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Assessment of organochlorine pesticide residues in fish samples from the Okavango Delta, Botswana.

A thesis submitted in fulfilment of the requirement for the degree of Master of Science in

Chemistry

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Supervised by

Professor Nelson Torto

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With God's Glory things are possible! He has plans for all of us.

Dedication

To my wife and Sons

Seitiso, Lindani, Andy and little **Jeelie**.

Abstract

This thesis presents an evaluation of the dispersive solid-phase extraction (d-SPE) method referred to as the quick, easy, cheap, effective, rugged and safe (QuEChERS) method for the determination of four organochlorine pesticide residues in fish samples. The pesticides investigated in this study were o, p′-DDT, p, p′-DDE, aldrin and dieldrin. The combined use of Gas Chromatography with an Electron Capture Detector (GC-ECD) and sensitive Time of Flight (TOF) mass detector facilitated the identification of the target analytes. In the absence of certified reference material, the overall analytical procedure was validated by systematic recovery experiments on spiked samples at three levels of 2, 5 and 10 ng/g. The targeted compounds were successfully extracted and their recovery ranged from 76 to 96% with relative standard deviations of less than 13%. The optimum QuEChERS conditions were 2 g of fish powder, 10 ml acetonitrile and 1 min shaking time. The optimal conditions were applied to assess the levels of chlorinated pesticides in blunt-tooth catfish (*Clarias ngamensis*), tigerfish *(Hydrocynus vittatus*), *Oreochromis andersonii* and red-breasted tilapia (*Tilapia rendalli*) from the Okavango Delta, Botswana.

Dieldrin, p, p′-DDE and aldrin were detected in all the analysed samples with a concentration range of 0.04 – 0.29, 0.07 – 0.33, 0.04 – 0.28 and 0.03 – 0.24 ng/g per dry weight in *O. andersonii, C. ngamensis, T. rendalli* and *H. vittatus* respectively. These concentrations were below the US-EPA 0.1 µg/g allowable limit in edible fish and the Australian Maximum Residue Limit (MRL) of 50 - 1000 ng/g in fresh fish. DDT was not detected in all the fish species investigated.

The mean lipid content recorded in the fish samples were 1.24, 2.16, 2.18 and 4.21% for *H. vittatus, T. rendalli, O. andersonni* and *C. ngamensis* respectively. No systematic trend was observed between fish age and pesticide levels in fish. Acetylcholinesterase (AChE) activity assays were performed to assess the effects of organochlorine pesticides in *Clarias ngamensi*s. The enzyme activity recorded in *Clarias ngamensis* from the Okavango Delta and the reference site was 12.31 µmol of acetylcholine iodide hydrolysed/min/g brain tissue. The enzyme activity remained the same indicating no enzyme inhibition.

The conclusions drawn from this study are that the QuEChERS method is applicable for the determination of organochlorine pesticide residues in fish matrices. The fish from the Okavango Delta are safe for human consumption.

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LIST OF ABBREVIATIONS

AChE	acetylcholinesterase
Ah	aryl hydrocarbon
ALAD	aminolevulinic acid dehydratase
AOAC	Association of Official Analytical Chemists
ATC	acetylthiocholine
ATCI	acetylthiocholine iodide
CAT	catalase
CB	carbamates
CBNRM	community-based natural resources management
CRM	certified reference material
DDD	dichlorodiphenyldichloroethane
DDE	dichlorodiphenylchloroethylene
DDT	dichlorodiphenyltrichloroethane
DPX	distyrene phosphate xylene
DTNB	5, 5′-dithiobis-2-nitrobenzoic acid
DWA	Department of water affairs
d-SPE	dispersive solid-phase extraction
EI	electron impact
EPA	Environmental protection agency
EROD	ethoxyresorufin-o-deethylase
EU	European union
E	east

EDCs	endocrine disrupting chemicals
eV	electron volt
g	gram
GIGO	garbage-in garbage-out
GC-µECD	gas chromatography- micro electron capture detector
GC-MS	gas chromatography-mass spectrometry
GSH	glutathione
GPx	glutathione peroxidase
GR	glutathione reductase
GST	glutathione-s-transferase
HCB	hexachlorobenzene
HPLC	high performance liquid chromatography
IRS	indoor residual spraying
kHz	kilohertz
LLE	liquid-liquid extraction
LD_{50}	lethal dose 50%
LC-MS	liquid chromatography-mass spectrometry
LOD	limit of detection
LOQ	limit of quantification
LPO	lipid peroxidase
mm	millimetre
mL	millilitre
M	moles per litre

MAE	microwave-assisted extraction
MeCN	acetonitrile
MFO	mixed function oxygenate
MPA	megapascal
MRL	maximum residue limit
MSPD	matrix solid-phase dispersion
m/z	mass to ion charge ratio
nm	nanometre
NIST	National institute of standards and technology
OBT	odour baited target
OCP	organochlorine pesticide
ODMP	Okavango delta management plan
OKACOM	Okavango river basin water commission
PLE	pressurised liquid extraction
PAHs	polycyclic aromatic hydrocarbons
PCA	polychlorinated alkanes
PCB	polychlorinated biphenyls
PCDDs	polychlorinated dibenzo-*p*-dioxins
PCDFs	polychloplorinated dibenzofurans
PCN	polychlorinated napthalene
PSA	primary secondary amine
POPs	persistent organic pollutants
QuEChERS	quick, easy, cheap, effective, rugged and safe

rpm	revolutions per minute
RSD	relative standard deviation
S	south
SFE	supercritical fluid extraction
SPAD	solid-phase analytical derivatives
SPE	solid-phase extraction
SRM	standard reference material
TEQ	toxic equivalents
UNEP	United nations environmental programme
USDHHS	United states department of health and human services
UV	ultraviolet visible
μgL^{-1}	microgram per litre
µl	microlitre
µm	micrometre
WHO	World health organisation

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Chapter 1

Introduction

1.1 Background

The Okavango River rises in the highlands of central Angola by merging of the Cubango and Cuito rivers. The river then flows southeast approximately 600 km towards the centre of the southern African subcontinents where it terminates in the vast flood plain of the Okavango Delta, northwest Botswana (**Figure 1.1)**. The Okavango Delta (18° 45′ 0″ S 22° 45′ 0″ E), is one of the world's largest inland wetland regions (McCarthy and Ellery, 1994). This is reflected by its designation as a wetland of international importance protected as a Ramsar site (Kgathi *et al*., 2006). The Okavango Delta is described as a large alluvial fan created and maintained by the annual flooding of the Okavango River (Mendelsohn and El Obeid, 2004).

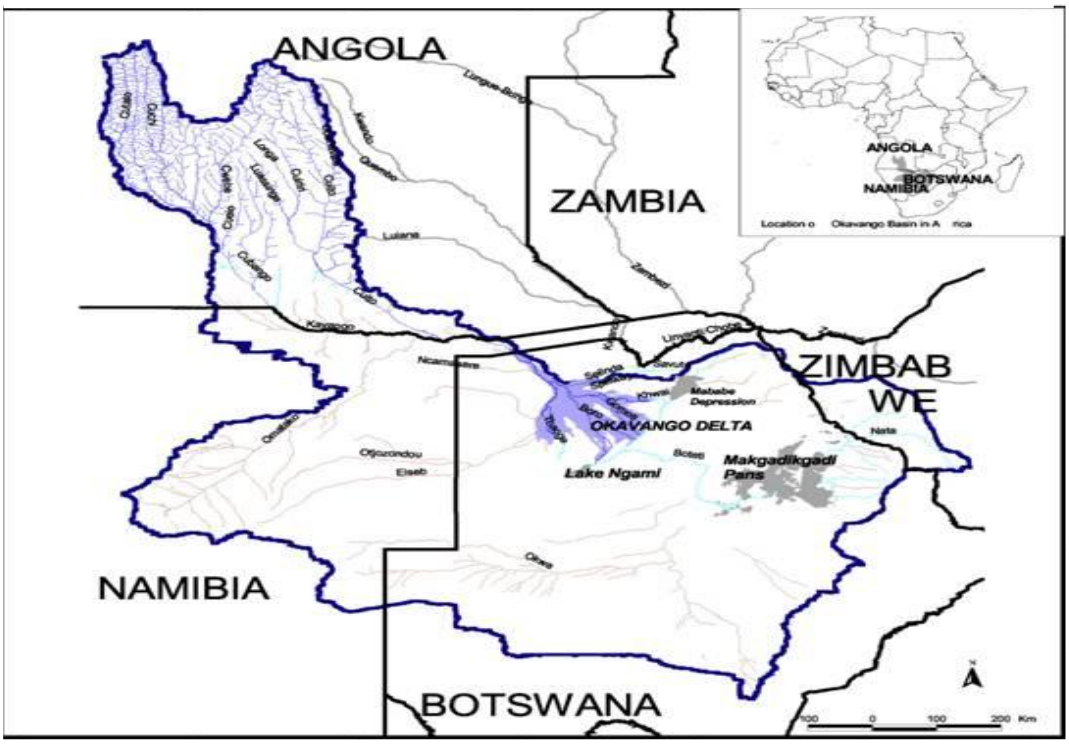


Figure 1.1: Cuito and Cubango rivers that form the Okavango Delta, Botswana (Kgathi *et al*., 2006)

Annually, the floods peak in the upper delta around April only to reach the distal end of the delta five months later between June and August. This is the period when the floods are receding in the upper delta (Gieske, 1997). The wetted delta ranges seasonally in size from 8000 to 16,000 km^2 (Turton *et al*, 2003a, Mendelsohn and El Obeid, 2004). In wet periods it reaches approximately 28,000 km^2 (Ramberg *et al*, 2006).

