DEVELOPMENT OF A SOLID PHASE EXTRACTION-GAS CHROMATOGRAPHY METHOD FOR THE DETERMINATION OF ENDOCRINE DISRUPTING PESTICIDES IN WATER FROM WATER TREATMENT PLANTS.

A MINI THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

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ABSTRACT

A solid phase extraction (SPE) method for the gas chromatography analysis of pesticides was developed, optimised and validated for the simultaneous determination of pesticides from the Gammams water treatment plant (GWTP) and the Unique Janitorial & Maintenance Services (UJAMS) wastewater treatment plant (UWWTP). Parameters including, sample volume, elution solvent and ionic strength were optimised to improve the performance of the SPE method. After optimisation, the method gave good sensitivity with a detection limit ranging from 0.0009-0.0270 μg/mL, a good repeatability with a relative standard deviation ≤13 % and acceptable recoveries of 76 % and above. For most of the pesticides the method was linear in the range of 0.00625-0.15 μg/mL with R^2 values ranging from 0.9800-1.000, except for atrazine (**6**) and δ-HCH (**7**) where the R^2 values were 0.9558 and 0.9608, respectively. The validated method was subsequently applied to the determination of pesticides in water from the selected water treatment plants. Five herbicides namely, prometon (**5**), atrazine (**6**), alachlor (**9**), metolachlor (**11**) and butachlor (**14**) were detected in water samples from the GWTP inlet sampling point. Alachlor (**9**) and metolachlor (**11**) were present in the highest concentrations at 0.1858 and 0.2301 μg/mL, respectively. Prometon (**5**) and butachlor (**14**) were found to be present at concentrations of 0.0460 and 0.0100 μg/mL, respectively, while atrazine (**6**) was present at the lowest concentration of 0.0040 μg/mL. No pesticides were detected in water samples from the UWWTP, which could be attributed to the fact that the inflow of wastewater to this plant is strictly from industrial activities.

Keywords: pesticides, SPE, GC-FID

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LIST OF ABBREVIATIONS AND ACRONYMS

AchE	Acetylcholinesterase
AhR	Aryl Hydrocarbon Receptor
AP	Alkylphenol
AR	Androgen Receptor
BBP	Butyl Benzyl Phthalate
BPA	Bisphenol A
DBP	Dibutyl Phthalate
DCM	Dichloromethane
DDD	Dichlorodiphenyldichloroethane
DDE	Dichlorodiphenyldichloroethylene
DDT	Dichlorodiphenyltrichloroethane
DEHP	Diethylhexyl Phthalate
DEP	Diethyl Phthalate
DES	Diethylstilbestrol
DI-HS-SPME	Direct Immersion- Head Space-Solid Phase Microextraction
DINP	Di-Isononyl phthalate
DI-SPME	Direct Immersion- Solid Phase Microextraction
DLLME	Dispersive Liquid-Liquid Microextraction
DMP	Dimethyl Phthalate
DVB	Divinylbenzene
ECD	Electron capture detector
ECETOC	European Center for Ecotoxicology and Toxicology of Chemicals

ED	Endocrine Disruptor
EDP	Endocrine Disruptor Pesticide
EPA	Environmental Protection Agency
ER	Estrogen Receptor
EU	European Union
GABA	Gama Amino Butyric Acid
GC	Gas Chromatography
GC-FID	Gas Chromatography-Flame Ionisation Detection
GC-MS	Gas Chromatography-Mass Spectrometry
GSTP	Gammams Sewage Treatment Plant
GWTP	Gammams water Treatment Plant
GWRP	Gammams water Reclamation plant
HCB	Hexachlorobenzene
HCH	Hexachlorocyclohexane
HHG	Hypothalamic Pitituary Gonagal
HPLC	High Perfomance Liquid Chromatography
HS-SPME	Head Space-Solid Phase Microextraction
LC	Liquid chromatography
LC-MS	Liquid chromatography-Mass Spectrometry
LLE	Liquid-Liquid Extraction
LOD	Limit of Detection
LOQ	Limit of Quantification
LSE	Liquid-Solid Extraction
MS	Mass Spectrometry
NP	Nonylphenols

OCP	Organochlorine Pesticides
OPs	Organophosphorus
OP	Octylphenol
OPP	Organophosphorus Pesticides
PBB	Polybrominated Biphenyl
PBDE	Polybrominated Diphenyl Ethers
PCB	Polychlorinated Biphenyl
PDMS	Polydimethylsiloxane
PG	Progesterone
POP	Persistent Organic Pollutant
PPCP	Pharmaceuticals and Personal Care Products
PR	Progesterone Receptor
RSD	Relative Standard Deviation
SDME	Single Drop Microextraction
SGE	Sun Grid Engine
SPE	Solid Phase Extraction
SPME	Solid Phase Microextraction
TBT	Tributyltin
TCDD	Tetrachlorodibenzodioxin
TPhT	Triphenyltin
TTR	Transthyretin
UJAMS	Unique Janitorial & Maintenance Services
UNAM	University of Namibia
USGS NWQL	United States Geological Survey National Water Quality Laboratory

UV	Ultraviolet
UWWTP	UJAMS Wastewater Treatment Plant

DECLARATION

I, Hilaria Hakwenye, declare hereby that this study is a true reflection of my own research, and that this work, or part thereof has not been submitted for a degree in any other institution of higher education.

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..... Date.....04/04/2018.....

Hilaria Hakwenye

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CHAPTER ONE

1 INTRODUCTION

1.1 Orientation of the study

Numerous adverse health effects of endocrine disrupting compounds (EDCs) have been reported globally (1). In Namibia, a study was performed on sewage water (untreated and treated) from the Gammams Sewage Treatment Plant (GSTP) and the Goreangab Water Reclamation Plant (GWRP) using enzyme-linked immune-sorbent assays (ELISA) and chromogenic tests for the determination of natural hormones (also EDCs) (2). In this study, the level of estradiol in sewage treatment inlet was found to be 78 pg/mL, while the concentration of estrone ranged between 10 to 161 pg/mL and that of testosterone ranged between 162 and 405 pg/mL at the plant inlet. However, the presence of other types of potential EDCs, such as pesticides was not determined. Currently the types of pesticides present in water sources around Windhoek have not been identified. In Windhoek pesticides and herbicides are used for the removal of weeds in paved areas, such as car parks, road pavement, sport facilities etc. Pesticides and insecticides are also used in commercial farming in the areas of Tsumeb, Grootfontein and Otavi as well as the Okavango and Zambezi regions. Rain water and floods flush contaminants including pesticides from these areas into the downstream, distributing pesticides in rivers which later may be percolated down into aquifers (3). The European Union (EU) directive on the quality of drinking water has set the maximum permissible concentration of individual

pesticides at 0.0001 μg/mL and the total concentration of all pesticides at 0.0005 μg/mL (4,5).

1.2 Statement of the problem

Windhoek is the most heavily populated city in Namibia, with highly diversified industrial activities. Common water contaminants in Windhoek water sources include pesticides, heavy metals, industrial chemicals and suspended soil particles (6). Even though persistent pesticides (of which mostly are endocrine disruptors) have been banned in Namibia, there is evidence that they were imported into the country as late as 2013 (7), and therefore might still be present in the environment including water sources. Nowadays pesticides are mostly used in the agricultural sector which is among the primary industries in Namibia. Currently, it is not known which endocrine disrupting pesticides (EDPs) and other contaminants are present in the water sources around Windhoek. In addition, the levels at which these contaminants are present and the potential risk to the health of the population is unknown. A good starting point is to assess whether or not pesticides are present in the water treatment plants in Windhoek where water from this whole area is received. A mixture of halogenated pesticides and pesticides containing amine groups were chosen for this study to cover a wide range of expected pesticide contaminants in Namibian water sources. To the best of our knowledge no solid phase extraction (SPE) method was previously developed for the particular mixture of pesticides analysed in this study. Hence, this study was aimed at developing a method for the determination of these EDPs present in water from water treatment plants in Windhoek.

1.3 Objectives of the study

The main objectives of the study were to:

a). Develop and validate a solid phase extraction (SPE) method for the extraction of pesticides from water.

b). Apply the validated method to the determination of pesticides present in water samples from the water treatment plants, UWWTP and GWTP, using gas chromatography-mass spectrometry (GC-MS) and gas chromatography-flame ionization detection (GC-FID).

1.4 Significance of the study

By determining which EDPs are present in a number of water sources in Windhoek and at what concentrations they occur, the risk of these pollutants to the health of affected communities can be assessed. In addition, the analytical method developed in this study can be implemented in the Analytical Service Laboratory at UNAM for the routine determination of EDPs and other organic pollutants in water samples sourced from all over Namibia.

CHAPTER TWO

2 LITERATURE REVIEW

2.1 The endocrine system

The endocrine system is a combination of glands and the hormones produced by the glands, which are essential for biological reproduction, growth, development, and behaviour of animals as well as humans. An endocrine system is found in almost all animals, mainly mammals, non-mammalian vertebrates such as fish, amphibians, reptiles, birds and invertebrates: snails, lobsters, insects and other species. Endocrine systems keep human and animal bodies in balance, maintaining homeostasis and guiding normal growth and development. The main glands of the endocrine system are the hypothalamus, pituitary, thyroid, parathyroid, adrenals, pancreas, pineal body, and the reproductive organs (ovaries and testes) as shown in Fig 2.1 (8). These glands produce and pump hormones into the blood to act as the body's chemical messengers where they direct communication and coordination among other tissues throughout the body (9). Hormones are peptide or proteins, lipids or amino acid derivatives that are produced and secreted into the body by different glands to cause reactions within respective receptors (10). The unifying nature of hormones is the existence of specific receptors on target cells, which bind a specific hormone with high attraction and stereo-specificity (11).

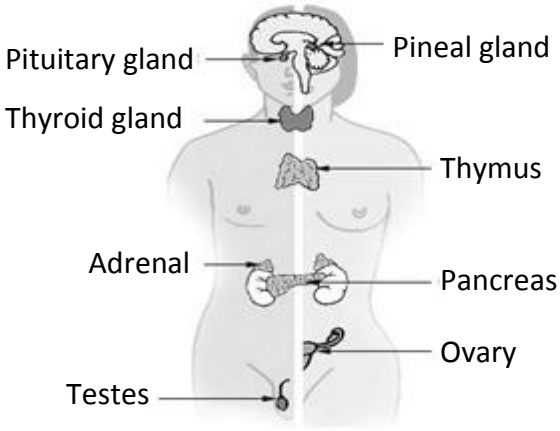


Figure 2.1 Main endocrine glands in humans (8)

2.2 Endocrine Disrupting Compounds (EDCs)

An endocrine disruptor is an exogenous substance or mixture of substances that changes the functions of the endocrine system and as a result cause adverse health effects in an organism (12,13). For a compound to be classified as an endocrine disruptor, the mechanism of action should be known or at least be suggested (14). The European centre for ecotoxicology and toxicology of chemicals (ECETOC) was established to identify and evaluate endocrine activity, and to provide scientific criteria that can be used in this regard (15). More than 70 000 chemicals have been identified to be EDCs (16).

EDCs mimic or block the action of natural hormones, hence affecting biological functions in organisms (13). A mimicking action also known as an agonistic effect happens once a chemical bind to a cellular receptor, leading to an unwanted response by initiating the cell's normal response to the natural occurring hormone at the wrong time or to an excessive extent (17). Blocking on the other hand, happens when EDCs bind to the receptor without activating it and its presence on the receptor preventing the binding of the natural hormone (antagonistic effect) (17).

Table 2.1: A list of potential EDCs (11)

Compound class	**Compound**	
Pesticides (synthetic)	Alachlor Aldrin Atrazine Benomyl Butachlor Carbaryl α-hexachlorohexane (α-HCH) β-hexachlorohexane (β-HCH) γ-hexachlorohexane (γ-HCH) 4,4'-Diclorodiphenyltrichloroethane (4,4'-DDT) and its metabolites 4,4'-Dichlorodiphenyldichloroethylene (4,4'-DDE) 4,4'-Dichlorodiphenyldichloroethane (4,4'-DDD) Dicofol Dieldrin/aldrin endosulfan α -endosulfan β-endosulfan Endrin aldehyde Endrin	Kepone (Chlordecone) Lindane Malathion Mancozeb Methomyl Methoxychlor Metolachlor Mirex Parathion Pentachlorophenol Permethrin Prometon Simazine Toxaphene Trifluralin Vinclozolin Heptachlor Heptachlor-epoxide Hexachlorobenzene (HCB) Iprodione

Organohalogens (synthetic)	Dioxins and furans Polychlorinated Biphenyls (PCBs) 2,4-Dichlorophenol	Polybrominated biphenyls (PBBs) Polybrominated diphenyl
Alkylphenols (synthetic)	Nonylphenols Octylphenols Pentaphenols	Nonylphenol ethoxylates Octylphenol ethoxylates Butylphenols
Heavy metals	Cadmium Lead	Mercury Arsenic
Organotins (synthetic)	Tributyltin (TBT)	Triphenyltin (TPhT)
Phthalates (synthetic)	Di-ethylhexyl phthalates Butyl benzyl phthalate Di-n-butyl phthalate Di-n-pentyl phthalate	Di-hexyl Phthalate Di-propyl phthalate Dicyclohexyl phthalate Diethyl phthalate
Pharmaceuticals (synthetic)	Diethylstilbestrol (DES)	Tamoxifen
Phenols (synthetic)	Bisphenol A	
Aromatic hydrocarbons (synthetic)	Benzo(a)pyrene Benza(a)anthracene Benzo(b/h)fluoranthene	Anthracene Pyrene Phenanthrene
Synthetic hormones	Ethynylestradiol Mestranol	Androgens
Natural hormones	17β-Estradiol Estrone	Estriol Testesterone

(natural EDCs)		
Phytoestrogens **(natural EDCs)**	Isoflavonoids Coumestans	Zearalenone β-sitosterol

EDCs may disturb endocrine functions by interfering with the synthesis, secretion and signalling of peptide as well as steroid hormones (18). They bind to the protein responsible for transport in blood, thus altering the amount of natural hormones that are present in circulation and this may interfere with the metabolic processes in the body that affect the synthesis, or rates of breakdown and release of natural hormones (17). More modes of action of EDCs on the natural hormones in an organism are listed in Table 2.2. Experiments performed on animal models revealed that early prenatal and/or perinatal exposure to EDCs could lead to long-term effects on reproduction and development, which can become evident later at sexual maturity or at adulthood (19).

Table 2.2: General classes of EDCs present in the food and the environment with the effect and mode of action on female reproductive system (19).

Chemicals	**Pathways of exposure**	**Mode of action**
PCBs	Food chain (fat-containing food, e.g. milk and derivatives, fatty fish etc), living environment	Change steroid hormone metabolism/transport, capability to bind with thyroxin transport protein, transthyretin (TTR), interaction with thyroid hormone receptors, neuroendocrine effects
Dioxins and dioxin-like PCBs	Food Chain (fat-containing food), living environment	Aril hydrocarbon receptor interaction leading to changed steroid hormone metabolism and neuroendocrine effects
DDT and metabolites	Food chain, living environment and workplace (esp. in developing countries)	Mainly estrogenic activity
Triazoles, Imidazoles	Food chain (agricultural and zoo-technical fungicides) workplaces (agricultural area)	Inhibition of steroid hormone biosynthesis
Triazines	Food chain (herbicides, living environment and workplaces (agricultural	Effects on HHG (hypothalamic pituitary gonagal) axis

	area)	
Ethylenethiourea (ETU) **Benzimiadazoles**	Food chain (agricultural and zootechnical fungicides), living environment and workplaces (agricultural areas)	Thyreostatic effects
Industrial products and daily-use-products **Nonylphenols and octylphenols**	Detergent by-products: food chain (seafood) and consumer products	Estrogen agonists-ER (estrogen receptor) alpha
Organochlorine insecticides	Food chain, living environment and work place	Homeostasis of steroid hormones (estrogenic and or anti-androgenic effects, interaction with PR)
Bisphenol A	Food chain (e.g. food in contact with plastics), consumer products (deodorants, adhesive etc.)	Estrogen agonist-ER alpha
Some phthalates	Food chain (food in contact with plastics), consumer (deodorants, adhesive etc.),	Agonists of pregnane X receptor (PXR), effects on steroid hormone biosynthesis
Polybrominated flame retardants	Food chain (milk, fatty fish), ecosystem, factories, electronic devices etc.	Interaction with PXR leading to changed steroid thyroid hormone homeostasis
Organotins	Food chain (sea food), products such as antifouling agents	Aromatase inhibition

Perfluoroctane Sulphonate	Food chain (bioaccumulation in animal tissues), products such as plastics, carpets	Change HHG axis
Parabens	Major cosmetics, toiletries and pharmaceutical preservatives	Estrogen agonist-ER alpha and beta
UV-screen (benzophenone 2, 4-methylbenzylidene camphor)	Cosmetics used to protect against UV radiation	Estrogen agonist-ER alpha
Cadmium	Food chain (flour, rice, sugar seafood), cigarette smoking	Estrogen agonist-ER alpha
Phytoestrogens Isoflavones, lignans etc.	Food chain (vegetables, soy-based food), cosmetics	Selective ER modulators high affinity

EDCs can enter the environment through direct discharge or through the use of pharmaceutical and chemicals in households, farming activities and industries (13). There are various types of EDCs found in the air, soil and water, which fall into two broad categories, synthetic and natural (Table 2.1). Synthetic EDCs consist of xenobiotic substances such as persistent organics pollutants (POPs), pesticides, synthetic hormones and phenol compounds which are widespread in food chains and in the environment (19). These pollutants are also present in a large number of products commonly used in households, including some plastic bottles and containers, liners of metal food cans, detergents, flame retardants, food, toys, cosmetics and pesticides (9). Natural EDCs are the natural steroid hormones, a group of biologically active compounds bio-synthesised from cholesterol and contain a cyclopentano-perhydrophenanthrene ring (20).

2.2.1 Organohalogens

Organohalogens are a group of organic compounds that contain at least one halogen (such as, fluorine, chlorine, bromine or iodine) bonded to a carbon atom (21). Organohalogens such as polychlorinated biphenyls (PCBs), dioxins, furans, and polybrominated diphenyl ethers (PBDEs), are classified as EDCs. PCBs, dioxins, furans and PBDEs may disrupt the normal endocrine function by different mechanisms. Exposure to PCBs causes fatty liver, hyper-excitability and infertility (22). In general, PCBs and dioxins are found to have estrogen-disrupting (agonistic or antagonistic) effects (23). Dioxins bind to the cytoplasmic aryl hydrocarbon receptor (AhR) in turn interfering with the steroids nuclear receptors (10,19). The pharmaceutical and personal care product (PPCP) triclosan, once released into wastewater reacts with residual chlorine leading to the formation of chlorine derivatives which once photo-degraded form a toxic dioxin, 2,3,7,8-tetrachlorodibenzodioxin (2,3,7,8-TCDD) (24). PBDEs and PCBs are discharged into sewage systems from households, as well as industrial and commercial facilities (8), while dioxins and furans enter water through forest fires, volcanic activity, incinerations, industrial activities and the combustion of fossil fuels (25). The manufacture of chlorinated pesticides can also be a source of dioxin pollution (26).

2.2.2 Phenols and alkylphenols

Phenols (or phenolics) and alkylphenols (APs) are synthetic compounds, a class of weakly acidic water-soluble compounds related to the organic chemical compounds also naturally present in most foods (23). Phenolic compounds are some of the lignin degradation products which can leach down from plant foliage/litter into the

ecosystem (27). APs primarily have an antagonistic effect on estrogenic receptors (14). They bio-accumulate and may cause estrogenic effects to aquatic organisms (25). In the study performed by Meier (23) it was determined that high concentrations up to 220 μg/mL of APs may be found in fish, mussels and rats.

One phenolic compound of particular relevance is bisphenol A (BPA), an ingredient commonly used in the production of many plastics and resins used for the manufacture of food and drink containers, the internal linings of cans and dental enamels (9,28). The Ministry of the Environment of Japan has classified BPA as a compound with endocrine disruptive action in fish and shellfish. In addition, endocrine disruption effects by BPA in mammals (rats and mice) and estrogen-like activity have been reported (29). BPA was detected in streams or rivers, bridge run-off and storm water at high concentrations of 0.00914 μg/mL in King County (USA) (30). BPA has also been detected in human urine in the United States, while phthalates used as plasticisers were found in breast milk (31). Another phenol is 4-nitrophenol, used in the manufacture of drugs (e.g. acetaminophen), fungicides, consumer dyes and methyl and ethyl parathion insecticides (32). Exposure to this phenol occurs mainly through contaminated air, water and soil (32).

2.2.3 Heavy metals

Several metals such as cadmium, chromium, cobalt, copper and lead possess potential endocrine disruption properties in humans and may possibly be present in drinking water (11). Cadmium is found in soil, surface, and ground water through contamination from metallurgical and electroplating industries (32). Cr occurs naturally in the earth's crust and it can be found in all environments where it is released from the metal industries, or from the combustion of coal and oil and from

cement work (32). High levels of Cr exposure have been reported to cause adverse health effects including liver and kidney toxicity as well as lung and sino-nasal cancers (32). Co occurs naturally in the environment, it is used in gas turbine aircraft engines. Other sources of Co include burning of fossil fuels, fertilizers, mining and smelting of Co ores (32). Exposure to high levels of Co is reported to cause asthma, interstitial lung disease and wheezing (17,33).

Cu occurs naturally in the environment and is utilised as a pure metal or an alloy. Copper sulphate is used as fungicides, algicide and as a nutritional supplement. Too much exposure of Cu has negative health effects including liver and kidney damage, anaemia and immunotoxicity (32). Sources of Pb are batteries, gasoline, paint, rubber, and plastics. Exposure to Pb causes various neurological disorders for instance, in children it prevents the development of the brain cells. And it also inhibits the absorption of iron by the human body, resulting in anemia (34). Humans can be exposed to heavy metals through consuming contaminated foods such as meat fish and fruits, or from direct skin contact with heavy metal or consumer products containing the chemicals (15).

2.2.4 Phthalates

Phthalates are a group of industrial chemicals that are used in a wide range of products including cosmetics, toys, car interiors and medical devices (18,35). A number of phthalates namely, dibutyl phthalate (DBP) diethyl phthalate (DEP), dimethyl phthalate (DMP), di-ethylhexyl phthalate (DEHP), di-isononyl phthalate (DINP) and butyl benzyl phthalates (BBP) are classified as EDCs (Table 2.1). DBP is commonly used as a polymer plasticizer, in adhesives and printing inks. DBP may be present in large number of products including food wrap and packaging (32,36).

DEP and DMP are used mainly in cosmetic productions, while DEHP and DINP are used in plastic products (18,37). Phthalates are frequently detected in fatty food from packaging or leaching from medical devices (35).

DBP, DEHP and DHP compete with 17β-estradiol to bind to the estrogen receptor (35). Phthalate esters have an inhibitory effect on Sertoli cells (found in seminiferous tubules) (14), thus it affect fertility in males. It was established that prenatal exposure to phthalates resulted in negative effect on anti-androgenic activity (18,38). Phthalates enter the environment through leaching and incinerations (35).

2.2.5 Pharmaceuticals and Personal care products

Pharmaceutical and personal care products (PPCPs) are a group of compounds which include pharmaceutical drugs, ingredients in cosmetics, food supplements and active ingredients in soaps, detergents, perfumes, skin, hair and dental care products (39), plus their respective metabolites (28). PPCPs are persistent and biologically active compounds classified as EDCs (33). Pharmaceuticals are drugs ranging from analgesics and antibiotics and contraceptive to lipid regulators. The highest sources of PPCPs are sewage effluents, hospital waste, and animal waste. PPCPs detected from sewage treatment plants include clofibric acid, naproxen, ibuprofen, caffeine, triclosan and antibiotics which are not entirely removed during water treatment (11). Several studies have detected residues of PPCP and their metabolites in sewage effluent and aquatic environments which may affect water quality and potentially impact drinking water supplies (11,28). A study done by Kasprzyk-Horden, Dinsdale & Guwy (33) found PPCPs in river water and effluents at concentrations ranging from 0.1-300 μg/mL and 30-800 μg/mL, respectively. A variety of PPCPs has been reported in surface water and wastewater treatment (40).

2.2.6 Synthetic Steroids

Steroids are important sex hormones in animals and humans. Synthetic steroids, a group that mainly consist of oral contraceptives as well as steroids used for the substitution therapy during menopause, physiological replacement therapy in deficiency states and treatment of cancers (41,42). Steroids, namely 17α-ethynyl-estradiol (EE2) (the main ingredient in contraceptive pills) (41) and conjugated estrogens are commonly detected in recycled water and municipality wastewater treatment plant effluents (25). Several studies reported the occurrence of steroids in wastewater treatment plant inlets, which are effectively removed during water treatment processes such as ozonation (2,25,43–45).

2.2.7 Natural endocrine disrupting compounds

There are two kinds of natural EDCs namely, organisms' and plants' estrogenic hormones. Organisms natural estrogenic hormones are synthesised from reproductive and adrenal glands (41,46). Natural hormones such as 17α- and 17β-ethynyl-estradiol (EE2), 17α- or 17β-estradiol (17α-E2 or 17β-E2) and estriol (E3) (35) have been identified as endocrine disruptors (EDs) (47). These steroids have negative health effects on the reproductive system of the exposed organism (48).

