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Assessing the carbamate decay kinetics in post-mortem intoxication cases with reference to matrix storage conditions

by

EDB Radebe

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Dedication

Dedicated to:

My parents (Mr and Mrs Radebe) my best friends and support system

and

Vuyelwa Buque, this journey is filled with so many priceless memories with you, but I am glad that you are in a peaceful place. Thank you for everything as well as your advices. You will be forever remembered my friend. Rest in peace.

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Abstract

Pesticide poisoning is a global health concern with approximately three million cases being reported on an annual basis. The latter includes both intentional and unintentional poisonings. Organophosphorus and carbamate insecticides are frequently found to be 'responsible' for pesticide poisoning in developing countries. In South Africa, aldicarb is the most potent carbamate pesticide and is sold in the informal markets as Temik. It is colloquially known as "Two step" or "Galephirimi" resulting in numerous cases of acute poisoning, especially in urban areas.

Underreporting of suspected or confirmed pesticide poisoning cases has been a problem encountered in the national notification systems. Although a number of carbamate poisonings have been identified at the Pretoria Medico-Legal Laboratory, the presence of carbamates in post-mortem samples is rarely confirmed analytically. This may be ascribed to insufficient sample preparation, analytical methods not being sensitive enough or storage conditions not being optimal or too long before analysis takes place.

It is well documented that most analytical errors occur during the pre-analytical phase, leading to a high prevalence of inconclusive results being attained. This may possibly be due to pre-analytical degradation, binding to biological matrix or the analytical method not been sensitive enough for detection in collected samples. Post-mortem redistribution factors such as physicochemical properties of the xenobiotic compounds (pH, volume of distribution, protein binding affinity, bacterial biotransformation and lipophilicity), characteristics of the matrix, specimen collection procedure and the use of preservatives may also influence the carbamate stability.

The primary aim of the study was to optimise the sample preparation and analysis of biological matrices for select carbamates using LC-MS/MS method. Additionally, to

analyse pesticide samples sold by street vendors as well as post-mortem samples collected from suspected cases of carbamate intoxication to determine whether the developed method can detect carbamates in real samples.

Assessment of the aldicarb decay kinetics was done by spiking biological samples (whole blood, plasma, urine) collected from consenting healthy volunteers. Post-mortem samples (blood, urine, stomach content) of suspected carbamate poisoning cases, were screened for possible carbamate compounds and their metabolites or breakdown products. Optimisation and validation of the method was performed using a high-performance liquid chromatography (HPLC) system coupled to a triple quadrupole mass spectrometer following different extraction methods. The system was operated in positive electrospray ionisation (ESI+) mode. Different columns, mobile phase buffers and cartridges were used to compare the chromatographic separation of the carbamate compounds. Validation according to ICH guidelines was done for aldicarb. A set of matrix-matched standard calibration curves, was constructed using Analyst (version 1.5.2) software.

Initial sample preparation of carbamate pesticides using three different SPE cartridges proved to be unreproducible with poor recoveries of specific compounds due to the wide range of carbamate pesticide polarities, so this was abandoned for the stability testing and forensic samples tested.

About 85% reduction of the concentration of aldicarb was seen in whole blood only at ambient temperature but was stable at lower temperatures. Stability proved to be better in plasma compared to whole blood, for aldicarb and its oxidation products. Aldicarb was stable in urine stored with boric acid preservative. The ideal storage temperature for biological samples containing these carbamate compounds was found to be -80°C.

During analysis of forensic samples, unknown peaks were consistently detected which are believed to correspond to adulterants and diluents which are added to “backstreet” pesticides. A possible match of an organophosphate, terbufos, found in some “backstreet” pesticide products was detected in some of the post-mortem samples.

Considering their different physicochemical properties and that several factors can influence the biodegradation of carbamate compounds, no extrapolation of results from one carbamate compound to another can be formulated.

The development and validation of an analytical method to quantify aldicarb and its oxidation products (aldicarb sulfoxide and sulfone) in whole blood, plasma and urine, using the protein precipitation method and LC-MS/MS was successful. Method validation to quantify ten standard carbamate pesticides using SPE and UPLC-q-TOF/MS was unsuccessful.

The LC-MS/MS technique was found to be a suitable tool for the quantitation of aldicarb and its oxidation products in typical post-mortem sample matrices.

Study outputs

Conference proceedings

Poster presentation

- Radebe, EDB; Leuschner, M; Cromarty, AD; Saayman, G; Steenkamp, V. Development of a solid-phase and liquid-liquid extraction based high resolution tandem mass spectrometry method for detection of carbamate pesticides and metabolites in suspected poisoning cases. Health Sciences Faculty day, University of Pretoria, South Africa, 20 - 21 August 2019.

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Podium presentation

- E.D.B Radebe; L du Toit-Prinsloo; G Saayman. Carbamate and organophosphate fatalities at the Pretoria Medico-Legal Laboratory (2011-2015). 56th International Congress of the Federation of South African Societies of Pathology (FSASP-PathCape 2018). Spier Stellenbosch (Cape Town) South Africa, 16 - 18 August 2018.

- E.D.B Radebe; L du Toit-Prinsloo; G Saayman. Carbamate fatalities at the Pretoria Medico-Legal Laboratory (2011-2015). Health Sciences Faculty day, University of Pretoria South Africa, 22 - 23 August 2017.

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List of abbreviations

A

ACN	Acetonitrile
ACh	Acetylcholine
AChE	Acetylcholinesterase
AM	Ante-mortem
APP	Acute pesticide poisoning
ATP	Adenosine triphosphate

B

BDMC	4-Bromo-3,5-dimethylphenyl-N methylcarbamate
BChE	Butyrylcholinesterase

C

ChE	Cholinesterase
CID	Collision-induced dissociation
CNS	Central nervous system

D

DIA	Data-independent acquisition
DNA	Deoxyribonucleic acid
DR	Death registration

E

EDTA	Ethylenediaminetetraacetic acid

F

FIFRA	Federal Insecticide, Fungicide and Rodenticide Act
FMLL	Forensic Medico-Legal Laboratories

G

GC	Gas chromatography
GC-MS	Gas chromatography-mass spectrometry

H

HPLC	High performance liquid chromatography

I

IS	Internal standard
IPA	Isopropyl alcohol

L

LC-MS/MS	Liquid chromatography tandem mass spectrometry
LLE	Liquid-liquid extraction
LMICs	Low-middle income countries

M

MS	Mass spectrometry
MeOH	Methanol
MRM	Multiple reaction monitoring

N

nAChR	Nicotinic acetylcholine receptor
NaCl	Sodium chloride
NaF	Sodium fluoride
NHLS	National Health Laboratory Services
NPDS	National Poison Data System

O

OC	Organochlorines

P

PPB	Parts per billion
PFCL	Pretoria Forensic Chemistry Laboratory
PMLL	Pretoria Medico-Legal Laboratory
PMR	Post-mortem redistribution

S

SA	South Africa
SAPS	South African Police Services
SOP	Standard operation procedure
SPE	Solid phase extraction
SPME	Solid phase micro-extraction

T

TLC	Thin layer chromatography
TOF/MS	Time-of-flight mass spectrometer

U

UPLC-MS	Ultra performance liquid chromatography mass spectrometry
UPLC-q-TOF/MS	Ultra performance liquid chromatography quadrupole-time of flight mass spectrometry
UFLC-MS/MS	Ultra-fast liquid chromatography tandem mass spectrometry

W

WHO	World Health Organization

Symbols and numerical values

°C	Degrees Celsius
s	seconds
hr	hours
%	Percentage
kV	Kilovolts
L/hr	Litres/hour
µg/mL	Micrograms per millilitre
µL	Microliter
mM	Millimolar
g/kg	Milligram per kilogram
ng/mL	Nanograms per millilitre
V	Voltage
W	Watt

Statistical significance indicators

*	$P \leq 0.05$
**	$P \leq 0.01$
***	$P \leq 0.001$
§	$P \leq 0.0001$

1 Introduction

1.1 Pesticide poisoning

Pesticide poisoning is defined as a toxic condition due to excessive exposure to pesticides that results in illness or death.[1,2] Pest control contributes substantially to pesticide poisoning, as it results in use of highly toxic compounds for their erradication.[3,4] Due to resistance, pest control relies on the development of new potent synthetic organic chemical toxins as pesticides. However, potent pesticides are not selective to only insects or rodents but pose the potential to be fatal to all living organisms, including humans.[4] Pesticide intoxication is considered a health hazard for the general population.[4,5]

Pesticide poisoning incidents are divided into occupational and non-occupational exposures.[6] Occupational exposures are mostly agricultural work-related incidences, whilst non-occupational exposures are incidences that can occur with pesticides used for household pest control. Both these categories can result in fatalities from accidental or intentional exposure to pesticides.[6] Accidental exposures may be due to inhalation of fumes or powder of the pesticide or ingesting food without the sanitising of hands after pesticide application.[7,8,9] The majority of paediatric pesticide poisoning cases are accidental, where children gain access to and ingest the pesticides stored in unlabelled containers, or ingest bait mixtures (such as bread or maize) used for the trapping of rats or insects.[7,9,10,11] Intentional cases are mostly associated with deliberately ingesting the pesticide with the intention of committing suicide.[8,12] Despite pesticides typically being used for criminal intent in animal-related poisoning cases and for suicidal and homicidal purposes in humans, the majority of pesticide poisoning cases are not reported. However, there is a legal obligation to report pesticide poisoning [5,13,14]

The World Health Organization (WHO) acknowledges that pesticide poisoning is one of the most common methods of suicide, accounting for about one third of all suicide cases worldwide.[15] Approximately 250 000 - 370 000 people commit suicide using pesticides

annually.[16,17,18] Pesticide poisoning is reported to be a major global public health concern, with an estimated annual incidence of more than three million cases.[17,18,19] Of these, approximately one million cases are due to unintentional pesticide poisoning.[20] The majority of pesticide poisoning cases (approximately 60%) are reported in Africa, Asia, Central and South America.[12,15]

Pesticides are a large and diverse group of chemical compounds used to repel and/or control pest infestations.[4] Pesticides are classified as insecticides, herbicides, rodenticides, fungicides, nematicides, molluscicides and acaricides.[21] These classifications depend on the field of pesticide use.[21]

Among the numerous pesticides that can result in human death, organophosphorus insecticides are the major cause of acute pesticide poisoning (APP).[18] In humans, organophosphorus pesticides have been reported to result in adverse chronic health effects and are associated with diseases such as cancer, hormone disruption, asthma, allergies, hyperactivity disorders, and neurological conditions such as Parkinson's and Alzheimer's disease.[1,4] Carbamates are systemic neuro-toxicant insecticides which are also used as nematicides.[3,18] These insecticides are generally used as surface sprays or baits to ensure a hygienic, pest-free environment.[22] It has been reported that carbofuran (a carbamate compound) is the most misused pesticide in Africa.[14] Carbofuran inhibits glycolysis and decreases adenosine triphosphate (ATP) levels, leading to the energy impairment of the neurons and reduced spine density of pyramidal neurons in the hippocampus.[23] It also induces deoxyribonucleic acid (DNA) fragmentation, resulting in apoptosis and loss of hippocampal neurons.[23]

1.2 Acute pesticide poisoning

Acute pesticide poisoning (APP) is defined as an illness or adverse health occurring within 48 hours of pesticide exposure.[24] APP may be a result of accidental, homicidal or deliberate ingestion of harmful pesticides.[7] According to the Bulletin Report of the WHO, APP is a major contributor to morbidity and mortality worldwide.[24] Studies conducted in

developing countries have estimated the incidence of APP to be 18.2 cases per 100 000 in agricultural workers and 7.4 cases per million in school children.[24]

In low-middle income countries (LMICs) of America (Brazil, Mexico, and Colombia), an estimated 3100 cases of pesticide-related suicide cases were reported in 2015, accounting for 8.8% of suicides attributed to pesticide ingestion globally.[25] A prospective case study of poisoning by illegal acetylcholinesterase inhibitors (rodenticides) was conducted at the Campinas Poison Control Centre in Brazil between 2009 and 2010. The main cause of death in these years was found to be pesticides, specifically Chumbinho (a Portuguese name meaning small pellet, which in SA is sold as Temik).[26] Sixty-eight of the 76 reported cases resulted in hospitalisation and the poison identified had a physical appearance of grey or black granules. Aldicarb was identified in 8 samples and in some of the cases the product was described as an unknown liquid. Almost all reported exposures (92.1%) were as a result of pesticide ingestion related to attempted suicide.[26]

Brazil is known as an intensive agricultural country with easy access to pesticides, with a high incidence of human poisoning cases especially in the south-eastern region. The majority of the reported cases were suicides. Other reported exposures were accidental, work related, environmental or unknown.[5] A retrospective study was conducted to determine the incidence of human intoxication by agrochemicals between 1999 and 2014 in the southern region of Brazil.[27] The number of pesticide poisoning cases in urban areas over this period totalled 11478 compared to the 9040 in rural areas. Of these cases approximately 34% were suicide attempts, 33% were accidental exposures and 30% were occupational exposures.[27]

China is known to be the largest user, producer and exporter of pesticides.[20,28] In general, approximately 53 300 to 123 000 people are intoxicated annually.[20] It has been reported that more than 160 000 people commit suicide in China annually.[28] The most common method of suicide in this country between 2006 and 2013 was pesticide poisoning (4.9 per 100 000 males and 4.2 per 100 000 in females).[29] Acute pesticide poisoning in Sri Lanka has been ascribed to the use of unregulated pesticides.[30] The incidence of APP in

adults is between 426–446 per 100 000 population in Sri Lanka.[31] Acute paediatric poisoning cases in 45 hospitals between 2011 and 2013 were 60.4 per 100 000 in children younger than 12 years.[31]

In Turkey, 70 of the 10 720 autopsied cases conducted between 2001 and 2011 were attributed to fatal pesticide poisoning. Insecticides were the highest cause of fatal pesticide poisonings (94%) with organophosphates (63%) being the most common.[16] Between 2006 and 2009, a total of 54 pesticide poisoning cases were identified and reported in the Izmir Branch of Council of Forensic Medicine in Turkey, which were mainly ascribed to suicide.[32] In Romania, carbofuran (Furadan) was banned as an insecticide due to the high usage of the compound in the country and its toxicity to humans.[33]

In Africa, there is a paucity of published data regarding pesticide poisoning, however, the available data indicates that pesticide poisoning cases commonly occur in Tanzania, Kenya, Uganda and Zimbabwe.[25] The rate of APP due to occupational exposure in 10 facilities in Tanzania, ranged between 84.3 - 279.9 cases per 1 000 000 of farmers in 2006.[34] The rate of all pesticide poisonings (occupational and non-occupational) ranged between 97.4 - 290.29 cases per 1 000 000.[34] In Uganda a total of 739 poisoning cases were documented for 5 hospitals between 2010 and 2014. Of these cases, 212 were due to pesticide poisoning. Approximately 91.8% of the pesticide poisoning cases were due to organophosphates and the majority of the cases were suicide attempts.[35] In a case series in Uganda, 3 of 7 patients died after ingesting chapatti (flattened bread made from wheat flour water and oil) bought on the street, which was contaminated with organophosphates.[36]

In Kenya (Kenyatta National Hospital) during August 1972 to April 1978, of the 2 135 admissions, 72 were poisoning cases. Organophosphates accounted for the highest number of deaths.[37] In a study conducted in various hospitals of Zimbabwe, of the 6 018 admissions, 606 cases were a result of pesticide poisoning, of which 75% were deliberate and 21% were accidental.[37]

Insufficient regulation, lack of surveillance systems, reduced regulation enforcement, lack of training and poorly maintained or non-existent personal protective equipment collectively contribute to the lack of data regarding the trends of pesticide poisoning in African countries.[24] Consequently, the available figures may be a misrepresentation or

underestimation of the actual statistics.[24,25] Furthermore, the high incidence of pesticide poisoning cases in the above-mentioned countries may be attributed to the readily available pesticides on illegal street markets, which is a loophole due to low governance standards and corruption in most African regulatory boards.[24]

In the South African medico-legal setting, there is a paucity of data regarding the number of fatalities ascribed to pesticide poisoning, the pathological features and the diagnostic verification of these cases by toxicological analysis.[38] There are three state forensic chemistry laboratories (FCLs) providing analytical services for all nine provinces in respect of forensic toxicology. The FCLs are in Cape Town, Johannesburg and Pretoria.[39] As only three FCLs are operating in South Africa (SA), the routine service workload from the medico-legal mortuaries has resulted in a huge backlog of sample analysis.[39] Underreporting of pesticide poisoning data has been a problem encountered in the national notification system (a passive surveillance system with a mandate for the notification of certain medical conditions).[38] Most death notification forms and referral forms (from the hospitals) that accompany the decedents to the mortuary, refer to the cause of death based on symptoms with no actual reference to the probable agent that caused the death.[40]

Underreporting of pesticide poisoning cases has been corroborated in a retrospective study conducted at the Pretoria Medico Legal Laboratory (PMLL) (by Radebe EDB-unpublished data). A number of cases have been admitted to the PMLL, where post-mortem findings were suggestive of organophosphate or carbamate poisoning. In the 5-year retrospective study of possible carbamate fatalities that was conducted at the PMLL in 2016 (unpublished data, presented at Health Sciences Faculty day, University of Pretoria South Africa), a total of 160 autopsied cases were suggestive of organophosphate or carbamate poisoning. Of these, 154 cases were sampled and submitted for pesticide analysis. As of January 2019, 112 (72.7%) of these cases still had pending toxicology results, 33 (21.4%) tested negative and 9 (5.8%) positive for the presence of pesticides. A few positive or toxicologically-confirmed cases of fatal carbamate poisoning were seen over the study period. This analytical outcome raised a

concern, as it appeared to be in conflict with the reported autopsy findings in many cases. There is a vast backlog in the Pretoria Forensic Chemistry Laboratory (PFCL). This backlog has impacted on the majority of the cases requiring laboratory testing to confirm outcome. Questions that arose were: are errors occurring due to the extended pre-analytical phase of analyses; and is the analytical technique used sensitive enough to detect the pesticide of interest in autopsy samples of various biological fluids?

In a retrospective study conducted in four hospitals in the KwaZulu-Natal and Gauteng regions of SA, 15.8% (of 423 cases) of cases over a 6-month period in 2005 were found to be due to agricultural chemicals.[16] Of these, organophosphates accounted for 20% of the fatal cases, and these were furthermore all reported to be suicidal cases.[41]

In an epidemiological study conducted between 2012 and 2014 on organophosphate poisoning in the Tshwane District of SA, a total of 207 pesticide poisoning cases from health facilities were reviewed, which included hospitals and community health centres. Of these, 51.7% were due to intentional poisoning, 21.7% as a result of accidental poisoning, with the rest being unknown.[38] Suicide accounted for 50.2% of pesticide poisoning notifications.[38]

The WHO recognizes pesticide poisoning as the single most important means of suicide.[15] There is a need to identify the exact cause of death, as SA has existing legislations regarding pesticide poisoning notification. In terms of the Regulations Regarding Communicable Diseases, R27 (drafted in terms of the National Health Act 61 of 2003) pesticide poisonings must be notified or reported.[42]

1.3 Pesticide classes

Pesticides are classified according to their toxicity, where Type 1a is considered extremely hazardous; and type U as the least hazardous (Table 1.1).[46] Most Type-1 technical grade pesticides are banned or strictly controlled in developed countries, but not in developing countries.[16] Pesticides sold on the streets are usually highly toxic and generally illegally imported products.[47]

Table 1.1: Classification of pesticides according to their toxicity by the WHO.[46]

Pesticide class		LD_{50} in rats (mg/kg body weight)	
Type	**Toxicity level**	**Oral**	**Dermal**
Ia	Extremely hazardous	< 5	< 50
Ib	Highly hazardous	5 - 50	50 - 200
II	Moderately hazardous	50 - 200	200 - 2000
III	Slightly Hazardous	> 2000	> 2000
U	Unlikely to present an acute hazard	≥ 5000	

The second classification is based on the purpose of the pesticide. The four major classes of pesticides are insecticides, herbicides, fungicides and rodenticides.[43] Pesticides may be organic or inorganic compounds. Organic pesticides are natural or synthetic compounds, whereas inorganic pesticides are derived from minerals or inorganic chemical compounds. The latter are environmentally stable and dissolve readily in water. Natural organic pesticides are derived from naturally occurring sources such as plants.[1] Organic synthetic pesticides are produced through chemical reactions and began in the middle of the twentieth century. Pesticides can further be classified according to their physiological mechanisms such as nerve agents (affects the nervous system) or cholinesterase inhibitors (nerve signal enhancers), as denoted in Table 1.2.[44,45] The main subclasses of insecticides are organochlorines (OC), anticholinesterase inhibitors (organophosphates and carbamates), pyrethroids and neonicotinoids.[43]

Table 1.2: Pesticide classification according to their mechanism of action.[37]

Classification based on primary site of action	Chemical sub-group	Active ingredient
1 **Acetylcholinesterase (AChE) inhibitors** Nerve action	**1A** Carbamates	Alanycarb, Aldicarb, Bendiocarb, Benfuracarb, Butocarboxim, Butoxycarboxim, Carbaryl, Carbofuran, Carbosulfan, Ethiofencarb, Fenobucarb, Formetanate, Furathiocarb, Isoprocarb, Methiocarb, Methomyl, Metolcarb, Oxamyl, Pirimicarb, Propoxur, Thiodicarb, Thiofanox, Triazamate, Trimethacarb, XMC, Xylylcarb
	1B Organophosphates	Acephate, Azamethiphos, Azinphos-ethyl, Azinphosmethyl, Cadusafos, Chlorethoxyfos, Chlorfenvinphos, Chlormephos, Chlorpyrifos, Chlorpyrifos-methyl, Coumaphos, Cyanophos, Demeton-S-methyl, Diazinon, Dichlorvos/ DDVP, Dicrotophos, Dimethoate, Dimethylvinphos, Disulfoton, EPN, Ethion, Ethoprophos, Famphur, Fenamiphos, Fenitrothion, Fenthion, Fosthiazate, Heptenophos, Imicyafos, Isofenphos, Isopropyl *O*-(methoxyaminothio-phosphoryl) salicylate, Isoxathion, Malathion, Mecarbam, Methamidophos, Methidathion, Mevinphos, Monocrotophos, Naled, Omethoate, Oxydemeton-methyl, Parathion, Parathion-methyl, Phenthoate, Phorate, Phosalone, Phosmet, Phosphamidon, Phoxim, Pirimiphos- methyl, Profenofos, Propetamphos, Prothiofos, Pyraclofos, Pyridaphenthion, Quinalphos, Sulfotep, Tebupirimfos, Temephos, Terbufos, Tetrachlorvinphos, Thiometon, Triazophos, Trichlorfon, Vamidothion
2 **GABA-gated chloride channel antagonists** Nerve action	**2A** Cyclodiene Organochlorines	Chlordane, Endosulfan
	2B Phenylpyrazoles (Fiproles)	Ethiprole, Fipronil
3 **Sodium channel modulators** Nerve action (Strong evidence that action at this protein is responsible for insecticidal effects)	**3A** Pyrethroids Pyrethrins	Acrinathrin, Allethrin, d-*cis-trans* Allethrin, d-*trans* Allethrin, Bifenthrin, Bioallethrin, Bioallethrin Scyclopentenyl isomer, Bioresmethrin, Cycloprothrin, Cyfluthrin, *beta*-Cyfluthrin, Cyhalothrin, *lambda*-Cyhalothrin, *gamma*-Cyhalothrin, Cypermethrin, *alpha*-Cypermethrin, *beta*-Cypermethrin, *theta*cypermethrin, *zeta*-Cypermethrin, Cyphenothrin, (1*R*)-*trans*-isomers], Deltamethrin, Empenthrin (*EZ*)-(1*R*)- isomers],

		Esfenvalerate, Etofenprox, Fenpropathrin, Fenvalerate, Flucythrinate, Flumethrin, *tau*-Fluvalinate, Halfenprox, Imiprothrin, Kadethrin, Permethrin, Phenothrin [(1*R*)-*trans*- isomer], Prallethrin, Pyrethrins (pyrethrum), Resmethrin,Silafluofen, Tefluthrin, Tetramethrin, Tetramethrin[(1*R*)-isomers], Tralomethrin, Transfluthrin,
	3B Dichlorodiphenyltrichloroethane Methoxychlor	Dichlorodiphenyltrichloroethane, Methoxychlor
4 **Nicotinic acetylcholine receptor (nAChR) agonists** Nerve action	**4A** Neonicotinoids	Acetamiprid, Clothianidin, Dinotefuran, Imidacloprid, Nitenpyram, Thiacloprid, Thiamethoxam,
4 **Nicotinic acetylcholine receptor (nAChR) agonists** Nerve action	**4B** Nicotine	Nicotine
	4C Sulfoxaflor	Sulfoxaflor
	4D Butenolides	Flupyradifurone
5 **Nicotinic acetylcholine receptor (nAChR) allosteric activators** Nerve action	Spinosyns	Spinetoram, Spinosad
6 **Chloride channel activators** Nerve and muscle action	Avermectins, Milbemycins	Abamectin, Emamectin benzoate, Lepimectin, Milbemectin
7 **Juvenile hormone mimics** Growth regulation	**7A** Juvenile hormone analogues	Hydroprene, Kinoprene, Methoprene
	7B Fenoxycarb	Fenoxycarb
	7C Pyriproxyfen	Pyriproxyfen

1.4 Street pesticides

Illegal pesticides also known as “street pesticides” are referred to as pesticides that are obtained from informal markets (street vendors), repackaged and sold unlabelled for domestic use.[3,47,48,49] Global warming is a contributory factor to pest infestation and has led to increased usage of chemicals to control pests.[47] The unending pest infestation has further led to the trade of toxic, cheap and effective illegal pesticides.[3,48] Street pesticides are classified into two groups. The first category of these pesticides is the legally registered pesticides for agricultural use that are commonly decanted into common drinking or medicinal bottles or transparent plastic bags and sold illegally to the public by street vendors. The second category is manufactured and packaged (most often in China) and imported despite not being legally registered in SA.[3,47] In some of these pesticides, the active ingredient is not indicated, however, it has been reported that the majority of the products are neurotoxins (Figure 1.1).[47] The trade of street pesticides has also been reported in other countries (United States, Brazil, Zimbabwe, Mozambique) and these pesticides have been linked to acute pesticide poisoning in humans.[3,48,49,50]

In SA, aldicarb (which has resulted in numerous cases of acute poisoning, especially in urban areas, is the most potent carbamate pesticide and commonly sold as Temik. Temik is colloquially known as “Two step” (referring to the number of steps a rodent takes after ingesting the pesticide) or “Galephirimi” (meaning you will never see the sun rise).[3,49] Aldicarb is commonly sold illegally in the informal sector as a rodenticide in the form of

fine black to brown or purple granules, similar in appearance to coffee grounds or poppy seeds (Figure 1.1 A).[11] Other street pesticides sold in SA are depicted in Figure 1.1 B.

Figure 1.1: (A) "Two step" packaged in transparent plastic bags; (B) street pesticides sold in Pretoria, South Africa.

1.5 Anticholinesterase inhibitors

Anticholinesterase inhibitors are derived from phosphoric, thiophosphoric and carbamic acids.[43,51] Relative to other pesticide classes, cholinesterase inhibitors are considered to act more acutely but break down rapidly.[43] Pesticides that have anticholinesterase activity are classified by the WHO as extremely and highly hazardous.[16] These pesticides are commonly used as surface sprays or baits for the control of household pests and are typically distributed as granulated material.[52]

Examples of cholinesterase inhibitors are the organophosphates and carbamate insecticides.[45] Terbufos (an organophosphate) and aldicarb (a carbamate) are considered the most toxic cholinesterase inhibitors. These insecticides continue to be the cause of APP in developing countries.[43] During the metabolism phase, the sulphur bond of these compounds is converted to oxygen known as the oxon form, which is a potent cholinesterase inhibitor in humans. These metabolites and breakdown products are excreted in the urine, in free-form or having been bound to glucuronic acid or sulfate.[51] Fatalities of probable or suspected anticholinesterase pesticides are mostly seen at the

Forensic Medico-Legal Laboratories (FMLL) and pathology settings in SA. Aldicarb (rated as extremely hazardous) is commonly implicated from grey or black granules in the stomach content of fatalities admitted to the PMLL. The observation of these granules in the stomach at autopsy suggests pesticide poisoning. Even though aldicarb is one of the pesticides that is banned in SA (since the year 2012), it remains readily available in the street markets in the informal sector.[53]

1.6 Carbamates

Carbamate pesticides are derived from carbamic acid (Figure 1.2A) originally extracted from the calabar bean (*Physostigma venenosum*) that grows naturally in tropical West Africa.[54] Carbamates are used therapeutically (physostigmine) to treat glaucoma.[33] Carbamates fall into two distinct structural classes; the methylated N-methylcarbamates which are generally used as insecticides (Figure 1.2B) or the N-allyl carbamates used as herbicides (Figure 1.2C).[55] Carbamates in general are chemically unstable compounds that break down in the environment within weeks.[52] The different carbamate pesticide compounds with their respective toxicity index are presented in Table 1.3. Some carbamate compounds are very toxic, have teratogenic effects and may lead to the development of other abnormalities (particularly at high pesticide dosages). The abnormalities in different organ systems depends on the hosts genetic properties (especially in animals) and the structure of the carbamate compound.[56]

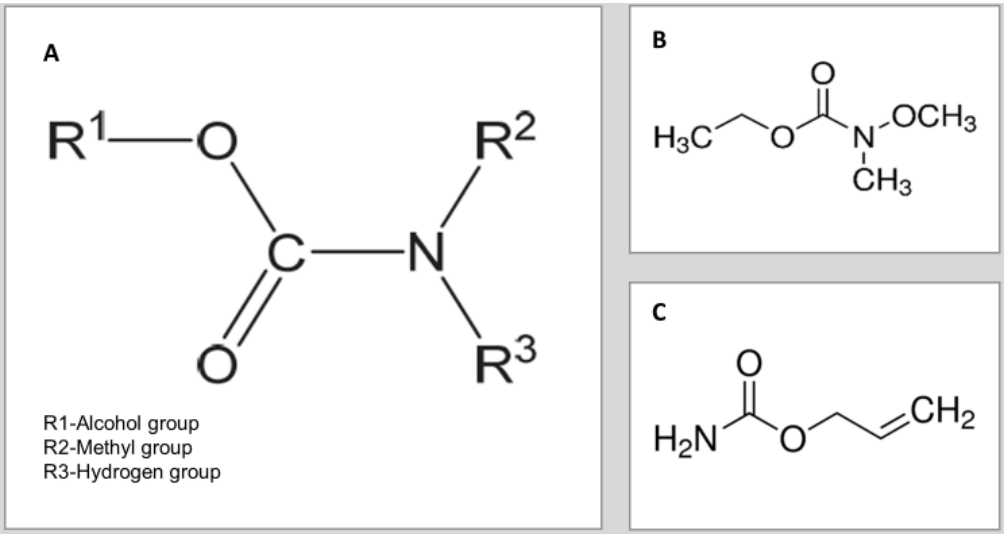


Figure 1.2: The general chemical structure showing the pharmacophore of (A) carbamates, (Figure available through open access IntechOpen Limited[57]) (B) N-methylcarbamate,[58] and (C) allyl carbamate.[59]

Table 1.3: The main carbamate insecticides classified according to their relative toxic potency (estimated human values).[60]

High Toxicity (LD_{50} < 50 mg/kg)	**Moderate toxicity** (LD_{50} = 50-200 mg/kg)	**Low toxicity** (LD_{50} > 200 mg/kg)
Aldicarb (Temik)	Bufencarb (Bux)	BPMC (Fenocarb)
Aldoxycarb (Standak)	Carbosulfan	Carbaryl (Sevin)
Aminocarb (Metacil)	Primicarb (Pirimor)	Isoprocarb (Etrofolan)
Bendiocarb (Ficam)	Promecarb	MPMC (Meobal)
Carbofuran (Furadan)	Thiocarb (Larvin)	MTMC (Metacrate, Tsumacide)
Dimetan (Dimetan)	Trimethacarb (Broot)	XMC (Cosban)
Dimetilan (Snip)		
Dioxacarb (Elecron, Famid)		
Formetanate (Carzol)		
Methiocarb (Mesurol)		
Methomyl (Lannate, Nudrin)		
Oxamyl (Vydate)		
Propoxur (Baygon)		

1.6.1 Mechanism of action

Carbamates inhibit acetylcholinesterase (AChE), an enzyme responsible for the hydrolysis of the neurotransmitter acetylcholine (ACh), into choline and acetic acid (Figure 1.3).[21] The inhibition of AChE results in the accumulation of ACh at neural synapses and neuromuscular junctions, causing excessive stimulation at the sites. The accumulation of the acetylcholine further leads to muscarinic, nicotinic and central nervous system (CNS) effects, which are collectively referred to as the acute cholinergic crisis.[18,22,61] This inhibition known as carbamylation is reversible.[37,43]

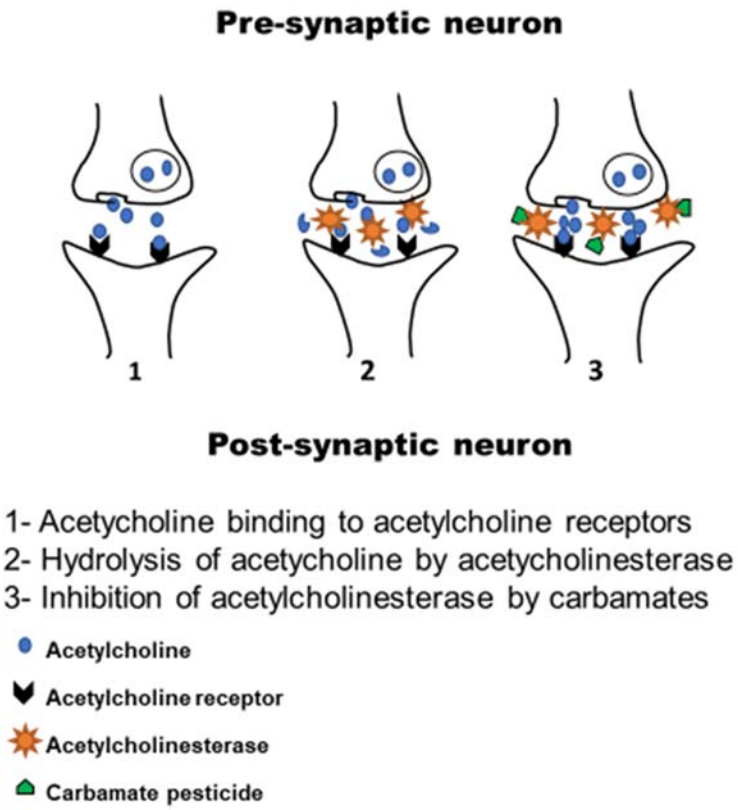


Figure 1.3: Mechanism of action of carbamates (created using power point. Adapted from https://www.cdpr.ca.gov/docs/legbills/reports/reg/cholinesterase/appendix).