The future of the delta depends on the activities and developments taking place in the fringes of the delta. Possible future plans to abstract water from the Okavango River and the use of water for developmental projects in Angola and Namibia will reduce the quantity of water reaching the delta. This poses a threat to the delta's future and the life it supports. An advisory committe was formed between the three states (Botswana, Namibia and Angola) in 1994. This body is known as the Okavango River Basin Water Commission (OKACOM), it acts as a technical advisor of the contracting states on matters relating to the conservation, development and utilisation of the resources of common interest in the basin. The three countries recognise the implications that developments upstream the river can have on the resources downstream. Other than OKACOM, Botswana has a number of government departments that help in the management of the delta. For instance, the Department of Water Affairs (DWA) oversees the hydrology and water resources component in the Okavango Delta Management Plan (ODMP). One key responsibility of this component is to monitor the quantity and quality of water that flows into the delta. Its information base, aims to address the impact of water resources development upstream, abstraction of water and physical interventions in the delta.

Use of pesticides by farmers in the basin could adversely affect the quality of the water and all forms of life if it goes unchecked. In this regard, there is need that the science community joins hands with the government in monitoring the levels of organic pollutants in the delta. The generated scientific data on levels of pollutants could assist the government to make informed decisions on management aspects of the delta.

1.2 Livelihood of the riparian communities of the Okavango Basin

The main natural resource-based livelihood activities in the Okavango Basin include arable farming, livestock farming, collection of veld products, basket making, fishing and tourism. Important livelihood opportunities for the riparian communities in the basin are the outcome of a combination of the natural resources available in the area, primarily water, land and living biological resources. According to Mendelson and El Obeid (2004), more people are

dependent on farming in Angola than in other parts of the basin. They estimated that in Angola there were 60 000 farmers involved in small scale arable and livestock farming compared to 18 000 and 8500 farmers in Kavango and Ngamiland respectively. In the Angolan part of the basin, the majority of farmers cultivate their crops on dry land and other households practice flood recession agriculture. Arable farming in Ngamiland is also in the form of dry-land and flood recession cultivation. In 1997 and 1998, the proportions of farmers who practiced flood recession farming accounted for 27% and 16% of all the farmers in Ngamiland, whereas those who practiced dry-land farming accounted for 73% and 84% respectively.

The challenges faced by the riparian communities include drought, diseases (animals, crops and humans), floods and desiccation of the river channels. The other main challenge encountered by these communities is reduced access to land and natural resources as some areas in the basin have been declared no-go zones. In the Okavango delta, the establishment of community-based natural resource management (CBNRM) projects have reduced the access of the riparian communities to their land and natural resources (Taylor, 2000). In addition, the introduction of cordon fences by the veterinary department for disease control purposes in Ngamiland has had an adverse effect on livelihoods; access to water, pasture, fishing and gathering of veld products by the communities have been reduced (Ministry of Agriculture, 2002, Kgathi *et al*., 2004). There is no information on the impact of land tenure institutions on access to natural resources on the Angolan and Namibian part of the basin. However, the proposed expansion of irrigation schemes in the Kavango may reduce access by the local communities to land and its natural resources.

1.3 Fish species of the Okavango Delta

The delta has more than 71 fish species and these support subsistence, commercial and recreational fisheries (Merron and Bruton, 1995; Mosepele and Kolding, 2003). In the lower delta, there are about 62 fish species with different fish assemblages in permanent and seasonal swamps (Mosepele and Mosepele, 2005). The permanent swamp populations are characterised by high abundance of tiger fish *(Hydrocynus vittatus),* sharp tooth catfish *(Clarias gariepinus)* and the three spotted tilapia *(Oreochromis andersonii).*The seasonal swamp populations are dominated by silver catfish *(Schilbe intermedius)* and the African pike *(Hepsetus odoe)* (Merron and Bruton, 1995).

Recreational fishery is concentrated in the upper delta, while commercial fishery is more wide spread and involves about 40 full-time fishermen (Kgathi *et al.,* 2005). According to Mosepele and Kolding (2003), the five most important species in the recreational fishery are tiger fish, nembwe, three-spot tilapia, deep deck bream *(Sargochromis greenwoodii),* and thin largemouth *(Serranochromis angusticeps).* The principal commercial species are three-spotted tilapia, red breasted tilapia, green-head tilapia, nembwe, thin face largemouth and hump back *(Serranochromis altus).* The catfishes are also harvested but are rarely target species as are the tilapia (Mosepele and Kolding, 2003, Mosepele and Mosepele, 2005). The subsistence fishery involves about 3000 fisherman who mainly target small fish species like small tilapia and cyprinids. However they do harvest other species of different sizes as well.

The tiger fish (*Hydrocynus vittatus*) is endemic to Africa. It belongs to the Characidae family which is one of the largest freshwater fishes found in Africa and the neotropics (Kenmuir, 1972; Skelton, 1993). The tiger fish is restricted to the open waters in the upper region of the Okavango Delta known as the panhandle. The species represents the major piscovorous fish and mainly feeds on cichlid fishes, African pike and characids (Winemiller and Kelso-Winemiller, 1994). The tiger fish has a well-deserved reputation as a most sought-after sport fish in the Okavango delta and the African continent (Kotze *et al*., 1998). The common tilapia species of the Okavango Delta are the three spotted tilapia (*Oreochromis andersonni*) and the red-breasted tilapia (*Tilapia rendalli*). These species belong to the Cichlidae family. The tilapia is benthopelagic and is adapted to fast flowing waters. The species also prefer fairly deep quiet waters with some weed cover. They are herbivores and feed on fine particulate matter including algae, diatoms, detritus and zooplanktons (Skelton, 1993). The three spotted tilapia is characterised by three prominent black spots on either flank. Usually these spots are visible from an early age of the fish. The red-breasted tilapia is olive on the surface and has a red throat and under belly. The catfish belongs to the Clariidae family, the air breathing fishes. The catfish have large accessory breathing organs composed of modified gill arches that enable them to survive outside water. The most common and popular catfishes in the delta are the sharp tooth catfish (*Clarias gariepenus*) and the blunt tooth catfish (*Clarias ngamensis*).

One of the major threats to the delta's biodiversity especially fish is the use of pesticides that has been ongoing for several decades. A number of studies have been conducted on ecotoxicological effects of endosulfan on aquatic invertebrates in the delta (Douthwaite, 1982, Fox and Matthiessen, 1982). Mbongwe *et al* 2003, reported concentrations of DDT and

its metabolites in water and fish tissue. With all these endeavours, still no extensive investigations of pesticide levels have been carried out in biota of the delta. Increased health awareness associated with environmental contaminants has stimulated further investigations of toxic compounds especially persistent organic pollutants (POPs) to be carried out in biota. Studies to assess the levels of persistent organic pollutants in fish tissue and plant material are in progress and investigations of these compounds in crocodile blood and in human milk are soon to follow. It is envisaged that these studies will generate data that could be of great use in the formulation of strategies that will protect the delta's environment and its biodiversity from contaminants.

1.4 Persistent organic pollutants (POPs)

Persistent organic pollutants are organic compounds that are highly resistant to photolytic, biological and chemical degradation. They originate almost entirely from anthropogenic sources associated with manufacture, use and disposition of certain chemicals. Natural sources of organochlorine compounds are known to exist as well.

Persistent organic pollutants are halogenated and most often chlorinated compounds. The carbon-chlorine bonds in these compounds are very stable towards hydrolysis (Stimman *et al*., 1985). The greater the number of chlorine substitutions and/or functional groups in the compound the more it resists degradation. The chlorine atom attached to an aromatic (benzene) ring is more stable to hydrolysis than the chlorine atom in the aliphatic structure. Chlorinated persistent organic pollutants are typically ring structures with a chain or branched chain framework. By virtue of their high degree of halogenation, persistent organic pollutants have very low water solubility and are readily soluble in fat. Being soluble in lipid, persistent organic pollutants are able to pass through the lipid structure of biological membranes and accumulate in fat deposits.

