van farmaseutiese middels en pestisiede deur van vloeistof chromatografie tandem massa spektrometrie (LC-MS/MS) gebruik te maak. 'n Aantal van hierdie middels is gekies in konsultasie met beide TPIC, wat op 'n daaglikse basis met vergiftigings werk, en dokters in die Divisie van Kliniese Farmakologie by Tygerberg wie gereeld met pasiente werk wat vergiftig is. CropLife SA het baie kennis oor watter pestisiede in Suid-Afrika gebruik word en Dr Gerhard Verdoorn is gekonsulteer oor die keuse van pestisiede wat ingesluit is in die studie. Daar is besluit op 37 farmaseutiese middels en 10 pestisiedes wat gereeld verdink word as oorsake van vergiftigings en oordosisse. Twee metodes is ontwikkel: 'n ongeteikende (LC-TOFMS) metode vir die farmaseutise middels en 'n geteikende LC-MS/MS metode vir die pestisiede.

Metodes

Die middels is uit die plasma ge-ekstrareer deur van proteïen presipitasie (PP) gebruik te maak. 'n Waters Synapt G2 Kwadrupool Vlugtyd (QTOF) massaspektrometer (MS) met 'n Acquity uiters hoë werkverrigting

 vloeistofchromatograaf (UHPLC) is gebruik met 'n Waters HSS T3 (1.8 µm, 2.1 ×150 mm) kolom. Die mobiele fase het bestaan uit 0.1% mieresuur in beide water (A) en in asetonitriel (B) teen 'n vloeispoed van 0.25 mL/min. Beide positiewe and negatiewe ionisasie modus was gebruik (ESI +/-); Stikstof was die desolvasie gas teen 650 L/h; die desolvasie temperatuur was 275˚C. Data was gegenereer in MS^E mode. Die metode was 37 minute lank. Daar is vier verskillende konsentrasies ingespuit om al die moontlike vlakke van farmakokinetiese topvlakke van die verskillende middels in te sluit (10, 50, 100, 1000 ng/mL). Daar is ook bewys hoe hierdie metode in die studie van aanlyn biblioteke gebruik kan maak om onbekende middels in die plasma te identifiseer,

Daar is twee skeidingsmetodes gebruik vir die geteikende metode op die Shimadzu 8040 LC-MS/MS instrument. Die eerste gebruik 'n Agilent Poroshell 120 EC-C18 (2.7 µm, 4.6 ×100mm) kolom met die gradient. Die tweede is 'n isokratiese metode waar 'n Restek Raptor Biphenyl (2.7 µm, 2.1 x 100 mm) kolom gebruik word. Beide metodes gebruik dieselfde mobiele fase as die ongeteikende metode (0.1% mieresuur water (A) en in asetonitriel (B)) met 'n vloeispoed van 0.5 mL/min. Die LC program van die Poroshell metode begin met 30% (B) vir 2 minute gevolg deur 'n 0.5 minute oorgang na 90% (B); 2.5 minute by 90% (B); en oor 0.5 minute terug na 30% (B) om te ekwilibreer vir 2.5 minute. Die metode is 9 minute lank. Die biphenyl metode gebruik (65% A:35% B, v/v) vir 1.75 minute. Die kalibrasiekurwe was kwadraties met 1/x regressie vir al die middels oor 'n konsentrasiegebied van 20 tot 600 ng/mL. Die metodes was gedeeltelik gevalideer om te verseker dat dit toepaslik en akkuraat is.

Resultate

Die ongeteikende metode kon 92% van die farmaseutiese middels waarneem waarna gekyk is. Matrikseffekte en verskillende ionisasiepotensiale het veroorsaak dat sommige middels nie by die laagste vlakke gesien kon word nie. Drie middels kon nie met sukses geanaliseer word nie agv van hul polariteit en dus onvermoë om teruggehou te word deur die kolom of swak ionisasie: epilizine (valproate), epikepp (levetiracetam) en diaphage (metformin).

A metode om tergelyke tyd 'n aantal pestisiede (7) te analiseer is opgestel. Drie middels is laat vaar omdat dit nie bepaal kon word met die tegniek nie (glifosaat, paraquat en deltamethrin). 'n Ander tipe kolom en mobiele fase is nodig vir die analise daarvan en van hulle (glifosaat) sal eers gederivatiseer moet word om dit te

kan meet. Die FDA en EMA voorskrifte is gevolg om die metode gedeeltelik te valideer.

Drie onafhanklike lopies was gedoen om die akkuraatheid en presisie te bepaal. Matriks effekte, herwinning, proses effektiwiteit, op toonbank stabiliteit, vries en ontvries stabiliteit, storingsstabiliteit en heelbloed stabiliteit was suksesvol bepaal vir die sewe pestisiede. Die kalibrasie kurwe was reglynig oor die konsentrasiegebied van 20 tot 600 ng/mL. Die helling van die kurwe se standaardafwyking was minder as 5% vir al 7 verbindings.

Gevolgtrekking

In reaksie op die las wat vergiftigings op die medici in hospitale veroorsaak het hierdie studie effektiewe skandeermetodes opgestel vir relevante farmaseutiese middels en pestisiede in menslike plasma. Die pestisied metode is gedeeltelik gevalideer om sy betroubaarheid en akkuraatheid te bevestig sodat dit gebruik kan word om die gifstowwe wat gebruik is in vergiftigde pasiente te bepaal.

Dedication

To my Grandmother who is turning a 100 years this year, my grade one and two teacher, Bhabha Josephine Siwela

Thank you for giving me a strong start and a solid foundation.

To my parents, Kadefuna and Phumla

You have always been my biggest fans and greatest cheerleaders. You taught me never to let go of my dreams and aspirations

_____

If I have seen further, it is by standing upon the shoulders of giants

– Isaac Newton

Acknowledgements

It literally took an entire village to get me through this journey. I, therefore, wish to acknowledge and thank the following:

First and foremost I would like to thank God. Your grace and love are what sustain me.

My entire family, thank you for your unconditional love, support, and encouragement.

Prof. Helmuth Reuter, the events that unfolded following a single meeting with you have been life-changing. Thank you very much for giving me this opportunity.

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Clinicians of Clinical Pharmacology Division, the Tygerberg Poisons Information Centre staff, and the Central Analytical Facilities staff, thank you for all your intellectual contributions and constant encouragement and support.

Dr. Gerhard Verdoorn and Crop Life SA, thank you for your financial support through bursaries and scholarships. Your support has been unwavering through this journey, for that, I am extremely grateful.

The National Research Foundation (NRF), thank you for your financial support through bursaries and scholarships.

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~	Approximately
%CV	Coefficient of variation
ACN	Acetonitrile
ADHD	Attention deficit hyperactivity disorder
ASD	Antiseizure drugs
ATSDR	Agency for toxic substances and disease registry
CDER	Center for drug evaluation and research
Cmax	Pharmacokinetic peak concentrations
CNS	Central nervous system
COX	Cyclooxygenase
CPD	Drug free citrate phosphate dextrose
EMA	European medicines agency
ESI	Electrospray ionisation
FAO	Food and agriculture organization of the United Nations
FDA	USA food and drug administration
FL	Fluorescence
GC	Gas chromatography
GC-MS	Gas chromatography coupled to mass spectrometry
GIT	Gastrointestinal tract
HILIC	Hydrophilic interaction liquid chromatography
HIV	Human immunodeficiency virus and acquired immunodeficiency syndrome
HPLC	High performance liquid chromatography
IAs	Immunoassays
ICP-MS	Inductively coupled plasma mass spectrometry
LC	Liquid chromatography
LC-MS	Liquid chromatography coupled to mass spectrometry
LC-MS/MS	Liquid chromatography linked to tandem mass spectrometry

LC-TOFMS	Liquid chromatography coupled to quadrupole time-of-flight mass spectrometry
LOQ	Limit of quantitation
LLOQ	Lower limit of quantitation
MCPA	2-methyl-4-chlorophenoxyacetic acid
MeOH	Methanol
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
ng/mL	Nano grams per millilitre
PFP	Pentafluorophenyl
ppm	Parts per million
QC	Quality control
r^2	Correlation coefficient
RCWMCH	Red cross war memorial children's hospital
SOP	Standard operating procedure
STDEV	Standard deviation
TDM	Therapeutic drug monitoring
TPIC	Tygerberg poison information centre
TIC	Total ion count
TOF	Time-of-Flight
UV	Ultra violet
Vol	Volume
WHO	World Health Organisation
WS Soln	Working stock solution

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CHAPTER 1

Introduction and literature review

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Introduction

1.1. Toxicology and analytical toxicology

Toxicology can loosely be defined as the study of poisons, and more accurately, "the study of how chemicals interfere with the normal function of a biological system". Paracelsus, the father of toxicology, said "All substances are poisons; there is none which is not a poison. The right dose differentiates a poison and a remedy" (Sullivan *et al.*, 2005). Today, toxicology as a discipline contributes to the growth of many specialised scientific and health disciplines (Hodgson & Smart, 2001). Toxicology has over the years evolved from being merely the science of poisons to being "the study of adverse effects of chemical, physical or biological agents on living organisms" (Gulumian, 2009).

So what is analytical toxicology? In a paper prepared by Dr. R. Flanagan for the World Health Organisation (WHO), he defines it as the ability to detect, measure and identify drugs and unknown analytes in biological specimens. This analysis is necessary for the adequate treatment of poisoning and overdose cases. For the ability to determine the nature of a particular compound and measure the amount thereof, analytical toxicology has been regarded as being very useful in many countries (Flanagan, 2004). The application of new molecular techniques continue to add gains in our understanding of how toxicants are able to produce their adverse effects and how best to treat them (Hodgson & Smart, 2001).

1.2. Clinical aspects of analytical toxicology

A large percentage of patients admitted to hospital are due to acute poisoning. Up to 17% of South African hospital cases in a ward involve children admitted for acute poisoning. Furthermore, children below the age of 10 years make up 80% of all poisoning cases (Malangu & Ogunbanjo, 2009). Also alarming are acute poisoning cases involving infants (<1 year of age) also making their way into emergency centres. At ages between 8 and 12 months, infants start becoming mobile and curious about their surroundings and thus ending up putting substances that are potentially harmful into their mouths (Marks & van Hoving, 2016).

When a suspected case of acute poisoning is presented at a hospital, a physical exam has to be performed to determine the agent responsible for the poisoning.

There are quite a number of clinical features that are linked to common poisons that can assist in making a diagnosis (Table 1).

Table 1. Clinical features associated with specific poisons (WHO, 1995)

	Clinical Feature	Poison
Central Nervous System	Ataxia	Thallium, phenytoin, bromide, hypnotics/sedatives, ethanol, carbamazepine
	Convulsions	Theophylline, amitriptyline, orphenadrine and other tricyclic antidepressants, strychnine
	Coma	Opioids, hypnotics/sedatives, tranquilizers, alcohols
Respiratory Tract	Respiratory depression	Sedatives, tranquilizers, alcohols, opioids
	Pulmonary oedema	Opioids , irritant (non-cardiogenic) gases, organic, solvents, paraquat, acetylsalicylic acid, herbicides
	Hyperpnoea	Ethylene glycol, herbicides, isoniazid, methanol, hydroxybenzonitrile, pentachlorophenol, acetylsalicylic acid
Heart and Circulation	Hypertension	Sympathomimetic, anticholinergics
	Bradycardia	Opioids, cholinergics, digoxin, /J-blockers
	Hypotension	Opioids, hypnotics/sedatives, tranquillizers ethanol
	Tachycardia	Anticholinergics, sympathomimetic
	Arrhythmias	β-Blockers, digoxin, antidepressants, phenothiazines, quinidine, theophylline, cyanide chloroquine
Eyes	Miosis	Opioids, pesticides, phencyclidine, phenothiazines, carbamate pesticides, organophosphates
	Mydriasis	Amphetamine, atropine, cocaine, tricyclic antidepressants
	Nystagmus	Carbamazepine, ethanol, phenytoin
Body Temperature	Hyperthermia	Dinitrophenol pesticides, pentachlorophenol, procainamide, quinidine, acetylsalicylic acid, hydroxybenzonitrile herbicides
	Hypothermia	Ethanol, opioids, phenothiazines, hypnotics/sedatives, tricyclic antidepressants, carbon monoxide
Skin, Hair and Nails	Acne	Organochlorine pesticides, bromides
	Hair loss	Thallium
Gastrointestinal Tract	Hypersalivation	Strychnine, cholinesterase inhibitors
	Gastrointestinal bleeding	Caustic compounds (strong acids/bases), coumarin anticoagulants, indomethacin, acetylsalicylic acid
	Liver damage	Phosphorus (white), amanita toxins, paracetamol, carbon tetrachloride
	Constipation	Opioids, lead, thallium
	Dry mouth	Antidepressants, atropine, phenothiazines, opioids
	Diarrhoea	Cholinesterase inhibitors, laxatives, arsenic
Urogenital Tract	Urinary retention	Opioids, tricyclic antidepressants , atropine
	Incontinence	Carbamate pesticides, organophosphorus pesticides
	Kidney damage	Mercury, amanita toxins, carbon tetrachloride, ethylene glycol, cadmium, paracetamol

From Table 1 above, it is apparent that there are a number of different "poisons" which can exhibit the same or similar clinical features. With that in mind, the approach of handling acute poisoning cases with the hopes of linking a particular toxidrome to a particular drug can be inaccurate. Different drugs can elicit similar effects on the body. Furthermore, one must also consider alternative diagnoses other than poisoning. For instance, a coma can be induced by uncontrolled diabetes as well as poisoning. It is therefore imperative that a poisoning should be verified using reliable and robust analytical methods. The screening tools employed in analytical toxicology enable medical practitioners to make rapid, informed decisions regarding the best course of treatment for the patient (WHO, 1995).

1.3. Analytical methods

There are many toxicological screening methods currently in use by toxicology laboratories. The most widely used methods include immunoassays and gas chromatography coupled to mass spectrometry (GC-MS) (Li & Tse, 2010). In the past, immunoassays and GC-MS were regarded as the gold standards for the identification of compounds (Rentsch, 2016). However, due to labour intensive sample preparation, as well as the challenge of identifying thermolabile and very polar compounds, these methods are fast loosing favour to liquid chromatography coupled to mass spectrometry (LC-MS) (Li & Tse, 2010).

Separation of a broad range of toxic compounds is not an easy task as they have extreme differences in chemical and physical properties which cannot easily be detected under generic conditions. It is because of this wide distribution that one needs to develop a separation method where all analytes are retained on the column and effectively separated from the matrix to enable the identification of these toxins (Pasch, 2013).

Let us briefly discuss the above-mentioned analytical techniques:

1.3.1. Immunoassays

Most laboratories make use of immunoassays (IAs) for the general testing of unknowns. The advantage of using IAs is that they do not require any pre-treatment of specimens and the results are obtained rapidly. They comprise of kits that allow for automation and batch processing which results in a high turnover of results over a short period. Technologists working at routine

laboratories are able to be trained on the use of these kits very easily and quickly. That being said, it cannot be ignored that there are many disadvantages to IAs. Perhaps the biggest disadvantage is limited sensitivity and specificity and not being able to detect new designer drugs (Pedersen *et al.*, 2013). Furthermore, samples can easily be adulterated using household chemicals which ultimately leads to the detection of false negatives (Brent *et al.*, 2005). Due to a high degree of cross-reactivity among structurally related and unrelated compounds, IAs can yield false positive results (Saitman *et al.*, 2014). Therefore positive IA findings often require further analysis in order to identify the specific drugs and metabolites (Rosano *et al.*, 2011).

1.3.2. Gas chromatography

Biological specimens and drugs are made up of a series of highly complex mixtures. The human body on its own is thought to be made up of over 100 000 different compounds. In order to analyse these complexities one needs to have a separation method capable of screening the various substances of interest (Bartle & Myers, 2002).

Martin and Synge (1941), developed a method that use gas-liquid partitioning of a sample. As depicted in Figure 1, the carrier gas, also called the eluent, is introduced into the system. As the name suggests, the carrier gas is what is used to carry the sample through the system. The instrument has a pressure regulator, gas filters and flow controller, all to regulate the amount of gas that enters the system. Regulation of flow rate is important as it impacts the level of separation of the different compounds in the sample. The gas enters the machine through the inlet port. This is where gas meets the sample that is being injected in the heated injection port. The sample instantly vaporises into a gaseous form. The gaseous sample separates out into its constituent gases at different speeds inside the column which are recorded by the detector. The computer acquires this data and produces a chromatogram comprising of different peaks, representative of the different analytes observed (Chromacademy.com, 2019)

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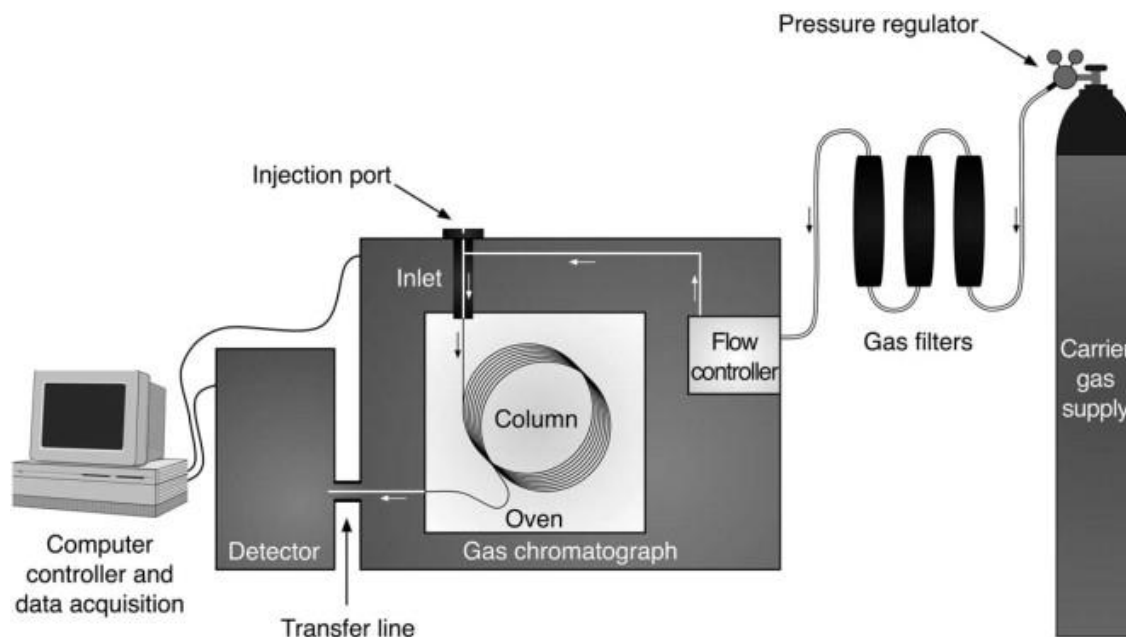
Figure 1. Basic components of a Gas Chromatograph (Stauffer *et al.*, 2008)

1.3.3. High performance liquid chromatography

As in gas chromatography, high performance liquid chromatography (HPLC) is also a separation method that is employed for separating highly complex mixtures. As seen in Figure 2, the system is regulated by the pumps, solvents (mobile phase) are pumped into the system at a continuous flow rate. The sample gets introduced into the flowing mobile phase by the injector. The sample is then carried by the mobile phase into the narrow column packed with a stationary phase which interacts with the analytes. Depending on the polarity of the sample being analysed, some components of the mixture will have a higher affinity to adsorb to the column stationary phase than the others. Some components will have a higher affinity for the mobile phase, therefore having less retention on the column and this is how separation is achieved. The elution of the different components from the column is then recorded by the detector and a chromatogram is produced (Chromacademy.com, 2019).
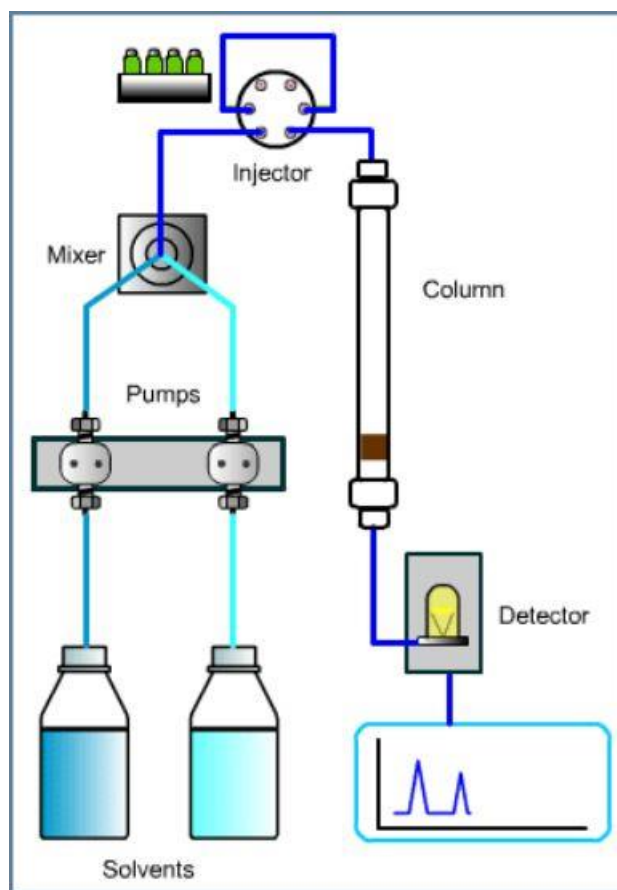
Figure 2. Basic component of HPLC (Chromacademy.com, 2019)

1.4. Instruments for detection

Detectors are designed to make use of the chemical attributes of either the solute or the mobile phase to measure and monitor substances after their separation. Choosing which detector to use is dependent on many characteristics. A compound with no chromophore groups (double bonds, aromatic rings etc.) will have no UV absorbance, and will therefore need a different means of detection (de Rijke *et al.*, 2006). There are also hyphenated techniques. These refer to the coupling of a separate independent analytical technology to an HPLC system. Liquid chromatography coupled to mass spectrometry (LC-MS) is the most common of these (Swartz, 2010).

1.4.1. Ultra violet detectors

Ultra violet (UV) detectors are compound specific property detectors that respond to compounds that absorb UV light at a particular wavelength. They are

used to measure the absorbance (190 nm to 600 nm) of light in the UV or visible range. Suitable analytes for UV detectors must contain an unsaturated bond, aromatic groups or heteroatom functional groups (Swartz, 2010).

1.4.2. Fluorescence detectors

Compounds that are naturally fluorescent or those that can be derivatised to fluoresce are excited to a higher energy wavelength and their optical emissions of light are measured by fluorescence (FL) detectors. FL detectors are much more sensitive than UV detectors for compounds with the ability to fluoresce. This sensitivity makes them ideal for the analysis of trace elements or in cases where a sample is very limited (Swartz, 2010).

1.4.3. Electrochemical detectors

Electrochemical detectors are particularly useful and sensitive for compounds that can be oxidized and reduced. They work by measuring the electrochemical reaction (oxidation or reduction) taking place across three electrodes (Swartz, 2010).

1.4.4. Mass spectrometry

Mass spectrometry (MS) is an advanced technique that makes use of ionisation methods that lead to the separation and identification of charged molecules. The basis of ionisation is being able to add or remove protons or electrons from a neutral molecule and thereby creating cationic or anionic species. Mass spectrometers are usually used in conjunction with HPLC and GC instruments (Chromacademy.com, 2019).

1.4.4.1.   Quadrupole Mass Analysers

Quadrupole mass analysers have a mass-selective action generated by an alternating electric field. The electric field is produced by four parallel rods with varying voltages that aid in the separation of ions according to their mass to charge ratio. As depicted in Figure 3, depending on the voltage running on the poles, some ions make it through to the analyser and some are deflected out (Batey, 2014).
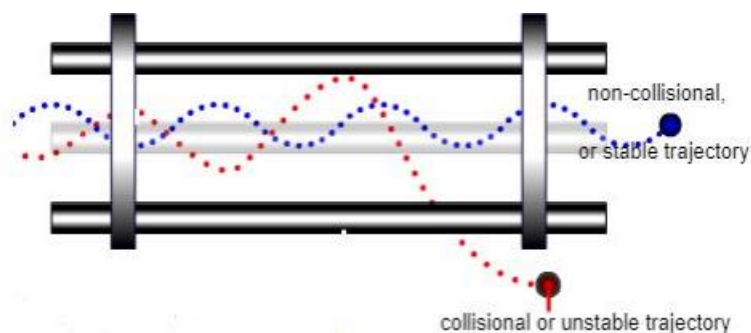
Figure 3. Stable ions moving through a quadrupole towards the analyser while unstable ions are deflected out (Chromatocademy.com, 2019)

1.4.4.2. Time-of-Flight Mass Analysers

Time-of-flight (TOF) mass analysers involve the production of ions within the ion source and subjecting these ions to an accelerating voltage. These accelerated ions fly down an evacuated tube of measured length. The main difference of TOF analysers to quadrupole mass analysers is the fact that there is no focusing device that selects particular ions to a particular flight path on TOF analysers. The bigger the ion the slower it will travel to the source (chromatocademy.com, 2019).

1.4.4.3. Ion Trap Mass Analysers

An ion trap mass analyser makes use of electric or magnetic fields to trap or capture ions. Depending on the amplitude of voltage the desired ions get stored in a "trap" while undesired ions leave the trap by a given trajectory applied by an electrostatic field (Stauffer et al., 2008).

1.4.4.4. Orbitrap

The orbitrap consists of three electrodes. On the outside facing each other are two cup shaped electrodes. A secured dielectric central ring separates the two electrodes apart. Ions are pumped into the space between the central and outer electrodes along a tangent. The right amount of voltage enables the ions to remain on a circular spiral inside the trap. The conical shape of the electrodes pushes ions to the widest part of the trap. The outer electrodes act as receiver plates for image current detection (Zubarev & Makarov, 2013).

1.4.4.5. Tandem mass spectrometry

Tandem mass spectrometry (MS/MS) is a method that involves different stages of mass analysis. As can be seen if Figure 4, the first analyser (Q1) isolates the precursor ion. The precursor ion undergoes fragmentation in the second analyser (Q2), the collision cell. The collision cell is operated at a mode that allows for ions to be focused (radio frequency – mode). It is usually filled with a neutral inert gas such as nitrogen or argon. The third analyser (Q3) is responsible for analysing yield product ions from the fragmentation that took place (Amorim Madeira & Helena, 2012).
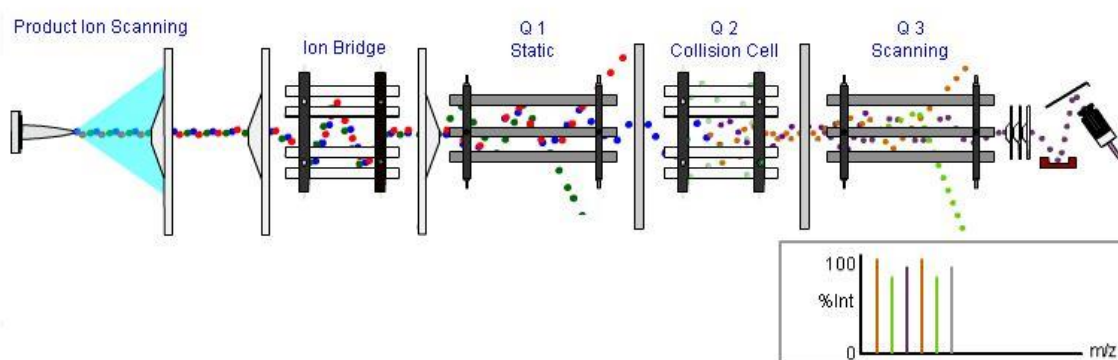
Figure 4. Principles of tandem mass spectrometry (chromacademy.com, 2019)

1.5. Pesticides

According to the Food and Agriculture Organization of the United Nations (FAO) a pesticide is "any substance or mixture of substances intended for preventing, destroying or controlling any pest, including vectors of human or animal disease". There are clear positive economic benefits to pesticide use. However, there are even greater negative health risks associated with them if one considers the very nature and design of pesticides, which is to cause harm or terminate certain organisms (Damalas & Eleftherohorinos, 2011).