1.7 Diagnosis of carbamate poisoning

1.7.1 Clinical signs and symptoms of toxicity

There are several common symptoms of carbamate toxicity namely: salivation, lacrimation, urination, defecation, gastrointestinal distress, emesis, (represented by the acronyms SLUDGE or DUMBELS [diarrhoea, urination, miosis, bronchospasm and bradycardia, emesis, lacrimation, salivation]).[52] The onset of characteristic signs and symptoms of carbamate poisoning depends on the amount of ingested pesticide as shown in Table 1.3 above, and is estimated to present within 15 to 30 min after exposure to the pesticide.[33] Failure to initiate immediate treatment of critical symptoms of pesticide poisoning (such as respiratory failure), can lead to relatively rapid death.[18] The mechanism of death is typically asphyxia, but cardiovascular failure cannot be excluded.[33] Patients who present with carbamate poisoning have similar signs and symptoms to those intoxicated by organophosphates. Contrary to organophosphate poisoning, the symptoms presented by carbamate poisoning may tend to exhibit shorter duration of toxicity due to the reversible enzyme inhibition while causing events of sysmptoms.[18,52]

1.7.2 Acetylcholinesterase as a biological marker

Measurement of the inhibition of AChE activity is used as a biomarker in suspected AChE pesticide poisonings.[62] This has been a routine biomarker used for assessing accidental pesticide exposure and can give an early warning of pesticide exposure of individuals. This biomarker can also further indicate both acute and chronic exposure to organophosphorus pesticides, as well as specific effects at cellular and molecular levels.[63] The classes of cholinesterase enzymes inhibited by carbamate pesticides include AChE and pseudocholinesterase (also known as butyrylcholinesterase - BChE).[62] The AChE enzyme is found abundantly in the membranes of the red blood cells, nerve motor endplates and the grey cerebral matter of the brain. Pseudocholinesterase is produced in the liver and is predominantly found in plasma, white matter of the brain, the heart and pancreas.[52]

Low levels of AChE in whole blood are used as a biomarker for the diagnosis of exposure to anticholinesterase pesticides. Pseudocholinesterase levels fall more rapidly than those

of the red blood cell AChE and are preferred for clinical diagnosis.[52] Acetylcholinesterase levels are more reliable for post-mortem diagnosis as they represent the true cholinesterase (ChE) levels.[52] The interpretation of the results may be complex due to the relation of the dose-response *in vivo* which may be modified by the pharmacokinetic parameters of the pesticide compound. The ChE values may also be influenced by the timing of sample collection since exposure, with pesticide present but not showing any alteration of normal ChE levels.[63] Certain factors must be considered during the interpretation of acetylcholinesterase activity results as these can influence ChE levels. These include pregnancy, concomitant therapeutic drugs intake (e.g., anti-malarial drugs), allergic diseases, liver or heart diseases.[64,65] Toxicants such as cocaine, carbon disulfide, benzalkonium salts, ciguatoxins, solanines and organic mercury compounds can also reduce enzyme activity, especially BChE. It is reported that red blood cell AchE activity can be affected by haemolytic anaemia.[63] Due to the carbamylation of carbamates, the ChE inhibitor complex is prone to produce false negative results when ChE levels are detected.[63]

AChE levels are measured by assessing ChE activity,[63] with the normal range being 4620-11500 U/L in red blood cells. However, some individuals may have their own 'normal' acetylcholinesterase concentration that differs from the standardised normal range. This can be due to different genetic polymorphism of individuals or exposure to environmental pollutants. Therefore, it is advised that a baseline level of enzyme activity be recorded, especially in people working in the agricultural sector.[33,35] The latter can assist with the monitoring of these concentrations in persons who may be at risk. Unfortunately baseline values are rarely available for members of the general population.[64,66]

1.7.3 Toxicokinetics

Carbamates enter the human body via inhalation, ingestion (food and water) and dermal absorption.[52] These compounds are rapidly absorbed by the gastrointestinal tract after ingestion and into the blood stream after inhalation.[26,67] Symptoms of toxicity are already

noted after ingestion of 50–500 mg/kg of this class of pesticide.[33,68] Carbamates accumulate in adipose tissue, after which they can then be redistributed to other body tissues.[60] These compounds are generally metabolised in the liver by the CYP 450 enzymes.[43,52] Metabolites of products formed through hydrolysis and oxidation include; sulfoxide, sulfone, oxime and nitrile products, with elimination mainly via the kidneys into the urine within 24 hours of ingestion, as well as excretion in faeces.[26,52,55,61] The biotransformation of carbamates is divided into two distinct phases. Phase I reaction where oxidation or hydrolysis takes place and phase II where sulpho-conjugation or glucuronidation occurs, resulting in the formation of a water soluble product, which is excreted via the kidneys.[33] The metabolites are mostly found in high concentrations in biological samples and have severe toxicity compared to the parent compounds, which for aldicarb are aldicarb sulfoxide and aldicarb sulfone.[26,67] Fatal poisonings due to methomyl, which is a thiodicarb metabolite (carbamate compound) have been reported.[69]

1.8 Medico-legal aspects of carbamate fatalities

Deaths deemed unnatural or unexplained, are subjected to a medico-legal investigation in order to determine the cause and manner of death (natural, suicidal, homicidal, accidental or undetermined).[70,71] In SA, the Inquests Act 58 of 1959 provides for the investigation of unnatural deaths and in addition, whilst the notification of pesticides poisoning cases is required by the National Health Act 61 of 2003.[42,72]

The medico-legal investigation of death usually includes a post-mortem examination and an autopsy.[42] Post-mortem findings in cases of carbamate poisoning may include externally visible features such as salivation, emesis and defecation, whereas the internal findings are mostly not specific (vascular congestion, pulmonary oedema and features of multi-organ failure). Dark poppy seed-like granules in the stomach (depending on the commercial preparation ingested) may be a very helpful feature at autopsy (Figure 1.4).[52] Furthermore, a strong odour (sometimes described as garlic or almond odour) may also be evident. At autopsy, biological samples such as blood, urine, stomach contents, bile and vitreous humour and organ tissue may be collected for toxicological analysis.[52]

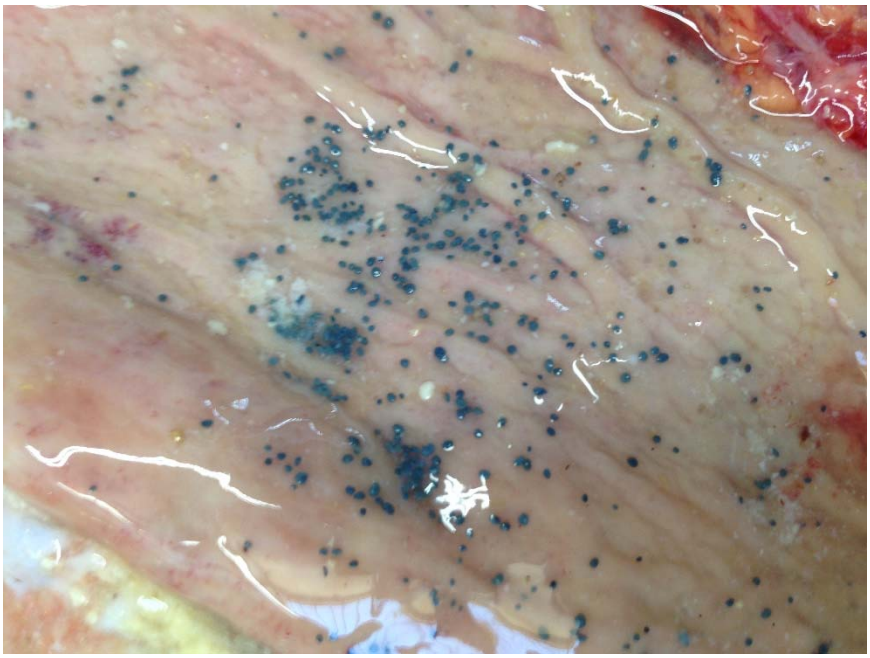


Figure 1.4: Dark granules on the stomach lining indicating the probable presence of carbamate pesticides.

1.9 Confirmation of toxicity in biological matrices

1.9.1 Biological matrices used in post-mortem analysis

Various biological matrices are used for post-mortem toxicological analysis.[73,74] Blood is commonly collected for analysis.[75] However, post-mortem blood differs from ante-mortem blood with a lower pH and higher viscousity.[75] Liquefaction of the tissues influences post-mortem redistribution of xenobiotics and may result in blood haemolysis.[74] To minimize the risk of sample contamination it is recommended that blood sampling be done before evisceration of the body.[74]

Previous studies on deaths resulting from methomyl (carbamate pesticide) poisoning indicate that this compound is usually detected at very low concentrations or not detected at all in blood samples, as it is rapidly metabolised in this matrix; however it is detected in high concentrations in the vitreous humour.[6,76] A recent study has shown that methomyl is decomposed by bacterial species post-mortem and is therefore difficult to detect in

post-mortem human blood.[76] Urine is also an important biological fluid for toxicological analysis, as it contains the unchanged toxic compound (parent compound) as well as its metabolites.[71,77]

Stomach content is one of the important samples that is used for toxicological analysis in suspected oral poisoning fatalities.[78] The stomach content contains the parent compound of the drug or poison, the concentration of which is dependent on the type and quantity of food present in the stomach.[55,69,77,79] According to Hoizey *et al.*[69] thiodicarb (parent toxic compound) is only detected in gastric content, and not in other post-mortem tissues or fluids.[69] In a case study, Chumbinho was observed as black granules in the stomach content, which was confirmed through toxicity testing. In spite of the advanced putrefaction of the body, the concentration of aldicarb in the stomach content was 62.3 µg/mL while in the blood it was 7.8 µg/mL.[67]

Cerebrospinal fluid (CSF) is useful for the screening and detection of drug and toxic metabolites that have crossed the blood brain barrier. This matrix is less prone to contamination and bacterial invasion.[75] However, there is limited reference data published for the quantification of xenobiotics in CSF and the interpretation of the analytical findings.[55,75]

Certain considerations should be taken into account when selecting and collecting ante-mortem and post-mortem toxicology specimens. For instance, the site of blood collection for ante-mortem specimens differs from that at which post-mortem blood is collected. This may also have an influence on the concentration of the toxin.[73,75,77] Peripheral blood is recommended for post-mortem toxicology analysis, as it should contain the concentrations of the toxin responsible for death but may also show low concentrations due to post-mortem degradation or redistribution into surrounding tissue through diffusion.[73] The advantages and disadvantages of the various specimens used in forensic toxicological analysis are summarised in Table 1.4. In cases where death occurred very rapidly, there may be no or very low concentration of the toxicant in vitreous humour, bile or urine. If the survival period is prolonged (for example, when patients are hospitalised) the toxin concentration may be higher in these matrices.[78,80,81]

1.10 Stages during sample analysis

The reliability of laboratory analytical results may be influenced by factors related to one of three phases in the total testing process; pre-analytical, analytical or post-analytical (Figure 1.5).[82] Errors that occur during this process impact on the accuracy of the results.[82]

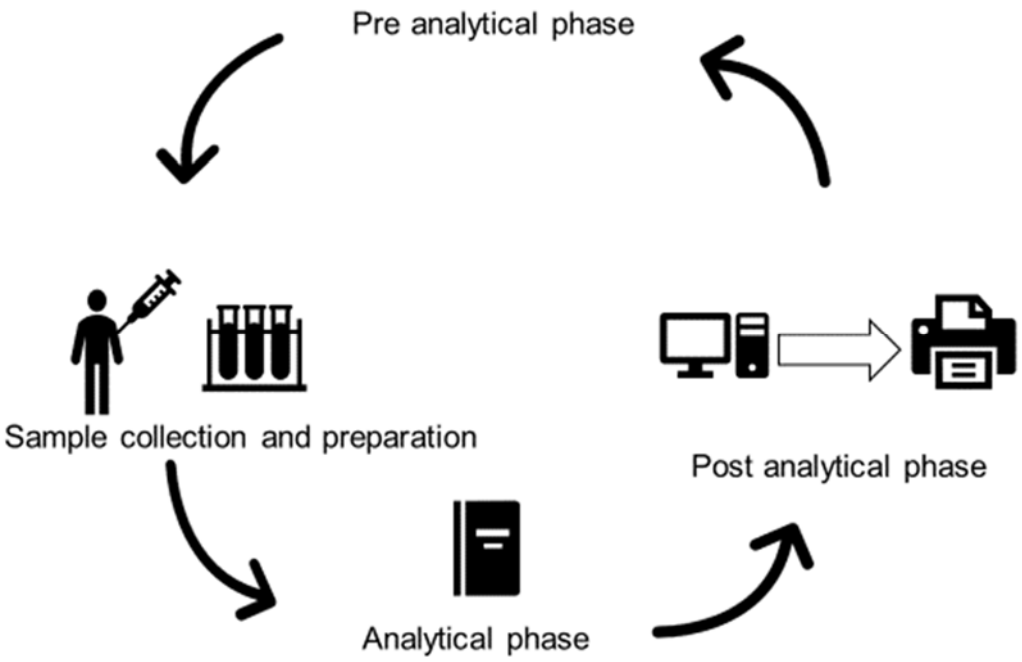


Figure 1.5: The stages of biological sample analysis.

Table 1.4: Advantages and disadvantages of ante-mortem (AM) and post-mortem (PM) biological specimens used in toxicology.[75]

Specimen	Advantages	Disadvantages
Blood (AM)	• Widely accepted matrix • Determines recent drug use (hours–days) • Related to the true drug concentration (pharmacological effect) • Large reference data available	• Fixed collection site • Shorter detection window
Blood (PM)	• Widely accepted matrix • Reference data available • Central/peripheral blood drug ratios known for some drugs • Cardiac blood in large volume but requires caution with interpretation	• Susceptible to post-mortem redistribution (central) • Susceptible to post-mortem artefacts and interferences • Susceptible to contamination (e.g. trauma) • Quality of specimen highly dependent on collection protocol • Limited volume of peripheral blood
Urine (AM and PM)	• Widely accepted matrix • Easy collection • Amenable to automated analysis • Longer detection window than blood (days–weeks)	• Minimal parent drug • Not useful for quantitative analysis • Not related to impairment or pharmacological effect
Gastric content (AM and PM)	• Identification of acute ingestion/delayed absorption • Identification of fragments possible • Particularly useful for orally administered drugs/poisons	• Non-homogeneous matrix • Complex matrix • Requires sample preparation/pre-treatment • Requires total specimen collection for interpretation
CSF (AM and PM)	• Determines recent drug use (hours–days) • Minimal sample preparation • Relatively few interferences	• Fixed collection site • Limited reference data

1.10.1 Pre-analytical phase

The pre-analytical aspects include variables such as sample collection, handling and transportation, sample preparation and storage conditions.[83,84] Before a sample can be 'inserted' into any analytical instrument, it requires sample preparation that includes but is not limited to homogenisation, extraction, pre-concentration and final analysis.[46] This is important to ensure precision and quality in the analytical laboratory.[85]

The majority of errors leading to inaccuracy of results appears to be related to the pre-analytical phase. This naturally has a knock-on effect.[83] Approximately 93% of errors in diagnostic processes were reported to be due to the lack of standardised procedures that addressed these variables.[85] Human error is also reported to be high, as many laboratories experience increasing workloads while there is a reduction in qualified personnel.[86] These errors include: i) improper requests, ii) incorrect sample labelling, iii) time of sampling not recorded, iv) insufficient sample volumes of specimen, v) *in vitro* haemolysis, vi) incorrect specimen tube or container used, vii) inappropriate specimen handling.[85]

The major reasons for erroneous analytical results are reported to be due to *in vitro* haemolysis of samples and the use of incorrect sample collection tubes.[85] This is due to blood cell haemolysis interfering with the analytical method due to the cell constituents being released from erythrocytes and platelets, resulting in low sensitivity and low reproducibility.[87] Post-mortem specimens pose additional challenges when compared to ante-mortem samples. These challenges include variable post-mortem drug redistribution and concentration gradients. The time interval between death, sample collection and the processing of specimens is of critical importance.[88] For example; In post-mortem drug analyses, whole blood is used, as separation of red blood cells from serum is usually not possible.[88] The factors influencing post-mortem redistribution include passive drug release from different organ tissues after death, autolysis of cells and putrefaction. Xenobiotics with a large volume of distribution, such as basic lipophilic analytes are specifically susceptible, resulting in the misinterpretation of toxicological results.[88]

1.10.2 Analytical phase

Several screening methods have been employed in clinical and forensic laboratories for the identification of xenobiotics in biological samples. Analytical techniques used for the detection of pesticides in biological samples include; thin layer chromatography (TLC), gas chromatography (GC), gas chromatography with electron ionisation and mass detection (GC-MS), high-performance liquid chromatography (HPLC), ultra fast liquid chromatography tandem mass spectrometry (UFLC-MS/MS) and ultra-performance liquid chromatography connected to a mass detector (UPLC-MS).[22,71,89,90,91,92,93,94] The application of GC-MS has been used for the qualitative and quantification analyses of pesticides in various samples at low concentrations.[95] However, due to the thermal instability of the N-methylcarbamates, this class is not easily detectable using GC analysis due to the high temperature that is required for the GC separation during analysis. Many of the new synthetic pesticides are not responsive to GC due to thermal instability and high polarities.[96] Carbamate compounds are known to be polar and sensitive to heat.[97] Liquid chromatography tandem mass spectrometry (LC–MS/MS) has been reported to have increased sensitivity and selectivity in various applications, especially when operated in multiple reaction monitoring (MRM) mode.[95,96,98] LC-MS/MS methods have been used for the quantification and confirmation of pesticides and their metabolites at low parts per billion (ppb) concentrations.[96]

Tandem mass spectrometry (MS/MS or MS^e) has become a major detection technique, due to its confirmation of peak identity and quantitation abilities.[99] It has been reported that high resolution mass spectrometry instruments, such as the time-of-flight mass spectrometer can selectively determine the molecular weight of compounds with accuracy of less than 5 ppm. It also provides full MS sensitivity and mass spectral information.[96] LC–MS/MS thus allows for both targeted and untargeted screening, providing for a large scope of analytes to be identified.[100] The UPLC-MS/MS is considered the gold standard for quantification of pesticides.[101] The ultra-performance liquid chromatography tandem quadrupole time of flight mass spectrometer (UPLC-q-TOF-MS/MS) combines the advantages of UPLC and q-TOF-MS/MS, resulting in faster and more accurate analysis albeit less sensitive.[95]

Various sample preparation methods for biological samples are used prior to analysis of pesticides.[102] These include simple protein crash with MeOH or ACN, liquid-liquid extraction (LLE), solid phase micro-extraction (SPME) and solid phase extraction (SPE). SPE is one of the useful techniques for sample preparation that results in higher selectivity and cleaner extracts. This technique is useful for targeting and isolating relevant analytes present in biological samples.[102]

Liquid-liquid extraction is also one of the common sample preparation techniques employed for the determination of pesticide residues. LLE is an easy method to use, however it may require large volumes of organic solvents and samples. Low selectivity and efficiency and poor recovery of analytes due to high matrix effect have been reported for this extraction method.[94,99,103,104]

1.10.3 Post analytical phase

In this phase, the results are reported and interpreted (in the current setting for medico legal decisions). Errors such as validation of analytical data, incorrect data entry and turnaround time (the period from when the test is requested till the results are reported) may be noted, and may influence the quality of the results.[105,106] There is, however, limited information available on post-analytical phase errors, as most errors are reported in the pre-analytical phase.[105]

1.11 Parameters that influence sample stability

1.11.1 Post-mortem redistribution

Post-mortem redistribution (PMR) is defined as the changes of drug or xenobiotic concentration in a tissue or biological sample that take place after death.[80,88] This takes place after xenobiotic metabolism. Xenobiotic metabolism has three phases: i) oxidation, reduction and hydrolysis, ii) conjugation reaction of compounds, iii) secondary conjugation through transport molecules. However, the third phase cannot take place after death as it requires energy to transport conjugates out of a cell. [107]

The mechanisms that may have an influence on the artificial increase or decrease in drug concentration during the post-mortem period can be encompassed in PMR.[80,88] The post-mortem concentrations do not represent the actual concentration at the time of death for compounds that can easily diffuse or are chemically unstable. Factors that affect PMR are the physicochemical, pharmacokinetic properties of xenobiotics as well as environmental conditions (Table 1.5). The body organ, type of cell and tissue decomposition may also have an effect post-mortem redistribution.[80,88] The vascular pathway and passive diffusion or pH gradient may be the main routes of the initiation of post-mortem redistribution.[80,88] Though there is limited data regarding factors influencing PMR of carbamates, the post-mortem influences that may affect the concentration of xenobiotics in biological samples are: i) the xenobiotics' metabolism progression in the body in the early post-mortem phase, ii) the non-metabolic degradation of xenobiotics and their metabolites, iii) post-mortem microbial metabolism degradation of xenobiotics and their metabolites, iv) the formation of xenobiotics or endogenous compounds in post-mortem through putrefaction and microbial processes.[107]

After death, bodily enzymes remain active to further metabolise xenobiotics. The xenobiotic concentration may either increase or decrease dependent on the metabolic enzyme present, especially in blood.[107] There is limited data regarding the metabolites of toxins. The comparison of the parent compound and its metabolite is important in post-mortem toxicology of xenobiotics that are subjected to PMR.[107]

Degradation of compounds containing moieties such as esters, sulphur atoms and oxidised structures may occur from enzyme metabolic processes. The enzyme metabolic processes may be due to certain conditions introduced through sampling or sample preparation.[107]

The colonisation of bacteria and fungus in a corpse during putrefaction can catalyse various metabolic changes of xenobiotics as well as their metabolites. For example, many drugs and poisons are subjected to metabolism by polymorphic enzymes. If a xenobiotic is metabolised by microorganisms, degradation may take place, resulting in low

concentrations in post-mortem samples.[107] However, if phase two metabolites produced through conjugation reactions are degraded by microorganisms, this may result in high concentrations of the parent drug or phase 1 metabolite. Bacteria reported to be identified in post-mortem samples include different genera of *Bacillus, Escherichia, Pseudomonas, Staphylococcus and Streptococcus*, whereas fungi include *Aspergillus, Candida and Penicillium*.[107]

The proficiency to preserve the initial analyte concentration in a collected sample within specified limits is described as stability.[81] Physicochemical properties of the carbamate compounds (pH, volume of distribution, protein binding affinity, bacterial biotransformation action and lipophilicity) during post-mortem specimen collection procedure, characteristics of the matrix, and the use of preservatives can influence the compounds stability.[75] The reason being that; post-mortem drug concentration changes may vary due to anatomical collection sites and that sample collection is time dependent. The site-dependent changes can arise from passive diffusion from other organs and blood vessels as well as incomplete distribution of a drug at the time of death.[88]

It is well known that metabolites may be more toxic than the parent compound, which holds true for post-mortem specimens.[88] The effect that time and temperature has on metabolite degradation is essential to consider when analysing and reporting results, especially in the forensic setting.[81]

Table 1.5: Factors that influence post-mortem redistribution of drugs.[80,88]

Physico-chemical and pharmacokinetic properties of the drug	• Size, shape, charge, pKa-value and partition coefficient of the drug • Apparent volume of distribution • Binding to proteins, blood cells and/or tissues Residual enzyme activity during the early post-mortem time period
Environmental conditions	Initial concentration, pH-value, orientation of solute flux, temperature, time, blood coagulation and hypostasis, blood movement due to fluidity changes and pressure, position of the corpse, lysosomal enzyme activities, bacterial invasion

1.11.2 Preservation and storage

The general procedure for specimen preservation and storage is that once a specimen is collected, aliquots should be stored in tightly sealed containers at 4°C should analysis be carried out short term and -20°C or -80°C for long-term storage.[74] This applies to compounds known to be unstable (which are often matrix dependent).[75] For example, in toxicological sampling, blood is usually collected in tubes containing sodium fluoride and potassium oxalate. Vitreous humour and urine are stored in tubes with or without preservative, depending on the toxicology kit used. Gastric content is stored without preservative whereas tissue from liver and brain is stored in specimen cups.[100] Post-mortem metabolic and chemical degradation may occur with the carbamates, thereby reducing the apparent concentration of the carbamate in biological specimens.[75] The complexity of post-mortem matrices is due to the increased presence of bacteria (as mentioned previously).[75] Also, even after specimen collection the enzymes from biological samples may remain active and continue to degrade the compound *in vitro* after specimen collection.[75] Instability of ester moieties or sulphur containing functional groups is well documented. Sodium fluoride (which is commonly used as a preservative in post-mortem fluid or blood samples) must not be used in specimens to be tested for organophosphorus compounds as this preservative is reported to catalyse the breakdown of organophosphorus compounds.[74,108,109] The rapid degradation of carbamate compounds in biological samples (especially blood) is well known, as they are unstable compounds and the degradation increases based on temperature and extended storage

time.[22,61,79,90,109] Furthermore, although it is recommended that samples should be frozen, transported and stored in tightly sealed containers at -20°C or preferably at -80°C, prior to and until analysis, this is not the routine practice in many laboratories.[52,73,109]

1.12 Aim and objectives

1.12.1 Aim

The primary aim of the study was to optimise the sample preparation and analysis of biological matrices for select carbamates using LC-MS/MS method.

A secondary aim was to assess the stability of aldicarb in biological matrices over time when stored at various temperatures and to analyse post-mortem samples collected from suspected cases of carbamate intoxication to assess the sensitivity of the assay in real samples.

1.12.2 Objectives

The objectives of the study were:

- To optimise a LC method of common carbamates.
- To optimise sample preparation methods for the extraction of carbamates from spiked biological samples. To optimise and validate a quantitative LC-MS/MS method for the determination of aldicarb and its metabolites (aldicarb sulfone and aldicarb sulfoxide) in these biological samples.
- To determine the stability of aldicarb and its oxidation products, over a 28 Day storage period when specimens were stored at four different storage temperatures, using the validated LC-MS/MS method.
- To determine whether preservatives added to whole blood, plasma and urine affect the stability of aldicarb and its metabolites.
- To determine whether aldicarb can be detected in “Street pesticides” obtained from street vendors as well post-mortem samples.
- To determine whether aldicarb and its metabolites can be detected in post-mortem samples.

2 Materials and methods

2.1 Study design

The study was of an analytical nature, involving *in vitro* experiments to determine the kinetic stability of specific carbamate compounds (aldicarb, aldicarb sulfone, aldicarb sulfoxide) but included the assessment of chromatographic separation of other common carbamate pesticides; carbaryl, carbofuran, 3–hydroxycarbofuran, methiocarb, methomyl, oxamyl and propoxur in spiked antemortem biological samples.

A stable isotope labelled internal standard based quantitative LC-MS/MS method was applied to samples collected from post-mortem cases from the Pretoria Medico-Legal Laboratories (PMLL) between December 2017 and July 2019 where carbamate poisoning was suspected. The post-mortem samples collected included blood, stomach content and urine.

2.2 Study setting

This study was conducted at the PMLL, the Department of Forensic Medicine and the Departments of Pharmacology and Chemistry of the University of Pretoria.

2.3 Ethical consideration

Ethical approval for this study was obtained from the Health Sciences Research Ethics Committee of the University of Pretoria with approval number: 4/2018 (Appendix I).

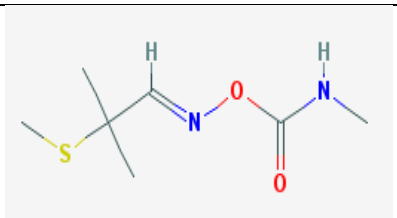
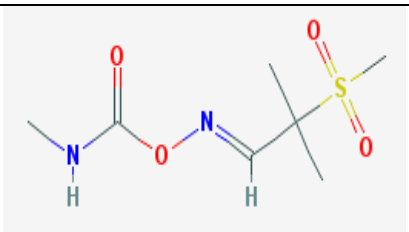
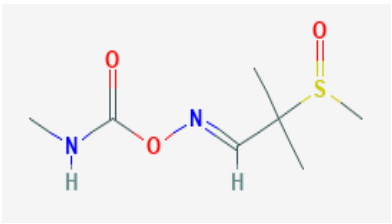
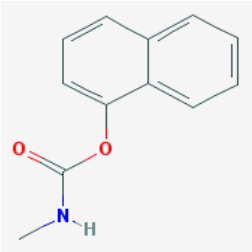
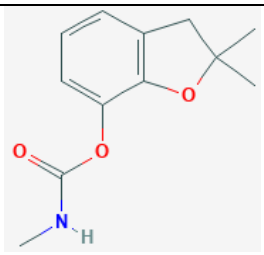
2.4 Carbamates under study

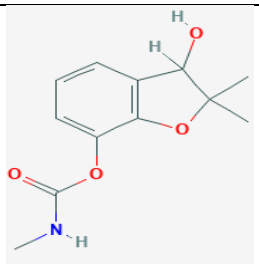
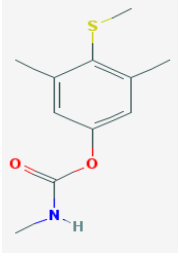
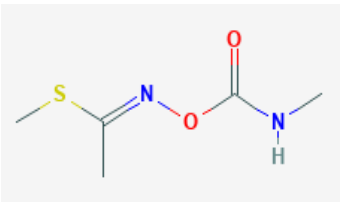
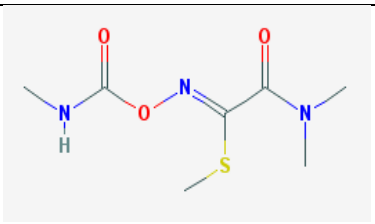
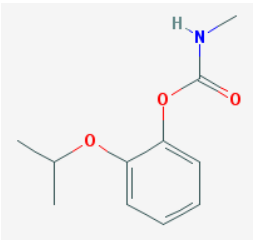
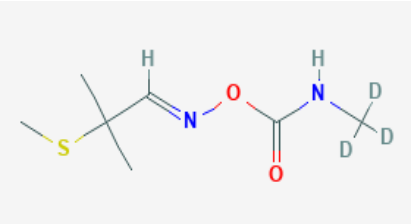
A list of the reagents and solutions used for the different extractions and sample preparation in the study and their preparation is provided in Appendix II.

A standard mixture of ten carbamate pesticide at concentrations of 100 µg/mL in methanol was used as a stock solution (RESTEK Corporation, Lot number: A0135315) (Table 2.1

and Appendix III). The standard stock solution was stored at -20°C until used and calibration standard mixes, freshly prepared for each analytical batch. Aldicarb-D3 (Industrial Analytical, lot number: 7-JMR-163-1) was used as stable isotope labelled internal standard. (Table 2.1 and Appendix III).

Table 2.1: Chemical structures of the ten carbamate pesticides investigated in this study. Permission for re-print was provided under the National Centre for Biotechnology Information PubChem database open source copyright law.[110]

Carbamate compound	Chemical structure
Aldicarb	
Aldicarb sulfone	
Aldicarb sulfoxide	
Carbaryl	
Carbofuran	

3 – Hydroxycarbofuran	
Methiocarb	
Methomyl	
Oxamyl	
Propoxur	
Aldicarb-D3 (used as a stable isotopically internal standard)	

A second experiment testing aldicarb stability in different biological matrices was conducted using aldicarb isolated from Temik by acetone extraction and recrystallization from xylene.

2.5 Sample collection

2.5.1 Sample collection from healthy volunteers for stability study

Biological samples (blood, plasma and urine) were collected from 25 consenting healthy volunteers. Approximately 8 - 10 mL of venous blood was collected from each participant using a vacutainer tube (Becton–Dickinson Vacutainer) with ethylenediaminetetraacetic acid (EDTA) or ethylenediaminetetraacetic acid (EDTA) plus sodium fluoride (NaF) as respective anticoagulant or glycolysis inhibitor (±4 mL blood per tube). Urine (3 mL) was collected into a urine specimen container and was decanted into plain test tubes (Becton–Dickinson) and 4 mL boric acid added before been stored. All containers were sealed and labelled (the type of sample, anonymous participant number and date of collection). For stability testing, well mixed samples were divided into 500 µL aliquots and spiked with 12.5 µL of aldicarb to a final concentration of 25 µg/mL then kept under different storage temperature conditions; ambient temperature 19°C, fridge temperature 4°C; -20°C and -80°C and analysed at different storage times time; Day 1, Day 3, Day7, Day 14, Day 21 and Day 28.

2.5.2 Sample collection from post-mortem suspected poisoning cases

Post-mortem specimens (blood, urine, stomach content), were collected from suspected poisoning cases at the PMLL by the principal investigator under the supervision of the forensic pathologist conducting the post-mortem examination. Blood, urine (when available) and stomach content (3 - 5 mL) from the decedents were collected using sterile and disposable syringes. Samples were placed in a standard toxicology kit (containing a 20 mL McCartney bottle for post-mortem blood with NaF as a preservative, a 20 mL urine container with boric acid preservative and a 10 mL stomach content container). All these samples were appropriately labelled with an anonymous study number and stored until analysed at -80°C. Street pesticides were used as a reference control for the post-mortem

samples as the decedents would most likely have ingested the pesticide granules purchased from the informal street market.

2.6 Sample preparation

2.6.1 Optimisation of pre-analytical sample preparation of spiked biological samples for stability testing

2.6.1.1 The initial solid phase extraction method for biological samples

2.6.1.1.1 Blood and plasma

A volume of 220 µL of whole blood was spiked with 30 µL of 100 µg/mL BDMC solution (RESTEK Corporation Lot number: A0128234) as a carbamate based internal standard (IS), mixed by inversion at least 10 times then diluted 1:1 with a 0.05 M $ZnSO_4$ solution and sonicated in a Bransonic 52 ultrasonic water bath (Branson, Danbury, USA) for 5 min. The mixture was then centrifuged using an Allegra X-12R centrifuge (Beckman Coulter, Brea, USA) at 2 000 x *g* for 5 min before loading 400 µL of the supernatant slowly onto a pre-conditioned Waters Oasis Prime HLB cartridges (3 cc, 150 mg; MicroSep, Johannesburg) for solid phase extraction (SPE). The cartridge was washed twice with 250 µL of 5% MeOH and sucked dry for 20 sec. Elution of carbamate analytes was done using two aliquots of 25 µL ACN:isopropyl alcohol (IPA) (90:10) and sucking the cartridge dry after each addition. The eluate was diluted with 50 µL distilled water for UPLC-q-TOF-MS/MS analysis. The same pre-analytical SPE method was followed for carbamate mixture spiked plasma samples.

2.6.1.1.2 Urine.

A volume of 440 µL of urine, was spiked with 60 µL of a 100 µg/mL BDMC solution as the IS, then diluted 1:1 with a 0.05 M $ZnSO_4$ solution and sonicated in a Bransonic 52 ultrasonic water bath (Branson, Danbury, USA) for 5 min. The mixture was then centrifuged (2 000 x *g* for 5 min) using an Allegra X-12R centrifuge (Beckman Coulter, Brea, USA) before loading 400 µL of the supernatant slowly onto pre-conditioned Waters Oasis Prime HLB cartridges (3 cc, 150 mg) for SPE. The cartridge was washed twice with

250 µL of 5% MeOH. Elution was done using two aliquots of 25 µL ACN:IPA (90:10). The eluate was diluted with 50 µL distilled water for UPLC-q-TOF-MS/MS analysis.

2.6.1.2 Final SPE method

Four different SPE methods for preparation of samples were evaluated (Table 2.2). The sample preparation method was as follows:

A volume of 440 µL of whole blood, was spiked with 60 µL of 100 µg/mL carbamate mix (RESTEK Corporation Lot number: A0135315), then diluted with 1:1 0.05 M Z_nSO_4. The sample was gently vortex mixed with a vortex mixer (Gemmy Industrial Corporation, Taiwan) for 5 min and allowed to equilibrate for at least 1 h. A volume of 1 mL of 50:50 acetone:H_2O and 1 mL 90:10 ACN:MeOH was added to the blood solution. The matrix was vortex mixed for at least 1 min then sonicated in a Bransonic 52 ultrasonic bath (Branson, Danbury, USA) for 5 min and centrifuged (2 000 x *g* for 5 min) using a Allegra X-12R centrifuge (Beckman Coulter, Brea, USA) before loading 1000 µL onto the cartridge.

Table 2.2: Four different SPE cartridges tested during the extraction of carbamate compounds from biological matrices.

Cartridge brand	**Preconditioning step**	**Initial pass through sample volume (µL)**	**Wash condition**	**Elution volume** (90:10 ACN:MeOH)
Oasis Prime HLB 3 cc (150 mg)	2 mL of MeOH, 2 mL H_2O, 2 mL $ZnSO_4$	1000	2 mL of 5% MeOH	2 mL
Strata C8 cartridge	3 mL of MeOH,3 mL H_2O, 3 mL $ZnSO_4$	1000	3 mL of 5% MeOH	3 mL
Strata C18 cartridge	3 mL of MeOH, 3 mL H_2O, 3 mL $ZnSO_4$	1000	3 mL of 5% MeOH	3 mL
Sep-pak Vac C18-1 cc	1 mL of MeOH,1 mL H_2O, 1 mL $ZnSO_4$	1000	1 mL of 5% MeOH	1 mL

2.6.1.3 Protein precipitation method for analysis of aldicarb only in blood, plasma and urine

A volume of 500 µL biological sample (blood, plasma and urine) was spiked with 25 µg/mL of the aldicarb standard, where after it was mixed by inverting the sample tubes at least 10 times. The samples were allowed to stand for a minimum of an hour at 4°C to equilibrate. An aliquot of 150 µL of each sample was used for extraction in 1.5 mL Eppendorf tubes. A volume of 75 µL aldicarb-D3 IS in MeOH was spiked into each individual sample to a final concentration of 0.75 µg/mL. The sample was vortex mixed with a Vortex-Genie 2 (Scientific Industries, USA) for 1 min and sonicated in a Bransonic 52 ultrasonic water bath (Branson, Danbury, USA) for 10 min. A volume of 75 µL MeOH was added to the sample and vortex mixed for 1 min and sonicated for 10 min. A further volume of 150 µL of MeOH was added, vortex mixed for 1 min and sonicated again for 10 min. The sample was then centrifuged (16 000 x *g* for 15 min) using a Microfuge 16 (Beckman Coulter, Brea, USA). A volume of 300 µL supernatant was transferred to a new Eppendorf tube and evaporated to dryness under reduced pressure in a centrivap vacuum concentrator system (Labconco Corporation, Kansas City, MO) at 30°C. The dried sample was reconstituted with 100 µL of 50:50 ACN:H_2O with vortex mixing for 2 min and centrifuging at 16 000 x g for 15 min. A volume of 50 µL of the matrix was used for LC-MS/MS analysis on a Sciex 4000QTrap triple quadrupole mass spectrometer. Acetonitrile was also tested as a protein precipitation solvent using the same volumes and method but showed no advantage over the MeOH in terms of recovery.

2.6.2 Preparation and extraction of post-mortem samples

2.6.2.1 Stomach contents

A 2 g aliquot of the stomach content was mixed with 10 mL of ACN (containing 5 µL BDMC (IS) at a concentration of 100 µg/mL) followed by homogenisation with an ultrasonic homogeniser (BioLogics, Inc, Manassas, USA) at 1 500 W, with the pulser function set at 60% and timer set for 5 min. Thereafter, the sample was centrifuged at 2 500 x *g* for 5 min to obtain a clear supernatant solution. The supernatant was removed,

and the sediment subjected to a further two repetitions of the extraction steps. The three acetonitrile extracts per sample were combined and mixed with 80 mL of a 2% sodium chloride (NaCl) solution and 25 mL of an n-hexane/ethyl acetate mixture (1:1 v/v) in a 250 mL volume separating funnel. The funnel was shaken for 10 min with an orbital shaker (Gemmy Industrial Corporation, Taiwan). The n-hexane/ethyl acetate layer was collected and evaporated to dryness under reduced pressure in a centrivap vacuum concentrator system (Labconco Corporation, Kansas City, MO) at 30°C. The residue was dissolved and reconstituted in 100 µL methanol.

2.6.2.2 Blood and urine

A volume of 1 mL (whole blood and urine) was centrifuged (10 min at 2 000 x *g*) with a Microfuge 16 (Beckman Coulter, Brea, USA). A volume of 200 µL of each of the supernatant and the sediment of the samples was each diluted with 400 µL of ice cold 50:50 ACN:MeOH which was stored at -20°C. The matrix was vortex mixed with Vortex-Genie 2 (Scientific Industries, USA) for 5 min then centrifuged (16 000 x *g* for 15 min) with a Microfuge 16 (Beckman Coulter, Brea, USA). A volume of 100 µL of the matrix was used for UPLC-q-TOF/MS analysis.

2.7 UPLC-q-TOF-MS/MS instrumentation and method development

2.7.1 UPLC-q-TOF-MS/MS instrumentation

The chromatographic separation and detection of the carbamate calibration mix were performed with a Waters Acquity Ultra Performance Liquid Chromatography (UPLC) system coupled a to Waters Synapt G2 high definition quadrupole-time-of-flight (q-TOF) mass spectrometry system (HDMS) with MassLynx software (version 4.1) used for system control and data acquisition and processing.

The mass spectrometer was operated in both positive (ESI+) and negative (ESI-) electrospray ionisation mode to achieve optimised source conditions fragmentation was performed using MS^e technique with collision energy set at 5 V for low energy acquisition and ramped from 10 to 30 V for high energy collision-induced dissociation (CID)

acquisition within the transfer cell of the mass spectrometer. The mass spectral scans were collected every 0.2 s covering a range of 50-600 mass to charge ratio.