Laboratory studies concluded that steroid estrogens including EE2 can cause inter-sex, a group of conditions where there is an inconsistency between the external genitals and the internal reproductive organs of an organism (e.g. with gonads containing male and female tissues and/or feminized reproductive ducts) (31,49). The presence of steroids in water has been reported to affect growth and development, decrease fertility and feminization in aquatic life, and these effects

may be passed on to the future generation (50). High concentrations of estrone and 17β-E2 were reported in sewage outflow in King County up to 0.000056 μg/mL and 0.000141 μg/mL, respectively (31).

Plants estrogenic compounds are called phytoestrogens, they are natural hormones found in plants and fruits. The Environment Protection Agency (EPA) considers phytoestrogens as potential EDCs, because of the effect they have on the endocrine system (10). There are a number of phytoestrogens including, genistein, daidzein, coumestrol, and β-sitosterol (25). Genistein and daidzein are mostly found in soy-derived products (49). Since some baby products are made of soy, there has been detection of phytoestrogens in these products and this is of great concern. (51). It was found that prenatal exposure to genistein decreases progesterone (PG) levels in adult female rats but not in males, it also impedes allopregnanolone in invertebrates which could result in psychiatric disease (28). In the human body phytoestrogens can imitate estrogens, though the reactions are not favourable. For instance they can provoke estrogenic and anti-estrogenic activities intersecting cell communication pathways (52). Phytoestrogens can also interrupt brain sexual differentiation, which is likely to affect the reproductive physiology and the behaviour in future of the exposed animal or human (51).

Natural hormones are deposited into water sources through recycled, septic systems, aquaculture, and application of sewage sludge and manure to farming land (25). Natural estrogens contained in effluents are normally effectively removed by biological process (53).

2.2.8 Pesticides

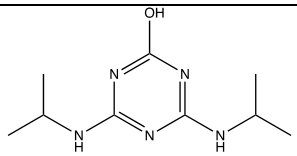
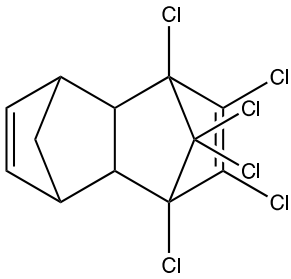
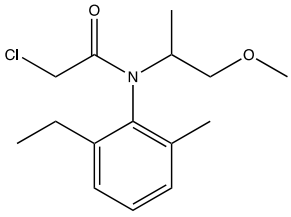
Pesticides are generally described as chemicals used in the agricultural sector for protecting plants from pests, insects and unwanted weeds (54). Several pesticides, which include fungicides, insecticides, and herbicides, are classified as endocrine disrupting compounds (Table 2.1). The two main groups of pesticides are organochlorine and organophosphate pesticides (55) which are broadly used in agricultural practices. Their residues may be found in food crops (56) and they are also one of the sources of water contaminants that pose serious threats to human and animal health. The use of pesticides triggers changes in the ecosystem, with harmful consequences to the environment and agriculture due to the spreading of new pests and diseases (57).

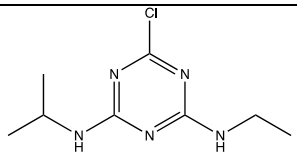
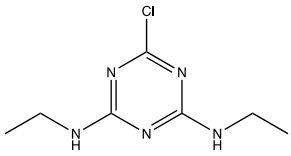
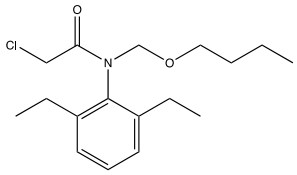
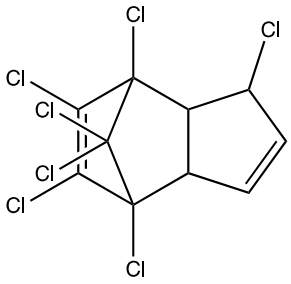
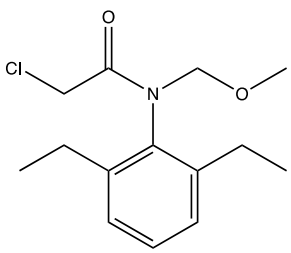
There is a connection between human occupational exposure to some pesticides during pregnancy and consequent stillbirth, as well as testicular cancer (58). Carbamate pesticides inhibit acetylchlolinesterase, which could affect nerve impulse transmission, provoking dramatic toxicological effects to the unborn child. Furthermore, carbamates and their metabolites are found to be carcinogens and mutagens (59). Chlorpyrifos, fonofos and phorate strongly inhibit the cytochrome enzymes, CYP1A2 and CYP3A4, that are responsible for the metabolism of estradiol, estrone and testosterone in the liver (51). Hence, it is possible that exposure to these compounds can interfere with the metabolism of steroid hormones, disturbing the normal hormonal balance which may contribute to increased chances of developing prostate cancer (51).

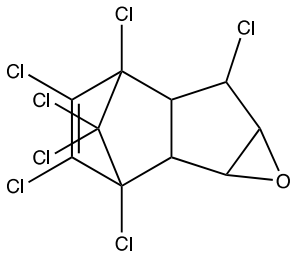
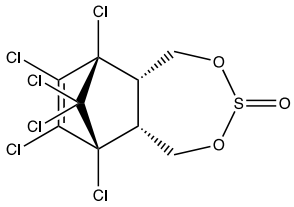
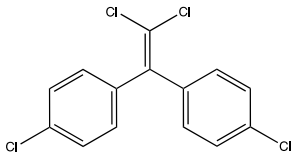
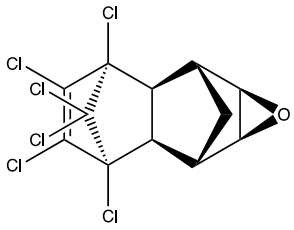
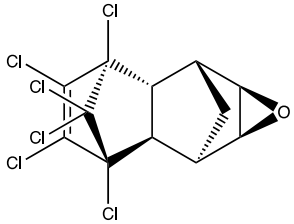
The fungicides vinlozolin and procymidone and the herbicide linuron block the androgen receptor and also disturb the neuroendocrine regulation of estrogenic

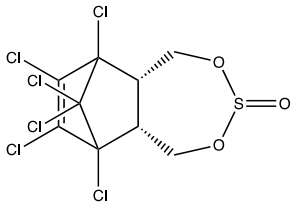
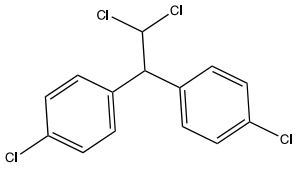
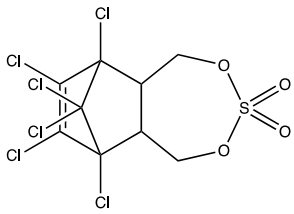
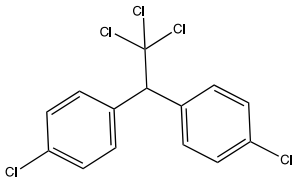
production (14). Atrazine, a chlorotriazine herbicide, is an endocrine disruptor which can inhibit peptide hormone synthesis (18). In addition, simazine and other triazines are bio-persistent, and humans and animals are exposed to these chemicals through contaminated food and drinking water. A list of pesticides with their modes of action and effects are listed in Table 2.3.

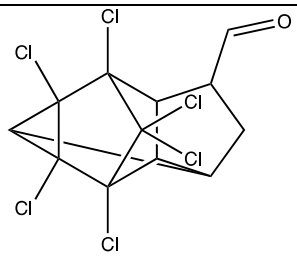
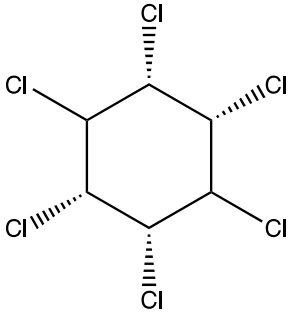
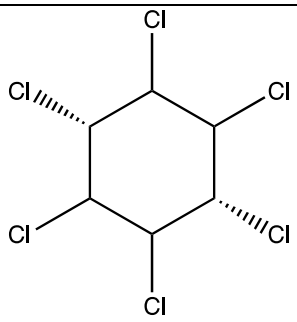
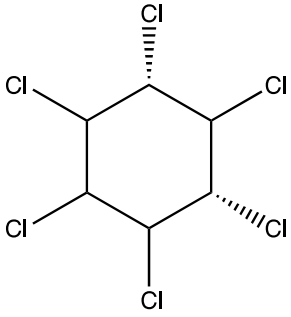
Table 2.3: Properties, structures and endocrine disruption effects of the pesticides analysed in the current study (12,18,60,61)

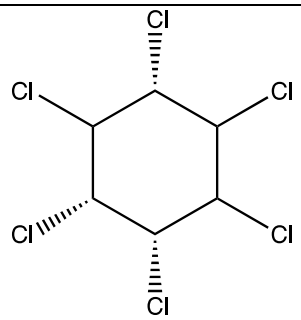
Pesticides	**Chemical structure**	**Endocrine disruptor effect**
Prometon MW=225.0g/mol pKa =4.3 log K_{ow}= 2.99		Embryo-toxin
Aldrin MW=364.909g/mol pKa= n.a log K_{ow} = 6.50		Nerve poison. Attacks ganglia. Competes with androgen receptors.
Metolachlor MW= 283.8g/mol pKa= n.a log K_{ow} = 3.13		Activation of pregnane X cellular receptor.
Atrazine MW=215.685g/m		Inhibit androgen, weakening estrogenic effect.

ol pKa=1.6 log K_{ow} =2.61		Damages adrenal glands and reduces steroid hormone metabolism.
Simazine MW=201.658g/mol pKa= 1.62 log K_{ow} =2.18		Initiation of aromatase activity. Increase the production of estrogen.
Butachlor MW= 311.85g/mol pKa=n.a log K_{ow} = 4.5		Estrogenic effect.
Heptachlor MW= 373.3g/mol pKa=n.a log K_{ow} = 6.10		Bind to estrogen and androgen receptors.
Alachlor MW=269.8g/mol pKa=n.a log K_{ow} =3.09		Competes with estrogen and progesterone (PG) receptors. Hinder with steroid hormone metabolism.

Heptachlor epoxide MW= 389.3g/mol pKa= n.a log K_{ow} = 5.40		Liver damage, increase risk of cancer
α-endosulfan MW= 406.90g/mol pKa=n.a log K_{ow} = 3.83		Competes with androgen receptors. Estrogenic effect. Inhibits aromatase activity.
4,4'-DDE MW= 318.02g/mol pKa=n.a log K_{ow} = 6.51		Inhibitory effect on prostaglandin synthesis.
Dieldrin MW= 380.9g/mol pKa=n.a log K_{ow} = 5.40		Competes with androgen receptors. Estrogenic effect.
Endrin MW= 380..9g/mol pKa=n.a log K_{ow} = 5.20		Competes with androgen receptors.

β-endosulfan MW= 406.90g/mol pKa= n.a log K_{ow} = 3.62		Competes with androgen receptors. Estrogenic effect. Inhibits aromatase activity.
4,4'-DDD MW= 320.03g/mol pKa= n.a log K_{ow} = 6.02		Competes with estrogenic receptors.
Endosulfan sulfate MW= 422.90g/mol pKa= n.a log K_{ow} = 3.66		Anti-ecdysteroidal compound.
4,4'-DDT MW= 354.48g/mol pKa= n.a log K_{ow} = 6.91		Competes with androgen receptors. Estrogen receptor agonist and PR antagonistic effect.
Endrin aldehyde MW=380.9g/mol pKa= n.a		Neurotoxin, antagonize gamma-aminobutyric acid (GABA)

log K_{ow} =4.80		
γ-HCH MW=290.81g/mol pKa= n.a log K_{ow} = 3.61-3.72		Reduces oestrus cycles and luteal progesterone amount. Increase estradiol blood serum amount. Competes with AR, ER and PR.
δ-HCH MW= 290.81g/mol pKa= n.a log K_{ow} = 3.61-3.72		Reduces oestrus cycles and luteal progesterone amount. Increase estradiol blood serum amount. Competes with AR, ER and PR.
β-HCH MW= 290.81g/mol pKa= n.a log K_{ow} = 3.61-3.72		Reduces oestrus cycles and luteal progesterone amount. Increase estradiol blood serum amount. Competes with AR, ER and PR.

α-HCH MW= 290.71g/mol pKa= n.a log K_{ow} =3.61-3.72		Reduces oestrus cycles and luteal progesterone amount. Increase estradiol blood serum amount. Competes with AR, ER and PR.

2.2.8.1 Organochlorine Pesticides

Organochlorine Pesticides (OCPs) are man-made pesticides that contain chlorine, and sometimes also oxygen (62). Common OCPs include, DDT, lindane, endosulfan, endrin, aldrin, methoxychlor and heptachlor epoxide. OCPs are neurotoxic and some of them are also suspected to be carcinogens and endocrine disruptors (51). They are used as herbicides, insecticides and pesticides in different forms from pellet application to spray in seed and grain storage (63,64). DDT and endosulfan were used to control mosquitoes that were spreading malaria and acarus that were infesting animal and lice that was transmitting typhus. Aldrin was used mainly to control insects in the soil, while dieldrin was used on crops to protect cattle and sheep from ectoparasites (65).

Most OCPs are banned in the United States of America due to their unacceptably slow degradation (63) as they tend to accumulate in living organisms (66). OCPs are also banned in Namibia, except DDT which is still used by the Ministry of Health and Social services to control malaria vector (7). Even though the use of OCPs has been abolished, they can persevere and bio-accumulate, presenting a toxicological threat to organisms. OCPs break down very slowly once released into the

environment. This persistence cause them to be incorporated into the ecosystems and food chains where they remain for years (67). As a result, OCPs are still being detected in the environment, although they have been banned from being use (66). One of the characteristics of persistent OCPs is volatility which enable their transportation from the source to remote areas and enrichment in water (64). They are also hydrophobic, lipophilic and extremely stable which make it easier for them to be absorbed into the tissues of living organisms, causing harmful effects (63).

Exposure to OCPs has been linked to endometriosis, a condition in which endometrial glands and stroma occur in locations outside the uterine lining (41). DDT and methoxychlor agonise the estrogenic receptor and also disturb the neuroendocrine regulation of estrogenic production (13). Cyclodiene insecticides, e.g. aldrin, endrin, dieldrin inhibit the function and activation of chloride channels by blocking γ-aminobutyric acid (GABA) (22). Reproductive disorders in alligators exposed to OCPs, including DDT and its metabolites, were discovered by researchers in Florida (1).

2.2.8.2 Organophosphorus pesticides

Organophosphorus or Organophosphate Pesticides (OPPs) are some of the most common and toxic pesticides used worldwide. This is due to their favourable characteristics such as biodegradable and short persistence compared to the organochlorine pesticides (69). There are at least 13 groups of OPPs including phosphate, phosphonates, phosphinates, phosphorothioates, phosphonothioates, phosphorodithioates, phosphorotrithioates, phosphoramidothioates (70).

In general, OPPs are less persistent, however some such as ethions are found to accumulate in soil (71). OPPs are sprayed over crops or soils, and their residues can be detected in the surface, groundwater, fruits, vegetables and in drinking water (69). Short-term effects exposure to OPPs has been studied mainly on the nervous system. They were found to be highly neurotoxic as they impede the acetylcholinesterase (AchE) enzyme that controls the function of the nervous system (71). Human exposure to OPPs is mostly assessed by measurements of decrease in AchE activity (70).

2.3 Removal of pesticides and other EDCs from water

EDCs have been detected in fresh and marine water, posing a potential hazard on animals human beings (21). Drinking water treatments require a series of methods to effectively remove such contaminants. Water treatment processes such as coagulation or flocculation followed by sedimentation and filtration alone are not capable of removing EDCs and PPCPs at the parts-per-trillion concentration level. However, the removal of these contaminants from water are found to be successfully removed through chlorination, ozonation and addition of chlorine dioxide treatment of drinking water (25). Ozone and advance oxidation process (AOP) can remove a wide range of hydrophobic PPCPs and EDCs including steroid hormones (25). Other methods which are less effective in removing EDCs include UV radiation, lower pressure membranes (microfiltration, and ultrafiltration (UF)) (25). With the right operating conditions there is a 90 percent success rate of removing organochlorines, (the most stable pesticides) (61).

Faul *et al* (2) studied the presence of EDCs, specifically steroids in the inlet and outlets of the Goreagab Water Reclamation Plant (GWRP) and Gammams Sewage

Treatment Plant (GSTP), and the removal efficiency of such contaminants. Results from that study indicated that GSTP was not successful in removing EDCs, however EDCs were completely removed by further treatment at GWRP (2).

2.4 Human exposure to pesticides and other EDCs

Human beings may be exposed to these harmful chemicals through food, beverages, medicines they take, cosmetics they use and pesticides they apply to their crops. Hence the exposure may be through the diet, air, skin and mostly water (drinking, and or swimming), as listed in Table 2.2 (9). Since pesticides enhance quantity and the quality of crops, many farmers resort to using them, however their residues remain in crops, and this may pose a health hazard to the consumers of fresh and processed fruits as well as drinks (72).

Grapevine used to produce wine are vulnerable from attack from harmful insects such as grapevine moth and mealy bugs. To maintain high quality wines produce, farmers use pesticides to control pests and diseases. Pesticides are gradually eliminated during the winemaking process. However, data showed residual amounts of pesticides in wines which possibly survived the fermentation process (72). Since wine is the most consumed drink in the world, this has potetially resulted in the consumption of these pesticides by a large number of people (73). Honey, wax, propolis and royal jelly could be polluted with pesticides residues that remained on treated beehives or plants where bees collect nectars (74).

2.5 Analysis of pesticides

Various sample preparation techniques have been used to extract and concentrate pesticides from water samples including liquid-liquid extraction (LLE), single drop

microextraction (SDME), dispersive liquid-liquid microextraction (DLLME), solid phase extraction (SPE), solid phase microextraction (SPME) and stir bar-sorptive extraction (SBE) (13).

The determination of several classes of pesticides such as OCPs, OPPs, triazines, pyretroides and acetamides is usually performed using gas chromatography (GC) or high performance liquid chromatography (HPLC) (66). Detectors that are used for the GC analysis of pesticides include flame ionisation detectors (FID), electron capture detectors (ECD) and mass spectrometry (MS). The presence of chlorine atoms in OCP structures makes them excellent target for electron capture detectors (ECD). ECD is sensitive, cheaper and easier to operate than other detectors such as mass spectrometry (67). On the other hand, mass spectrometry (MS) has an added advantage, because it is not only for quantification but also for the identification of pesticides in complex samples (66). Since pesticides are usually present in low concentrations in water samples, it is essential to combine extraction procedures that can effectively pre-concentrate these analytes, with highly sensitive analysis techniques.

2.5.1 Solid phase microextraction

SPME consists of a coated fibre and a syringe like handling device (Figure 2.2) that is used to isolate and concentrate analytes of interest (69). The fibre coating is normally an immobilized polymer, a solid adsorbent or a combination of both (56). This technique takes less time and no organic solvent consumption. SPME was developed by Pawliszyn and co-workers (67,75) and nowadays it is used to extract traces of organic compounds from different sample matrices, followed by the desorption of retained substances into an analytical instrument. There are two

different types of SPME, namely manual and automated SPME. SPME is sensitive and rapid but requires a lot of work when analyzing many samples; hence, automated SPME was developed to address this problem. Automated SPME shortens the total analysis time, therefore improving productivity and provide better accuracy and precision when compared to the manual technique (75).

Many automated SPME applications are focused on direct immersion (DI-SPME) or head-space extraction (HS-SPME) (75). HS-SPME is used for the analysis of highly volatile compounds and DI-SPME for the determination of compounds with lower volatilities. While a combination of the two extraction modes in a single procedure (DI-HS-SPME) has recently been introduced for the analysis of organic pollutants from different samples (76). SPME has numerous advantages such as simplicity, it takes less time, low cost in terms of solvent (solvent-free) than other consumables. It has been used routinely in combination with GC and GC/MS to analyse a wide variety of compounds (75). However, SPME fibers have some serious complications such as fibre connatural properties and the likelihood of carry-over. In addition, SPME fibers are expensive and have a limited life time compared to other techniques and they tend to degrade with repeated usage (77).

A fully automated SPME was developed for the determination of EDCs and steroid hormones from water. Applying the developed method OP, NP and DES were detected at 0.00013, 0.00503 and 0.00002 μg/mL, respectively, while estrone, 17β-estradiol and testosterone were at 0.00019, 0.00011 and 0.00622 μg/mL, respectively in river water in Guangdong, China (75).

Figure 2.2 Solid phase microextraction (SPME) device (78).

2.5.2 Single-drop microextraction

SDME is a method in which the extraction solvent is a single drop. Advantages of SDME include, the fact that it is cheap, needs simple equipment, easy to operate, make use of very low quantities of organic solvents and is amenable to in situ derivatisation of the analytes (84). In addition, SDME has the advantage of combining pre-concentration and sample introduction steps into a single-step extraction (79). However, the methods have disadvantages such as instability of the drop and the small surface of the drop slows the kinetics of the extraction (80). Extraction solvents should meet a number of characteristics, mainly: it should have a high boiling point, the right viscosity, should be compatible with the extracted analytes, with the chromatographic system and hydrophobic for aqueous extraction analyses (65). The type and amount of organic solvent drop, extraction time and temperature, salting out and agitation rate are other factors that could affect SDME methods (80). SDME can be operated in various modes such as direct immersion SDME (DI-SDME), headspace SDME (HS-SDME) and three phases SDME (36,76).

SDME has been used to analyse different groups of analytes in various matrices, with an emphasis on the determination of pesticides from water (81). Using the SDME procedure developed by Pinheiro et al (79) to analyse pesticides from the San Francisco river in Propria city, the LOD and LOQ values range between 0.05-0.40 μg/mL and 0.10-0.20 μg/mL, respectively were reported.

2.5.3 Dispersive liquid-liquid microextraction

Dispersive liquid-liquid microextraction (DLLME) was developed by Assai and co-workers in 2006 for the analysis of pesticides from food and water samples (81). It involves a rapid addition of a mixture of organic solvents to an aqueous sample, forming a cloudy solution of small droplets of the extraction solvent dispersed throughout the aqueous solution (82). Target analytes are then trapped into these droplets and after centrifugation, the extract phase is settled at the bottom of the tube, which is ready for direct injection into GC, or LC (83).

DLLME is fast, simple and consumes small quantities of organic solvent and offers high recovery, and enrichment factors (84). The extraction efficiency of DLLME is affected by various factors including the type and volume of the dispersion solvent, extraction time, salt addition, and temperature (71). In addition, the extraction solvent should satisfy two major parameters. One, it should have a higher density than water, and the other is that it should have the capability to extract the compounds of interest from the sample (85). The main disadvantage of DLLME is that its efficiency is limited by solvent selection (i.e. they should be capable of forming a dispersive phase), which limits the method's application scope (86).

DLLME has been used to extract and identify pesticides from water samples. Bidari et al (82) developed a DLLME method for the analysis of OPPs from tomato

samples. Methylparathion and ethion were detected at concentration levels of 34.3 and 0.0289 μg/mL, respectively (82). In another study, DLLME and GC-MS were used to analyse OCPs from water, and recoveries of 81-113 percent and relatively low LODs ranging from 0.000001-0.0006 μg/mL, were achieved (87).

2.5.4 Solid phase extraction

Solid phase extraction (SPE) is a sample preparation technique used to extract polar to non-polar and non-volatile analytes from different matrices (64). A SPE method make use of a solid phase and a liquid phase to selectively isolate certain types of analytes from a sample solution (88), by means of polarity or ionic interactions. In general, a sample solution containing the analyte of interest is loaded onto the SPE cartridges that contain the solid phase (sorbent), and the desired analytes are then retained on the phase. Finally, an eluent (mobile phase) is used to wash the analytes of interest off the solid phase into a collection tube (83). The resulting solution is concentrated and is eventually analysed by GC, HPLC on other analytical equipment (Fig 2.3).

The most common retention mechanisms in SPE are based on van der Waal forces (non-polar interactions), hydrogen bonding, dipole-dipole forces (polar interactions) and cation-anion interactions (ionic interactions) (88). Reversed phase SPE is performed using sorbents that are non-polar, made from carbon-based, polymer coated and bonded silica media, and is used in combination with a polar mobile phase. In this case, analytes of interest have to be moderately polar to polar. On the other hand, normal phase SPE involves a moderate to nonpolar mobile phase, a polar stationary phase (e.g. modified silica gel) and non-polar analytes (88).