Farmers and farm workers working directly with pesticides or those working on treated fields are at a greater health risk. The general population, wildlife and ecosystem is also at risk through continuous exposure to pesticide residues on food and contaminated drinking water (Damalas & Eleftherohorinos, 2011).
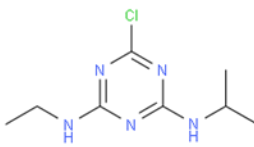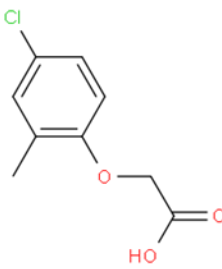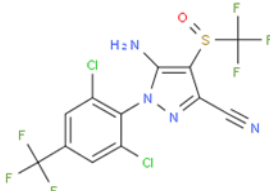
As is the case in many developing countries, South Africa has a huge problem with an increasing informal market for selling pesticides on the streets. An explorative study showed that toxic pesticides such as aldicarb, methamidophos and chlorpyrifos could be accessed easily on the street markets of Cape Town. According to the study, these markets include, but are not limited to, taxi stations, train stations, on trains and door-to-door. More concerning is the fact that it is predominantly the youth that is responsible for the sale and distributions of these pesticides on the streets. In South Africa, this market is increasing and will continue to rise because of high rates of unemployment and poverty. Poverty related conditions such as informal settlements, inadequate sewerage provisions, improper toilet facilities and uncollected refuse result in pest infestations. These issues in turn increase the demand for street market pesticides as they are seen as the cheaper and more effective option as opposed to the commercially available products. Street pesticides are more effective in the control of pests because they are sold in stronger formulations. The active ingredients are at a higher concentration than legally permitted. This subsequently results in a higher health risk than would normally be observed with commercially available product. Furthermore, they are sold in inappropriate and unsafe containers used for beverage, which can lead to accidental consumption by small children (Rother, 2010). According to the WHO, over 30% of the world's disease burden in children can be attributed to pesticides (WHO, 2006). A survey conducted at the Red Cross War Memorial Children's Hospital (RCWMCH) in Cape Town (South Africa) showed a total of 311 pesticide poisoning cases seen over a single year in children younger than 14 years. Accidental ingestion of pesticides in children is high due to their lack of maturity and inability to assess risks. Children are less able to detoxify or expel harmful chemicals and therefore are more vulnerable to adverse health effects of pesticides (Lekei *et al.*, 2017). Another study conducted over a 3 year period in the Tshwane District (South Africa) reported a total of 207 organophosphate poisoning cases. Of these, 51% were intentional poisoning cases, 21.7% were unintentional cases and 26.5% were of unknown circumstances. Data collected from this study was dependent on a national surveillance system where medical practitioners and nurses are required by the law to report any agricultural or stock remedy poisoning. However, due to time constraints and lack of knowledge and diligence, this reporting didn't always happen. Therefore, the magnitude and severity of poisoning in South Africa is misrepresented due to the underreporting of cases (Razwiedani & Rautenbach, 2017).
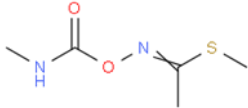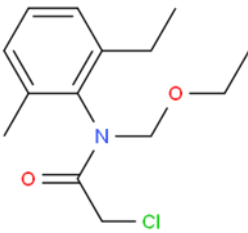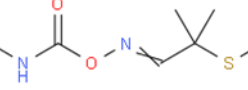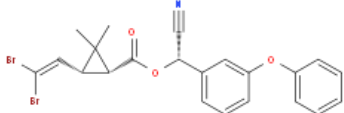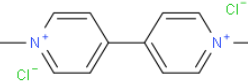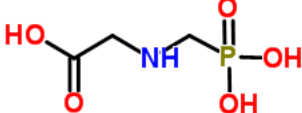
Effects associated with short-term pesticide exposure include blindness, headaches, rashes, dizziness, blisters, nausea, and stinging eyes (Farmworker Justice, 2013). Mid- to long-term pesticide exposures can lead to adverse health risks such as carcinogenicity, neurotoxicity and reproductive toxicity (Schummer *et al.*, 2012).

The process of correctly and accurately assessing levels of exposure to organic toxicants such as pesticides is not an easy one. There are many different approaches that can be considered when assessing this exposure. However, being able to detect chemicals and their metabolites in biological matrices, also called biomonitoring, remains the most effective. Biomonitoring offers the advantage of giving the internal dose of exposure and also takes into account all possible sources and routes of entry (Appenzeller *et al.*, 2017).

Guided by Crop Life SA, an organisation that has the knowledge of which pesticides are currently in use in South Africa, and the Tygerberg Poison Information Centre (TPIC), a centre which deals with poisoning cases on a daily basis, a list of pesticides to be included in this study was compiled. The list comprised of the following pesticides: Atrazine, 2-methyl-4-chlorophenoxyacetic acid (MCPA), imidacloprid, fipronil, methomyl, acetochlor, aldicarb, glyphosate, deltamethrin and paraquat.

Table 2. List of pesticides considered for the screening tool

Compound name	IUPAC name	Chemical Structure
Atrazine	2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine	
MCPA	2-Methyl-4-chlorophenoxyacetic acid	
Fipronil	(±)-5-amino-1-(2,6-dichloro-α,α,α-trifluoro-p-tolyl)-4-trifluoromethylsulfinylpyrazole-3-carbonitrile	

Methomyl	methylN-(methylcarbamoyloxy)ethanimidothioate	
Acetochlor	2-chloro-N-(ethoxymethyl)-N-(2-ethyl-6-methylphenyl) acetamide	
Aldicarb	2-Methyl-2-(methylthio)propanal O-(N-methylcarbamoyl)oxime	
Deltamethrin	(-(S)-α-cyano-3-phenoxybenzyl (1R,3R)-3-(2,2-dibromovinyl)-2,2-dimethylcyclopropanecarboxylate)	
Paraquat	(N,N′-dimethyl-4,4′-bipyridinium dichloride)	
Glyphosate	(N-(phosphonomethyl) glycine)	

1.5.1. Atrazine

Atrazine is a synthetic odourless white powder and is used as an herbicide to kill weeds. It is mainly used for the following crops: sugarcane, corn, pineapples, sorghum and on evergreen forests. Due to the fact that atrazine is one of the most popularly used herbicides, people living in areas close to where these crops are grown become exposed. In many cases, atrazine is found in high amounts in the air that is close to waste disposal sites. Atrazine and its metabolites can be detected in urine, plasma and organ tissues (ATSDR, 2003).

1.5.2. MCPA

MCPA is a synthetic herbicide used to kill weeds both on land and in water. It is available in a liquid and powder form. People come into contact with MCPA while walking or playing on treated lawns, golf courses, swimming pools, for example. MCPA enters the blood stream through absorption from the skin and gastrointestinal tract (GIT) (ATSDR & EPA, 2017). Most common symptoms of

MCPA poisoning include headaches, nausea, weakness and dizziness (Karanth, 2014).

1.5.3. Imidacloprid

Imidacloprid is designed to replace the orgaphosphorus pesticides. It is commonly used on potatoes, sunflowers, rice, maize, rape, fruits, vegetables, and sugar beets (Suchail *et al.*, 2000).

There is very little data on the toxicity of imidacloprid. However, an HPLC analysis study done on 56 Imidacloprid poisoning cases was able to describe acute imidacloprid poisoning in plasma in relation to clinical outcomes. While there were no reported deaths, most patients only had mild symptoms such as nausea, vomiting, headache and diarrhoea (Mohamed *et al.*, 2009).

1.5.4. Fipronil

Fipronil is used both domestically and agriculturally to treat pests (Das *et al.*, 2006). Domestically, fipronil is mostly used in veterinary medicine to treat dogs for fleas and ticks. There is little work done on fipronil toxicity in humans. A recent study conducted by Peluso (2018) was able to detect fipronil and its metabolite in dog plasma by HPLC-UV (Bell, 2018). An older study was also able to make use of LC-UV/MS/MS to quantify fipronil and its metabolite in rat plasma (Lacroix *et al.*, 2010).

1.5.5. Methomyl

Methomyl is a broad-spectrum insecticide used through foliar treatment of vegetables and fruits for the control of ticks and spiders (Farré *et al.*, 2002). Methomyl has been classified as a chemical of public health concern by the WHO (WHO, 2014). Methomyl toxicity is mainly reported in accidental and suicidal cases. In one such case, methomyl was detected in the blood six hours post ingestion (International Programme On Chemical Safety, 1996).

1.5.6. Acetochlor

Acetochlor is a herbicide primarily used for broad leaf crops. There is very little data available that describes acetochlor toxicity on the general population and the farming community. Liu *et al.*, (2017) studied the various toxic effects of acetochlor on zebrafish. Due to the strong genetic similarities (up to 70%)

between zebrafish genes and humans, characterizing toxicity on zebrafish is useful. This study showed that the primary toxic target organ was the heart. Acetochlor reduced the heart rate, induced thrombosis and a histopathological examination confirmed that acetochlor-treated zebrafish had a malformed heart (Liu *et al.*, 2017).

1.5.7. Aldicarb

Aldicarb, also known as Temek™, is a carbamate insecticide used to control nematodes and insects on crops such as citrus fruits, ornamental plants, sugar cane, cotton and potatoes (El-Alfy & Schlenk, 1998).

As with methomyl, aldicarb has also been classified as a chemical of public health concern by the WHO. It has been classified under pesticide class 1A - pesticides that are extremely hazardous (WHO, 2014).

Carbamate insecticides such as aldicarb are absorbed across the lungs, the gut and the skin and get into the blood. They are known to inhibit pseudo cholinesterase and acetylcholinesterase activity resulting in the inactivation of acetylcholine. This results in the accumulation of acetylcholine at nerve junctions (Micromedex, 2019). Consequently this accumulation may result in convulsions and death (El-Alfy & Schlenk, 1998).

1.5.8. Glyphosate

Glyphosate, also known as Roundup™, is a one of the most popular and widely used herbicides in the world (Shaner*et al.*, 2012). This can be attributed to its effectiveness on weed control and its use results in cleaner crops during the harvest period (Huber, 2007).

Glyphosate has a moderate toxicity and is poorly absorbed into the body through dermal and oral routes. In mammals, glyphosate is mostly excreted unchanged. Mice fed glyphosate for 90 days showed a small body weight loss and microscopic changes in liver and kidneys. High levels of human exposure to glyphosate can lead to nephrotoxicity. This has been shown in patients who had developed acute kidney injury due to glyphosate ingestions (Patocka, 2018).

1.5.9. Paraquat

Paraquat, also known as Methyl Viologen ™, is an organophosphorus non-selective herbicide that is relatively inexpensive resulting in its widespread use in many developing countries.

Paraquat is considered to be highly toxic. It is the most common pesticide for suicide cases in developing countries. This trend is not only seen in developing countries, as paraquat was shown to be responsible for up to 56% of all pesticide deaths in England and Wales (Gawarammana & Buckley, 2011). It is associated with a high mortality due to respiratory failure as the primary target organs are the lungs and kidneys (Ntshalintshali & Manzini, 2017).

1.5.10.   Deltamethrin

Deltamethrin is an insecticide used for the treatment of a wide range of insects in gardens, lawns and golf courses. However, the greatest use of deltamethrin is in cotton farming and it has been classified as a hazardous chemical falling under Class II (WHO, 2014).

Deltamethrin is absorbed mainly through the oral and dermal routes and is said to cause ataxia, loss of coordination, hyperexcitability, convulsions and can lead to paralysis (Patro *et al.*, 2009).

1.6. Therapeutic drug toxicity

In developing countries self-poisoning with therapeutic drugs is very common. Self-poisoning is when an individual ingests or inhales an amount of substance that has a great potential to cause harm. Accidental self-poisoning can also occur when an individual has little understanding of the substance they might be exposing themselves to (Camidge *et al.*, 2003). People intentionally self-poison themselves to escape from complex and harsh realities they might be dealing with. Intentionally self-poisoning happens worldwide and across all income brackets. In 2015, intentional self-poisoning was ranked 6th of all non-natural deaths in South Africa (van Hoving *et al.*, 2018).
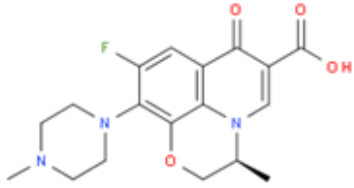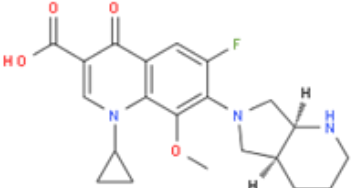
On presentation, clinicians often have to rely on information given by the patient, friends and family about the ingested agent. To test the reliability of patient history,
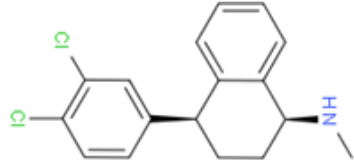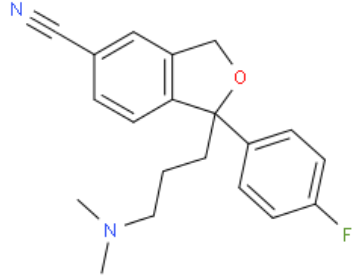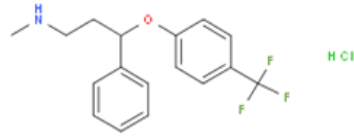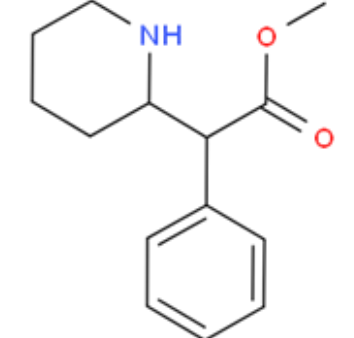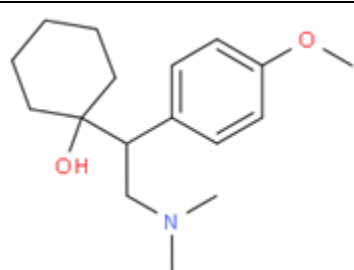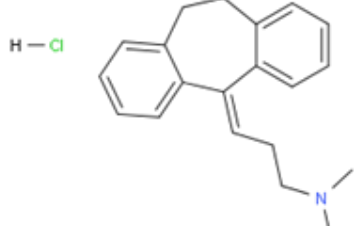
Pohjola-Sintonen *et al.* (2000) conducted a study using 51 cases of acute, deliberate drug poisonings. The study found that only 27% of all the cases had toxicological results in agreement with the information gathered on admission. The over-reliance on history has the potential to put patients in danger as it is used to guide clinical management. The employment of toxicological screening tools will decrease the cost involved in effectively treating poisoned patients and could improve and facilitate the optimal treatment of acute poisoning cases (Pohjola-Sintonen *et al.*, 2000).
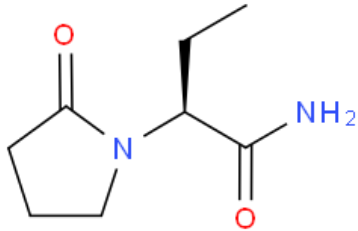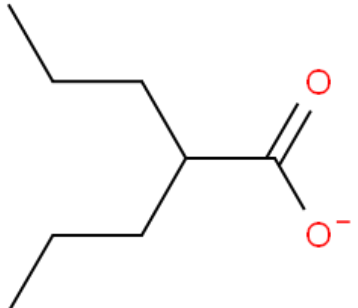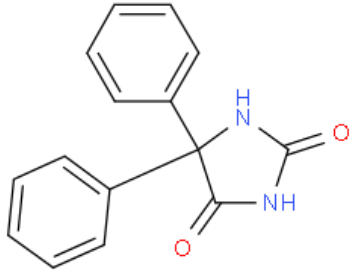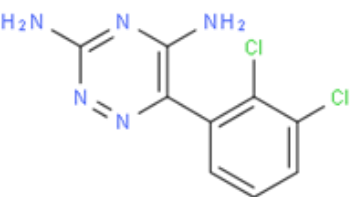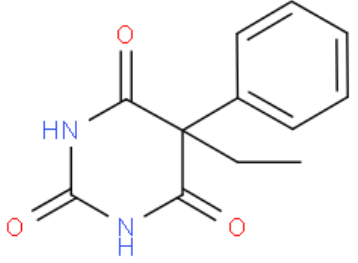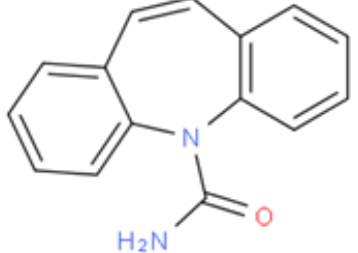
Due to the abundance and diversity of therapeutic drugs on the market today, a need was identified for a toxicological screening tool relevant to South Africa. In choosing which drugs were relevant, the Tygerberg Poison Information Centre (TPIC), a centre which deals with poisoning cases on a daily basis, was consulted. Furthermore, clinicians in the Division of Clinical Pharmacology at Stellenbosch University, who also deal with patients admitted at Tygerberg Hospital daily, were also consulted with regards to the most frequent observed agents involved in poisonings or overdoses.

Due to the large number of drug classes being investigated, the information has been divided between Tables 2 and 3. Inorganic compounds such as arsenic, lead, and thallium were initially added to the tables but were subsequently removed as they would require inductively coupled plasma mass spectrometry (ICP-MS) for analysis. Table 2 below indicates the list of five therapeutic drug categories considered for the toxicological screening method.
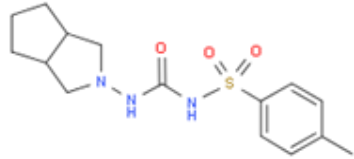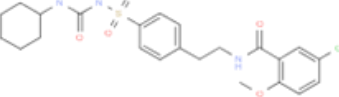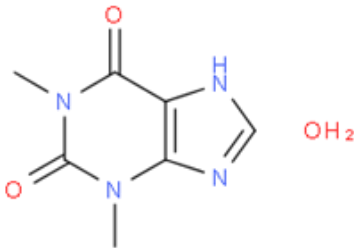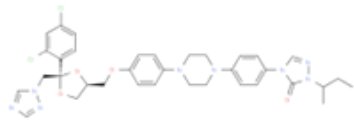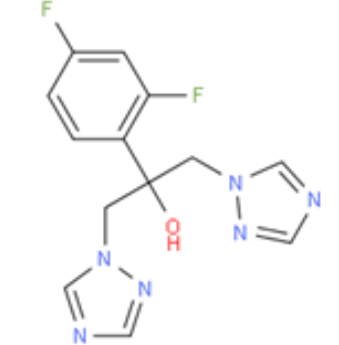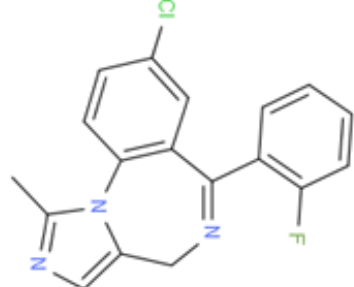
Table 3. List of therapeutic drugs considered for the screening tool
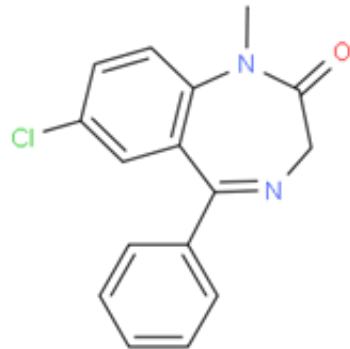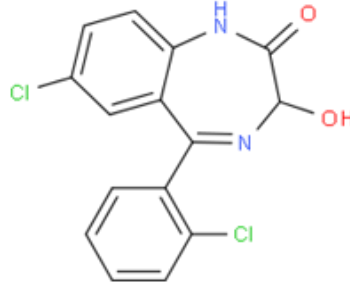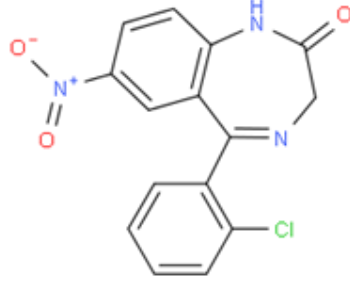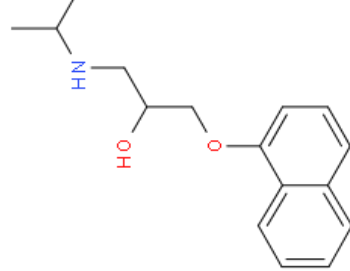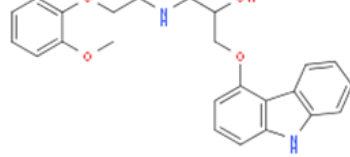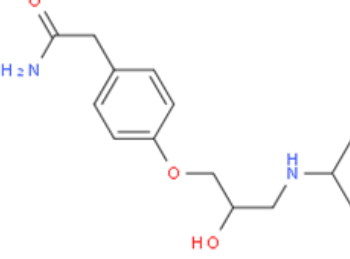
Class	Compound name	IUPAC name	Chemical Structure
Antibiotics	Levofloxacin	(2*S*)-7-fluoro-2-methyl-6-(4-methylpiperazin-1-yl)-10-oxo-4-oxa-1-azatricyclo[7.3.1.05,13]trideca-5(13),6,8,11-tetraene-11-carboxylic acid	
	Moxifloxacin	7-[(4a*S*,7a*S*)-1,2,3,4,4a,5,7,7a-octahydropyrrolo[3,4-b]pyridin-6-yl]-1-cyclopropyl-6-fluoro-8-methoxy-4-oxoquinoline-3-carboxylic acid	
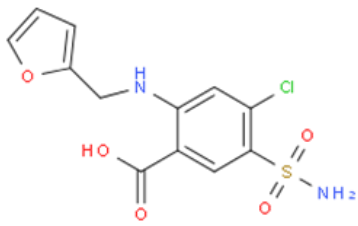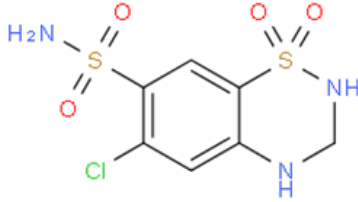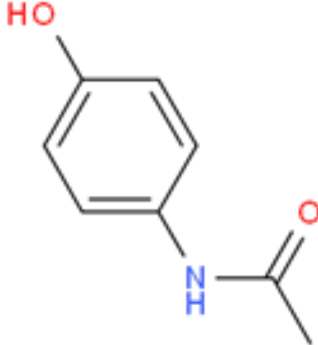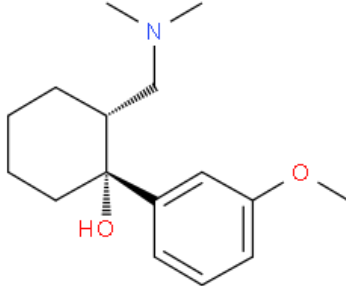
Psychiatric	Sertraline	(1*S*,4*S*)-4-(3,4-dichlorophenyl)-N-methyl-1,2,3,4-tetrahydronaphthalen-1-amine	
	Citalopram	1-[3-(dimethylamino)propyl]-1-(4-fluorophenyl)-3H-2-benzofuran-5-carbonitrile	
	Fluoxetine Hydrochloride	N-methyl-3-phenyl-3-[4-(trifluoromethyl)phenoxy]propan-1-amine;hydrochloride	
	Methylphenidate	methyl 2-phenyl-2-piperidin-2-ylacetate	
	Venlafaxine	1-[2-(dimethylamino)-1-(4-methoxyphenyl)ethyl]cyclohexan-1-ol	
	Amitriptyline Hydrochloride	N,N-dimethyl-3-(2-tricyclo[9.4.0.03,8]pentadeca-1(15),3,5,7,11,13-hexaenylidene)propan-1-amine;hydrochloride	

Anticonvulsants	Levetiracetam	(2S)-2-(2-oxopyrrolidin-1-yl)butanamide	
	Valproic Acid	2-propylpentanoic acid	
	Phenytoin	5,5-diphenylimidazolidine-2,4-dione	
	Lamotrigine	6-(2,3-dichlorophenyl)-1,2,4-triazine-3,5-diamine	
	Phenobarbital	5-ethyl-5-phenyl-1,3-diazinane-2,4,6-trione	
	Carbamazepine	benzo[b][1]benzazepine-11-carboxamide	

Atypical Antipsychotics	Olanzapine	2-methyl-4-(4-methylpiperazin-1-yl)-10H-thieno[2,3-b][1,5]benzodiazepine	
	Aripiprazole	7-[4-[4-(2,3-dichlorophenyl)piperazin-1-yl]butoxy]-3,4-dihydro-1H-quinolin-2-one	
	Clozapine	3-chloro-6-(4-methylpiperazin-1-yl)-11H-benzo[b][1,4]benzodiazepine	
	Risperidone	3-[2-[4-(6-fluoro-1,2-benzoxazol-3-yl)piperidin-1-yl]ethyl]-2-methyl-6,7,8,9-tetrahydropyrido[1,2-a]pyrimidin-4-one	
	Haloperidol	4-[4-(4-chlorophenyl)-4-hydroxypiperidin-1-yl]-1-(4-fluorophenyl)butan-1-one	
	Quetiapine	2-[2-(4-benzo[b][1,4]benzothiazepin-6-ylpiperazin-1-yl)ethoxy]ethanol	
Hypoglycaemics	Metformin	3-(diaminomethylidene)-1,1-dimethylguanidine	

	Gliclazide	1-(3,3*a*,4,5,6,6*a*-hexahydro-1*H*-cyclopenta[c]pyrrol-2-yl)-3-(4-methylphenyl)sulfonylurea	
	Glibenclamide	5-chloro-N-[2-[4-(cyclohexylcarbamoylsulfamoyl)phenyl]ethyl]-2-methoxybenzamide	
Bronchodilators	Theophylline	1,3-dimethyl-7H-purine-2,6-dione	
Antifungals	Itraconazole	2-butan-2-yl-4-[4-[4-[4-[[2-(2,4-dichlorophenyl)-2-(1,2,4-triazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy]phenyl]piperazin-1-yl]phenyl]-1,2,4-triazol-3-one	
	Fluconazole	2-(2,4-difluorophenyl)-1,3-bis(1,2,4-triazol-1-yl)propan-2-ol	
Psychoactives	Midazolam	8-chloro-6-(2-fluorophenyl)-1-methyl-4H-imidazo[1,5-a][1,4]benzodiazepine	

	Diazepam	7-chloro-1-methyl-5-phenyl-3H-1,4-benzodiazepin-2-one	
	Lorazepam	7-chloro-5-(2-chlorophenyl)-3-hydroxy-1,3-dihydro-1,4-benzodiazepin-2-one	
	Clonazepam	5-(2-chlorophenyl)-7-nitro-1,3-dihydro-1,4-benzodiazepin-2-one	
Cardiovascular	Propranolol	1-naphthalen-1-yloxy-3-(propan-2-ylamino)propan-2-ol	
	Carvedilol	1-(9H-carbazol-4-yloxy)-3-[2-(2-methoxyphenoxy)ethylamino]propan-2-ol	
	Atenolol	2-[4-[2-hydroxy-3-(propan-2-ylamino)propoxy]phenyl]acetamide	

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	Furosemide	4-chloro-2-(furan-2-ylmethylamino)-5-sulfamoylbenzoic acid	
	Hydrochlorothiazide	6-chloro-1,1-dioxo-3,4-dihydro-2H-1λ6,2,4-benzothiadiazine-7-sulfonamide	
Analgesics	Paracetamol	N-(4-hydroxyphenyl)acetamide	
	Tramadol	(1R,2R)-2-[(dimethylamino)methyl]-1-(3-methoxyphenyl)cyclohexan-1-ol	

In the following section, a brief overview has been given of each of the drug classes included. The vast majority of the drug discussions below were obtained from Micromedex. Micromedex is one of the largest online reference databases and has been active for over 45 years (Micromedexsolutions.com). Micromedex is a comprehensive resource that is utilized by both the PIC and by the clinicians of the Division Clinical Pharmacology, Stellenbosch University. This resource has information for all FDA approved drugs and some that were approved by EMA and Health Canada.