The source temperature was set at 140°C and desolvation temperature at 300°C with the sampling cone voltage at 10 V and extraction cone voltage at 3.0 V. The desolvation and flow gas was set to 100 L/h and 650 L/h respectively. Quantitative data-independent acquisition (DIA) data was collected (MS^e- experiment).

Four different columns (Kinetex C18 2.1 x 100 mm, 1.7 µm; Acquity HSS T3 2.1 x 100 mm, 1.8 µm; Acquity BEH C18 2.1 x 100 mm, 1.7 µm and RESTEK Raptor ARC-18 2.1 x 100 mm, 2.7 µm) were used to optimise a separation method for the carbamates using different mobile phases and gradient elution programs at a flow rate of 350 µL/min and a run time of 15 min, with the sample injection volume set at 5 µL. The column oven temperature was set at 40ºC. The mobile phase conditions are presented in Table 2.3.

Table 2.3: Different mobile phase compositions used for separate injections on each column.

Mobile phase condition 1	
Aqueous	0.1% formic acid in HPLC grade H_2O
Organic	Acetonitrile with 0.1% formic acid
Mobile phase condition 2	
Aqueous	10 mM ammonium acetate with 0.1% formic acid in HPLC grade H_2O
Organic	Methanol with 10 mM ammonium acetate and 0.1% formic acid
Mobile phase condition 3	
Aqueous	2 mM ammonium formate in HPLC grade H_2O with 0.2% formic acid
Organic	2 mM ammonium formate in methanol with 0.2% formic acid

2.7.2 UPLC-q-TOF-MS/MS method optimisation

2.7.2.1 The initial phase of method optimisation

Different volumes of standard carbamate mixture (Restek Corporation, Lot number: A0135315) were aliquoted into Eppendorf tubes to a final concentration of 1 – 1 000 ng

after which they were evaporated to dryness using a centrivac dryer (Labconco Corporation, Kansas City, MO) at room temperature and reconstituted with exactly 100 µL pure MeOH. The Kinetex C18 column 100 x 2.1 mm, 1.7 µm, was used for the chromatographic separation. Mobile phase buffers used were 0.1% formic acid in HPLC grade H_2O (aqueous phase) and acetonitrile with 0.1% formic acid (organic phase). For negative mode ionisation, the mobile phase buffers used were 10 mM ammonium acetate in H_2O (mobile A) and 10 mM ammonium acetate in methanol (mobile B). The column temperatures tested ranged between 22.4°C (minimum column temperature) and 50.1°C (maximum column temperature). The optimised target column temperature was 40°C with an autosampler tray temperature at 4°C. The injection volume was 5 µL and the total run time was 15 min. The optimisation of the flow rate was between 0.350 - 0.500 µL/min.

2.7.2.2 Second phase of method optimisation

Mass spectrometer optimisation of the detection parameters was done by direct infusion of 500 ng/mL carbamate standard mix via the built in fluidic system of the Waters Synapt G2 mass spectrometer. An internal LockMass reference, leucine enkephalin (m/z 555.2693), was infused directly into the source at time intervals of 10 s during the run to ensure mass accuracy and to compensate for mass drift during the run. The source was operated in positive mode with the capillary voltage set at 2.75 kV. The injection volume was 5 µL. Different gradients were used for different elution strengths of the solvents.

2.7.2.3 Third phase of method optimisation

Mixed carbamate standard solutions 1 µg/mL and 5 µg/mL were prepared and transferred into tapered glass inserts and placed in amber autosampler vials. Analyte separation of the carbamate mix were performed on a Waters Synapt G2 q-TOF-MS operated in positive electrospray ionisation (ESI+) mode at optimised source conditions (Table 2.4). Low and high energy scans were aligned at specified time points to predict the fragment ions and precursor ions acquiring the full mass spectrum simultaneously with the mass spectrometry method (Table 2.5). Three columns using two different mobile phases with gradient elution (Table 2.6) were used to compare the chromatographic separation of the

carbamate analytes using a Waters Acquity UHPLC system. The column temperature was kept at 40°C and the sample temperature at 4°C. The total run time was 15 min that included a re-equilibration step of 2.5 min. The product ions' retention time windows were 20 s. The injection volume was 5 µL.

Table 2.4: Waters SYNAPT-G2 q-TOF tune file parameters.

Mass spectrometry method setup	
Acquisition mass range (m/z)	50 – 600
Scan time (s)	0.2
Data format	Continuum
Low energy acquisition (V)	5
Transfer cell energy (V) ramp start	10
Transfer cell energy (V) ramp stop	30

Table 2.5: Mass spectrometry source conditions.

Source parameters	
Capillary voltage (kV)	2.75
Desolvation gas temperature (°C)	300
Sampling cone voltage (V)	10
Cone gas flow (nitrogen L/hr)	100
ESI source temperature (°C)	140
Desolvation gas flow (nitrogen L/hr)	650
Extraction cone voltage (V)	3

Table 2.6: UPLC gradient elution method.

Time (min)	Flow Rate (mL/min)	%A	%B	Curve
Initial	0.500	85.0	15.0	Initial
0.10	0.500	85.0	15.0	6
11.00	0.500	15.0	85.0	6
11.10	0.500	0.0	100.0	6
12.40	0.500	0.0	100.0	6
12.50	0.500	85.0	15.0	6
15.00	0.500	85.0	15.0	6

2.8 UPLC-q-TOF-MS/MS method validation for targeted analysis of spiked biological samples to assess decay kinetics

2.8.1 Linearity, Limit of Detection and Limit of Quantitation

Matrix matched calibration curves were used to assess linearity of the ten carbamate analytes at seven different concentrations. A series of seven calibration standards were prepared at 100 ng/mL, 250 ng/mL, 500 ng/mL, 1 µg/mL, 1.5 µg/mL, 2 µg/mL and 2.5 µg/mL carbamate standard mixes in 50:50 MeOH:H_2O with 0.1% formic acid and in each of the different biological matrices obtained from healthy volunteers. The standards were processed as unknown samples and the final eluents made up to 100 µL and transferred to inserts in amber autosampler vials. The mobile phases used for the chromatography were 2 mM ammonium formate in LC grade water with 0.2% formic acid (Mobile phase A) and 2 mM ammonium formate in methanol with 0.2% formic acid (Mobile phase B). The matrix-matched calibration curves for each of the ten carbamate compounds were constructed using the TargetLynx (version 4.1) software with a 1/x weighting for the regression to compensate for the carbamate compounds at low concentrations.

2.8.2 Matrix effects and recovery

Matrix effect was evaluated by comparing the response and the ratio of peak areas of analytes spiked into biological samples before extraction with the response obtained from

the analytes at the same concentrations prepared in solvent but not processed through the sample preparation steps. Recovery was evaluated by comparing the mass spectrometric response of analytes spiked into a pre-extraction control matrix and analytes spiked into a post-extracted matrix.

2.9 LC-MS/MS method development and validation of a targeted quantitative method for aldicarb

Aldicarb-D3 stable isotope labelled internal standard (IS) (LGC-TRC A514652, Lot number: 7-JMR-163-1) was used for method validation and quantitative analysis of aldicarb concentrations in biological matrices and to assess the stability of aldicarb using a targeted analysis on a triple quadrupole mass spectrometer. Exactly 1,0 mg of the aldicarb-D3 standard was dissolved in 50:50 acetone:MeOH with 0.1% formic acid to make up 1,0 mg/mL in a 1,0 mL volumetric flask (Grade A). A working stock solution of the IS was made using 350 µL the IS solution with 27.375 g of MeOH by weight (equivalent to 34.65 mL) in a 50 mL volumetric flask. A volume of 75 µL of the IS stock solution was used to spike the samples to a final concentration of 0.75 µg/mL. Direct infusion using a 5 µg/mL solution of the aldicarb-D3 was done via a Harvard syringe pump into a triple quadrupole (Sciex 4000 QTrap) mass spectrometer (Table 2.7) at 10 µL/min to optimise the source and mass spectrometer parameters. Stock solutions were prepared by making up six-point calibration concentrations of 5, 10, 15, 20, 25, and 30 µg/mL. Analyte separation was performed on an Agilent Rapid Resolution XDB 4.6 x 50 mm, 1.8 µm column. Two mobile phase buffers were used, 2 mM Ammonium formate with 0.2% formic acid in H_2O (Mobile phase A) and MeOH (Mobile phase B) and 10 mM ammonium formate with 0.2% formic acid in H_2O (Mobile phase A); ACN with 0.1% formic acid (Mobile phase B) to compare chromatographic separation. The initial run time was 10 min but the final run time chosen was 8.5 min using the gradient shown in Table 2.8.

Table 2.7: Source parameters of the Sciex 4000 QTRAP mass spectrometry.

Source parameters	
Curtain plate	23
Declustering potential	40 V
Entrance potential	10 V
Collision energy	18 V medium gas flow
Desolvation temperature	550°C
IonSpray Voltage	+5500 V
Ion source gas 1	40
Ion source gas 2	36

Table 2.8: LC-MS/MS gradient elution method.

Time (min)	Flow Rate (mL/min)	Module	Event	Parameter
0.01	0.8	Pumps	Total A flow	0.8
0.25	0.8	Pumps	AB concentration	15
4.00	0.8	Pumps	AB concentration	70
4.75	0.8	Pumps	AB concentration	95
6.00	0.8	Pumps	AB concentration	95
6.25	0.8	Pumps	AB concentration	15
8.50	0.8	Pumps	AB concentration	15
8.51	0.8	Controller	Stop	

2.9.1 LC-MS/MS method validation for targeted analysis of spiked biological samples to assess aldicarb decay kinetics

2.9.1.1 Linearity, Limit of Detection and Limit of Quantitation

Calibration curves were assessed for linearity of aldicarb response at 7 different concentrations (0, 5,10,15,20,25,30 µg/mL). The limit of detection (LOD) was measured based on the lowest detectable concentration of the analytes with signal to noise (S/N) ratio of >3. The limit of quantification (LOQ) was measured based on the lowest concentration that could be quantified and reproducibly detected with a S/N ration >10. The matrix-matched standard derived calibration curves for aldicarb was constructed using the Analyst (version 1.5.2) software.

2.9.1.2 Accuracy and precision

Accuracy and precision of the method were determined by comparing the QC standards that were made up separately from the standards for the calibrations and replicate data of intra- and interday analyses. The intraday variability was assessed in both solvent and matrix matched samples by analysing batches of relevant standards injected in triplicate on the same day. Interday variability was assessed using triplicate injections of standards at two-day intervals using freshly made-up standards in both solvent and matrix.

2.9.1.3 Matrix effects and recovery

Matrix effect was evaluated by comparing the response and the ratio of peak areas of analytes spiked into biological samples before extraction with the response obtained from the analytes at the same concentrations prepared in solvent. Recovery was evaluated by comparing the mass spectrometric response of analytes spiked into a pre-extraction control matrix, which represented 100% recovery.

2.9.1.4 Stability assay

A pilot study was conducted to determine the time range to be used for the stability assays and to determine whether the carbamates accumulated in erythrocytes or became bound to plasma proteins. A 25 µg/mL concentration of aldicarb standard was spiked into ante-mortem (fresh) whole blood, pre-prepared plasma and urine collected from three healthy volunteers and stored at ambient temperature (left on a bench to mimic possible worst-case storage conditions) for the duration of the experiment. This was to determine the kinetics of chemical decay and was initially conducted for 28 days. Samples were collected into tubes containing EDTA or EDTA with NaF, for blood samples, whereas for urine collection the tubes contained either boric acid preservation or no additive. Samples were stored under four different storage conditions. Recovery, stability and matrix effects were assessed for the analytes.

2.10 Untargeted LC-MS/MS and UPLC-q-TOF-MS/MS method for screening of post-mortem biological samples and street pesticide positive control

Approximately 30 g of the unregulated street pesticides (black granules) were purchased from street vendors. Amounts of 10 g of each of the purchased pesticides were extracted using 50 mL of different solvents (acetonitrile, alkaline aqueous, chloroform, methanol, methanol/chloroform) in suitable test tubes to obtain extracts of the pesticide. The extracts were filtered through a 0.22 µm, 13,25 mm diameter PVDF syringe filters (Labotech, Midrand, South Africa) and 2 mL aliquots dried in a centrivap drier (Labconco, Corporation, Kansas City, MO). Each extract was reconstituted in 2 mL MeOH containing 0.1% formic acid for direct infusion into the mass spectrometer. Direct infusion was done via a Harvard syringe pump into a triple quadrupole (Sciex 4000QTrap) mass spectrometer to identify possible analytes contained within the street pesticide extracts in reference to the post-mortem samples. The source parameters are shown in Table 2.9.

The same solutions were analysed by a Shimadzu LC-MS/MS (operated in positive mode), using an Agilent Rapid Resolution XDB C18 4.6 x 50 mm, 1.8 µm column with 2 mM ammonium formate; 0.2% formic acid in water (Mobile phase A) and MeOH (Mobile phase B) or 10 mM ammonium formate with 0.2% formic acid in H_2O (Mobile phase A); ACN with 0.1% formic acid (Mobile phase B) set at a gradient elution as indicated in Table 2.10. The total run time was 10 min with an injection volume of 4 µL.

Analyte separation and detection of the street pesticide extracts were also performed on a Waters UPLC Synapt G2 q-TOF-MS/MS operated in positive (ESI+) mode at optimised source conditions (Table 2.11). The Acquity HSS T3, 2.1 x 100 mm, 1.8 µm column was used with the following mobile phases; 2 mM ammonium formate with 0.2% formic acid in water (Mobile phase A) and MeOH (Mobile phase B) with a gradient elution (Table 2.12). The column temperature was kept at 40°C and the sample tray temperature at 4°C. The total run time was 20 min which included an equilibration step of 2 min. The product ions retention time window was ±10 sec. The injection volume was 5 µL. The elemental composition reporting function of the MassLynx software program was used to produce a list of possible compounds for a given mass detected from the data independent

acquisition and possible chemical formulas used for the comparison of elemental isotope clusters in post-mortem samples to the spectrum and ranked by a fit confidence percentage.

Table 2.9: Source parameters of the Sciex 4000 QTRAP mass spectrometry.

Source parameters	
Curtain plate	23
Declustering potential	40 V
Entrance potential	10 V
Collision energy	25 V
Temperature	550°C
IonSpray Voltage	5500 V
Ion source gas 1	40
Ion source gas 2	36

Table 2.10: LC-MS/MS gradient elution flow.

Time (min)	Flow Rate (mL/min)	Module	Event	Parameter
0.01	1.000	Pumps	Total A flow	1
0.50	1.000	Pumps	AB concentration	15
6.00	1.000	Pumps	AB concentration	90
7.00	1.000	Pumps	AB concentration	90
7.25	1.000	Pumps	AB concentration	15
10.00	1.000	Pumps	AB concentration	15
10.02	1.000	Pumps	Stop	

Table 2.11: Waters SYNAPT-G2 q-TOF tune file setup.

Source parameters	
Capillary voltage (kV)	2.75
Desolvation gas temperature (°C)	300
Sampling cone voltage (V)	25
Cone gas flow (nitrogen L/hr)	10
ESI source temperature (°C)	120
Desolvation gas flow (nitrogen L/hr)	650
Extraction cone voltage (V)	2.5

Table 2.12: UPLC gradient elution method.

Time (min)	Flow Rate (mL/min)	%A	%B	Curve
Initial	0.350	90.0	10.0	Initial
0.10	0.350	90.0	10.0	6
15.00	0.350	5.0	95.0	6
16.40	0.350	5.0	95.0	6
18.00	0.350	90.0	10.0	6
20.00	0.350	90.0	10.0	6
Initial	0.350	90.0	10.0	Initial

2.11 Statistics

Raw mass spectrometer data was stored in electronic format on DVD or external hard drives and appropriately labelled for long term storage and included a record of all methodology and analysis parameters. Raw data was exported in csv format and captured using Microsoft Excel (Microsoft) and statistical analyses performed with GraphPad Prism 8.0 (GraphPad Software, San Diego California USA). Quantitative data was expressed as mean ± standard error of the mean (SEM) with a minimum of three separate samples analysed per condition. Data analysis was done using the non-parametric Kruskal-Wallis test. To describe the relationship between variables, the Tukey's multiple comparisons of 2-way ANOVA test was used. Where the data failed to meet the criteria for the paired t-test, the Wilcoxon's signed rank test was carried out. Multiple pairwise comparisons were carried out using the critical p-value of 0.05/n for statistical significance, where n is the number of comparisons attempted. Statistical analysis was done with the assistance of the statistician from the University of Pretoria.

3 Results and discussion

3.1 UPLC-q-TOF-MS chromatography method optimisation

Method optimisation for the ten carbamate compounds was achieved by adjusting chromatographic separation of the ten reference carbamate compounds by using different mobile phases and different vendors reverse phase columns changing gradient conditions while using optimised mass spectrometer parameters. Obtaining the optimised chromatographic conditions started by testing several analytical columns and solvents to select best selectivity and resolution of the analytes.

During identification of the carbamate pesticide in post-mortem samples by UPLC-q-TOF/MS, the choice of both stationary and mobile phase is critical as they influence separation and mass spectrometric response of the compounds. This is important for optimal detection and quantitation during screening in suspected poisoning cases. Figures 3.1 - 3.4 depict extracted ion chromatograms of the ten carbamates in the standard mix (Table 3.1) at a concentration of 1 µg/mL each for the given chromatographic conditions on four different stationary phase column chemistries.

The Acquity HSS T3 column gave better retention and separation of the small more polar hydrophilic carbamate compounds. Improved peak shape was observed when the stationary phases were paired with methanol containing 10 mM ammonium acetate, however, peak intensities remained low, possibly as a result of ionisation suppression. This apparent reduced ionisation could be improved using lower buffer strength in the mobile phases. The advantage of better resolution and separation outweighed the slightly longer run times found with methanol in comparison to acetonitrile. Mobile phases containing 2 mM ammonium formate with 0.2% formic acid in water (Mobile phase A) and 2 mM ammonium formate with 0.2% formic acid in methanol (Mobile phase B) showed increased sensitivity while maintaining chromatographic separation and low peak tailing. Good resolution of the ten carbamate compounds was observed with the final chosen method using an Acquity HSS T3 column when paired with these chromatographic mobile phase conditions (Figure 3.4). 4-Bromo-3,5-dimethylphenyl N-methyl carbamate used as

internal standard proved to be an unstable compound as very poor recovery was obtained.

Table 3.1: Carbamate analytes in order of elution.

Analyte elution order on an Acquity HSS T3 column	
1	Aldicarb sulfoxide
2	Aldicarb sulfone
3	Oxamyl
4	Methomyl
5	3-Hydroxycarbofuran
6	Aldicarb
7	Propoxur
8	Carbofuran
9	Carbaryl
10	Methiocarb

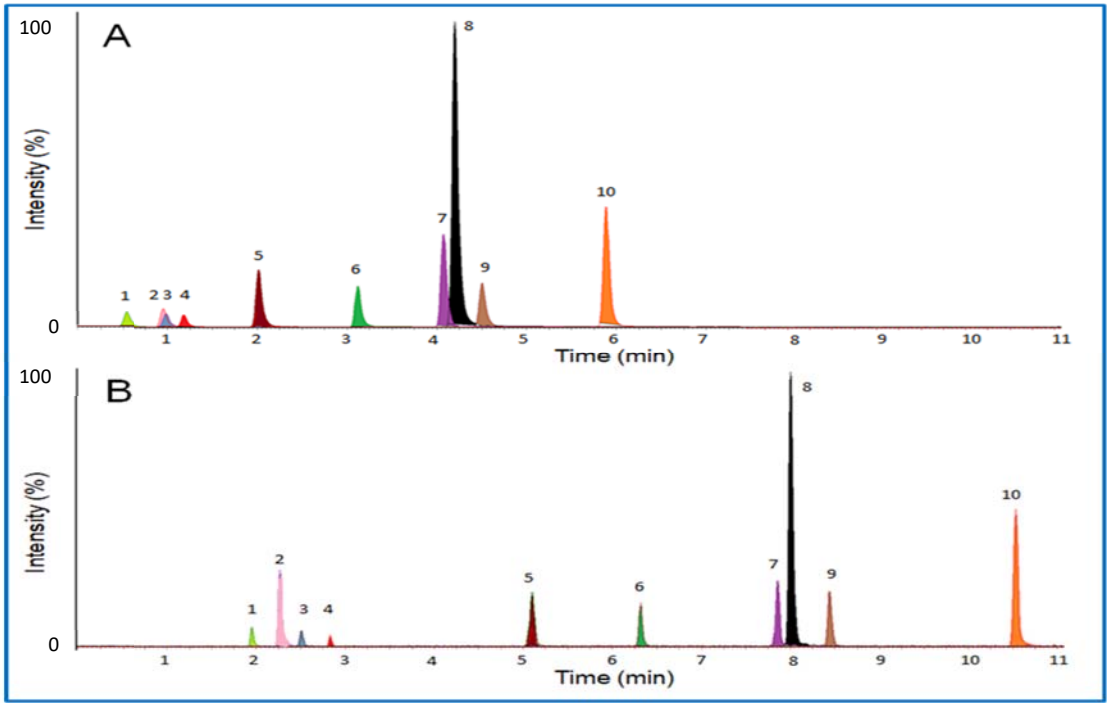


Figure 3.1: Extracted ion chromatograms comparing separation on an Acquity HSS T3 (2.1 x 100 mm, 1.8 µm) column with (A) acetonitrile and (B) methanol. [Refer to Table 3.1 for analyte identities]

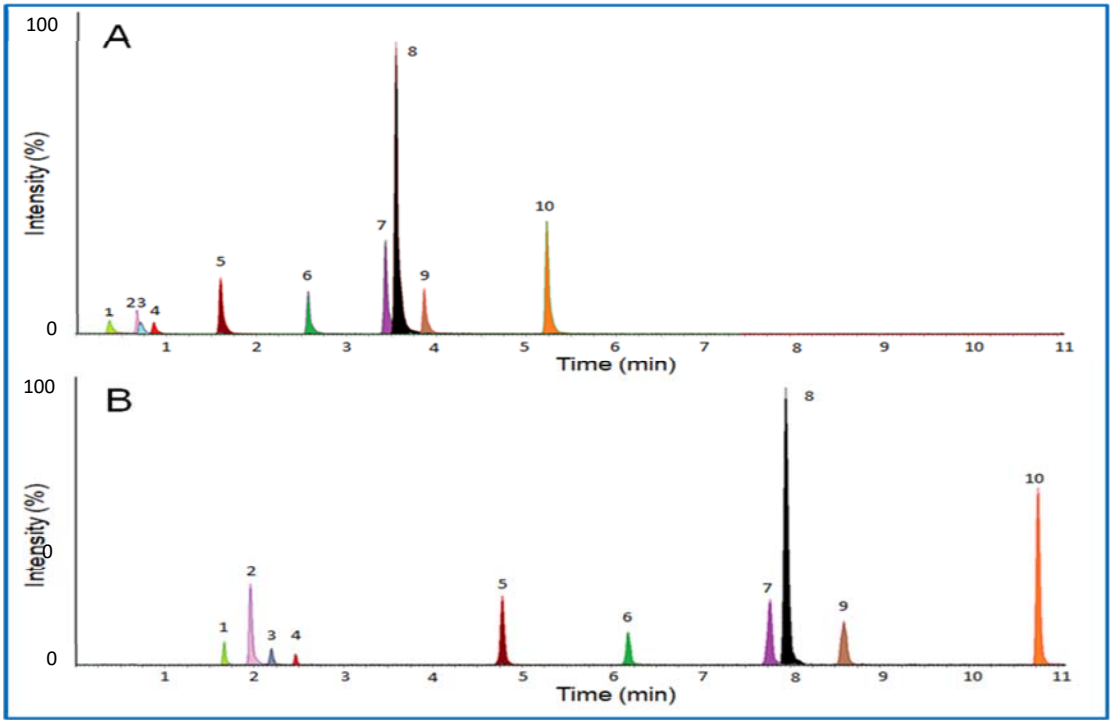


Figure 3.2: Extracted ion chromatograms comparing separation on an Acquity BEH C18 (2.1 x 100 mm, 1.7 µm) column with (A) acetonitrile and (B) methanol. [Refer to Table 3.1 for analyte identities]

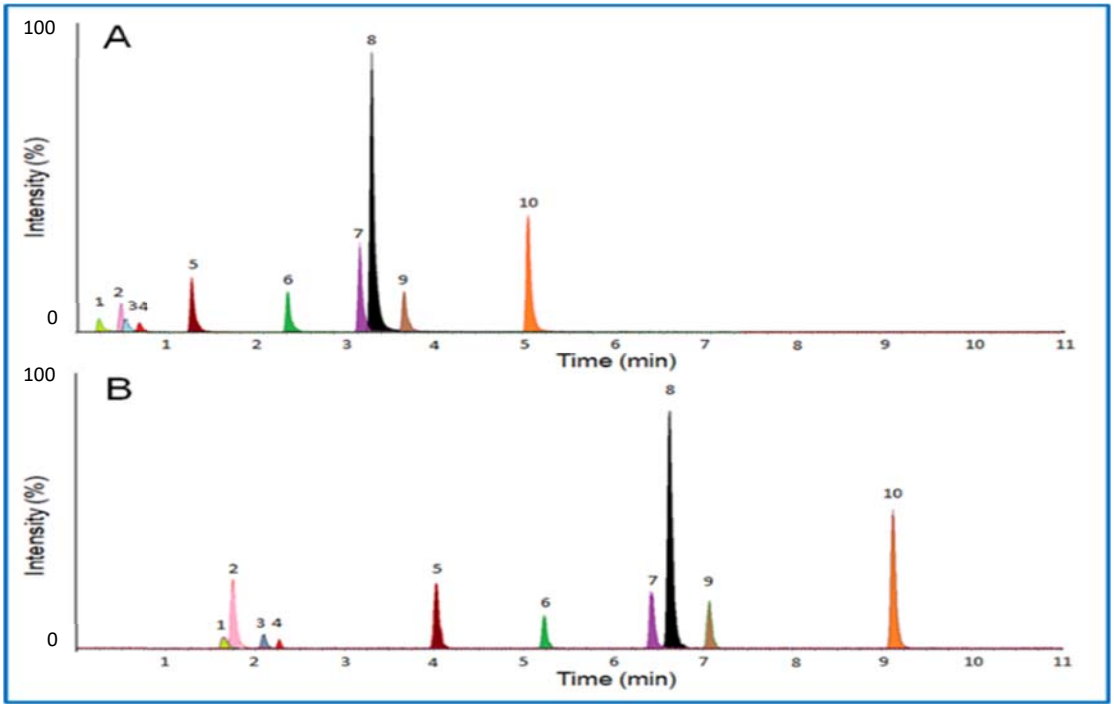


Figure 3.3: Extracted ion chromatograms comparing separation on a RESTEK Raptor ARC -18 (2.1 x 100 mm, 2.7 µm) column with (A) acetonitrile and (B) methanol. [Refer to Table 3.1 for analyte identities]

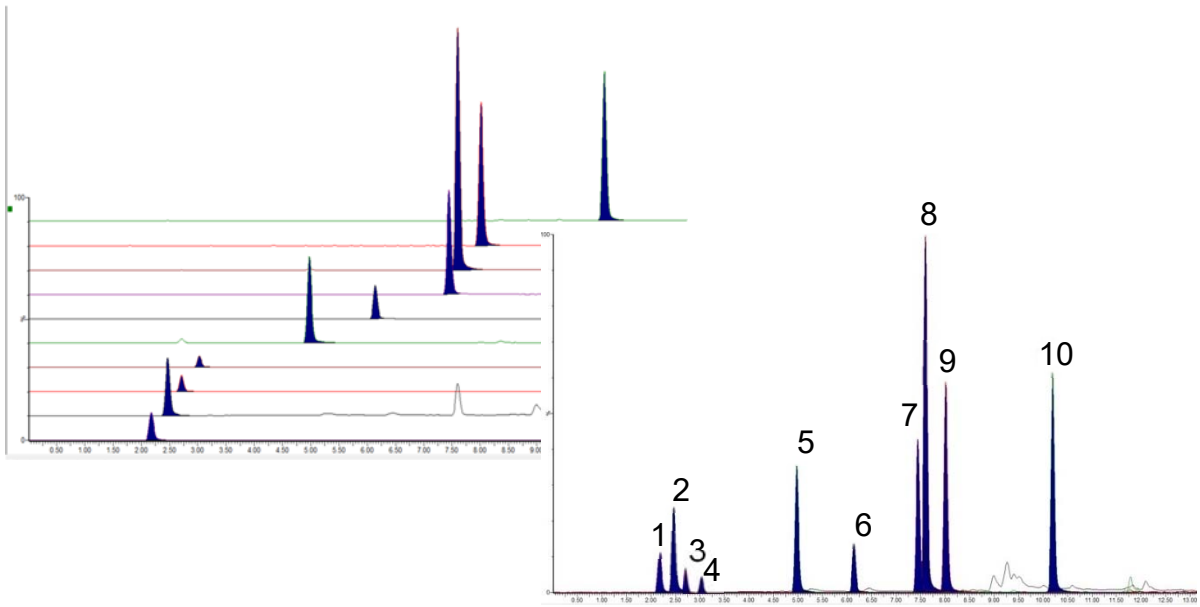


Figure 3.4: Extracted ion chromatograms of the final optimised chromatographic method showing separation on an Acquity HSS T3 (2.1 x 100 mm, 1.8 µm) column using 2 mM ammonium formate with 0.2% formic acid in water and methanol. [Refer to Table 3.1 for analyte identities]

Comparing the three columns used in this study, the best analyte resolution was achieved using the Acquity HSS T3 2.1 x 100 mm, 1.8 µm column. This column gave a shorter run time and lower back pressure changes during the solvent gradient. Regarding the comparison of mobile phase buffers used; 10 mM ammonium acetate as a mobile phase additive resulted in the best peak shape and resolution, but the intensity of the peak signal was low. Better peak symmetry was observed when methanol was used as an organic phase compared to acetonitrile. The presence of formic acid in the mobile phases increased mass spectrometer responses. After testing several mobile phases, 2 mM ammonium formate showed good chromatographic separation and peak parameters for all analytes. These results corroborated with published literature where, Rezende *et al.*[95] and Gao *et al.*[95,111] used mobile phase buffers containing 10 mM ammonium acetate with 0.1% formic acid. It was observed that methanol gave better separation of the analytes due to stronger retention, compared to acetonitrile, with added formic acid increasing detector sensitivity. Mobile phases combining ammonium formate and formic acid gave good analyte separation parameters for the tested analytes.[95,112]

3.2 UPLC-q-TOF-MS/MS SPE method for targeted analysis of spiked biological samples to assess decay kinetics

This SPE method showed effective extraction of the analytes as some compounds were detected at high intensity in different cartridges. The SepPak C18 1 cc cartridge resulted in the largest peaks areas followed by the Strata C18 cartridge. Only four out of ten compounds were eluted from three different SPE cartridges. The recovery data is summarised in Figure 3.5. Retention times of the detected compounds are summarised in Table 3.2.

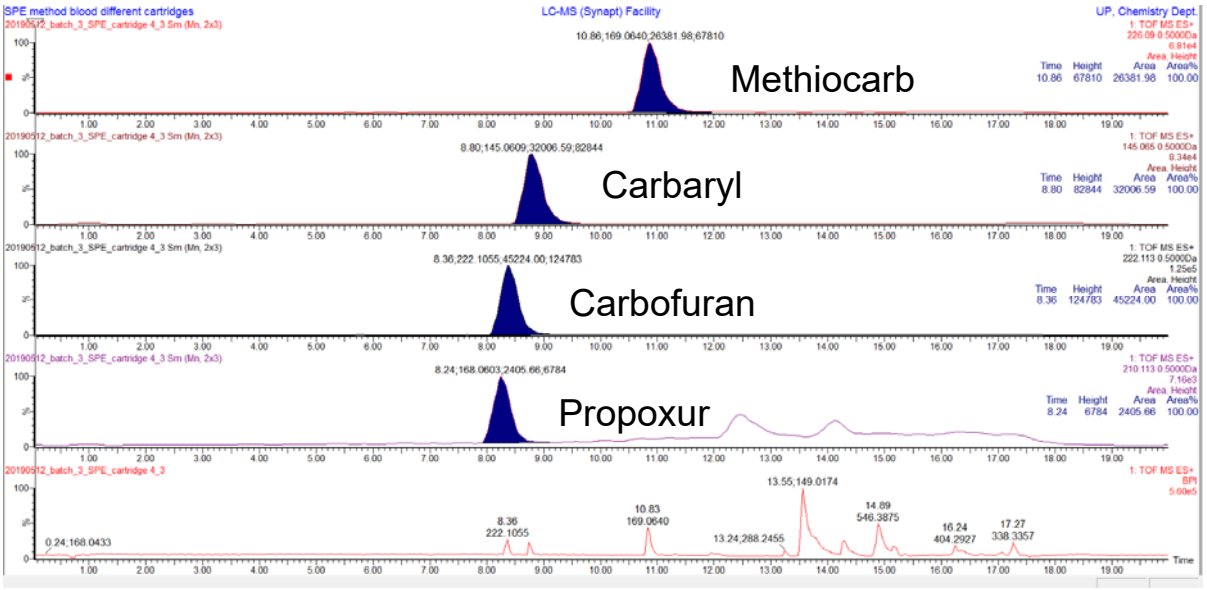


Figure 3.5: Integrated extracted ion chromatogram of the four carbamate compounds from the elution fraction of the SepPak C18 cartridge showing peak height and area.

Table 3.2: Dominant compounds extracted (elution fraction) using the different cartridges with their LC-MS/MS retention time.

Cartridge	Carbamate compounds detected	Retention time (min)
Oasis HLB prime	Carbofuran	8.40
	Carbaryl	8.36
	Methiocarb	10.86
Strata C8	Propoxur	8.24
	Carbofuran	8.36
	Carbaryl	8.80
	Methiocarb	10.86
Strata C18	Propoxur	8.24
	Carbofuran	8.36
	Carbaryl	8.80
	Methiocarb	10.86
SepPak C18	Propoxur	8.24
	Carbofuran	8.36
	Carbaryl	8.80
	Methiocarb	10.86

Selectivity is a quality of the combination of the stationary phase, mobile phase and analyte that can be achieved and measured using different solvent ratios and can be manipulated to achieve elution from different sample matrices without any interferences or co-eluting substances present in the sample.[111]

Chromatographic resolution of the ten carbamate compounds extracted from solvent could be achieved using a C18 reverse phase column. When comparing the four SPE cartridges used for analyte extraction, the highest intensity and symmetrical peaks were observed after extraction with SepPak C18 cartridges (Appendix VI). Constructing matrix matched calibration curves in the mentioned biological matrices was not without challenges. This may be due to the binding of carbamates to erythrocytes or phospholipids in biological samples which resulted in the compounds appearing as if there was lack of sensitivity in biological matrices. As a result, accurate quantification of the ten carbamate compounds in biological samples was not successful. With an added

re-equilibration time of 2 min to the analytical chromatographic method, no analyte retention time shifts were observed.

The effectiveness of the Oasis HLB and SepPak SPE cartridges in extraction and analysis of pesticides in biological samples is well documented.[99,102,113] In this study the standard SPE method recommended by the companies (conditioned and unconditioned cartridges) was not an accurate or reliable extraction method for 50% of the carbamate compounds. The recommended SPE method was only effective for five compounds; 3-hydroxycarbofuran, propoxur, carbofuran, carbaryl and methiocarb. Papoutsis *et al.*[22] reported that during the optimisation of the extraction procedure of pesticides in blood samples, unconditioned SPE cartridges were used. The recovery of several pesticides, especially organophosphates, such as azinphos methyl, dialifos, methamidophos, methidathion and methomyl was below 20%. The SPE proved not to be an effective extraction procedure.[22]

Extraction capacity refers to the amount of analyte an SPE cartridge can bind. Extraction capacity is an important factor to consider in developing SPE methods.[95] The reason being, that if the extraction capacity is too low, the targeted analytes will not be extracted from the matrix and if the extraction capacity is very high, unexpected co-elution of compounds may be observed.[95] In this study six organic solvents (90:10 ACN:IPA, 90% MeOH with 0.1% formic acid, 90% MeOH with 20 mM ammonium formate, pure MeOH and acetone, 90:10 ACN:MeOH and IPA) were investigated as elution solvents for the SPE method. These six solvents did not produce good recoveries. Pure acetone was evaluated as an elution solvent, testing the SPE eluate using direct infusion in the MS. Acetone yielded good recoveries as most of the compounds could be detected in the elution fraction (Figure 3.6).

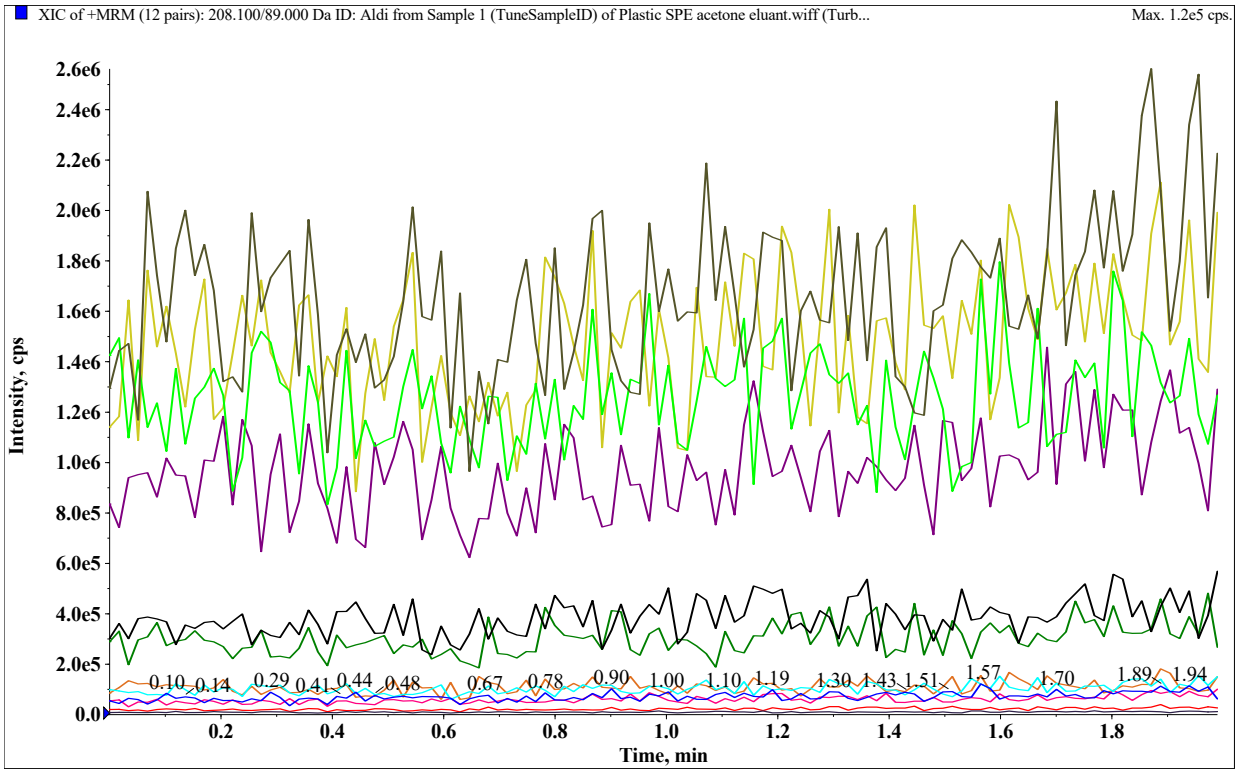


Figure 3.6: A two-minute direct infusion of a solvent based mixture of the ten carbamate compounds using the Sciex 4000 QTRAP MS/MS.