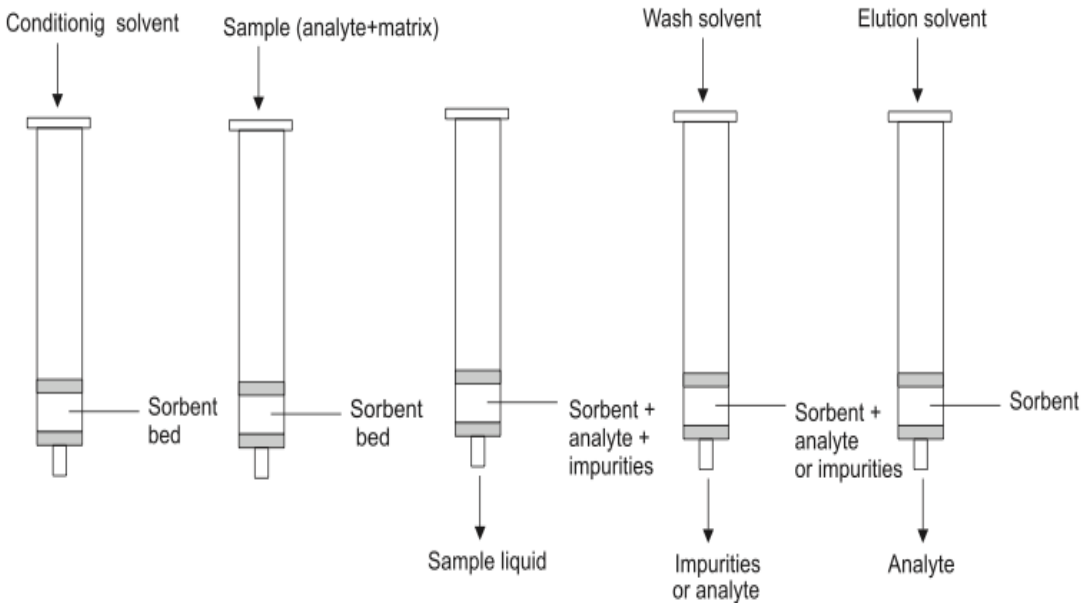


Figure 2.3 SPE method steps (88)

On the other hand, the ion exchange SPE retention mechanism is based on the electrostatic attraction of the charged functional groups of the compounds to the charged group that is bonded to the silica surface. For instance, anionic (negatively charged) compounds can be isolated on an aliphatic quaternary amine group that is bonded to the silica surface, while cationic (positively charged) compounds can be isolated by using the silica with aliphatic sulfonic groups that are bonded to the surface (89,90).

In the past, contaminants were extracted from a variety of environmental samples using liquid-solid (LSE) or liquid-liquid extraction (LLE) with organic solvents, followed by sample cleanup and pre-concentration steps (83). However, these methods are laborius, time consuming, costly and require discarding of toxic solvents. Faster and friendlier extraction methods have since been developed and one of these methods is SPE (83). This method has been recommended by the U.S Geological Survey National Water Quality Laboratory (USGS NWQL) as an official

method for the analysis of known and or suspected EDCs (91). Nowadays SPE is the most preferred pretreatment technique for the analysis of pesticides from water samples due to many advantages, such as wide availability of selective sorbents, lower consumption of organic solvents, reduced cost and analysis times and because it can easily be automated (77). It is considered as one of the most powerful technique for isolating trace amounts of organic compounds such as pesticides from water and other environmental samples (64). SPE methods are suitable for multi-residue analysis of compounds covering a wide range of polarities and physico-chemical properties (13), therefore it was chosen as the preferred method for the current study.

SPE method was applied to the analysis of EDCs from drinking water, river and wastewater in northern Morocco and southern Spain, and the highest concentration of analytes were reported in wastewater samples (13). Bin et al (43), developed a SPE method for the determination of steroid EDCs, the method was subsequently applied to determine steroids in Dianchi Lake (China) water samples. In this study, recoveries ranged between 83-95 percent, while LOD and LOQ values were 0.00000010-0.00000130 μg/mL and 0.00000030-0.00000420 μg/mL, respectively. Maldaner et al (92) developed and validated SPE method for the analysis of pharmaceuticals and pesticides from drinking water samples, this study reported higher recoveries of up to120%) for all target compounds. The removal efficiency of biocides from wastewater was studied using SPE in Regensdorf, Switzerland, the study established that the average removal for all compounds were below 50 percent (54).

CHAPTER THREE

3 MATERIALS AND METHODS

3.1 Chemicals and materials

All organic solvents used were LiChrosolv® HPLC grade, i.e. acetone, hexane, ethyl acetate, dichloromethane (DCM) and methanol, obtained from Merck (Darmastadr, Germany through Biodynamics, Namibia). Pesticide standards, OP mix standard (1000.00 µg/mL in acetone) and EPA pesticide mix standard (2000 µg/mL in hexane) were purchased from Sigma-Aldrich (Germany) and kept in a freezer (-20 °C). Biphenyl (Sigma-Aldrich, Germany) was dissolved in methanol to prepare an internal standard solution of 100 µg/mL and the solution was stored at -20 °C. The working internal standard solution was prepared at a concentration of 10 µg/mL in a 1:1 mixture of ethyl acetate and n-hexane. Purified water from a Millipore® Elix Milli-Q water purification system was used to prepare all aqueous solutions. Strata C_{18}-E, 55 µm, 70 Å, 200 mg/3mL reversed phase cartridges (Phenomenex Inc) were obtained through Landulamed (Windhoek, Namibia).

3.2 Instrumentation

3.2.1 GC-MS

Identification of the pesticides present in the water treatment plant samples was performed using a Thermo Scientific Focus GC coupled to a Thermo Scientific ITQ 700 mass spectrometer. A SGE Analytical Science DB-5MS capillary column (30.00 m x 0.25 mm, film thickness: 0.25µm) (Genmed, Namibia) with a 5% diphenyl, 95%

dimethyl polysiloxane stationary phase was used for the analyses. Helium of 99.999% purity (Afrox, Namibia) was used as the carrier gas at a flow rate of 1.00 mL/min. The injector temperature was set at 250 °C. The column oven temperature was programmed to increase from 50 °C to 120°C at a rate of 10 °C/min and then increased to 200 °C at a rate of 3 °C/min held for 5 min afterwards increased to 219 °C at a rate of 3 °C/min and then increased to 300 °C at 10 °C/min and finally held for 10 min. Using a 10 µL microsyringe, samples (1 µL) were injected (manually) in split mode with a split ratio of 10:1. The total analysis time was 62 min including the equilibration time of 0.5 min. The MS was operated in positive electron ionization (EI^+) mode at 70 eV. The transfer line and MS source temperatures were maintained at 250 °C and 200 °C, respectively. A mass range of *m/z* 25-525 was recorded in full-scan mode. Analytes were identified based on the comparison of their mass spectra and retention times to those of the pesticide standards.

3.2.2 GC-FID

Quantitative analyses were performed on a Perkin-Elmer Clarus 580 gas chromatograph equipped with a flame ionisation detector. The detector was operated at 280 °C. A SGE Analytical Science HP-1 capillary column (30 m x 0.32 mm, film thickness: 0.25 µm) (Genmed, Namibia) with a 100% dimethyl polysiloxane stationary phase was used for the quantitative analysis. Hydrogen, (Afrox, Namibia) was used as the carrier gas at a flow rate of 1.40 mL/min. Samples (1 µL) were injected manually, using a 10 µL microsyringe. The GC inlet was operated in split mode with a split ratio of 10:1 and the injector port was maintained at 250 °C. Different oven temperatures programmed in the ranges of 60-280°C, at different ramping speeds were investigated for complete separation of all compounds. Unless otherwise specified, the analyte response is expressed as the ratio of the analyte peak

area vs. the internal standard peak area. Analytes were identified by comparison of their retention times with those of the pesticide standards analysed under the same conditions (after confirmation of their presence by GC-MS).

3.3 Sample collection

Water samples were collected in capped glass Schott bottles, from both the inlets and the outlets of the water treatment plants, GWTP and UWWTP. Prior to sample collection, the bottles were washed and subsequently heated in an oven for two days at 250 °C to remove any organic contamination. Before collecting the samples, bottles were rinsed with water from the sampling site. Samples were then transported on ice and stored at -4 °C in the laboratory until analysis.

3.4 Preparation of standard and sample solutions

A stock solution was prepared from the commercially available pesticides mixtures using n-hexane as the diluent. The final concentration of the pesticides in the stock solution was 200.00 μg/mL. The stock solution was kept in the freezer (-20°C) for the duration of the study, and the other standard solutions were freshly prepared from the stock solution prior to analysis. A series of standard solutions (0.25, 1.25, 2.50, 5.00, 10.00, 20.00, 30.00, 40.00 and 50.00 μg/mL) were prepared using the internal standard solution as the diluent. Water samples were prepared by spiking a certain amount of a pesticide solution into different volumes of Milli-Q water as described in the method development and validation sections.

3.5 Solid phase extraction method

The SPE cartridges were conditioned by sequential addition of 5.00 mL n-hexane, 5.00 mL ethyl acetate and 5.00 mL Milli-Q water. Spiked water samples were added to the cartridges directly after they were conditioned. The water samples were loaded at a flow rate of 10.00 mL/min with the aid of a Millipore vacuum pump (Merck, Billerica). Subsequently, the cartridges were dried under vacuum for 60 minutes. In all the experiments performed prior to the elution solvent optimisation experiments, elution was performed with 2.00 mL n-hexane followed by 2.00 mL ethyl acetate (90). The extracts were dried under a stream of nitrogen and the residue was re-dissolved in 500.00 μL of a mixture (1:1) of ethyl acetate and n-hexane containing 10 μg/mL of an internal standard solution. Finally, 1.00 μL of the extract was injected into the GC for analysis. All analyses were performed in triplicate.

3.5.1 Optimisation of sample volume

In order to examine the effect of sample volume on the recoveries of the pesticides using SPE, the use of three different sample volumes was evaluated, 100.00, 250.00 and 500.00 mL (5,85). Milli-Q water samples were spiked with the pesticide standards and subjected to SPE. The water samples were spiked with 0.10, 0.04 and 0.02 μg/mL (respectively) of the pesticides in order to have target concentrations of 20 μg/mL at the end of the SPE procedure. The influence of the sample volume was determined based on the extraction recoveries. All analyses were performed in triplicate. The sample volume that gave the best recoveries was used in all subsequent experiments.

3.5.2 Selection of eluent type

The effect of the type of eluent on the recovery of pesticides that were extracted from water using SPE was evaluated. The following solvent systems were tested: elution of the pesticides by DCM only (solvent system 1), sequential elution of the pesticides with methanol followed by acetone (solvent system 2) and the sequential elution of the pesticides with n-hexane followed by ethyl acetate (solvent system 3). A volume of 4 mL of each solvent system was used for each experiment. Each eluate was dried in a N_2 atmosphere and re-dissolved in the internal standard solution and analysed by GC-FID. The efficiency of the eluents to quantitatively elute the pesticides from SPE cartridges was examined by comparing the recoveries of each pesticide individually. The solvent system that gave higher recoveries for most of the pesticides was used for all subsequent experiments.

3.5.3 Optimisation of eluent volume

In order to study the effect of eluent volume on the extraction recoveries of the pesticides, different amounts of solvent were tested. The use of three different volumes, 2.00, 5.00 and 10.00 mL was evaluated. The eluent volume that gave the best recoveries was used for the subsequent experiments.

3.5.4 Optimisation of ionic strength

In order to study the effect of ionic strength on the extraction efficiency of pesticides from water samples, different amounts of NaCl were added to the samples before extraction. Mass concentrations of 0, 2, 5 and 10 % (w/v) were prepared by adding

different masses of NaCl ranging from 0-10 g. The ionic strength condition that gave the highest recoveries for most of the pesticides was selected.

3.6 Method validation

Using the optimised GC-FID method, the injection repeatability, linearity, LOQ and LOD of the instrument were determined. Injection repeatability was evaluated by analysing a pesticide standard solution (20.00 μg/mL) seven times. The linearity was determined by analysing reference standard solutions at different concentration levels, ranging between 0.25-50.00 μg/mL (94). The LOQs and LODs of the instrument were determined as the concentrations that gave signal to noise ratios (S/N) of 10:1 and 3:1, respectively and were calculated for each pesticides (Fig. 4.1).

Subsequently, the optimised SPE method was validated by evaluating its selectivity, linearity, LOQs, LODs, extraction recoveries and precision (86). Using the optimised SPE method, blank Milli-Q water samples were analysed in order to evaluate the method's selectivity. In addition, Milli-Q water samples spiked with pesticides (0.05 μg/mL) were analysed in the same way (in duplicate), and the chromatograms were compared to that of the blank water sample analysis. To examine the method linearity, Milli-Q water samples were spiked with pesticide standards at different concentration levels (0.00125, 0.00625, 0.0125, 0.025, 0.05, 0.10 and 0.15 μg/mL) and subsequently pre-concentrated the pesticides to levels ranging from 0.25 - 30 μg/mL, using SPE. The LOQs and LODs were determined as described for the instrument method validation.

Water samples spiked at 0.10 μg/mL were analysed in triplicateto evaluate method intra-day precision. Repeatability (precision) was expressed in percentage relative standard deviation (% RSD):

% RSD = (standard deviation/ mean) × 100 *[1]*

Extraction recovery (% ER) was defined as the percentage of the analyte ratio response (*analyte peak area/internal standard peak area*) in relation to the standard ratio response (*standard peak area area/internal standard peak area*):

% ER = [(*analyte peak area/internal standard peak area) / (standard peak area area/internal standard peak area)] x 100* *[2]*

3.7 Application of the method

The response factor (RF) of each pesticide was determined by analysing standard solutions at concentrations ranging between 0.25-30.00 μg/mL. From the data of the analysis of the standards, the RFs were calculated using the following formula:

$RF = C_iA/CA_i$ *[3]*

Where *C* is the concentration of the selected compound in the standard solution; A_i is the area of the internal standard; C_i is the concentration of the internal standard and *A* is the area of peak of the selected compound (94).

Re-arranging equation [3] analyte concentrations in the samples were calculated with the resulting Eq. [4] (94):

$C = (C_i\ A)/ (A_i\ RF)$ *[4]*

Where *C* is the concentration of the selected compound in the sample solution; *A* is the area of the peak of the selected compound; A_i is the area of the internal standard peak; *RF* is the response factor calculated using Eq [3]; and C_i is the concentration of the internal standard.

Once a target analyte has been qualitatively identified in a sample, the concentration of that analyte in the sample was calculated based on the dilution factor from the sample volume (100 mL) to the extract volume (0.50 mL):

$$C_{sample} = C \times (0.5\ mL/100\ mL) \quad [5]$$

Where C_{sample} is the concentration of the compound in the water sample and C is the final (extract) concentration of the identified compound.

Subsequently, the validated SPE method was applied to the analysis of pesticides in water samples from the inlet and outlet sampling points of the water treatment plants, GWTP and UWWTP.

CHAPTER FOUR

4 RESULTS

4.1 GC method development

The mixture of 22 pesticides (Table 4.1) was first analysed using an initial oven temperature of 80 ^{o}C followed by an increase at a rate of 5 ^{o}C/min to 280 ^{o}C. However, a number of pesticides were not resolved (Fig. 4.1 A), therefore the temperature programme was optimised to improve the separation. The GC method was optimised by first varying the initial temperature and then the temperature at which the oven temperature is held constant as well as the programmed rate. Using the optimised method, all 22 pesticides were separated from each other (Fig. 4.1 B).

4.2 Instrument method validation

4.2.1 Injection repeatability

The precision of the analysis method was assessed in terms of its injection repeatability, which repeatability was determined by analysing the standards repeatedly (20.00 μg/mL, n = 7) under the same conditions on the same day. The precision was in the range of 0.02-5.70 percent RSD with an average of 3.40 percent (Table 4.1). The method precision is acceptable when the % RSD is <10 % (61,71,95).

4.2.2 Instrument linearity, LOD and LOQ

The linear detection range of the GC instrument was determined by the analysis of the pesticides standard solutions at nine concentration levels ranging between 0.25-50.00 µg/mL. The response of most of the analytes were found to be linear in the range of 0.25-20.00 µg/mL with correlation coefficients higher than 0.9972 (Table 4.1). The linearity curves appear in Appendix 1.

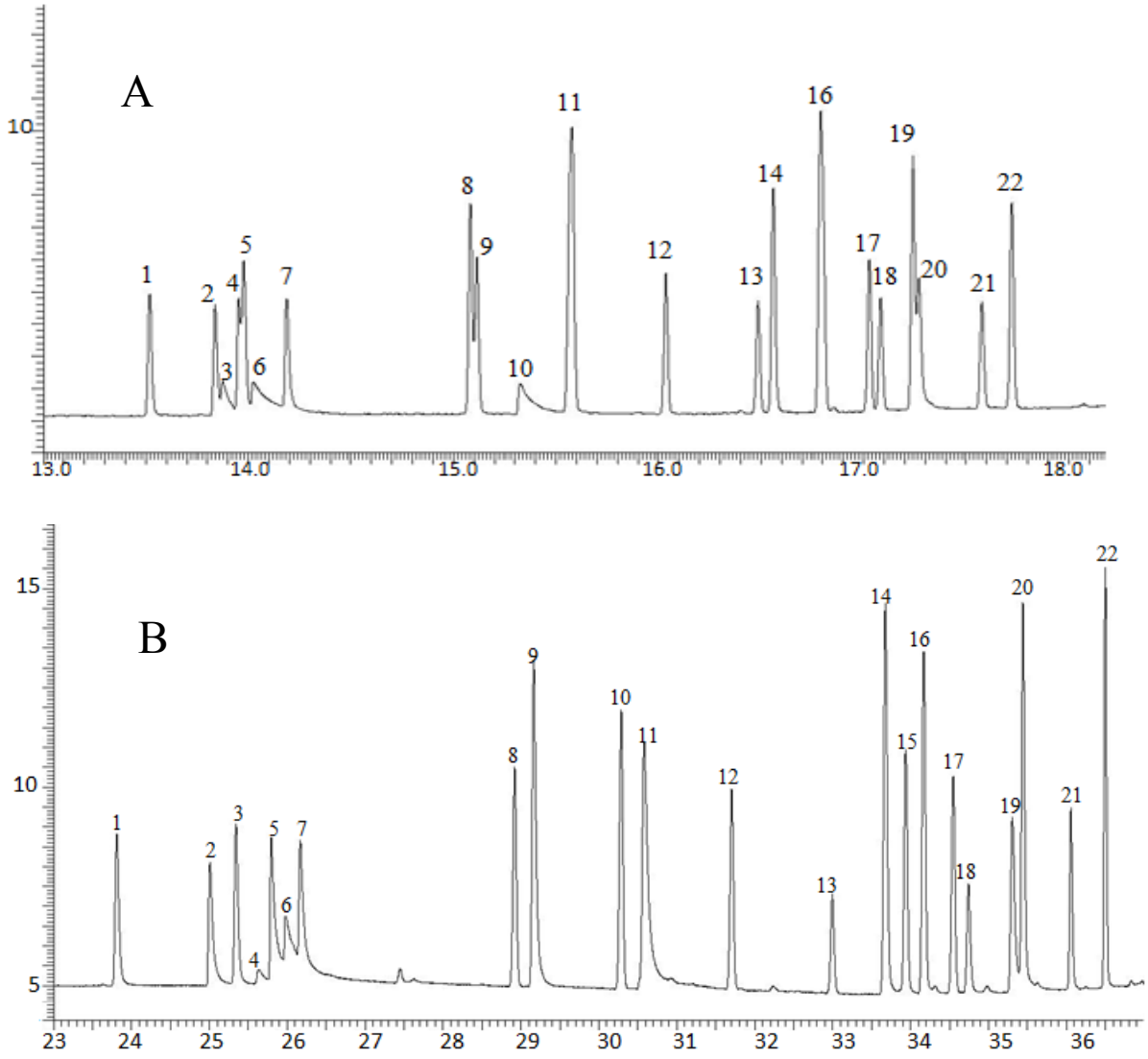


Figure 4.1 GC-FID chromatograms of the analysis of the mixture of 22 pesticides, using temperature programs A: 80ºC to 280 ºC at 5min and B: 80ºC to 125ºC at 2

ºC/min (hold 5 min); then to 200 ºC at 5 ºC/min then to 280ºC at 15 ºC/min (hold 4 min).

Table 4.1: Linear ranges, detection and quantitation limits of the instrument.

Peak	**Retention**	**Compound**	**% RSD**	**R^2**	**Linear**	**LOD**	**LOQ**
1	23.82	α-HCH	3.90	0.9951	0.25-20.00	0.29	0.99
2	25.04	β-HCH	5.62	0.9966	0.25-50.00	0.08	0.25
3	25.36	Lindane	3.74	0.9916	0.25-10.00	0.20	0.66
4	25.57	Simazine	2.30	0.9977	5.00-30.00	1.6	5.39
5	25.79	Prometon	4.04	0.9996	2.50-20.00	0.84	2.80
6	28.06	Atrazine	1.83	0.9978	0.25-40.00	0.07	0.23
7	28.24	δ-HCH	3.50	0.9990	0.25-50.00	0.04	0.14
8	28.93	Heptachlor	4.40	0.9987	1.25-20.00	0.45	1.50
9	29.16	Alachlor	4.73	0.9961	0.25-20.00	0.07	0.24
10	30.30	Aldrin	1.60	0.9968	1.25-20.00	0.87	2.90
11	30.57	Metolachlor	5.70	0.9969	0.25-40.00	0.06	0.21
12	31.71	Heptachlor	5.10	0.9919	0.25-20.00	0.20	0.67
13	33.00	α-Endosulfan	0.92	0.9974	0.25-20.00	0.42	1.41
14	33.68	Butachlor	3.23	0.9945	0.25-20.00	0.27	0.90
15	33.95	4,4'-DDE	4.82	0.9976	0.25-20.00	0.39	1.30
16	34.18	Dieldrin	3.60	0.9951	1.25-20.00	0.75	2.50
17	34.56	Endrin	3.90	0.9965	1.25-20.00	0.42	1.40
18	34.75	β-Endosulfan	1.23	0.9986	0.25-20.00	0.54	1.80
19	35.31	4,4'-DDD	2.70	0.9981	0.25-20.00	0.37	1.24
20	35.45	Endrin	0.02	0.9972	0.25-20.00	0.39	1.29
21	36.06	Endosulfan	3.91	0.9992	0.25-40.00	0.05	0.15
22	36.50	4,4'-DDT	3.70	0.9980	0.25-20.00	0.31	1.03
Ave.			**3.40**	**0.997**		**0.39**	**1.32**

The instruments LODs and LOQs were determined using the data of the instrument linearity experiments. Plus LOD and LOQ values were calculated using signal-to-noise ratios (Fig. 4.2). A number of examples of how the signal-to-noise ratios were determined appear in Appendix 4. Instrument LOD values ranged from 0.04 μg/mL (δ-HCH, **7**) – 1.60 μg/mL (simazine, **4**), while the LOQ values were between 0.14 (δ-

HCH, **7**) and 5.39 μg/mL (simazine, **4**). A list of LOD's and LOQ's appear in Table 4.1.

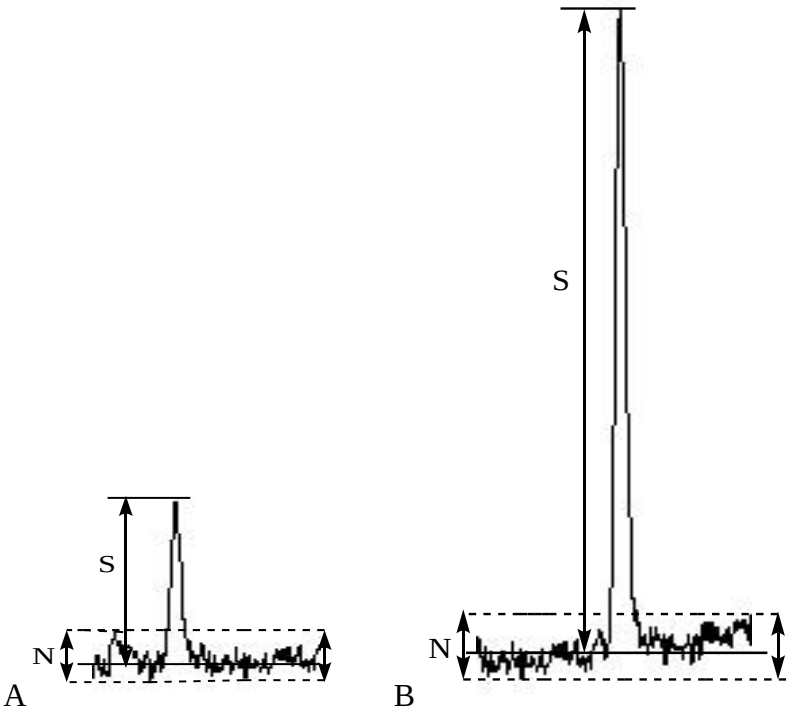


Figure 4.2 Examples of signal-to-noise measurements used to calculate LOD and LOQ values: (A) signal-to-noise ratio 3:1 and (B) signal-to-noise ratio 10:1.