1.6.1. Antibiotics

Antibiotics, also called antimicrobials, are a group of drugs used to treat of a whole host of bacterial infections. They can be taken orally as tablets or as vaginal, ophthalmic, and topical skin preparations (Caravati, *et al.* 2004).

Levofloxacin and moxifloxacin are quinolone antibiotics particularly for urinary tract infections, bacterial induced diarrhoea and bacterial prostatitis. Their introduction has not only increased coverage for the treatment of traditional Gram-negative bacterial infections, but also for certain Gram-positive organisms (Caravati, *et al.* 2004). South Africa has a very high burden of multi-drug resistant tuberculosis (MDR TB), and levofloxacin and moxifloxacin are indicated in the national treatment guidelines for the treatment of MDR TB (Naidoo *et al.*, 2017).

The mechanism of action of levofloxacin and moxifloxacin is the inhibition of bacterial topoisomerase IV and DNA gyrase. These are the enzymes required in DNA replication, transcription, repair and recombination. Overdoses and side effects include nausea, vomiting, dizziness, drowsiness, disorientation, slurred speech, acute renal failure and distal nephron apoptosis (Micromedex.com, 2019).

1.6.2. Psychiatrics

The most common mental illnesses affecting ~15% of the population are depression and anxiety. Safer and more selective antidepressants and anxiolytics have been developed and as a result, psychiatry as an exclusive domain has shifted to other medical specialities. It is possible for depression and anxiety to affect a patient simultaneously, which suggest that they might have a common pathophysiological mechanism (Brunton *et al*, 2018).

Drugs listed under this category are antidepressants used for depressive disorders, panic attacks, anxiety, obsessive-compulsive disorders, premenstrual dysphoric syndrome, bulimia nervosa and posttraumatic stress disorders (Micromedex.com, 2019).

Sertraline's mode of action is to inhibit the central nervous system (CNS) neuronal uptake of serotonin. Severe toxicity is marked by CNS depression (Micromedex.com, 2019).

Citalopram potentiates serotonergic activity in the CNS and has greater selectivity than other antidepressants. Severe toxicity can cause rigidity, hypertension, seizures, hyperthermia or hypotension, influence electrical properties of the heart, coma, and in rare instances, can cause death (Micromedex.com, 2019).

Fluoxetine hydrochloride is a "second generation" antidepressant that specifically inhibits serotonin reuptake while having little effect on the noradrenergic system. Severe poisoning can lead to significant CNS depression and seizures (Micromedex.com, 2019).

Methylphenidate is a moderate CNS stimulant that has properties similar to amphetamines. It is mainly used for ADHD and narcolepsy control. Severe toxicity is characterized by life-threatening hyperthermia (Micromedex.com, 2019).

Venlafaxine inhibits the neuronal reuptake of serotonin and norepinephrine in the CNS. An increase in serotonin can lead to cardiovascular toxicity and seizures (Micromedex.com, 2019).

Amitriptyline hydrochloride is a tricyclic antidepressant primarily used for major depression and has sedative properties for chronic pain syndromes. It acts by blocking the membrane pump that is responsible for the absorption of serotonin and norepinephrine. Severe toxicity can lead to a coma, seizures, QRS prolongation with ventricular dysrhythmias, respiratory failure, and hypotension (Micromedex.com, 2019).

1.6.3. Anticonvulsants

Anticonvulsants also called antiseizure drugs (ASD), are used in the treatment of myoclonic seizures, partial onset seizures, and generalized tonic-clonic seizures. Ideally, a drug with the potential to suppress all seizures is always selected during treatment. However, current drugs fail to control seizures in ~33% of patients and frequently lead to unwanted adverse effects. These effects can range from a mild impairment of the CNS to death from aplastic anaemia or hepatic failure. In order to minimize toxicity, it is always recommended that a single ASD be administered at a time (Brunton *et al.*, 2018).

Levetiracetam was first developed as a medication for Alzheimer's disease. Although the exact mechanism of action remains largely unknown, it has been shown that levetiracetam acts via an unknown stereoseletive binding site in the brain. Severe toxicity can lead to a coma, bradycardia, hypotension, and respiratory depression (Micromedex.com, 2019).

Valproic acid is an anticonvulsant used to treat a broad range of seizure disorders and is also used as a mood stabiliser in the treatment of bipolar disorder. It acts by inhibiting the voltage-gated sodium channels, the T-type calcium channels and GABA transaminase. An overdose can lead to bone marrow suppression and severe poisoning can lead to severe CNS depression, coma, miotic pupils, tachycardia, hypotension, QTc prolongation, and respiratory depression (Micromedex.com, 2019).

Phenytoin is an anticonvulsant that is used in the prevention of epilepticus and for seizures. It acts by increasing the sodium efflux from the neurons of the motor cortex. Severe toxicity is characterized by CNS depression, coma, and, rarely, respiratory depression (Micromedex.com, 2019).

Lamotrigine is used in the treatment of seizures and Bipolar I Disorder. It has a pharmacological profile that is very similar to phenytoin. Although the exact mechanism is not fully understood, just like phenytoin, it is proposed that it inhibits sodium channels. Severe toxicity leads to tachycardia and conduction disturbances, hypokalaemia, oculogyric crisis, seizures, rhabdomyolysis, encephalopathy, coma, and respiratory depression (Micromedex.com, 2019).

Phenobarbital acts as a long-acting barbiturate by depressing the CNS and results in sedation and a reduction in seizures. Severe toxicity includes coma, hypotension, decreased myocardial contractility, hypothermia and respiratory failure (Micromedex.com, 2019).

Carbamazepine is a primary drug used for the treatment of generalized tonic-clonic, focal-to-bilateral tonic-clonic, focal seizures and trigeminal neuralgia (Brunton *et al.*, 2018). Its mechanism of action is unknown and severe toxicity is marked by seizures, coma and respiratory depression (Micromedex.com, 2019).

1.6.4. Atypical antipsychotics

Psychosis is a form of mental illness marked by the lack of sense of reality. There are a variety of causes that can lead to psychosis all of whom each require a unique treatment approach. The most common psychotic disorders are mood disorders, substance induced psychosis, dementia with psychotic features, delusional disorder, delirium and schizophrenia (Brunton *et al.*, 2018). The drugs in this category are mainly used to treat schizophrenia patients and bipolar I disorder patients and is used for rapid sedation in patients with undifferentiated and acute agitation.

Olanzapine's mechanism of action is unknown. It is believed to be mediated through dopamine and serotonin type 2 antagonism in schizophrenia. Severe toxicity is marked by seizures, delirium, coma, respiratory depression, hypotension, oculogyric crisis, and central diabetes insipidus (Micromedex.com, 2019).

Aripiprazole has a partial agonist activity at the dopamine D2 and serotonin 5-HT2 receptors. Overdose results in hypotension, coma, and neuroleptic malignant syndrome (Micromedex.com, 2019).

Clozapine is used for treatment-resistant schizophrenia. In addition to the same mechanism of action as in aripiprazole, clozapine also slightly reduces dopamine binding at D1, D3, and D5 receptors, and moderately at D4 receptors. Severe toxicity involves the CNS and cardiovascular system and may include, ventricular dysrhythmia, coma, hyperthermia, severe hypotension, delirium myoclonus, muscle rigidity and seizures (Micromedex.com, 2019).

Risperidone is a benzisoxazole derivative that has a high antagonist affinity for dopamine (D2) and serotonin (5-HT2) receptors. Severe toxicity results in QTc prolongation, respiratory depression, seizure, coma and neuroleptic malignant syndrome (Micromedex.com, 2019).

Haloperidol is a butyrophenone whose mechanism of action is not clearly understood. Severe toxicity results in CNS depression that can lead to a coma (Micromedex.com, 2019).

Quetiapine's mode of action is not known. However, the results it induces in schizophrenia and its mood stabilization properties are also due to the antagonism of D(2) and 5HT(2) receptors. Severe poisoning is marked by CNS depression, pronounced sinus tachycardia, urinary retention, seizures and/or myoclonic jerks (Micromedex.com, 2019).

1.6.5. Hypoglycaemics

Hypoglycaemia is a low blood sugar condition mainly affecting people with diabetes. Hypoglycaemia is usually as a result of over dosage with insulin, sulfonylureas or other antidiabetic drugs (Caravati, *et al.*, 2004).

Metformin is used to improve glucose tolerance in patients affected by type II diabetes mellitus. It acts by lowering the basal and postprandial plasma glucose. Severe toxicity results in confusion, severe lactic acidosis, mental status depression, hypothermia, hypotension and renal failure. In rare instances it may result in respiratory insufficiency, ventricular dysrhythmias and death (Micromedex.com, 2019).

Gliclazide is a second-generation sulphonylurea oral hypoglycemic drug, meaning that it is more potent than the first-generation drugs. The mechanism of action is the stimulation of insulin secretion from pancreatic beta cells, and possibly via direct effects on intracellular calcium transport. It can accumulate in patients with severe hepatic and renal dysfunction resulting in dizziness, lack of energy, drowsiness, and headaches (Micromedex.com, 2019).

Glibenclamide acts by lowering the blood glucose by activating the release of insulin from the pancreas. Toxicity results in severe CNS symptoms such as seizures, altered mental status, delirium, focal neurologic effects, and coma (Micromedex.com, 2019).

1.6.6. Bronchodilators

This group of drugs is responsible for reversing airway obstruction by relaxing the smooth muscles in the constricted airways in asthma (Brunton *et al.*, 2018).

Theophylline is used to treat asthma and chronic obstructive pulmonary disease. It is mainly used to treat neonatal apnoea of preterm infants. As mentioned above, theophylline induces bronchodilation through smooth muscle relaxation

and suppression of airway stimuli. Poisoning is uncommon and severe toxicity includes seizures, rhabdomyolysis, hypotension, and ventricular dysrhythmias (Micromedex.com, 2019).

1.6.7. Antifungals

The kingdom fungi comprises of over 200 000 known species. Human fungal infections contribute to significant morbidity and mortality. Life-threatening fungal infections have increased over the years. This is the case because of a large number of patients that are immunocompromised due to other illnesses such as cancer, organ transplantation as well as those patients affected by the human immunodeficiency virus and acquired immunodeficiency syndrome (HIV/AIDS) (Brunton *et al.*, 2018).

Itraconazole and fluconazole are used in the treatment of Cryptococci meningitis and oropharyngeal, oesophageal, and vaginal candidiasis. They act by inhibiting cytochrome P450 enzymes and the demethylation of ergosterol, all of which are responsible for fungistatic activity. Overdoses and poisoning are rare and unexpected. Adverse effects include seizures, vomiting, nausea, diarrhoea, abdominal pain, thrombocytopenia, visual changes, hypokalaemia, dizziness, hepatotoxicity, congestive heart failure, and delirium (Micromedex.com, 2019).

1.6.8. Psychoactives

Psychoactives also known as benzodiazepines are used for variety of medical conditions and their therapeutic effects results from their work on the CNS. The main therapeutic effects of benzodiazepines are sedation, hypnosis, reduction in anxiety, muscle relaxation, anterograde amnesia, and anticonvulsant activity (Brunton *et al.*, 2018).

Benzodiazepines are in the top 200 generic drugs ranked by number of prescriptions written. Lorazepam, clonazepam and diazepam were ranked 17, 23 and 41 respectively. Physiological and psychological dependence has been shown to lead to misuse and abuse (Marin *et al.*, 2008). They are amongst the most common drugs used in self-poisoning cases in the world and have been shown to contribute up to 55% of fatal poisoning cases (Caravati, *et al.* 2004). Benzodiazepine overdose is very common and is usually in combination with other drugs (Micromedex.com, 2019).

The mechanism of action of benzodiazepines is not fully understood but is thought to potentiate GABAergic neurotransmission and results in the binding of the GABA-A receptor binding site. An overdose may result in a coma, respiratory depression, hypotension, hypothermia, and rhabdomyolysis (Micromedex.com, 2019). The following benzodiazepines were considered: Midazolam, diazepam, lorazepam and clonazepam.

1.6.9. Cardiovascular

The following drugs were selected under this category: propranolol, carvedilol, atenolol, furosemide and hydrochlorothiazide. These drugs are used for a variety of heart related conditions.

Propranolol, carvedilol and atenolol are β-blockers used in the treatment of kathisia, essential tremor, hypertrophic subaortic stenosis, pheochromocytoma, stable angina, tachydysrhythmias, thyrotoxicosis, congestive heart failure, congenital heart conditions, migraine headache prophylaxis and variceal haemorrhage prophylaxis. β-blockers's mechanism of action is the blockage of cardiac beta-receptors. This blockage results in a negative inotropic and chronotropic effect. As a result the cardiac output becomes reduced causing a reduction in blood pressure (Brunton *et al.*, 2018).

Furosemide and hydrochlorothiazide are diuretics. They are used to treat hypertension and congestive heart failure. Furosemide is an inhibitor of Na^+, K^+ and $2Cl^-$ symport, while Hydrochlorothiazide is an inhibitor of Na^+ and $2Cl^-$ symport.

1.6.10. Analgesics

Analgesic are drugs used in the management of pain. They exert their action by actively acting on the pain receptors.

Paracetamol is the most commonly used analgesic in the world and is responsible for a large percentage of the overall overdose and poisoning cases worldwide (Chiew *et al.*, 2018; Cairns *et al.*, 2019; Thanacoody & Anderson, 2020). Paracetamol is said to reduce pain by inhibiting cyclooxygenase (COX) which results in the elevation of pain threshold. Furthermore, paracetamol also leads to a reduction in fever by inhibiting the formation and release of prostaglandins in the CNS and by inhibiting the thermoregulatory center. Severe

toxicity includes liver failure, renal injury and a massive overdose can lead to a coma, hyperglycaemia and lactic acidosis (Micromedex.com, 2019).

Tramadol is an opioid analgesic for the treatment of pain that is commonly abused for euphoric effects. For a quick euphoric effect, abusers may inject, snort or smoke this drug. Severe toxicity leads to respiratory depression leading to apnea, hypoxia, coma, possibly hypotension or bradycardia, non-cardiogenic pulmonary edema (Micromedex.com, 2019).

1.7. The impact of poisoning on the healthcare system

South Africa has a health system that is overloaded with many cases of HIV/AIDS-related infections, maternal and child mortalities, and high incidents of violence-related injuries (Rowe, 2016; van Hoving *et al.*, 2018). In 2018, the former minister of Health Dr. Aron Motswaledi acknowledged that the country's health system was very distressed and attributed the ailing system to the exponential growth of the burden of disease and the severe shortage of human resources (Dhai & Mahomed, 2018). Large volumes of acute poisoning cases, estimated to be as much as 500000 annually, are becoming a heavy load on this overburdened system (Marks & van Hoving, 2016). At the forefront of these poisonings are pharmaceuticals and pesticides (Veale *et al.*, 2013). To diagnose and treat these cases, health workers have to physically examine patients and most often have to rely on the information given by the patient, friends, and family about the ingested agent. Determining which poisoning agent is involved is crucial in deciding which medical course of action needs to be followed. Different drugs can induce similar effects on the body and information about the patient's history is often incomplete or unreliable. It is for these reasons that analytical screening tools have to be employed to enable medical practitioners to make informed decisions regarding the best course of treatment for patients (WHO, 1995; Pohjola-Sintonen *et al.*, 2000). About 84% of patients in South Africa access healthcare in the public sector (Dhai & Mahomed, 2018). These are the most vulnerable and poorest of the population. This makes it clear that not only must these analytical screening tools be efficient, rapid, and robust, they must also be affordable.

1.8. Aims and objectives

A survey conducted at Tygerberg Poison Information Centre (TPIC) over a single year period showed that the TPIC dealt with 4 771 consultations related to human

exposure to various poisonous substances. Of these substances, 35.2% were medicines, and 34.8% were pesticides (Veale *et al.,* 2013). In 2004 the WHO estimated a total of 346 000 people globally to have died due to unintentional poisoning. Of these deaths, it is estimated that 91% occurred in low and middle-income countries. Based on a previous population-based study, Marks & van Hoving (2016) were able to extrapolate and estimate that South Africa might have a total of 500 000 poisoning cases each year (Marks & van Hoving, 2016).

Aims

To adequately respond to the medical burden imposed by the growing number of poisoning cases, this project aims to develop effective screening tools for both medicines and pesticides in human plasma. The information generated on the poisoning agent's identity, dose absorbed, and degree of exposure will lead to successful treatment poisoning cases with improved clinical outcomes (Yuan *et al.,* 2018).

Objectives

1. To develop an untargeted screening method capable of simultaneous determination of multiple drugs in plasma.
2. To develop a semi-quantitative targeted screening method capable of simultaneous detection of several pesticides in plasma.
3. To perform a partial validation of the targeted analysis method to ensure its robustness and accuracy.

CHAPTER 2

UNTARGETED ANALYSIS OF THERAPUETIC DRUGS IN PLASMA

2.1. Introduction

Liquid chromatography coupled to time-of-flight mass spectrometry (LC-TOFMS) is one of the most useful methods employed for the accurate determination of compounds in complex mixtures (Ojanperä *et al.*, 2006). Targeted analysis allows for the quantification of known compounds in biological mixture. On the other hand untargeted analysis allows for the detection of both known and unknown compounds in a mixture (Wang *et al.*, 2015). There is a general acceptance in the capabilities of LC-TOFMS to achieve accurate mass measurements without losing sensitivity. Furthermore, high resolution and accuracy MS data that is generated allows for the calculation of elemental composition (Li *et al.*, 2009).

In the present study we aimed to develop a LC-TOFMS method with the potential of simultaneously carrying out screening for a wide range of compounds. In Chapter 1, a list of all pesticides and drugs to be considered was discussed. Pesticides were obtained as high-grade reference standards, but due to budget constraints it was not also possible to obtain high-grade reference standards for the therapeutic drugs. As proof of concept, drugs were purchased from Stelkor Pharmacy and only a few deuterated internal standards were purchased. An LC-TOFMS method was developed to identify compounds based on accurate mass, elemental composition, fragment ions and retention time.

For its ability to observe a large number of known and unknowns within single injection LC-TOFMS is able to generate useful data (Zhu *et al.*, 2013). This study seeks to demonstrate how this information can be used in the identification of unknowns using online search libraries.

2.2. Materials and Methods

2.2.1. Reference Standards

Pesticides

Atrazine, MCPA, imidacloprid, fipronil, methomyl and acetochlor were donated to the project by Villacrop Protection (Pty) Ltd (Kempton Park, South Africa). Atrazine-D5, aldicarb and paraquat were purchased from Sigma Aldrich (Schnelldorf, Germany).

Drugs:

Due to the high cost of high purity reference standards, drugs were purchased in tablet form from Stelkor Pharmacy (Stellenbosch, South Africa): 200 mg Levofloxacin, 400 mg moxifloxacin, 50 mg sertraline, 20 mg citilopram, 20 mg fluoxetine hydrochloride, 10 mg methylphenidate, 75 mg venlafaxine, 1 mg risperidone, 25 mg amitriptyline hydrochloride, 5 mg haloperidol, 100 mg quetiapine, 250 mg levetiracetam, 300 mg valproate, 100 mg lamotrigine, 100 mg phenytoin, 200 mg carbamazepine, 10 mg olanzapine, 15 mg aripiprazole, 100 mg clozapine, 500 mg metformin, 80 mg gliclazide, 5 mg glibenclamide, 250 mg theophylline, 100 mg itraconazole, 150 mg fluconazole, 15 mg midazolam, 10 mg diazepam, 1 mg lorazepam, 2 mg clonazepam, 40 mg propranolol, 500 mg paracetamol, 50 mg tramadol, 10 mg enalapril, 12.5mg carvedilol, 50mg atenolol, 40mg furosemide and 12.5mg hydrochlorothiazide.

Buffers, Solvents and Matrix

Formic Acid was purchased from Kimix Chemicals (Cape Town, South Africa). Methanol and acetonitrile were purchased from Romil Limited (Cambridge, England). A Milli-Q water purification system (Millipore, Milford, USA) was used for all water needed.

Plasma was selected as the biological matrix of choice since many drugs show instability in whole blood. Whole blood haemolyses when frozen and therefore negatively impacts the accuracy of the measured compounds. Plasma is very widely used and easy to separate from whole blood, allowing for it be frozen or tested immediately (de Castro *et al.*, 2008; Tang & Thomas, 2012; Fisher *et al.*, 2013). Drug free Citrate Phosphate Dextrose (CPD) plasma was purchased from the Western Province Blood Transfusion Services (Tygerberg, South Africa) and whole blood was collected from heathy volunteers.

2.2.2. Instruments

The Synapt G2 Quadrupole Time-of-Flight Mass Spectrometer by Waters (Milford, USA) was used for the untargeted method development and optimization for both drugs and pesticides. The system was connected to an Acquity ultra-high performance liquid chromatograph (UHPLC) and electrospray ionisation was used.

2.2.3. Preparation of drug stock solutions

The entire tablet was crushed into a fine powder using a mortar and pestle. The powder was then dissolved into measured volumes of methanol to a final

concentration of 2.00 mg/mL. The weight and contents of excipients was not taken into account, and the concentration is that of the active substance. All the stock solutions were stored at ~-80˚C until required.

2.2.4. Preparation of drug calibration standards

Calibration standards were prepared for the therapeutic drugs, as described in Table 4 below. Briefly, the crushed drugs were used to spike an initial working solution (WS1) in methanol, which was in turn used to prepare working solutions 2 to 4 in methanol by means of serial dilution. All of the therapeutic drugs being investigated were not combined into a single working solution, but were divided into groups based on therapeutic function. The working solutions were then used to prepare calibration standards by spiking 100 µL into 900 µL of plasma. These calibration standards were aliquoted in microcentrifuge tubes and stored at approximately -80 °C until analysis. The concentrations in Table 4 were selected based on the pharmacokinetic peak concentrations (Cmax) of each drug. As to be expected, there is a wide distribution in Cmax values across all listed drugs. The vast majority of the drugs fell within the concentration ranges in Table 4. Detection information acquired at this range will not only identity the ingested drugs but also tell whether the ingestion should be considered sub-therapeutic, therapeutic or toxic.

Table 4. Preparation of drug calibration standards

Working solutions (WS)	Blank solvent (methanol) volume (µL)	Spiking solution	Spiking solution volume (µL)	WS Soln (µg/mL)	Vol into 0.900 ml plasma (µL)	STD	Concentration in Plasma (ng/mL)
WS1	426.5	SS2	500	10.0	100	STD 1	1000
WS2	900	WS1	100	1.000	100	STD 2	100
WS3	400	WS2	400	0.5000	100	STD 3	50.0
WS4	800	WS3	200	0.1000	100	STD 4	10.0

Abbreviations: WS = Working Stock; WS Soln: = Working Stock Solution; STD = Standard

2.2.5. Preparation of pesticide stock solutions

Based on the solubility of each pesticide, a suitable solvent was chosen. As described in Table 5, a concentration of 2.00 mg/mL was achieved by adjusting the weighed mass to purity. All the stock solutions were stored at ~-80 ˚C until required.

Table 5. Preparation of 2.00 mg/mL pesticide stock solutions

Analyte	% Purity	Solvent	Weighed mass (mg)	Adjusted Mass (mg)	Solvent Volume (ml)
Glyphosate	95.8	ACN/Water (1:1)	2.73	2.62	1.310
Atrazine	97.5	ACN	2.31	2.25	1.125
MCPA	96.9	ACN/Water (1:1)	2.49	2.41	1.205
Imidacloprid	97.1	ACN/Water (1:1)	2.02	1.96	0.980
Fipronil	95.1	ACN/Water (1:1)	2.26	2.15	1.075
Deltamethrin	98.7	ACN/Water (1:1)	2.19	2.16	1.040
Alpha-Cypermethrin	98.7	ACN/Water (1:1)	2.38	2.34	1.080
Methomyl	98.0	ACN/Water (1:1)	2.46	2.46	1.205
Acetochlor	95.3	ACN/Water (1:1)	2.00	1.90	0.950
Paraquat Dichloride	98.0	ACN/Water (1:1)	1.95	1.91	0.955
Atrazine-D5	99.7	ACN	2.21	2.20	1.000
Aldicarb	98.0	MeOH	2.60	2.55	1.275

Abbreviations: ACN = Acetonitrile; MeOH = Methanol

2.2.6. Extraction protocol

The samples were subjected to protein precipitation. After allowing plasma to thaw at room temperature, 50.0 µL samples of plasma were precipitated with 350 µL of methanol containing the internal standards. The samples were then vortexed for 30 sec and centrifuged at 15000 ×g for 10 min. The supernatant was transferred into 2.00 mL glass vials with inserts.

2.2.7. Preparation of Pesticide calibration standards

The calibration standards for the pesticides in Table 6 were prepared similarly to that described in Table 4 for the therapeutic drugs, in that working stock solutions were first prepared and used to spike plasma. These were aliquoted into microcentrifuge tubes and stored at approximately -80 °C until analysis. In order to work out the detection limits of the LC-TOFMS, the calibration standards were prepared in the same manner as in the target analysis method.

Table 6. Preparation of pesticide calibration standards

Working Solutions (WS)	Blank solvent (methanol) volume (μL)	Spiking solution	Spiking solution volume (μL)	WS Soln (μg/mL)	Vol into 0.98 ml plasma (μL)	STD	Concentration in Plasma (ng/mL)
WS1	100	SS2	100	143	20.0	STD 1	2860
WS2	100	WS1	100	71.5	20.0	STD 2	1430
WS3	151	WS2	109	30.0	20.0	STD 3	600
WS4	100	WS3	140	17.5	20.0	STD 4	350
WS5	250	WS4	100	5.00	20.0	STD 5	100
WS6	135	WS5	200	3.00	20.0	STD 6	60.0
WS7	200	WS6	100	1.00	20.0	STD 7	20.0

Abbreviations: WS = Working Stock; WS Soln: = Working Stock Solution; STD = Standard

2.2.8. Liquid chromatography and time-of-flight mass spectrometry

2.2.8.1. LC-TOFMS analysis method

Chromatography

Chromatographic separation and quantification was achieved by using a reverse phase Waters HSS T3 (1.8 μm, 2.1 ×150 mm) column (Waters, Milford, USA). The mobile phase used was 0.1% formic acid in both water (A) and in acetonitrile (B). A gradient was created at a flow rate of 0.25 mL/min. The LC program starts at 100% (A) for 22 min; 72% (A) for 8 min; 40% (A) for 1 minute; 100% (B) for 2 min and equilibration at 100% (A) for 4 min. The total run time is 37 min.