This finding showed the effect of the sample matrix on the analytes of interest. Matrix effect is evident when some matrix components are co-extracted with the analytes of interest, leading to the alteration of the signal response in the electrospray ionisation mass spectrometry, causing either ion suppression or enhancement. Matrix effects can also result in poor analytical reproducibility, accuracy and linearity.[114,115] Enhanced peak height and area was observed for 3-hydroxycarbofuran, propoxur, carbofuran, carbaryl and methiocarb. Therefore, no significant matrix interference was observed for the quantification of the aforementioned compounds. Matrix effects are also dependent on the characteristics of matrix components and the physicochemical properties of the pesticides.[5]

3.3 LC-MS/MS method development and validation of a targeted quantitative method for aldicarb

The aldicarb standard spiked in solvent was detected at a good intensity signal. The mobile phases with 10 mM ammonium formate; 0.2% formic acid in water and acetonitrile using an Agilent Rapid Resolution XDB 4.6 x 50 mm, 1.8 µm column showed good chromatographic retention of aldicarb which was well separated from the two oxidation products aldicarb sulfoxide and aldicarb sulfone (Figure 3.7).

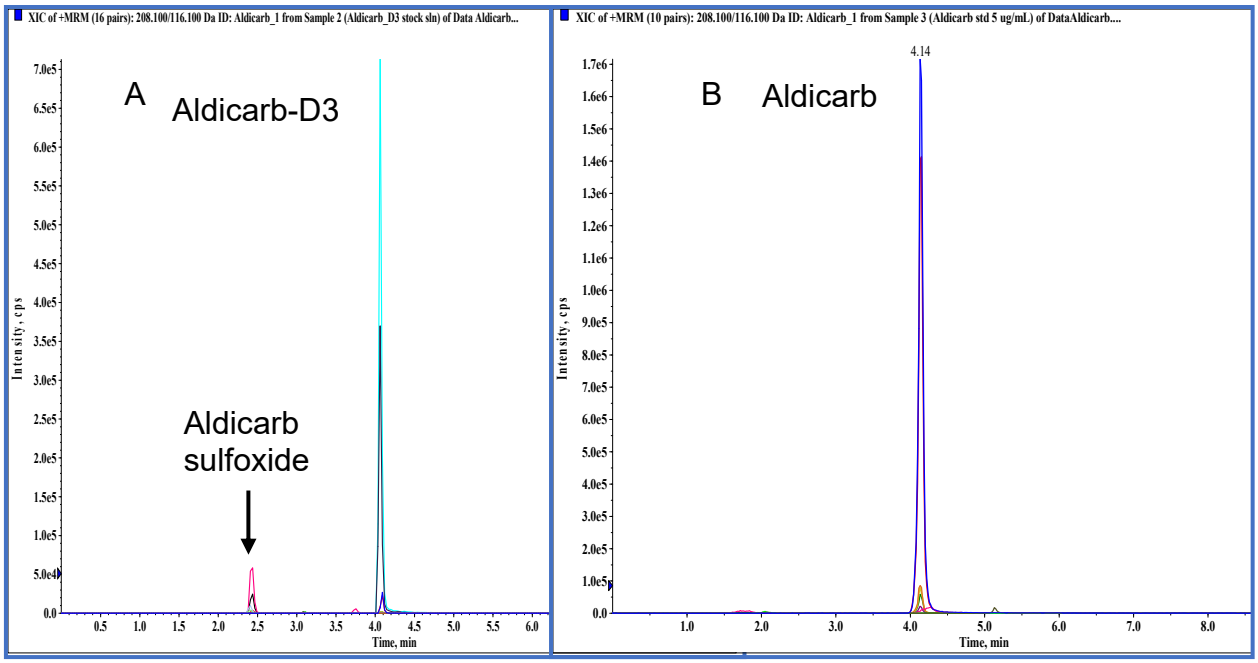


Figure 3.7: Chromatograms showing the elution of aldicarb on an Agilent RR XDB C18 4.6 x 50 mm, 1.8 µm column using 10 mM ammonium formate with 0.2% formic acid in H2O with MeOH with 0.1% formic acid. (A) Aldicarb-D3 (IS) and aldicarb sulfoxide; (B) Aldicarb.

The mobile phase and sharp gradient showed good separation and determination of compounds. It was observed that the increased acid concentration in the mobile phases improved peak tailing and sensitivity. Therefore, 10 mM ammonium formate with 0.2% formic acid and ACN with 0.2% formic acid was used and the aldicarb ammonium adduct ion targeted as the precursor ion. The results corroborated with Gao *et al.,* who observed that a mobile phase containing 10 mM ammonium formate showed good chromatographic

separation of polar carbamates with good peak symmetry.[95] The high acetonitrile wash improved retention time reproducibility for plasma and whole blood samples.

3.4 Protein precipitation method validation for targeted LC-MS/MS analysis of aldicarb spiked biological samples to assess decay kinetics

Serum and plasma were spiked with a concentration of 5 µg/mL of the aldicarb analyte before extraction to measure the recovery percentage from the matrix. Aldicarb and its common oxidation products (aldicarb sulfoxide and aldicarb sulfone) were detected in these samples (Figure 3.8). Aldicarb indicated a high recovery of approximately 100%. Good peak signal and separation was also obtained from the different matrices.

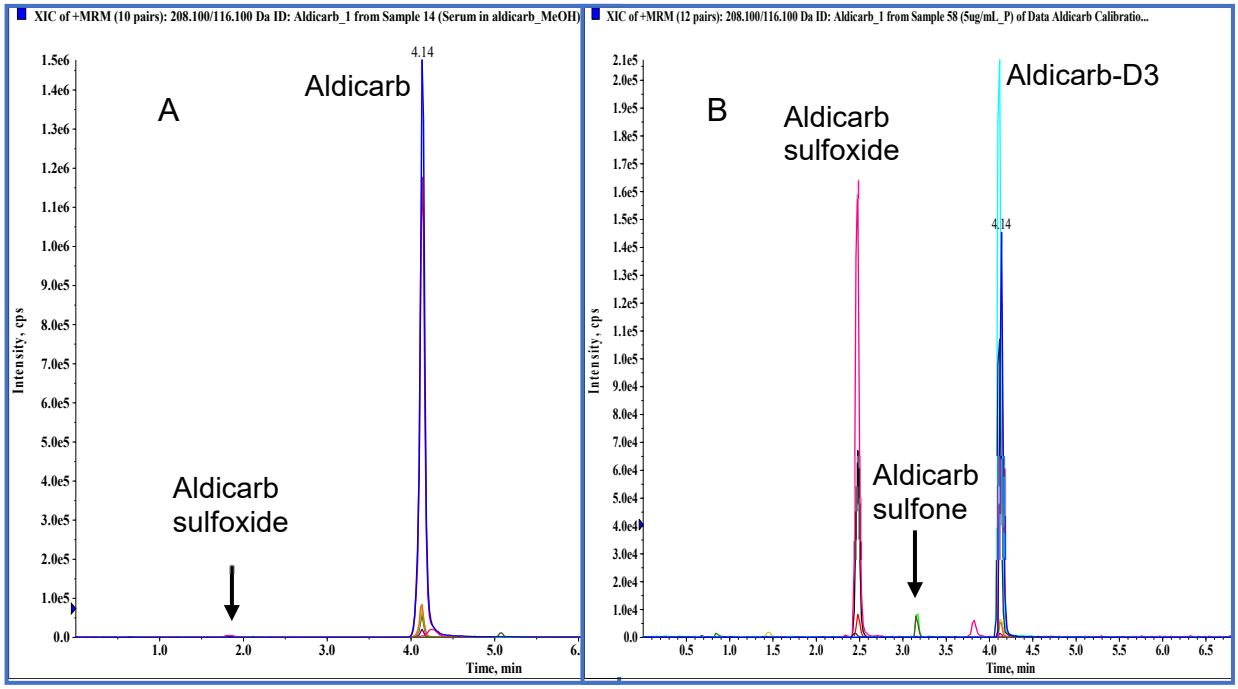


Figure 3.8: Chromatograms showing (A) Spiked serum with aldicarb at 5 µg/mL concentration; (B) Plasma spiked with aldicarb (5 µg/mL) and IS. Aldicarb break down products are also observed.

The stepwise protein precipitation method using methanol as a solvent, provided efficient protein precipitation efficiency and approximately 100% recovery. Mikayelyan *et al.*[116] and

Cohier *et al.*[117] reported that performing protein precipitation method followed by LC-MS/MS proved to be an efficient and sensitive method for the detection of clobazam in human plasma.[116,117]

3.4.1 Linearity, LOD and LOQ

Linear calibration curves were obtained for aldicarb spiked in solvent as well as biological samples, additionally with good precision when comparing triplicate analyses of the standards. The linear regression equation (LRE) and correlation coefficient (r) is presented in Table 3.3 and Appendix IV.

Table 3.3: Linear through zero regression and correlation coefficient of the calibration curves of aldicarb, aldicarb sulfone and sulfoxide obtained in plasma.

Compound	r	LRE
Aldicarb	0.9975	y=0.123x
Aldicarb sulfone	0.9919	y=0.402x
Aldicarb sulfoxide	0.9975	y=0.595x

LRE = linear regression equation; r = correlation coefficient

Linear best fit lines passing through the origin were obtained when plotting the ratio of analyte to internal standard peak areas versus the analyte concentration for aldicarb. This concentration range was spiked into plasma for analysis of spiked samples as a part of the stability experiment. The lowest proven limit of quantification in this study was 5 µg/mL with a signal to noise ratio of ≥ 10.

3.4.2 Accuracy and precision

Accuracy was expressed as the mean measured concentration × 100 or percent bias, using the calibration curves of the analyte. Precision was expressed as the relative standard deviation. Precision and accuracy data of aldicarb, and its metabolites (aldicarb sulfone and sulfoxide) is presented in Tables 3.4 and 3.5, respectively.

Table 3.4: Precision and accuracy data of triplicate analyses of aldicarb in solvent and plasma.

Aldicarb concentration (µg/mL) in solvent											
5		10		15		20		25		30	
PRE %	ACC %	PRE %	ACC %	PRE %	ACC %	PRE %	ACC %	PRE %	ACC %	PRE %	ACC %
7,2	**104,3**	**2,8**	**101,5**	**6,9**	**104,5**	**9,6**	**98,1**	**7,1**	**96,8**	**4,9**	**94,9**
Aldicarb concentration (µg/mL) in plasma											
5		10		15		20		25		30	
PRE %	ACC %	PRE %	ACC %	PRE %	ACC %	PRE %	ACC %	PRE %	ACC %	PRE %	ACC %
3,7	**110,8**	**2,1**	**103,6**	**7,4**	**104,1**	**4,2**	**98,7**	**3,4**	**103,4**	**3,1**	**104,0**

Table 3.5: Precision and accuracy data of triplicate analyses of aldicarb sulfone and aldicarb sulfoxide in solvent.

Aldicarb sulfone concentration (µg/mL) in solvent											
5		10		15		20		25		30	
PRE %	ACC %	PRE %	ACC %	PRE %	ACC %	PRE %	ACC %	PRE %	ACC %	PRE %	ACC %
5,5	**96,4**	**7,2**	**119,9**	**4,4**	**109,3**	**1,5**	**107,6**	**6,6**	**107,9**	**10,7**	**86,3**
Aldicarb sulfoxide concentration (µg/mL) in solvent											
5		10		15		20		25		30	
PRE %	ACC %	PRE %	ACC %	PRE %	ACC %	PRE %	ACC %	PRE %	ACC %	PRE %	ACC %
6,3	**101,0**	**3.9**	**106,2**	**1,0**	**105,4**	**4,6**	**104,7**	**2,1**	**102,8**	**3,5**	**93,8**

PRE % = Precision, ACC % = Accuracy

3.4.3 Stability assay

3.4.3.1 Whole blood

The concentrations of aldicarb and its oxidation products (aldicarb sulfone and aldicarb sulfoxide) spiked into whole blood on Day 1 were compared to those of samples stored for 3, 7, 14, 21 and 28 days at each of four different storage temperatures. Spiked whole blood stored at room temperature (19°C) in EDTA blood tubes showed significant ($P \leq 0.0001$) decreases in the aldicarb concentration from Day 1 through to Day 28 (where a loss of approximately 85% was evident) (Figure 3.9). Associated with the decreasing aldicarb concentration, a significant increase in the concentrations of aldicarb sulfoxide ($P \leq 0.05$) and aldicarb sulfone ($P \leq 0.001$) was observed (Figures 3.10 and 3.11).

The spiked whole blood stored in EDTA vacutainers at 4°C showed no significant decrease in aldicarb or aldicarb sulfone concentration, although significant ($P \leq 0.05$) increases were observed in the equivalent concentrations of aldicarb sulfoxide from Day 14 to Day 28.

In spiked whole blood stored in EDTA vacutainers at -20°C, there were no significant changes in the concentrations of aldicarb between the initial samples (Day 1) and those analysed up to Day 28. Significant differences ($P \leq 0.0001$) were observed between aldicarb sulfone and sulfoxide in the peak area between Day 1 and Day 7 and Day 14 and Day 28 (Figures 3.10 and 3.11).

In spiked whole blood, stored in EDTA vacutainers at -80°C, there were no significant changes in the concentrations of aldicarb between the initial samples (Day 1) and those analysed up to Day 28. Significant differences were observed in the concentrations of aldicarb sulfone ($P \leq 0.001$) and sulfoxide ($P \leq 0.01$) between Day 1 and Day 14 (~20% decrease). A significant difference was also observed in aldicarb sulfone ($P \leq 0.01$) and sulfoxide ($P \leq 0.001$) between Day 14 and Day 28 (Figures 3.10 and 3.11).

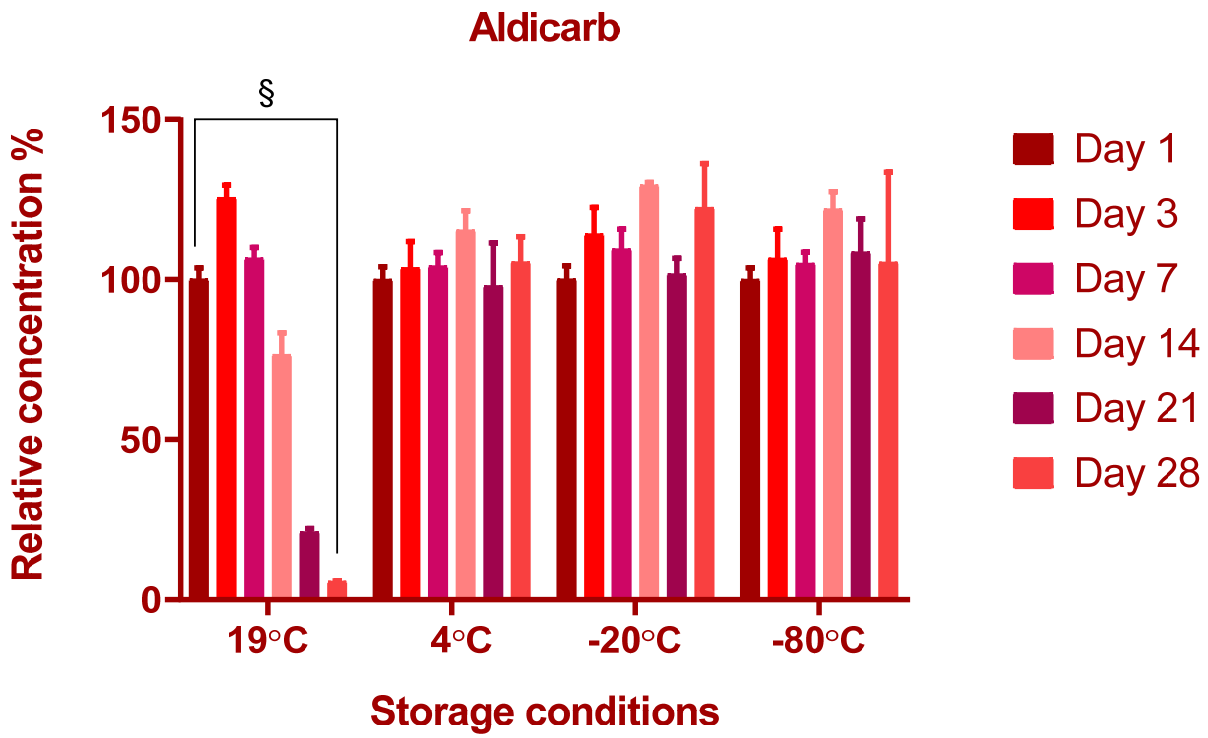


Figure 3.9: A bar graph showing the normalised effect of storage temperature on aldicarb concentration in whole blood preserved with EDTA. § = P ≤ 0.0001. Statistical significance was calculated using 2-way ANOVA with Tukey's test.

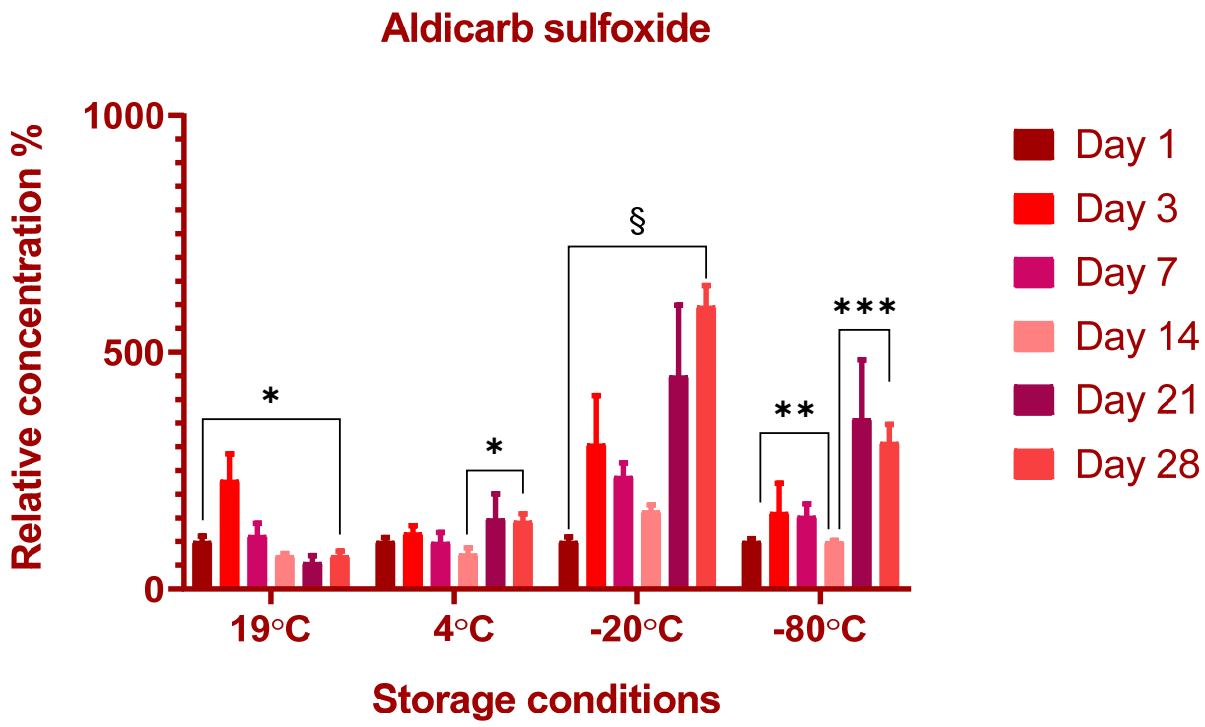


Figure 3.10: Bar graph showing the normalised effect of storage temperature on relative aldicarb sulfoxide concentration in whole blood preserved with EDTA. Day 1 represents 100% * = P ≤ 0.05, ** = P ≤ 0.01, *** = P ≤ 0.001, § = P ≤ 0.0001. Statistical significance was calculated using 2-way ANOVA with Tukey's test.

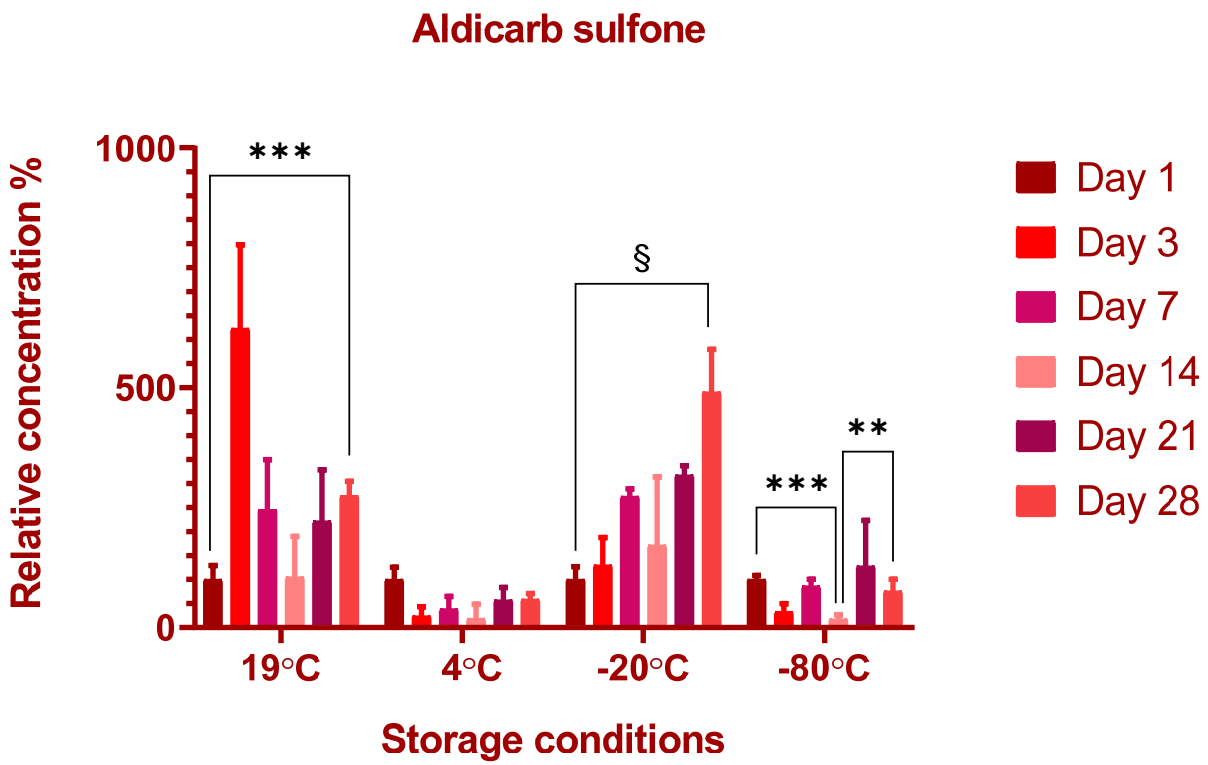


Figure 3.11: Bar graphs showing the normalised effect of storage temperature on aldicarb sulfone concentration in blood preserved with EDTA. ** = P ≤ 0.01, *** = P ≤ 0.001, § = P ≤ 0.0001. Statistical significance was calculated using 2-way ANOVA with Tukey's test.

The two oxidation products of aldicarb were added to the analysis as the oxidation reaction has been reported to occur spontaneously in dilute solutions of aldicarb. The two oxidation products were detected in low concentrations in the spiked whole blood stored in EDTA at room temperature where the percentage decrease in aldicarb was approximately 85%. Aldicarb appeared to be stable when stored at -80°C. Papoutsis *et al.*[22] determined the stability of 11 pesticides in whole blood spiked with EDTA. The samples were stored at 4°C for 3 days and at -20°C for a week. The most unstable pesticide compounds were found to be the organophosphates, methamidophos (4°C) and malathion (freeze-thaw cycles) and the carbamate, carbofuran (-20°C).[22] A study on the stability of organophosphate insecticides in fresh blood, found that 12 of the 14 organophosphate compounds were unstable at room temperature over a 24 h period. At 4°C, the concentration of seven compounds decreased by approximately 10-14%.[90]

Additionally, Júnior *et al.*[118] determined the stability of drugs and pesticides in post-mortem blood samples (without any preservative) at 25 ± 5°C after a period of 48 h. The analytes which included carbaryl, carbofuran and methiocarb were deemed stable with ≥ 80% of the analyte still being detectable after 48 h.

Aldicarb in spiked whole blood, stored in NaF containing tubes at room temperature, were shown to be unstable, with concentrations decreasing by approximately 60% from Day 1 to Day 28 (Figures 3.12). Aldicarb showed a similar gradient of degradation (~ 20%) at 4°C, -20°C and -80°C. (Figure 3.12).

Aldicarb sulfoxide showed no significant change in concentration between Day 1 and Day 28 at 19°C (Figure 3.13). Concentration increases in aldicarb sulfoxide, were also noted in blood stored in tubes containing NaF and stored at 4°C ($P \leq 0.001$), -20°C and -80°C ($P \leq 0.0001$) , between Day 14 versus Day 28. (Figure 3.13). Aldicarb sulfone was also not stable in blood stored in NaF containing tubes at the four temperature conditions. Significant difference ($P \leq 0.0001$) in aldicarb sulfone concentration was observed when stored at the four different temperatures (Figure 3.14).

Significant ($P \leq 0.01$) decreases in concentrations for especially aldicarb in blood stored in vacutainer tubes containing NaF were found after 28 days, irrespective of storage temperature. This result implies that carbamate compounds degrade more rapidly in specimen containers containing NaF, compared to EDTA. Ethylenediamine tetra acetic acid has been reported to prevent the loss or degradation of some organophosphorus compounds.[22]

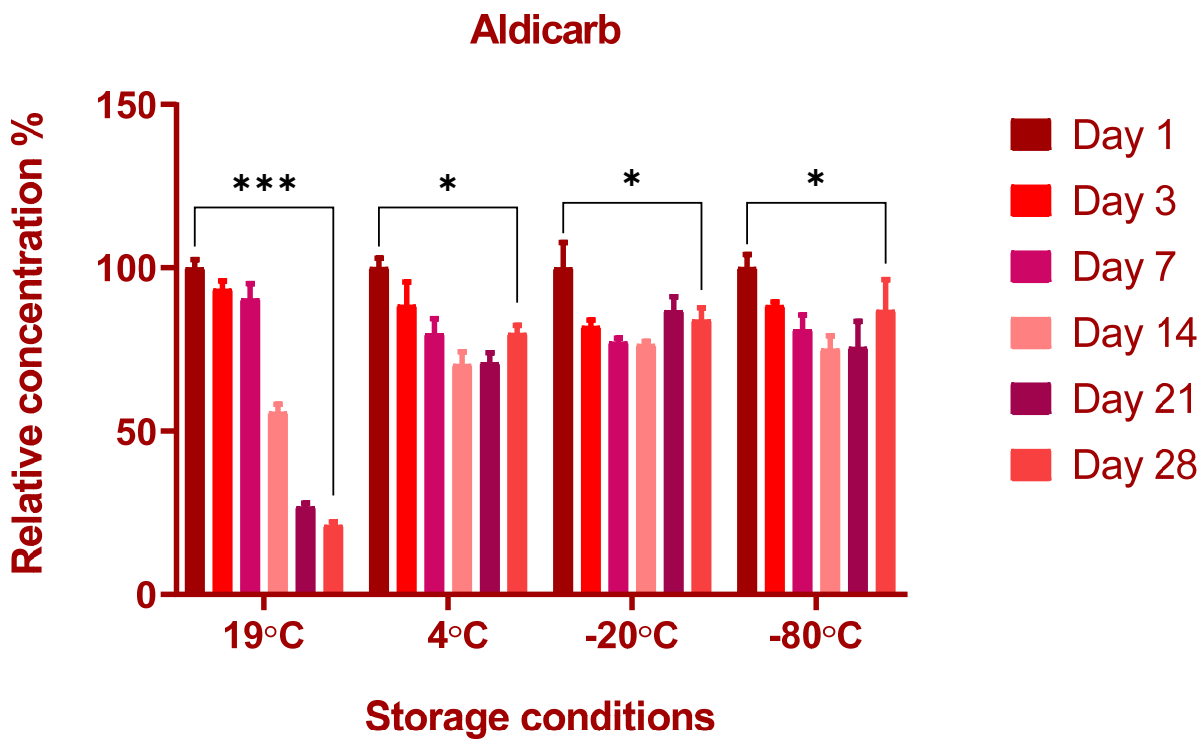


Figure 3.12: Bar graphs showing the normalised effect of NaF on aldicarb concentration in spiked whole blood. * = P≤ 0.05, *** = P ≤ 0.001. Statistical significance was calculated using 2-way ANOVA with Tukey's test.

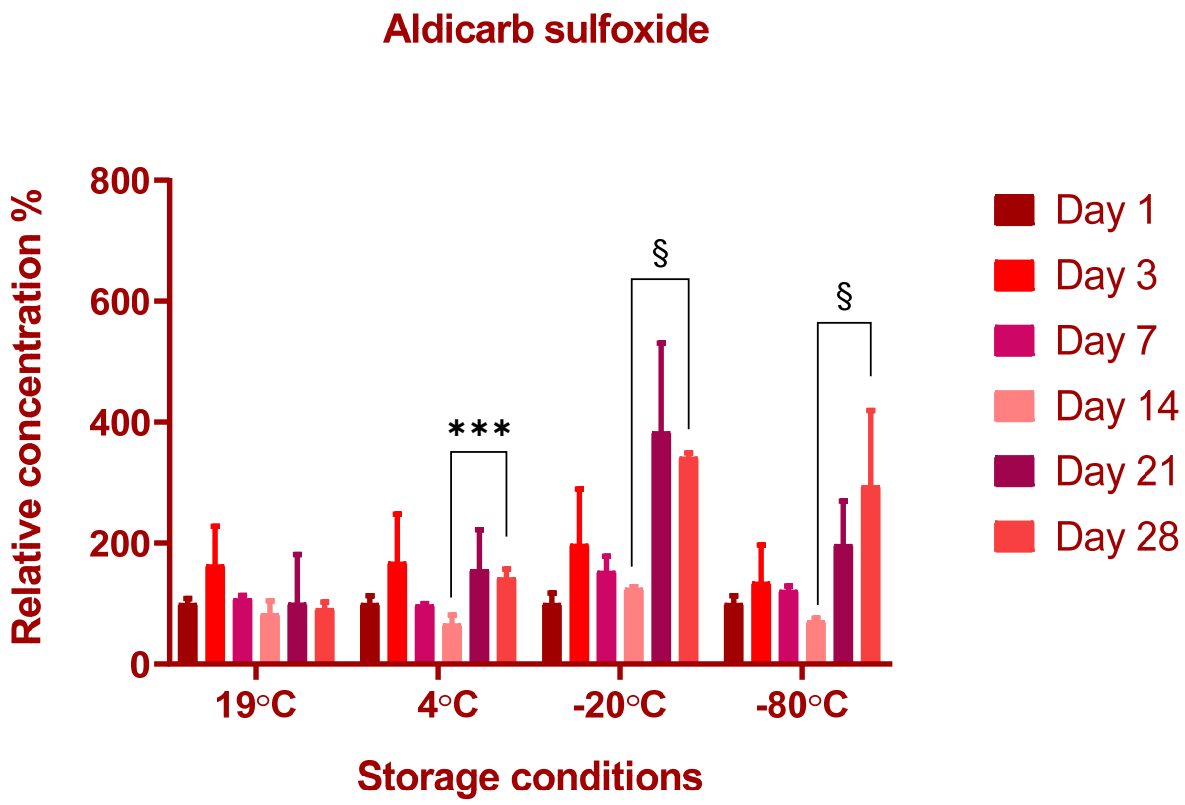


Figure 3.13: Bar graphs showing normalised changes in aldicarb sulfoxide concentration of whole blood spiked with aldicarb and stored in NaF containing tubes at different temperature conditions. *** = P ≤ 0.001, §= P ≤ 0.0001. Statistical significance was calculated using 2-way ANOVA with Tukey's test.

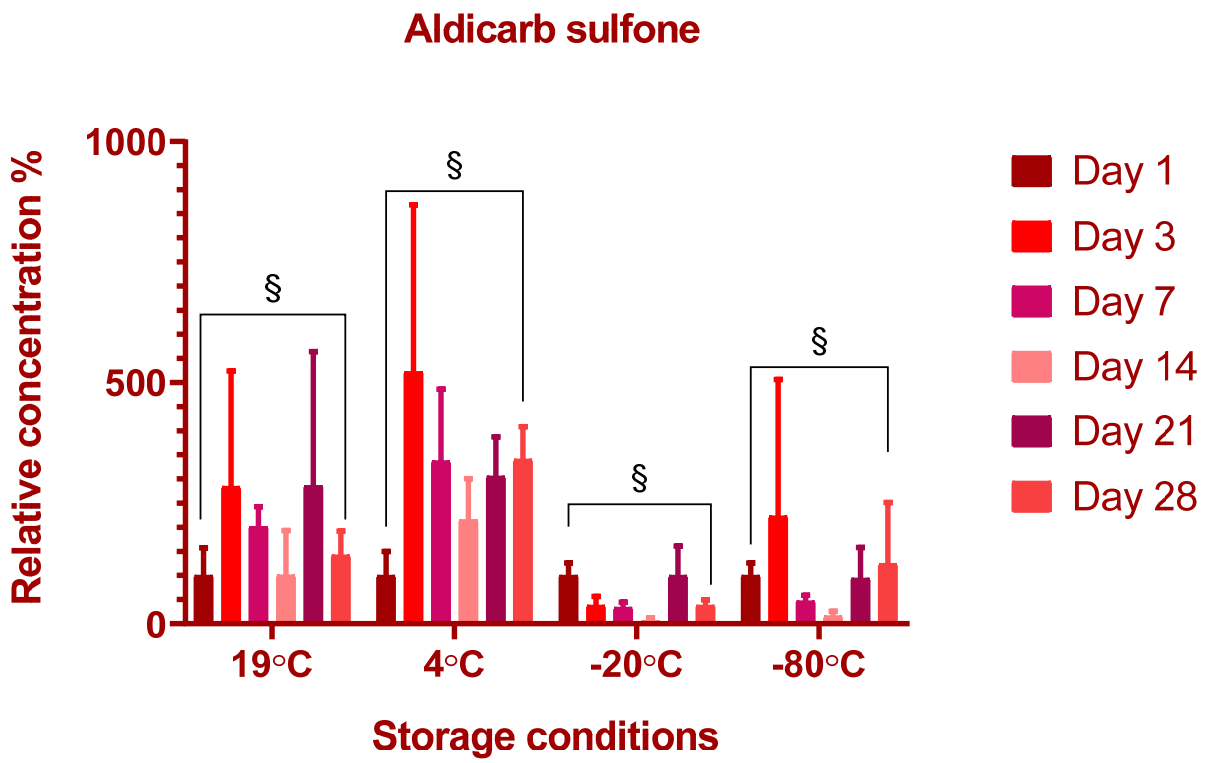


Figure 3.14: Bar graph showing the normalised changes in aldicarb sulfone concentration of whole blood spiked with aldicarb and stored in NaF containing tubes at different temperature conditions. §= P ≤ 0.0001. Statistical significance was calculated using 2-way ANOVA with Tukey's test.

3.4.3.2 Plasma

Degradation of aldicarb was also assessed in spiked plasma. There was no significant difference in aldicarb concentrations between Day 1 and Day 28 with regards to the four temperature conditions (Figure 3.15), when plasma was stored in EDTA. A significant ($P \leq 0.0001$) increase of ~50%, was noted for the peak area of aldicarb sulfoxide after Day 28 compared to Day 1 in plasma samples stored in vacutainer blood tubes containing EDTA the four different temperature conditions (Figure 3.16). Aldicarb sulfone showed a significant difference between Day 1 and Day 7 ($P \leq 0.01$) and Day 14 to Day 28 ($P \leq 0.001$), at both 19°C and 4°C. Aldicarb sulfone appeared to be unstable in plasma samples stored in vacutainer tubes containing EDTA at -20°C and -80°C. There was a significant difference ($P \leq 0.0001$) between Day 1 and day 14 as well as Day 14 and Day 28 (Figure 3.17).