Persistent organic pollutants are semi-volatile, a property that subjects them to long-range transport. These compounds are capable of moving over great distances through the atmosphere to reach regions far away from their point of release. They are ubiquitous, have been measured on every continent, at sites representing every major climatic zone and geographic sector throughout the world (Wania *et al*., 1993). These include remote regions such as open oceans, the deserts, the Arctic and Antarctica where no significant local sources exist. The only reasonable explanation for their ubiquity is long-range transport from other parts of the world. A large variety of organochlorine compounds including polychlorinated

biphenyls (PCBs), dichlorodiphenyltrichloroethane (DDT), toxaphene and chlorobenzenes were detected in Arctic cod, polar bears and ringed seals from the east-central Canadian Arctic (Muir *et al.*, 1998).

Dioxins and furans are aromatic hydrocarbons that have between one and eight chlorinated substituents. They are formed unintentionally in a wide range of manufacturing and waste combustion processes (Van Leeuwen and De Boer, 2008). Other recorded sources are paper production and by-products in pesticide/herbicide production (Pereira, 2004; Fiedler, 1996; WHO, 1998). There are 75 PCCD and 135 PCDF substituted forms (congeners) for a total of 210. The most toxic and extensively studied of the dioxins is 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). To simplify the assessment of toxicity data for PCDDs and PCDFs, a system has been developed to compare the relative toxicity of the congeners. The toxicity of 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin is used as a reference in relating the toxicity of the other 209 compounds. The toxicity of airborne mixture of PCDDs and PCDFs are expressed in terms of 2, 3, 7, 8-tetrachlorodibenzo-p-dioxins toxic equivalents (TEQ) in mg/m^3 (EPA, 1997).

Polychlorinated biphenyls (PCBs) have been used for a number of decades, e.g. as plasticizers, fire resistant liquid in closed circuits, dielectric in transformers and capacitors, (WHO, 1993). There are 209 polychlorinated biphenyl congeners but the number of these compounds found in the environment is much lower. The analysis of other polychlorinated biphenyls is often limited to a selection of six or seven compounds referred to as the "indicator PCBs". This selection consists of the congeners 28, 52,101,118, 138 and 180 (WHO, 1993).

Polychlorinated napthalenes (PCNs) have been synthesised from melted naphthalene and chlorine in the presence of a catalyst. The application of PCNs is similar to that of PCBs and includes application as dielectrics for flame proofing and insulation in various industries, additives to rubber products, flame retardant and in lubricants (Falandysz, 1998). PCNs are also found as impurities in PCB technical mixtures and can be formed in thermal processes e.g. solid waste burning (Falandysz, 1998). PCNs can be potent inducers of ethoxyresorufin-O-deethylase (EROD) and the aryl hydrocarbon (Ah) receptor. PCNs have been found in the environment worldwide, mostly in concentrations lower than those of other persistent organic pollutants (Evenset *et al.*, 2005; Jannson *et al.*, 1984; Wang *et al.*, 2007).

Short-chain polychlorinated alkanes (PCAs) have found their application as extreme pressure additives in lubricants, cutting oils, plasticizers and flame retardants. They were also used as

replacements for PCBs (Reth *et al.*, 2007). PCAs are produced by the chlorination of *n*-paraffin or paraffin wax. Their widespread use has resulted in a ubiquitous distribution in the environment (Stejnarova *et al.*, 2005).

Polycyclic aromatic hydrocarbons (PAHs) are a natural occurring class of persistent organic pollutants. They are found in crude oil and coal deposits. They are also formed by incomplete combustion of carbon-containing fuel such as wood, coal, diesel fat and tobacco. Polycyclic aromatic hydrocarbons consist of fused rings with no substituents. The simplest of these compounds are phenanthrace, and anthracene which both have three fused aromatic rings.

The organochlorines are the most important group of persistent organic pollutants. These compounds are noted for their environmental persistence, long half-lives and their potential to bioaccumulate and biomagnify in organisms once dispersed into the environment. Twelve of the known persistent organic pollutants, referred to as the "dirty dozen" (**Table 1.1**), have been officially registered by the United Nations Environmental Programme (UNEP) under the Stockholm Convention in 2001 as priority pollutants. The member states of the Convention aim to eliminate or reduce levels of these compounds in the environment. DDT is classified as a restricted compound and can only be used for disease vector control in the Indoor Residual Spraying (IRS) programme initiated by the World Health Organisation (WHO). The IRS programme is rolled out to all epidemiologic settings including unstable, epidemic-prone areas with seasonal transmissions, and stable-hyperendemic areas with seasonal or perennial transmission (WHO, 2006a). In this strategy, DDT is sprayed on the walls and other surfaces inside dwellings where female *Anopheles* mosquitoes land and rest before and after a blood meal. The WHO recommended standard dosage is 1 - 2 g of active ingredient per square metre at six months intervals (WHO, 2006b). Sufficient contact with DDT- sprayed surfaces kills malaria vectors. More importantly, DDT has an excitorepellent effect, deterring entry into and promoting exit from sprayed dwellings (Roberts, 1994). It is argued that the combined mosquito toxicity and excitorepellent effects of DDT may maintain its continued efficacy in areas where there is resistance against the insecticide (Roberts, 1994; Curtis, 2002). A study in India which assessed the impact of IRS with DDT on malaria transmission corroborated the results of earlier studies which reported marked reductions in vector densities and malaria incidences (Sharma *et al*, 2005).

Table 1.1 Chlorinated compounds registered by the Stockholm Convention as priority persistent organic pollutants.

Compound	Class	Stockholm Convention Classification
Aldrin	Organochorine insecticide	. Elimination
Dieldrin	Organochlorine insecticide	. Elimination
Endrin	Organochlorine insecticide	. Elimination
Chlordane	Organochlorine insecticide	.Elimination
Heptachlor	Organochlorine insecticide	. Elimination
HCB	Organochlorine insecticide	Elimination
Mirex	Organochlorine insecticide	Elimination
Toxaphene	Organochlorine insecticide	Elimination
DDT	Organochlorine insecticide	Restricted
PCB	Industrial Chemical	Source reduction
Dioxins	Combustion byproduct	Source reduction
Furans	Combustion byproduct	Source reduction

Nine of the Stockholm Convention priority pollutants compounds (DDT, dieldrin, toxaphene, endrin, aldrin, hexachlorobenzene, chlordane, heptachlor and mirex) are insecticides and are used in agriculture as pesticides. Pesticides are substances or mixtures of substances intended to prevent, destroy, repel or mitigate pests. Pests can be insects, mice, unwanted plants (weeds), fungi or microorganisms such as bacteria and viruses. Though often misunderstood to refer only to insecticides, the term pesticide also applies to fumigants, fungicides, herbicides, rondenticides and various other substances used to control pests. Chemical classification of pesticides can be based on the functional groups on their molecular structures or their specific biological activity on target species (Van der Hoff and Van Zoonen, 1999). Insecticides are a group of pesticides whose target is insects. Insecticides are

divided into three main groups; acetylcholinesterases, pymethrins and organochlorine pesticides (OCPs). The main feature of organochlorine pesticides is the presence of the chlorine atoms attached to the aromatic ring which renders them resistant to degradation, hence their persistence in the environment. **Figure 1.2** shows chemical structures of some organochlorine pesticides.