Mass spectrometry

Ionisation mode was set for both positive and negative (ESI +/-) as two separate runs; nitrogen as the desolvation gas was set at a flow rate of 650 L/h; desolvation temperature was set at 275 ˚C. The instrument was operated in the MS^E mode which obtains both low collision energy (6V) and high collision energy (scanning from 20 to 60V) fragmentation data.

2.2.9. Data analysis

Data was acquired using Masslynx version 4.1 software (Waters Corporation, Milford, USA) and quantified using TargetLynx application manager (Waters Corporation, Milford, USA). Identification was based on each individual compound's accurate mass, elemental composition, fragment ions and retention time.

2.3. Results and Discussion

Shown below are tables for the different drug categories that were spiked into plasma and analysed using the LC-TOFMS analysis method. All drugs, except for hydrochlorothiazide, were detected in positive ionisation mode. The tables contain retention times, various masses of the precursor ions, fragment ions of each drug, and the concentration range at which each drug was detected. No quality controls were ran on the method, therefore no limits of quantification were determined. However, the presence of a drug at any particular concentration level implies that the limit of detection is likely below that actual value. ND denotes - not detected and D denotes - detected. For further identification, Masslynx v4.1 was used to extract $[M+H]^+/[M-H]^-$ ions of each drug, the extracted mass chromatograms of the highest measured level were also embedded on the tables. All drugs at this concentration level had a signal to noise ratio above 10. On the y-axis the chromatograms have percentage (%) and on the x-axis is retention time (min). Each peak is annotated with the retention time (on the top), the peak top scan number (middle) and peak *m/z* (bottom).
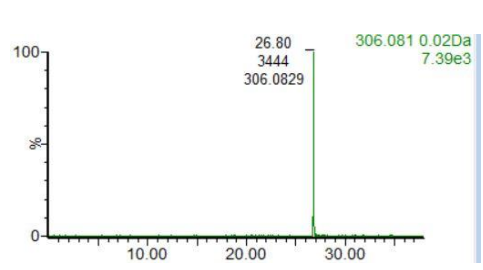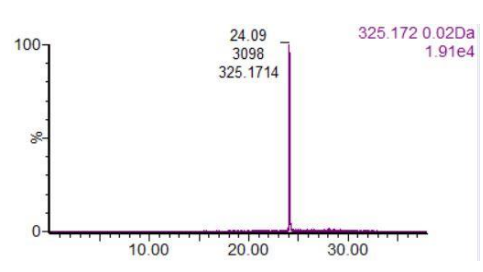
Table 7. Determination of antibiotics in plasma

Drugs	Retention time (min)	M+H	MSE Fragments	Concentration Range (ng/mL)				Chromatogram at 1000 ng/mL
				10	50	100	1000	
Levofloxacin	14.20	362.1516	318.1638 261.0908 221.0764	ND	D	D	D	
Moxifloxacin	19.13	402.1860	358.1918 384.1731 261.1042	D	D	D	D	

As can be seen in Table 8, moxifloxacin was detected across the entire concentration range. This means that 10 ng/mL is not necessarily the lowest limit of quantitation for moxifloxacin. Levofloxacin on the other hand had a limit of quantitation (LOQ) of 50 ng/mL. Tilea *et al.* (2013) and Meredith *et al.*, (2012) both conducted LC-MS/MS methods for the quantification of levofloxacin in human plasma. The lower limit of quantitation (LLOQ) values from the two studies were 78 and 100 ng/mL, both above 50 ng/mL (LOQ)

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obtained in our method (Meredith *et al.*, 2012; Ţilea *et al.*, 2013). Furthermore, therapeutic plasma levels of levofloxacin are between 500 and 600 ng/mL meaning that clinically an LOQ of 50 ng/mL is sub-therapeutic (Ţilea *et al.*, 2013).

Table 8. Determination of psychiatric drugs in plasma

Drugs	Retention time (min)	M+H	MSE Fragments	Concentration Range (ng/mL)				Chromatogram at 1000 ng/mL
				10	50	100	1000	
Sertraline	26.80	306.0807	275.0405 277.0377 158.9792	D	D	D	D	
Citalopram	24.09	325.1720	109.0448 234.0714 262.1027	D	D	D	D	

Lorien (Fluoxetine Hydrochloride)	26.61	310.1420	255.1197 123.0823 184.0736	ND	D	D	D	
Methylphenidate	16.40	234.1499	149.0261 129.0709 84.0829	ND	D	D	D	
Venlafaxine	20.06	278.2120	260.2014	D	D	D	D	

Risperidone	19.71	411.2226	191.1116	ND	ND	D	D	
Amitriptyline Hydrochloride	26.21	278.1922	203.0863 233.1338 191.0872	ND	D	D	D	
Haloperidol	24.92	376.1499	123.0255 165.0729 358.1379	ND	D	D	D	

| Quetiapine | 22.28 | 384.1749 | 253.0805 221.1091 210.0384 | ND | D | D | D | |

Discussion: As can be seen in Table 9 above, sertraline, citalopram and venlafaxine were detected across the entire concentration range. This means that 10 ng/mL is not necessarily the lowest limit of quantitation for these drugs. In fact, a study done to determine nine antidepressants in oral fluid and plasma via LC-MS/MS showed LLOQ values of 2 ng/mL for sertraline, citalopram and venlafaxine (de Castro *et al.*, 2008). Fluoxetine hydrochloride, methylphenidate, amitriptyline hydrochloride, haloperidol and quetiapine all had an LOQ of 50 ng/mL and could not be detected at 10 ng/mL. The LOQ for risperidone was 100 ng/mL and could not be detected at 50 and 10 ng/mL. Many methods in literature have reported quantitation limits lower than the ones observed in Table 9 (Čabovska *et al.*, 2007; de Castro *et al.*, 2008; Rosano, Wood & Swift, 2011). However, these studies made use of targeted methods using triple quadrupole systems that have a better quantitative ability than the LC-TOFMS method used in the current study (Pang *et al.*, 2016). Instead of trying to determine the lowest limit of detection for each drug, this study focused on the simultaneous detection of many drugs at sub-therapeutic, therapeutic and overdose ranges. Unlike the other studies, matrix effects were not evaluated in the present study and could have resulted in the decreased sensitivity of this method. Matrix effects can be defined to be the difference in response between an analyte in a standard solution compared to the same analyte in a biological matrix such as plasma. The different components of biological matrix can co-elute with the targeted analyte or affect its ionisation. Matrix effects can have an effect on the accuracy, precision and robustness of bioanalytical method (Chambers *et al.*, 2007). Matrix components have the ability of co-eluting with the analytes and therefore reduce the ion intensity (Matuszewski *et al.*, 1998). Furthermore, protein precipitation was employed in the present study protein as opposed to the more efficient solid phase extraction (SPE). Protein precipitation method has the potential to

contaminate the electrospray ionisation (ESI) source rapidly, and therefore negatively affect detection. However, it comes at a low cost and is particularly useful when working with a large number of chemicals of variable nature and polarity (Shah *et al.*, 2010).

Table 9. Determination of anti-convulsant drugs in plasma

Drugs	Retention time (min)	M+H	MSE Fragments	Concentration Range (ng/mL)				Chromatogram at 1000 ng/mL
				10	50	100	1000	
Lamotrigine	15.18	256.0157	210.9831 149.0240	D	D	D	D	
Valproate	ND	ND	ND	ND	ND	ND	ND	
Levetiracetam	ND	ND	ND	ND	ND	ND	ND	
Phenytoin	26.32	253.0992	217.0347 200.2400 166.5378	ND	ND	ND	D	

| Carbamazepine | 26.46 | 237.1035 | 179.0735 194.0976 203.0960 | ND | D | D | D | |

Depicted in table 10, Lamotrigine is the only anti-convulsant drug that could be detected across the entire concentration range. In this study we were able to detect lamotrigine at an LOQ of 10 ng/mL, compared to 25 ng/mL described in a study by Shah *et al.* (Shah *et al.*, 2010). However, more recently the limit of detection for lamotrigine was found to be 2.50 ng/mL (Reddy *et al.*, 2016). Carbamazepine showed an LOQ of 50 ng/mL and could not be detected at 10 ng/mL. Using chloroform in a liquid-liquid extraction, another study obtained an LOQ of 10 ng/mL (Rajender, *et al.*, 2009). Phenytoin could only be detected at 1000 ng/mL. Phenytoin has a high protein binding affinity and liquid-liquid and protein precipitation have been reported to be more efficient than protein precipitation. These methodologies were however associated with long sample preparation and analysis time. A more recent study has made use of a SPE extraction technique and also incorporated a deuterated internal standard for phenytoin (phenytoin-d10). The study recorded an LLOQ of 101.437 ng/mL (Alegete *et al.*, 2017). Valproate and levetiracetam were not detected at all. Valproate is a low molecular weight compound that is difficult to analyse as it does not produce any stable fragments or product ions after collision energy has been applied. To further illustrate the complexity of valproate analysis, it is omitted from commercially available Waters[TM] Toxicological libraries. G-CMS has been shown to be more reliable in detecting valproate due to its ability to detect low molecular weight compounds (Pope *et al.*, 2017). Lu *et al.* (2015) and Pope *et al.* (2017) showed that it was more reliable to rather scan for Valproate metabolites as they have a higher molecular weight and therefore greater detectability via LC-MS methods (Lu *et al.*, 2016; Pope *et al.*, 2017).

Reverse phase chromatography makes use of a polar mobile phase and a non-polar stationary phase. An increase in the aqueous phase results in greater adsorption of non-polar compounds onto the column. However, a polar compound such as levetiracetam has

a greater affinity for the aqueous mobile phase than for the hydrophobic C18 stationary phase. It is because of these interactions that no retention was observed for levetiracetam. In an attempt to overcome the hydrophilic character of levetiracetam, Hamdan *et al.* (2017) showed that using a C8 column, which is less hydrophobic than the C18 column, yielded better retention.

Table 10. Determination of atypical antipsychotic drugs in plasma

Drugs	Retention time (min)	M+H	MSE Fragments	Concentration Range (ng/mL)				Chromatogram at 1000 ng/mL
				10	50	100	1000	
Olanzapine	11.30	313.1494	157.0785 246.1718 213.0500	ND	D	D	D	
Aripiprazole	26.26	448.1584	285.0916 176.0725	ND	ND	ND	D	

| Clozapine | 20.63 | 327.1390 | 164.0736 227.0421 | D | D | D | D | |

From the results of atypical antipsychotic drugs shown in Table 11, clozapine is the only drug that could be detected across the entire concentration range. While olanzapine had an LOQ of 50 ng/mL, another study was able to record an LLOQ of 0.100 ng/mL for olanzapine. The said study experienced difficulty recovering olanzapine and experienced matrix effects when employing liquid-liquid extraction and protein precipitation techniques. To overcome this, they made use of a SPE method on Waters Oasis HLB catridges. This as a result improved their recoveries significantly (Bonde *et al.*, 2014). Aripiprazole could only be detected at 1000 ng/mL. Although there are many methods developed to determine aripiprazole, they are usually not sensitive and specific enough. The effect of other drugs used in combination with aripiprazole makes the determination of aripiprazole difficult in plasma (Zuo *et al.*, 2006). The most common extraction procedure for difficult analytes often includes SPE. More recently Zhong *et al.*, (2016) demonstrated that an on-line SPE–UHPLC–MS/MS procedure to be more sensitive and selective. They made use of a reverse phase SPE column Shim-pack MAYI-C8 (G) that selectively concentrates the analyte whilst removing endogenous impurities in the plasma. As a result, they manage to produce an LOQ of 102 ng/mL for aripiprazole (Zhong *et al.*, 2016).

Table 11. Determination of hypoglycaemic drugs in plasma

Drugs	Retention time (min)	M+H	MSE Fragments	Concentration Range (ng/mL)				Chromatogram at 1000 ng/mL
				10	50	100	1000	
Gliclazide	30.20	324.1390	127.1240 110.0980	ND	ND	ND	D	
Diaphage (Metformin)				ND	ND	ND	ND	
Glycomin (Glibenclamide)	31.58	494.1516	338.3418 304.0711	ND	D	D	D	

Table 12 shows that metformin is the only hypoglycaemic drug that was not detected on the method. Due to the polar structure of metformin, the compound has a greater affinity for the aqueous mobile phase than for the hydrophobic C18 stationary phase. On an application note written by W. Faulkner (2011) for Thermo Fisher Scientific, the high polarity of metformin was overcome by making use of a pentafluorophenyl (PFP) Stationary phase (Faulkner , 2011). Glibenclamide displayed an LOQ of 50 ng/mL. Gedeon *et al.*, (2008)

showed that glibenclamide could be determined using liquid chromatography and atmospheric pressure chemical ionisation (APCI). Their method was able to provide an LOQ of 1.50 ng/mL for glibenclamide. It is also worth noting that their injection volume was 50.0 µL (Gedeon *et al.*, 2008). Due to the fact that protein precipitation used in the current study could potentially contaminate the source, we opted to only inject 10 µL. Gliclazide could only be detected at 1000 ng/mL. Gliclazide also has a high protein binding affinity like phenytoin (Hu *et al.*, 2009).
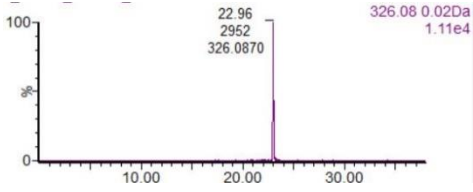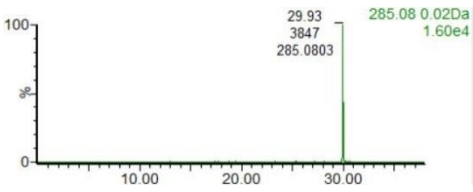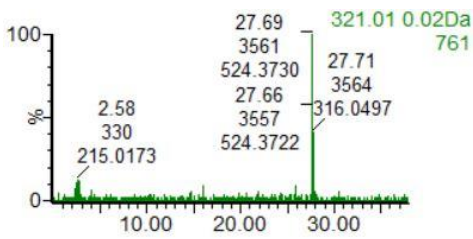
Table 12. Determination of anti-fungal drugs in plasma

Drugs	Retention time (min)	M+H	MSE Fragments	Concentration Range (ng/mL)				Chromatogram at 1000 ng/mL
				10	50	100	1000	
Itraconazole	32.60	705.2479	663.4445 593.3288	ND	ND	ND	D	
Fluconazole	17.52	307.1104	220.0929 238.0870 169.0446	ND	D	D	D	

As can be seen in Table 13 above, Itraconazole could only be detected at the highest concentration tested, namely 1000 ng/mL. This could be due to the fact that mere protein precipitation may not produce a sufficiently clean sample to avoid ion suppression of certain molecules. Previous studies have shown that other techniques such as the use of functionalized ferromagnetic micro-particles or even the use of liquid-liquid extraction yielded cleaner extracts and as a result had fewer matrix effects (Kousoulos *et al.*, 2006; Vogeser *et*

al., 2008). Fluconazole on the other hand had an LOQ of 50 ng/mL. This LOQ is however still below the normal Cmax value of fluconazole of 10.6 +/- 0.4 µg/mL (Brunton *et al.*, 2018).

Table 13. Determination of psychoactives in plasma

Drugs	Retention time (min)	M+H	MSE Fragments	Concentration Range (ng/mL)				Chromatogram at 1000 ng/mL
				10	50	100	1000	
Midazolam	22.96	326.0870	291.1184 249.0844 209.0661	ND	D	D	D	
Diazepam	29.93	285.0803	184.0748 193.0892 222.1150	ND	ND	ND	D	
Lorazepam	28.59	321.0497	229.1397 269.1690 179.1104	ND	ND	ND	D	

Clonazepam	27.74	316.0512	179.0866 221.1332 137.0647	ND	ND	ND	D	

Table 14 shows that diazepam, lorazepam and clonazepam were only detected at 1000 ng/ml. While midazolam had an LOQ 50 ng/mL. Characteristics of different drugs have a direct influence in how they get distributed. These characteristics include the drug's ability to bind to plasma proteins, including lipid solubility and molecular size. Diazepam, lorezepam and clonazepam have low lipid solubility. In fact when administered intravenously, they have to be prepared in a solution with propylene glycol for them to be water soluble (Griffin *et al.*, 2013).

Table 14. Determination of cardiovascular drugs in plasma

Drugs	Retention time (min)	M+H	MSE Fragments	Concentration Range (ng/mL)				Chromatogram at 1000 ng/mL
				10	50	100	1000	
Propranolol	21.79	260.1656	155.0867 183.0804 234.1498	D	D	D	D	

Enalapril	22.12	377.2076	234.1506 117.0710	ND	D	D	D	
Carvedilol	25.49	407.1981	222.0930 194.0980	ND	D	D	D	
Atenolol	8.94	267.1727	149.0240 190.0876	ND	D	D	D	
Austell-Furosemide (Furosemide)	26.05	329.0011	283.2613 204.9737	ND	ND	D	D	

Hydrochlorothiazide	11.21	295.9566	77.9655 126.0161	ND	ND	ND	D	

As presented in Table 15, propranolol could be detected across the entire concertation range, while enalapril, carvedilol and atenolol all showed an LOQ of 50 ng/mL. Furosemide had an LOQ of 100 ng/mL and hydrochlorothiazide could only detected at 1000 ng/mL. A recent study reports that its authors were able to develop an assay that could detect 52 cardiovascular drugs in plasma. They were able to show that protein precipitation using trichloroacetic acid (TCA) improved sensitivity when compared to acetonitrile and methanol (Punt *et al.*, 2019). Achieving good sensitivity at lower concentrations for hydrochlorothiazide using LC-MS/MS in human plasma has been a challenge. An earlier study by Kumar *et al.,* (2012) overcame this challenge by employing SPE using a methanol preconditioned Oasis cartridge and a modular HPLC system coupled to an API 3000 mass spectrometer. On an isocratic run they were able to produce an LLOQ of 1 ng/mL for hydrochlorothiazide (Kumar *et al.*, 2012).

Table 15. Determination of analgesic drugs in plasma

Drugs	Retention time (min)	M+H	MSE Fragments	Concentration Range (ng/mL)				Chromatogram at 1000 ng/mL
				10	50	100	1000	
Tramadol	16.36	264.1952	149.0243	ND	D	D	D	
Paracetamol	9.27	152.0707	110.0612	D	D	D	D	

Discussion: Paracetamol could be detected across the entire concentration range with an LOQ of 10 ng/mL. There are many studies that describe the detection of paracetamol in plasma. One study performed a simultaneous determination of paracetamol, pseudoephedrine and chlorpheniramine in human plasma. That study observed an LLOQ of 20 ng/mL for paracetamol, above the LOQ observed by this study (Liao *et al.*, 2008). Tramadol had an LOQ of 50 ng/mL. Another study was, however able to develop a more sensitive method for tramadol in human plasma using an isocratic LC-MS/MS method. They managed to obtain an LOQ of 12.5 ng/mL for tramadol. It goes without saying, like in all other studies mentioned in this section, they made use of pure standards and tramadol-d6 as an internal standard to account for any matrix effects (Tanaka *et al.*, 2016).

Table 16. Determination of Theophylline in plasma

Drugs	Retention time (min)	M+H	MSE Fragments	Concentration Range (ng/mL)				Chromatogram at 1000 ng/mL
				10	50	100	1000	
Theophylline	9.12	181.0697	152.0719	D	D	D	D	

Discussion: As presented in Table 17, theophylline was detected across all concentration levels. Theophylline is polar and thus was not expected to have good retention on the column. Previous studies have been able to measure theophylline using hydrophilic interaction liquid chromatography (HILIC). A recent study was able to quantify theophylline in guinea pig plasma at an LOQ of 500 ng/mL, well above the LOQ determined by this study (Kertys *et al.*, 2018).

2.4. Identification of unknown compounds using online search libraries.

In order to demonstrate how an unknown would be identified using online search laboratories, we made use of a standard operating procedure (SOP) compiled by M. Malcolm Taylor from the Central Analytical Facilities (CAF), Stellenbosch University. To aid in the demonstration, it was assumed that carbamazepine is an unknown compound within the mixture of drugs detected in Table 7. Figure 5 shows the steps that were followed for identifying a compound, such as carbamazepine as example, from sampling, to sample preparation, to LC separation, to MS detection and to actual compound identification.
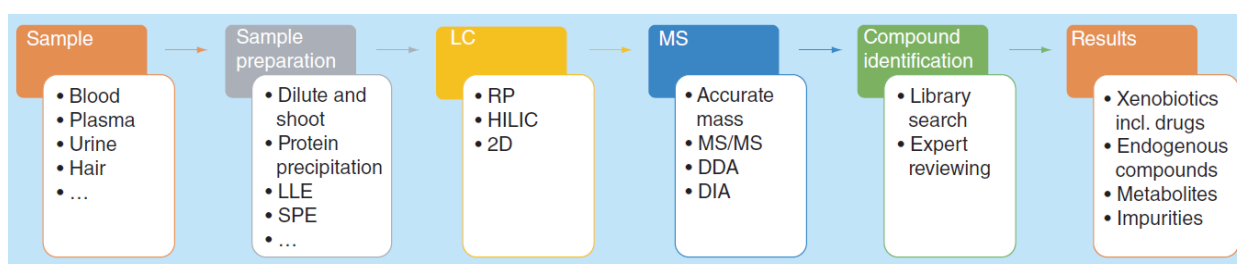
Figure 5. Steps involved in LC–MS-based screening and confirmation analysis (Oberacher & Arnhard, 2015).

The software chosen for this purpose is MZmine (http://mzmine.github.io/download.html). It is a software that is freely available and can open Waters instrument data files without the need to convert them first. Waters data files can then be imported into the software and a total ion count (TIC) of the sample can be viewed. Figure 6 below shows the TIC of the mixture of 24 therapeutic drugs analysed in the sample.
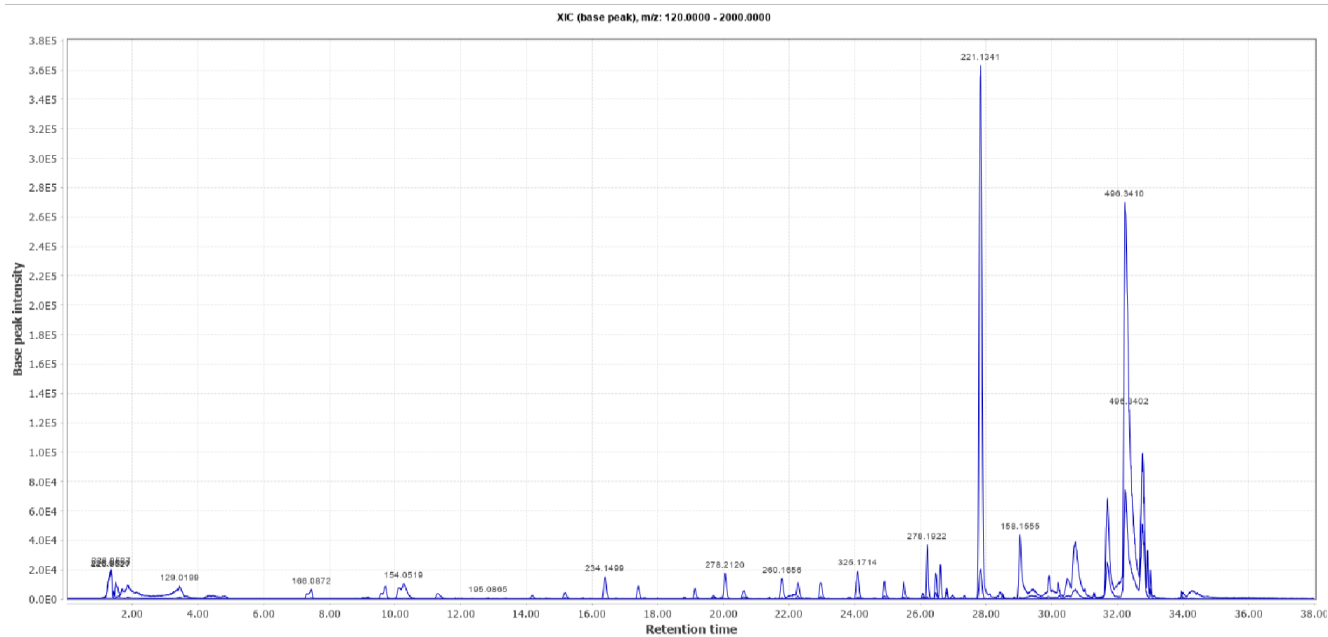
Figure 6. Total Ion Count MS-Chromatogram of a mixture of therapeutic drugs

By zooming in and selecting a desired peak one is able to get product ion and molecular ion data for that analyte. For the purposes of this illustration we selected the peak with [M+H] value of 237.1034 as seen in Figure 7.
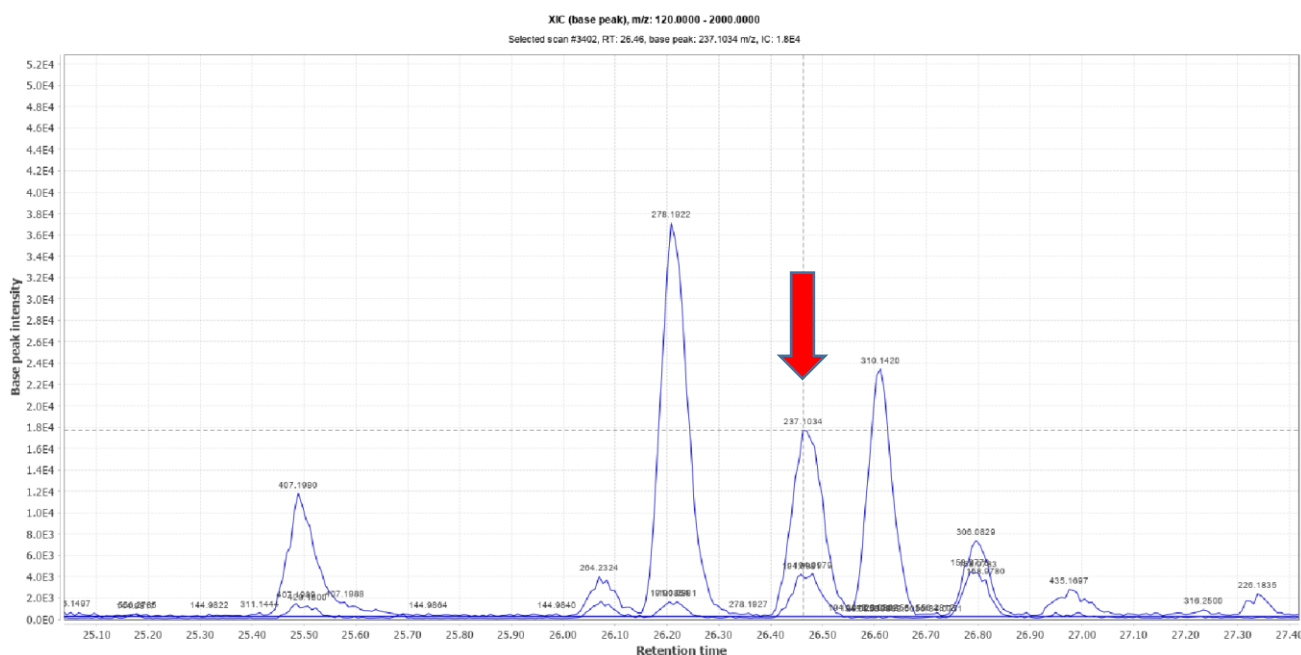
Figure 7. Selection of unknown peak by putting cross-hairs