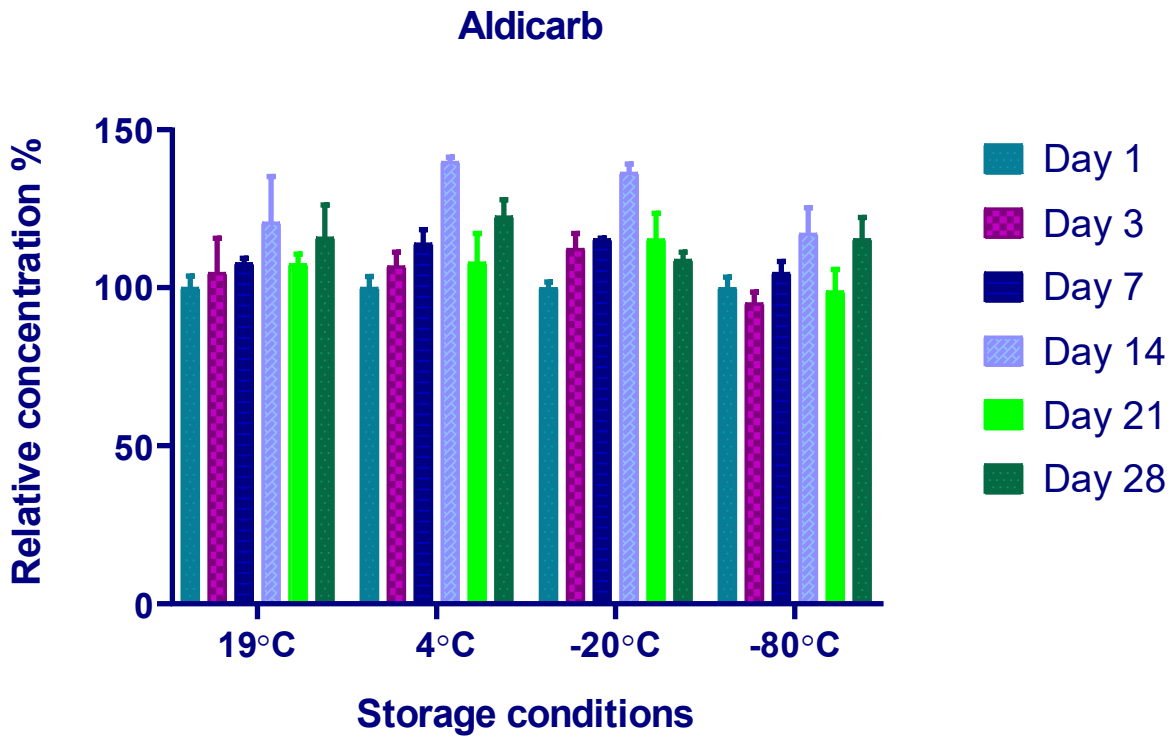


Figure 3.15: Bar graph showing the normalised effect of storage temperature on aldicarb concentration in plasma spiked with aldicarb and containing EDTA. Statistical significance was calculated using the 2-way ANOVA with Tukey's test.

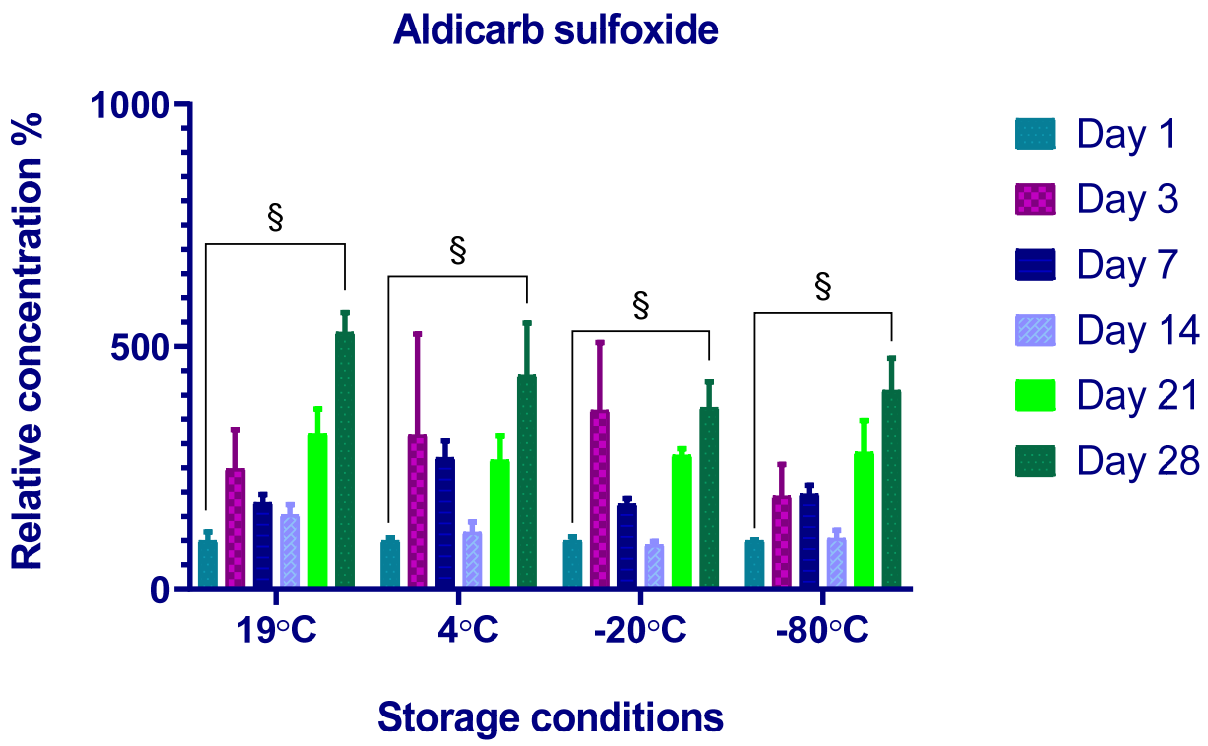


Figure 3.16: Bar graph showing the normalised effect of storage temperature on aldicarb sulfoxide concentration in aldicarb spiked plasma containing EDTA. § = P ≤ 0.0001. Statistical significance was calculated using 2-way ANOVA with Tukey's test.

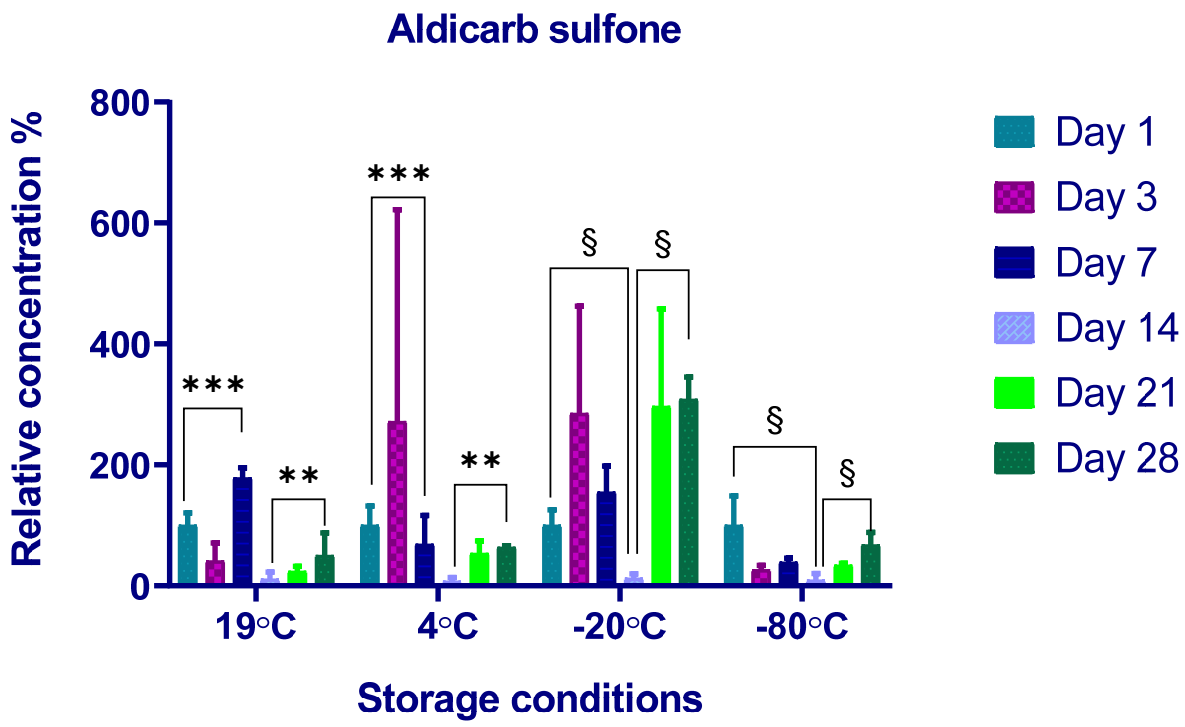


Figure 3.17: Bar graph showing the normalised effect of storage temperature on spiked aldicarb sulfone concentration in aldicarb spiked plasma containing EDTA. ** = P ≤ 0.01, *** = P ≤ 0.001, § = P ≤ 0.0001. Statistical significance was calculated using 2-way ANOVA with Tukey's test.

Plasma spiked with aldicarb and stored in vacutainer blood tubes containing NaF showed no significant difference when stored at the four temperature conditions (Figure 3.18). An increase in concentrations (P ≤ 0.0001) of aldicarb sulfoxide in plasma containing NaF and stored at the four different temperature conditions was observed between Day 1 and Day 28 (Figure 3.19). There was on average a >10% degradation in aldicarb sulfone in plasma stored in tubes containing NaF for the four storage temperatures from Day 1 to Day 28 when measured as peak area (Figure 3.20). A similar gradient was observed for each of the three carbamate compounds between the days of stability.

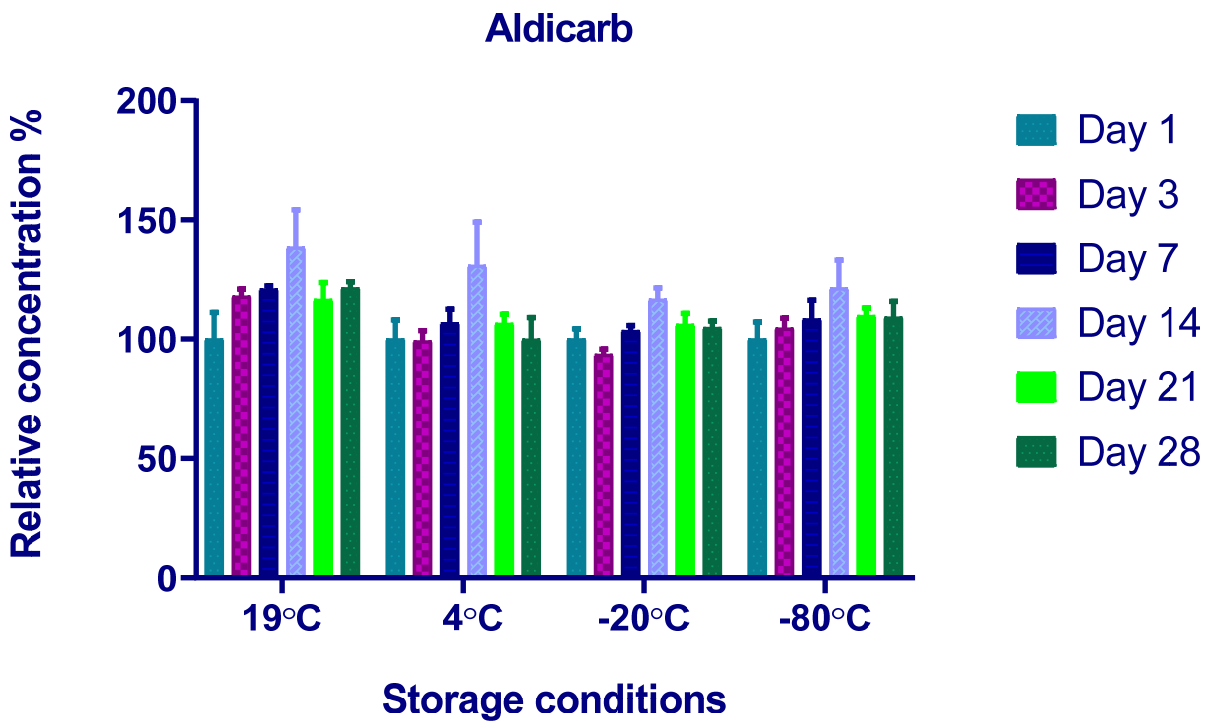


Figure 3.18: Bar graph showing the normalised effect of storage temperature on spiked concentration of aldicarb when stored in tubes containing NaF. Statistical significance was calculated using 2-way ANOVA with Tukey's test.

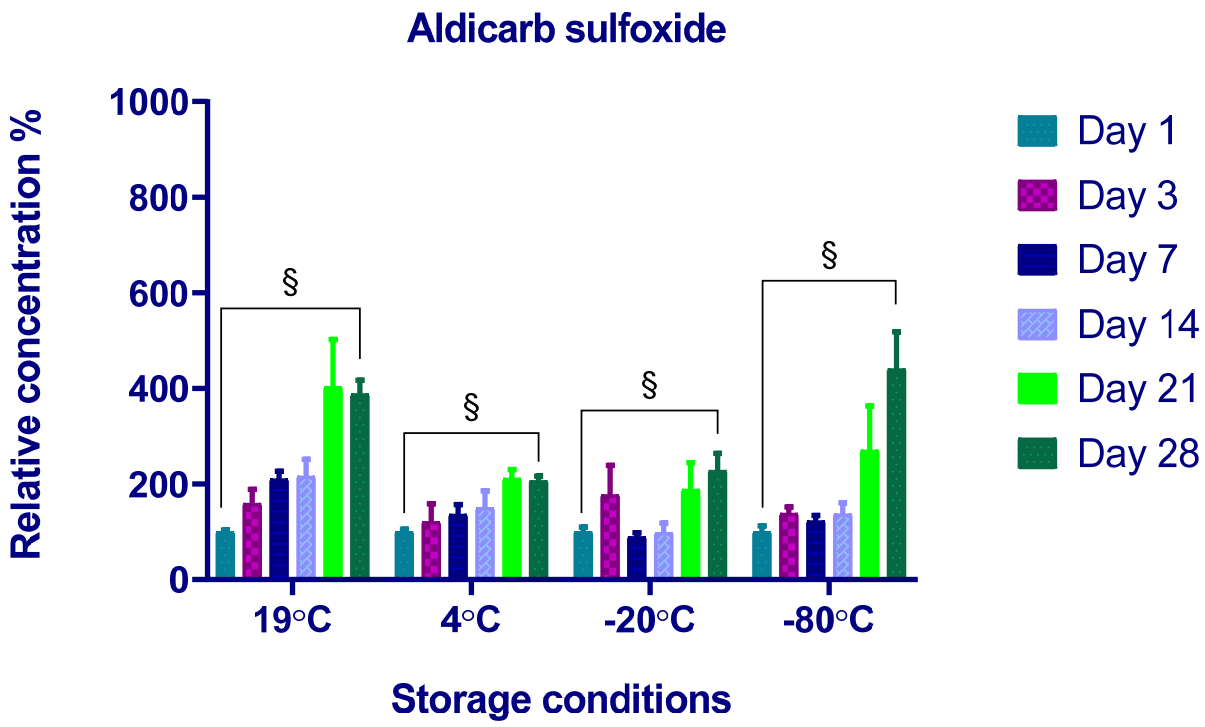


Figure 3.19: Bar graph showing the normalised effect of storage temperature on aldicarb sulfoxide concentration in aldicarb spiked plasma containing NaF. § = P ≤ 0.0001. Statistical significance was calculated using 2-way ANOVA with Tukey's test.

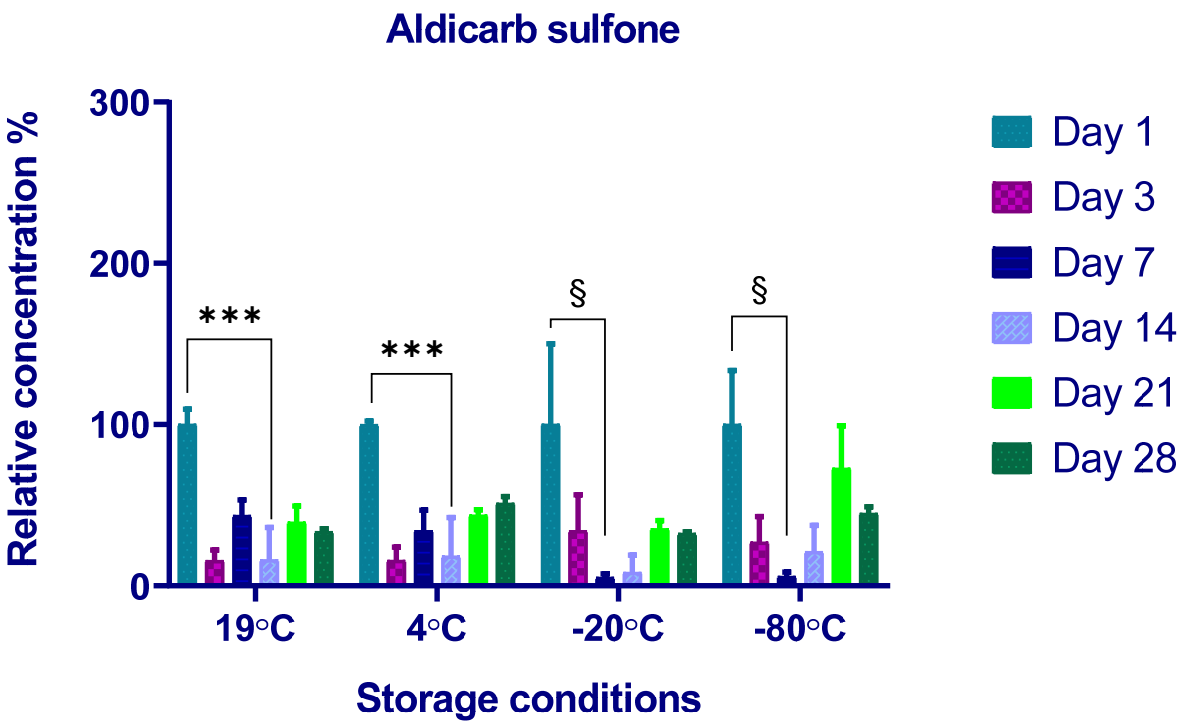


Figure 3.20: Bar graph showing the normalised effect of storage temperature on aldicarb sulfone concentration in aldicarb spiked plasma containing NaF. *** = P ≤ 0.001, § = P ≤ 0.0001. Statistical significance was calculated using 2-way ANOVA with Tukey's test.

From the results it was evident that plasma appeared to be a better matrix compared to whole blood with regards to the stability of aldicarb and its oxidation products. Ethylenediaminetetraacetic acid and NaF appeared to be a better plasma additive for aldicarb when stored at 19°C, 4°C, -20°C and -80°C. When assaying carbamate compounds, NaF appeared to promote carbamate instability especially in blood.

A factor to be considered during analysis is the freeze-thaw cycles of the matrix. In this study the samples had 4 freeze-thaw cycles. Pinto *et al.*[119] reported that EDTA was suitable for plasma collection. In a study on the stability of lipid components in plasma, after 5 freeze-thaw cycles indicated significant changes in these components. Furthermore, pesticides that are generally lipophilic are known to have a strong binding affinity for erythrocytes and lipids, therefore isolation of plasma or serum by centrifugation may result in grossly underestimated levels of pesticides.[22]

3.4.3.3 Urine

Aliquots of urine were stored in containers with and without boric acid as a preservative. Urine stored in boric acid showed no significant difference in aldicarb concentration over the 28-day storage period when stored under any of the four temperature conditions (Figure 3.21). There was a significant ($P \leq 0.0001$) difference in aldicarb sulfoxide concentration between Day 1 and Day 28 for all four of the storage conditions (Figure 3.22). The concentrations of aldicarb sulfone in urine stored in boric acid at 19°C and -20°C decreased significantly ($P \leq 0.0001$) by ~50% from Day 1 to Day 14. A significant difference ($P \leq 0.001$) in aldicarb sulfone concentration in urine stored in boric acid was also observed at 4°C and -80°C between Day 1 and 14 as well as Day 14 and Day 28 (Figures 3.23).

Urine stored at room temperature in containers without any preservative showed no significant difference in aldicarb concentrations in urine stored at 4°C, 19°C-20°C and -80°C. (Figure 3.24). There was however, a significant ($P \leq 0.0001$) increase in aldicarb sulfoxide concentration between Day 1 and Day 28 for the four storage conditions (Figure 3.25). Contrary, there was an ~50% degradation in the aldicarb sulfone concentration in urine stored in unpreserved containers under the four different storage conditions between Day 1 and Day 7. A significant difference ($P \leq 0.01$) in aldicarb sulfone concentration in urine stored in unpreserved containers was also observed between Day 14 and Day 28 for the four storage conditions (Figure 3.26). All three compounds were unstable in urine that was stored without preservative.

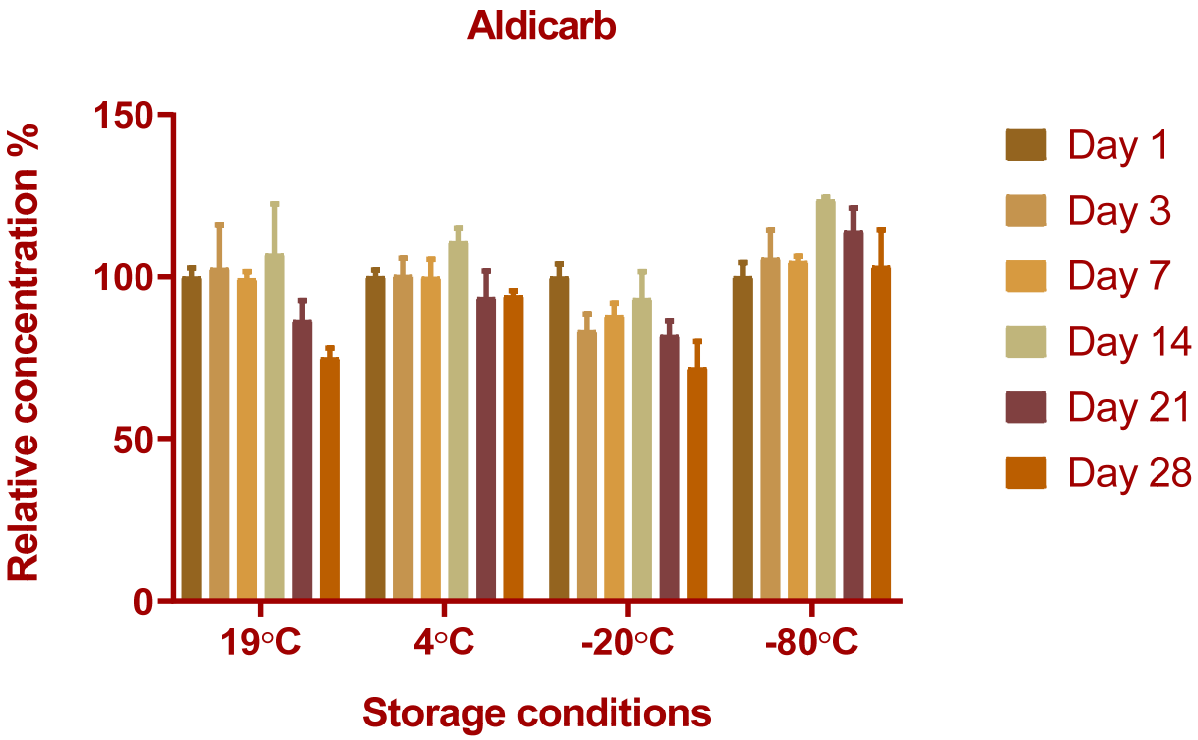


Figure 3.21: Bar graph indicating the stability of aldicarb spiked into boric acid preserved urine stored at different temperatures for up to 28 days. Statistical significance calculated using 2-way ANOVA with Tukey's test.

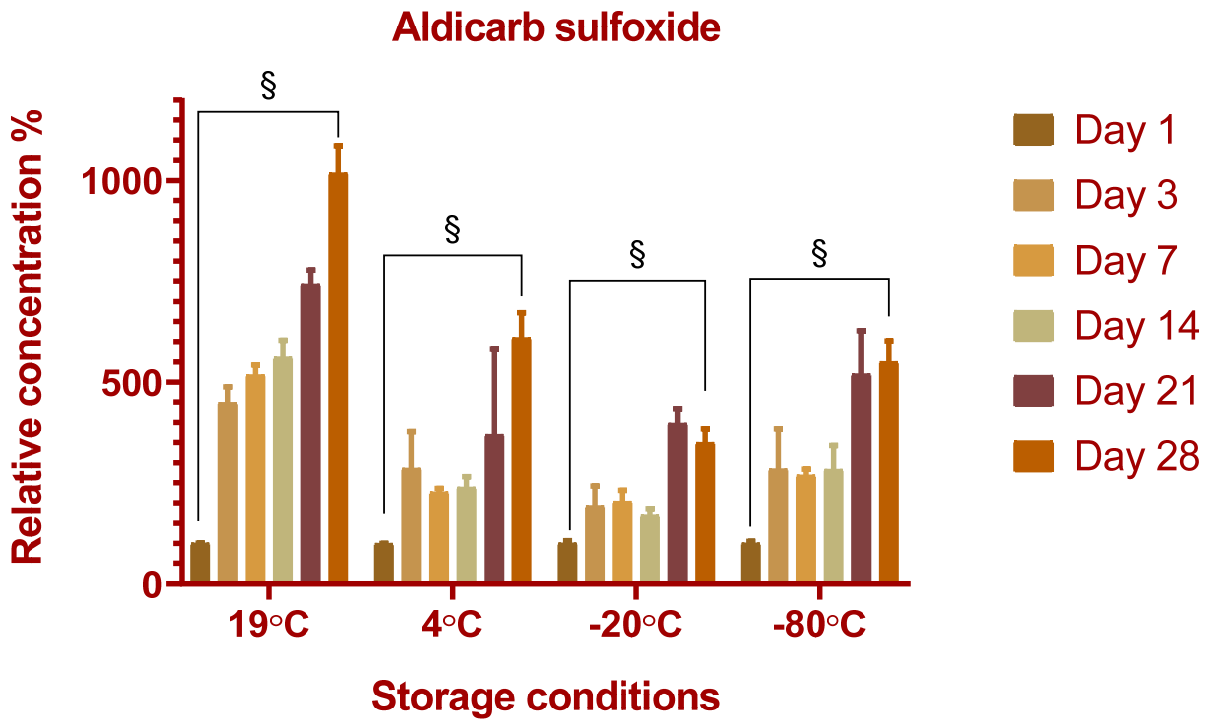


Figure 3.22: Bar graph indicating the concentration of aldicarb sulfoxide in aldicarb spiked boric acid preserved urine stored at different temperatures for up to 28 days. § = P ≤ 0.0001. Statistical significance calculated using 2-way ANOVA with Tukey's test.

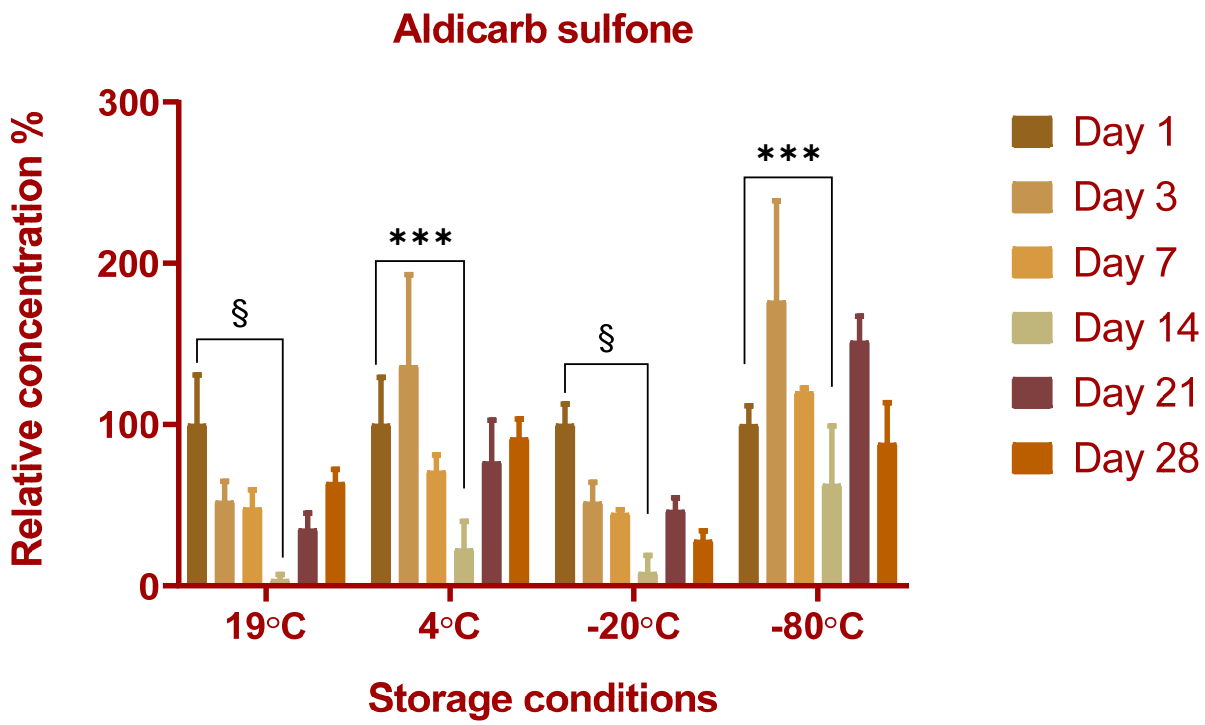


Figure 3.23: Bar graph indicating the concentration of aldicarb sulfone in aldicarb spiked boric acid preserved urine stored at different temperatures for up to 28 days. *** = P ≤ 0.001, § = P ≤ 0.0001. Statistical significance calculated using 2-way ANOVA with Tukey's test.

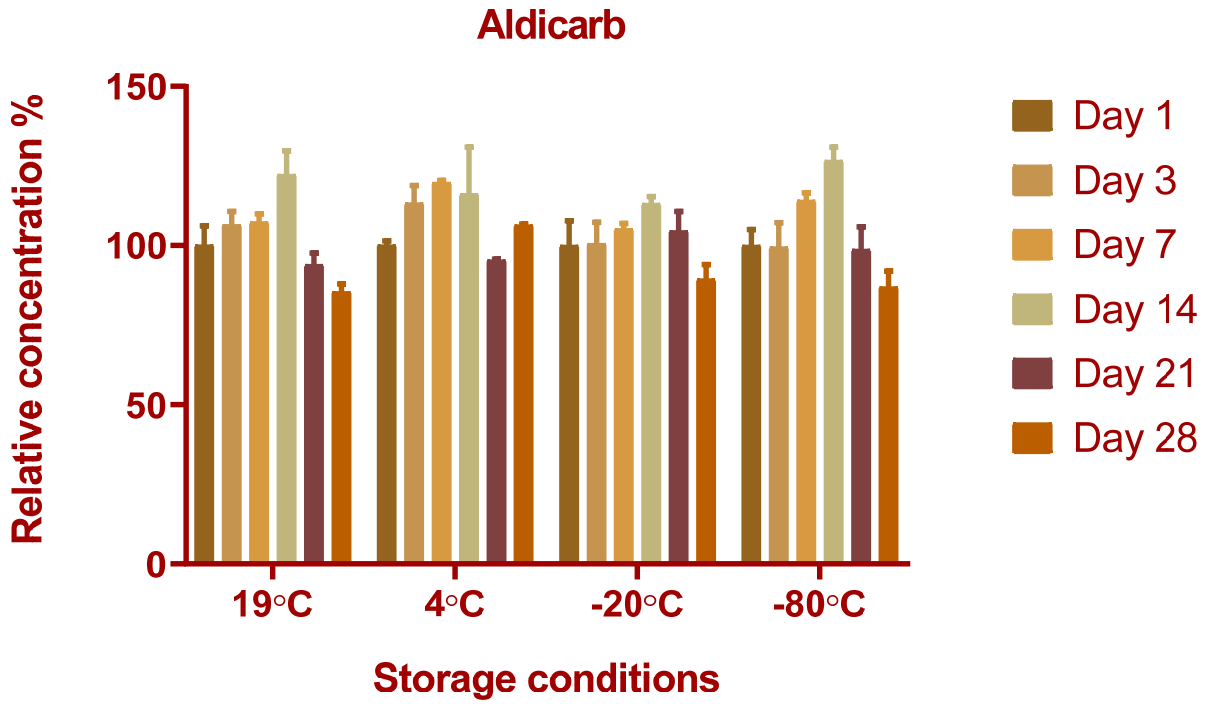


Figure 3.24: Bar graph indicating the concentration of aldicarb in spiked urine containing no preservative stored at different temperatures for up to 28 days. Statistical significance calculated using 2-way ANOVA with Tukey's test.

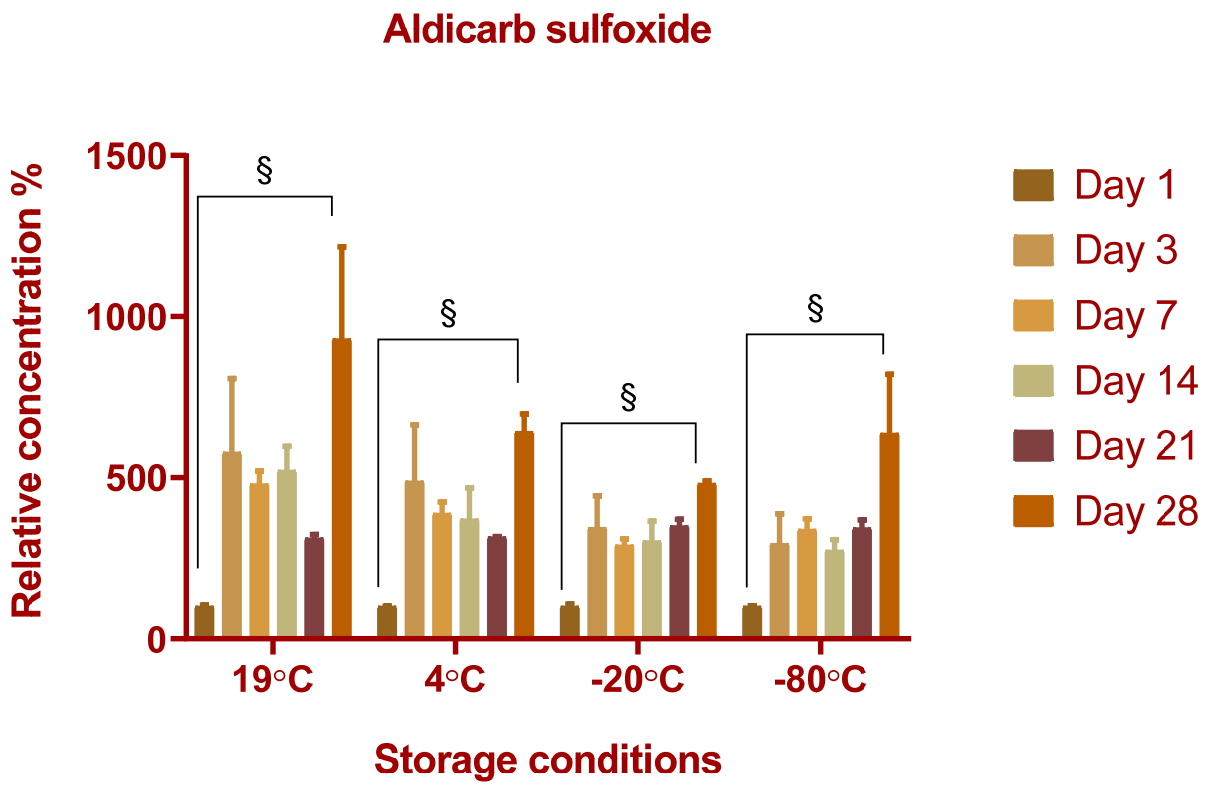


Figure 3.25: Bar graph indicating the concentration of aldicarb sulfoxide in aldicarb spiked urine containing no preservative stored at different temperatures for up to 28 days. § = P ≤ 0.0001. Statistical significance calculated using 2-way ANOVA with Tukey's test.

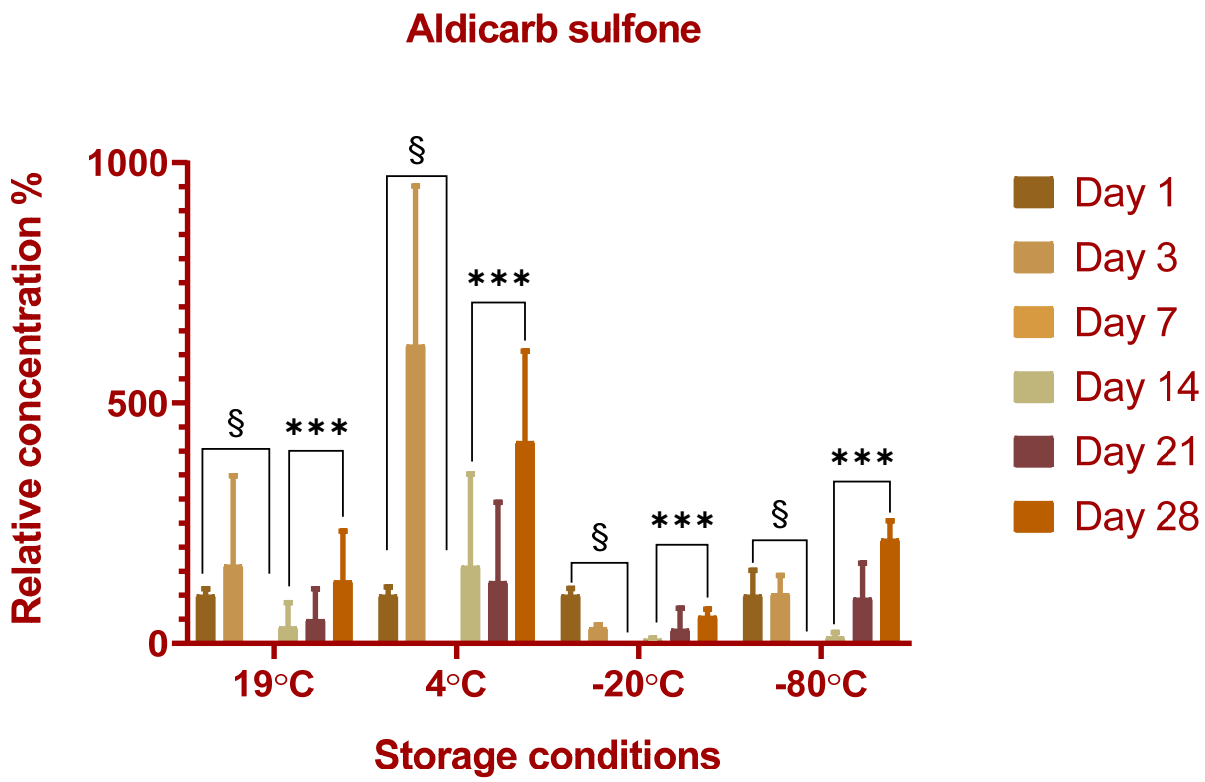


Figure 3.26: Bar graph indicating the concentration of aldicarb sulfone in aldicarb spiked urine containing no preservative stored at different temperatures for up to 28 days. *** = P ≤ 0.001, § = P ≤ 0.0001. Statistical significance calculated using 2-way ANOVA with Tukey's test.