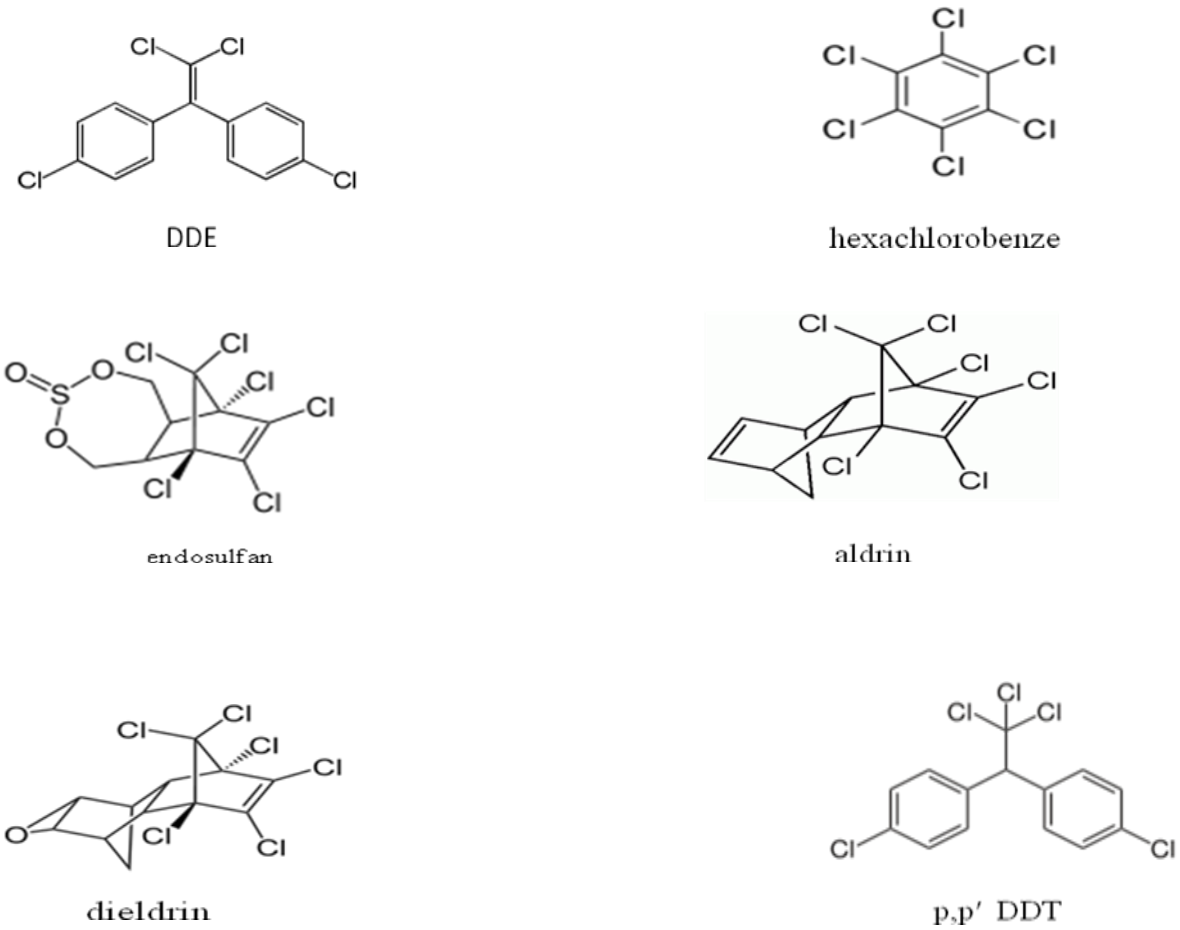


Figure 1.2: Chemical structures of some organochlorine pesticides

1.4.1 Mode of action and toxicity of pesticides

Pesticides act by poisoning the nervous system of the target and non-target organisms. The basic mode of action of most pesticides/ insecticides is inhibition of the normal functioning of the nervous system. These compounds alter the transfer of signals along nerve fibres and across synapses from one nerve to another or from a nerve to a muscle fibre. The transfer of a signal along a nerve occurs by changes in the electrical potential across the nerve cell membrane which is created by the movement of ions in and out of the cell. At the terminal end of a nerve, the signal is transferred across a synapse to the next nerve cell by the release

of neurotransmitters such as acetylcholinesterase (ACh). Different classes of pesticides inhibit this process in different ways, but the end result is an alteration in the normal nerve propagation.

Organochlorine pesticides act primarily by altering the movement of ions across the nerve cell membranes, thus changing the ability of the nerve to fire. For example, organisms exposed to low doses of DDT exhibit tremors and in-coordination as a result of repetitive discharge (over-firing) of the nerves. Studies by Dutta and Arends 2003, showed inhibition of brain acetylcholinesterase activity in juvenile bluegill sunfish exposed to endosulfan (an organochlorine pesticide). Therefore organochlorine pesticides also have the same effect as organophosphate and carbamate insecticides. Organophsphates and carbamates exert their toxicity by blocking the breakdown of acetylcholine (a neurotransmitter) by the enzyme acetylcholinesterase (AChE) at the synaptic junction between communicating cells.

One challenge faced by pesticides regulatory bodies is that pesticides are marketed under different trade names which often makes it difficult to identify or recognise the pesticide by just reading the label. Banned pesticides are likely to find their way back into the market under different names. All the same, to protect the public, the World Health Organisation (WHO) developed a hazard classification system which is used to label pesticide containers to warn users of the hazards carried by these compounds. The hazard system was based on LD_{50} of the pesticides in rats under either oral or dermal exposure conditions (**Table 1.2**).

Table 1.2 The WHO recommended classification of pesticide hazard (Copplestone, 1988).

		LD_{50} for the rat (mg/kg body weight)			
		Oral		Dermal	
Class	Toxicity	Solid	Liquid	Solid	Liquid
I a	Extremely hazardous	≤ 5	≤ 20	≤ 10	≤ 40
I b	Highly hazardous	5 – 50	20 – 200	10 – 100	40 – 400
II	Moderately hazardous	50 – 500	200 – 2000	100 – 1000	400 – 4000
III	Slightly hazardous	> 500	> 2000	> 1000	> 4000
III +	Unlikely to present hazard in normal use	> 2000	> 3000	-	-

1.4.2 Properties of DDT and its metabolites

Dichlorodiphenyltrichloethane (DDT) is one of the oldest and most well known organochlorine pesticides. It was first synthesized in 1874 and its insecticidal properties were not discovered until 1939 (Robert *et al*, 2000). It was used successfully in the second half of World War II to control malaria and typhus among troops and civilians. After the war, DDT was used as an agricultural insecticide and soon its production and use increased significantly. DDT does not occur naturally, it is produced by the treatment of chloral with chlorobenzene in the presence of sulphuric acid as a catalyst (**Eqn 1.1**).

$$Cl_3CCHO + 2\ C_6H_5Cl \xrightarrow{H_2SO_4} Cl_3CCH(C_6H_4Cl)_2 + H_2O \quad (1.1)$$

DDT is a colourless crystalline solid with a weak chemical odour. The technical grade DDT is a mixture of about 85% p,p′-DDT and 15% o,p′- DDT (UNEP, 2003). The trade names under which DDT has been marketed include Anofex, Cezarex, Chlorophenenothane, Dicophane, Gesarol, Guesapon, Guesanol, Gyron, Ixodex, Neocid and Zendane (WHO, 1979).

In the atmosphere, about 50% of DDT is adsorbed to particulate matter and 50% exists in the vapour form (Bidleman, 1988). DDT remains in the atmosphere for a short time as it eventually photooxidises to carbon dioxide, hydrochloric acid and hydroxyl radicals. In water DDT adsorbs to particulate matter in the water column and partitions into the sediments. In soils, DDT adsorbs strongly on the soil particles. The routes though which DDT is lost or degrades in the environment include runoff, volatilization, photolysis, both aerobic and anaerobic decomposition. DDT degrades into two additional forms, DDE (dichlodiphenylchloroethane) and DDD (dichlorodiphenyldichloroethane). DDE is DDT's main metabolite and also the most persistent form of the three compounds. Though DDD is less persistent than DDE it is independently used as a pesticide in some instances. **Figure 1.3** shows the degradation process of DDT to DDD and DDE by elimination of hydrochloric acid and reductive dechlorination respectively.

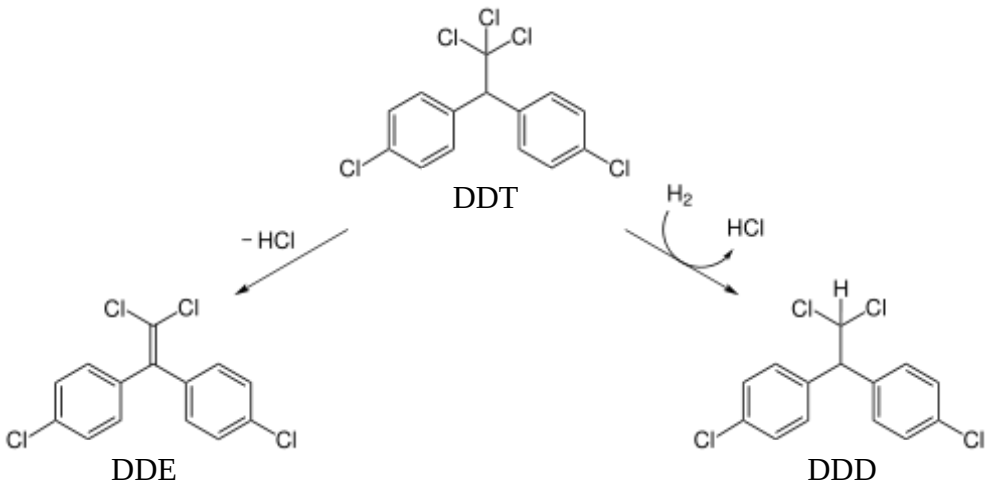


Figure 1.3: Degradtion of DDT to DDE and DDD by elimination of HCl and reductive dechlorination.