4.3 SPE method development

4.3.1 Optimisation of sample volume

The breakthrough volume is the volume at which the SPE cartridge become saturated with the analyte solution, subsequently the analytes are no longer adsorbed and they begin to co-elute with the mobile phase (97). Various study experiments were performed to determine the breakthrough volume for the extraction of pesticides from water samples using SPE (5,38,100). In the present study, the 100.00 mL

sample volume gave extraction recoveries (on average) of 73 percent compared to 70 and 68 percent for 250.00 and 500.00 mL, respectively (Fig. 4.3). A sample volume of 100 mL was therefore used in all subsequent experiments.

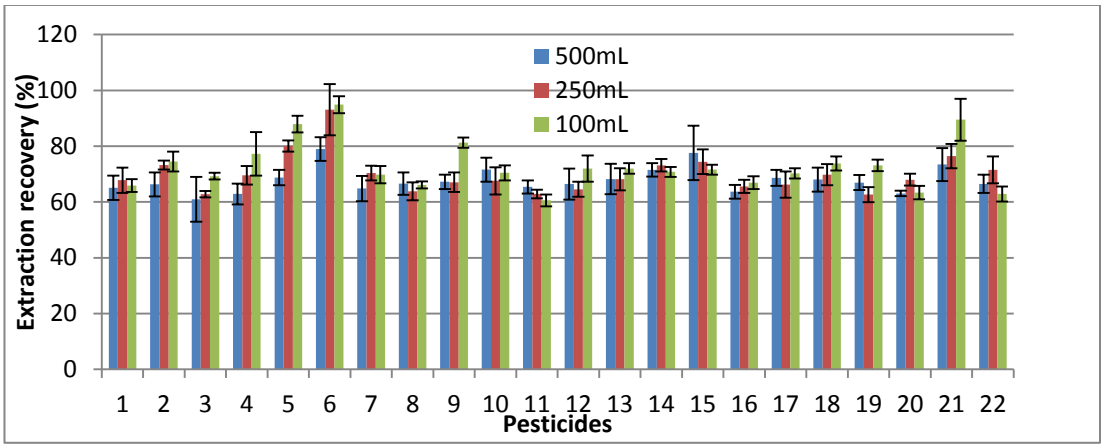


Figure 4.3 The effect of sample volume on the extraction recoveries of the pesticides (n=3).

4.3.2 Selection of eluent type

The polarities of the pesticides that were analysed in this study are within a wide range, making it vital to evaluate organic solvents of different polarities for the efficient elution of all the pesticides from the SPE sorbent. Of the three elution solvent systems that were evaluated, n-hexane followed by ethyl acetate (solvent system 3) yielded the highest recoveries for most of the pesticides (Fig. 4.4). On average, solvent system 3 gave an extraction recovery of 87 percent, compared to 81 and 64 percent for solvent systems 1 and 2, respectively. Therefore, elution solvent system 3 was used for all the subsequent experiments.

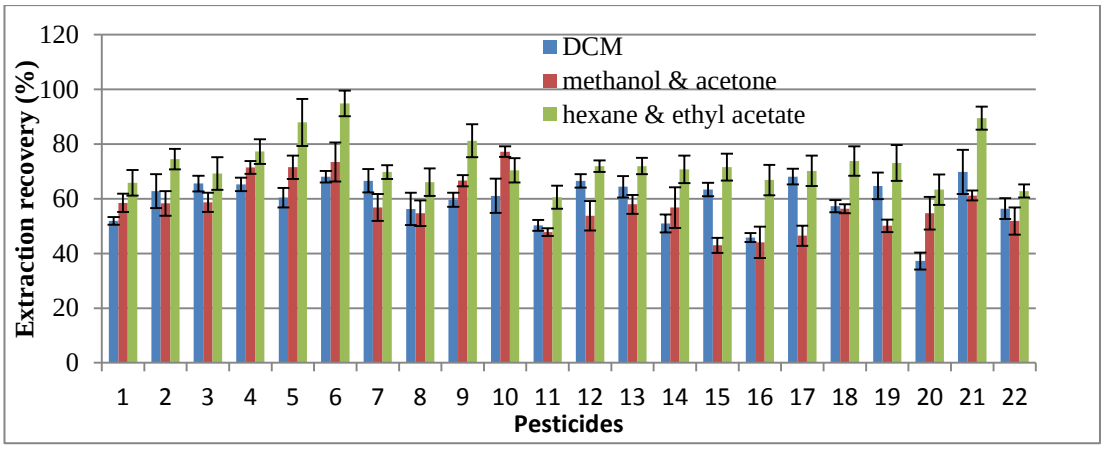


Figure 4.4 The effect of the eluent type on the extraction recoveries of the pesticides (n=3).

4.3.3 Optimisation of eluent volume

The volume of the elution solvent plays a major role in the extraction efficiency of a SPE method. The eluent amount should be sufficient to desorb and recover all the analytes from the cartridges. The effect of elution solvent volume on pesticides recovery was investigated at 2.00, 5.00 and 10.00 mL. The results indicated that recovery increased as the volume of elution solvent was increased (Fig 4.5). For most pesticides under study, the best recoveries were obtained with elution by 10 mL of the eluent, hence it was used for all subsequent experiments.

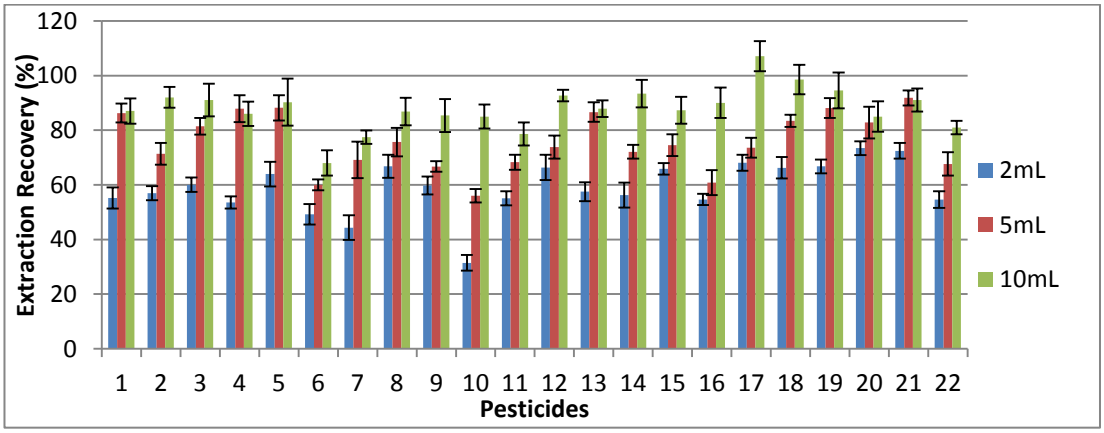


Figure 4.5 The effect of the eluent volume on the extraction recoveries of the pesticides (n=3).

4.3.4 Optimisation of ionic strength

To investigate the effect of ionic strength on the recovery of the analytes from water samples, different amounts of sodium chloride were added to the water samples to give final concentrations ranging from 0 to 10 percent (w/v). The results indicated that adding 5 percent of salt to the water samples significantly increased the extraction recoveries of most pesticides and was therefore chosen for all subsequent extraction experiments (Fig. 4.6).

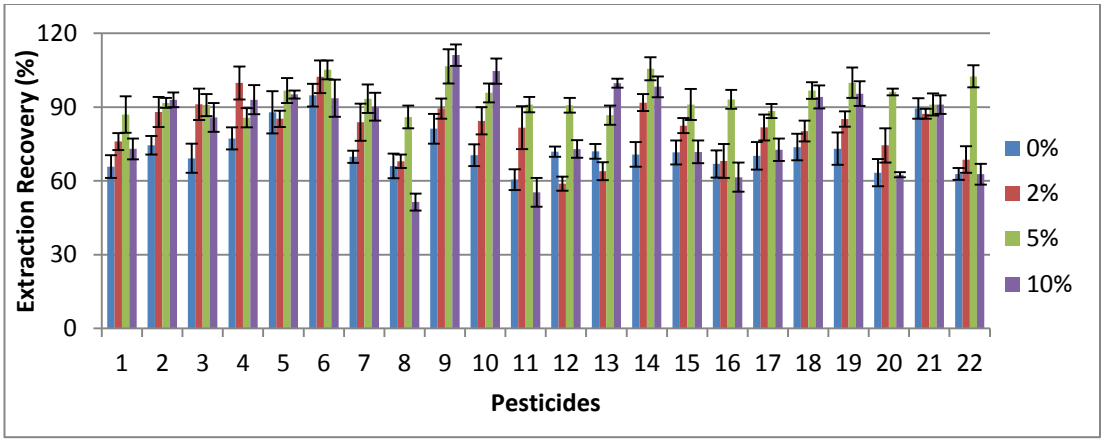


Figure 4.6 The effect of ionic strength on the extraction recoveries of the pesticides (n=3).

4.4 Effect of optimised parameters on each analyte

The effects of the optimised parameters on each pesticide appear in Appendix 2. The trends show that the optimization of these parameters had improved the recovery efficiency of the SPE method of most of the pesticides. A few irregular cases were observed, such as prometon (**5**), heptachlor epoxide (**12**), endrin (**17**), β-endosulfan (**18**) and endosulfan sulfate (**21**), where changing the ionic strength did not increase the extraction recovery of these pesticides. Furthermore, for δ-HCH (**7**) and metolachlor (**11**) the extraction recoveries decreased when the optimum elution

solvent volume (i.e the optimum for all other pesticides) were used.

4.5 SPE method validation

Method validation is essential for confirming that an analytical method developed is effective in measuring the parameters it intended to measure. The optimised method was validated by determining its selectivity, linear range, LOD, LOQ extraction recoveries and precision. Results for this section are summarised in Tables 4.2 and 4.3.

4.5.1 Method selectivity

The selectivity of the method was evaluated by comparing the chromatograms of a blank sample with that of a spiked Milli-Q water sample (Fig 4.7). The absence of chromatographic peaks at the same retention times as the analytes of interest indicated that there were no major matrix interferences or contamination that may interfere with peak identification in the spiked sample analyses.

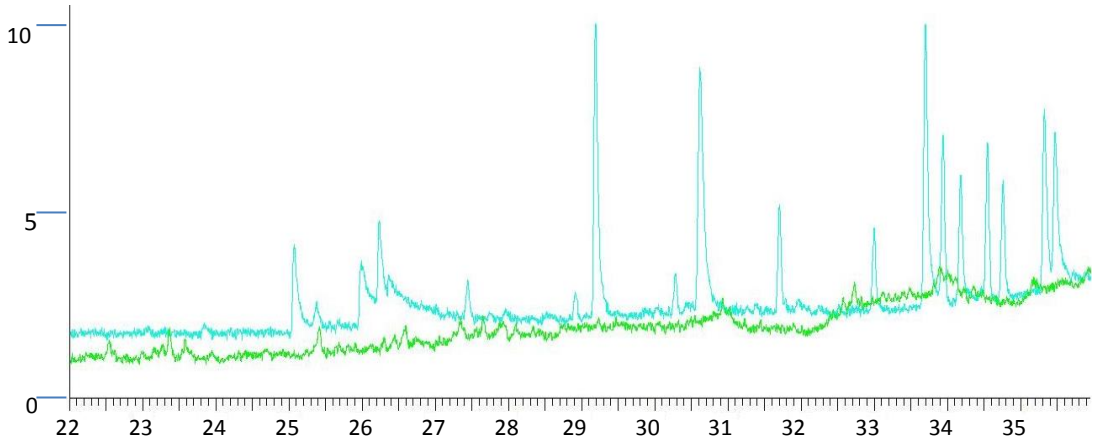


Figure 4.7 An overlay of the GC-FID chromatograms of a spiked (0.05 μg/mL) (blue) and a blank (green) water sample, analysed using the optimised SPE method.

4.5.2 Method linearity, LOQ and LOD

The method was found to be linear across different concentration ranges for the individual pesticides, but most linear ranges were between 0.00625 and 0.15000 μg/mL (Table 4.2). A number of analytes, including α-HCH (**1**), simazine (**4**) and prometon (**5**) could only be detected at concentrations higher than 0.05 μg/mL. Regardless, most analytes showed good linearity for SPE in the studied working range, with correlation coefficients (R^2) greater than 0.99 (acceptance criteria $R^2 \geq 0.99$) (5,96), except for prometon (**5**) atrazine (**6**), and δ-HCH (**7**), where values of 0.9866, 09558 and 0.9608 respectively were obtained. The linearity curves for each analyte appear in Appendix 3. The LOD values for most analytes were equal to or lower than the lowest linear range data point ranging between 0.0009 – 0.0270 μg/mL, while LOQs ranged from 0.0030 to 0.0900 μg/mL (Table 4.2). Moreover, the average LOD and LOQ values for the pesticides were 0.0070 and 0.0263 μg/mL, respectively.

Table 4.2: Linear range, detection and quantitation limit of the method.

Peak #	**Pesticide**	**R^2**	**Linear range**	**LOD (μg/mL)**	**LOQ (μg/mL)**
1	α- HCH	0.9938	0.05000-0.15000	0.0150	0.0500
2	β- HCH	1.0000	0.05000-0.15000	0.0090	0.0300
3	Lindane	0.9926	0.01250-0.15000	0.0038	0.0125
4	Simazine	0.9996	0.05000-0.15000	0.0270	0.0900
5	Prometon	0.9866	0.05000-0.15000	0.0120	0.0400
6	Atrazine	0.9558	0.01250-0.10000	0.0021	0.0710
7	δ- HCH	0.9608	0.01250-0.10000	0.0070	0.0230
8	Heptachlor	0.9991	0.02500-0.15000	0.0105	0.0350
9	Alachlor	0.9903	0.00625-0.15000	0.0021	0.0071

10	Aldrin	0.9995	0.02500-0.15000	0.0195	0.0650
11	Metolachlor	0.9913	0.00625-0.15000	0.0018	0.0061
12	Heptachlor epoxide	0.9941	0.00625-0.10000	0.0099	0.0326
13	α-Endosulfan	0.9906	0.00625-0.05000	0.0090	0.0300
14	Butachlor	0.9995	0.01250-0.10000	0.0016	0.0054
15	4,4'-DDE	0.9918	0.00625-0.05000	0.0040	0.0133
16	Dieldrin	0.9991	0.00625.0.10000	0.0060	0.0200
17	Endrin	0.9914	0.00625-0.05000	0.0022	0.0074
18	β-Endosulfan	0.9976	0.00625-0.15000	0.0043	0.0142
19	4,4'DDD	0.9993	0.02500-0.15000	0.0016	0.0053
20	Endrin aldehyde	0.9970	0.00125-0.15000	0.0024	0.0081
21	Endosulfan sulfate	0.9999	0.00125-0.01250	0.0009	0.0030
22	4,4'-DDT	0.9946	0.00125-0.15000	0.0027	0.0090
	Average	**0.9924**	**-**	**0.0070**	**0.0263**

4.5.3 Precision and extraction recovery of the method

The precision of the method was assessed by analysing spiked water samples containing known concentrations of the pesticides, in triplicate. This was achieved by using different concentration levels (0.00625 - 0.15000 μg/mL) and calculating the % RSD of the results of the triplicate experiments at each concentration level (Table 4.3). In general, the % RSD values ranged from 3 to 13 percent for the individual pesticides across all the concentration levels, and on average it ranged from 6 to 10 percent, which is within the acceptable limit of % RSD $\leq$ 10. The results presented in Table 4.3, indicate that on average the recoveries were between 76 – 93% in all.

Table 4.3: Pesticide recovery and precision values at different concentration levels.

Peak #	**Pesticide**	**RF**	**Concentrations (µg/mL) (n=3 for each concentration level)**														**Average**	
			0.00125		**0.00625**		**0.0125**		**0.02**	**0.05**			**0.1**		**0.15**			
			%	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%
1	α- HCH	5.00	-	-	-	-	-	-	-	-	6	**73**	10	**76**	9	**78**	***8***	***76***
2	β- HCH	21.6	-	-	11	**74**	6	**71**	5	**77**	8	**91**	4	**94**	6	**91**	***7***	***83***
3	Lindane	4.60	-	-	-	-	4	**70**	10	**74**	7	**86**	6	**95**	5	**91**	***6***	***83***
4	Simazine	0.04	-	-	-	-	-	-	-	-	6	**93**	8	**94**	5	**86**	***6***	***91***
5	Prometon	0.15	-	-	-	-	-	-	-	-	5	**92**	9	**90**	5	**97**	***6***	***93***
6	Atrazine	0.15	-	-	-	-	10	**81**	6	**87**	8	**94**	7	**64**	4	**105**	***7***	***86***
7	δ- HCH	5.60	-	-	10	**72**	6	**84**	7	**104**	6	**90**	7	**76**	6	**93**	***7***	***87***
8	Heptachlor	9.90	-	-	-	-	-	-	11	**80**	7	**71**	6	**87**	7	**68**	***8***	***77***
9	Alachlor	0.25	-	-	8	**81**	9	**95**	6	**87**	8	**72**	7	**85**	6	**107**	***7***	***88***
10	Aldrin	2.40	-	-	12	**73**	11	**86**	9	**77**	12	**73**	5	**79**	8	**89**	***10***	***80***
11	Metolachlor	0.24	-	-	-	-	-	-	-	-	9	**87**	5	**72**	7	**102**	***7***	***87***
12	Heptachlor epoxide	5.6	-	-	12	**75**	9	**68**	7	**85**	8	**70**	7	**95**	5	**92**	***8***	***81***
13	α-Endosulfan	8.8	-	-	13	**83**	10	**81**	11	**80**	6	**91**	7	**86**	4	**87**	***9***	***85***
14	Butachlor	0.17	-	-	6	**111**	10	**72**	9	**75**	7	**92**	5	**93**	6	**105**	***7***	***91***

15	4,4’-DDE	5.0	-	-	13	**70**	8	**80**	10	**82**	6	**72**	6	**87**	7	**91**	***8***	***80***
16	Dieldrin	8.0	-	-	-	-	11	**67**	11	**86**	8	**66**	6	**90**	4	**93**	***8***	***80***
17	Endrin	6.5	-	-	11	**75**	8	**78**	10	**72**	6	**73**	5	**107**	7	**86**	***8***	***82***
18	β-Endosulfan	7.0	-	-	5	**66**	9	**74**	6	**70**	5	**94**	7	**98**	4	**97**	***6***	***83***
19	4,4’DDD	15.3	-	-	7	**85**	10	**62**	9	**82**	5	**95**	7	**95**	6	**100**	***7***	***87***
20	Endrin aldehyde	2.5	7	77	12	**76**	7	**77**	8	**92**	7	**65**	7	**85**	6	**95**	***8***	***81***
21	Endosulfan sulfate	9.8	11	82	10	**61**	9	**73**	9	**82**	4	**91**	5	**91**	5	**91**	***8***	***82***
22	4,4’-DDT	6.1	3	70	7	**87**	9	**78**	7	**90**	7	**63**	7	**83**	4	**103**	***6***	***82***
	Average	**-3**	**7**	**76**	**10**	**78**	**9**	**76**	**8**	**82**	**7**	**82**	**7**	**87**	**6**	**93**		

concentrations for all the pesticides. All pesticides showed recoveries of ≥78 percent at the highest concentration level, except heptachlor (**8**), for which it was 68 percent. On average, the best extraction recoveries were obtained for the experiments performed at higher concentration levels. Most of the pesticides were not detected at the lowest concentration level (0.00125 μg/mL), except endrin aldehyde (**20**), endosulfan sulfate (**21**) and 4,4'-DDT (**22**). For these compounds, extraction recoveries and % RSD values of 77 % (7 %), 82 % (11 %) and 70 % (3 %), respectively, were obtained at 0.00125 μg/mL.

4.6 Application of the method

The validated SPE method was applied to the analysis of water samples from GWTP and UWWTP. A total of 28 water samples were collected from inlets and outlets (7 samples from each) of these water treatment plants. All samples were subjected to the validated SPE method and the resulting extracts were analysed using the validated GC-FID method after confirmation of the presence of the target compounds using GC-MS. The identities of the compounds were confirmed by comparing their retention times and mass spectra to those of the authentic reference standards (Figures 4.8 – 4.18). The mass spectra of the pesticides identified in some of the water samples and the mass spectra of the corresponding reference standards appear in Figures 4.8 - 4.17. Once the presence of a number of pesticides was confirmed in this way, their peaks were located in the GC-FID chromatograms of the samples by retention time comparison with the pesticide standards analysed under the same conditions (Fig. 4.19). Pesticides were only detected in 4 of the 7 water samples collected from GWTP inlet point. These were prometon (**5**), atrazine (**6**), alachlor (**9**), metolachlor (**11**) and butachlor (**14**) and they are all herbicides (Table

4.4). No pesticides were detected in any of the UWWTP samples or in the GWTP outlet samples (Figures 4.20 – 4.22). The former could be attributed to the fact that UWWTP is an industrial wastewater treatment plant where pesticides are not expected to be found.

Table 4.4 Herbicides detected in water samples from the GWTP inlet point.

Peak ♯	**Compound**	**Concentration (µg/mL)**	**% RSD**
5	Prometon	0.0460	14
6	Atrazine	0.0040	10
9	Alachlor	0.1858	10
11	Metolachlor	0.2301	9
14	Butachlor	0.0100	10

The experimentally determined RF values (Table 4.3) were used to calculate the concentrations of the pesticides identified in the real samples analyses. On average, alachlor (**9**) and metolachlor (**11**) exhibited the highest concentrations of 0.1858 and 0.2301 µg/mL respectively, followed by prometon (**5**) with a concentration of 0.046 µg/mL. All other herbicides detected in the water were present below 0.010 µg/mL, with atrazine (**6**) present at the lowest concentration of 0.0040 µg/mL (Table 4.4).

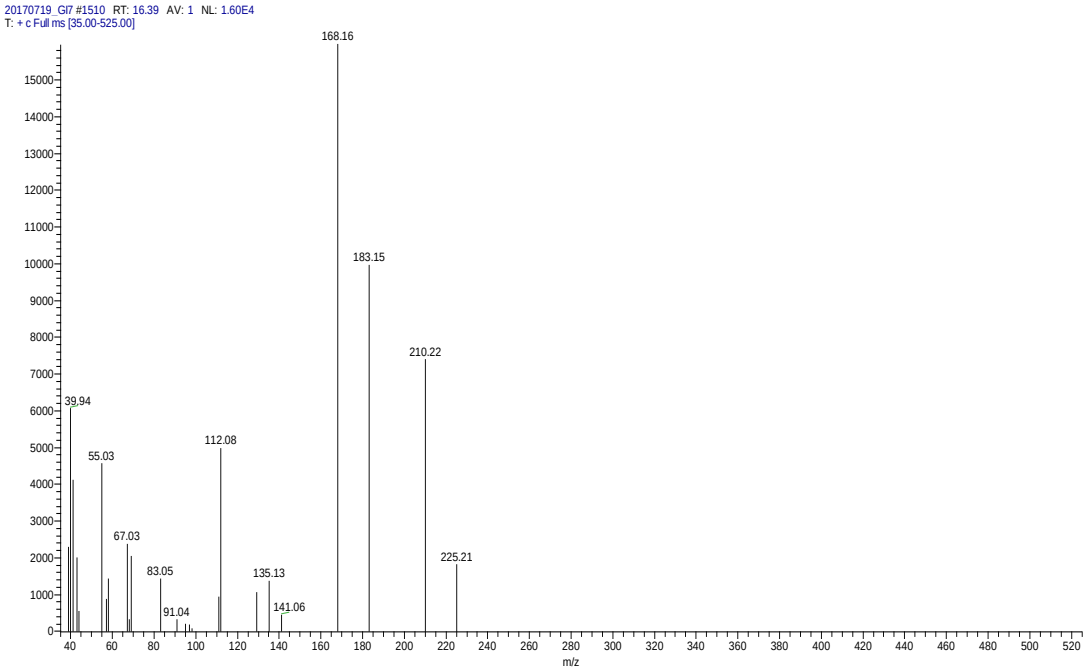


Figure 4.8 Mass spectrum of compound **5**, identified as prometon in GWTP inlet water samples.

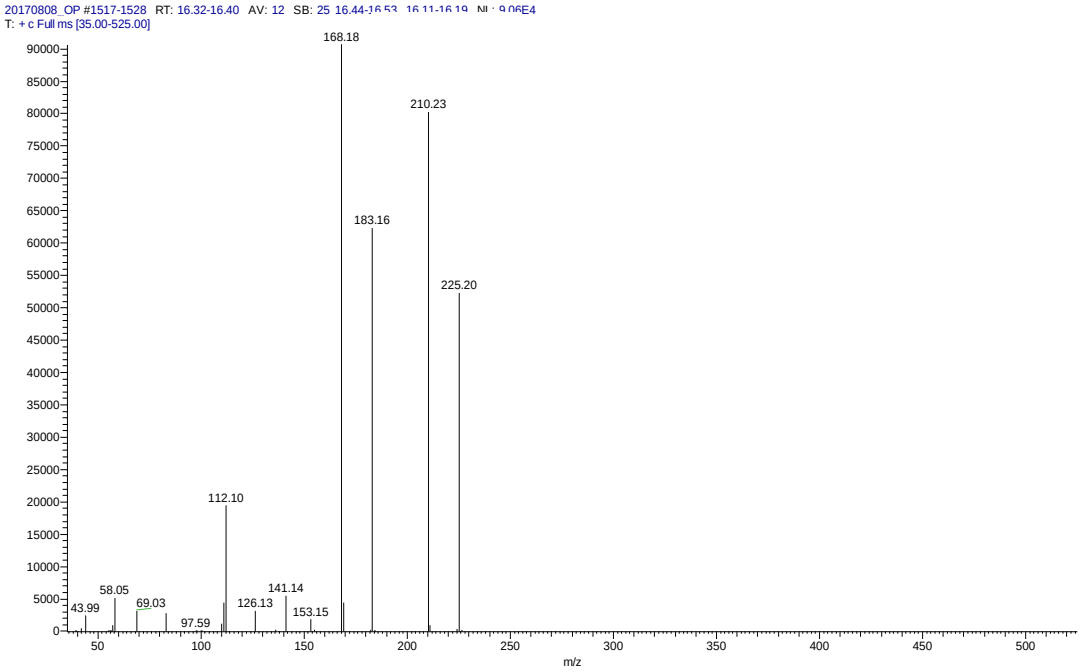


Figure 4.9 Mass spectrum of the prometon (**5**) reference standard.