Boric acid was found to inhibit the degradation of carbamate compounds to some extent, regardless of the storage temperature. The slight decrease in aldicarb concentrations in the samples stored in boric acid could be due pre-analytical conditions or even due to the initial dilution, due to the boric acid solution. A study on the stability of urine specimens stored with and without preservatives at room temperature and on ice, prior to urinalysis, found that keeping preservative tubes on ice caused a significant decrease in white blood cell clumps and calcium oxalate formation.[120] Lippi *et al.*[121] reported that boric acid retains the pH of urine and inhibits bacterial growth. Bacteria of the *Proteus* genus produces urease which leads to the increase of pH in urine samples, causing leukocyte lysis which may also occur at higher pH values or low density.[120,121]

The stability of carbamate compounds in different biological matrices, it is evident that the compounds were not stable in either whole blood or urine, even when the samples were stored at -80°C with a preservative. Degradation of the carbamate compounds was observed after 28 days especially in whole blood. It should be noted that oxidation products for only aldicarb were screened for in this study and extrapolation between different carbamate compounds stability cannot be done as stability depends on the compound properties, the matrix components and presence of preservatives or inhibitors in the storage container.[111]

3.5 Untargeted LC-MS/MS and UPLC-q-TOF-MS/MS method for screening of post-mortem biological samples and street pesticide positive control

The pesticides that were purchased from street vendors were extracted with different solvents and the extracts clarified by centrifugation showed different visual colours (Figure 3.27). Each of the street pesticide extracts were initially analysed by direct infusion of dilutions of the extracts in 80:20 methanol:0.1% formic acid in MS grade water into the mass spectrometer in both positive and negative mode ESI with a typical untargeted precursor ion mass spectrum as shown in Figure 3.28.

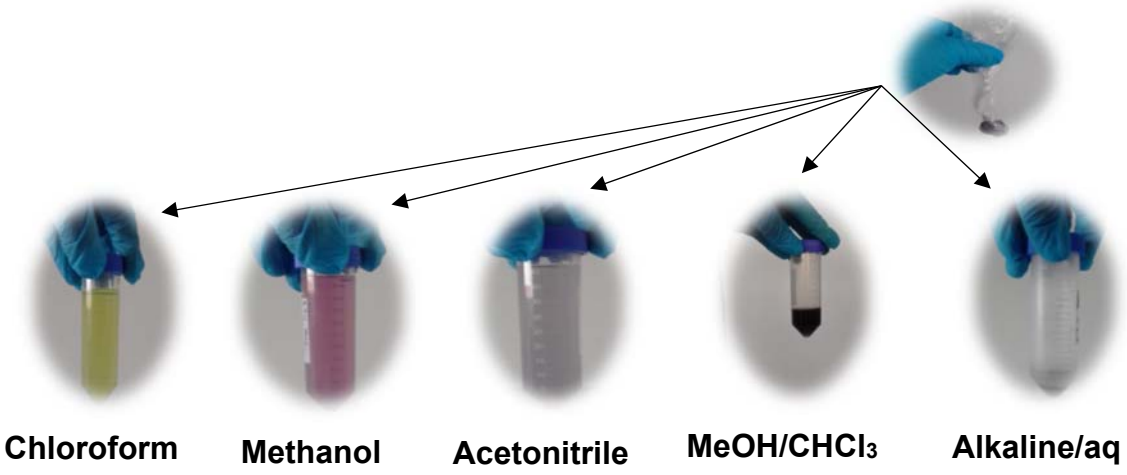


Figure 3.27: Extracts of street pesticides prepared using different solvents.

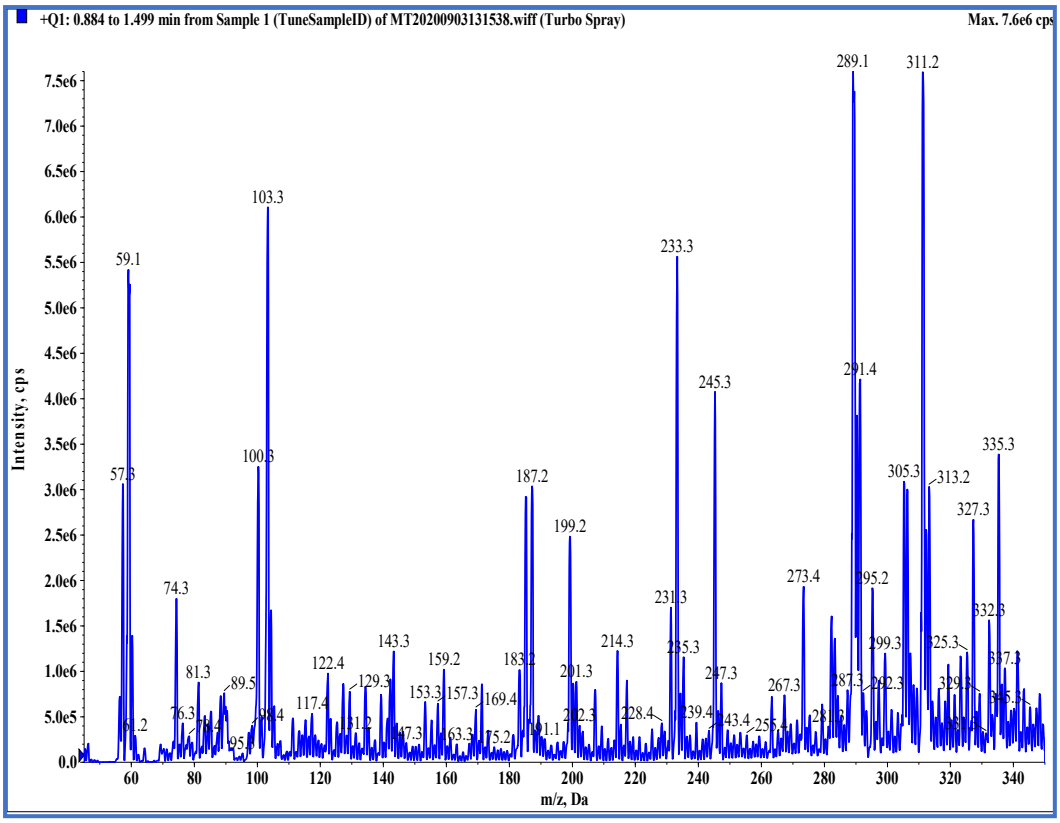


Figure 3.28: Untargeted positive mode precursor scans for all compounds detected in the street pesticide extracts using direct infusion in the Sciex 4000QTRAP.

Peaks with masses of 57.3, 103.3, 187.9, 199.2, 232.0, 233.9, 289.1, 311.2, 322.97,335.3 and 338.96 m/z were found to be present in four of the extracts. However, these masses did not correspond to any of the ten standard carbamate compounds included in this study during either analyses using LC-MS/MS or UPLC-q-TRAP-MS (Figures 3.28 and 3.29). The masses were initially scanned using untargeted analysis (triple quadrupole in Q1+ scan mode and MRM). The precursor with a high intensity mass of 289.1 had a possible match of an organophosphate insecticide, terbufos, which corresponded with both the precursor and product ion masses (Figures 3.30 and 3.31).

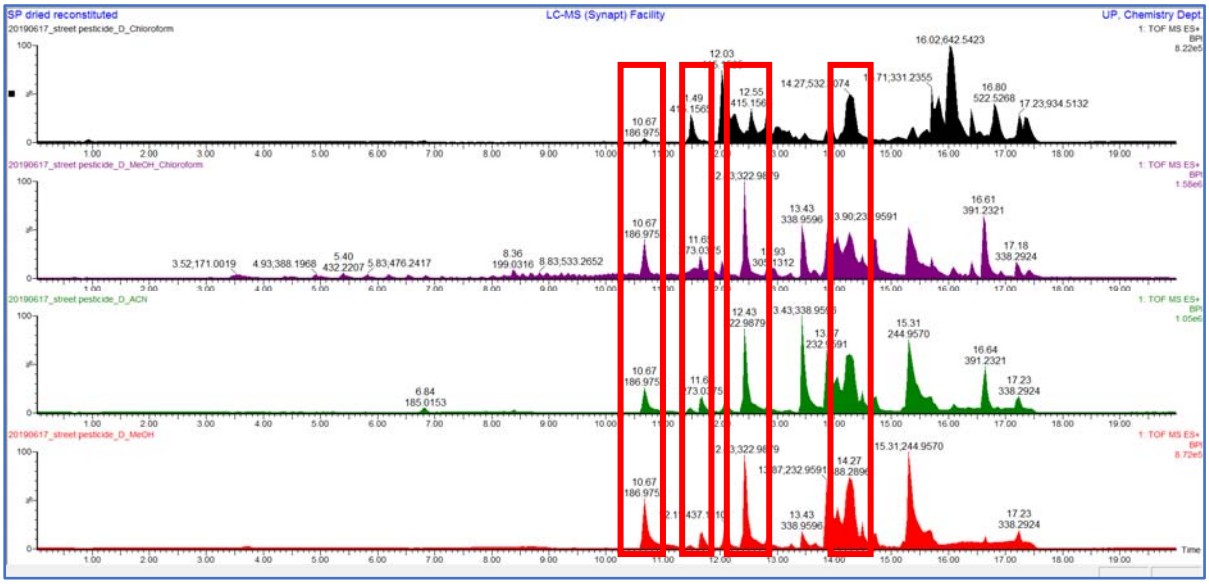


Figure 3.29: A series of chromatograms showing the consistent peaks in the four street pesticide extracts using the UPLC q-TRAP-MS.

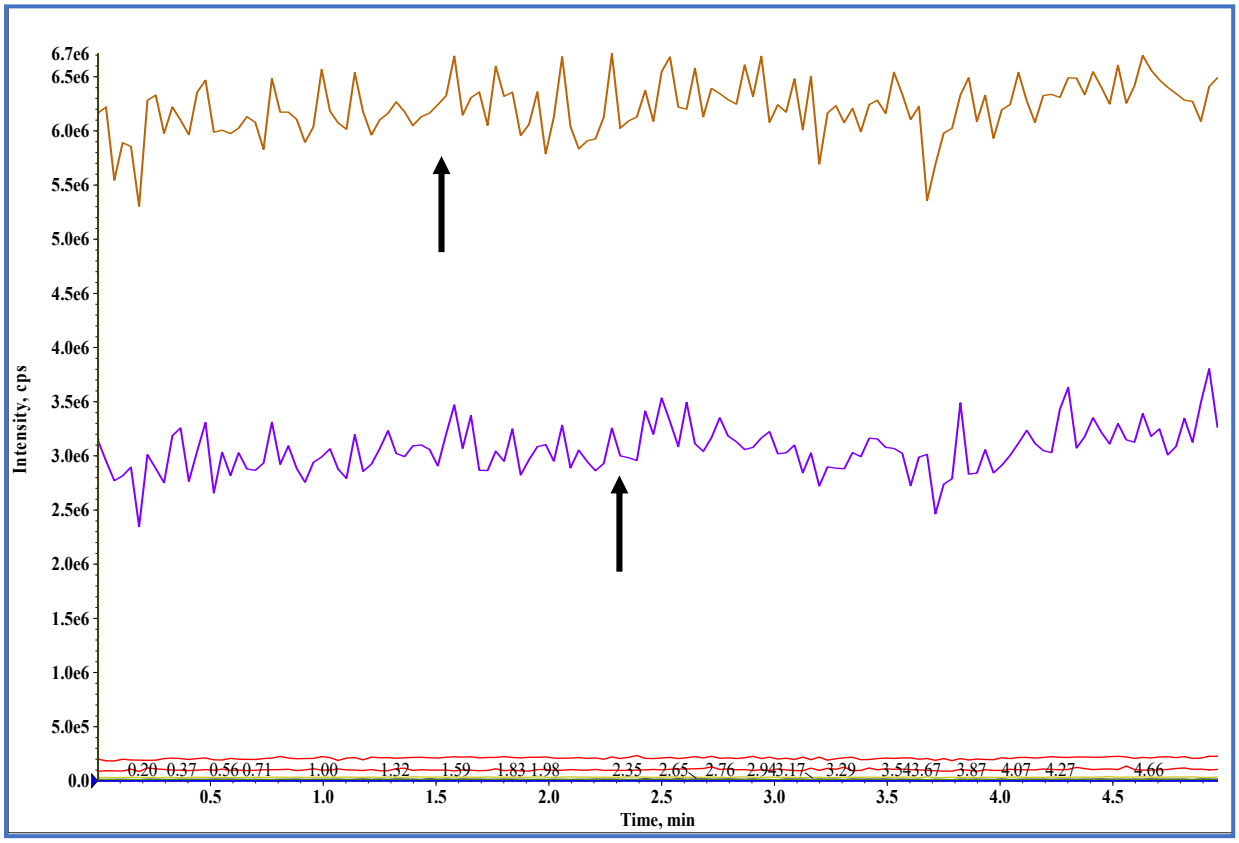


Figure 3.30: An infusion of the street vendor pesticide using the Sciex 4000 QTRAP/MS/MS in MRM scan mode with transitions 289.1 > 57.0 and 289.1 > 103.0, which matched the mass transitions reported for terbufos.

Extracts of the stomach content of suspected poisoning cases were also screened for the presence of carbamates as well as their potential breakdown products. The probable ten reference carbamate compounds were detected at low intensity in most of the cases (Appendix V). Interestingly, when the mass of aldicarb sulfoxide was extracted from the chromatogram, a match of a peak with a mass of 132.1089 was identified (Figures 3.32 and 3.33). This monoisotopic mass was suggestive of a product ion of aldicarb sulfoxide. Elemental composition was done to find the possible match for the mass (Figure 3.34). The first chemical formula ($C_6H_{14}NO_3$) with a chemical identification confidence of only 52.46% gave a match for three possible isomeric carbamate compounds which are? pentan-2-yl carbamate, 3-methylbutyl carbamate and ethyl N-propyl carbamate. As the mass spectrometer is operated in positive mode, the ionisation process adds a proton and a positive charge to the ions detected. To obtain the actual chemical formula of the

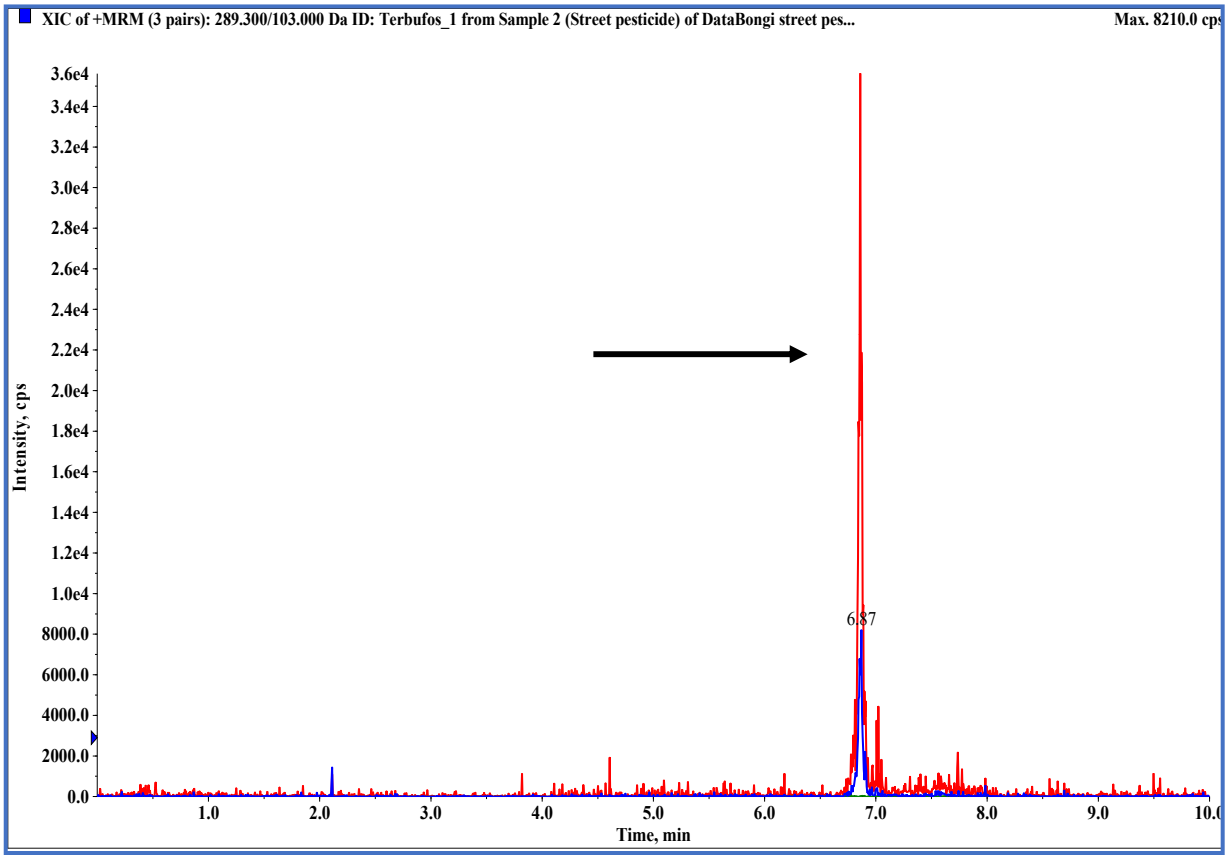


Figure 3.31: Untargeted screening of street pesticide showing a match for terbufos using the triple quadrupole LC-MS/MS.

ions, a hydrogen mass must be removed from the detected mass to obtain the chemical formula from the ion's elemental composition. The chemical formula $C_6H_{13}NO_3$ matched the three carbamate compounds mentioned above.

The different stomach content extracts had peaks with consistent retention times which did not match any of the ten carbamate compounds monoisotopic masses. The peaks had masses of, 187.01 226.96, 218.22, 246.25, and 274.28 m/z (Figure 3.35).

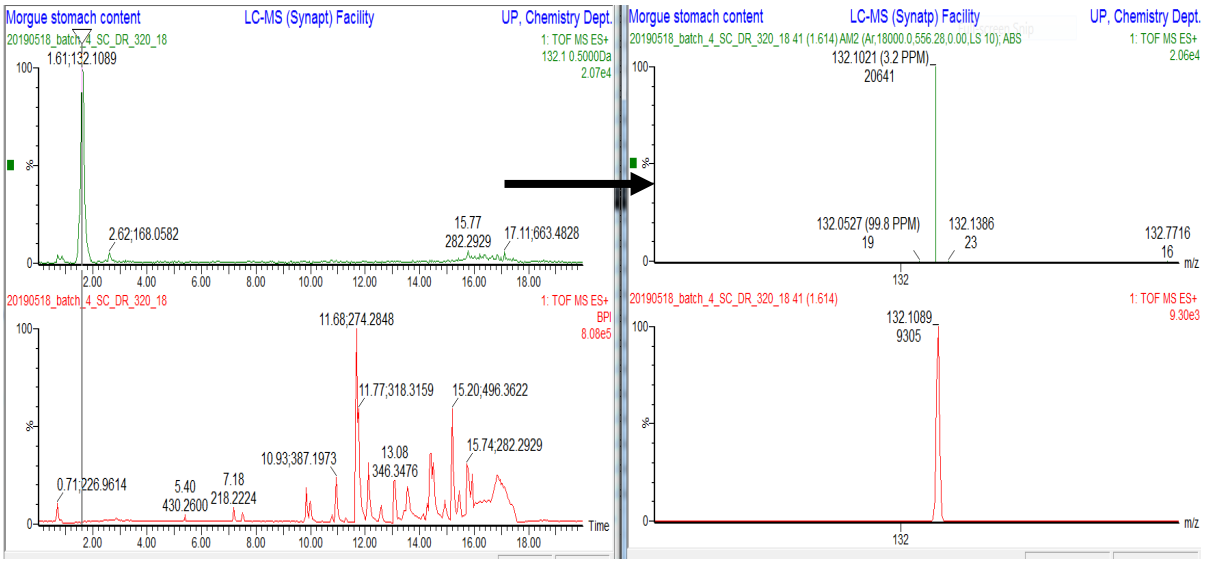


Figure 3.32: An extracted chromatogram showing the peak suggestive of aldicarb sulfoxide (product ion) in stomach content.

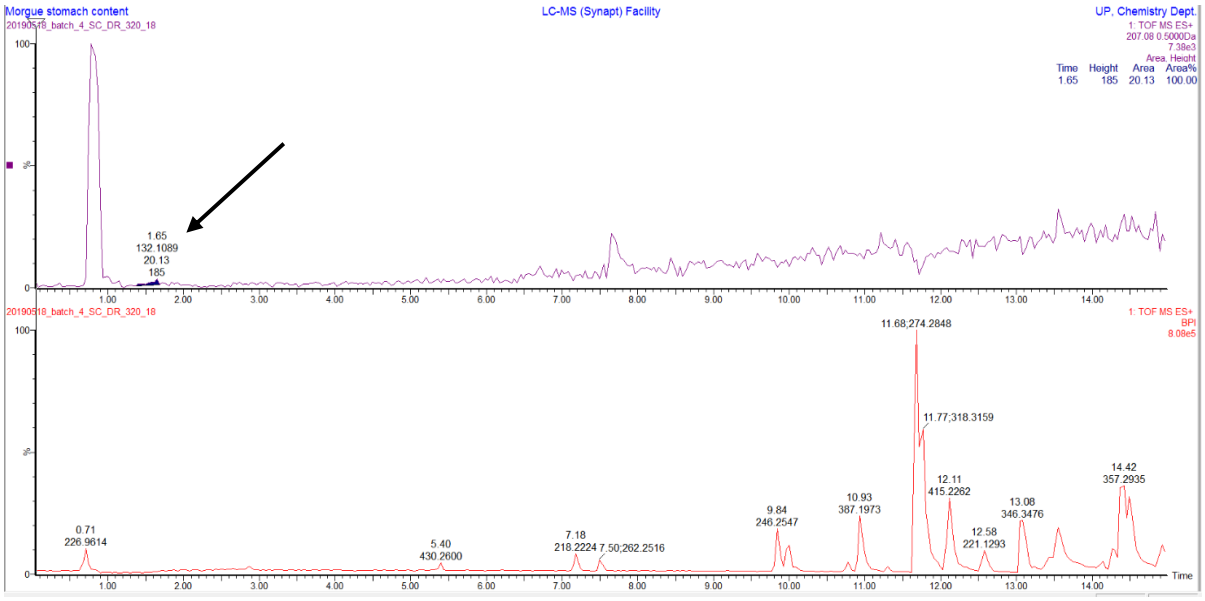


Figure 3.33: An integrated chromatogram showing a possible match of aldicarb sulfoxide (product ion) in stomach content.

Figure 3.34: Elemental composition of the peak suggestive of aldicarb sulfoxide (in source breakdown ion) in stomach content.

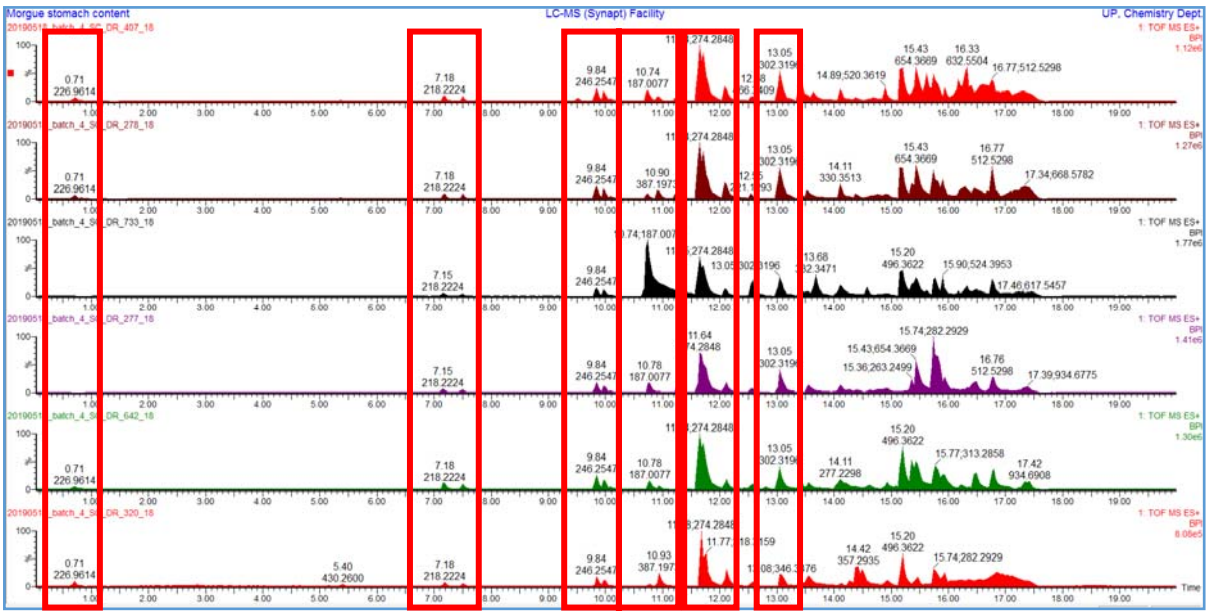


Figure 3.35: A series of chromatograms from different post-mortem stomach content samples from six suspected poisoning cases that show consistent unidentified peaks.

Post-mortem blood and urine samples from suspected poisoning cases were extracted using the solvent protein precipitation method. Consistent peaks in the samples were identified by mass and retention time (summarised in Appendix V). In some cases, detected peaks were assayed for elemental composition then identified by searching against a carbamate database using compound fit confidence percentage to identify possible carbamate toxins. In the blood sample of case 2, a peak with a mass of 404.1122 m/z and a retention time of 3.87 min was suggestive of a carbamate compound. The mass of 404.1122 m/z gave more than 200 possible matches of compounds. Of the >200 compounds, only 40 had feasible structures. Elemental composition identified the chemical formula with a high fit confidence of 96.6% as $C_{19}H_{25}N_5O_5$. A compound search on PubChem identified the compound as [(4S)-11-(dimethylaminomethylideneamino)-7-methoxy-5,12-dimethyl-10,13-dioxo-2,5-diazatetracyclo[7.4.0.02,7.04,6]-trideca-1(9),11-dien-8-yl] methyl carbamate, an unlikely synthetic molecule.

In case 3 (blood sample), elemental composition of the peak eluting at 14.37 min with a mass of 576.3392 m/z (Figure 3.36) showed a mass envelope suggestive of a typical polyethylene glycol compound (Figure 3.37). The chemical formula $C_{29}H_{45}N_5O_7$ with 55.62% fit confidence (Figure 3.38) gave a match of the carbamate compound (methyl N-[1-[5-amino-6-hydroxy-3,3,8-trimethyl-9-[(2-morpholin-4-yl-2-oxoethyl)amino]-9-oxononanoyl]-3,4-dihydro-2H-quinolin-3-yl] carbamate).

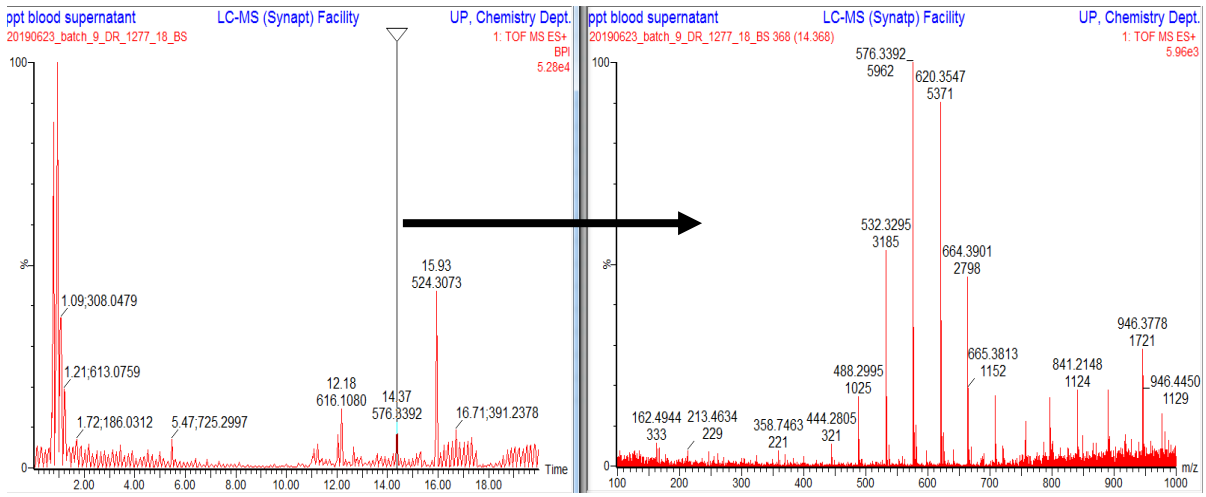


Figure 3.36: An ion chromatogram indicating the retention time of the compound with a mass envelope with most abundant mass of 576.3392 m/z found in the blood of case 3.

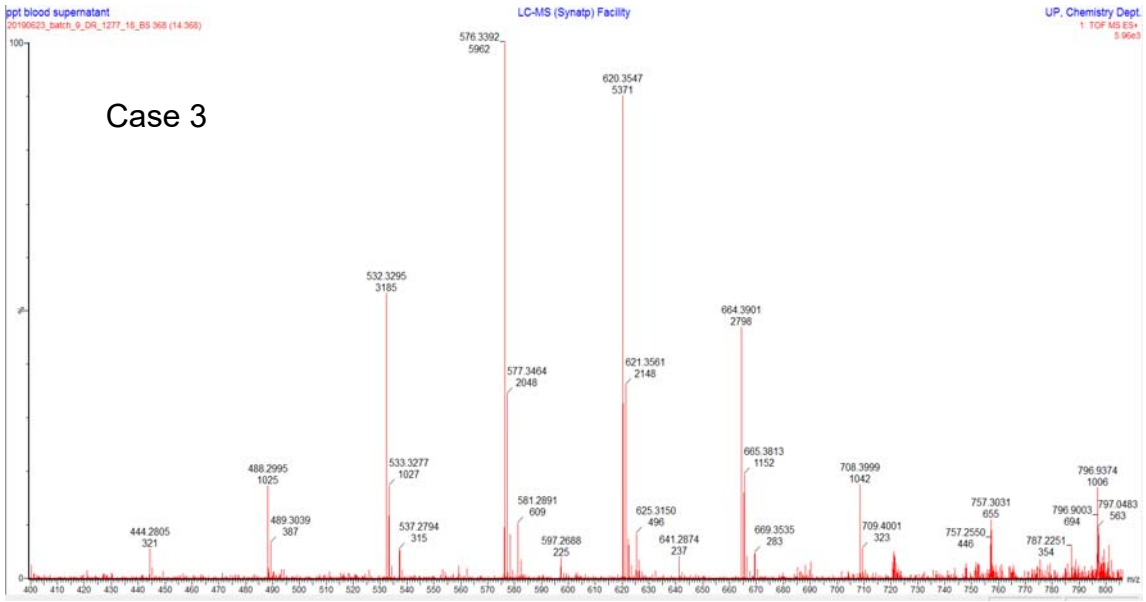


Figure 3.37: An expanded view of the ion chromatogram showing a mass envelope of the detected compound suggestive of a polyethylene glycol conjugate in the blood of case 3.

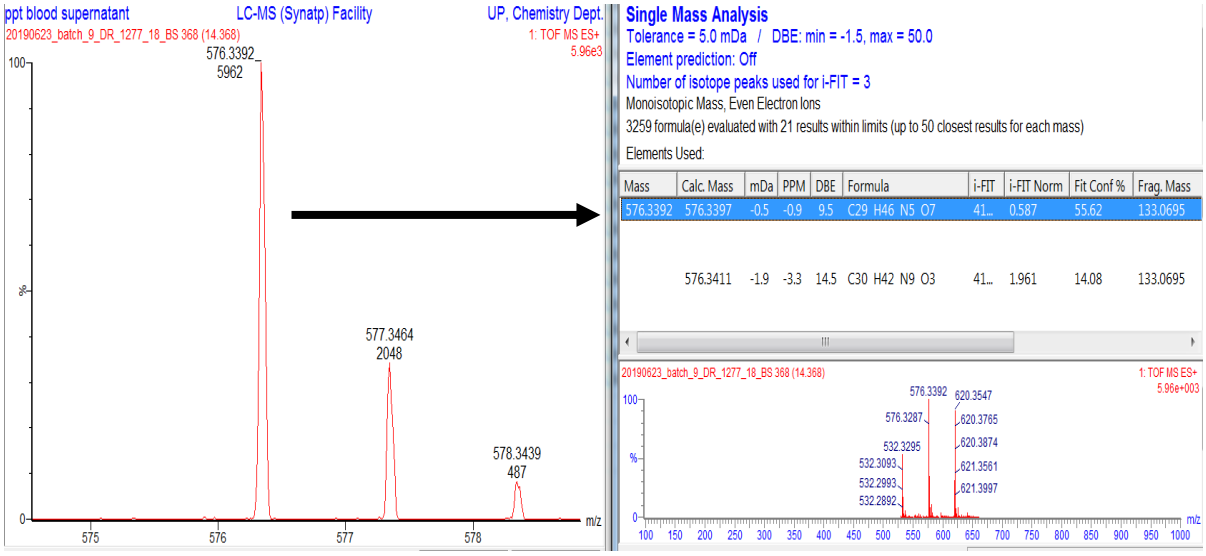


Figure 3.38: Elemental composition of the compound with m/z 576.3392 in blood of case 3.

In case 04 (urine sample), a peak with a mass of 166.0777 m/z was detected at 2.18 min. The mass of 166.0777 m/z had a 99.99% fit with the carbamate compound with a chemical formula of $C_9H_{11}NO_2$; ethyl N-phenyl carbamate. The same carbamate compound was detected in the blood sample of case 4. There was a slight shift of 0.1 min in the retention time of the peak detected in blood.

Two peaks with retention times of 3.90 and 3.99 min, with the masses of 180.0623 and 265.1071 m/z, respectively, in a urine sample of case 5 were identified as carbamate compounds by elemental composition (Figure 3.39). The compounds, $C_9H_9NO_3$, (3-formylphenyl) N-methylcarbamate (m/z 180.0623) with a fit confidence of 99.97% and $C_{13}H_{16}N_2O_4$, tert-butyl N-(1,3-benzodioxol-5-yl methylidene amino) carbamate (m/z 265.1071) with a 99.94% fit confidence, were identified (Figures 3.40 and 3.41). According to the PubChem database (3-formylphenyl) N-methylcarbamate is related to carbaryl.

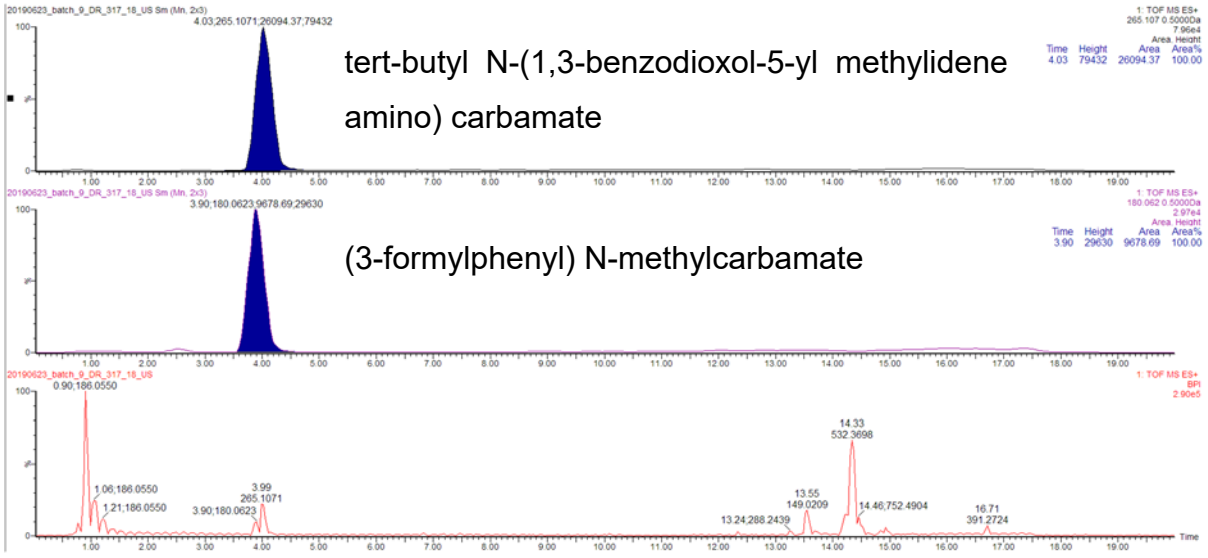


Figure 3.39: An ion chromatogram showing the two integrated carbamate peaks detected in the urine sample of case 5.