Un-fragmented spectra can be obtained by double clicking while the cross hairs are still on the upper peak (Figure 8). This data can be exported to a Microsoft Excel spreadsheet.
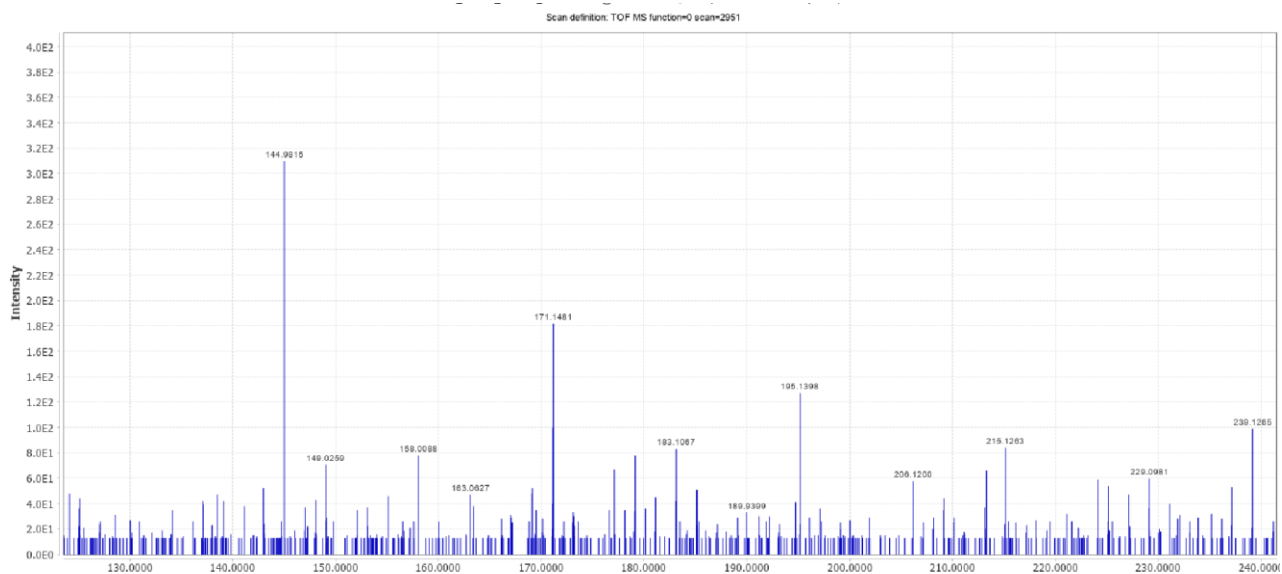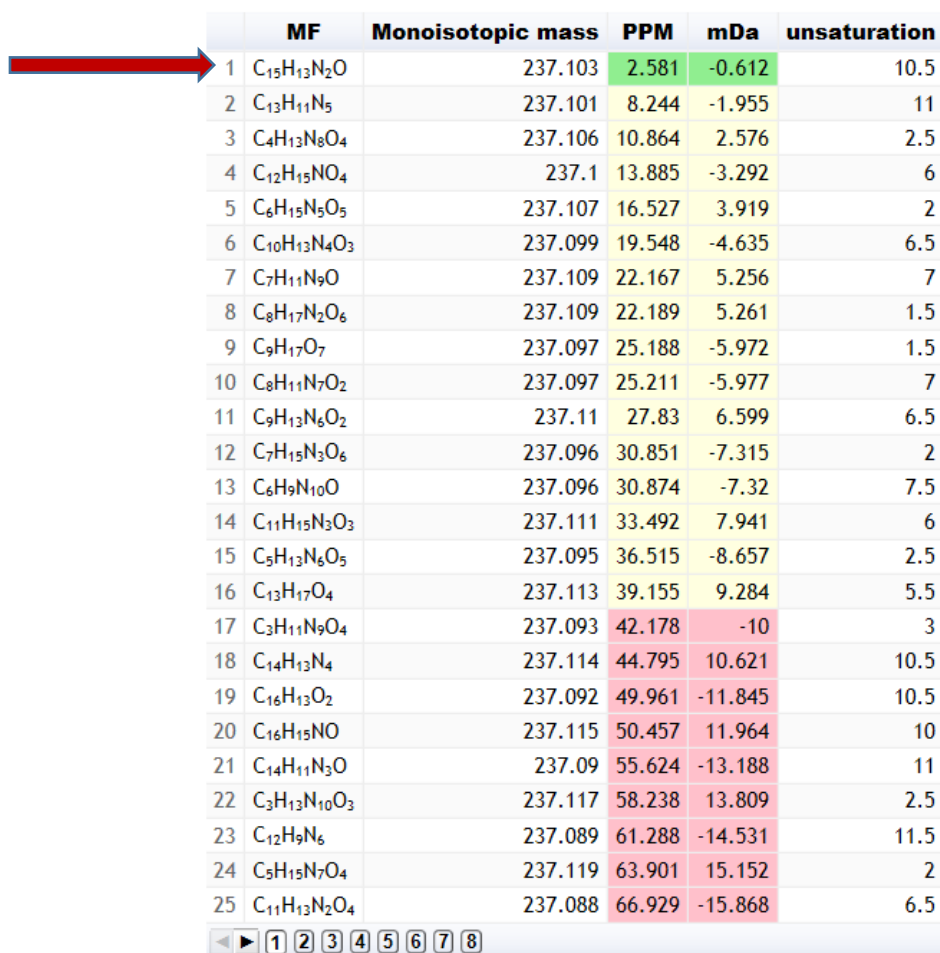
Figure 8. MS spectrum of low energy trace containing primarily molecular ions.

To determine the elemental composition of the compound the [M+H] value of 237.1034 obtained from mzMine is used. This mass is then analysed on ChemCal (https://www.chemcalc.org/mf_finder/mfFinder_em_new) a web-based software. This software is able to predict the compound's molecular formula as seen in Figure 9 in order of most likely possibility. According to the SOP the part per million (ppm) mass error should be less than 5. As seen in Figure 9 the ppm value for the compound is within the acceptable limits.

Results:

Color by difference: <=0.0010 <=0.01 <=1.0

Number of results: 180. Brute force iterations: 244420. Real iterations: 4210.

	MF	Monoisotopic mass	PPM	mDa	unsaturation
1	$C_{15}H_{13}N_2O$	237.103	2.581	-0.612	10.5
2	$C_{13}H_{11}N_5$	237.101	8.244	-1.955	11
3	$C_4H_{13}N_8O_4$	237.106	10.864	2.576	2.5
4	$C_{12}H_{15}NO_4$	237.1	13.885	-3.292	6
5	$C_6H_{15}N_5O_5$	237.107	16.527	3.919	2
6	$C_{10}H_{13}N_4O_3$	237.099	19.548	-4.635	6.5
7	$C_7H_{11}N_9O$	237.109	22.167	5.256	7
8	$C_8H_{17}N_2O_6$	237.109	22.189	5.261	1.5
9	$C_9H_{17}O_7$	237.097	25.188	-5.972	1.5
10	$C_8H_{11}N_7O_2$	237.097	25.211	-5.977	7
11	$C_9H_{13}N_6O_2$	237.11	27.83	6.599	6.5
12	$C_7H_{15}N_3O_6$	237.096	30.851	-7.315	2
13	$C_6H_9N_{10}O$	237.096	30.874	-7.32	7.5
14	$C_{11}H_{15}N_3O_3$	237.111	33.492	7.941	6
15	$C_5H_{13}N_6O_5$	237.095	36.515	-8.657	2.5
16	$C_{13}H_{17}O_4$	237.113	39.155	9.284	5.5
17	$C_3H_{11}N_9O_4$	237.093	42.178	-10	3
18	$C_{14}H_{13}N_4$	237.114	44.795	10.621	10.5
19	$C_{16}H_{13}O_2$	237.092	49.961	-11.845	10.5
20	$C_{16}H_{15}NO$	237.115	50.457	11.964	10
21	$C_{14}H_{11}N_3O$	237.09	55.624	-13.188	11
22	$C_3H_{13}N_{10}O_3$	237.117	58.238	13.809	2.5
23	$C_{12}H_9N_6$	237.089	61.288	-14.531	11.5
24	$C_5H_{15}N_7O_4$	237.119	63.901	15.152	2
25	$C_{11}H_{13}N_2O_4$	237.088	66.929	-15.868	6.5

◄ ► ① ② ③ ④ ⑤ ⑥ ⑦ ⑧

Figure 9. Molecular formula (elemental composition) determination on ChemCal

For additional confirmation the use of the saved fragmented data can be uploaded onto a Massbank database such as Metfrag (https://msbi.ipb-halle.de/MetFrag/). As seen in Figure 10, the mass bank will give a list of possible molecules associated with the uploaded fragmented data. A score close or equal to one indicates the likeliest possibility.
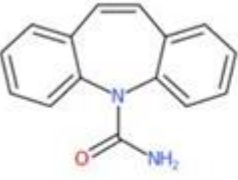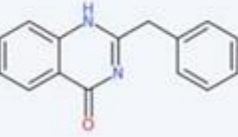
81

Figure 10. List of compounds associated with the fragmentation data used

2.5. Summary and Conclusion

In this chapter we were able to describe the development of an LC-TOFMS method for the determination of drugs in plasma. Taking into account that different molecules have different ionisation patterns, a reverse phase method was developed for their separation. Reverse phase chromatography makes use of a polar mobile phase and a non-polar stationary phase, ideal for non-polar compounds.

In total 37 drugs were included in the screening method. The study made use of four concentration levels for detection based on the pharmacokinetic peak concentrations of each drug. As there was a wide distribution in Cmax values across all listed drugs, it was important that the detection information acquired would at least inform whether an ingestion was sub-therapeutic, therapeutic or toxic.

This method was able to detect and identify 92% (34/37) of the drugs included on the screen. Some drugs could not be detected at lower concentrations. Due to the high cost of high-grade standards, this study procured drugs in a tablet form instead. The use of tablets instead of high-grade standards also poses a challenge when developing a screening method. This is because drug formulation involves the mixing of the drug with different excipients. This is usually done to improve the

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drug's manufacturability whilst increasing its ability to be administered effectively. The negative side to this is that the chemical nature and stability of a drug can be vastly affected by the physical and chemical interactions happening between the drug and the excipients. Residual excipients could also result in ion suppression, which could lead to impaired analyte detection (Bharate *et al.*, 2010). Furthermore, many similar studies advocate for the use of a cleaner extraction protocols such as liquid-liquid, ultra-filtration, SPE, and automatic online SPE methods (Rajender *et al.*, 2009; Shah *et al.*, 2010; Bonde *et al.*, 2014; Zhong *et al.*, 2016; Alegete *et al.*, 2017). This is because protein precipitation used in the drug extraction method has been known to leave residual components that can contaminate the electrospray ionisation (ESI) source rapidly and therefore have a negative impact on detection. This study, however, still chose to make use of protein precipitation as it was the most cost-effective given that this test is to be implemented in a laboratory within a developing country. Besides, a single SPE method compatible with such a wide variety of drugs would be a challenging undertaking. The method has to be rapid, accuracy, robustness and relatively low costing.

Only three drugs namely, valproate, levetiracetam and metformin were not detected by this method. Valproate has been shown to be difficult to analyze since it does not produce any stable fragments or product ions after collision energy has been applied. Other studies have shown that GC-MS is more reliable in detection of valproate (Pope *et al.*, 2017). Whilst others have advocated valproate metabolites analyses rather, since they have a higher in molecular weight and therefore greater detectability via LC methods (Lu *et al.*, 2016).

Levetiracetam and metformin were the most polar compounds of all the drugs analyzed. Just based on polarity alone, these compounds were not expected to do well in reverse phase chromatography. Hydrophilic compounds have a greater affinity for the aqueous mobile phase than for the hydrophobic C18 stationary phase therefore making them difficult to be retained. Making use of a C8 column that is less hydrophobic than the C18 column, another study showed good retention for Levetiracetam, while another study showed that the use of a PFP Stationary phase worked for the determination of metformin (Faulkner, 2011; Hamdan *et al.*, 2017).

Many studies cited in the discussion made use of high-grade standards. These methods compromised of just a few drugs compared to the 37 used in this study. Furthermore, these studies made use of targeted mass spectrometry with short

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isocratic runs (Kumar *et al.*, 2012; Reddy *et al.*, 2016; Tanaka *et al.*, 2016). Targeted analysis requires that each analyte be specifically optimized on the instrument and can result in a complex method development for multiple analytes (Allen & Mcwhinney, 2019). Analysing one compound at a time would defeat the purpose of this kind of study. The main aim is to develop a rapid screening tool for the simultaneous determination of multiple drugs in plasma that will reduce any delay in the diagnosis and treatment of poisoning/overdose cases. As the identity of a toxicant is often unknown and due the fact that clinicians often have to rely on information from second parties about the ingested agent, a simultaneous method able to detect multiple drugs would be very useful.

Despite all limitations and uncertainties surrounding excipients, lack of high-grade standards, and the application of protein precipitation, this study was still able to develop an untargeted method able to detect 92% of the drugs listed. Furthermore, this study was also able to demonstrate how unknown compounds could be accurately identified using online search libraries.

Pesticides, on the other hand, were mostly polar and were more of a challenge to detect on the developed method. Due to the vast amounts of drugs already included into the method and in the interests of time management, it was decided that a more robust targeted method would be developed for the seven pesticides namely atrazine, MCPA, imidacloprid, fipronil, methomyl and acetochlor and aldicarb. Details on this targeted approach can be viewed in Chapters 4 & 5.

CHAPTER 3

TARGETED ANALYSIS OF PESTICIDES IN PLASMA

3.1. Introduction

Liquid chromatography linked to tandem mass spectrometry (LC-MS/MS) has become the leading analysis method in the identification of pesticides. This can be attributed to the increase in sensitivity and selectivity, faster analysis times and wide detection limits, offered by the analysis method. To further add to this, manufacturers are also regularly offering new software and hardware capabilities to enhance data-handling features of these instruments (Kmellár *et al.*, 2010). LC-MS/MS provides better selectivity due to its employment of chromatographic separation and tandem MS of the compound of interest from interfering components in the sample matrix (Li & Tse, 2010).

Apart from the instrumental analysis part, another critical step that is often overlooked in the analysis of compounds is sample preparation. Sample preparation can be the most laborious and time consuming part of the analysis. An efficient sample preparation becomes vital when pesticides have to be measured in biological matrices. It becomes even more challenging when developing a multi-analyte method. In a multi-analyte method one has to consider the various classes of different compounds with different polarities, solubilities and volatilities that must be extracted simultaneously and analyzed (Margariti *et al.*, 2007).

A common misconception is that LC-MS/MS guarantees specificity with little to no sample preparation and requires minimal chromatographic separation. In reality, for a robust LC-MS/MS one needs to extensively validate and properly assess the assay's specificity. This would entail also doing matrix effects, recovery and process efficiency experiments to ensure analytes are extracted efficiently and that there is good chromatographic separation (Matuszewski *et al.*, 1998).

Matrix effects due sensitivity of electrospray ionisation (ESI) are a huge problem in LC-MS/MS pesticide analysis. To properly account and compensate for matrix effects, the use of isotopically labelled internal standards for each pesticide can be useful. However, finding and purchasing isotopically labelled internal standards for many compounds comes at a huge cost (Kmellár *et al.*, 2010).

In the present study, we made use of LC-MS/MS with ESI for the identification and quantification of pesticides. Precursor ion scanning mode and multiple reaction monitoring (MRM) were used. Two internal standards structurally similar to the pesticides were selected.

3.2. Materials and Methods

3.2.1. Reference Standards

Standards

Atrazine, MCPA, imidacloprid, fipronil, methomyl and acetochlor were donated to the project by Villacrop Protection (Pty) Ltd (Kempton Park, South Africa). Atrazine-D5, aldicarb and *para*-aminosalicylic acid (PAS) were purchased from Sigma Aldrich (Schnelldorf, Germany).

Buffers, Solvents and Matrix

Formic Acid was purchased from Kimix Chemicals (Cape Town, South Africa). Methanol and acetonitrile were purchased from Romil Limited (Cambridge, England). A Milli-Q water purification system (Millipore, Milford, USA) was used for all water needed.

Drug free citrate phosphate dextrose (CPD) plasma was purchased from the Western Province Blood Transfusion Services (Tygerberg, South Africa) and consent was obtained from healthy volunteers for the collection of whole blood.

3.2.2. Instruments

Method development and validation was done on the Shimadzu 8040 Liquid Chromatograph Tandem Mass Spectrometer with LC-20AD pumps, a DGU-20A3 degasser, SIL-20AC auto sampler, CTO-20A column oven, and an electrospray ionisation source (Kyoto, Japan).

3.2.3. Preparation of stock solutions

The same methodology as described in Chapter 2, Table 5, was used for the preparation of the stock solutions.

3.2.4. Calibration standards and quality controls

Equal volumes of the seven (2.00 mg/mL) standards were mixed together to obtain a final concentration of 286 µg/mL. This cocktail was serially diluted to prepare the working solutions required for plasma spiking. As this was semi-quantitative method, only three calibration standards were selected. A volume of 20 µL of each working solution was spiked into 980 µL of plasma or whole blood at three calibration levels (20 ng/mL, 100 ng/mL and 600 ng/mL) and at two quality control levels (60 ng/mL and 350 ng/mL).

3.2.5. Extraction protocol

The extraction method of choice was protein precipitation because of simplicity of operation and low cost. Two internal standards (atrazine-d5 at 333 ng/mL and PAS at 1 µg/mL) were dissolved in methanol. After allowing plasma to thaw at room temperature, 50 µL samples of plasma were precipitated with 350 µL of methanol containing internal standard. The double blank is the only sample precipitated with methanol free from internal standards. The samples were then vortexed for 30 seconds and centrifuged at 15000 × g for 10 min. The supernatant (300 µL) was transferred into polypropylene vials (2 ml) and a further 300 µL of 50% acetonitrile (v/v) containing 0.1% formic acid was added. The injection volume was 10 µL.

3.2.6. Liquid chromatography tandem mass spectrometry (LC-MS/MS)

Two different methods were developed:

3.2.6.1.   Method 1

This method was developed and optimized for the identification of methomyl, aldicarb, atrazine, acetochlor, MCPA and fipronil on the Shimadzu 8040 LCMS, where atrazine-d5 was used as an internal standard.

<u>Chromatography</u>

Chromatographic separation and quantification was achieved by using an Agilent Poroshell 120 EC-C18 (2.7 µm, 4.6 ×100 mm) column (California, USA). The mobile phase used was 0.1% formic acid in water (A) and in acetonitrile (B). A gradient was created at a flow rate of 0.5 mL/min. The LC program starts at 30% (B) for 2 min followed by a 0.5 minute ramp to 90% (B); 2.5 min at 90% (B); 0.5 min to 30% (B), equilibrate at 30% (B) for 2.5 min. The total run time is 9 min. The following retention times were observed as shown in Figure 11: methomyl 3.24 min (**A**); aldicarb 4.77 min (**B**); atrazine 5.14 min (**C**); atrazine-D5 5.12 min (**D**); acetochlor 5.81 min (**E**); PAS 3.27 min (**F**); MCPA 5.039 min (**G**) and fipronil 5.46 min (**H**).

<u>Mass Spectrometry</u>

Ionisation mode was set for both positive and negative (ESI +/-); nebulizing gas flow rate was set at 3 mL/min; Drying gas flow rate set at 15 mL/min; desolvation line (DL) temperature was set at 250˚C and the heating block temperature set at

500˚C. The instrument was operated in the scheduled multiple reaction monitoring (sMRM) mode and the loop time was set at 0.5 seconds. A quadratic curve fit was chosen for all of the analytes with 1/x regression over the range of 20 to 600 ng/mL. As can be seen in Table 18 below, the MRM transitions of the protonated precursor ion and the product ion were obtained.

3.2.6.2.　　　Method 2

This method was also developed on the Shimadzu 8040 LCMS for the identification of imidacloprid where PAS was used as an internal standard.

Chromatography

Chromatographic separation and quantification was achieved by using the Restek Raptor Biphenyl (2.7 μm, 2.1 x 100 mm) column (Bellefonte, USA). An isocratic mobile phase of 0.1% formic acid in water (A) and in acetonitrile (B) (65%:35%, v/v) was used at a flow rate of 0.5 mL/min for a run time of 1.75 min. As can be seen in Figure 12 below, the following retention times were observed: Imidacloprid 1.183 min and PAS 0.85 min.

Mass Spectrometry

Ionisation mode was set for both positive and negative (ESI +/-); nebulizing gas flow rate was set at 4 mL/min; drying gas flow rate set at 15 mL/min; DL temperature was set at 250˚C and the heating block temperature set at 400˚C. The instrument was operated in the multiple reaction monitoring (MRM) mode and the loop time was set at 0.5 seconds. A quadratic curve fit was chosen for all of the analytes with a 1/x regression over the range of 20 to 600 ng/mL. As seen in method 1, Table 19 below also shows the MRM transitions of the protonated precursor ion and the product ion.

3.3. Method Validation

The following validation experiments for both methods were carried out on the Shimadzu 8040 LCMS: Inter- and intra-day validation batches over three days, matrix effects, recovery, process efficiency, freshly prepared standards versus stored quality control samples, storage stability, long-term stability, freeze/thaw stability, benchtop stability and whole blood stability.

3.3.1. Intra-day validation batches

Inter- and intra-day validation batches were performed to test accuracy and precision by validating the calibration curve over three days. As this method is aimed at being semi-quantitative, only three standard levels and two quality controls were validated. Two standards at each level were analyzed, namely STD-High at 600 ng/mL, STD-Medium at 100 ng/mL and STD-Low at 20 ng/mL. Six repeats of each Quality Control was also analyzed within each validation batch, namely QC-High at 350 ng/mL and QC-Low at 60 ng/mL.

3.3.2. Matrix effects

Six different sources of CPD plasma were obtained for the matrix effects experiment. These six sources of un-spiked plasma were subjected to protein precipitation using methanol according to the finalised method. The supernatant from the extracted blank samples was then spiked with dilution solution at three theoretical concentration levels at high, medium and low containing both standards and internal standards. For each individual matrix, peak area ratios at three concentration levels in a single matrix were used to generate regressions to evaluate the impact of matrix effects on the robustness of the method.

3.3.3. Recovery

Six different sources of CPD plasma were obtained and spiked at three concentration levels (high 600 ng/ml, medium 100 ng/ml, and low 20 ng/ml). Recoveries were calculated by comparing the peak areas from this data to the peak areas of the samples that were spiked post extraction (used in the matrix effects experiment, as described above 4.3.2.).

3.3.4. Process efficiency

Samples containing only the theoretical concentrations at three levels in injection solution (used to reconstitute the matrix effect samples) were analysed and compared to the spiked QC samples used in the recovery experiment.

3.3.5. Freshly prepared standards versus stored quality control samples

This experiment was performed to determine the impact of freezing on the analytes. Spiked QC samples were aliquoted and frozen. They were then extracted in a batch and compared to freshly prepared samples.

3.3.6. Storage stability

The stability of the analytes during various storage conditions was evaluated. Freshly prepared standards and quality controls were compared to those stored at ~-80°C overnight, ~4°C for 6 hrs, ~-20°C for 6 hrs, and for a longer term of two weeks at ~ -80°C.

3.3.7. Freeze/thaw stability

To evaluate the effects of freezing and thawing on sample stability, QCs stored at ~ -80°C were subjected to three freeze thaw cycles and analyzed. The samples were stored for a minimum of 12 hrs at ~-80°C and were thawed for a maximum of one hour at room temperature during each cycle of the experiment.

3.3.8. Benchtop stability

To determine the stability of the analytes at room temperature, as would occur during sample processing and extraction prior to analysis, QCs were left on the bench for 6 hrs and analyzed against freshly prepared standards for stability.

3.3.9. Whole blood stability

In order to mimic the sample conditions upon receipt from Tygerberg Hospital, whole blood stability was performed.

Validation results are presented and discussed next in Chapter 5.

Chapter 4

Validation results and discussion

4.1. Method validation

Validation is a very important aspect in LC-MS/MS method development as it gives a measure of how reliable the methods are. Reliability is measured by testing the method's accuracy, precision, specificity, sensitivity and reproducibility. A validation becomes even more crucial when the assay method is to be applied within a clinical setting. It is important that the results be reliable since they would have an impact on the diagnosis given and how a patient should be managed. The main aim of developing these methods was to particularly assist with cases of poisoning, overdose and suicide. A semi-quantitative method was therefore deemed appropriate for patient diagnosis and management.

For the purposes of this study, a partial validation was carried out by performing three intra- and inter-day validations to measure method accuracy and precision, together with matrix effects, recovery, process efficiency, whole blood stability, and storage and benchtop stability.
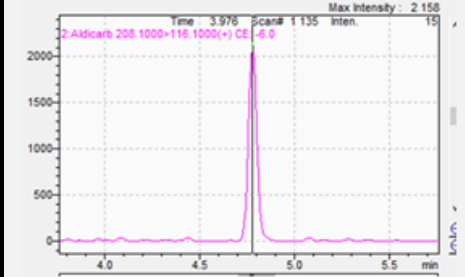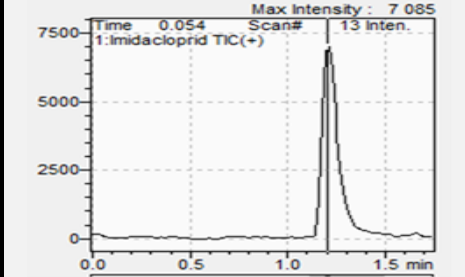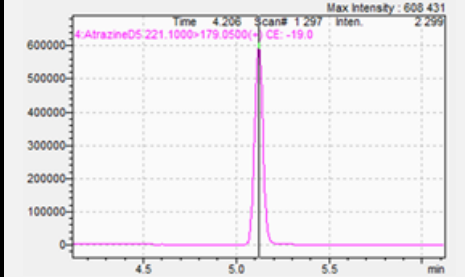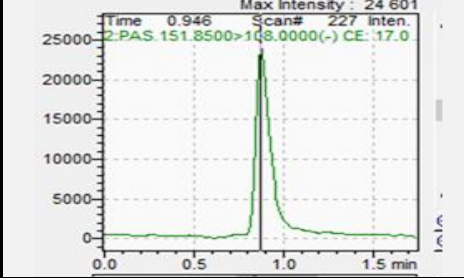
A validation was done to test the reliability and reproducibility of the method. Matrix effects, recovery, process efficiency, whole blood stability, storage and benchtop stability were all measured and tested against accepted criteria. Acceptable criteria used in this validation was applied as recommended by the international guidelines such as the USA Food and Drug Administration (FDA) as well as the European Medicines Agency (EMEA) (EMA, 2011; FDA, 2018).

4.2. Specificity

For specificity, an MRM of each compound was determined. This was done by optimizing the cone voltages and the collision energies of the MS/MS apparatus for each compound. Any compound that does not produce the specific parent ion and product ion as on the MRM will be excluded. Presented in Table 19 is a summary of the multiple reaction monitoring transitions that were obtained for each compound. Furthermore, the extracted mass chromatograms of the highest measured level have also shown.

Table 17. Summary of the multiple reaction monitoring transitions.