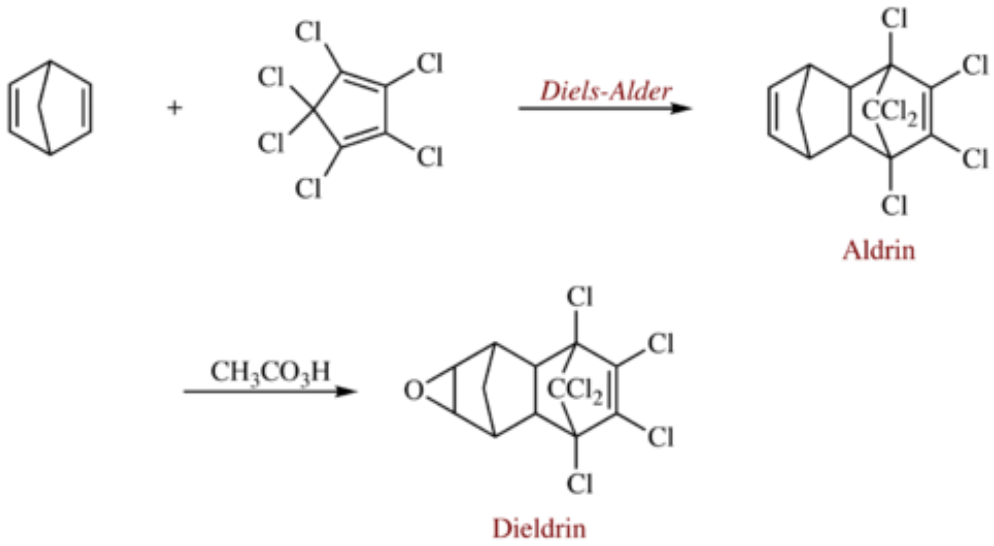
The WHO has classified DDT as a class II pesticide based on its LD_{50} of 250 mg/kg. The distribution and fate of DDT and its metabolites is variable in different media in the environment (**Table 1.3**).

Table1.3 Distribution and fate of DDT in the environment (USDHHS, 1993).

DDT and its metabolites				
Transformation and degradation/ persistence (chemical changes and distribution)				
Water	Soils	Sediments	Air	Biota
○ Half-life; 7 to 350 days ○ Mainly photolysis	○ Half-life; 2 to 15 yrs ○ Very persistent ○ Slow degradation	○ Half-life;16.6 days to 31.1 yrs ○ Slow biodegradation	○ Half-life; 16.6 days to 31.3 yrs ○ Slow biodegradation	○ Very persistent

1.4.3 Properties of aldrin and dieldrin

The compounds aldrin and dieldrin were first formulated from a waste product of synthetic rubber, cyclopentadiene. Their first production was in 1948 (Jorgenson, 2001). The two compounds are cyclodienes synthesized by the Diels-Alder reaction, hence their names. **Scheme 1.1** shows the synthesis of dieldrin from a mixture of norbornadiene and hexachlorocylopentadiene followed by epoxidation of the norbornene ring to produce dieldrin.



Scheme 1.1: Synthesis of dieldrin via a diels-Alder reaction (wikipedia.org/wiki/file: dieldrin synthesis.png).

Aldrin and dieldrin exist as white crystalline solids in pure form. The technical grade of aldrin and dieldrin contain not less than 85% of aldrin and dieldrin respectively. The trade names for aldrin include Aldrex, Aldrec, Drinox, Octalene, Seedrin and Compound 118. Adlrin has been used as a soil insecticide in agriculture and in foundations in the construction industry abate termites. Dieldrin has been used in agriculture soil and seed treatment. Other uses of dieldrin included control for mosquitoes, tsetse fly, veterinary treatment of animals and wood treatment against termites. The trade names for dieldrin are Alvit, Dieldrex, Octalox, quintox and Red shield. Both aldrin and dieldrin are class Ib pesticides according to the WHO classification. In the environment and body systems, aldrin is converted to dieldrin through biodegradation and it is the active compound i.e. it is more toxic than aldrin. In general aldrin undergoes photolysis to dieldrin, which in turn maybe degraded by ultraviolet radiation or microbial action into the more persistent photodieldrin. **Table 1.4** gives a summary of the distribution and fate of aldrin and dieldrin in various media in the environment.

Table 1.4 Distribution and fate of aldrin and dieldrin in the environment (USDHHS, 1993).

Aldrin/Dieldrin			
Transformation and degradation/ Persistence (Chemical changes and distribution)			
Water	Soil and Sediments	Air	Biota
○ Hydrolysis not important ○ Persistent in water (associated with particulate phase)	○ Half-life; 1 month to 5 yrs ○ Epoxidation of aldrin to dieldrin ○ biodegradation (dielrin is more resistant to degradation than aldrin)	○ Epoxidation of aldrin to dieldrin ○ Photodecomposition of dieldrin to photodieldrin	○ Aldrin is converted to dieldrin in plant and animal tissues

Organochlorine pesticides have contributed greatly in the increase of food production in agriculture and improved both human and animal health. However, these successes have been marred by side-effects resulting from the action of pesticides on non-target species (Barlas, 2002). Some toxic effects associated with organochlorine pesticide residues include cancer, immunosuppression, reproductive and developmental disorders (Garabrant *et al*., 1992). Studies have shown that dieldrin, DDT and its metabolites are some of the numerous endocrine disrupting chemicals (EDCs) that exhibit estrogenic and anti-androgenic effects (Mckinney and Walker, 1994). These effects may alter the functioning of the endocrine system and in so doing result in adverse effects on the health of the exposed organism. Similarly, epidemiological studies have suggested an etiological relationship between exposure to organochlorine pesticides and Parkison's disease (Fleming *et al.*, 1994). The observed adverse effects of DDT exposure on wildlife include: reproductive abnormalities in birds, mammals and the feminisation of males (Guilett *et al*., 2001).

1.5 Pathways and use of organochlorine pesticides

Organochlorine pesticides contamination pathways to water bodies are likely to be nonpoint sources via runoff, atmospheric deposition and leaching due to agricultural applications, vector pest control and improper waste disposal. Sediments act as a sink for persistent organic pollutants; hence increase the compounds bioavailability and accumulation in the food chain through re-suspension. This exposes fish and other aquatic organisms to the pollutants through ingestion, dermal absorption and respiration. Consumption of fish from polluted water systems is considered to be one of the important routes through which humans get exposed to pesticides (Johansen *et al.,* 1996).

Some developing countries still use organochlorine pesticides because of their low cost, versatility in industry, agriculture and the public health (Tanabe *et al.,* 1994). In Vietnam, the prohibition of these substances was first issued in 1993, but some studies showed that DDT residues were detected in their highest concentration (Hung *et al.,* 2002). In Taiwan, a study by Doong *et al.,* 2003 indicated the existence of a variety of some organochlorine pesticide residues in the rivers in which DDT and HCHs were the dominant compounds. In Germany the prohibition of organochlorine pesticides was effected many years ago, but DDT residues were still detected in canal waters many years after. In USA, Europe (Germany and Russia), Asia (China) and Africa (Egypt) the presence of organochlorine pesticide residues in surface water, sediments biota and vegetation have been investigated in detail (Castilho et al 2000; Jiang *et al.,* 2000; Zholidov *et al.,* 2000; Samia *et al.,* 2000).

In the Okavango Delta, organochlorine pesticides have been employed to control mosquitoes (*Anopheles sp*) which cause malaria and tsetse fly *(Glossina morsitans)* which causes sleeping sickness in humans and nagana in cattle. In the 1930's, Botswana began tsetse elimination strategies in the delta which until the 1960's mainly involved game culling and bush destruction (Grant and Crick, 1987). In 1967, these techniques were replaced by the spraying of DDT and other pesticides (Davies, 1976). The 1970's saw the development and aerial spraying of endosulfan, a highly toxic chlorinated hydrocarbon (Meynell, 2001). In 1991, all pesticide use was discontinued and for the next ten years, tsetse fly control was dependent on Odour Baited Target (OBT) technology (Meynell, 2001). The technology exploits the flies' odour and colour detection strategies (Scott Wilson, 2001). Poor maintenance of these targets as well as their destruction by wildlife resulted in their failure and the spread of the tsetse in the delta (Meynell, 2001). An outbreak of trypanosomiasis in

cattle in 1999 led the government to reintroduce aerial spraying of pesticides in the delta (Scott Wilson, 2001). This time the more persistent deltamethrin replaced endosulfan which was used prior 1991. Deltamethrin was chosen because it is rapidly metabolised by birds and mammals and stands very little chance of biomagnification (Perkins and Ramberg, 2002). DDT was eventually banned in 1997 as a result of pressure from international organisations on the use of this compound. Towards the end of 2009 the government of Botswana through the Ministry of health reintroduced the use of DDT for indoor spraying against mosquitoes. This step was taken after observing the reduction in vector susceptibility to pyrethroids which are currently being used against the vector.

Recent studies conducted in both water and sediments in the Okavango Delta showed significant levels of organochlorine pesticide residues. Hexachlorobenzene, trans-chlordane, 4, 4′ DDD and DDE concentrations in water were 61.4, 3.2, 2.4 and 5.5 µg/L respectively (Mmualefe *et al.,* 2008). These concentrations were far much higher than the 0.1 µg/L limit for drinking water set by the European Community Directive of 1998. The concentration range of hexachlorobenzene, aldrin and 4, 4′ DDT were 1.1 – 30.0, 0.5 – 15.2 and 1.4 – 55.4 µg/g in sediments (Mmualefe *et al.,* 2009).

The work conducted in this study contributes to the scientific data on persistent organic pollutants in the delta. Therefore objectives of the study were;

- to optimise and evaluate the QuEChERS method for the extraction of organochlorine pesticides in fish samples.

- to determine the baseline concentration levels of organochlorine pesticide residues in fish from the Okavango Delta.

- to assess the effects of organochlorine pesticides on fish of the Okavango Delta through AChE activity assay.