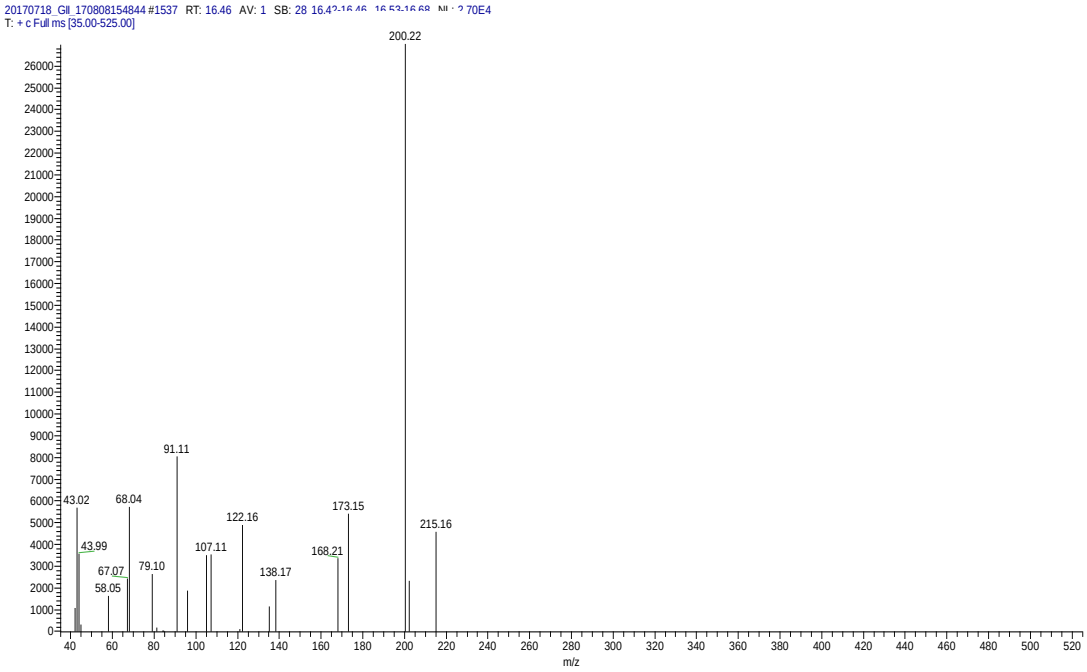


Figure 4.10 Mass spectrum of compound **6**, identified as atrazine in GWTP inlet water samples.

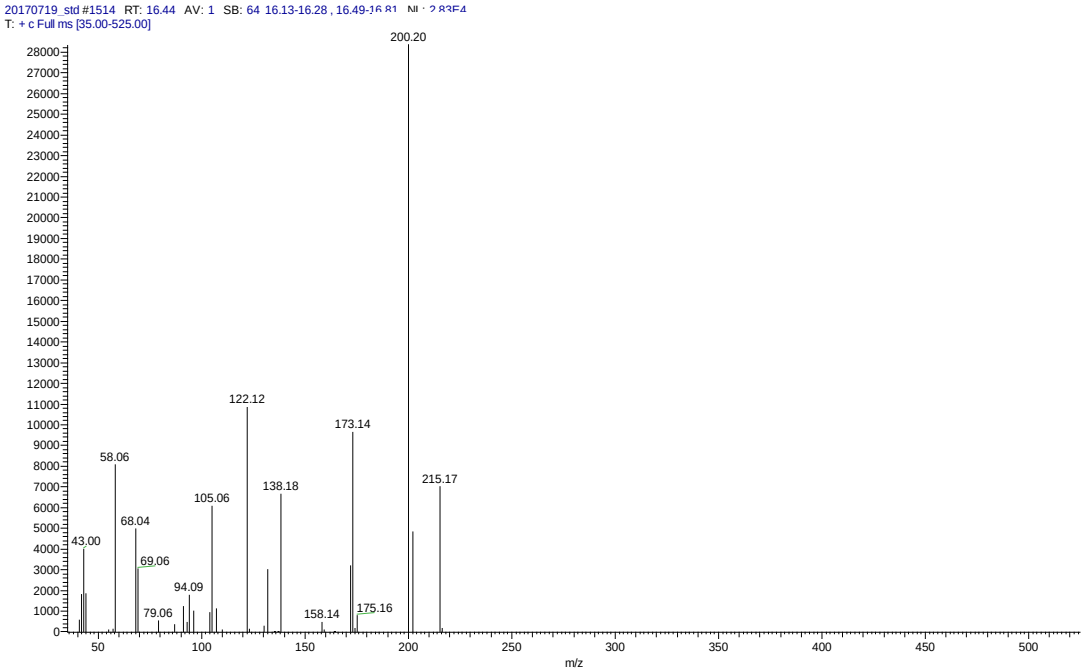


Figure 4.11 Mass spectrum of the atrazine (**6**) reference standard.

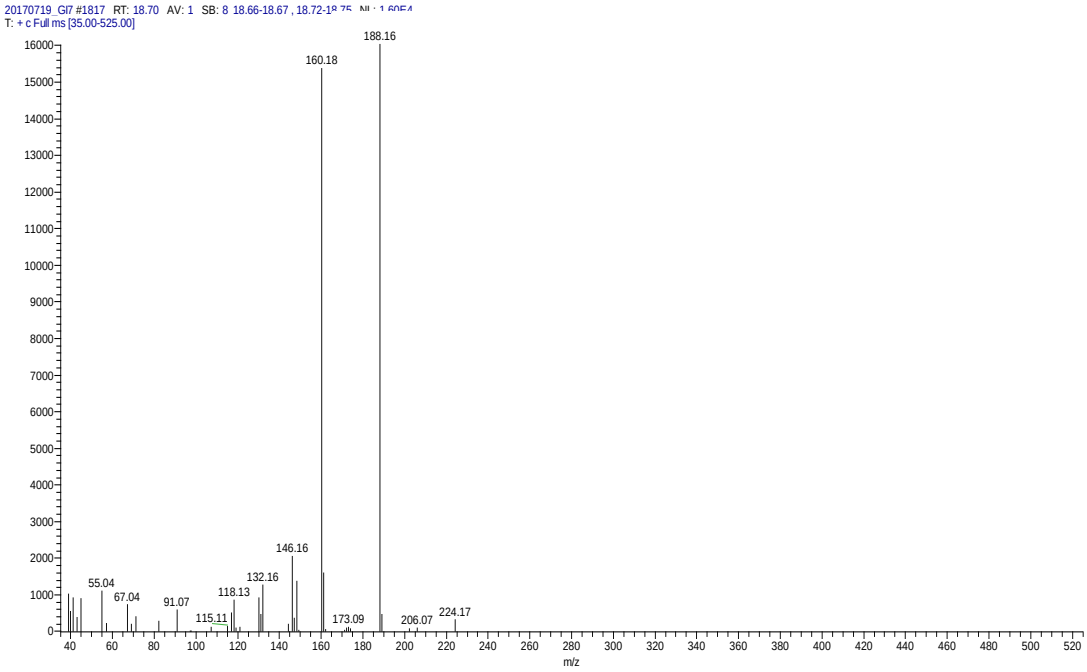


Figure 4.12 Mass spectrum of compound **9**, identified as alachlor in GWTP water samples.

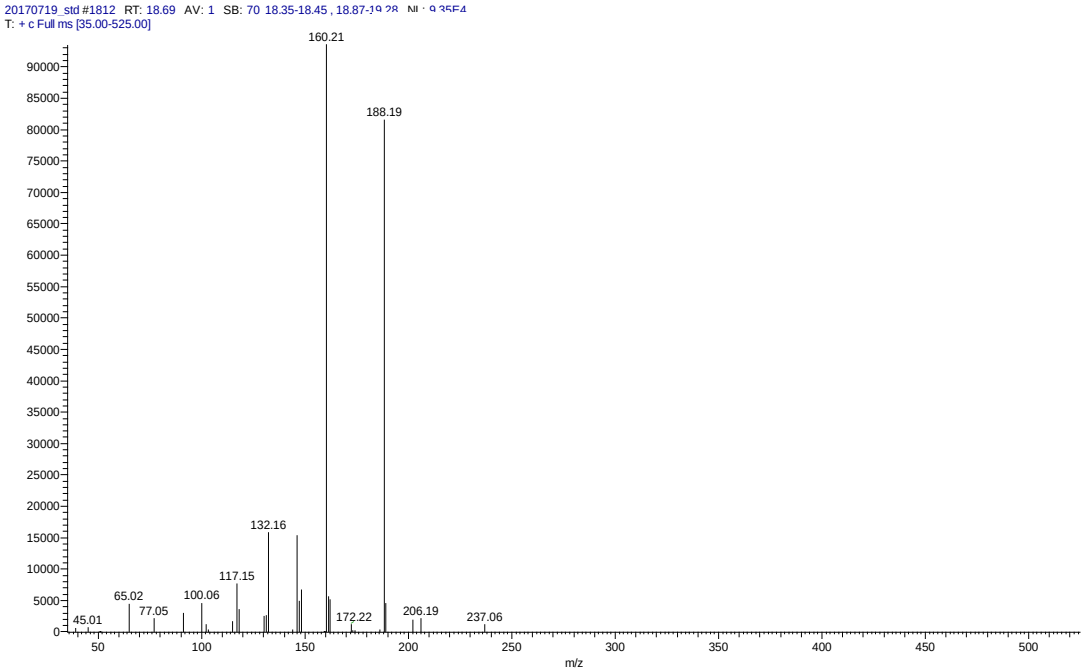


Figure 4.13 Mass spectrum of the alachlor (**9**) reference standard.

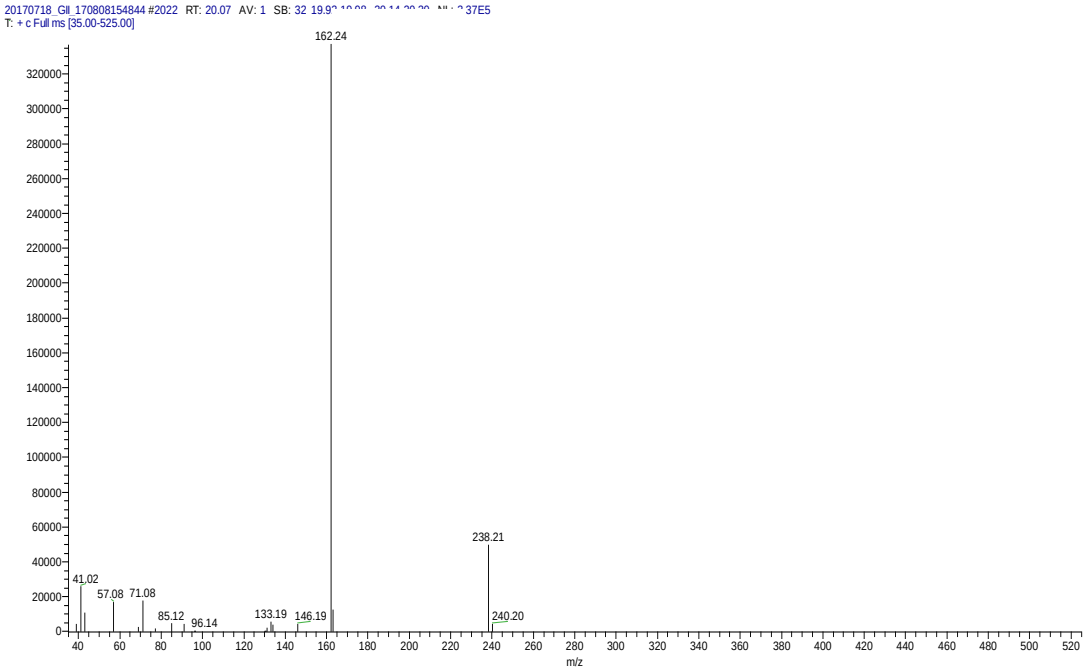


Figure 4.14 Mass spectrum of compound **11**, identified as metolachlor in GWTP inlet water samples.

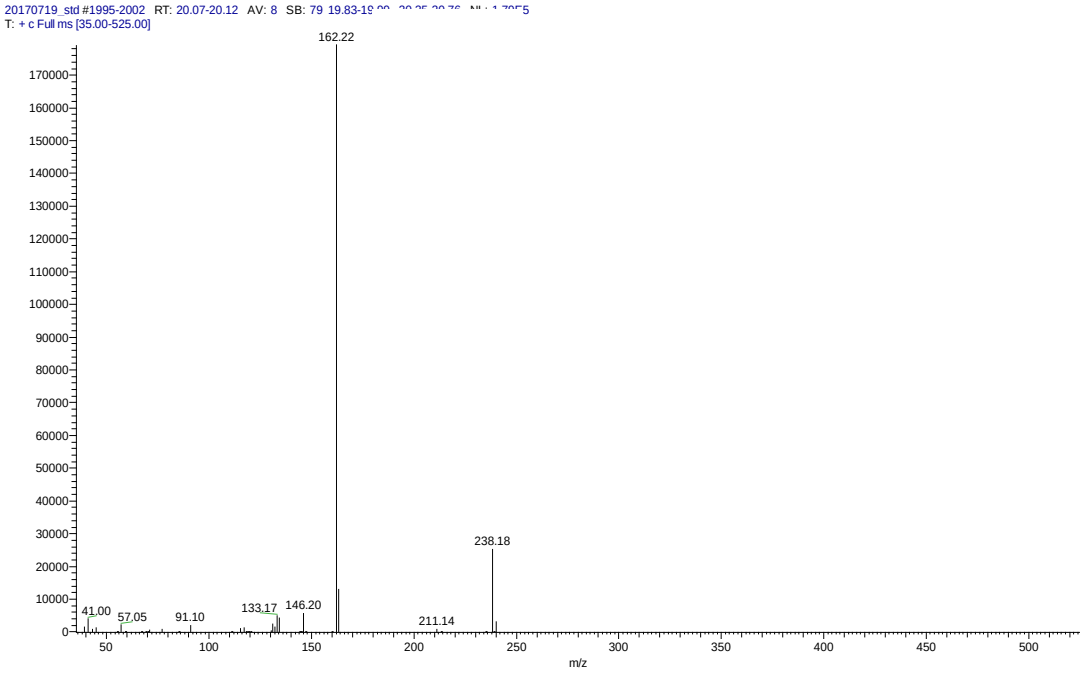


Figure 4.15 Mass spectrum of the metolachlor (**11**) reference standard.

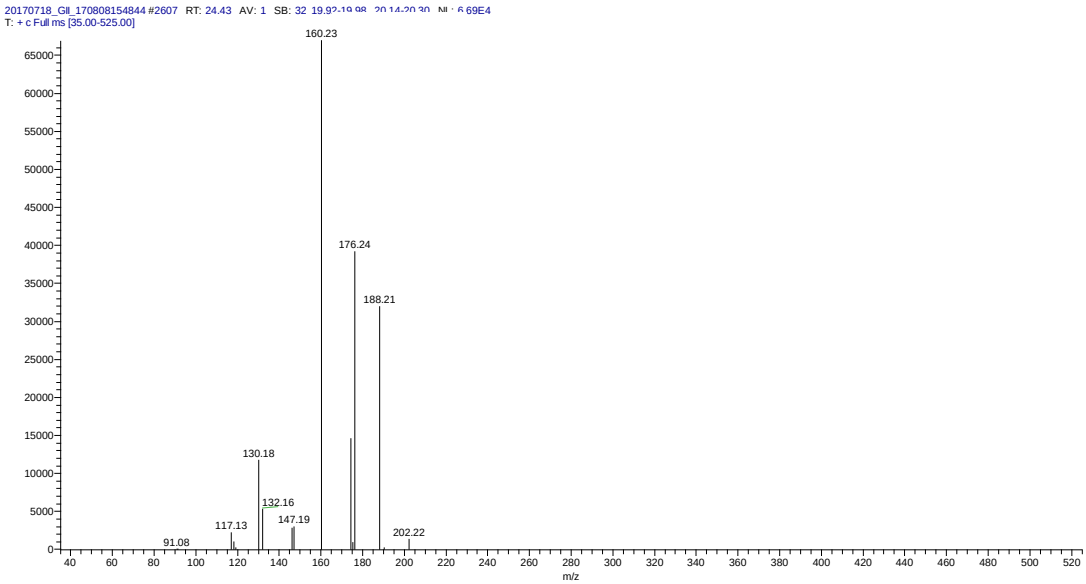


Figure 4.16 Mass spectrum of compound **14**, identified as butachlor in GWTP inlet water sample.

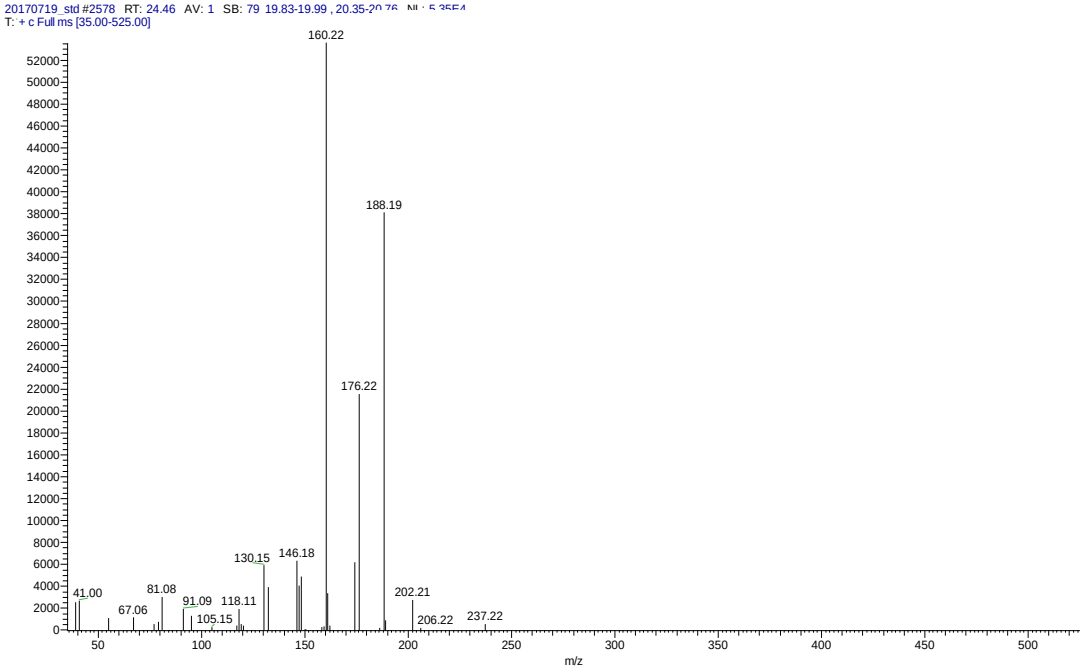


Figure 4.17 Mass spectrum of the butachlor (**14**) reference standard.

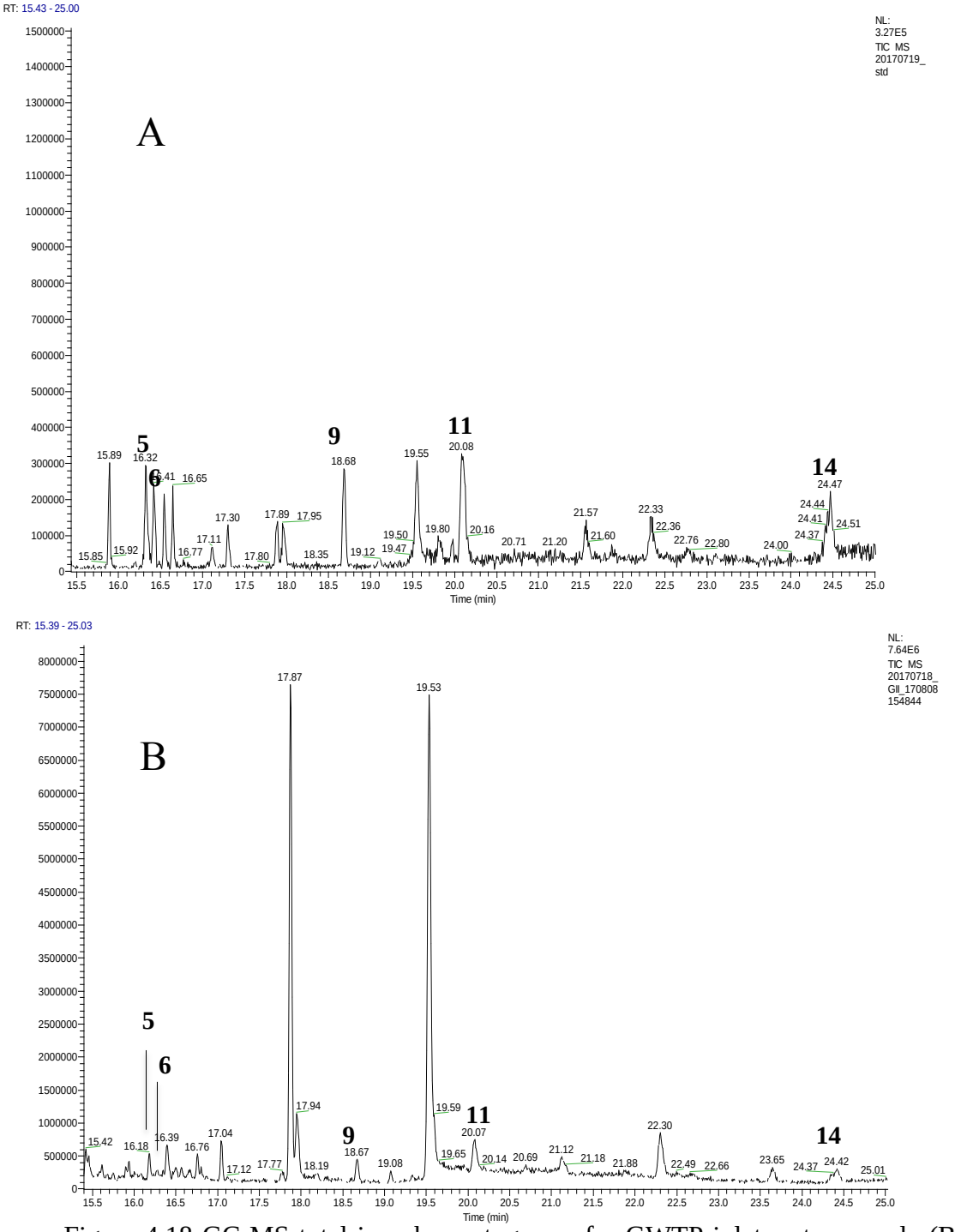


Figure 4.18 GC-MS total ion chromatogram of a GWTP inlet water sample (B), in comparison with GC-MS total ion chromatogram of the standards (A) showing the identified pesticides (Table 4.4).