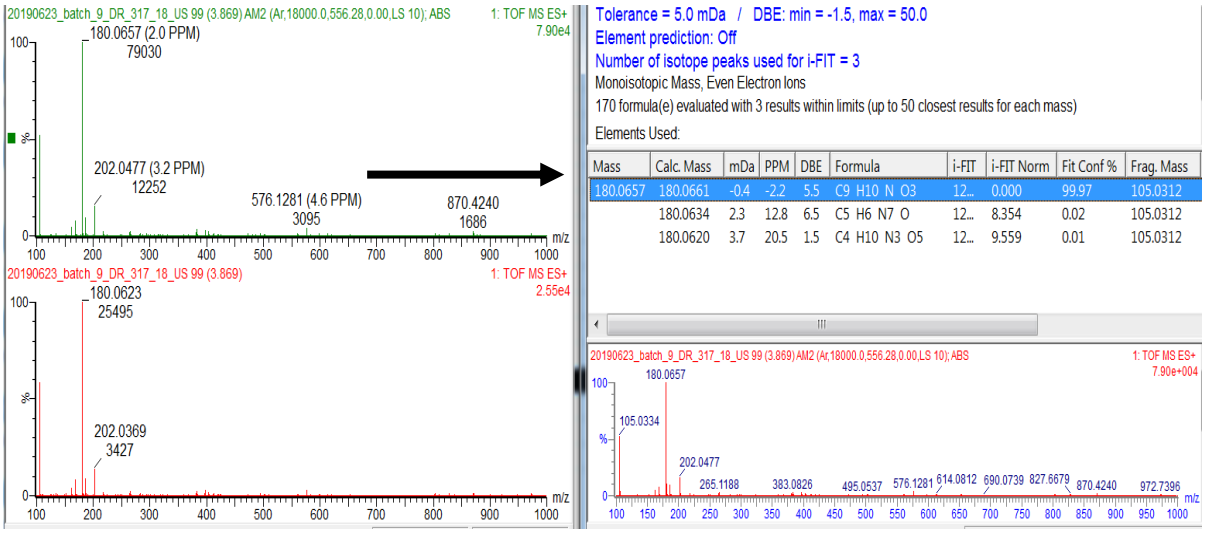


Figure 3.40: Elemental composition of compound with m/z 180.0623 in urine of case 5.

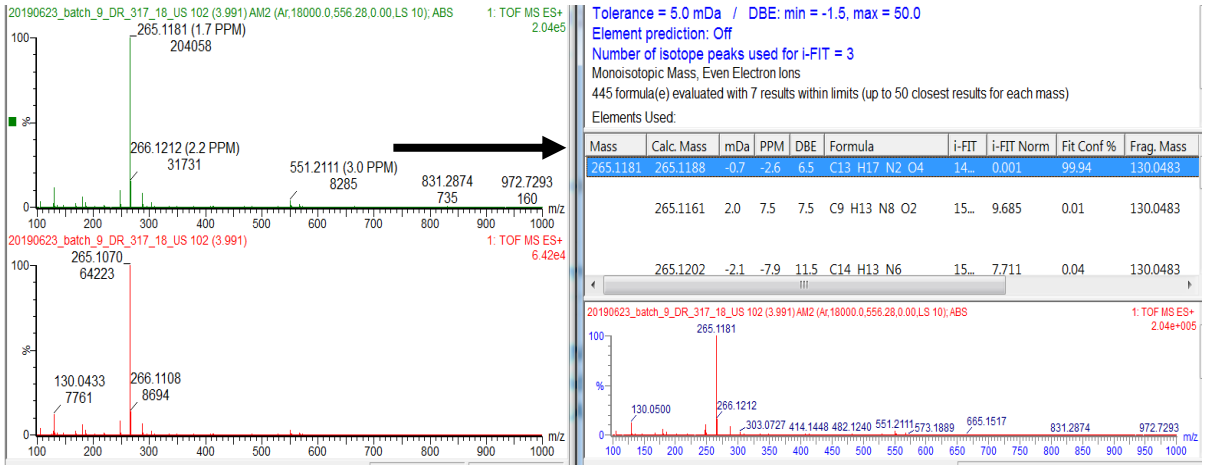


Figure 3.41: Elemental composition of compound with m/z 265.1071 in urine of case 5.

Three peaks with the masses and retention times of m/z 132.0900 (1.56 min),166.0662 (2.19 min) and 231.1461 (3.37 min) in the blood sample of case 6 were identified (Figure 3.42) as carbamate compounds with formulas; $C_6H_{13}NO_2$ (100.00%), $C_9H_{11}NO_2$ (100.00%) and $C_{11}H_{22}N_2O_3$ (100.00%) (Figures 3.43 and 3.45). The chemical formula; $C_6H_{13}NO_2$ had 13 isomeric carbamate compound matches, $C_9H_{11}NO_2$ had six isomeric carbamate compounds and $C_{11}H_{22}N_2O_3$ had two isomeric carbamate compounds as indicated in the PubChem database (Table 3.6). In the urine sample from the same case, a peak with a m/z of 288.2306 and retention time of 13.24 min was identified. Elemental composition was determined and the chemical formula with the highest fit confidence was $C_{16}H_{33}NO_3$ (99.98%) (Figures 3.46 and 3.47). The chemical formula gave a match of seven isomeric carbamate compounds.

Post-mortem cases that had a match for carbamates compounds present in both blood and urine are summarised in Table 3.7. Blood samples from cases 7, 8, 9, 10 and 11 did not have any significant peaks that corresponded to carbamate compounds.

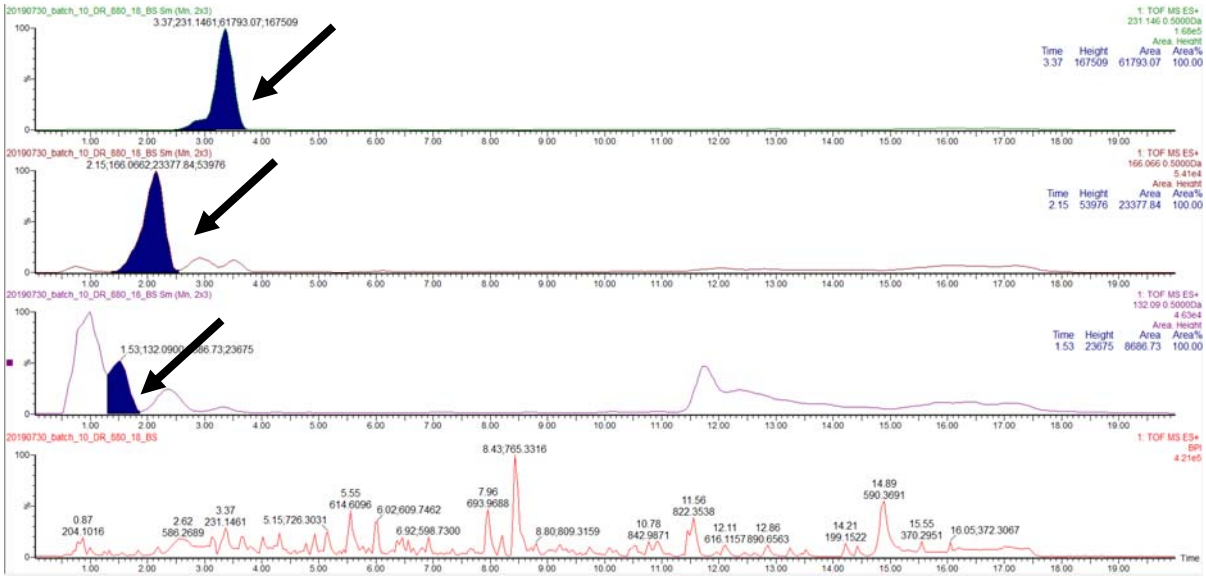


Figure 3.42: An ion chromatogram indicating integrated carbamate peaks detected from the blood sample of case 6.

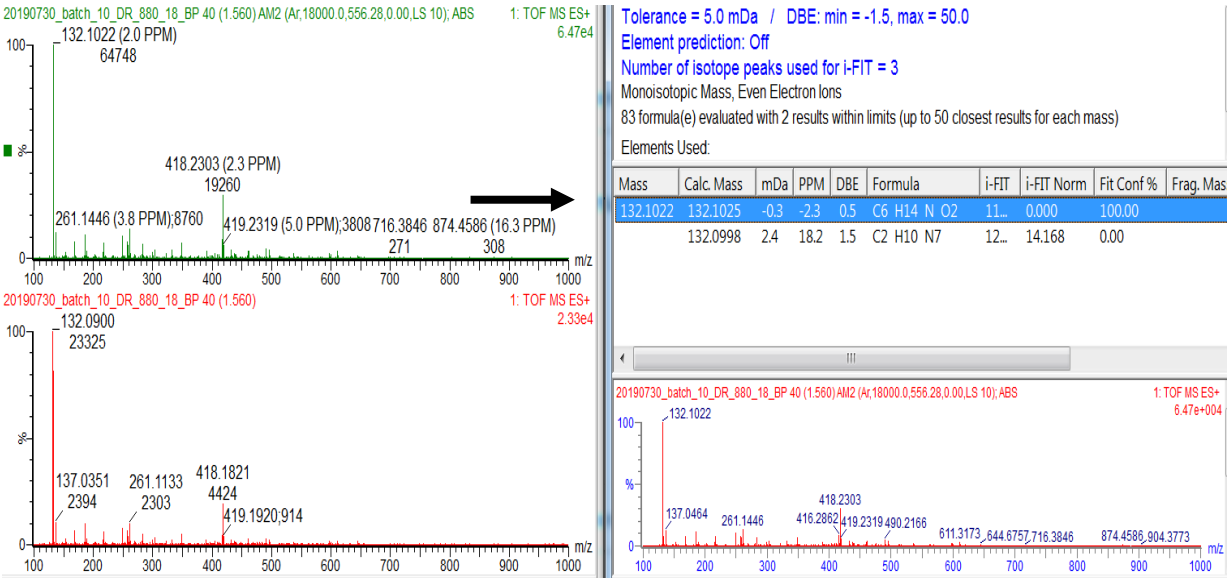


Figure 3.43: Elemental composition of compound with m/z 132.0900 in blood of case 6.

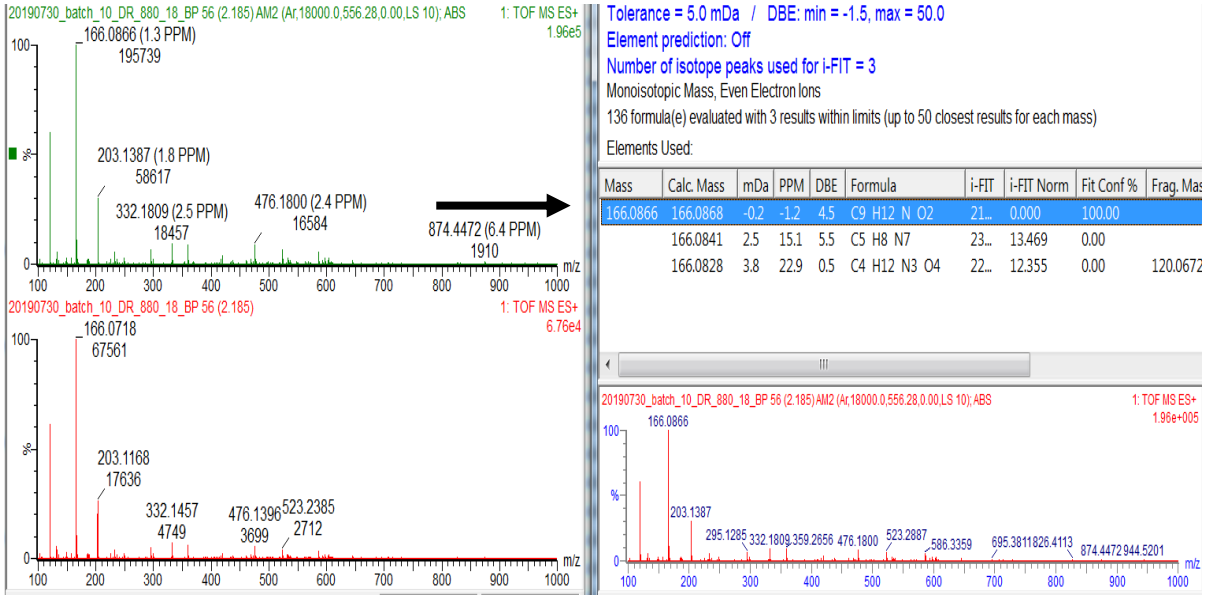


Figure 3.44: Elemental composition of compound with m/z 166.0662 in blood of case 6.

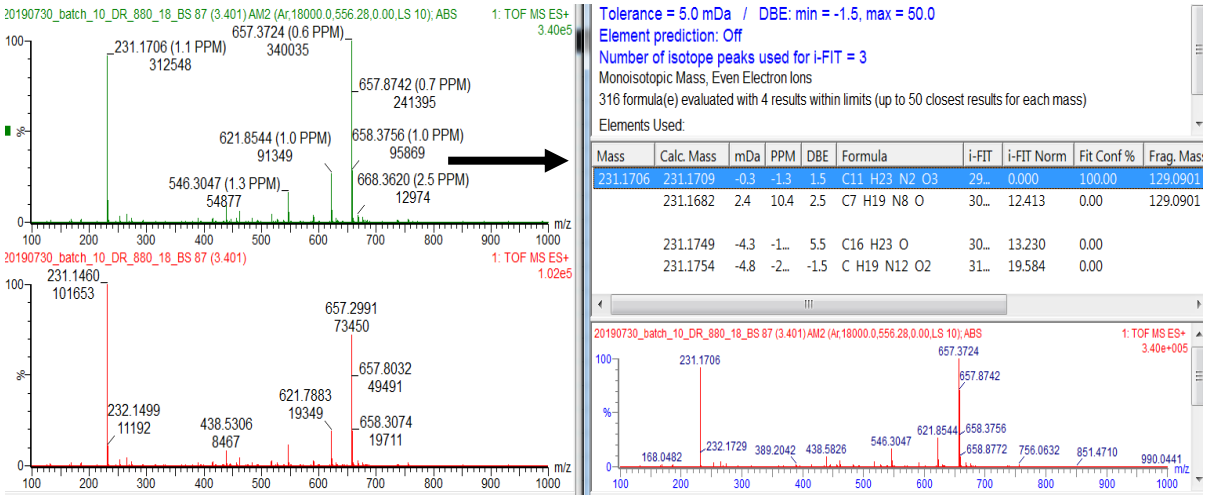


Figure 3.45: Elemental composition compound with m/z 231.1461 in blood of case 6.

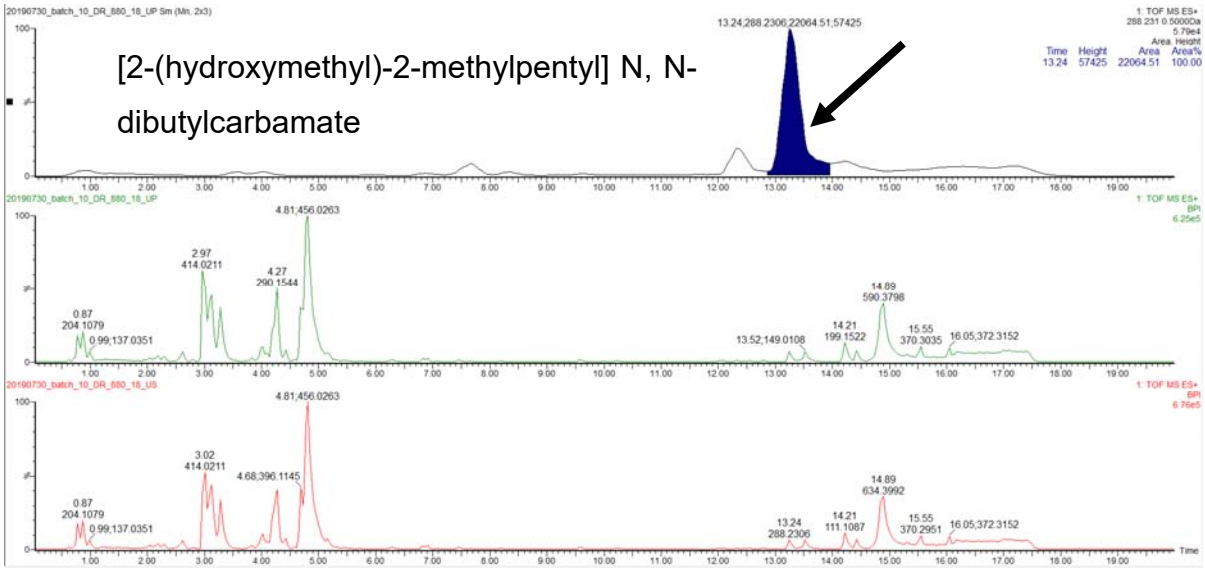


Figure 3.46: An ion chromatogram showing integrated carbamate peaks detected in the urine sample of case 6.

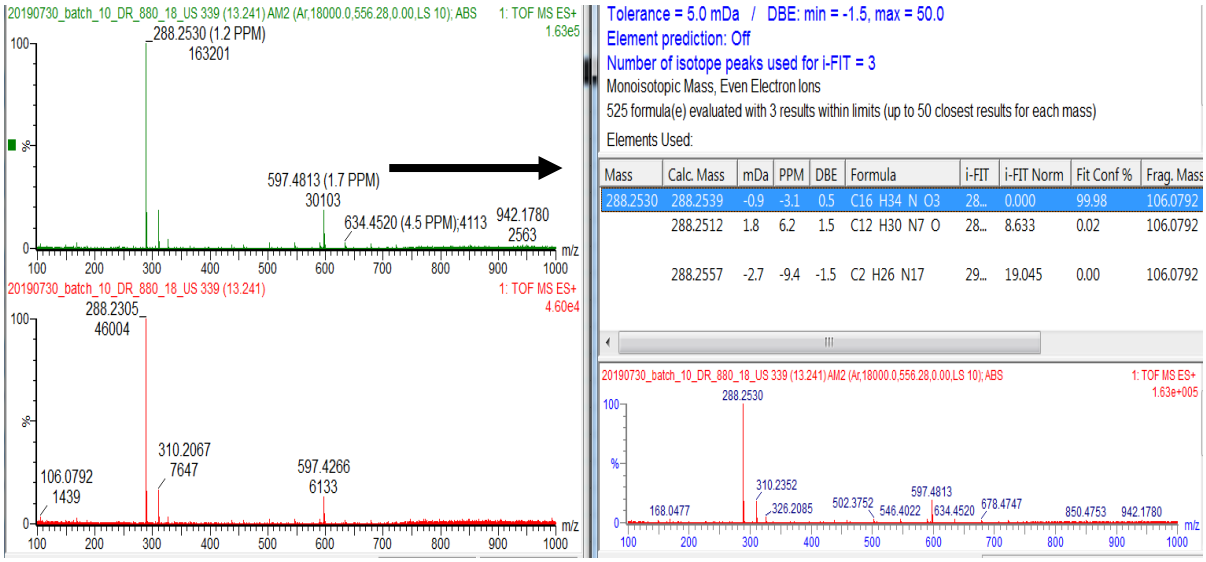


Figure 3.47: Elemental composition compound with m/z 288.2306 in urine of case 6.

Table 3.6: Potential carbamate compounds detected in blood and urine samples of case 6.

Chemical formulas (mass m/z)	Retention time (min)	Probable carbamate compounds detected	Fit confidence %
$C_6H_{13}NO_2$ (132.0900)	1.56	pentan-2-yl carbamate, 3-methylbutyl carbamate, ethyl N-propylcarbamate, ethyl N-propan-2-ylcarbamate, methyl N-butan-2-ylcarbamate, methyl N-butylcarbamate, 2-methylbutan-2-yl carbamate, pentyl carbamate, 2,2-dimethylpropyl carbamate, methyl N,N-diethylcarbamate, methyl N-(2-methylpropyl)carbamate, propan-2-yl N,N-dimethylcarbamate, propan-2-yl N-ethylcarbamate,	100
$C_9H_{11}NO_2$ (166.0662)	2.19	ethyl N-phenylcarbamate (3-methylphenyl) N-methylcarbamate (2,3-dimethylphenyl) carbamate (2,5-dimethylphenyl) carbamate (2-methylphenyl) N-methylcarbamate (4-methylphenyl) N-methylcarbamate	100
$C_{11}H_{22}N_2O_3$ (231.1461)	3.37	propan-2-yl N-(3-morpholin-4-ylpropyl)carbamate tert-butyl-N-[2-(diethylamino)-2-oxoethyl]carbamate	100
$C_{16}H_{33}NO_3$ (288.2306)	13.24	[2-(hydroxymethyl)-2-methylpentyl] N,N-dibutyl-carbamate, octyl N-(hexoxymethyl)carbamate, tert-butyl N-(5-hydroxy-2,8-dimethylnonan-4-yl)carbamate, butyl N-(5-hydroxy-2,8-dimethylnonyl)carbamate, tert-butyl carbamate; 3-(4-ethylcyclohexyl)propan-1-ol, tert-butyl carbamate;4-(4-methylcyclohexyl)butan-1-ol, tert-butyl N-(11-hydroxyundecyl)carbamate	99.98

Table 3.7: A summary of the carbamate compounds detected in the different post-mortem cases.

Case number (sample type)	Compound mass (m/z)	Retention Time (min)	Chemical formula	Carbamate compound/s	Fit (%)
10 (urine)	185.1197	2.62	$C_9H_{16}N_2O_2$	4-(dimethylamino)but-2-ynyl N,N-dimethylcarbamate	99.96
	265.1022	4.03	$C_{13}H_{16}N_2O_4$	tert-butyl N-(1,3-benzodioxol-5-ylmethylideneamino) carbamate	
				(3-methyl-6-propan-2-ylcyclohex-3-en-1-yl) N-(2-nitrophenyl) carbamate,	57.71
	319.1483	4.62	$C_{17}H_{22}N_2O_4$	(1,7,7-trimethyl-2-bicyclo[2.2.1]heptanyl) N-(2-nitrophenyl)carbamate,	80.09
				(1,3,3-trimethyl-2-bicyclo[2.2.1]heptanyl) N-(2-nitrophenyl)carbamate,	
				3,7-dimethylocta-1,6-dien-3-yl N-(2-nitrophenyl)carbamate,	
				(5-methyl-2-prop-1-en-2-ylcyclohexyl) N-(2-nitrophenyl)carbamate	
				tert-butyl N-[3-(1,3-dioxolan-2-yl)-6-hydroxyhexyl]-N-methylcarbamate	
	304.1892	5.78	$C_{15}H_{29}NO_5$	2-(2-hydroxyethoxy)ethyl N,N-dicyclohexylcarbamate	93.92
				[2-(hydroxymethyl)-2-methylpentyl] N,N-dibutylcarbamate,	
	314.2124	9.68	$C_{17}H_{31}NO_4$	2-hydroxypropyl N-dodecylcarbamate,	57.84
	288.2379	13.24	$C_{16}H_{33}NO_3$	1-hydroxypropan-2-yl N-dodecylcarbamate	99.98
11 (urine)	232.1433	2.03	$C_{11}H_{21}NO_4$	(2,2-dipropyl-1,3-dioxolan-4-yl)methyl carbamate,	96.60
				(2-methyl-2-pentyl-1,3-dioxolan-4-yl)methyl carbamate	
				4-(dimethylamino)but-2-ynyl N,N-dimethylcarbamate	
				(3-formylphenyl) N-methyl carbamate	
	185.1197	2.62	$C_9H_{16}N_2O_2$	tert-butyl N-(1,3-benzodioxol-5-ylmethylideneamino) carbamate	100.00
	180.0556	3.87	$C_9H_9NO_3$	(3-methyl-6-propan-2-ylcyclohex-3-en-1-yl) N-(2-nitrophenyl)carbamate,	100.00
				(1,7,7-trimethyl-2-bicyclo[2.2.1]heptanyl) N-(2-nitrophenyl)carbamate,	
	265.1022	3.99	$C_{13}H_{16}N_2O_4$	(1,3,3-trimethyl-2-bicyclo[2.2.1]heptanyl) N-(2-nitrophenyl)carbamate,	99.75

	319.1483	4.62	$C_{17}H_{22}N_2O_4$	3,7-dimethylocta-1,6-dien-3-yl N-(2-nitrophenyl) carbamate, (5-methyl-2-prop-1-en-2-ylcyclohexyl) N-(2-nitrophenyl)carbamate	99.69
	314.2124	9.68	$C_{17}H_{31}NO_4$	2-(2-hydroxyethoxy)ethyl N,N-dicyclohexylcarbamate	98.65
	288.2379	13.24	$C_{16}H_{33}NO_3$	[2-(hydroxymethyl)-2-methylpentyl] N,N-dibutyl carbamate, 2-hydroxypropyl N-dodecylcarbamate, 1-hydroxypropan-2-yl N-dodecylcarbamate	98.61
12 (blood)	166.0777	2.19	$C_9H_{11}NO_2$	ethyl N-phenylcarbamate (3-methylphenyl)N methylcarbamate	97.10
13 (blood)	433.3051	7.65	$C_{22}H_{42}N_4O_5$	tert-butyl N-[(2R)-1-[[(2S)-1-[[(2S)-6-amino-1-oxohexan-2-yl]amino]-4-methyl-1-oxopentan-2-yl]amino]-3-methyl-1-oxobutan-2-yl]carbamate	66.64
14 (urine)	265.1071	4.03	$C_{13}H_{16}N_2O_4$	tert-butyl N-(1,3-benzodioxol-5-ylmethylidene amino) carbamate	92.31
15 (blood)	232.1233	2.15	$C_{11}H_{21}NO_4$	(2,2-dipropyl-1,3-dioxolan-4-yl)methyl carbamate	75.35
	443.2632	7.65	$C_{22}H_{42}N_4O_5$	(2-methyl-2-pentyl-1,3-dioxolan-4-yl)methyl carbamate tert-butyl N-[(2R)-1-[[(2S)-1-[[(2S)-6-amino-1-oxohexan-2-yl]amino]-4-methyl-1-oxopentan-2-yl]amino]-3-methyl-1-oxobutan-2-yl]carbamate	98.44
16 (urine)	180.0623	3.90	$C_9H_9NO_3$	(3-formylphenyl)N-methylcarbamate	98.36
17 (blood)	253.1010	2.90	$C_{12}H_{16}N_2O_4$	[3-(propan-2-yloxycarbonylamino)phenyl] N-methylcarbamate, [3-[methoxycarbonyl(methyl)amino]phenyl]N,N-dimethyl carbamate, [2-(dimethylcarbamoyloxy)phenyl] N,N-dimethyl carbamate, [4-(dimethylcarbamoyloxy)phenyl] N,N-dimethyl carbamate, (3,4,4-trimethyldioxetan-3-yl)methyl N-pyridin-4-ylcarbamate, ethyl N-[2-(2-hydroxyethylcarbamoyl) phenyl] carbamate, (3-carbamoyloxy-2-methyl-2-phenylpropyl) carbamate	72.30
17 (urine)	152.0602	2.50	$C_8H_9NO_2$	benzyl carbamate	99.98
	166.0775	2.19	$C_9H_{11}NO_2$	phenyl N-methylcarbamate ethyl N-phenylcarbamate	98.60
	232.1366	2.03	$C_{11}H_{21}NO_4$	(2,2-dipropyl-1,3-dioxolan-4-yl)methyl carbamate,	80.61
	265.1022	4.03	$C_{13}H_{16}N_2O_4$	(2-methyl-2-pentyl-1,3-dioxolan-4-yl)methyl carbamate	96.80

				tert-butyl N-(1,3-benzodioxol-5-ylmethylideneamino) carbamate	
18 (blood)	232.1366 253.1010	2.15 2.90	$C_{11}H_{21}NO_4$ $C_{12}H_{16}N_2O_4$	(2,2-dipropyl-1,3-dioxolan-4-yl)methyl carbamate, (2-methyl-2-pentyl-1,3-dioxolan-4-yl)methyl carbamate 3-(propan-2-yloxycarbonylamino)phenyl] N-methylcarbamate, [3-[methoxycarbonyl(methyl)amino]phenyl]N,N-dimethyl carbamate, [2-(dimethylcarbamoyloxy)phenyl] N,N-dimethyl carbamate, [4-(dimethylcarbamoyloxy)phenyl] N,N-dimethyl carbamate, (3,4,4-trimethyldioxetan-3-yl)methyl N-pyridin-4-ylcarbamate, ethyl N-[2-(2-hydroxyethylcarbamoyl) phenyl]carbamate, (3-carbamoyloxy-2-methyl-2-phenylpropyl) carbamate	100.00 99.97
19 (blood)	232.1366	2.15	$C_{11}H_{21}NO_4$	(2,2-dipropyl-1,3-dioxolan-4-yl)methyl carbamate, (2-methyl-2-pentyl-1,3-dioxolan-4-yl)methyl carbamate	99.97
19 (urine)	132.0950 166.0775 188.0612 265.1022	1.02 2.19 3.09 4.03	$C_6H_{13}NO_2$ $C_9H_{11}NO_2$ $C_{11}H_9NO_2$ $C_{13}H_{16}N_2O_4$	pentan-2-yl carbamate, 3-methylbutyl carbamate, ethyl N-propyl carbamate ethyl N-phenyl carbamate naphthalen-1-yl carbamate tert-butyl N-(1,3-benzodioxol-5-ylmethylideneamino) carbamate	99.86 99.94 99.74 98.77
20 (blood)	166.0775 232.1366	2.19 2.15	$C_9H_{11}NO_2$ $C_8H_{17}N_5O_3$	ethyl N-phenylcarbamate (3-azido-2-methoxypropyl) N-(ethylaminomethyl) carbamate	100.00 70.01
20 (urine)	288.2379	13.24	$C_{16}H_{33}NO_3$	[2-(hydroxymethyl)-2-methylpentyl] N,N-dibutyl carbamate, 2-hydroxypropyl N-dodecylcarbamate, 1-hydroxypropan-2-yl N-dodecylcarbamate	99.31
21 (blood)	166.0775	2.15	$C_9H_{11}NO_2$	ethyl N-phenylcarbamate	99.98

Post-mortem samples (blood, stomach content and urine) were re-analysed using the LC-MS/MS with protein precipitation method. In several of the post-mortem samples a peak with a monoisotopic mass of 289.1 was detected, which could be a possible match for terbufos due to the product fragments detected (Figure 3.48). A summary of the other post-mortem samples where a possible match for terbufos and other compounds were detected is shown in Table 3.8.

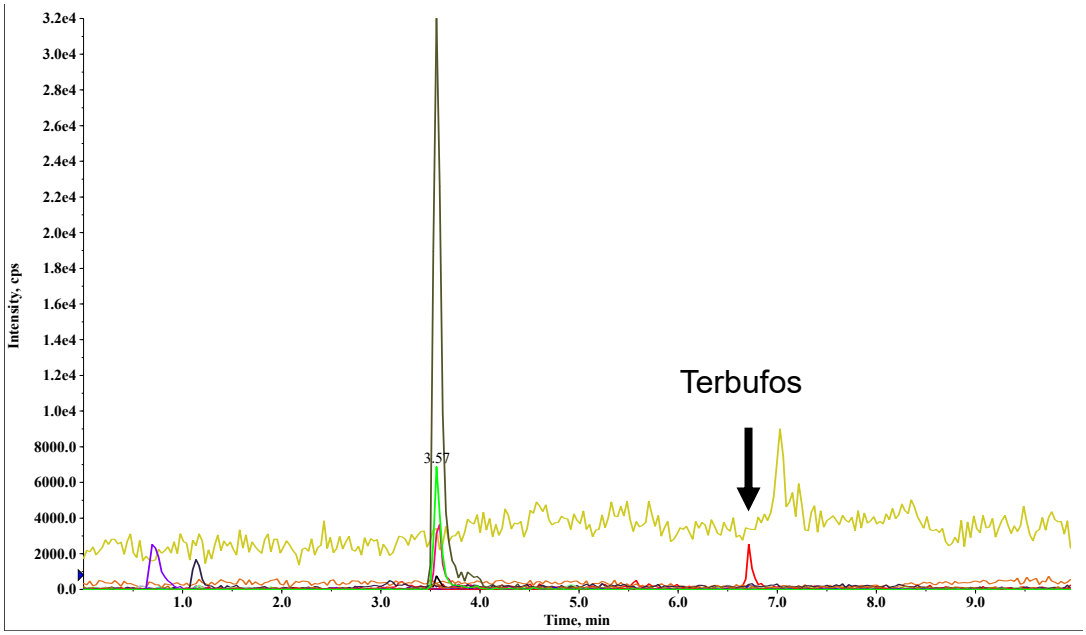


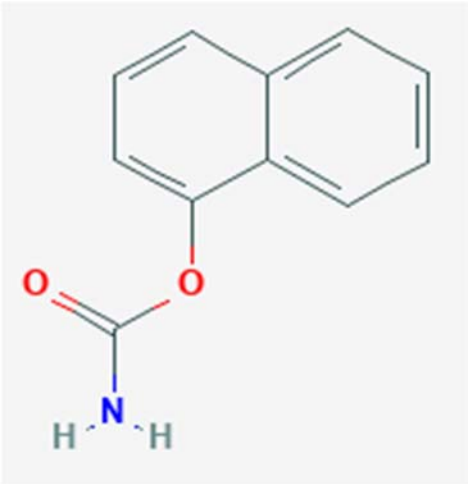
Figure 3.48: An extracted chromatogram showing a match of terbufos in stomach content of case 10.

The protein precipitation method used for preparing the blood and urine post-mortem samples was successful and provided better separation of the more polar lipophilic compounds. Elemental composition was determined for a number of peaks seen in the extracts and some of these detected compounds, in both blood and urine samples, were related to carbamate compounds. For example, (3-formylphenyl) N-methylcarbamate is associated with carbaryl. Ethyl N-phenylcarbamate is associated with lidocaine which is a local anaesthetic. Pentan-2-yl carbamate is associated with methomyl. Naphthalen-1-yl carbamate is associated with carbaryl, with the only difference in chemical structures of the compounds being that naphthalen-1-yl carbamate lacks an N methyl group (Figure 3.49).

Table 3.8: Possible compound matches detected in post-mortem samples using the LC/MS/MS.

Case number (sample type)	Possible compound match
4 (stomach content)	Aldicarb sulfoxide
5 (stomach content)	Terbufos and aldicarb sulfoxide
5 (urine)	Terbufos and aldicarb sulfoxide
10 (stomach content)	Terbufos
11 (blood)	Aldicarb sulfoxide
14 (stomach content)	Terbufos
16 (stomach content)	Terbufos
17 (blood)	Terbufos
18 (stomach content)	Terbufos
21 (stomach content)	Terbufos
22 (stomach content)	Terbufos
24 (stomach content)	Terbufos and aldicarb sulfoxide

A



B

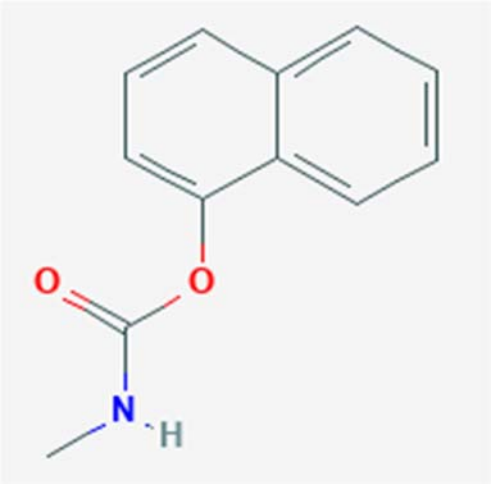


Figure 3.49: The difference in chemical structures of (A) Naphthalen-1-yl carbamate and (B) Carbaryl.[110] Permission for re-print provided under the National Center for Biotechnology Information PubChem database open-source copyright law.

Regarding the cases where there was no match of carbamate peaks, some patients were hospitalized where atropine (an antidote of carbamate poisoning) was infused intravenously

as an antidote prior to death. In these cases the toxin concentration may still have been high in selected matrices, such as vitreous humour, as the toxin could have been unevenly distributed.[81,122]

During the analysis of the stomach contents using LLE, up to ten different carbamates could be detected at low intensities from different cases. All cases showed consistent unidentified non-carbamate peaks. The detection of possible carbamate compounds in the post-mortem samples could be coincidental as many other compounds with the same molecular masses to these compounds exist. Street pesticides manufactured in clandestine laboratories may contain very high or low concentrations of active ingredients or any number of contaminants (adulterants and diluents) or no active ingredient at all.[123] For example, in one case a mass envelope suggestive of polyethylene glycol was identified. Polyethylene glycol chains are used in many compounds to increase solubility in aqueous media and in detergents indicating that a high concentration of a formulated pesticides may have led to the acute toxicity. The consistent peaks may represent adulterants and diluents commonly added to “backstreet” pesticides and may or may not contribute to the cause of death. This could also be one of the reasons that carbamates are not detected in most of the toxicology cases that were suggestive of carbamate poisoning.

The increase of online trading of the street pesticides at affordable prices has led to the easy availability of clandestine pesticides.[67,123,124] Informal trading has also led to the illegal importation and transportation of the pesticides to be misused and sold as poison even in countries where pesticides are strictly regulated.[67] Informal pesticide trading is prevalent in South Africa but is also reported in the USA and Brazil, where Chumbinho or *Tres Pasitos* is sold clandestinely.[26,50,67] The availability and illegal selling of street pesticide continues to be a public health problem. Due to the clandestine trade and lack of quality control of the pesticides, it is to be expected to find adulterants in street pesticides, such as anticoagulants, graphite, ground corn or sand, which may also influence toxicity of the product.[26,67,124]

During sample preparation and extraction of the street pesticides, the black or grey granules did not dissolve in any organic or aqueous solvent. The non-dissolvable black or grey

granules raised questions on the formulation and composition of these street pesticides. As street pesticides consist of many compounds and include crude synthetic intermediates, and several bulking additives, few laboratories have reference samples or libraries that can be used for comparison to suspect products.[123]

In medico legal cases, the forensic aetiology must consider the combination of the history of a case and the external and internal examinations at autopsy . The key external findings in all the post-mortem cases cited in this study included urination, defecation, emesis and bloody fluid in the mouth and nostrils. The key internal findings were dark coloured granules in the stomach. Internal organs were generally congested in most cases and gastric erosion was commonly noted. These are typical symptoms of poisoning. , Autopsy pathologists submitted blood samples to the National Health Laboratory Services for analysis of acetylcholinesterase levels for the different cases.