Chapter 2

Sample preparation and analytical techniques

2.1 The analytical process

The development of a complete analytical method includes a number of steps from sample collection to data handling. The intermediate steps of a complete analytical process include sampling, sample preparation, separation, detection, identification and quantification of the target compounds (Figure 2.1).

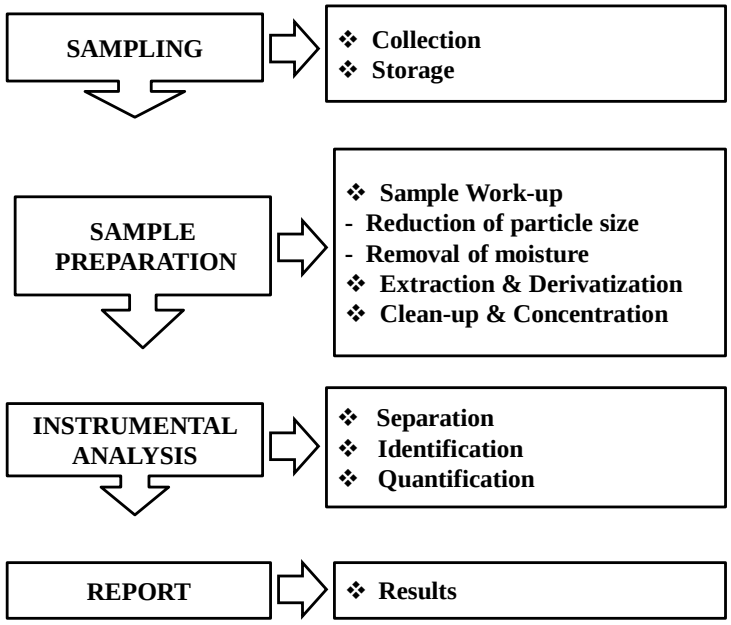


Fig 2.1: Stages of a complete analytical process.

2.1.1 Sampling

Material bound for analysis cannot be taken to the laboratory as a whole and be analysed in its entirety (with the exception of small plants, animals and microorganisms). Normally, a small representative portion (sample) is collected and sent for analysis. To achieve this, sampling techniques are required to obtain representative, laboratory-sized primary samples ahead of subsequent subsamples. The amount of sample required for an analytical procedure varies from a fraction of a gram to several grams. The required sample size is defined in part by the nature of the target analyte, that is, the extent to which it is retained in the matrix.

Depending on the objective of the analysis, different parts of the material to be analysed are considered. Fresh produce maybe separated into core, inner and outer tissues. Shells are usually separated from nut kernels and pits from stone fruits. In the case of plants, roots, stems and leaves are sampled separately. For animals e.g. fish, are dissected and the target organs retrieved. While awaiting further work-up, samples need to be stored in appropriate environments so that analytes of interest are not lost and to avoid contamination. Fresh animal samples are kept frozen at subzero temperatures while plant and soil samples are kept at room temperature in well ventilated rooms.

2.2 Sample preparation

Pesticides are generally found in complex matrices at trace levels. Their exact determination has been a challenging task to analytical chemists for many years. Their low levels usually necessitate an extraction and a concentration step before introducing the sample into a detection device. Chromatography is the most widely used separation technique in pesticide analysis. A gas chromatograph equipped with an electron capture detector and often coupled to mass spectrometer is often best suited for organochlorine pesticide analysis.

Sample preparation is a multi-step process most often carried off-line. This process usually accounts for over 60% of the total analysis time, and the quality of these steps largely determine the success of an analysis from complex matrices (Chen *et al.*, 2010). Sample preparation is manually laborious, tedious, prone to loss of analytes and contamination. As such, choosing an appropriate sample preparation method and getting the preparation stages correct influence the reliability and accuracy of a given analysis (Smith 2003).

Historically, sample preparation has not been an important factor of method development. Emphasis has always been placed on instrumentation i.e. chromatographic separation and detection rather than on the actual extraction and clean-up of the analytes from the various matrices. For instance, the past two decades have seen a boom in the development of sophisticated instruments all primarily dedicated to the separation and detection of chemical compounds in various matrices. By comparison, advances in the field of sample preparation have been a neglected area and given much less attention.

The recognition of the poor performance of the traditional methods with respect to time of analysis, precision and solvent usage has called for major strides in this area. Sample preparation is now coming to the forefront in the area of separation science. The main aim of

sample preparation is to make a complex sample suitable for chromatographic analysis. The increase in numbers of analytical samples reaching monitoring laboratories has necessitated the development of fast dissolution techniques and clean-up procedures in order to increase sample throughput. To be accepted in the analytical community the sample extraction and clean-up procedure should be applicable to a wide range of sample types and should be 'non' matrix dependent. Most samples require that the analyte be isolated from a matrix before the detection and quantification processes. This is important, especially in trace analysis where the complexity of many matrices and low concentrations of analytes of interest have to be identified and quantified in the midst of many other compounds. The basic concept of sample preparation is to convert a complex matrix into a form suitable for instrumental analysis. This can be achieved by a wide range of techniques many of which have remained unchanged for over many years (Smith 2003). All sample preparation methods are aimed at;

- extracting the analyte of interest from the matrix.
- bringing the analyte to a suitable concentration level (pre-concentration).
- removal of possible interferences (sample clean-up) and,
- converting the analytes into a detectable form.

Preceding this step samples are sized-up and dried in a step that could be referred to as the sample work-up.

2.2.1 Sample work-up

2.2.1.1 Reduction of the particle sizes of the sample

Once a sample has been collected, a suitable method is employed to make the material less heterogeneous. Various approaches may be utilised for reducing the particle size in a primary sample, so that smaller subsamples are taken for a representative analysis of a whole. The methods for the reduction of solid and semi-solid samples include grinding, mixing, rolling, agitating, stirring, chopping, crushing, macerating, mincing, pressing or pulvurization. During the sample reduction process, it should be borne in mind that the equipment used is inert enough to prevent contamination of the sample. Care should be taken to prevent release of volatile constituents if there are the target compounds. Particle size is an important parameter for reproducible results as the extent to which the matrix is broken down influences extraction rates. The analyte is desorbed from the matrix and dissolved into a solvent or fluid.

The extraction of the analyte is therefore influenced by solubility, penetration of the sample by the solvent (mass transfer) and matrix effects (Mitra, 2003).

2.2.1.2 Removal of moisture from the samples

Samples like animal or fish tissue and similarly moist or wet solids pose a challenge. When treated as whole, the sample particles form aggregates that prevent the extraction media to enter the sample. Dispersion can be used to avoid aggregation of the sample particles and ensure a good solvent penetration. Where non-polar solvents are used as extraction media drying the sample becomes important. Moisture remaining in the sample can reduce the extraction efficiency of the solvent. Moisture exists in biological samples in three forms (Aurand *et al.,* 1987);

- as a solvent or dispersing agent.
- adsorbed on the internal or external surfaces or as fine capillaries by capillary condensation and,
- as water of hydration.

Solvent or free moisture is easily removed from samples. The rate at which moisture is removed from samples is affected by drying temperature, particle size, vacuum, crust formation on the surface and the surface area of the sample (Aurand *et al.,* 1987). Moisture bound to the sample is difficult to remove and normally requires a vacuum process. Vacuum drying is preferred since it accelerates drying time and prevents samples from deterioration. A general rule of thumb for sample drying is that it should be rapid and performed at low temperatures should heating be required. Vacuum methods that can be used to dry samples include vacuum ovens and lyophilisation or freeze-drying. Desiccants such as sodium sulphate and diatomaceous earth or cellulose can also be used to dry samples. Once dry, the sample is directly ground into powder before liquid or solvent extraction. After the extraction step the analytes of interest are obtained in an organic or aqueous solution, which requires concentration or additional clean-up. The extract solutions are then treated as liquid samples.

2.3 Extraction

The role of extraction in sample preparation is to access the analyte of interest from the matrix. Extraction is applied primarily as means of separating analytes from compounds in preparation for chromatographic analysis. Liquid-liquid extraction (LLE) has been known for over one hundred years as a method of separation in analytical procedures. In liquid-liquid extraction the analytes to be extracted are partitioned between two immiscible liquids. However, due to its limitations liquid-liquid extraction is being replaced by solid phase extraction (SPE). The solid-phase extraction technique is capable of combining sample clean-up, enrichment, class fractionation and derivatization of compounds in one single step. Solid-phase extraction is discussed in detail towards the end of this chapter.