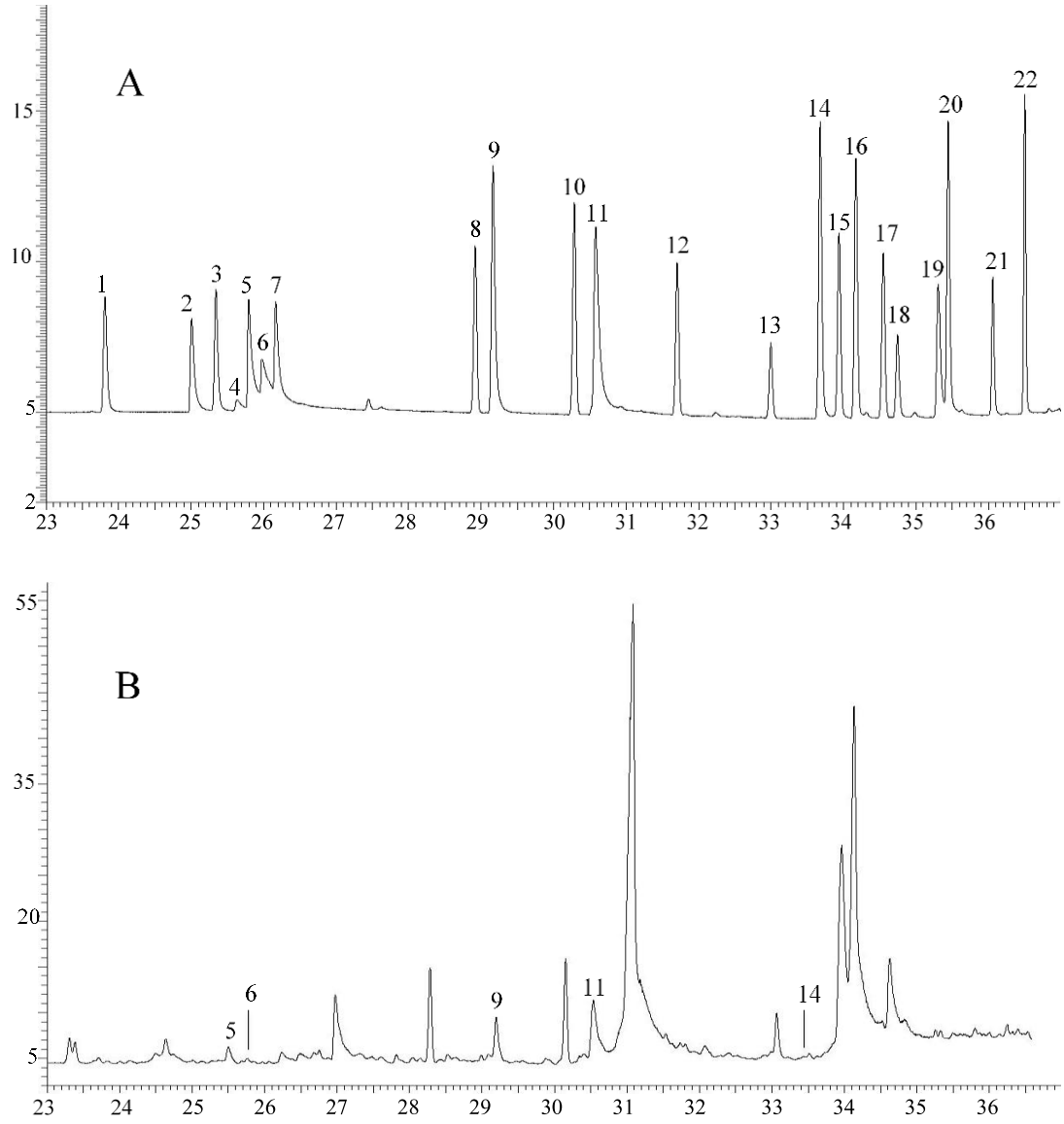


Figure 4.19 GC-FID chromatograms of a 30 μg/mL pesticides standard solution (A) and a GWTP inlet water sample (B). The numbers refers to the identified pesticides listed Table 4.4.

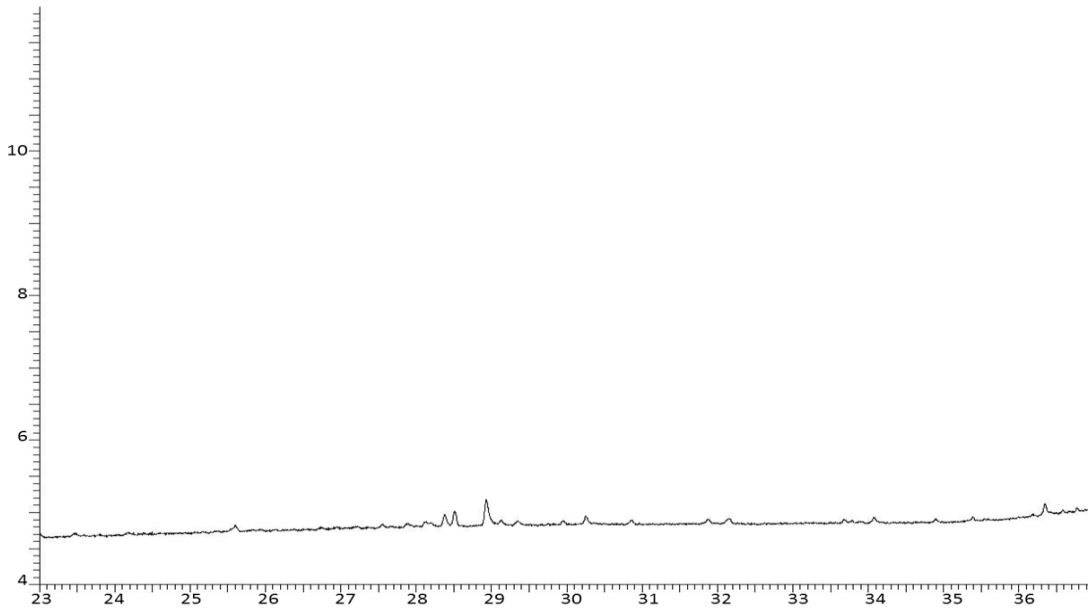


Figure 4.20 GC-FID chromatogram of a GWTP outlet water sample.

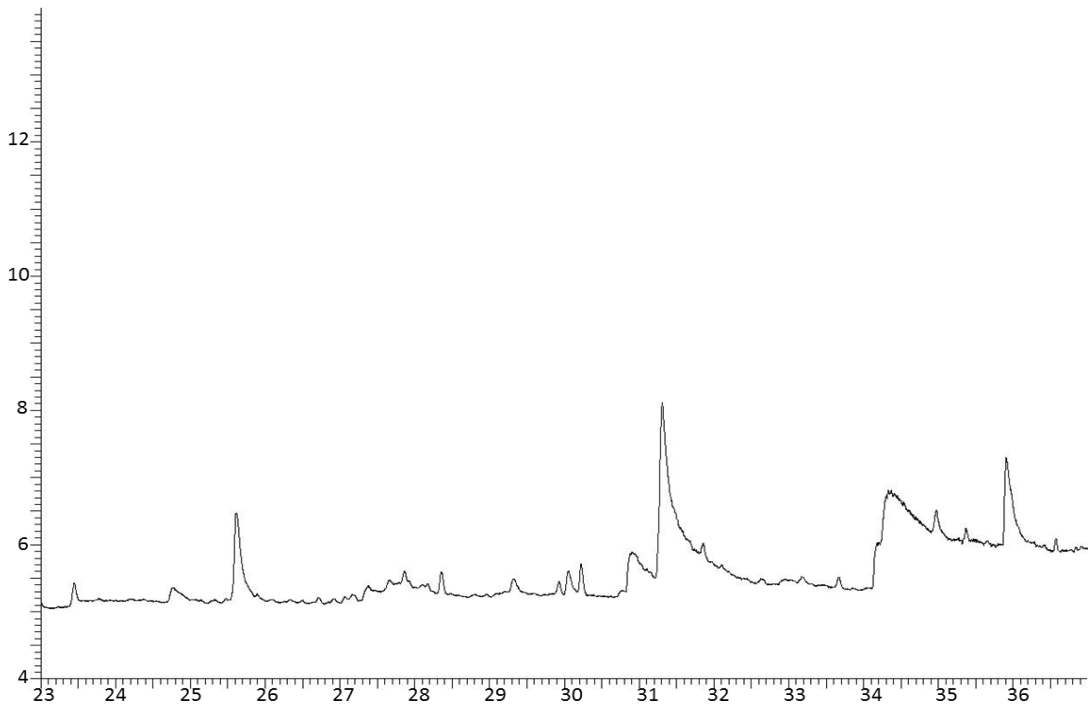


Figure 4.21 GC-FID chromatogram of an UWWTP inlet water sample.

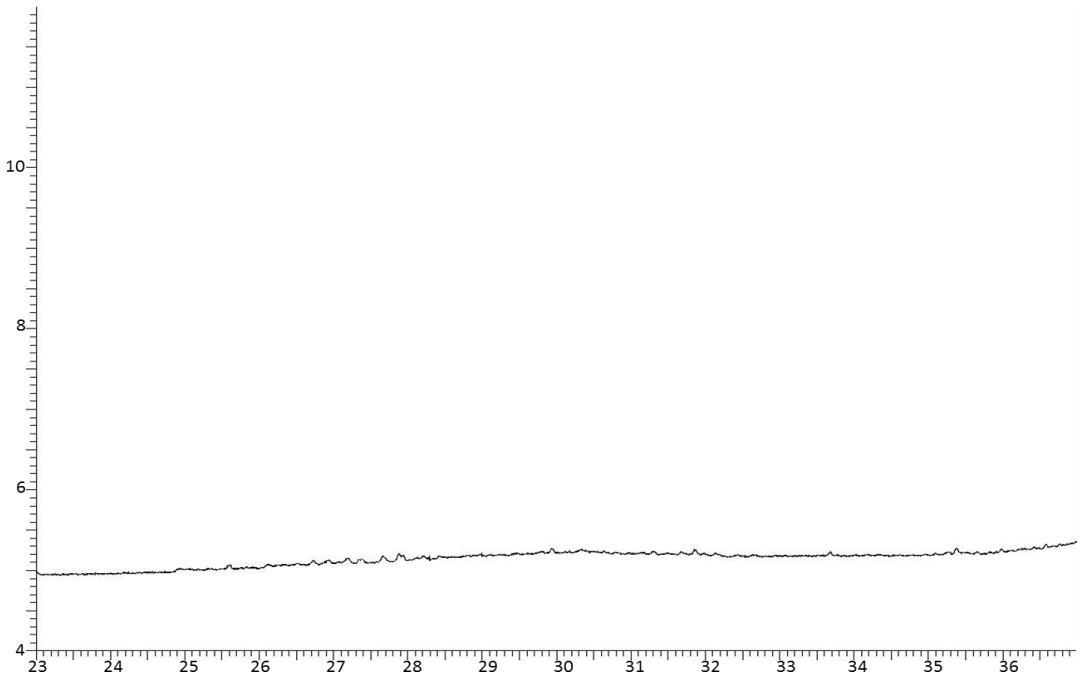


Figure 4.22 GC-FID chromatogram of an UWWTP outlet water sample.

CHAPTER FIVE

5 DISCUSSION

The SPE methods used for the analysis of pesticides and organic pollutants from different matrices have been widely studied (37,42,75,76,89,103). Currently, the most commonly used SPE sorbent for the extraction of pesticides from water samples is C_{18}. In addition, it has been applied widely in the research of environmental pollution chemistry (90). It was also reported that increasing the sample temperature had a negative effect on the extraction recoveries, which is to some extent due to the exothermic effect of the adsorption interaction between the target compounds and the sorbent phase (97). Therefore a C_{18} SPE sorbent was used in this study and extractions were performed at room temperature. The use of many different eluent types, eluent volumes and ionic strength conditions have been reported in the literature, depending on the type of analytes that were investigated (37,42,75,76,89,103). Therefore, it was important for the effects of these parameters on the extraction efficiency of the SPE method to be evaluated, in order to find the optimal conditions for the particular pesticides analysed in this study.

5.1 SPE method development

5.1.1 Optimisation of sample volume

Sample volume is one of the fundamental factors in SPE analytical procedures that have an influence on the efficient recovery of analytes from water samples. Breakthrough volume refers to the volume at which a particular target analyte loaded

onto a SPE column begins to elute (97). Analytes are adsorbed on the SPE column as the sample is loaded, up to the point of saturation at which the column reaches its retention capacity. Further addition of analyte solution after that point (retention capacity) the analyte will not be retained by the column (104)**.** As seen in Fig. 4.3, there was no apparent difference in extraction recoveries with sample volumes tested for most of the pesticides analysed, except for aldrin (**10**), 4,4'-DDE (**15**) and 4,4'-DDT (**22**), which 500 mL yielded 65 percent, 78 percent and 66 percent, respectively, compared to 61 percent, 72 percent and 63 percent for 100 mL, respectively.

5.1.2 Selection of eluent type

The ideal elution solvent should be strong enough to efficiently elute all the targeted compounds. The elution strength of the organic solvent depends on the type of sorbent used and the target analytes (97). Elution solvents desorb analytes while the remaining matrix components should be retained on the SPE column (103). Pesticides are often eluted from C_{18} cartridges with hexane, ethyl acetate, acetone, methanol, dichloromethane (DCM) or mixtures of these solvents (5,85,104).

In preliminary experiments performed at the beginning of this work, hexane was used as the elution solvent and it was established that hexane alone was not suitable for efficient elution of the mixture of pesticides analysed in this study (results not shown). Usually, hexane is used to elute non-polar, DCM to elute intermediate polarity and ethyl acetate to elute polar pesticides (102). Therefore, for this study three solvent systems were tested for the elution of pesticides from the SPE sorbent, namely DCM only, methanol followed by acetone and hexane followed by ethyl acetate (85,104–106).

The results revealed that the solvent system where pesticides, eluted with hexane followed by ethyl acetate, which provided the highest recoveries for all pesticides, except for aldrin (**10**) for which slightly higher recoveries resulted when DCM was used (Fig. 4.3). Elution of the pesticides with hexane and ethyl acetate provided higher recoveries on average of 87 percent compared to 81 and 64 percent by DCM and methanol followed by acetone, respectively.

5.1.3 Optimisation of eluent volume

The amount of eluent investigated in this study was chosen according to previous studies which found volumes between 1 and 10 mL to be sufficient to desorb pesticides from a SPE cartridge (5,42,84,85,97). The effect of the eluent volume on the recovery of the pesticides was therefore investigated using total volumes of 2, 5 and 10 mL. The highest extraction recoveries were obtained for most of the pesticides when 10 mL of the eluent was used (5 mL of hexane followed by 5 mL ethyl acetate), except for simazine (**4**) and endosulfan sulfate (**21**) where 5 mL yielded marginally higher extraction recoveries.

5.1.4 The effect of ionic strength

Since most of the pesticides that were investigated in this study are somewhat hydrophilic (log K_{ow} < 10, see Table 2.3), ionic strength was regarded as a significant factor that may influence the methods' extraction efficiency. Adding various salts to water samples decrease the solubility of the analytes in water, thereby increasing the adsorption on the SPE sorbent (99). The effect of the certain salt addition on the extraction recovery of pesticides was investigated by adding different amounts of NaCl (from 0 to 10 percent, w/v) to the sample solutions. An increase in

extraction recovery was observed for most of the pesticides, as the amount of NaCl increased from 0 to 5 percent NaCl. On the other hand, increasing the concentration of NaCl to 10 percent had no significant impact on the extraction recovery of the pesticides from the water samples,this could be due to salting-out and or electrostatic interaction processes. When NaCl is added, the water molecules form hydration spheres that surround the ionic salt molecules (40). This process reduces the concentration of water molecules available to dissolve the analytes, driving the analytes into the extraction phase. However, as the amount of salt is increased, analytes may start partaking in electrostatic interactions with the salt ions in solution, thus reducing the ability to move into the extraction phase (97).

5.2 Method validation

A method is considered accurate and precise when accuracy data (% recovery) is between 70-120 percent and the method precision (% RSD) is not higher than 20 percent (62 ,91,98,104). The method developed during this study yielded average recovery values ranging from 76 – 93 percent (Table 4.3). Furthermore, good linearity was obtained over the studied working range, with correlation coefficients (R^2) greater than 0.99 for all but three of the pesticides. A few analytes have LOD values higher than the lowest linear range point, such as simazine (**4**), heptachlor epoxide (**12**), α-endosulfan (**13**), endrin aldehyde (**20**) and 4,4'-DDT (**22**). In these instances, it was still possible to detect the pesticides at lower levels in practice, even though their chromatographic peaks were smaller than those for the theoretical LOD value. This can sometimes occur if LOD values are determined through an empirical method (109), which was the case in the current study. LOQs values for most pesticides were equal or higher than the lowest calibration levels analysed in this

study, e.g. LOQ value of atrazine (**6**) was 0.0710 µg/mL and its lowest calibration level was 0.0125 µg/mL.

The results obtained in this study are comparable to the results of previous studies. Samadi *et al* (86) applied SPE coupled to GC-ECD and obtained a linear range from 0.000005 – 0.01 µg/mL and LOQ values of 0.00000033 – 0.000005 µg/mL. In contrast, better detection limits (0.000000004 – 0.00006 µg/mL) were reported by Qiu and Cai (97) using a combination of SPE and SPME coupled with GC-ECD, however, lower recovery rates were recorded (31 % to 44 %). In addition, average extraction recoveries similar to the ones obtained in the current study were previously reported (summarised in Table 5.1). For instance, the extraction recoveries that were previously reported for of atrazine (**6**) was 87 percent (current study 86 %), for aldrin (**10**) it was 80 percent (current study 80 percent), for α-endosulfan (**13**) it was 85 percent (current study 85 percent) and for butachlor (**14**) it was 94 % (current study 91 %) (92,102,105).

5.3 Application of the method

Water samples from GWTP and UWWTP were subjected to the validated SPE method. GC-MS analysis of the extracts revealed the presence of five herbicides, prometon (**5**), atrazine (**6**), alachlor (**9**), metolachlor (**11**) and butachlor (**14**) in the raw sewage entering GWTP. Using the validated SPE/GC-FID method, the concentrations of the herbicides could be determined.

The main effluent sources of UWWTP wastewater are tanneries, a brewery and abattoirs (92), which are unlikely to discharge pesticide pollutants. This could be the reason that no pesticides were detected in water samples from this plant.

The presence of herbicides in GWTP inlet water samples could be attributed to the agricultural and or urban run-off resulting from activities such as bush encroachment control and weed control. Prometon (**5**) is used for total flora control on industrial sites, it can also be used to inhibit weed breakthrough in driveways or parking areas (92). Atrazine (**6**), alachlor (**9**), metolachlor (**11**) and butachlor (**14**) are used to prevent and eradicate broadleaf and grassy weeds in crops such as sorghum, maize and sugarcane, it is also used on residential lawn and golf courses (61).

All herbicides detected from GWTP inlet water samples have a negative effect on organism's endocrine systems. Prometon (**5**) is an embryo-toxin, while metolachlor (**11),** butachlor (**14**) and alachlor (**9**) have estrogenic effects, i.e. they compete for the estrogen receptors, thereby preventing the natural hormones to bind and trigger the necessary biological processes, which could result in infertility. Alachlor (**9**) also compete with progesterone receptors, while metolachlor (**11)** activate pregnane X receptor and atrazine (**6**) inhibit and reduces steroid hormones metabolism in the body (69).

The concentrations at which these herbicides were detected in this study were relatively high in comparison with data reported for other sewage treatment plants, it was however below the EPA guidelines value of total herbicides in wastewater influent which is set at 11.00 μg/mL (110). Concentrations of metolachlor (**11**) at 0.00028 μg/mL was reported in the Willow Slough (USA) (92), alachlor (**9**) at 0.000074 μg/mL was reported in the Harbin sewage treatment plant (China) (92), atrazine (**6**) at 0.00003 μg/mL was reported in the Regensdorf wastewater treatment plant (Switzerland) (54), prometon (**5**) at 0.0000033 μg/mL in the Barcelona wastewater treatment plant (Spain) (92) and butachlor (**14**) at 0.0025 μg/mL in the Zibo wastewater plant (China), had been reported (92).

Table 5.1: Data from previous studies for the SPE analysis of the pesticides analysed in the current study (refer to Tables 4.2 & 4.3 for data of the current study).

Pesticide	**Linear range**	**R^2**	**%ER**	**%RSD**	**LOD**	**LOQ**	**Analytical method**	**Reference**
α- HCH	0.01-0.5	0.9990	90.9	2.5	-	0.000004	SPE/GC-MS	(5)
β- HCH	0.025-0.5	0.9990	92.4	1.3	-	0.00001	SPE/GC-MS	(5)
Lindane	-	0.9978	90	3.7	0.0003	0.0001	Matrix SPE/GC-μECD	(102)
Simazine	-	-	84.2	5.7	0.0015	-	SPE/GC-MS	(105)
Prometon	-	-	98.8	10.2	0.0028	-	SPE/GC-MS	(105)
Atrazine	-	-	87.4	6.2	0.0017	-	SPE/GC-MS	(105)
δ- HCH	0.025-0.5	0.9990	91.0	2.3	-	0.00001	SPE/GC-MS	(5)
Heptachlor	-	0.9962	84	2.0	0.00008	0.00028	Matrix SPE/GC-μECD	(102)
Alachlor	-	-	92.3	3.7	0.0011	-	SPE/GC-MS	(105)
Aldrin	-	-	80	6.0	0.000086	0.000286	SPE/UHPLC-MS-MS	(93)
Metolachlor	-	0.9981	91	3.6	0.00008	0.00028	Matrix SPE/GC-μECD	(102)
Heptachlor epoxide	-	0.9986	91	3.3	0.00006	0.0002	Matrix SPE/GC-μECD	(102)
α- Endosulfan	-	0.9986	85	1.9	0.00009	0.0003	Matrix SPE/GC-μECD	(102)

Butachlor	-	-	94.5	2.5	0.0043	-	SPE/GC-MS	(105)
4,4’-DDE	1-0.0156	0.9993	-	4.31	0.068	0.228	SPE/GC-MS	(64)
Dieldrin	-	0.9986	93	4.5	0.000001	0.0000032	Matrix SPE/GC-μECD	(102)
Endrin	0.00004-0.06	0.9997	44.67	13.3	0.000008	-	Matrix SPE/GC-μECD	(102)
β-Endosulfan	0.00004-0.06	0.9996	65.7	3.92	0.000005	-	SPE-HS-SPME/GC-ECD	(97)
4,4’DDD	-	-	97.9	4.2	0.0013	-	SPE/GC-MS	(105)
Endrin aldehyde	0.00004-0.06	0.9994	78.6	16.3	0.034	-	SPE-HS-SPME/GC-	(102)
Endosulfan sulfate	0.00004-0.06	0.9999	31.34	19.35	0.000015	-	SPE-HS-SPME/GC-ECD	(97)
4,4’-DDT	-	-	96.2	3.7	0.0013	-	SPE/GC-MS	(105)

CHAPTER SIX

6 CONCLUSION

In the present study, a SPE method coupled with GC-FID was optimised and validated for the preconcentration and quantification of 22 pesticides in water samples. The method is simple and produces low volumes of organic solvent waste. Under the optimised conditions, the method showed good recoveries of 76 % and above, on average, for the 22 pesticides and precision values of up to 13 % RSD.

The validated method was successfully applied to the analysis of pesticides in water samples collected from GWTP and UWWTP. The results demonstrate that agricultural and/or urban activities have an effect on wastewater. These results could draw attention to the necessity of monitoring pesticides used in Namibia both in rural and urban areas, especially in light of their endocrine disrupting properties, including interference with the steroids metabolism and the activation of pregnane X receptors, which could result in liver, kidney, brain damage and breast cancer.

6.1 Recommendations

The method developed during this study aimed to accommodate the simultaneous analyses of all 22 pesticides, hence the optimal conditions chosen are not necessary suitable for the individual compounds investigated. Therefore a method targeting only the observed herbicides should be developed and optimised. In addition, the use of a SPME method should be evaluated to compare the sensitivity with the SPE method. Since pesticides with a range of different polarities can be analysed with this method it could potentially also be applied to the identification and quantification of other pesticides and organic compounds with similar characteristics in future studies.

This is the first report on the study of pesticides in GWTP and UWWTP water samples. Therefore further investigation is required to determine if there are any other pesticides present in the water of these wastewater treatment plants. In addition, it should also be investigated whether there are any pesticides present in the surface and underground water sources around Windhoek and relevant parts of Namibia.