Compound	Monoisotopic Mass	[M+H]⁺; [M-H]⁻; [M+NH₄]	Product ion mass (*m/z*) Quantifier	Product ion mass (*m/z*) Qualifier	Chromatogram at 1000 ng/mL
Atrazine	215.09	[M+H]⁺ 216.10	174.10	104.05	
MCPA	200.62	[M-H]⁻ 199.00	141.00	105.20	
Fipronil	435.94	[M-H]⁻ 434.95	329.90	250.00	
Methomyl	162.05	[M+H]⁺ 163.05	88.10	106.10	
Acetochlor	269.12	[M+H]⁺ 270.30	224.10	148.05	

Aldicarb	190.08	[M+NH₄] 208.10	116.10	89.00	
Imidacloprid	255.05	[M+H]⁺ 256.05	209.05	175.15	
Atrazine-D5	220.13	[M+H]⁺ 221.10	179.05	101.10	
PAS	153.04	[M-H]⁻ 151.85	108.00	66.00	

4.3. Validation summary of calibration standards and quality controls

The overall accuracy and precision of a validation is determined by performing inter- and intra-day validation batches. Each of these validation batches must contain all the individual analytes. A summary of the overall accuracy is computed as % Accuracy. This is a measure of the closeness to the nominal concentration, while precision is expressed as the coefficient of variation (%CV). A total of 3 validations were done and are summarised below.

4.3.1. Criteria

A best fit curve representing the observed response vs nominal concentration must be used. For a valid method, the within- and between-batch accuracy over the entire

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calibration range has to within 15%, while the lower limit of quantitation (LLOQ) is allowed to be 20% of the nominal concentration. Inter- and intra-day precision has to be less than 15% over the calibration range and less than 20% at the LLOQ (FDA, 2018).

The following tables (Tables 20 to 33) show the overall summary of calibration standards and QCs for all seven pesticides. Each analyte has separate tables for calibration standards and for QCs. These tables contain accuracy and precision results of each analyte over three validations.

Table 18. Overall summary of calibration standard accuracy and precision: validation 1-3: methomyl

Validation Batch	Sample ID Nominal Conc. Replicates	STD 1 600 (ng/mL) Observed Conc.	STD 2 100 (ng/mL) Observed Conc.	STD 3 20 (ng/mL) Observed Conc.
Validation 1	1	617	97.6	18.6
	2	583	102	21.4
Validation 2	1	633	106	20.4
	2	567	93.6	19.6
Validation 3	1	603	98.1	19.0
	2	600	100	20.0
	n	6	6	6
	Average	601	100	19.8
	STDEV	23.5	4.39	0.99
	% CV	3.91	4.41	5.01
	% Accuracy	100.1	99.7	99.2

Table 19. Overall quality control accuracy and precision estimation: validation 1-3: methomyl

Validation Batch	Sample ID Nominal Conc. Replicates	QC - High 350 (ng/mL) Observed Conc.	QC - Low 60 (ng/mL) Observed Conc.
Validation 1	1	363	56.6
	2	367	61.7
	3	344	59.8
	4	362	60.4
	5	372	61.0
	6	313	59.1
Validation 2	1	386	61.5
	2	366	63.8
	3	364	61.3
	4	363	50.3
	5	346	56.6
	6	328	68.1
Validation 3	1	311	56.7
	2	343	60.8
	3	341	54.9
	4	326	57.7
	5	327	59.7
	6	360	53.7
	n	18	18
	Average	349	59.1
	STDEV	21.4	4.01
	% CV	6.14	6.79
	% Accuracy	99.7	98.5

Table 20. Overall summary of calibration standard accuracy and precision: validation 1-3: aldicarb

Validation Batch	Sample ID Nominal Conc. Replicates	STD 1 600 (ng/mL) Observed Conc.	STD 2 100 (ng/mL) Observed Conc.	STD 3 20 (ng/mL) Observed Conc.
Validation 1	1	577	98.2	23.4
	2	623	102	16.6
Validation 2	1	671	113	18.4
	2	524	86.9	21.6
Validation 3	1	558	103	17.0
	2	600	100	20.0
	n	6	6	6
	Average	592	100	19.5
	STDEV	51.4	8.32	2.65
	% CV	8.68	8.29	13.6
	% Accuracy	98.7	100.4	97.5

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Table 21. Overall quality control accuracy and precision estimation: validation 1-3: aldicarb

Validation Batch	Sample ID Nominal Conc. Replicates	QC - High 350 (ng/mL) Observed Conc.	QC - Low 60 (ng/mL) Observed Conc.
Validation 1	1	409	69.9
	2	391	71.3
	3	407	57.5
	4	378	69.1
	5	415	47.5
	6	339	78.0
Validation 2	1	304	97.1*
	2	360	55.8
	3	335	80.6*
	4	303	55.8
	5	334	59.4
	6	323	77.3
Validation 3	1	256	51.5
	2	294	55.3
	3	314	53.7
	4	318	56.9
	5	323	51.5
	6	349	49.7
	n	18	15
	Average	341.8	58.9
	STDEV	44.0	9.00
	% CV	12.9	15.3
	% Accuracy	97.7	98.1

Denotes QC's that are not within acceptance criteria. These have been omitted from the overall average

Table 22. Overall summary of calibration standard accuracy and precision: validation 1-3: atrazine

Validation Batch	Sample ID Nominal Conc. Replicates	STD 1 600 (ng/mL) Observed Conc.	STD 2 100 (ng/mL) Observed Conc.	STD 3 20 (ng/mL) Observed Conc.
Validation 1	1	619	103	18.6
	2	581	96.8	21.4
Validation 2	1	658	104	17.9
	2	542	95.8	22.1
Validation 3	1	513	97.0	19.4
	2	600	103	20.6
	n	6	6	6
	Average	586	100	20.0
	STDEV	52.7	3.84	1.66
	% CV	8.99	3.84	8.28
	% Accuracy	97.6	100.0	100.0

Table 23. Overall quality control accuracy and precision estimation: validation 1-3: atrazine

Validation Batch	Sample ID Nominal Conc. Replicates	QC - High 350 (ng/mL) Observed Conc.	QC - Low 60 (ng/mL) Observed Conc.
Validation 1	1	367	56.5
	2	378	59.2
	3	378	53.6
	4	345	58.1
	5	345	55.9
	6	291	56.1
Validation 2	1	382	59.5
	2	391	61.8
	3	383	60.6
	4	364	51.0
	5	348	54.7
	6	348	68.0
Validation 3	1	252	54.7
	2	294	58.7
	3	334	50.6
	4	339	60.6
	5	336	69.1
	6	330	56.7
	n	18	18
	Average	344.8	58.1
	STDEV	36.5	4.91
	% CV	10.6	8.46
	% Accuracy	98.5	96.8

Table 24. Overall summary of calibration standard accuracy and precision: validation 1-3: acetochlor

Validation Batch	Sample ID Nominal Conc. Replicates	STD 1 600 (ng/mL) Observed Conc.	STD 2 100 (ng/mL) Observed Conc.	STD 3 20 (ng/mL) Observed Conc.
Validation 1	1	644	101	19.3
	2	556	98.7	20.7
Validation 2	1	634	101	21.4
	2	566	98.7	18.6
Validation 3	1	450	92.7	19.3
	2	600	107	20.7
	n	6	6	6
	Average	575	100	20.0
	STDEV	70.7	4.76	1.11
	% CV	12.3	4.76	5.56
	% Accuracy	95.9	100.0	100.0

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Table 25. Overall quality control accuracy and precision estimation: validation 1-3: acetochlor

Validation Batch	Sample ID Nominal Conc. Replicates	QC - High 350 (ng/mL) Observed Conc.	QC - Low 60 (ng/mL) Observed Conc.
Validation 1	1	385	58.1
	2	398	59.5
	3	403	53.4
	4	324	53.4
	5	334	49.4
	6	287	56.2
Validation 2	1	339	59.2
	2	353	64.9
	3	342	58.8
	4	379	49.5
	5	349	52.7
	6	324	63.5
Validation 3	1	243	56.2
	2	297	60.2
	3	315	53.2
	4	359	65.2
	5	336	64.7
	6	384	57.5
	n	18	18
	Average	342	57.5
	STDEV	41.0	5.01
	% CV	12.0	8.72
	% Accuracy	97.7	95.9

Table 26. Overall summary of calibration standard accuracy and precision: validation 1-3: MCPA

Validation Batch	Sample ID Nominal Conc. Replicates	STD 1 600 (ng/mL) Observed Conc.	STD 2 100 (ng/mL) Observed Conc.	STD 3 20 (ng/mL) Observed Conc.
Validation 1	1	613	104	20.8
	2	587	95.7	19.2
Validation 2	1	645	104	21.6
	2	555	95.7	18.4
Validation 3	1	518	99.5	21.3
	2	600	101	18.7
	n	6	6	6
	Average	586	100	20.0
	STDEV	44.8	3.86	1.35
	% CV	7.64	3.86	6.77
	% Accuracy	97.7	100.0	100.0

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Table 27. Overall quality control accuracy and precision estimation: validation 1-3: MCPA

Validation Batch	Sample ID Nominal Conc. Replicates	QC - High 350 (ng/mL) Observed Conc.	QC - Low 60 (ng/mL) Observed Conc.
Validation 1	1	361	58.9
	2	413	58.2
	3	409	51.8
	4	353	58.8
	5	374	50.3
	6	303	58.7
Validation 2	1	333	52.7
	2	355	58.8
	3	339	64.6
	4	343	39.4*
	5	330	57.3
	6	326	64.9
Validation 3	1	220	62.9
	2	292	62.6
	3	313	50.1
	4	372	60.2
	5	315	65.6
	6	354	60.9
	n	18	17
	Average	339.3	58.7
	STDEV	44.1	4.95
	% CV	13.0	8.44
	% Accuracy	96.9	97.8

Denotes QC's that are not within acceptance criteria. Not included in the overall average

Table 28. Overall summary of calibration standard accuracy and precision: validation 1-3: fipronil

Validation Batch	Sample ID Nominal Conc. Replicates	STD 1 600 (ng/mL) Observed Conc.	STD 2 100 (ng/mL) Observed Conc.	STD 3 20 (ng/mL) Observed Conc.
Validation 1	1	613	100	19.8
	2	587	100	20.2
Validation 2	1	642	103	20.8
	2	559	96.8	19.2
Validation 3	1	575	93.8	20.3
	2	600	106	19.7
	n	6	6	6
	Average	596	100	20.0
	STDEV	29.4	4.44	0.56
	% CV	4.93	4.44	2.82
	% Accuracy	99.3	100.0	100.0

Table 29. Overall quality control accuracy and precision estimation: validation 1-3: fipronil

Validation Batch	Sample ID Nominal Conc. Replicates	QC - High 350 (ng/mL) Observed Conc.	QC - Low 60 (ng/mL) Observed Conc.
Validation 1	1	386	55.4
	2	351	57.6
	3	380	55.9
	4	349	58.1
	5	355	55.1
	6	309	56.9
Validation 2	1	373	58.6
	2	350	64.4
	3	337	60.0
	4	356	49.4
	5	342	56.9
	6	319	67.0
Validation 3	1	289	56.7
	2	330	55.3
	3	354	59.5
	4	352	58.0
	5	357	61.8
	6	373	51.1
	n	18	18
	Average	347.9	57.7
	STDEV	24.6	4.14
	% CV	7.08	7.18
	% Accuracy	99.4	96.1

Table 30. Overall summary of calibration standard accuracy and precision: validation 1-3: imidacloprid

Validation Batch	Sample ID Nominal Conc. Replicates	STD 1 600 (ng/mL) Observed Conc.	STD 2 100 (ng/mL) Observed Conc.	STD 3 20 (ng/mL) Observed Conc.
Validation 1	1	542	104	19.5
	2	659	95.7	20.5
Validation 2	1	586	106	16.9
	2	614	94.2	23.1
Validation 3	1	623	112	17.6
	2	577	88.1	22.4
	n	6	6	6
	Average	600	100	20.0
	STDEV	40.6	8.81	2.53
	% CV	6.77	8.81	12.6
	% Accuracy	100	100	100

Table 31. Overall quality control accuracy and precision estimation: validation 1-3: imidacloprid

Validation Batch	Sample ID Nominal Conc. Replicates	QC - High 350 (ng/mL) Observed Conc.	QC - Low 60 (ng/mL) Observed Conc.
Validation 1	1	337	56.9
	2	375	43.4
	3	313	57.8
	4	368	33.0*
	5	335	63.5
	6	316	71.9
Validation 2	1	383	61.5
	2	349	82.0
	3	357	66.7
	4	368	67.5
	5	343	69.1
	6	352	56.2
Validation 3	1	308	61.8
	2	347	59.9
	3	376	53.6
	4	339	60.5
	5	330	60.2
	6	313	48.5
	n	18	16
	Average	345.0	59.9
	STDEV	23.2	7.41
	% CV	6.73	12.4
	% Accuracy	98.6	99.9

Denotes QC's that are not within acceptance criteria. Not included in the overall average

4.3.2.   Validation summary and discussion

Methomyl, atrazine, acetochlor, MCPA, fipronil and imidacloprid had an observed overall accuracy and precision for calibration standards and quality controls that was within acceptable limits as set out on the criteria above (**4.3.1**).   Certain QC replicates for aldicarb, MCPA and imidacloprid were not within acceptable limits. However, more that 50% of QCs for each analyte at each level were still within the required 15% accuracy for each validation batch, and more than 67% of the total QCs within a batch passed, as per FDA guidelines.

Accuracy for both calibration standards and QCs for aldicarb was within acceptable limits (Tables 5.3 and 5.4). Precision on the other hand for the QC Low was 15.3%, slightly above the acceptable criteria of 15%.

Based on these validation results we can conclude the calibration range of 20 ng/mL to 600 ng/mL for the semi-quantitative analysis of all the analytes is valid. The calibration standards and QCs depicted an inter- and intra-day precision and accuracy that is acceptable.

4.4. Matrix effect

To assess the effects of matrix on the analytes Matuszewski strategies were employed (Matuszewski *et al.*, 2003). CPD plasma was extracted from six different sources and spiked at three theoretical concentration levels post-extraction (high - 600 ng/ml, medium - 100 ng/ml & low - 20 ng/mL). Table 34 below shows how theoretical concentrations were obtained when taking the various steps used during extraction into consideration.

4.4.1.   Criteria

A method may be considered valid when a minimum of five different lots of post-extraction spiked matrix have a slope variability (%CV) less than 5% across the high, medium and low concentration ranges.

Table 32. Calculation of theoretical concentrations

	High (600 ng/mL)	Medium (100 ng/mL)	Low (20 ng/mL)
EXTRACTION	600 ng/mL×50 µL = **C2**×350 µL **C2** = 85.7 ng/mL	100 ng/mL×50 µL = **C2**×350 µL **C2** = 14.3 ng/mL	20 ng/mL×50 µL = **C2**×350 µL **C2** = 2.86 ng/mL
DILUTION	85.7 ng/mL×300 µL = **C2**×600 µL **C2** = 42.85 ng/mL	14.3 ng/mL × 300 µL = **C2**×600 µL **C2** = 7.15 ng/mL	2.86 ng/mL×300 µL = **C2**×600 µL **C2**= 1.43 ng/mL

Six individual CPD matrixes was spiked at low, medium and high concentration levels. The internal standard was spiked at one concentration. Tables 35 to 41 below show the overall %CVs of the slopes calculated for each analyte.

Table 33. Matrix effects for methomyl showing regression results from different matrices

	High Concentration (600 ng/mL) Peak Area Ratio	Medium Concentration (100 ng/mL) Peak Area Ratio	Low Concentration (20 ng/mL) Peak Area Ratio	Area Ratio v Conc. Regression Slope
Matrix 1	0.217	0.0410	0.0101	0.000356
Matrix 2	0.211	0.0396	0.00919	0.000346
Matrix 3	0.217	0.0387	0.00933	0.000358
Matrix 4	0.214	0.0392	0.00872	0.000353
Matrix 5	0.215	0.0398	0.00881	0.000353
Matrix 6	0.217	0.0399	0.00859	0.000358
Average	0.215	0.0397	0.00913	0.000354
STDEV	0.00249	0.000785	0.000559	0.00000440
% CV	1.16	1.98	6.13	1.24

Table 34. Matrix effects for aldicarb showing regression results from different matrices

	High Concentration (600 ng/mL) Peak Area Ratio	Medium Concentration (100 ng/mL) Peak Area Ratio	Low Concentration (20 ng/mL) Peak Area Ratio	Area Ratio v Conc. Regression Slope
Matrix 1	0.0227	0.00392	0.000963	0.0000375
Matrix 2	0.0223	0.00469	0.000977	0.0000362
Matrix 3	0.0202	0.00339	0.000665	0.0000337
Matrix 4	0.0212	0.00416	0.00125	0.0000343
Matrix 5	0.0213	0.00356	0.000772	0.0000354
Matrix 6	0.0228	0.00372	0.000819	0.0000380
Average	0.0218	0.00391	0.000907	0.0000358
STDEV	0.00101	0.000470	0.000204	0.00000171
% CV	4.65	12.0	22.5	4.77

Table 35. Matrix effects for atrazine showing regression results from different matrices

	High Concentration (600 ng/mL) Peak Area Ratio	Medium Concentration (100 ng/mL) Peak Area Ratio	Low Concentration (20 ng/mL) Peak Area Ratio	Area Ratio v Conc. Regression Slope
Matrix 1	0.242	0.0429	0.0101	0.000399
Matrix 2	0.240	0.0435	0.00966	0.000395
Matrix 3	0.242	0.0435	0.00957	0.000399
Matrix 4	0.242	0.0430	0.0104	0.000398
Matrix 5	0.242	0.0434	0.0101	0.000398
Matrix 6	0.251	0.0446	0.00934	0.000416
Average	0.243	0.0435	0.00988	0.000401
STDEV	0.00426	0.000594	0.000417	0.00000767
% CV	1.75	1.37	4.22	1.91

Table 36. Matrix effects for acetochlor showing regression results from different matrices

	High Concentration (600 ng/mL) Peak Area Ratio	Medium Concentration (100 ng/mL) Peak Area Ratio	Low Concentration (20 ng/mL) Peak Area Ratio	Area Ratio v Conc. Regression Slope
Matrix 1	0.429	0.0743	0.0185	0.000709
Matrix 2	0.408	0.0744	0.0169	0.000672
Matrix 3	0.434	0.0746	0.0184	0.000718
Matrix 4	0.435	0.0774	0.0197	0.000717
Matrix 5	0.430	0.0749	0.0161	0.000713
Matrix 6	0.433	0.0788	0.0165	0.000714
Average	0.428	0.0757	0.0177	0.000707
STDEV	0.0102	0.00189	0.00141	0.0000175
% CV	2.39	2.50	7.98	2.48

Table 37. Matrix effects for MCPA showing regression results from different matrices

	High Concentration (600 ng/mL) Peak Area Ratio	Medium Concentration (100 ng/mL) Peak Area Ratio	Low Concentration (20 ng/mL) Peak Area Ratio	Area Ratio v Conc. Regression Slope
Matrix 1	0.0330	0.00599	0.00135	0.0000543
Matrix 2	0.0320	0.00626	0.00121	0.0000526
Matrix 3	0.0332	0.00579	0.00143	0.0000548
Matrix 4	0.0330	0.00611	0.00128	0.0000544
Matrix 5	0.0316	0.00572	0.00141	0.0000520
Matrix 6	0.0345	0.00593	0.00149	0.0000570
Average	0.0329	0.00597	0.00136	0.0000542
STDEV	0.00101	0.000201	0.000103	0.00000179
% CV	3.07	3.38	7.54	3.30

Table 38. Matrix effects for fipronil showing regression results from different matrices

	High Concentration (600 ng/mL) Peak Area Ratio	Medium Concentration (100 ng/mL) Peak Area Ratio	Low Concentration (20 ng/mL) Peak Area Ratio	Area Ratio v Conc. Regression Slope
Matrix 1	1.35	0.285	0.0750	0.00217
Matrix 2	1.32	0.282	0.0701	0.00212
Matrix 3	1.35	0.271	0.0714	0.00218
Matrix 4	1.30	0.282	0.0719	0.00209
Matrix 5	1.33	0.275	0.0664	0.00216
Matrix 6	1.34	0.274	0.0712	0.00217
Average	1.33	0.278	0.0710	0.00215
STDEV	0.0183	0.00553	0.00278	0.0000351
% CV	1.37	1.99	3.92	1.63

Table 39. Matrix effects for imidacloprid showing regression results from different matrices

	High Concentration (600 ng/mL) Peak Area Ratio	Medium Concentration (100 ng/mL) Peak Area Ratio	Low Concentration (20 ng/mL) Peak Area Ratio	Area Ratio v Conc. Regression Slope
Matrix 1	0.300	0.0622	0.0156	0.000485
Matrix 2	0.281	0.0501	0.0115	0.000464
Matrix 3	0.314	0.0565	0.0128	0.000519
Matrix 4	0.299	0.0530	0.0164	0.000488
Matrix 5	0.284	0.0574	0.0119	0.000463
Matrix 6	0.300	0.0470	0.0124	0.000499
Average	0.296	0.0544	0.0134	0.000486
STDEV	0.0122	0.00549	0.00205	0.0000211
% CV	4.12	10.1	15.3	4.35

4.4.2. Matrix effects discussion

The regression slope variability across all seven analytes was less than 5%. This shows that the internal standards used in the method were adequately compensating for any matrix effects present. This means that any co-extracted matrix components have no impact on the ionisation of the analytes and internal standards. The absence of matrix effects on this quantitation method therefore indicates method robustness (Matuszewski *et al.*, 2003).

4.5. Absolute recovery

To test whether a method's extraction is efficient, recovery is measured. The recovery tube contains the extracted spiked sample, as for the conventional standards and QCs, and it is compared to the matrix effects sample which contains extracted blank samples that are reconstituted in spiked injection solvent at theoretical concentrations. The recovery results for all of the analytes are displayed Tables 42 to 48 below.

4.5.1. Criteria

Analyte recovery should display consistency and precision. The percentage coefficient of variation (%CV) should not exceed 15% at any measured concentration, and the average percentage recovery across the concentration range should also have a %CV of less than 15%.

Table 40. Recovery of methomyl

	High (600 ng/mL)		Medium (100 ng/mL)		Low (20 ng/mL)	
	Extraction	Spiked Solution	Extraction	Spiked Solution	Extraction	Spiked Solution
	Peak Area	Peak Area	Peak Area	Peak Area	Peak Area	Peak Area
Sample 1	599560	552959	96242	109994	20948	25144
Sample 2	538865	581607	96702	106135	19727	23512
Sample 3	525671	602425	92657	108040	17723	23857
Sample 4	487850	574513	91551	107894	17708	21779
Sample 5	533517	608463	88676	110220	17787	24258
Sample 6	546411	573732	93169	111140	17764	21111
Average	538646	582283	93166	108904	18610	23277
STDEV	36182	20420	3000	1863	1393	1535
% CV	6.72	3.51	3.22	1.71	7.49	6.59
% Recovery		92.5		85.5		79.9

Average % Recovery		86.0
Average % CV		7.31

Table 41. Recovery of aldicarb

	High (600 ng/mL)		Medium (100 ng/mL)		Low (20 ng/mL)	
	Extraction	Spiked Solution	Extraction	Spiked Solution	Extraction	Spiked Solution
	Peak Area	Peak Area	Peak Area	Peak Area	Peak Area	Peak Area
Sample 1	70059*	57769	11387	10522	1545	2396
Sample 2	60718	61335	10022	12564	1440	2499
Sample 3	47530	56101	8455	9473	1263	1700
Sample 4	49110	56909	8538	11452	1374	3114
Sample 5	49991	60225	9149	9860	2075	2126
Sample 6	59725	60164	8937	10349	1428	2013
Average	53415	58751	9415	10703	1521	2308
STDEV	6286	2108	1118	1132	287	487
% CV	11.8	3.6	11.9	10.6	18.8	21.1
% Recovery		90.9		88.0		65.9

Denotes area not within acceptance criteria. Not included in the overall average

Average % Recovery		81.6
Average % CV		16.8

Table 42. Recovery of atrazine

	High (600 ng/mL)		Medium (100 ng/mL)		Low (20 ng/mL)	
	Extraction	Spiked Solution	Extraction	Spiked Solution	Extraction	Spiked Solution
	Peak Area	Peak Area	Peak Area	Peak Area	Peak Area	Peak Area
Sample 1	711721	615618	103811	115076	19553	25223
Sample 2	628081	659848	107807	116434	21093	24716
Sample 3	591849	670162	104937	121514	18274	24466
Sample 4	551917	647495	104291	118228	19522	26038
Sample 5	603314	684126	94551	120234	19573	27869
Sample 6	612482	663559	100472	124046	20539	22963
Average	616561	656801	102645	119255	19759	25213
STDEV	53235	23506	4609	3331	972	1649
% CV	8.63	3.58	4.49	2.79	4.92	6.54
% Recovery		93.9		86.1		78.4

Average % Recovery		86.1
Average % CV		9.00

Table 43. Recovery of acetochlor

	High (600 ng/mL)		Medium (100 ng/mL)		Low (20 ng/mL)	
	Extraction	Spiked Solution	Extraction	Spiked Solution	Extraction	Spiked Solution
	Peak Area	Peak Area	Peak Area	Peak Area	Peak Area	Peak Area
Sample 1	1173747	1092423	175352	199180	32775	46106
Sample 2	1022833	1123750	178569	199342	32185	43217
Sample 3	1057277	1204393	185055	208400	28991	47044
Sample 4	923829	1167446	164288	212715	28230	49237
Sample 5	1040693	1218974	168590	207400	27305	44275
Sample 6	1087322	1141148	178322	219268	30918	40599
Average	1050950	1158022	175029	207718	30067	45080
STDEV	81851	48408	7494	7772	2223	3046
% CV	7.79	4.18	4.28	3.74	7.39	6.76
% Recovery		90.8		84.3		66.7

Average % Recovery		80.6
Average % CV		15.4

Table 44. Recovery of MCPA

	High (600 ng/mL)		Medium (100 ng/mL)		Low (20 ng/mL)	
	Extraction	Spiked Solution	Extraction	Spiked Solution	Extraction	Spiked Solution
	Peak Area	Peak Area	Peak Area	Peak Area	Peak Area	Peak Area
Sample 1	91839	83853	15314	16055	2625	3364
Sample 2	86858	88210	14669	16774	4471	3103
Sample 3	80216	92039	14809	16179	2945	3650
Sample 4	72120	88455	14756	16812	3204	3188
Sample 5	79067	89617	13837	15839	2862	3885
Sample 6	82077	91105	14254	16501	3232	3664
Average	82030	88880	14607	16360	3223	3476
STDEV	6779	2877	507	398	652	306
% CV	8.26	3.24	3.47	2.43	20.22	8.80
% Recovery		92.3		89.3		92.7
					Average % Recovery	91.4
					Average % CV	2.06

Table 45. Recovery of fipronil

	High (600 ng/mL)		Medium (100 ng/mL)		Low (20 ng/mL)	
	Extraction	Spiked Solution	Extraction	Spiked Solution	Extraction	Spiked Solution
	Peak Area	Peak Area	Peak Area	Peak Area	Peak Area	Peak Area
Sample 1	3655139	3426343	674789	764596	163948	186490
Sample 2	3299077	3623883	665264	755142	157223	179370
Sample 3	3261457	3737746	649144	757855	143877	182567
Sample 4	2989092	3492468	619698	776844	144499	179357
Sample 5	3311771	3781520	622685	762065	142129	182769
Sample 6	3305293	3534902	649053	763128	142800	175009
Average	3303638	3599477	646772	763272	149079	180927
STDEV	211831	140321	22140	7522	9200	3923
% CV	6.41	3.90	3.42	0.99	6.17	2.17
% Recovery		91.8		84.7		82.4
					Average % Recovery	86.3
					Average % CV	5.66

Table 46. Recovery of imidacloprid

	High (600 ng/mL)		Medium (100 ng/mL)		Low (20 ng/mL)	
	Extraction	**Spiked Solution**	**Extraction**	**Spiked Solution**	**Extraction**	**Spiked Solution**
	Peak Area	**Peak Area**	**Peak Area**	**Peak Area**	**Peak Area**	**Peak Area**
Sample 1	37168	35882	5868	7436	1587	1892
Sample 2	33403	37615	6132	6130	1528	1427
Sample 3	33227	40161	6004	7150	1227	1586
Sample 4	30803	37060	5375	6651	1139	2046
Sample 5	34877	39135	5104	7301	1186	1491
Sample 6	34529	38159	5855	6499	1206	1480
Average	34001	38002	5723	6861	1312	1654
STDEV	2110	1517	397	513	193	254
% CV	6.21	3.99	6.94	7.48	14.7	15.4
% Recovery		89.5		83.4		79.3

					Average % Recovery	84.1
					Average % CV	6.06

4.5.2.   Absolute recovery discussion

All analytes displayed an average recovery ranging from 80.6% to 91.4%. According to Sonawane *et al.* (2014) recoveries need not be 100%, but should be consistent, precise and reproducible (Sonawawane *et al.*, 2014). None of the analytes exceed a coefficient variation of 15% except for aldicarb and acetochlor with a %CV of 16.8 and 15.4 respectively. Sinha *et al.* (2012) recommend the use of QuEChERS for the extraction of very polar samples such as aldicarb as they require little solvent and they were shown to achieve reproducible results with high levels of recoveries at low concentrations (Sinha *et al.*, 2012). Should isotopically labelled analogues of aldicarb and acetochlor had been included, the recoveries would have better consistency. As it stands, for a semi-quantitative method for pesticide poisoning, this was deemed acceptable by the laboratory management.