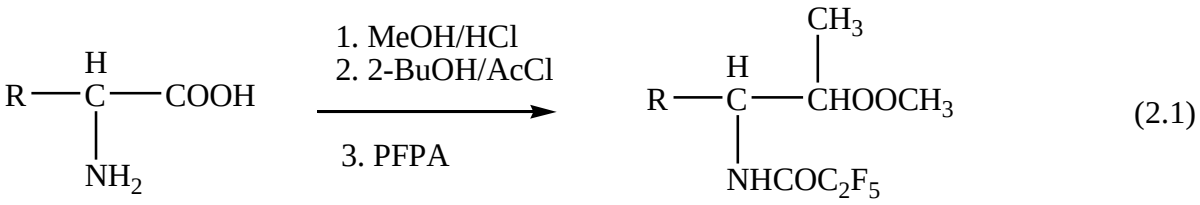
2.4. Derivatization

Derivatization is a process by which a compound's structure is chemically modified to produce a new compound that has amenable properties to a particular analytical method. In fact, analytical derivatization converts an analyte into a product with greater stability, superior chromatographic properties or one that can be detected at a higher sensitivity (Blau *et al.*, 1993 and Field, 1997). It is also a subset of functional group analysis. As such, it confers selectivity on quantitative determination by labelling only those compounds that react with the derivatizing reagent. In some instances, derivatization is essential for the isolation of the analytes from the matrix.

Analytical derivatizations are effective but present at least one additional step in the sample preparation scheme. The reactions can also produce side products that interfere with the analysis. One way of resolving the challenges of extra steps and interferences is to carry out analytical derivatization in solid phases i.e. solid-phase analytical derivatization (SPAD). Solid-phase analytical derivatization allows derivatization to occur concurrently with isolation from the matrix or can be carried out after sorption on the solid phase by reaction in-situ. The phases employed in solid-phase analytical derivatization are the standard commercially available materials applied to the isolation of organics by solid-phase extraction (SPE) or high-performance liquid chromatography (HPLC).

Some samples analysed by gas chromatography requiring derivatisation in order to make them suitable for analysis include non-volatile and thermally unstable compounds. Non-volatile compounds like polymers, sugars, amides and amino acids are either esterified,

salinated or acetylated using one of the numerous methods of derivatization. For example, acids can be esterified by treatment with an appropriate alcohol using an inorganic acid to catalyze the reaction. A general reaction would be to treat one or two milligrams of the acid with 125 mL methanol or ethanol that contains 3 M hydrochloric acid and heated at 65 °C for about 35 minutes. A stream of nitrogen is bubbled through the reaction mixture to remove residual alcohol. Amino acids are more difficult to derivatize but can also be esterified in a comparable manner. A few milligrams of the amino acid solution is mixed with 2 mL of 4 M alcoholic methanol and heated at 70 °C for two hours. Access methanol is removed by evaporation in a stream of nitrogen. Residual water is eliminated by the addition of a small quantity of dichloromethane (about 150 mL) and repeating the evaporation process. A typical general reaction for derivatization of amino acids using the reagents methanol/hydrochloric acid, 2-butanol/acetyl chloride and pentafluoro propionic anhydride is given below.



(2.1)

An example of thermally unstable compounds are carbamate insectides. Some carbamate insecticides decompose during gas chromatographic analysis to yield phenol and isocyanate (Zielinski *et al.*, 1965). Their analytical measurement usually involves hydrolysis followed by determination of the liberated phenol. Derivatization of the intact carbamates followed by gas chromatography analysis have been reported. Carbaryl (1-naphthyl *N*- methyl-carbamate) was reacted with acetic anhydride to give the *N*-acetyl derivative which was thermally stable during analysis by gas chromatograhphy (Suillivan *et al.*, 1967). Many carbamates have been converted to stable perfluoroa-cylated derivatives which have been utilized in sensitive assays employing gas chromatography with electron capture detection (Seiber, 1972).

Compounds that respond poorly to a specific detector may need to be derivatized by tagging with a different functional group to improve its detection. For example, tagging non-chlorinated compounds with chlorine can improve their response on an electron capture detector.

In liquid chromatography analyses, UV chromophores and fluorophores are often introduced into the sample to increase their sensitivity to UV absorption and florescence detection. Benzoyl chloride, m-tuluol chloride and p-nitrobenzoyl chloride are reagents that can add a benzene ring to a solute molecule and render it UV absorbing. Florescent derivatives of phenols, primary and secondary amine are prepared by the addition of dansyl chloride.

In some cases, the polarity of a solute is reduced in order to improve its chromatographic behaviour and reduction of peak tailing. For amino, hydroxyl and thiol groups reduction of polarity is achieved by acylation. Acylation is simply achieved by a direct reaction with acid anhydrides and acid chlorides e.g. acetylchloride and acetic anhydride.

Besides improving the suitability and response, derivatization can improve the resolution between co-eluting compounds and overlapping peaks in chromatographic analysis (Knapp, 1979).

2.5 Sample clean-up and concentration

An inherent difficulty in the extraction of biological samples is the co-extraction of matrix components that are also soluble in the extraction solvent. A common example is the co-extraction of lipids during the extraction of non-polar compounds from animal and vegetable matrices (Ali *et al.*, 2001; Kayali-Sayidi *et al.*, 2000; Amigo *et al.*, 2000; Yasumura *et al.*, 2001; Bjorkland *et al.*, 2000). The presence of matrix interferences in sample extracts can result in a multitude of challenges including;

- formation of emulsions.
- sample turbidity.
- contamination or plugging of the analytical instrument.
- masking of the analytical signal for the target analyte and,
- the consequent increase in the method limit of detection.

Co-extractives are frequently removed during the post-extraction clean-up step. The co-extracted substances in the extracts can be removed by destructive or non-destructive methods. Destructive methods e.g. sulphuric acid treatment or saponification efficiently remove the bulk lipids (triglycerides) from the exracts. However, some pollutants such as dieldrin and endrin degrade under strong acidic conditions. Saponification can cause

dechlorination of higher polychlorinated biphenyls and hexachlorobenzene from sewage sludge during alkaline saponification (Van der Valk *et al*., 1988). Efficient non-destructive removal of lipids can be obtained by adsorption on alumina (Hess *et al*., 1995).

Gel permeation chromatography (GPC) may serve as an alternative fat separation method. Polysterene-divinylbenzene copolymeric columns e.g. bio-beads SX-3 are the most commonly used ones (Kimmel *et al*., 1998, Yusa *et al*., 2006, Gouteux *et al*., 2002). However, rigid pluronic gels appear to be more efficient (De Boer, 2001). Gel permeation chromatography is not capable of removing all lipid-related substances e.g. sterols, therefore additional clean-up or repeated gel permeation chromatography (up to four in series) is required. Lipids may also be removed by freezing them out the extract and subsequent filtration. This very simple method allowed for 90% lipid removal from mackerel extract (Ahn *et al*., 2006).

2.6 Choice of extraction method

The choice of a sample preparation method to be employed is chosen partly, based on the initial sample type, whether it is solid or liquid. Liquid samples have an advantage over solid samples in that the matrix is already in liquid form. In some cases very little preparation may be required if the liquid sample is sufficiently free of matrix interferences. Methods that can be used to prepare liquid samples include dilution, evaporation, distillation, microdialysis, lyophilisation or liquid-liquid extraction (Snyder *et al*., 2009). For solid samples, preparation procedures consist of transfer of analytes from a primary solid matrix into a liquid matrix. A large number of techniques exist and for this purpose the most suitable method should be chosen.

2.7 Sample extraction methods

Great advancement has been made in order to achieve efficient extraction of analyte from a sample matrix with high selectivity and sensitivity. Different extraction methods are employed consisting of solvent extraction from solids and liquid-liquid extraction from solutions. The solvents may be organic liquids, supercritical fluids or superheated liquids. In some cases, the liquid extractant may be bonded to a support material. Selectivity may be achieved by manipulating the extraction temperature and pressure, by the choice of extraction solvent and the control of pH.

Analogous to the proverbial computer rule, garbage-in garbage-out (GIGO), poorly prepared extracts will invalidate the whole assay. This will make it impossible to gain a valid result even by use of the most powerful separation method. Correct sample preparation can be economically valuable as well as analytically important. The traditional methods for sample preparation are laborious, time consuming and usually involve large volumes of solvents which are expensive, generate large amounts of waste and may contaminate the sample. This therefore calls for more than one clean-up step prior to detection. This scenario has led analytical chemists to direct their attention to sample preparation methods which are geared towards;

- use of small initial sample sizes.
- use of small solvent volumes.
- easy to operate.
- have short run times and
- inexpensive.

Many attempts have been made to fulfil the above trend but most sample preparation methods still involve the use of expensive instruments and have long operating protocols. This increases the cost of analysis and as such, development of new or modification of existing methods need to be explored. Some of the past and current sample preparation methods for the determination of pesticides residues in food, environmental and biological samples are discussed in the next section.