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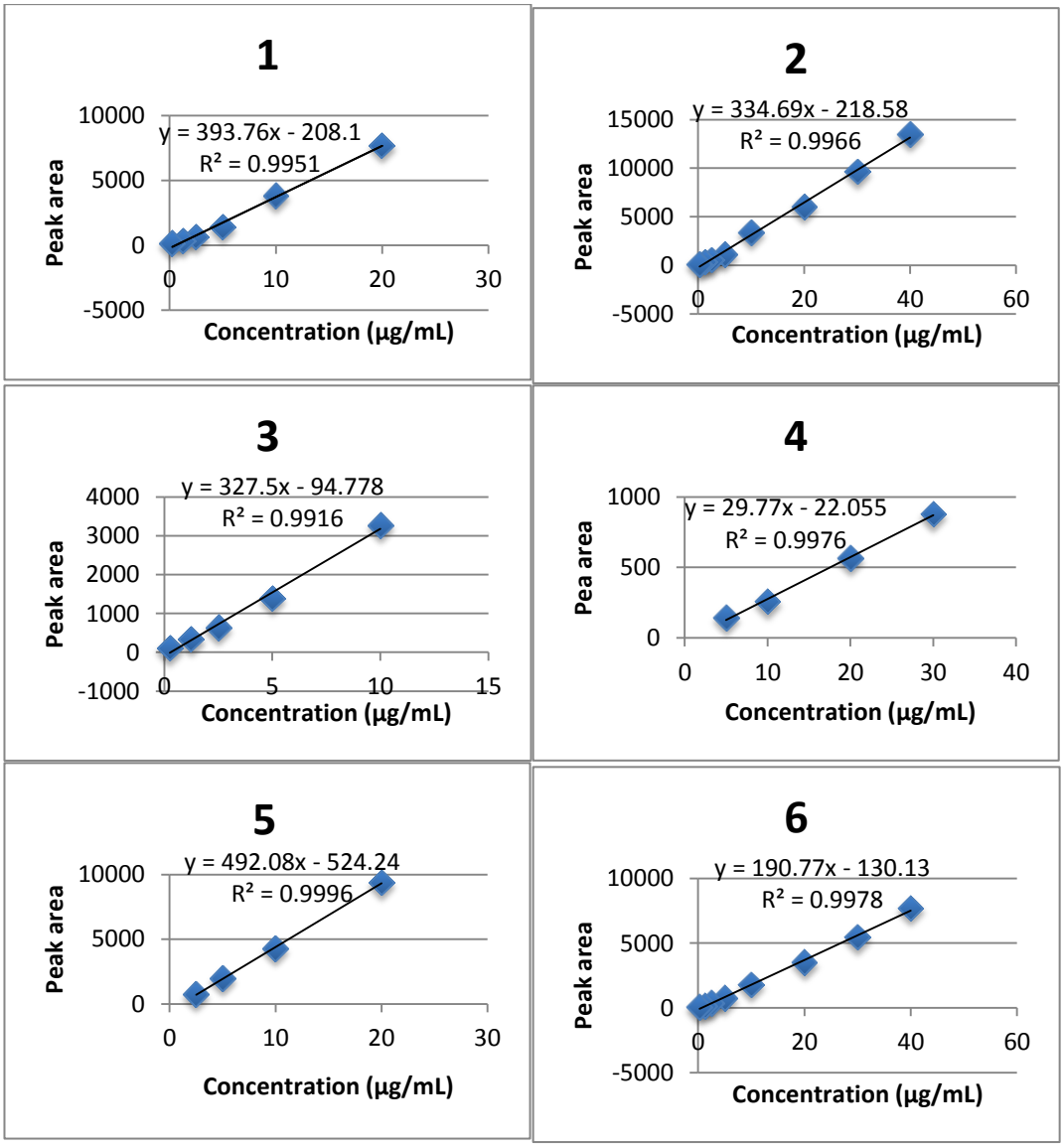
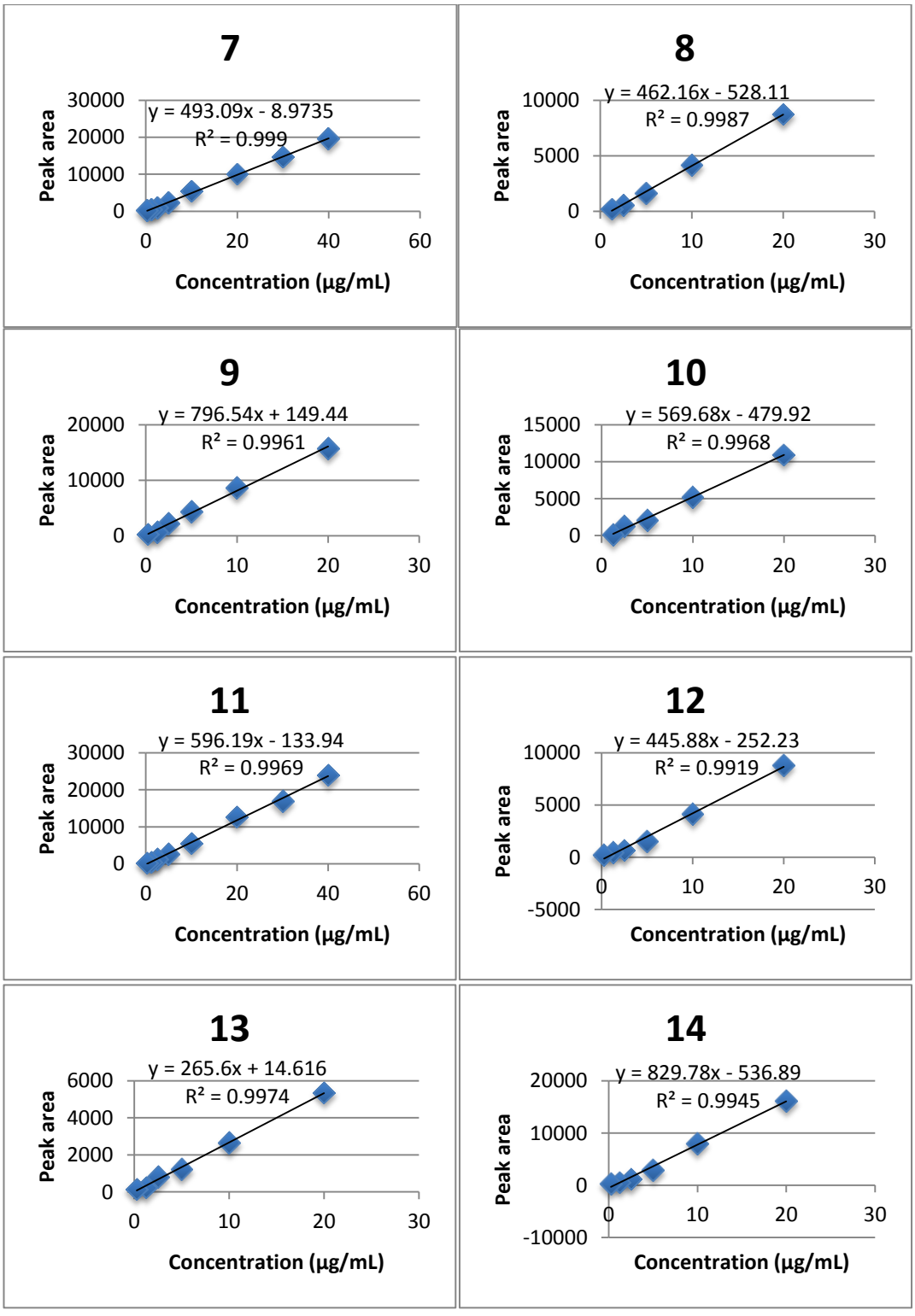
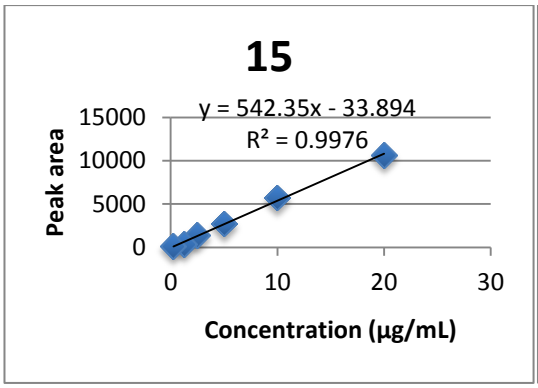
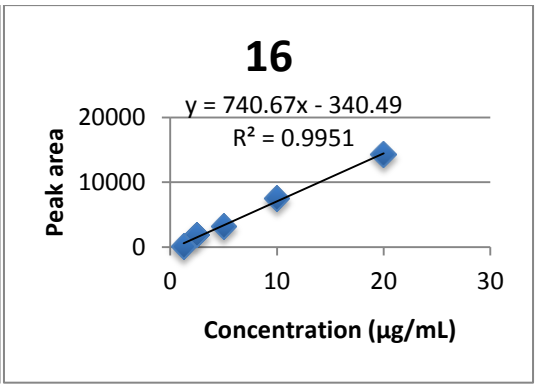
APPENDICES

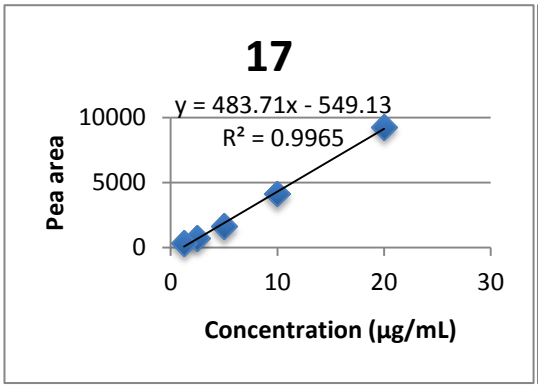
Appendix 1: Instrument linearity curves.

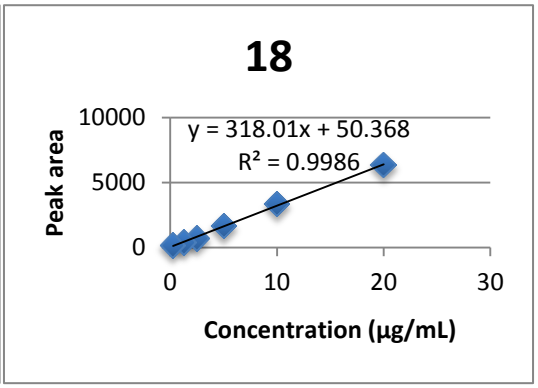


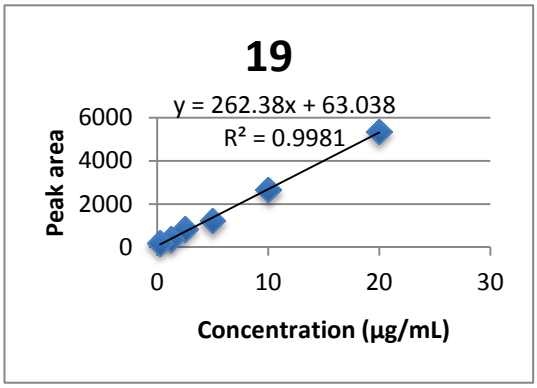

7
y = 493.09x - 8.9735
R² = 0.999
Peak area
Concentration (µg/mL)
8
y = 462.16x - 528.11
R² = 0.9987
Peak area
Concentration (µg/mL)
9
y = 796.54x + 149.44
R² = 0.9961
Peak area
Concentration (µg/mL)
10
y = 569.68x - 479.92
R² = 0.9968
Peak area
Concentration (µg/mL)
11
y = 596.19x - 133.94
R² = 0.9969
Peak area
Concentration (µg/mL)
12
y = 445.88x - 252.23
R² = 0.9919
Peak area
Concentration (µg/mL)
13
y = 265.6x + 14.616
R² = 0.9974
Peak area
Concentration (µg/mL)
14
y = 829.78x - 536.89
R² = 0.9945
Peak area
Concentration (µg/mL)

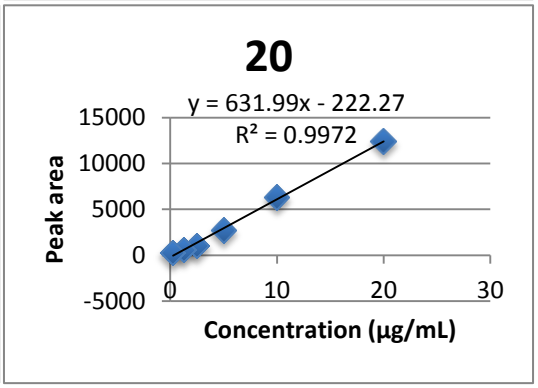

15
y = 542.35x - 33.894
R² = 0.9976
Peak area
15000
10000
5000
0
0
10
20
30
Concentration (µg/mL)

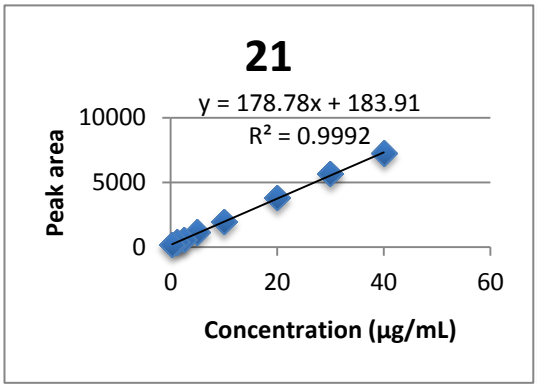

16
y = 740.67x - 340.49
R² = 0.9951
Peak area
20000
10000
0
0
10
20
30
Concentration (µg/mL)

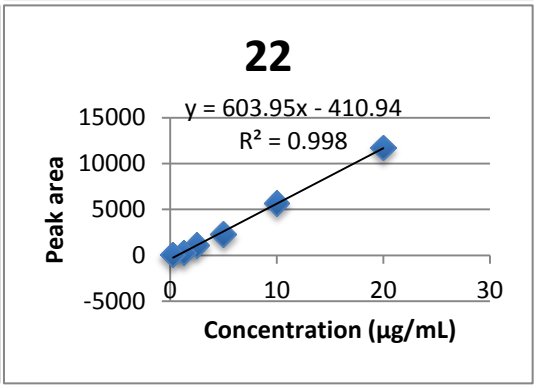

17
y = 483.71x - 549.13
R² = 0.9965
Pea area
10000
5000
0
0
10
20
30
Concentration (µg/mL)


18
y = 318.01x + 50.368
R² = 0.9986
Peak area
10000
5000
0
0
10
20
30
Concentration (µg/mL)

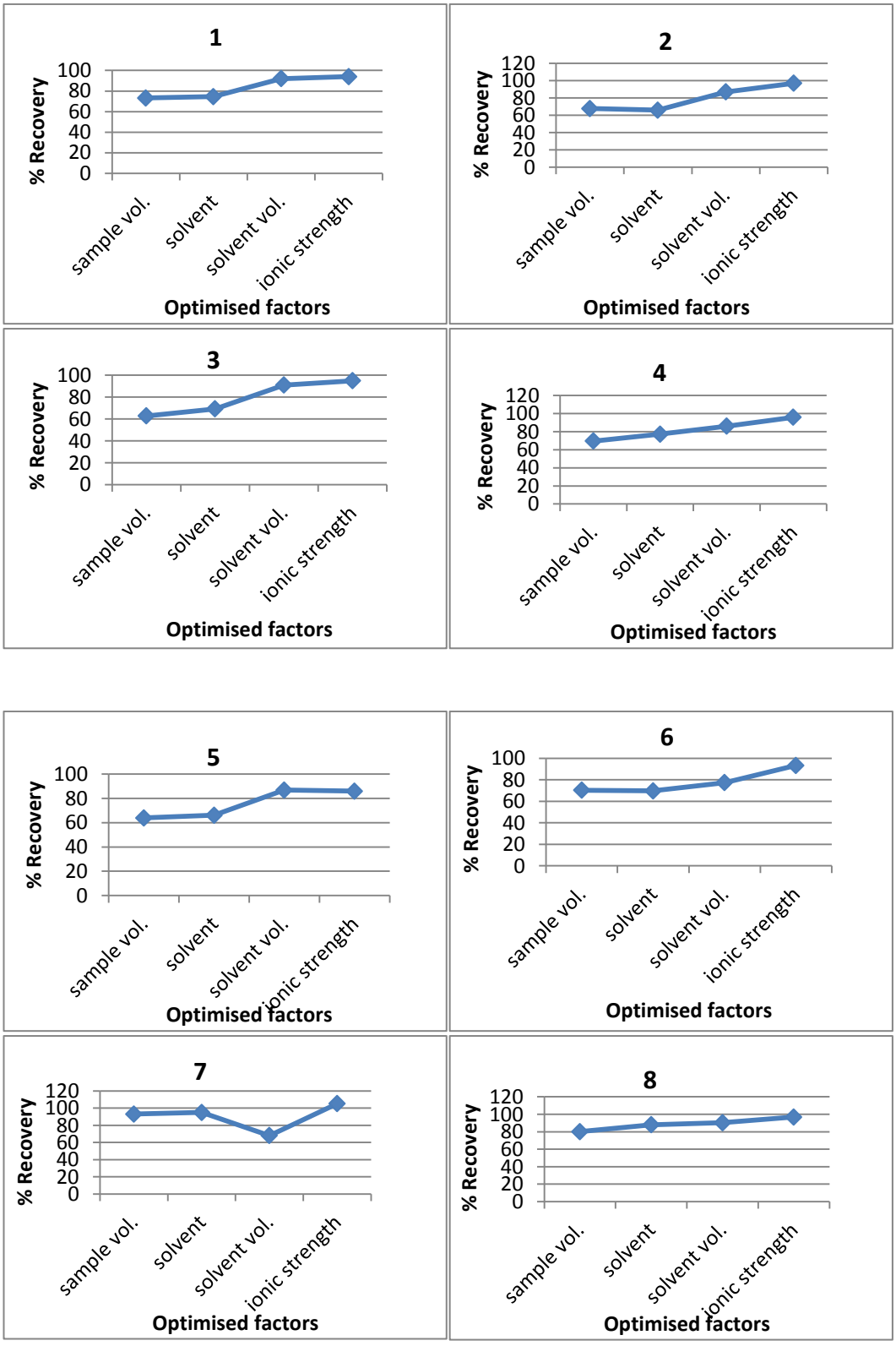

19
y = 262.38x + 63.038
R² = 0.9981
Peak area
6000
4000
2000
0
0
10
20
30
Concentration (µg/mL)

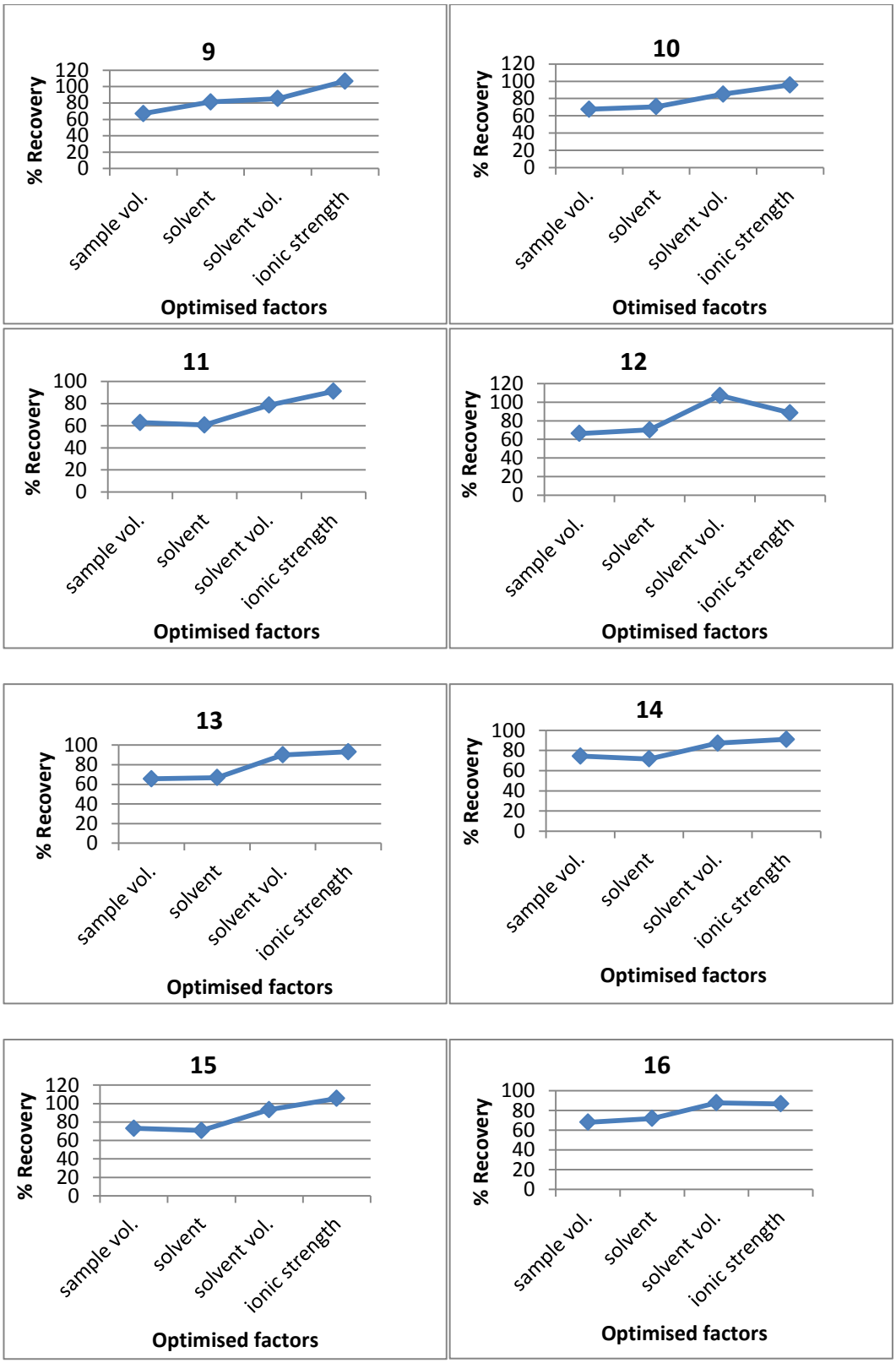

20
y = 631.99x - 222.27
R² = 0.9972
Peak area
15000
10000
5000
0
-5000
0
10
20
30
Concentration (µg/mL)

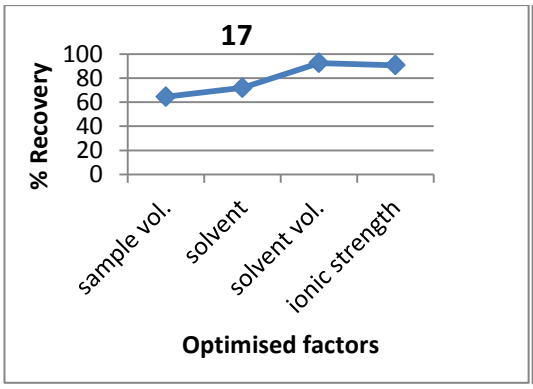

21
y = 178.78x + 183.91
R² = 0.9992
Peak area
10000
5000
0
0
20
40
60
Concentration (µg/mL)

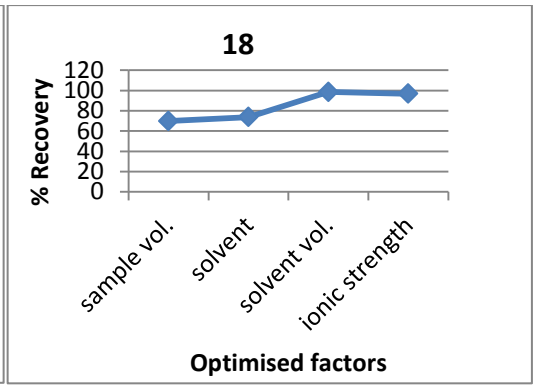

22
y = 603.95x - 410.94
R² = 0.998
Peak area
15000
10000
5000
0
-5000
0
10
20
30
Concentration (µg/mL)

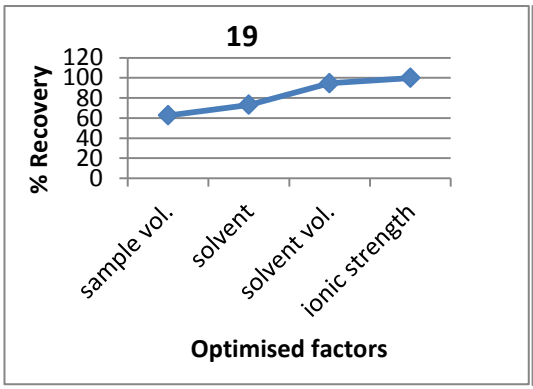
Appendix 2: The effect of each parameter on individual pesticides.