Liquid-liquid extraction of stomach content provided satisfactory results as consistent peaks were identified for the different post-mortem cases. Papoutsis *et al.*[22] compared different organic solvents during the optimisation of LLE method for pesticide extractions. Good recovery was observed when 4:1 v/v toluene/chloroform was used. In a study where the method development and validation of carbofuran analysis in biological samples was performed, 1:1 ether:chloroform was used as an organic solvent combined with phosphate buffered saline to enhance extraction.[5] Good recovery was observed from the biological samples used which included blood, liver, stomach content and vitreous humour. It should also be noted that different recoveries may be obtained depending on the LLE technique used.[5] The protein precipitation method using ice cold acetonitrile and methanol with ultrasonic extraction showed better extraction efficacy especially in post-mortem samples when compared to the other extraction methods. This result is also corroborated by a study where imatinib (a drug used to treat chronic myeloid leukaemia) was quantified in human serum using the protein precipitate method.[111]

The accurate mass q-TOF/MS analytical system is considered the “Gold standard” for untargeted screening for different compounds.[95] More information of precursor and product

ion fragments was obtained through the MS spectra in both targeted and non-targeted screening. In this study the post-mortem samples were successfully screened using both the validated targeted LC-MS/MS technique for the aldicarb and aldicarb oxidation products and the UPLC-q-TOF/MS for assessing possible other carbamate compounds in the biological matrices.

4 Conclusion, limitations and recommendations

4.1 Conclusion

The primary aim of this study was to assess the stability of aldicarb and its oxidation products in biological matrices under different storage conditions; including different temperature conditions and with or without preservatives. The secondary aim was to analyse samples of different biological matrices collected post-mortem from suspected carbamate poisoning cases to determine if aldicarb and its oxidation products could be detected. Method optimisation for the LC-MS/MS determination of up to ten common carbamate pesticides from a standard mix as well as carbamates present in street pesticides was successful. An LLE method used for extraction of gastric content produced satisfactory results with consistent unidentified peaks being noted in post-mortem cases. The simple solvent-based protein precipitation method provided acceptable recovery of the more polar lipophilic compounds from whole blood and plasma. A rapid, sensitive, and accurate method was developed and validated for the determination of aldicarb in whole blood, plasma and urine using the targeted LC-MS/MS method with the solvent-based protein precipitation method for sample preparation. The protein precipitation method gave good recovery for aldicarb and its two common oxidation products that still show toxic activity. The use of ethylenediaminetetraacetic acid alone as the anticoagulant appeared to be a better additive in whole blood and plasma samples with respect to the stability of aldicarb compared to sodium fluoride Boric acid proved to inhibit the degradation of carbamate compounds to some extent in urine samples.

Screening of street pesticides obtained from the street vendors in the Pretoria area confirmed the presence of toxic compounds belonging to the organophosphate pesticides but appeared not to contain aldicarb which could also account for some samples not showing aldicarb or aldicarb oxidation products in the post-mortem samples. The organophosphate pesticides block the same enzyme in nerve cell junctions as the carbamate pesticides which would show similar toxic effects of the pesticides.

Pesticide poisoning remains a major health concern with a high incidence of suicide cases using these substances (although unconfirmed analytically). As the circumstances and manner of death in the majority of post-mortem cases involved in this study were suggestive of suicide, it indicates the need for monitoring and controlling the usage and availability of pesticides in SA. Street pesticides are unregulated and appear to include toxic compounds other than carbamates despite label claims but still exhibit enhanced toxic effects of these products. The detection of other compounds in post-mortem samples provides an answer to one of the questions that arose during a retrospective study carried out on carbamate fatalities at the PMLL. This study has highlighted why a disparity exists between autopsy findings and analytical toxicology results obtained from the PFCL. It is advised that the PFCL perform untargeted screening as this may lead to the detection of compounds involved in pesticide poisoning cases as the methodology is sensitive enough to confirm the presence of toxic levels of the acetyl cholinesterase inhibitors. Although “two step” is reported to contain aldicarb, the street pesticides obtained and tested in this study had little aldicarb but showed high concentrations of carbofuran or terbufos (an organophosphate compound) and other compounds which could be toxic.

The fertilizers, farm feeds, agricultural remedies and stock remedies Act, 1947 (Act 36 of 1947 of the Republic of South Africa) stipulates the regulation or prohibition of the importation, sale, acquisition, disposal or use of fertilizers, farm feeds, agricultural remedies and stock remedies.[125] Section 7 of the Act further stipulates that no person shall sell any fertilizer, farm feed, agricultural remedy or stock remedy unless; (i) it is registered under the act and the name of a manufacturing company, (ii) the container in which it is sold, complies with the prescribe requirements and is sealed and labelled or marked in such manner as may be prescribed or, if it is not sold in a container, an invoice should be attached, (iii) it is of the composition and efficacy specified in the application for registration.[125] Street pesticides fail to meet any of the requirements of this Act. Violation of the Act results since pesticides are commonly decanted into common drinking or medicinal bottles and/or transparent plastic bags. Some of the pesticides sold on the streets are manufactured and packaged in China and are not registered in SA. South Africa is known as the “intersection” for drug shipments within the continental avenues. Lack of control and policing in SA has

resulted in the distribution of illicit drugs and pesticides from unrecognised or clandestine laboratories.[39] Illicit pesticides have also played a role in the restriction of the value of diagnostic capacity especially in the toxicology laboratories because of the contaminating synthetic byproducts.[39] Having the Toxicology Section controlled by the South African Police Services (SAPS) and not being affiliated with the National Department of Health Laboratories, has led to the lack of standardised diagnostic techniques and capability.[39]

It is still not clear how “two step’’ is commercialised as a rodenticide in SA, however this study has shown that it is most likely through the clandestine trading of illegally imported material. Enforcement of the law could assist in decreasing the availability of street pesticides as well as the restriction of the importation of the illegal products.

The development and validation of an analytical method to quantify aldicarb and its oxidation products (aldicarb sulfoxide and sulfone) using the solvent-based protein precipitation sample preparation method and targeted LC-MS/MS was successful. Analytical method validation to quantify the ten carbamate compounds using SPE and UPLC-q-TOF/MS was incomplete due to high variability of recoveries of the different polarities of the pesticides. However, the method was deemed feasible for the quantification of, carbofuran, carbaryl, methiocarb and propoxur based on the SPE cartridge elution profiles. Since carbamates can bind to erythrocytes and phospholipids in biological samples, this binding could have contributed to the compounds being detected at a very low intensities. Furthermore, pesticides are known to accumulate in adipose tissue of living organisms. The use of quick, easy cheap, effective, rugged, and safe (Quechers) dispersive SPE method in food and biological sample analysis results in the removal of lipids and coloured compounds from the matrix that leads to better extraction of a number of pesticides.[89]

Employing LLE using sodium chloride and n-hexane/ethyl acetate mixture allowed extraction of carbamates from stomach content. The protein precipitation method using acetonitrile and methanol showed higher extraction efficacy, especially for antemortem and post-mortem (whole blood, plasma and urine) samples compared to the other extraction methods.

Determination of elemental composition by means of accurate mass analysis and using the MassLynx software followed by PubChem database searching provided sensitive detection and positive identification of possible carbamate compounds in post-mortem samples.

The carbamate compounds were not stable at ambient temperature in any of the three matrices, whether EDTA as anticoagulant or boric acid as preservative for the blood and urine samples, respectively. The ideal storage temperature for both ante mortem and post-mortem blood samples containing carbamate compounds was found to be -80°C. Sodium fluoride was not found to lead to preservation of carbamate compounds especially in blood samples. Albumin is reported to hydrolyse carbamate and organophosphate pesticides.[45,90] In post-mortem cases of suspected pesticide poisoning, blood concentrations of the pesticides can decrease due to post-mortem interval.[90] Bacteria such as *B. cereus*, *P. aeruginosa* and different species of *Blastobacter, Arthrobacter, Achromobacter* and *Micrococcus* have the ability to break down a number of carbamate compounds.[45,76] The spreading of bacteria usually occurs during the putrefaction process of the body.[76]

Considering their different physicochemical properties and that several factors can influence the biodegradation of carbamate compounds, no extrapolation of results from one carbamate compound to another can be formulated. This is because one carbamate compound may be easily decomposed while others may be stable depending on the matrix.[56]

For laboratories where samples backup due to a lack of analytical equipment, pre-analytical parameters including storage conditions (temperature and length of storage) and sample preparation methodology are critical as these impact the reliability and accuracy of the final results.[126]

4.1 Limitations and recommendations

The validation of methods for many pesticides of the organophosphate and organochlorine classes in post-mortem blood have been reported, however, there is limited methods regarding analysis of carbamates in biological samples. Validation of a general method for

a number of common carbamates with a wide difference in polarities using the SPE method was not successful due to poor recovery of carbamate compounds in biological samples. There are a number of factors that could have led to poor recovery of the carbamate compounds from post-mortem samples. Diffusion and phospholipids and protein binding of the carbamate compounds and binding or penetration into the erythrocytes that is both concentration and time dependant can contribute to poor recovery of the more lipophilic carbamate compounds. Chemical or enzymatic instability of the more polar carbamate compounds can contribute to poor recovery as some of these compounds were not detected in the biological samples.

Further investigations with regards to the binding of carbamates to lipid fractions and erythrocytes as well as the chemical instability of carbamate compounds in biological matrices is required.

The analytical capacity exists in many academic and service laboratories in SA, but notable shortcomings are still encountered in forensic toxicology.[39] The validity of analytical results in long overdue cases becomes questionable, especially in the courts of law, highlighting the need for more infrastructure and capacity development in the forensic toxicology services in SA.

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Appendix I: Ethical Approval

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences

Institution: The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.

- FWA 00002567, Approved dd 22 May 2002 and Expires 03/20/2022.
- IORG #: IORG0001762 OMB No. 0990-0279 Approved for use through February 28, 2022 and Expires: 03/04/2023.

20 July 2020

Approval Certificate
Annual Renewal

Ethics Reference No.: 4/2018
Title: Assessing the carbamate decay kinetics in post-mortem intoxication cases with reference to matrix storage conditions

Dear Miss EDB Radebe

The **Annual Renewal** as supported by documents received between 2020-06-22 and 2020-07-15 for your research, was approved by the Faculty of Health Sciences Research Ethics Committee on its quorate meeting of 2020-07-15.

Please note the following about your ethics approval:

- Renewal of ethics approval is valid for 1 year, subsequent annual renewal will become due on 2021-07-20.
- Please remember to use your protocol number (4/2018) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely

Dr R Sommers
MBChB MMed (Int) MPharmMed PhD
Deputy Chairperson of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes, Second Edition 2015 (Department of Health)

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Fakulteit Gesondheidswetenskappe
Lefapha la Disaense tša Maphelo

Appendix II: Preparation of reagents and solutions

Chemicals and reagents

All general chemicals and solvents used were of analytical grade reagent or better purchased from reputable chemical vendors as indicated.

The reference standards of 10 carbamate pesticides (aldicarb, aldicarb sulfone, aldicarb sulfoxide, carbaryl, carbofuran, 3–hydroxycarbofuran, methiocarb, methomyl, oxamyl, propoxur) were purchased from RESTEK (SA, Lot number: A0135315). Aldicarb-D3 was used as a stable isotope labelled internal standard purchased from Toronto Research Chemicals; (Industrial Analytical, LGC Standards SA, Cat number TRC A514652, lot number: 7-JMR-163-1).

Oasis Prime HLB solid phase extraction cartridges were purchased from Waters (MicroSep, Johannesburg, SA). Street pesticides (in form of repackaged but unmarked black or grey granules) were purchased from the informal market (street vendors) in Pretoria. n-Hexane and ethyl acetate, sodium chloride (NaCl), and zinc sulphate ($ZnSO_4$) were purchased from Merck (Darmstadt, Germany). Acetonitrile (ACN), distilled (HPLC) water, methanol (MeOH) were mass spectrometry grade Romil products purchased from (MicroSep, Johannesburg SA). Ammonium acetate, ammonium formate, formic acid and isopropyl alcohol (IPA) were purchased from Sigma Aldrich (St Louis, Mo, USA).

Solutions

Preparation of ACN 0.1% formic acid buffer

A volume of 500 µL of formic acid was added into ACN into a 500 mL volumetric flask (Grade A) and accurately filled to the mark. The solution was transferred into a 500 mL Schott bottle for use as a mobile phase for HPLC.

Preparation of 0.1% formic acid in HPLC grade water buffer

A volume of 500 µL of formic acid was added into dH_2O in a 500 mL volumetric flask (Grade A) and accurately filled to the mark. The solution was transferred into a 500 mL Schott bottle for use as a mobile phase for HPLC.

Preparation of 10 mM ammonium acetate in deionized grade water buffer

A mass of 0.0708 g of ammonium acetate was weighed on an analytical balance (ATB 100-5NM, Kern, Balingen, Germany) using a weighing boat. The ammonium acetate was transferred into a 500 mL volumetric flask (Grade A). The volumetric flask was filled with deionized water till the calibration mark and mixed till dissolved completely. The solution was transferred into a 500 mL Schott bottle.

Preparation of 10 mM ammonium acetate in methanol buffer

A mass of 0.0708g of ammonium acetate was weighed using an analytical balance in a weighing boat. The ammonium acetate was transferred into a 500 mL volumetric flask (Grade A). The volumetric flask was filled with methanol till the calibration mark and mixed till dissolved completely. The solution was transferred into a 500 mL Schott bottle.

Preparation of 10 mM ammonium acetate with 0.1% formic acid in HPLC grade water buffer

A mass of 0.0708 g of ammonium acetate was weighed using an analytical balance in a weighing boat. The ammonium acetate was transferred into a 500 mL volumetric flask (Grade A) with about 400 mL HPLC water and 500 µL formic acid added. The volumetric flask was filled with HPLC water till the calibration mark and mixed till dissolved completely. The solution was transferred into a 500 mL Schott bottle.

Preparation of 10 mM ammonium acetate and 0.1% formic in methanol buffer

A mass of 0.0708 g of ammonium acetate was weighed using an analytical balance in a weighing boat. The ammonium acetate was transferred into a 500 mL volumetric flask (Grade A) with about 400 mL HPLC water and 500 µL formic acid added. The volumetric flask was filled with HPLC water till the calibration mark and mixed till dissolved completely.

The volumetric flask was filled with methanol till the calibration mark. The solution was transferred into a 500 mL Schott bottle.

Preparation of 2 mM ammonium formate and 0.2% formic acid buffer in HPLC water

A mass of 0.0630 g of ammonium formate was weighed using an analytical balance in a weighing boat. The ammonium formate was transferred into a 500 mL volumetric flask (Grade A) with about 400 mL HPLC water and 1000 µL formic acid added. The volumetric flask was filled with HPLC water till the calibration mark and mixed till dissolved completely. The volumetric flask was filled with HPLC water till the calibration mark. The solution was transferred into a 500 mL Schott bottle.

Preparation of 2 mM ammonium formate and 0.2% formic acid buffer in methanol

A mass of 0.0630 g of ammonium formate was weighed on an analytical balance using a weighing boat. The ammonium formate was transferred into a 500 mL volumetric flask (Grade A) with about 400 mL HPLC water and 1.0 mL formic acid added. The volumetric flask was filled with HPLC water till the calibration mark and mixed till dissolved completely. The volumetric flask was filled with MeOH till the calibration mark. The solution was transferred into a 500 mL Schott bottle.

Preparation of 10 mM ammonium formate and 0.2% formic acid buffer in double deionised H_2O

A mass of 0.630 g of ammonium formate was weighed on an analytical balance using a weighing boat. The ammonium formate was transferred into a 1000 mL volumetric flask with about 400 mL double deionised water and 2.0 mL formic acid added. The volumetric flask was filled with double deionised water till the calibration mark and mixed till dissolved completely. The solution was transferred into a 1000 mL Schott bottle.

Preparation of acetonitrile and 0.1% formic acid buffer

A volume of 1000 mL acetonitrile with 1 mL of formic acid was transferred into a 1000 mL Schott bottle.

Preparation of 2% NaCl solution

A mass of 10 g of NaCl was weighed using a weighing boat and an analytical balance (ATB 100-5NM, Kern, Balingen, Germany) and transferred into a 500 mL volumetric flask (Grade A). The volumetric flask was filled with double deionised water till the calibration mark and transferred to a Schott bottle.

Preparation of 500 mL n-hexane/ethyl acetate

A volume of 250 mL of n-hexane and 250 mL of ethyl acetate was placed in a measuring cylinder (Grade A) to make up 500 mL (1:1 V/V) of n-hexane/ethyl acetate. The solution was transferred into a 500 mL Schott bottle.

Preparation of 90:10 (500 mL) Acetonitrile (ACN): isopropyl alcohol (IPA)

A volume of 50 mL of IPA and 450 mL of ACN added into a measuring cylinder (Grade A) to make up 90:10 (ACN:IPA). The solution was transferred into a 500 mL Schott bottle.

Preparation of 5% methanol

A volume of 25 mL of MeOH was added to a measuring cylinder (Grade A) and double deionised water added to the 500 mL the mark. The solution was transferred into a 500 ml Schott bottle.

Preparation of 10% MeOH

A volume of 50 mL of MeOH and double deionised water added to the 500 mL mark in a measuring cylinder (grade A) and transferred into a 500 mL Schott bottle.

Preparation of 40% MeOH with 0.1% Formic acid

A volume of 200 mL of MeOH and 500 µL formic acid was added into a measuring cylinder (grade A). Double deionised water was added to the mark, mixed and transferred into a 500 mL Schott bottle.

50:50 MeOH:H_2O with 0.1% formic acid

A volume of 50 µL of formic acid was added into a 50 mL volumetric flask (grade A). Exactly 25 mL MeOH was added 25 mL and HPLC water added to the mark. The solution was transferred into a 100 mL Schott bottle and labelled.

5% MeOH with 0.1% formic acid (500 mL)

A volume of 25 mL of MeOH, and 500 µL formic acid was placed in a volumetric flask (grade A) with double deionised water added to the mark. The solution was transferred into a 500 ml Schott bottle.

MeOH with 20 mM ammonium formate (500 mL)

A mass of 0.600 g of ammonium formate was weighed on an analytical balance using a weighing boat. The ammonium formate was transferred into a 500 mL volumetric flask (grade A). The volumetric flask was filled with 25 mL of MeOH and double deionised water added till the calibration mark. The solution was transferred into a 500 mL Schott bottle.

Preparation of 0.9% saline (500 mL)

A mass of 4.5 g sodium chloride was weighed on an analytical balance using a weighing boat. The sodium chloride was then transferred into a 500 mL volumetric flask (grade A). The volumetric flask was then filled with double deionised water till the calibration mark. The solution was transferred into a 500 mL Schott bottle.

Preparation of 90% MeOH;0.1% formic acid

A volume of 450 mL of MeOH and 500 µL formic acid was placed in a 500 mL volumetric flask (grade A) then filled with double deionised water till the calibration mark. The solution was transferred into a 500 ml Schott bottle.

Preparation of 90% MeOH; 20 mM ammonium formate

A mass of 0.0631 g ammonium formate was weighed on an analytical balance using a weighing boat and transferred to a 500 mL volumetric flask that was then filled with double

deionised water till the calibration mark. The solution was transferred to a 500 mL Schott bottle.

Preparation of 90:10 ACN:IPA

A volume of 50 mL of IPA and 450 mL ACN was transferred into a 500 mL Schott bottle.

Preparation of 90:10 ACN:MeOH

A volume of 50 mL of MeOH and 450 mL ACN was transferred into a 500 mL Schott bottle.

Preparation of 50:50 Acetone:H_2O in a 100 mL Schott bottle

A volume of 50 mL of acetone and 50 mL H_2O was transferred into a 100 mL Schott bottle.

Preparation of 50:50 ACN:MeOH in a 100 mL Schott bottle

A volume of 50 mL of ACN and 50 mL MeOH was transferred into a 100 mL Schott bottle.

Preparation of 50:50 ACN: H_2O in a 500 mL Schott bottle

A volume of 250 mL of ACN and 250 mL double deionised water was transferred into a 500 mL bottle. The Schott bottle was labelled as 50:50 ACN:H_2O.

Preparation of 50:50 acetone:MeOH with 0.1% formic acid in a 100 mL Schott bottle

A volume of 50 mL of acetone and 50 mL MeOH with 100 µL of formic acid was added into a 100 mL Schott bottle.

Appendix III: Precursor and product ions of the ten carbamate compounds in positive electrospray ionisation (ESI +) mode

Table 1: Precursor and product ions of the ten carbamate compounds.

Carbamate compound	Precursor ion (m/z)	Product ions (m/z)
Aldicarb	208.1 $[M+NH_4]^+$	116.0 89.0
Aldicarb sulfone	223.1 $[M+H]^+$	86.1 148.0
Aldicarb sulfoxide	207.1 $[M+H]^+$	132.0 89.1
Carbaryl	202.1	145.1
Carbofuran	222.1	165.1
3 – Hydroxycarbofuran	238.1	220.1
Methiocarb	226.1	169.1
Methomyl	163.1	88.0
Oxamyl	237.1	72.0
Propoxur	210.1	168.1
Aldicarb-D3 (used as a stable isotope labelled internal standard)	211.2 $[M+NH_4]^+$	116.1 89.1

Appendix IV: Linearity and the concentration range of the aldicarb in solvent and serum

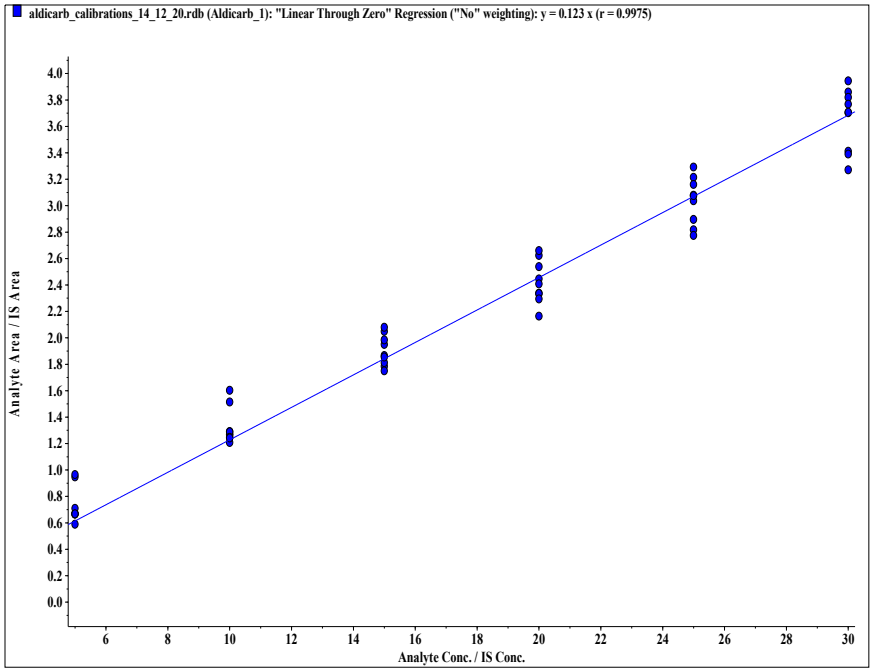

Figure A1: Aldicarb (mass 208.1/116.1) interday analysis.

Appendix V: Post-mortem cases

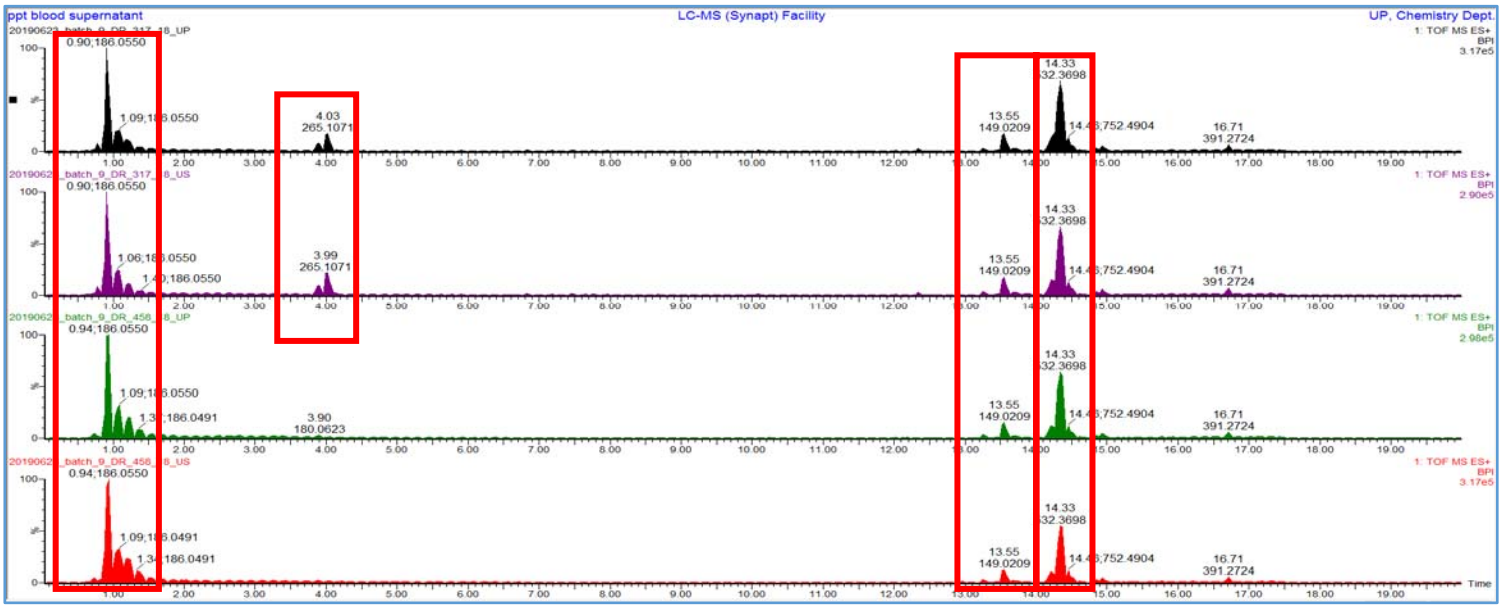

Figure A1: A series of chromatograms from post-mortem urine samples showing consistent unidentified peaks.

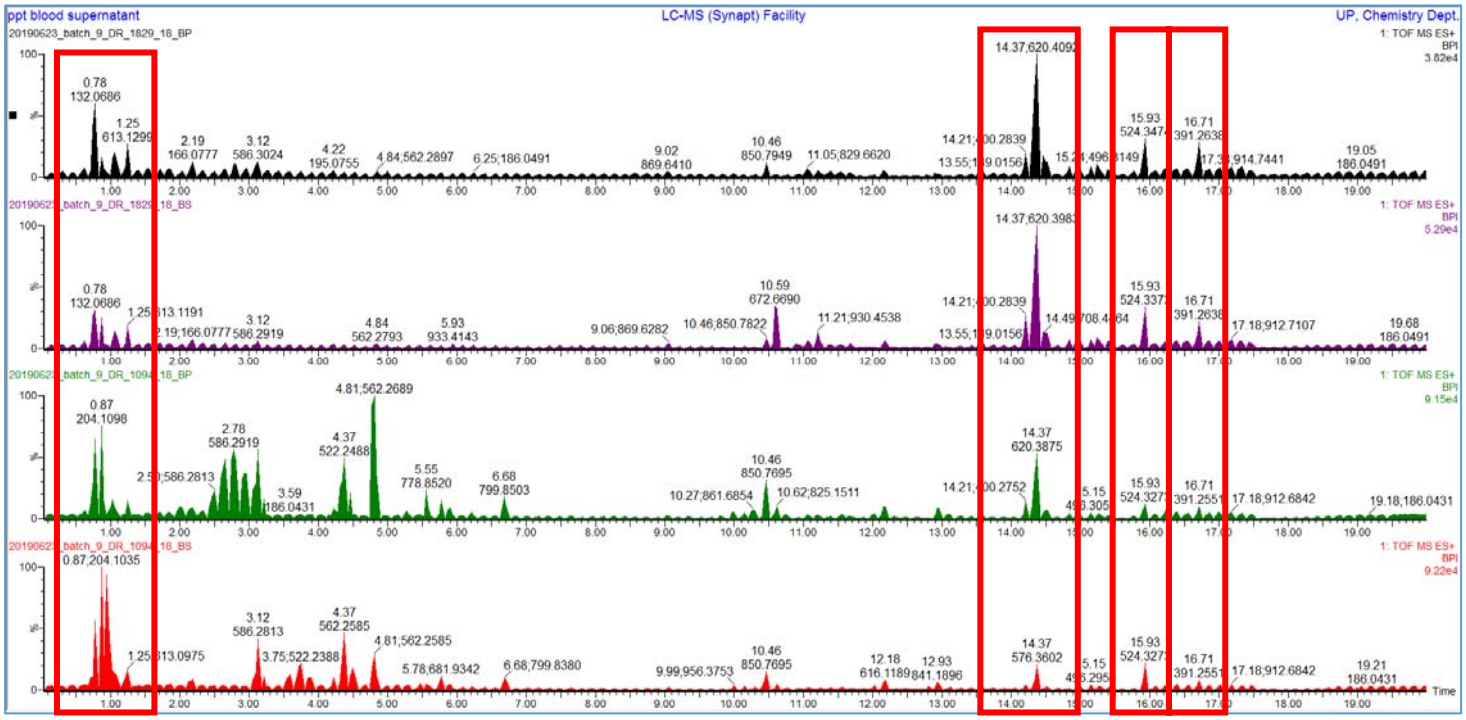

Figure A2: A series of chromatograms from different post-mortem whole blood samples showing consistent unidentified peaks.

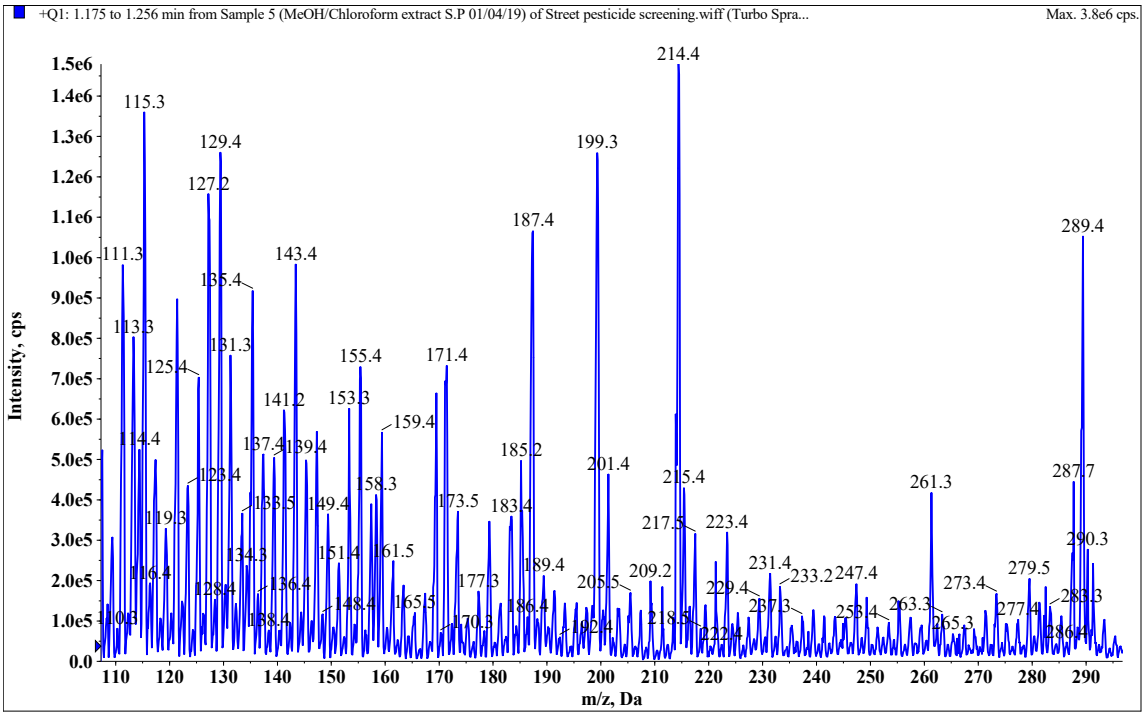

Figure A3: Detected compounds from the street pesticide extracts through direct infusion in the MS.

Appendix VI: SPE trouble shooting

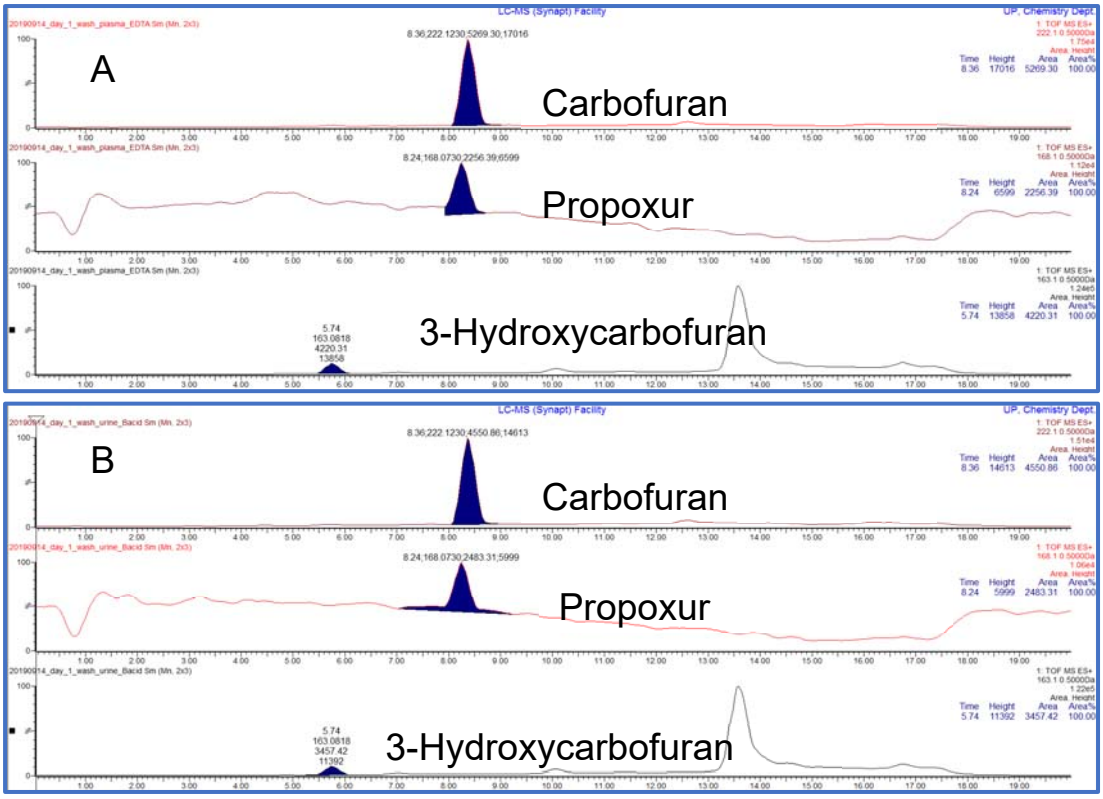

Figure A1: (A) Integrated extracted ion chromatogram of the three carbamate compounds in plasma wash fraction, (B) integrated extracted ion chromatogram of the three carbamate compounds in urine wash fraction from the SepPak C18 cartridge.

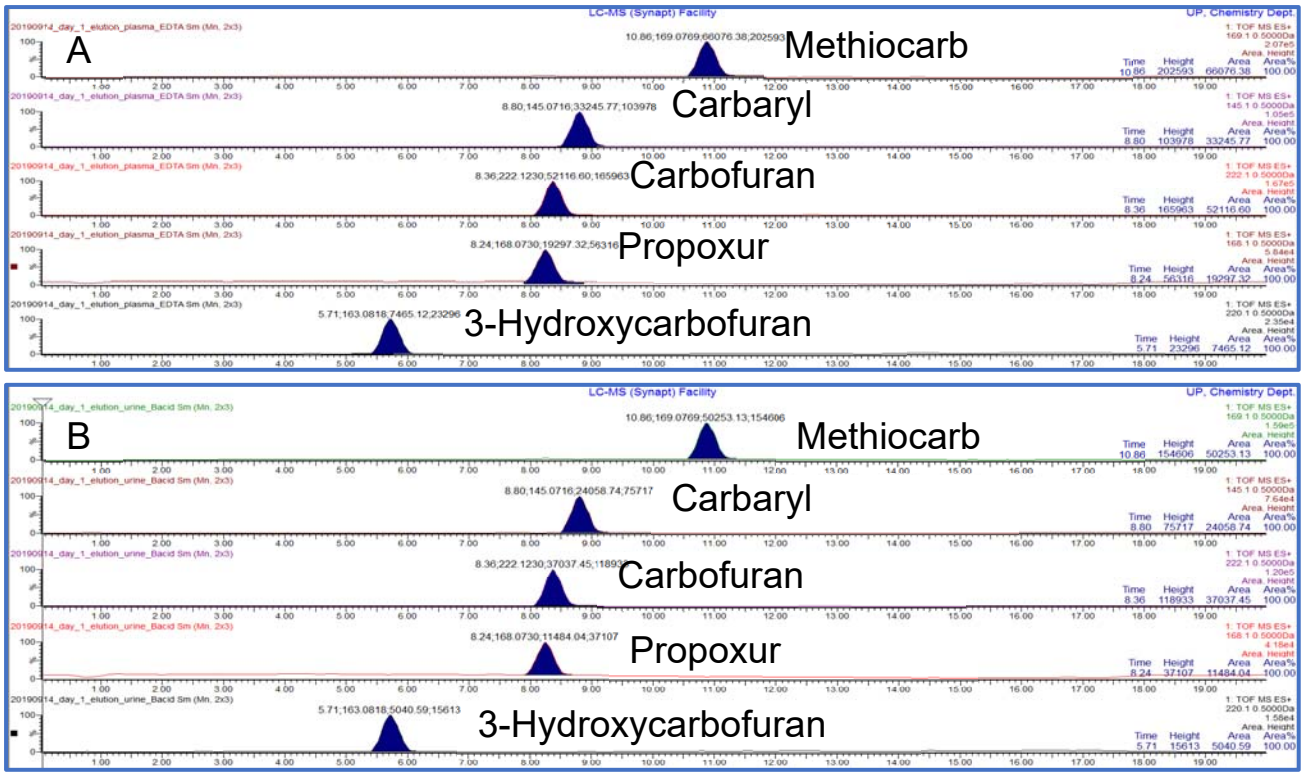

Figure A2: (A) Integrated extracted ion chromatogram of the five carbamate compounds in plasma elution fraction, (B) integrated extracted ion chromatogram of the five carbamate compounds in urine elution fraction from the SepPak C18 cartridge.