Based on the high recovery range observed, it can be concluded that the extraction methodology is effective and reliable at extracting these analytes from plasma.

4.6. Process efficiency

Process efficiency (PE) is performed to measure extraction and instrument efficiency. This is done by preparing theoretical concentrations of analytes in solvent and comparing them to spiked QCs used in the recovery experiment. The process efficiency results for all analytes are displayed Tables 49 to 55 below.

4.6.1.   Criteria

The process efficiency should be consistent across the three concentration levels tested for the matrix-free spiked injection solution and the recovery samples consisting of spiked and extracted plasma QC samples in 6 different matrices. The coefficient of variation at each level should be less than 15%, while the average of the process efficiencies across the concentration range should also be less than 15%.

Table 47. Process efficiency (PE) results for methomyl extracted from matrix compared to methomyl spiked into reconstitution solution free of matrix.

	High (600 ng/mL)		Medium (100 ng/mL)		Low (20 ng/mL)	
	Extraction	Spiked Solution	Extraction	Spiked Solution	Extraction	Spiked Solution
	Peak Area	Peak Area	Peak Area	Peak Area	Peak Area	Peak Area
Sample 1	599560	504729	96242	106702	20948	18542
Sample 2	538865	501038	96702	106711	19727	18754
Sample 3	525671	502094	92657	103965	17723	19029
Sample 4	487850	495351	91551	105216	17708	18691
Sample 5	533517	497697	88676	105963	17787	19438
Sample 6	546411	505363	93169	107767	17764	18816
Average	538646	501045	93166	106054	18610	18878
STDEV	36182	3923	3000	1331	1393	317
% CV	6.72	0.783	3.22	1.26	7.49	1.68
% Recovery		108		87.8		98.6

					Average % PE	98.0
					Average % CV	10.0

Table 48. Process efficiency (PE) results for atazine extracted from matrix compared to atrazine spiked into reconstitution solution free of matrix.

	High (600 ng/mL)		Medium (100 ng/mL)		Low (20 ng/mL)	
	Extraction	Spiked Solution	Extraction	Spiked Solution	Extraction	Spiked Solution
	Peak Area	Peak Area	Peak Area	Peak Area	Peak Area	Peak Area
Sample 1	711721	574332	103811	116577	19553	24302
Sample 2	628081	590867	107807	124195	21093	22649
Sample 3	591849	585399	104937	118518	18274	23313
Sample 4	551917	585156	104291	120158	19522	23171
Sample 5	603314	573482	94551	119426	19573	21786
Sample 6	612482	567494	100472	119888	20539	21853
Average	616561	579455	102645	119794	19759	22846
STDEV	53235	8978	4609	2515	972	958
% CV	8.63	1.55	4.49	2.10	4.92	4.19
% Recovery		106		85.7		86.5

Average % PE	92.9
Average % CV	12.6

Table 49. Process efficiency (PE) results for MCPA extracted from matrix compared to MCPA spiked into reconstitution solution free of matrix.

	High (600 ng/mL)		Medium (100 ng/mL)		Low (20 ng/mL)	
	Extraction	Spiked Solution	Extraction	Spiked Solution	Extraction	Spiked Solution
	Peak Area	Peak Area	Peak Area	Peak Area	Peak Area	Peak Area
Sample 1	91839	82810	15314	17633	2625	2951
Sample 2	86858	79760	14669	17402	4471	3092
Sample 3	80216	75516	14809	16688	2945	3169
Sample 4	72120	77548	14756	15996	3204	3093
Sample 5	79067	74033	13837	16231	2862	2885
Sample 6	82077	75815	14254	16661	3232	3412
Average	82030	77580	14607	16769	3223	3100
STDEV	6779	3227	507	641	652	185
% CV	8.26	4.16	3.47	3.82	20.2	5.96
% Recovery		106		87.1		104

Average % PE	98.9
Average % CV	10.4

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Table 50. Process efficiency (PE) results for fipronil extracted from matrix compared to fipronil spiked into reconstitution solution free of matrix.

	High (600 ng/mL)		Medium (100 ng/mL)		Low (20 ng/mL)	
	Extraction	Spiked Solution	Extraction	Spiked Solution	Extraction	Spiked Solution
	Peak Area	Peak Area	Peak Area	Peak Area	Peak Area	Peak Area
Sample 1	3655139	3108247	674789	765543	163948	155592
Sample 2	3299077	3189583	665264	720271	157223	159481
Sample 3	3261457	3141501	649144	746054	143877	166597
Sample 4	2989092	3246589	619698	739730	144499	161056
Sample 5	3311771	3100531	622685	746957	142129	165608
Sample 6	3305293	3151140	649053	756731	142800	168430
Average	3303638	3156265	646772	745881	149079	162794
STDEV	211831	54635	22140	15487	9200	4899
% CV	6.41	1.73	3.42	2.08	6.17	3.01
% Recovery		105		86.7		91.6

			Average % PE	94.3
			Average % CV	9.85

Table 51. Process efficiency (PE) results for imidacloprid extracted from matrix compared to imidacloprid spiked into reconstitution solution free of matrix.

	High (600 ng/mL)		Medium (100 ng/mL)		Low (20 ng/mL)	
	Extraction:	Spiked Solution	Extraction:	Spiked Solution	Extraction:	Spiked Solution
	Peak Area	Peak Area	Peak Area	Peak Area	Peak Area	Peak Area
Sample 1	37168	33086	5868	7017	1587	1130
Sample 2	33403	30865	6132	5664	1528	[1961]
Sample 3	33227	29140	6004	5968	1227	1539
Sample 4	30803	30720	5375	6795	1139	1550
Sample 5	34877	29083	5104	6129	1186	1603
Sample 6	34529	27475	5855	5343	1206	1290
Average	34001	30062	5723	6153	1312	1422
STDEV	2110	1935	397	646	193	203
% CV	6.21	6.44	6.94	10.5	14.7	14.3
% Recovery		113		93.0		92.3

			Average % PE	99.5
			Average % CV	11.9

A comparison of generated results was made comparing results without internal standard to results where atrazine-D5 was used as an internal standard. It became evident that acetochlor and aldicarb had more consistency and efficiency in the presence of an internal standard, using the ratio of the analyte to internal standard peak areas. Tables 54 and 55 below shows the PE results of the two analytes in the presence of the internal standard.

Table 52. Process efficiency (PE) results for acetochlor/ISTD extracted from matrix compared to acetochlor/ISTD spiked into reconstitution solution free of matrix

	High (600 ng/mL)		Medium (100 ng/mL)		Low (20 ng/mL)	
	Extraction	Spiked Solution	Extraction	Extraction	Spiked Solution	Extraction
	Peak Area Ratio	Peak Area Ratio	Peak Area Ratio	Peak Area Ratio	Peak Area Ratio	Peak Area Ratio
Sample 1	0.497	0.470	0.0742	0.0935	0.0166	0.0164
Sample 2	0.444	0.447	0.0764	0.0905	0.0146	0.0174
Sample 3	0.455	0.463	0.0799	0.0876	0.0127	0.0176
Sample 4	0.369	0.449	0.0705	0.0907	0.0129	0.0162
Sample 5	0.445	0.458	0.0716	0.0917	0.0119	0.0172
Sample 6	0.480	0.437	0.0782	0.0910	0.0139	0.0177
Average	0.448	0.454	0.0751	0.0908	0.0138	0.0171
STDEV	0.0442	0.0120	0.00371	0.00193	0.00167	0.000646
% CV	9.86	2.64	4.94	2.12	12.15	3.78
% Recovery		98.7		82.7		80.6

Average % PE	87.3
Average % CV	11.3

Table 53. Process efficiency (PE) results for aldicarb/ISTD extracted from matrix compared to aldicarb/ISTD spiked into reconstitution solution free of matrix

	High (600 ng/mL)		Medium (100 ng/mL)		Low (20 ng/mL)	
	Extraction:	Spiked Solution	Extraction:	Spiked Solution	Extraction:	Spiked Solution
	Peak Area Ratio	Peak Area Ratio	Peak Area Ratio	Peak Area Ratio	Peak Area Ratio	Peak Area Ratio
Sample 1	0.0297	0.0259	0.00482	0.00512	0.000783	0.000786
Sample 2	0.0263	0.0250	0.00429	0.00558	0.000654	0.00104
Sample 3	0.0204	0.0259	0.00365	0.00581	0.000555	0.00120
Sample 4	0.0196	0.0266	0.00366	0.00564	0.000627	0.00122
Sample 5	0.0214	0.0268	0.00388	0.00544	0.000906	0.00108
Sample 6	0.0263	0.0262	0.00392	0.00538	0.000642	0.000967
Average	0.0240	0.0261	0.00404	0.00549	0.000694	0.00105
STDEV	0.004050	0.000625	0.000448	0.000237	0.000127	0.000162
% CV	16.9	2.40	11.1	4.3	18.3	15.4
% Recovery		91.8		73.5		66.1

Average % PE	77.2
Average % CV	17.2

4.6.2. Process efficiency discussion

The results in the tables above show that methomyl, atrazine, MCPA, fipronil, imidacloprid all had a consistent process efficiency across all three concentration levels. This consistency was all in the absence of an internal standards. The

coefficient of variation at each level was less than 15%, while the average of the process efficiencies across the concentration range was also be less than 15%. As previously noticed and noted during absolute recovery, the coefficient of variation for acetochlor and aldicarb were slightly above 15%. However, with the addition of an internal standard, acetochlor had an improved process efficiency ≤15% at each level and across the range. Aldicarb, on the other hand, still had a %CV above 15 even with the addition of an internal standard, signaling that perhaps atrazine-d5 isn't a suitable internal standard for aldicarb. An isotopically labelled analogue for aldicarb would have been more suitable.

4.7. Stability assessment

To determine the effects that various storage conditions have on analytes, a total of seven stability experiments were performed.   As per FDA guidelines QCs must be used to assess the accuracy and precision of an assay and sample stability. The QCs are used to evaluate the performance of the various analytes under various conditions. These QCs must be prepared in the same matrix as would be used once the method has been validated. These assessments are performed to give a clear understanding how the samples should be collected, handled and stored (FDA, 2018).

4.7.1.   Fresh vs. frozen stability

This stability assessment was done in order to test the effect of freezing on the analyte by evaluating QCs that had been stored at ~-80°C against a calibration curve generated by freshly prepared standards.

4.7.1.1.   Criteria

For this experiment, the general criteria applicable to QCs is used. That is a measured accuracy between 85–115% and a precision below 15%.

Tables 56 and 57 give a consistency and accuracy account of the freshly prepared QCs vs stored QCs.   A total of six repeats were prepared and analyzed at every level for each analyte. Observed concentrations were then compared to the nominal concentration at each level.

Table 54. Summary of high quality controls to measure fresh vs. frozen stability

	Sample ID	Repeats	Nominal Conc. (ng/mL)	Observed Conc (ng/mL)	Mean Observed Conc. (ng/mL)	Std Dev	% CV	%Accuracy
Methomyl	QC H	Rep 1	350	399	367	17.3	4.71	114
		Rep 2		368				105
		Rep 3		349				100
		Rep 4		364				104
		Rep 5		368				105
		Rep 6		355				101
Aldicarb	QC H	Rep 1	350	476	397	55.3	13.9	136*
		Rep 2		389				111
		Rep 3		358				102
		Rep 4		321				91.8
		Rep 5		439				125*
		Rep 6		397				113
Atrazine	QC H	Rep 1	350	398	371	21.3	5.74	114
		Rep 2		364				104
		Rep 3		353				101
		Rep 4		356				102
		Rep 5		398				114
		Rep 6		357				102
Acetochlor	QC H	Rep 1	350	396	364	23.7	6.51	113
		Rep 2		351				100
		Rep 3		327				93.3
		Rep 4		364				104
		Rep 5		371				106
		Rep 6		377				108
MCPA	QC H	Rep 1	350	382	369	14.8	4.01	109
		Rep 2		359				103
		Rep 3		352				101
		Rep 4		386				110
		Rep 5		379				108
		Rep 6		357				102
Fipronil	QC H	Rep 1	350	391	358	19.0	5.31	112
		Rep 2		356				102
		Rep 3		337				96.3
		Rep 4		366				105
		Rep 5		354				101
		Rep 6		344				98.2
Imidacloprid	QC H	Rep 1	350	398	341	29.0	8.51	114
		Rep 2		330				94.3
		Rep 3		342				97.7
		Rep 4		328				93.8
		Rep 5		324				92.5
		Rep 6		321				91.7

*Denotes QC's that are not within acceptance criteria

Table 55. Summary of low quality controls to measure fresh vs. frozen stability

	Sample ID	Repeats	Nominal Conc. (ng/mL)	Observed Conc (ng/mL)	Mean Observed Conc. (ng/mL)	Std Dev	% CV	%Accuracy
Methomyl	QC L	Rep 1	60	60.1	62.6	2.35	3.75	100
		Rep 2		62.2				104
		Rep 3		66.1				110
		Rep 4		64.7				108
		Rep 5		60.7				101
		Rep 6		61.9				103
Aldicarb	QC L	Rep 1	60	77,0	59.1	11.7	19.8*	128*
		Rep 2		71.3				119*
		Rep 3		67.0				112
		Rep 4		94,5				157*
		Rep 5		48.2				80.3*
		Rep 6		50.0				83.4*
Atrazine	QC L	Rep 1	60	60.9	60.1	2.61	4.34	101
		Rep 2		61.3				102
		Rep 3		58.2				97.0
		Rep 4		63.6				106
		Rep 5		60.7				101
		Rep 6		56.1				93.6
Acetochlor	QC L	Rep 1	60	60.9	60.1	2.61	4.34	101
		Rep 2		61.3				102
		Rep 3		58.2				97.0
		Rep 4		63.6				106
		Rep 5		60.7				101
		Rep 6		56.1				93.6
MCPA	QC L	Rep 1	60	57.3	60.7	3.89	6.40	95.4
		Rep 2		66.0				110
		Rep 3		56.7				94.6
		Rep 4		62.3				104
		Rep 5		64.0				107
		Rep 6		58.0				96.7
Fipronil	QC L	Rep 1	60	57.8	62.3	2.58	4.14	96.4
		Rep 2		62.7				104
		Rep 3		64.4				107
		Rep 4		64.3				107
		Rep 5		60.7				101
		Rep 6		63.6				106
Imidacloprid	QC L	Rep 1	60	51.6	61.2	7.42	12.1	85.9
		Rep 2		65.3				109
		Rep 3		62.9				105
		Rep 4		71.4				119
		Rep 5		53.6				89.4
		Rep 6		62.6				104

Denotes QC's that are not within acceptance criteria

4.7.1.2. Fresh vs. frozen discussion

Six out seven analytes displayed QC High and QC Low results that had an overall precision (%CV) below 15% and the overall % Accuracy was within 15% (Table 5.37 and Table 5.38). Two QC High samples for aldicarb showed an accuracy that is not within acceptance criteria. However, these had no impact on the overall precision, and only a single QC Low sample was within acceptance criteria. This as a result had a big impact on the overall precision at the lower end. These results suggest that future work should be undertaken to investigate why aldicarb QCs failed. The use of a stable isotopically labelled analogue of Aldicarb may aid the reproducibility and accuracy of the assay.

With the exception of aldicarb we are able to conclude that for all other analytes, the process of freezing has no impact on the stability of the analyte in plasma. Analytes in CPD plasma can be stored at ~-80˚C and remain stable for at least four days.

4.7.2. Freeze - Thaw stability

The effects of freezing and thawing a sample has an impact on the stability of the analytes within. This experiment was conducted to assess the stability of the samples after a minimum of three freeze-thaw cycles. For the first cycle analytes must at least be frozen for period of at least 24 hrs. QCs samples must be frozen for at least 12 hrs during the freeze cycle, and for a minimum of 15 min during a thaw cycle at room temperature. These QCs are then compared to freshly prepared QCs and a freshly prepared calibration curve (FDA, 2018). This is intended to mimic intended sample handling conditions (Sonawawane *et al.*, 2014). In the present study the QCs were stored for 24 hrs at ~-80˚C, and thawed at room temperature for one hour for the remaining two cycles.

4.7.2.1. Criteria

Both the %CV and the %Difference have to be below 15%. A %Difference that is greater than 15% is an indication that the analyte is unstable due to freezing and thawing.

Tables 58 and 59 below show samples analyzed against a valid calibration curve to measure QC high and low. The tables represent nominal concentrations vs measured concentrations and calculated differences after three cycles.

Table 56. The stability of analytes at high QC concentrations after three freeze/thaw cycles.

	QC-High Methomyl	QC-High Aldicarb	QC-High Atrazine	QC-High Acetochlor	QC-High MCPA	QC-High Fipronil	QC-High Imidacloprid
Nominal	350 ng/mL	350 ng/mL	350 ng/mL	350 ng/mL	350 ng/mL	350 ng/mL	350 ng/mL
Replicates	Observed	Observed	Observed	Observed	Observed	Observed	Observed
1	374	484	322	253	394	359	357
2	354	464	328	244	365	347	326
3	353	456	337	243	404	342	302
4	354	419	365	261	221*	350	340
5	332	394	317	213	352	320	288
6	337	344	362	245	393	341	275
Average	351	427	338	243	381	343	315
STDEV	15.1	51.9	20.7	16.2	22.0	13.1	31.7
% CV	4.31	12.2	6.12	6.65	5.76	3.83	10.1
% Difference	0.20	21.9	-3.34	-30.5	9.00	-1.95	-10.1

Denotes outliers

Table 57. The stability of analytes at low QC concentrations after three freeze/thaw cycles.

	QC-Low Methomyl	QC- Low Aldicarb	QC- Low Atrazine	QC- Low Acetochlor	QC- Low MCPA	QC- Low Fipronil	QC- Low Imidacloprid
Nominal	60 ng/mL	60 ng/mL	60 ng/mL	60 ng/mL	60 ng/mL	60 ng/mL	60 ng/mL
Replicates	Observed	Observed	Observed	Observed	Observed	Observed	Observed
1	63.3	86.5	65.9	42.8	68.0	61.2	66.8
2	59.3	63.1	57.0	40.1	59.8	57.5	62.3
3	62.5	67.4	66.9	47.0	69.8	59.8	58.5
4	62.2	80.6	56.8	41.2	67.8	61.2	55.5
5	66.8	83.6	59.4	40.9	67.6	61.9	66.1
6	62.8	75.8	52.8	41.7	65.1	60.3	59.0
Average	62.8	76.2	59.8	42.3	66.3	60.3	61.3
STDEV	2.41	9.26	5.53	2.47	3.54	1.59	4.50
% CV	3.84	12.2	9.24	5.85	5.33	2.63	7.34
% Difference	4.68	27.0	-0.351	-29.6	10.6	0.552	2.25

4.7.2.2. Freeze – Thaw stability discussion

Methomyl, atrazine, MCPA, fipronil and imidacloprid all have %CVs and %Difference that were within the 15% threshold (Table 58 and Table 59). Aldicarb and acetochlor had reproducible results, but the %Difference for both analytes was above 15%. Although uncertain why this is the case, the possibility of experimental error possibly caused by incorrect spiking cannot be ruled out for the two compounds.

With the exception of aldicarb and acetochlor we therefore can conclude that the remaining analytes are stable in plasma for at least three freeze/thaw cycles. In order to rule out experimental error, future work should be undertaken to investigate 1-3 free/thaw cycles for those analytes that failed.

4.7.3.   Long-term matrix stability at ~-80˚C

This experiment was performed to test the long-term stability of the analytes in matrix. Long-term stability should mimic the amount of time that one anticipates that patient samples could be stored and the temperature at which it will be stored. A comparison is made between freshly prepared calibration curves and QCs stored long- term at approximately -80°C (FDA, 2018). In the present study QCs were stored over a period of two weeks.

4.7.3.1.   Criteria

Both the %CV and the %Difference have to be below 15%. A %Difference that is greater than 15% when compared to the nominal concentration indicates that the analyte in matrix is unsuitable for long – term storage.

Tables 60 and 61 present samples prepared for the long-term matrix stability assessment. These were prepared on 17 Sep 2019 and kept at ~ -80°C until extracted and analyzed on 01 Oct 2019. These samples were analyzed against a valid calibration curve to measure QC high and low. The tables represent nominal concentrations vs observed concentrations and calculated differences after two weeks.

Table 58. Long – Term matrix stability (QC High)

	QC-High Methomyl	QC-High Aldicarb	QC-High Atrazine	QC-High Acetochlor	QC-High MCPA	QC-High Fipronil	QC-High Imidacloprid
Nominal	350 ng/mL	350 ng/mL	350 ng/mL	350 ng/mL	350 ng/mL	350 ng/mL	350 ng/mL
Replicates	Observed	Observed	Observed	Observed	Observed	Observed	Observed
1	373	421	343	246	417	351	332
2	348	431	336	234	366	336	296
3	351	395	331	247	383	332	331
4	349	352	338	244	367	336	317
5	326	420	321	226	352	309	343
6	340	380	339	244	362	313	309
Average	348	400	335	240	374	329	321
STDEV	15.5	30.1	8.00	8.11	23.3	15.5	17.1
% CV	4.44	7.53	2.39	3.38	6.23	4.72	5.32
% Difference	-0.67	14.3	-4.38	-31.4	6.98	-5.87	-8.18

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Table 59. Long – Term matrix stability (QC Low)

	QC-Low Methomyl	QC- Low Aldicarb	QC- Low Atrazine	QC- Low Acetochlor	QC- Low MCPA	QC- Low Fipronil	QC- Low Imidacloprid
Nominal	60 ng/mL	60 ng/mL	60 ng/mL	60 ng/mL	60 ng/mL	60 ng/mL	60 ng/mL
Replicates	Observed	Observed	Observed	Observed	Observed	Observed	Observed
1	62.9	103*	64.7	47.5	67.6	63.3	64.2
2	59.3	38.9*	59.2	40.6	64.3	57.5	57.5
3	63.3	58.3	64.4	45.5	62.4	59.8	68.1
4	59.8	82.7	53.9	41.4	69.0	60.8	55.2
5	60.2	79.7	61.5	42.7	72.4	57.8	56.8
6	63.0	47.8	61.3	43.7	70.5	59.1	68.3
Average	61.4	67.1	60.8	43.6	67.7	59.7	61.7
STDEV	1.85	16.8	3.99	2.60	3.77	2.16	5.93
% CV	3.01	25.1	6.55	5.96	5.57	3.62	9.61
% Difference	2.34	11.9	1.40	-27.4	12.8	-0.462	2.78

Denotes outliers

4.7.3.2. Long – term matrix stability discussion

Methomyl, atrazine, MCPA, fipronil and imidacloprid all have %CVs and %Difference that were within the 15% threshold (Table 5.41 and Table 5.42). The high QCs for aldicarb had reproducible results and a %Difference was below the 15% cut off. The low QCs however did not have a %CV that was within acceptable limits. As previously discussed (**5.6.2**) the results obtained for aldicarb and acetochlor are potentially due to spiking errors and should be investigated in future.

Methomyl, atrazine, MCPA, fipronil and imidacloprid have long term matrix stability for at least two weeks.

4.7.4. Benchtop stability

Benchtop stability is performed to determine how stable samples are when placed on the bench during laboratory analysis. This is again done to mimic what actually happens in the laboratory in real time. Results of this analysis are important because they provide guidance as to whether samples must be placed on ice while on the bench or not (Sonawawane *et al.*, 2014). In the present study, QCs were left on bench for 5 hrs before analysis.

4.7.4.1. Criteria

Both the %CV and the %Difference have to be below 15%. A %Difference that is greater than 15% when compared to the nominal concentration indicates an on-bench instability.

Tables 62 and 63 represent samples prepared for the benchtop stability assessment. Samples were removed from ~-80˚C and placed on the bench at room temperature for ~5 hrs. These samples were analyzed against a valid calibration curve to measure QC high and low. The tables below represent nominal concentrations vs observed concentrations and calculated differences after two weeks.

Table 60. Benchtop Stability (QC High)

	QC-High Methomyl	QC-High Aldicarb	QC-High Atrazine	QC-High Acetochlor	QC-High MCPA	QC-High Fipronil	QC-High Imidacloprid
Nominal	350 ng/mL	350 ng/mL	350 ng/mL	350 ng/mL	350 ng/mL	350 ng/mL	350 ng/mL
Replicates	Observed	Observed	Observed	Observed	Observed	Observed	Observed
1	364	322	384	236	406	362	304
2	358	405	366	259	378	343	323
3	356	384	342	237	350	327	310
4	345	391	374	229	347	328	306
5	340	373	367	242	400	333	297
6	357	452	380	256	396	333	312
Average	353	388	369	243	380	337	309
STDEV	9.04	42.6	14.7	12.0	25.7	13.1	8.71
% CV	2.56	11.0	3.98	4.93	6.77	3.89	2.82
% Difference	0.96	10.8	5.37	-30.5	8.47	-3.58	-11.8

Table 61. Benchtop Stability (QC Low)

	QC- Low Methomyl	QC- Low Aldicarb	QC- Low Atrazine	QC- Low Acetochlor	QC- Low MCPA	QC- Low Fipronil	QC- Low Imidacloprid
Nominal	60 ng/mL	60 ng/mL	60 ng/mL	60 ng/mL	60 ng/mL	60 ng/mL	60 ng/mL
Replicates	Observed	Observed	Observed	Observed	Observed	Observed	Observed
1	64.8	65.9	59.4	38.7	60.6	60.4	53.1
2	65.3	107*	60.2	43.8	62.5	63.5	56.8
3	68.2	80.4	65.6	47.9	72.6	64.0	49.8
4	60.2	63.7	56.4	41.4	66.9	57.9	57.5
5	63.1	78.4	63.0	41.4	64.9	61.8	51.4
6	64.8	78.9	57.3	44.8	73.7	62.4	49.7
Average	64.4	73.4	60.3	43.0	66.9	61.7	53.1
STDEV	2.64	7.97	3.48	3.23	5.33	2.24	3.41
% CV	4.09	10.85	5.77	7.52	7.97	3.63	6.44
% Difference	7.29	22.4	0.530	-8.4	11.5	2.77	-11.6

*Denotes outliers

4.7.4.2. Benchtop stability discussion

Methomyl, atrazine, MCPA, fipronil and imidacloprid all have %CVs and %Difference that were within the 15% threshold (Table 62 and Table 63). Similarly to the previous assessment (5.6.3) the high QCs for aldicarb had reproducible results and a

%Difference was below the 15% cut off, while low QCs did not meet criteria. The opposite was observed for acetochlor where the QC High was above 15%, while the QC low was within criteria. As noted before, experiments for aldicarb and acetochlor should be repeated to investigate any experimental errors. Methomyl, atrazine, MCPA, fipronil and Imidacloprid have an on-bench stability of at least 5 hours.