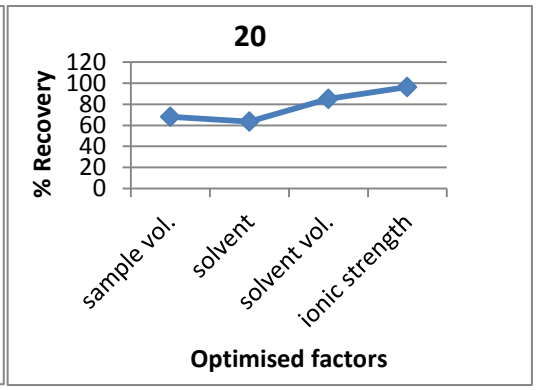


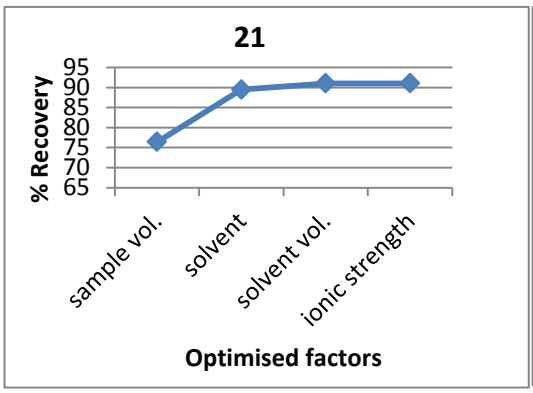

9
% Recovery
120
100
80
60
40
20
0
sample vol.
solvent
solvent vol.
ionic strength
Optimised factors
10
% Recovery
120
100
80
60
40
20
0
sample vol.
solvent
solvent vol.
ionic strength
Otimised facotrs
11
% Recovery
100
80
60
40
20
0
sample vol.
solvent
solvent vol.
ionic strength
Optimised factors
12
% Recovery
120
100
80
60
40
20
0
sample vol.
solvent
solvent vol.
ionic strength
Optimised factors
13
% Recovery
100
80
60
40
20
0
sample vol.
solvent
solvent vol.
ionic strength
Optimised factors
14
% Recovery
100
80
60
40
20
0
sample vol.
solvent
solvent vol.
ionic strength
Optimised factors
15
% Recovery
120
100
80
60
40
20
0
sample vol.
solvent
solvent vol.
ionic strength
Optimised factors
16
% Recovery
100
80
60
40
20
0
sample vol.
solvent
solvent vol.
ionic strength
Optimised factors

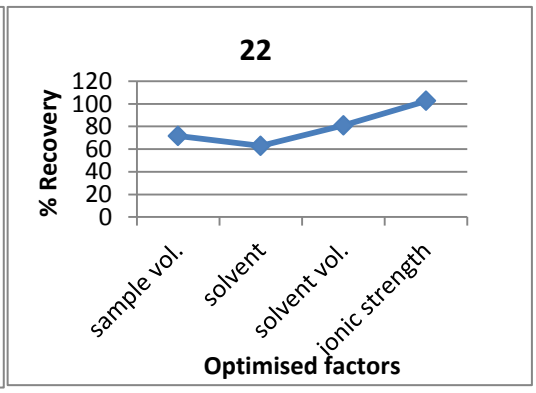

17
% Recovery
100
80
60
40
20
0
sample vol.
solvent
solvent vol.
ionic strength
Optimised factors


18
% Recovery
120
100
80
60
40
20
0
sample vol.
solvent
solvent vol.
ionic strength
Optimised factors

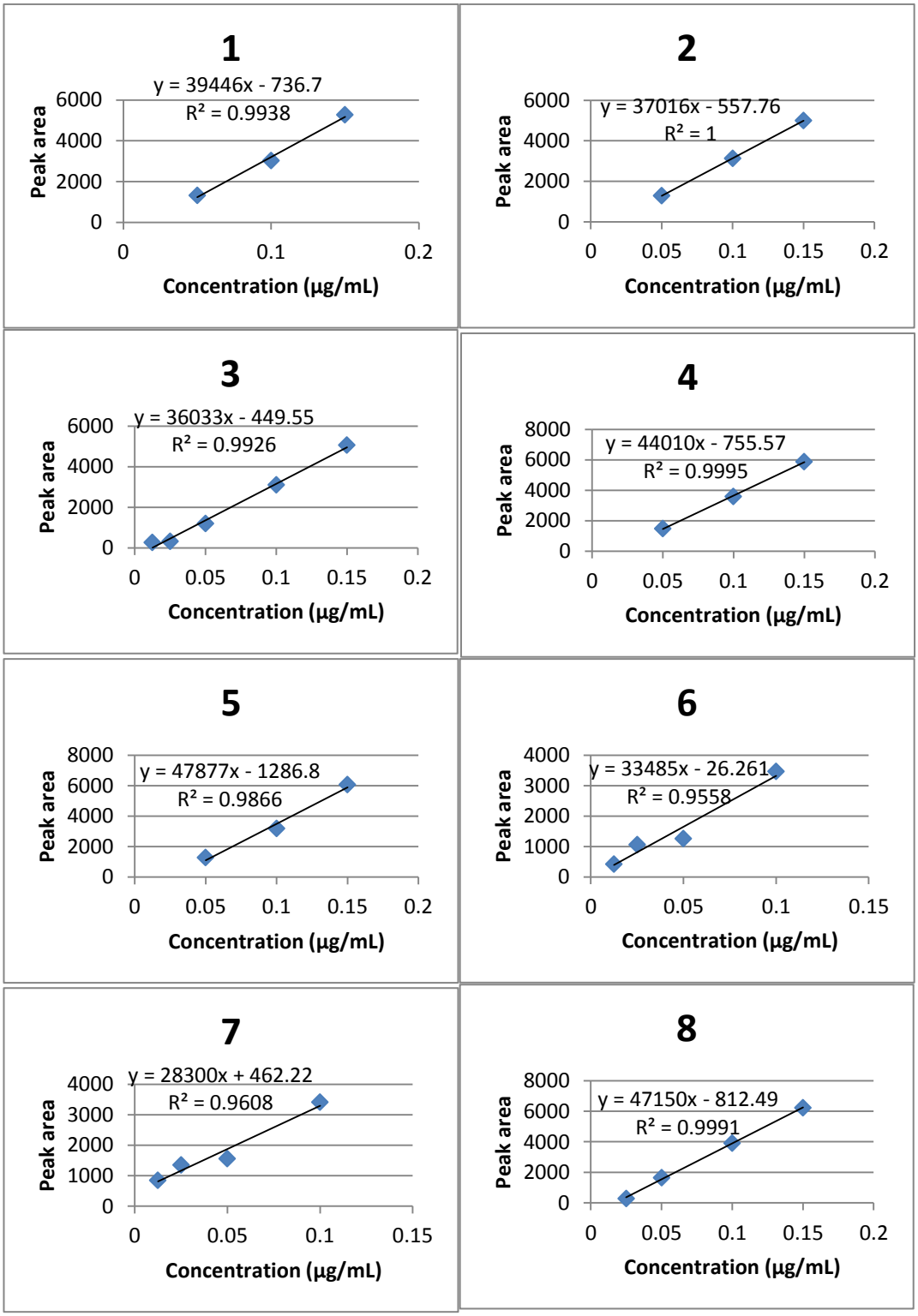

19
% Recovery
120
100
80
60
40
20
0
sample vol.
solvent
solvent vol.
ionic strength
Optimised factors

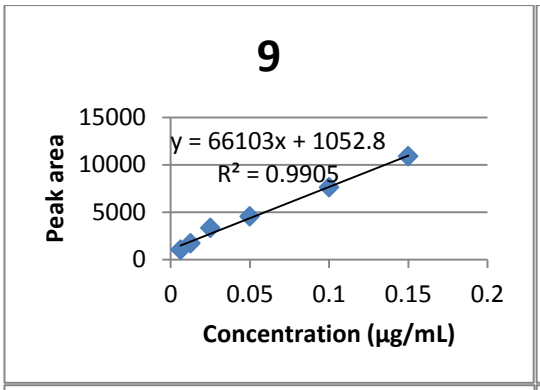

20
% Recovery
120
100
80
60
40
20
0
sample vol.
solvent
solvent vol.
ionic strength
Optimised factors

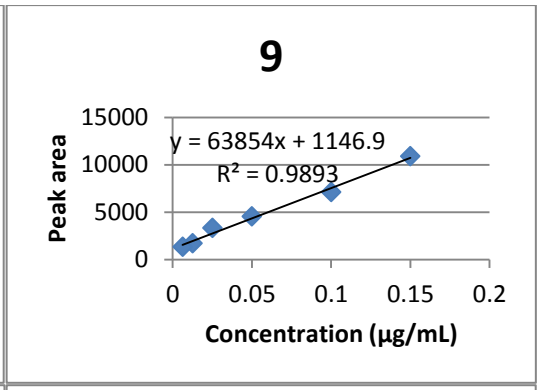

21
% Recovery
95
90
85
80
75
70
65
sample vol.
solvent
solvent vol.
ionic strength
Optimised factors

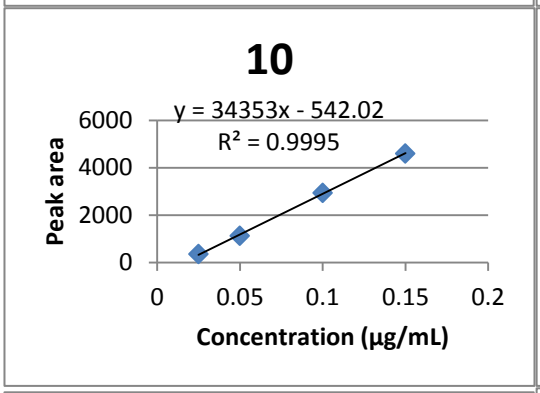

22
% Recovery
120
100
80
60
40
20
0
sample vol.
solvent
solvent vol.
ionic strength
Optimised factors

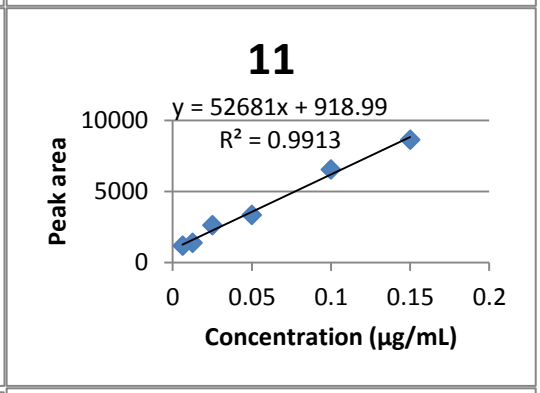
Appendix 3: Method linearity curves.

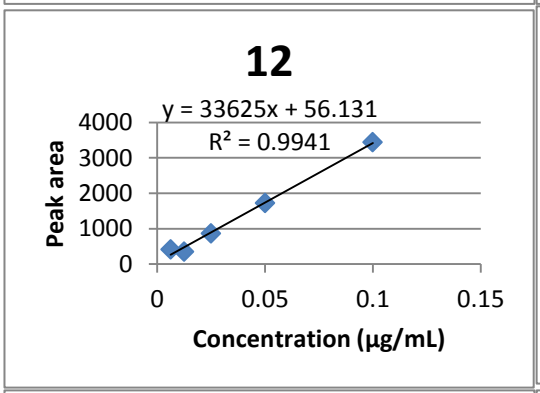


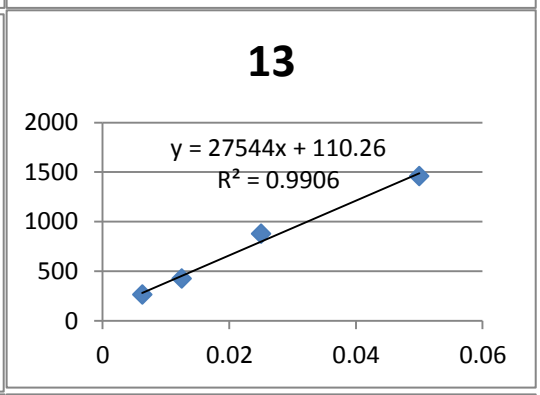

9
y = 66103x + 1052.8
R² = 0.9905
Peak area
Concentration (µg/mL)

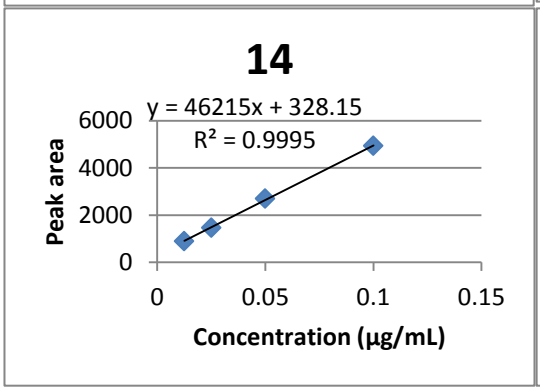

9
y = 63854x + 1146.9
R² = 0.9893
Peak area
Concentration (µg/mL)

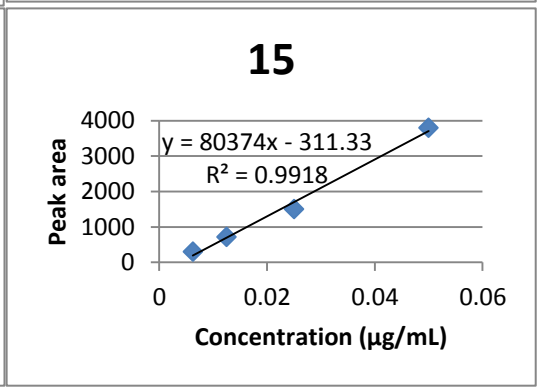

10
y = 34353x - 542.02
R² = 0.9995
Peak area
Concentration (µg/mL)

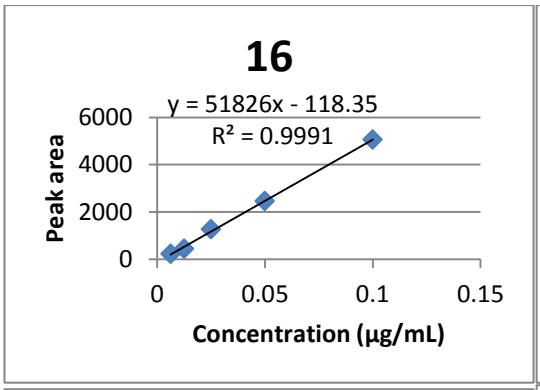

11
y = 52681x + 918.99
R² = 0.9913
Peak area
Concentration (µg/mL)

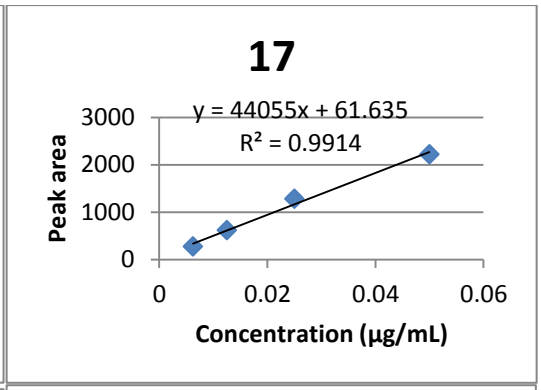

12
y = 33625x + 56.131
R² = 0.9941
Peak area
Concentration (µg/mL)

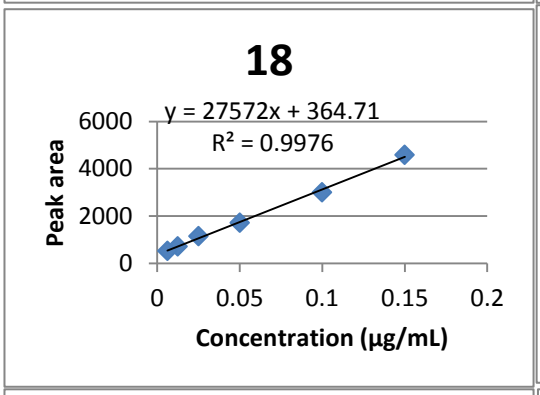

13
y = 27544x + 110.26
R² = 0.9906

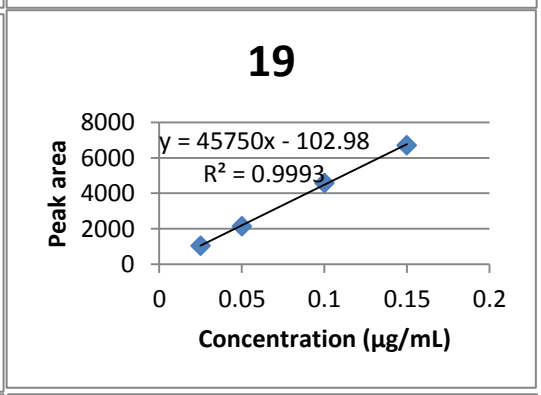

14
y = 46215x + 328.15
R² = 0.9995
Peak area
Concentration (µg/mL)

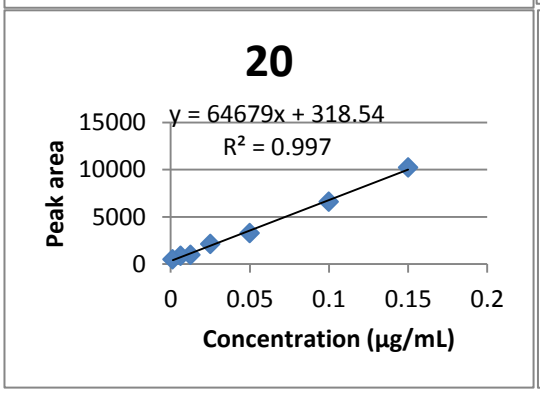

15
y = 80374x - 311.33
R² = 0.9918
Peak area
Concentration (µg/mL)

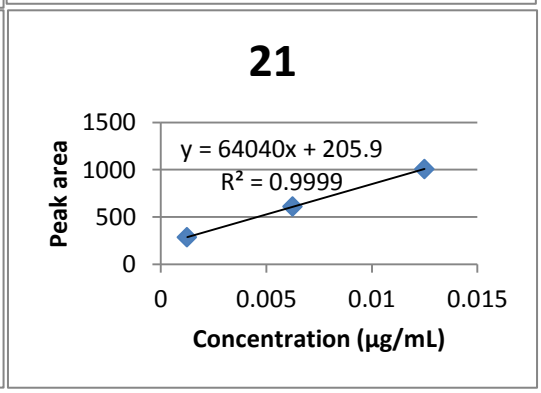

16
y = 51826x - 118.35
R² = 0.9991
Peak area
6000
4000
2000
0
0
0.05
0.1
0.15
Concentration (µg/mL)

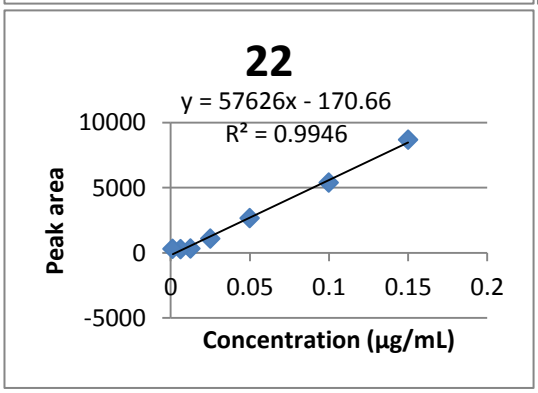

17
y = 44055x + 61.635
R² = 0.9914
Peak area
3000
2000
1000
0
0
0.02
0.04
0.06
Concentration (µg/mL)


18
y = 27572x + 364.71
R² = 0.9976
Peak area
6000
4000
2000
0
0
0.05
0.1
0.15
0.2
Concentration (µg/mL)

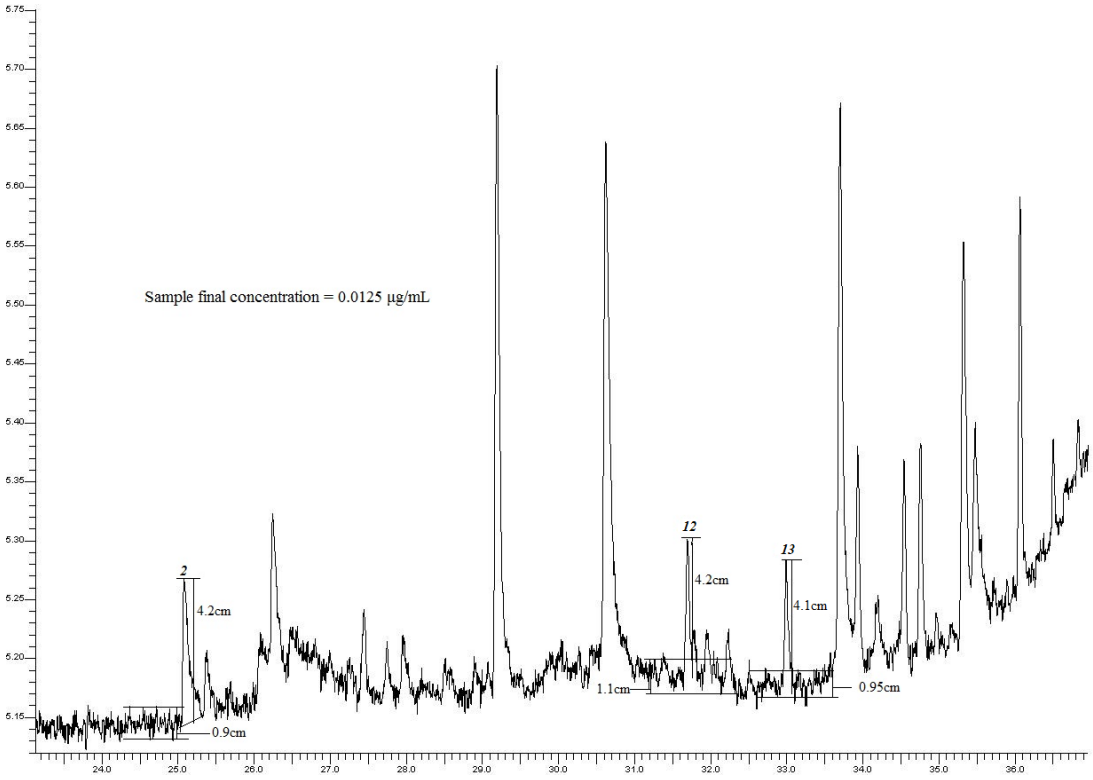

19
y = 45750x - 102.98
R² = 0.9993
Peak area
8000
6000
4000
2000
0
0
0.05
0.1
0.15
0.2
Concentration (µg/mL)


20
y = 64679x + 318.54
R² = 0.997
Peak area
15000
10000
5000
0
0
0.05
0.1
0.15
0.2
Concentration (µg/mL)


21
y = 64040x + 205.9
R² = 0.9999
Peak area
1500
1000
500
0
0
0.005
0.01
0.015
Concentration (µg/mL)


22
y = 57626x - 170.66
R² = 0.9946
Peak area
10000
5000
0
-5000
0
0.05
0.1
0.15
0.2
Concentration (µg/mL)

Appendix 4: Chromatograms illustrating examples of LOD and LOQ calculations.



Peak **2**: Signal/Noise (S/N)= 4.2cm/0.9cm=4.67

LOQ= (0.0125 µg/mL*10)/4.67

=0.0300 µg/mL

LOD: 0.0300 µg/mL/3.3 =**0.0090 µg/mL**

Peak **12: S/N =** 4.2cm/1.1cm =3.81

LOQ= (0.0125 µg/mL*10)/3.81

= 0.0300 µg/mL

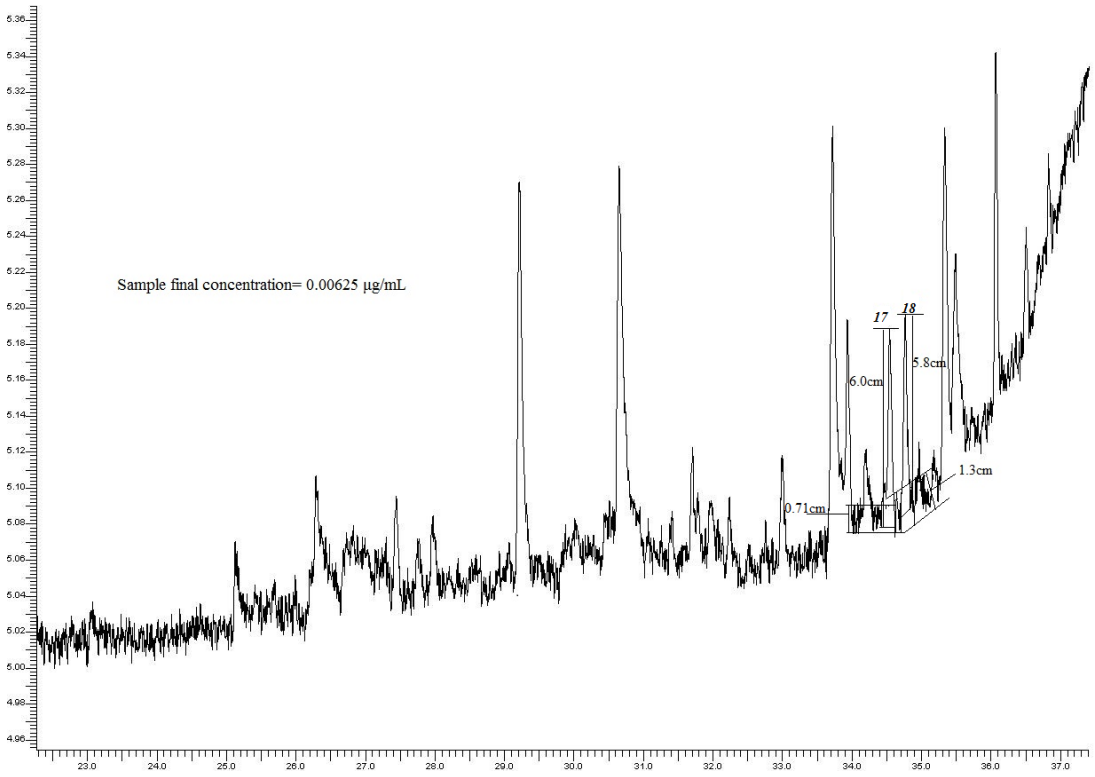
LOD=0.0300 µg/mL/3.3 =**0.0090 µg/mL**

Peak **13:** S/N= 4.1cm/0.95cm= 4.32

LOQ=(0.0125 µg/mL*10)/4.32

=0.0300 µg/mL

LOD= 0.0300 µg/mL/3.3= **0.0090 µg/mL**



Peak **17**: S/N=6cm/0.71cm=8.45

LOQ= (0.00625 µg/mL*10)/8.45

=0.0074 µg/mL

LOD=0.0074 µg/mL/3.3= **0.0022 µg/mL**

Peak **18: S/N=**5.8cm/1.3cm=4.46

LOQ=(0.00625 µg/mL*10)/4.46

=0.0140 µg/mL

LOD=0.0140 µg/mL/3.3=**0.0042 µg/mL**