4.7.5. Sample stability at ~4˚C and ~-20˚C

In order to mimic real-time laboratory conditions QCs were stored overnight at ~4˚C and ~-20˚C and analyzed. This was done to measure how stable the analytes would be at these temperatures overnight.

4.7.5.1. Criteria

Both the %CV and the %Difference have to be below 15%. A %Difference that is greater than 15% when compared to the nominal concentration would indicate instability at the different temperatures overnight.

Tables 64 to 67 represent samples stored overnight at ~4˚C and ~-20˚C. These samples were analyzed against a valid calibration curve to measure QC high and low. The tables below represent nominal concentrations vs observed concentrations and calculated differences.

Table 62. Stability at ~4˚C (QC High)

	QC-High Methomyl	QC-High Aldicarb	QC-High Atrazine	QC-High Acetochlor	QC-High MCPA	QC-High Fipronil	QC-High Imidacloprid
Nominal	350 ng/mL	350 ng/mL	350 ng/mL	350 ng/mL	350 ng/mL	350 ng/mL	350 ng/mL
Replicates	Observed	Observed	Observed	Observed	Observed	Observed	Observed
1	356	374	339	228	399	348	325
2	380	443	355	245	420	356	310
3	331	348	302	227	372	335	345
4	353	397	377	263	369	346	304
5	368	454	357	251	396	366	308
6	370	368	353	266	418	366	331
Average	360	397	347	247	396	353	321
STDEV	17.0	42.8	25.2	16.8	21.9	12.2	15.7
% CV	4.73	10.8	7.24	6.80	5.53	3.46	4.90
% Difference	2.77	13.5	-0.736	-29.5	13.0	0.791	-8.43

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Table 63. Stability at ~4°C (QC Low)

	QC- Low Methomyl	QC- Low Aldicarb	QC- Low Atrazine	QC- Low Acetochlor	QC- Low MCPA	QC- Low Fipronil	QC- Low Imidacloprid
Nominal	60 ng/mL	60 ng/mL	60 ng/mL	60 ng/mL	60 ng/mL	60 ng/mL	60 ng/mL
Replicates	Observed	Observed	Observed	Observed	Observed	Observed	Observed
1	67.3	73.3	70.4	47.3	70.7	66.5	59.1
2	64.9	62.2	70.7	49.7	3.83*	60.4	54.8
3	65.8	75.5	66.3	48.7	70.2	66.5	62.5
4	65.0	50.1	63.1	42.0	69.9	61.2	63.5
5	67.0	82.2	68.9	45.8	71.3	64.8	65.9
6	68.4	78.4	68.2	44.0	75.3*	68.1	62.5
Average	66.4	70.3	67.9	46.3	70.5	64.6	61.4
STDEV	1.41	12.0	2.84	2.93	0.623	3.13	3.89
% CV	2.13	17.0	4.18	6.32	0.883	4.85	6.34
% Difference	10.7	17.1	13.2	-22.9	17.6	7.64	2.32

Denotes Outliers

Table 64. Stability at ~20°C (QC High)

	QC-High Methomyl	QC-High Aldicarb	QC-High Atrazine	QC-High Acetochlor	QC-High MCPA	QC-High Fipronil	QC-High Imidacloprid
Nominal	350 ng/mL	350 ng/mL	350 ng/mL	350 ng/mL	350 ng/mL	350 ng/mL	350 ng/mL
Replicates	Observed	Observed	Observed	Observed	Observed	Observed	Observed
1	344	411	323	233	373	313	314
2	347	372	323	247	381	326	304
3	359	439	326	243	411	340	309
4	-----*	397*	194*	140*	195*	158*	156*
5	354	454	396	257	401	334	313
6	337	368	376	246	363	331	296
Average	348	397	349	228	386	329	307
STDEV	8.66	29.2	34.8	43.7	19.7	10.3	7.24
% CV	2.49	7.34	10.0	19.2	5.11	3.13	2.36
% Difference	-0.486	13.5	-0.323	-34.9	10.2	-6.09	-12.3

Denotes experimental error

Table 65. Stability at ~20°C (QC Low)

	QC- Low Methomyl	QC- Low Aldicarb	QC- Low Atrazine	QC- Low Acetochlor	QC- Low MCPA	QC- Low Fipronil	QC- Low Imidacloprid
Nominal	60 ng/mL	60 ng/mL	60 ng/mL	60 ng/mL	60 ng/mL	60 ng/mL	60 ng/mL
Replicates	Observed	Observed	Observed	Observed	Observed	Observed	Observed
1	63.5	58.3	56.9	41.5	71.7	59.0	49.8
2	61.8	66.9	57.4	40.5	56.7	56.4	56.3
3	62.5	50.6	54.9	43.4	62.5	59.4	59.9
4	63.9	50.1	59.1	43.4	68.2	59.3	58.2
5	62.3	82.2*	62.5	42.6	63.2	56.1	65.7
6	64.5	78.4*	66.2	45.7	67.8	65.9	61.9
Average	63.1	56.5	59.5	42.9	65.0	59.3	58.6
STDEV	1.04	7.87	4.16	1.79	5.34	3.55	5.41
% CV	1.66	13.9	7.00	4.17	8.21	5.98	9.22
% Difference	5.12	-5.86	-0.8	-28.6	8.35	-1.09	-2.28

*Denotes experimental error

4.7.5.2.   Sample stability at ~4˚C and ~-20˚C discussion

Methomyl, atrazine, fipronil and imidacloprid all had %CVs and %Differences that were within the 15% threshold at both ~4˚C and ~-20˚C storage (Table 5.45 to Table 5.48). The QC high for both Aldicarb and MCPA at ~4˚C overnight storage was within the 15% threshold, while the QC low for both compounds were slightly above 15%. Aldicarb and MCPA both had %CVs and %Differences within acceptable limits at ~-20˚C overnight storage. Acetochlor failed to meet acceptable criteria for both ~4˚C and ~-20˚C overnight storage, further indicating the need to have this compound re-evaluated.

It can therefore be concluded that methomyl, atrazine, fipronil and imidacloprid are stable for overnight storage at ~4˚C and ~-20˚C, while aldicarb and MCPA were shown to be stable for overnight storage at ~-20˚C only.

4.7.6.   Whole Blood Stability

This method has been developed in CPD plasma. Typically patient samples in hospital are collected as whole blood samples. It's unusual that a South African public hospital would have a centrifuge on the floor to first centrifuge the samples before sending them to the laboratory for analysis. To mimic the actual clinical conditions, this study undertook to do a whole blood stability assessment. This is to estimate the maximum amount of time that an analyte is stable in a whole blood sample before centrifugation to obtain plasma. In the present study whole blood samples were tested after two hours of being on the bench. These samples were compared to whole blood samples that were centrifuged immediately after spiking.

4.7.6.1.   Criteria

An analyte to internal standard peak ratio %Difference that is above 15% between samples at 0 min and at 120 min indicates that the analytes are unstable in whole blood.

As presented in Tables 68 to 72, whole blood stability was tested after 120 min for all analytes.

Table 66. Methomyl, atrazine and acetochlor after 120 min (QC High)

Sample ID	QC-High Methomyl		QC-High Atrazine		QC-High Acetochlor	
Storage	0 min	120 min	0 min	120 min	0 min	120 min
Repeats	Peak Area Ratio	Peak Area Ratio	Peak Area Ratio	Peak Area Ratio	Peak Area Ratio	Peak Area Ratio
1	0.125	0.0981	0.0587	0.0476*	0.0384	0.0314
2	0.135	0.124	0.0616	0.0594	0.0398	0.0383
3	0.130	0.111	0.0609	0.0534	0.0419	0.0339
4	0.135	0.178*	0.0641	0.0821*	0.0420	0.0537*
5	0.138	0.118	0.0366*	0.0308*	0.0425	0.0373
6	0.127	0.137	0.0604	0.0682	0.0394	0.0428
Avg	0.132	0.123	0.0611	0.0603	0.0406	0.0367
STDEV	0.005	0.0110	0.00198	0.00742	0.00168	0.00437
% CV	3.91	8.92	3.23	12.3	4.12	11.9
% Diff		-6.73		-1.32		-9.58

Denotes outliers

Table 67. Methomyl, atrazine and acetochlor after 120 min (QC Low)

Sample ID	QC- Low Methomyl		QC- Low Atrazine		QC- Low Acetochlor	
Storage	0 min	120 min	0 min	120 min	0 min	120 min
Repeats	Peak Area Ratio	Peak Area Ratio	Peak Area Ratio	Peak Area Ratio	Peak Area Ratio	Peak Area Ratio
1	0.0123	0.0094	0.00258	0.00249	0.000889	0.000889
2	0.0128	0.0108	0.00203	0.00225	0.000933	0.000848
3	0.0124	0.0104	0.00239	0.00245	0.00101	0.000967
4	0.0119	0.00987	0.00242	0.00268	0.00103	0.000825
5	0.0108	0.0110	0.00256	0.00249	0.00105	0.000724
6	0.0102	0.0105	0.00242	0.00247	0.000957	0.000914
Avg	0.0117	0.0103	0.00240	0.00247	0.000980	0.000861
STDEV	0.00101	0.000586	0.000199	0.000137	0.0000639	0.0000837
% CV	8.64	5.67	8.29	5.55	6.52	9.72
% Diff		-11.9		3.01		-12.1

Table 68. MCPA, Fipronil and Imidacloprid after 120 min (QC High)

Sample ID	QC-High MCPA		QC-High Fipronil		QC-High Imidacloprid	
Storage	0 min	120 min	0 min	120 min	0 min	120 min
Repeats	Peak Area Ratio	Peak Area Ratio	Peak Area Ratio	Peak Area Ratio	Peak Area Ratio	Peak Area Ratio
1	0.0144	0.0118*	0.459	0.366	0.0496	0.0642
2	0.0161	0.0150	0.460	0.454	0.0568	0.0578
3	0.0153	0.0131	0.487	0.412	0.0591	0.0641
4	0.0169	0.0211*	0.483	0,638*	0.125*	0.0668
5	0.0133	0.0107*	0.487	0.455	0.136*	0.0503
6	0.0149	0.0170	0.456	0.517	0.0563	0.0618
Avg	0.0151	0.0151	0.472	0.441	0.0555	0.0608
STDEV	0.00127	0.00196	0.0150	0.0561	0.00409	0.00596
% CV	8.37	13.0	3.18	12.7	7.38	9.81
% Diff		-0.5		-6.64		9.69

Denotes outliers

Table 69. MCPA, Fipronil and Imidacloprid after 120 min (QC Low)

Sample ID	QC- Low MCPA		QC- Low Fipronil		QC- Low Imidacloprid	
Storage	0 min	120 min	0 min	120 min	0 min	120 min
Repeats	Peak Area Ratio	Peak Area Ratio	Peak Area Ratio	Peak Area Ratio	Peak Area Ratio	Peak Area Ratio
1	0.00243	0.0125	0.0108	0.0106	0.0125	0.00243
2	0.00233	0.0123	0.0120	0.0112	0.0107	0.00233
3	0.00257	0.0123	0.0124	0.0099	0.0126	0.00257
4	0.00251	0.0117	0.0110	0.0128	0.0105	0.00251
5	0.00256	0.0104	0.0120	0.0109	0.0107	0.00256
6	0.00251	0.0107	0.0113	0.00905	0.00989	0.00251
Avg	0.00249	0.0117	0.0116	0.0107	0.0112	0.00249
STDEV	0.0000909	0.000893	0.000650	0.00126	0.00112	0.0000909
% CV	3.65	7.66	5.61	11.7	10.0	3.65
% Diff		-1.61		-0.795		3.89

Table 70. Aldicarb stability after 120 min (QC High and QC Low)

Sample ID	QC-High Aldicarb	QC- High Aldicarb	QC- Low Aldicarb	QC- Low Aldicarb
Storage	0 min	120 min	0 min	120 min
Repeats	Peak Area Ratio	Peak Area Ratio	Peak Area Ratio	Peak Area Ratio
1	0.00115	0.00100	0.0000358	0.000010*
2	0.00133	0.00140*	0.0000478	0.0000510
3	0.00124	0.000933	0.0000441	0.0000663
4	0.00127	0.00171*	0.000075*	0.0000613
5	0.00121	0.000991	0.000052*	0.0000313
6	0.00106	0.00121	0.0000349	0.000080*
Avg	0.00121	0.00103	0.0000406	0.0000525
STDEV	0.0000955	0.0001237	0.000006	0.0000155
% CV	7.88	12.0	15.6	29.5
% Diff		-14.8		29.1

Denotes outliers

4.7.6.2. Whole Blood Stability Discussion

All analytes except for Aldicarb had a %CV and %Difference that were within 15% at 120 min. The QC High for aldicarb fell within acceptable limit, while the QC low was above the acceptable limit. It is clear from the %CV of 29.5% that there was a variation within the six repeats at the lower end. Although not clear why this was the case, it was evident that this variation had a negative impact on %Difference (QC low), thereby warranting further investigation.

With the exception of aldicarb, all other analytes were stable in whole blood for up to 120 min.

4.7.6.3. Aldicarb Whole Blood Stability

Based on the previous whole blood assessment (**4.7.6.2.**) this study undertook to further investigate and understand what was happening with the stability of Aldicarb. To get a clearer understanding of Aldicarb stability over time, the initial 120 min was broken down to 30, 60, 90 and 120 min as presented in Tables 73 and 74 below.

Table 71. Aldicarb whole blood stability (QC High)

Sample ID	QC-High Aldicarb	QC-High Aldicarb	QC- High Aldicarb	QC- High Aldicarb	QC- High Aldicarb
Storage	0 min	30 min	60 min	90 min	120 min
Repeats	Peak Area Ratio	Peak Area Ratio	Peak Area Ratio	Peak Area Ratio	Peak Area Ratio
1	0.0161	0.0153	0.0136	0.0119	0.0107
2	0.0168	0.0140	0.0139	0.0125	0.0133
3	0.0167	0.0128	0.0138	0.0132	0.0114
4	0.0166	0.0142	0.0146	0.0126	0.0122
5	0.0155	0.0175	0.0121	0.0118	0.0133
6	0.0161	0.0136	0.0128	0.0141	0.0112
Avg	0.0163	0.0146	0.0135	0.0127	0.0120
STDEV	0.000502	0.00163	0.000896	0.000848	0.00110
% CV	3.08	11.2	6.66	6.68	9.12
% Diff		-10.7	-17.6	-22.2	-26.3

Table 72. Aldicarb whole blood stability (QC Low)

Sample ID	QC-Low Aldicarb	QC- Low Aldicarb	QC- Low Aldicarb	QC- Low Aldicarb	QC- Low Aldicarb
Storage	0 min	30 min	60 min	90 min	120 min
Repeats	Peak Area Ratio	Peak Area Ratio	Peak Area Ratio	Peak Area Ratio	Peak Area Ratio
1	0.00276	0.00204	0.00138	0.0137*	0.00223
2	0.00241	0.00256	0.00166	0.0118*	0.00164
3	0.00225	0.00188	0.00222	0.00203	0.00163
4	0.00194	0.00193	0.00211	0.00155	0.00155
5	0.00255	0.00179	0.00169	0.00174	0.00170
6	0.00198	0.00228	0.00213	0.00180	0.00144
Avg	0.00231	0.00208	0.00186	0.00178	0.00170
STDEV	0.000321	0.000291	0.000334	0.000195	0.000276
% CV	13.9	14.0	17.9	11.0	16.2
% Diff		-10.2	-19.5	-23.0	-26.7

Denotes incorrect sample injection

4.7.6.3.1. Aldicarb Whole Blood Stability Discussion

Whole blood stability assessment of aldicarb revealed that aldicarb was unstable and degraded over time. At 30 min aldicarb was relatively stable with %Difference within the required limits of less than 15%. The degree of degradation of aldicarb

caused results that did not meet criteria as early as 60 min. This is a significant finding and is very crucial as it has clinical implication. When a sample is not properly handled in a clinical setting it can result in instability and as a result can have an influence on concentration determination. This can minimize or underrepresent the actual amount of aldicarb present within a sample. An underrepresentation of results can lead to an incorrect treatment plan by clinicians and nurses (Pohjola-Sintonen *et al.*, 2000).

4.8. Summary and Conclusion

In this chapter we were able to develop an LC-MS/MS method for the determination of seven pesticides in plasma. In order to make the method applicable for a clinical setting a partial validation was done according to FDA guidance and EMA guidelines on bioanalytical method development (EMA, 2011; FDA, 2018). This was to ensure that the method reliable, accurate, precise, specific, sensitive and reproducible.

The method was initially developed for the determination of ten pesticides and two internal standards. However, due to difficulties with the optimization, three pesticides namely glyphosate, paraquat and deltamethrin, were omitted from the method and the study resorted to only including seven pesticides and two internal standards. The difficulty in developing a simple analytical method for the detection of glyphosate is due to many characteristics. These include its ionic character, very polar nature, it is insoluble in many common organic solvents, and its volatility is low and has a small mass. With the many laboratories available within the European Union (EU), only a small number of these laboratories are able to detect glyphosate. The detection of glyphosate is very difficult without any pre-treatment method and derivatization. This therefore makes it very difficult to develop a method that simultaneously analyses other different chemicals in the same assay (Valle *et al.*, 2019). MRMs were developed for paraquat and deltamethrin, however detection and optimization in the presence of CPD plasma proved to be difficult. HPLC methods for the determination of paraquat have usually relied on addition of ion-pair reagents such as sodiumheptanesulfonate, sodium octanesulfonate or potassium bromide to the mobile phase. Unfortunately these non-volatile salts are not suitable for use in the mobile phase of an LC-MS/MS system. Another study was able to establish a procedure for the determination of paraquat in urine and whole blood using heptafluorobutyric acid as an ion-pair reagent (Lee *et al.*, 2004). There are several bioanalytical methods that have been described for the determination of

deltamethrin. These, however, require large volume of sample for analysis and a tedious extraction procedure. Singh *et al.*, (2016) was able to develop a UPLC–MS/MS method for the determination of deltamethrin and six other pyrethroids in rat plasma using an ammonium adduct as a precursor ion. Due to all of these challenges, in the interest of time the study decided to omit glyphosate, paraquat and deltamethrin from the current method.

Three different columns were assessed to determine which produced good peak shapes and separation and shorter analytical runs. A Phenomenex Kinetex (2.1 mm I.D. x 100 mm L., 2.6 μm) column , a Restek Raptor Biphenyl (2.7 μm, 2.1 x 100 mm) column and an Agilent Poroshell 120 EC-C18 (2.7 μm, 4.6 ×100 mm) column. Two columns produced adequate separation and the resulting chromatography was not influenced by matrix effects, suggesting no co-eluting endogenous matrix components. Two different robust methods were therefore developed. One gradient method using the Agilent Poroshell column for the determination of methomyl, aldicarb, atrazine, MCPA, acetochlor and fipronil with atrazine-d5 as an internal standard was developed. A second isocratic method making the use of the Restek Raptor Biphenyl column for the determination of imidacloprid with PAS as internal standard was developed.

Due to low costs and ease of operation, this study employed protein precipitation for the extraction of analytes. To determine which solvent ensured adequate and clean recoveries, different assays were extracted using methanol, acetonitrile and a combination of both. The solvent that yielded the cleanest and best recoveries was methanol. Based on the matrix effect, recovery and process efficiency results, it was clear that the extraction employed in this study was effective and reliable at extracting analytes from plasma.

The method was partially validated and showed accuracy and robustness using 3-day inter- and intra- validations. The calibration range of 20 to 600 ng/mL was valid for all seven pesticides. Evaluation of matrix effects yielded regression slope variabilities that were within acceptable criteria for all seven pesticides, indicating that co-extracted matrix components had no negative impact on the quantitation method. Average recoveries for the seven pesticides ranged from 80.6% to 91.4%, while process efficiencies for methomyl, atrazine, MCPA, fipronil, and imidacloprid were within acceptable criteria in the absence of internal standards. More consistent process efficiencies were observed in the presence of atrazine-d5 for aldicarb and

acetochlor. Stable isotopically labelled analogues of aldicarb and acetochlor would probably have produced even better results. It was important to evaluate stability in the matrix, therefore the following stability assessments were undertaken, fresh vs. frozen, freeze-thaw, long-term matrix stability and benchtop stability, stability at ~ 4˚C, and ~-20 ˚C, and whole blood stability. While methomyl, atrazine, MCPA, fipronil, and imidacloprid passed all stability assessments, results obtained from aldicarb and acetochlor failed to meet acceptance criteria. Though unclear why aldicarb and acetochlor performed poorly, experimental errors could not be ruled out to have potentially contributed to the outcome of these two compounds.

The literature on the LC-MS determination of pesticides in water and agricultural food sources is in abundance but severely limited in human-based research. One study used a QuEChERS-based sample preparation approach on guinea pig blood, and showed a calibration range of 0.5 to 500 ng/mL for both methomyl and aldicarb. Precision for methomyl and aldicarb was <15% and had recoveries of 87% and 93%, respectively. No stability assessments were done on spiked samples though, but on stock solutions only. Furthermore, as in the current study, prominent signal suppression of aldicarb was also observed. This was overcome by the use of a suitable Aldicarb isotopically labelled analogue as internal standard ($^{13}C2$, d3-Aldicarb) (DeArmond *et al.*, 2015). Another recent study conducted the determination of 6 pesticides (atrazine and acetochlor included) in human plasma. Similarly to the current study, protein precipitation was the extraction method used. However, a GC-MS system instead of the LC-MS system applied in the current study was utilized. A calibration range (0.05 to 10 μg/mL), less sensitive than the one produced in the current method, and a sample volume (200 μL) four times greater than the current method was used (Yuan *et al.*, 2018). Accidental ingestion of pesticides in children is high, and ideally, you would want to use as small a sample volume as possible. Another study described an LC-MS method for the determination of MCPA and other pesticides in human serum samples. The study showed a calibration range for MCPA and other phenoxy acid herbicides of 10-100 μg/mL, above the range in the current method, and a lower average recovery of 76% when compared to the 91% average recovery in this study. Furthermore, no stability in matrix experiments was performed (Chen *et al.*, 2018).

Other studies have described HPLC and LCMS methods for the determination of imidacloprid and fipronil. The LCMS method was validated for the quantification of fipronil and its biomarkers in rat and human dry blood spots. Compared to the

current on-bench stability of 5 hrs for fipronil, Raju *et al.* (2016) showed longer on-bench stability of 24 hrs (Raju *et al.*, 2016). The HPLC method, on the other hand, made use of liquid phase extraction on goat plasma and achieved a calibration range of 10 to 40 ng/mL with an average recovery of 95%. With an isocratic runtime of 3.30 min, the method was longer compared to the 1.75 min runtime of the current method (Dewangan *et al.*, 2016).

To our knowledge, this is one of the first developed LC-MS/MS methods in South Africa to describe the detection of methomyl, aldicarb, atrazine, acetochlor, MCPA, fipronil and imidacloprid in human plasma. Furthermore, the developed method only requires a small sample volume of 50 µL and uses a cheaper sample extraction method, making it ideal for the analysis of acute poisoning cases in public healthcare institutions.

Chapter 5

Conclusion, limitations, and future work

5.1. Conclusion

With a clear aim of developing screening tools that would assist in the detection of common poisoning agents in South Africa, this study described how untargeted (LC-TOFMS) and targeted (LC-MS/MS) screening methods for the simultaneous determination of multiple drugs and pesticides in plasma were developed.

A total of 37 drugs were included in the untargeted screening method with detection limits set up to inform whether the ingestion was sub-therapeutic, therapeutic, or toxic. The untargeted method successfully detected and identified 34 of the drugs included, and was unable to detect valproate, levetiracetam and metformin. These three drugs lacked retention on the columns and had ionisation difficulties preventing them from producing stable fragments. This study furthermore illustrated how data derived from the method could be used to identify unknown compounds using online search libraries. This type of analysis could be useful in the identification of poisoning agents that are not included on this screening method.

The targeted screening method had initially considered 10 pesticides but due to difficulties with the optimization of glyphosate, paraquat, and deltamethrin, the study developed a method for the determination of seven pesticides. To ensure that the method was reliable, accurate, precise, specific, sensitive, and reproducible, a partial validation was done using the FDA guidance and EMA guidelines on bioanalytical method development as guidance (EMA, 2011; FDA, 2018). This was done through a 3 day inter- and intra-validations with a calibration range of 20 ng/mL to 600 ng/mL. Matrix effects showed to be within acceptable limits, while recoveries were between 80.6% and 91.4%. Process efficiencies in the absence of internal standards for methomyl, atrazine, MCPA, fipronil, and imidacloprid were within acceptable limits, but for aldicarb and acetochlor and ISTD had to be added. Furthermore, stability assessments were undertaken with methomyl, atrazine, MCPA, fipronil, and imidacloprid passing all assessments, while aldicarb and acetochlor failed to meet acceptable limits due to potential errors associated with spiking.

In response to the growing medical burden imposed by the increasing numbers of poisoning cases in the country, these developed screening methods will enhance how these cases are diagnosed and treated in our hospitals.

5.2. Limitations and future work

Due to the high cost of high purity reference standards and stable isotopically labelled internal standards, for the therapeutic drug screening method, this study made use of drugs purchased in tablet form. Future work should investigate physical and chemical interactions happening between the drugs and the excipients and the impact these have on the identification and detection of these drugs.

Although all pesticide standards were pure grade, only atrazine had a deuterated internal standard (atrazine-d5). The inclusion of stable isotopically labelled analogues as internal standards for other pesticides would have vastly improved robustness and sensitivity of the targeted method. Furthermore, compounds are actively metabolized upon ingestion whilst others are rapidly eliminated. Detection of the metabolites could further provide confirmation of the ingestion of a substance. Due to cost limitations, no drug nor pesticide metabolites were purchased to enhance the screening methods.

In addition to the re-analysis and re-evaluation of aldicarb and acetochlor stabilities, further validations should be done for all pesticides to evaluate stock solution stability and the influence of matrix anticoagulant on assay performance.

Further work should be undertaken to extend these screening methods to wildlife, livestock and poisoning of pets. Due to livestock losses farmers are resorting to the use of poisons and pesticides in the culling of predators. This poses an environmental threat with serious consequences to wildlife and biodiversity (Nattrass & Conradie, 2018). Furthermore, the validation of less invasive sampling methods such as dried blood spots (DBS) could result in the ease of storage and transportation of samples. DBS samples can minimize the risk of exposure to HIV/AIDS and other pathogens. Only a small sample volume (quick sampling) is required making DBS ideal for the collection of animal samples (Eibak, *et al.*, 2012).

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