2.7.1 Soxhlet extraction

Soxhlet extraction is a well-established technique developed in 1879 (Fildago-Used, 2007). The technique is based on exhaustive extraction of organic compounds in a Soxhlet system by an organic solvent, which is continuously refluxed through the sample contained in a porous thimble. The extracted analytes accumulate in the heated flask. Soxhlet extraction is the oldest method for the isolation of non-polar and semi-polar organic pollutants in different types of matrices, including biota samples (Lopez-Avila, 1999; Laque de Castro *et al.*, 1998). The technique has been used for decades and has been adopted by the U.S. Environmental Protection Agency (EPA) as method 3540C. The common Soxhlet procedures use 50 - 200

mL of the organic solvent to extract analytes from between 1 and 100 g of biological tissue (Barcelo, 1993). The advantages of Soxhlet extraction include use of large amounts of sample (e.g. 1 - 100 g), no filtration is required and the technique is not matrix depedent. Many Soxhlet extractors can be set up to perform unattended operations. The main disadvantages of the Soxhlet extraction are that it requires large amounts of solvent which must be evaporated to concentrate the analytes before determination. The process takes several hours or days to complete and the system generates extracts that need extensive clean-up (Lopez-Avila, 1999; Laque de Castro *et al.*, 1998).

2.7.2 Sonication-assisted extraction

This is a solid-liquid technique in which a solid sample is blended with an appropriate organic solvent and ultra-sonicated. The process is carried out in discrete systems using an ultrasonic bath or a closed extractor fitted with a probe. Sonication involves the use of sound waves to stir the sample immersed in an organic solvent. Energy in the form of acoustic sound waves in the ultrasound region above 20 kHz is employed. This energy accelerates mass transport and mechanical removal of analytes from the surface of the solid matrix by a process known as cavitation. This consists of the formation and implosion of vacuum bubbles through the solvent, thus creating microenvironments with high temperatures and pressures estimated up to 5000 °C and 100 MPA respectively (Preigo-Capote, 2004). This mechanical effect of the ultrasound induces a greater penetration of solvent into solid materials and improves mass transfer leading to enhanced sample extraction efficiency. Sonication-assisted extraction allows extraction of large amounts of samples with a relatively low cost. The extraction process takes between 5 - 30 minutes to complete. The method is labour intensive and uses about as much solvent as the Soxhlet extraction. Filtration is required after extraction. The extraction efficiency of the method depends on solvent polarity, nature and homogeneity of the sample matrix, sound frequency and sonication time (Lopex- Avila, 1999). Ultrasonic-assisted extraction has been carried out using a dynamic extraction set-up which continuously supplies fresh extraction solvent to the extraction vessel. A considerable reduction of extraction time, solvent consumption and sample handling with respect to the extraction in static way was reported (Perigo-Capote, 2004). Another feature of such dynamic arrangement is that the analytes are transferred from the extraction vessel as soon as they are extracted. This can avoid degradation of the anaytes due to sonication or if thermo-labile

analytes are extracted at higher temperatures and pressures. Domeno *et al*., 2006, used a dynamic sonication-assisted extraction procedure for extracting PAHs from lichens using hexane. The reported total extraction time was only 10 minutes versus 2 hours in the static extraction mode and 6 hours in Soxhlet extraction. However, the PAHs relative recoveries obtained by the three methods was similar.

2.7.3 Supercritical fluid extraction (SFE)

Supercritical fluid extraction is a technique that uses a solvent in its supercritical state. Supercritical fluids have similar densities to liquids, but lower viscosities and so analytes show higher diffusion coefficients. This combination of properties results in a fluid that is more penetrating, has higher solvating power and may extract solutes faster and more efficiently than liquids (Smith, 2003, Lopez-Avila, 1999, Camel, 2001). In addition, the density may be adjusted by varying the temperature and pressure affording the opportunity of theoretical performing highly selective extractions (Camel, 2001). Supercritical fluid extraction involves pumping the fluid at a pressure above its critical point through a sample placed in an inert extraction cell. The temperature of the cell is increased to overcome the critical value of the fluid. After depressurization, analytes are collected in a small volume of organic solvent or on a solid-phase cartridge. Extraction can be carried out in static, dynamic or recirculating mode. In the static mode, the cell containing the sample is filled with the supercritical fluid, pressurized and allowed to equilibrate. In the dynamic mode, the supercritical fluid is passed through the extraction cell continuously. Finally, in the circulating mode the same fluid is repeatedly pumped through the sample and, after the required number of cycles it is pumped out to the collection system.

Supercritical extractions use carbon dioxide (CO_2) because it reaches the supercritical state at a relatively low pressure (7 MPA) and low temperature (31.3 °C). It is non-toxic, non-flammable, non-corrosive, chemically inert and affordable. Though carbon dioxide is non-polar, its polarity can be adjusted with modifiers such as acetone and methanol.

Supercritical fluid extraction efficiency is affected by a wide range of paramaters such as nature of the supercritical fluid, temperature and pressure, extraction time, shape of the extraction cell, sample particle size, moisture content of the matrix, and the analyte collection system. Due to these numerous parameters affecting the extraction efficiency, supercritical fluid extraction affords a high degree of selectivity and the extracts are relatively quite clean. In fact, if coupled to solid sorbent traps, supercritical fluid extraction may provide a single-

step extraction and clean-up. However, the need to control the many operating parameters makes supercritical fluid extraction optimization tedious and difficult to practice. Other disadvantages of this technique are the limited sample size, and high cost of the equipment.

Supercritical fluid extraction has been adopted by the EPA as are reference method for extracting PAHs (Method 3561) and PCBs (Method 3562) from solid environmental samples (www.epa.gov). Several applications of supercritical extraction for the extraction persistent organic pollutants (POPs) in animal and plant material have been reported. For instance, Antunes *et al*, 2003, applied supercritical fluid extraction to extract PCBs and OCPs from fish muscle using three types of raw materials, fresh fillet, fresh fillet ground with anhydrous sodium sulphate and freeze-dried fillet. It was found that supercritical carbon dioxide extracted organochlorine compounds from freeze-dried fish fillets. The pressure had a significant effect on extraction, while temperature did not significantly affect the extraction efficiency.

2.7.4 Microwave-assisted extraction (MAE)

This technique uses microwave radiation as the source of heating a solid sample-solvent mixture (Camel 2000). Due to the effects of dipole rotation and ionic conductance, heating with microvaves is instantaneous and occurs in the heart of the sample leading to very fast extractions. Heat generation in the sample by microwave field requires the presence of a dielectric compound. The greater the dielectric constant, the more thermal energy is released and the more rapid the heating for a given frequency (Fidalgo-Used *et al*, 2007). The effect of microwave strongly depends on the nature of the solvent and the solid matrix. Usually, the extraction solvent has a high dielectric constant so that it strongly absorbs the microwave energy. In the case of thermo-labile compounds, the microwaves may only be absorbed by the matrix. This results in heating of the sample and release of the solutes into the cold solvent. The nature of the solvent is of great importance in microwave extraction. The solvent should selectively and efficiently solubilize the analyte in the sample. At the same time, it should absorb the microwaves without strong heating as this may degrade the analytes. A common practice is to use a binary mixture of solvents e.g. hexane-acetone in the ratio of one is to one. This allows only one of the solvents to absorb the microwaves. Other important parameters affecting the extraction process are applied power, temperature and extraction

time. The water content in the sample needs to be carefully controlled to avoid excessive heating and allow reproducible results.

The application of microwave energy to samples may be performed either in closed vessels with pressure and temperature control (pressurized MAE) or in open vessels at atmospheric pressure (focused MAE). In focused MAE, the temperature is limited by the boiling point of the solvent at atmospheric pressure and in pressurised MAE, the temperature may be elevated by simply applying adequate pressures (Camel, 2001). Some limitations of MAE are that it involves the use of expensive instruments and the extracts generatedneed to be filtered prior to determination.

An official EPA Method 3546, Microwave Extraction was approved for the extraction of organic compounds from the environmental samples. The technique is also considered as an alternative to Soxhlet extraction for persistent organic pollutants in biota and environmental solid samples because of its shorter extraction time and low solvent consumption. Barrida *et al*., 2003, carried out a comparative study between MAE and Soxhlet extraction of 21 OCPs from plants using *n*-hexane-acetone (1:1v/v) as solvent in both cases. Both techniques showed similar recoveries but the Soxhlet extraction was more laborious and required more solvent and longer extraction times.

2.7.5 Pressurised liquid extraction (PLE)

Pressurised liquid extraction was originally launched by Dionex Inc., in 1995 under the name accelerated solvent extraction (ASE^{TM}) (Fitzpatrick *et al.,* 2000, Richter *et al*., 1996). The technique is also known as pressurised fluid extraction (PFE). Pressurised liquid extraction is a technique carried out in closed-vessels at elevated temperatures (80 - 200 ℃) and pressures (10 - 20 MPA). It is similar to SFE and the only difference is the substitution of CO_2 by organic solvents to mitigate potential polarity challanges (Fitzpatrick *et al.,* 2000). The extraction process is performed under pressure to maintain the conventional organic solvent in liquid state while extracting at temperatures well above atmospheric boiling points. The solvent is kept below its critical conditions but gains solvation power and lower viscosities. This allows higher diffusion rates for the analytes, increases extraction efficiency, minimises solvent consumption and expedites the extraction process.

Both static and flow-through extraction systems can be used (Camel, 2001, Bautz, 2002). In the static mode the sample is loaded in an inert cell and pressurized with a solvent heated

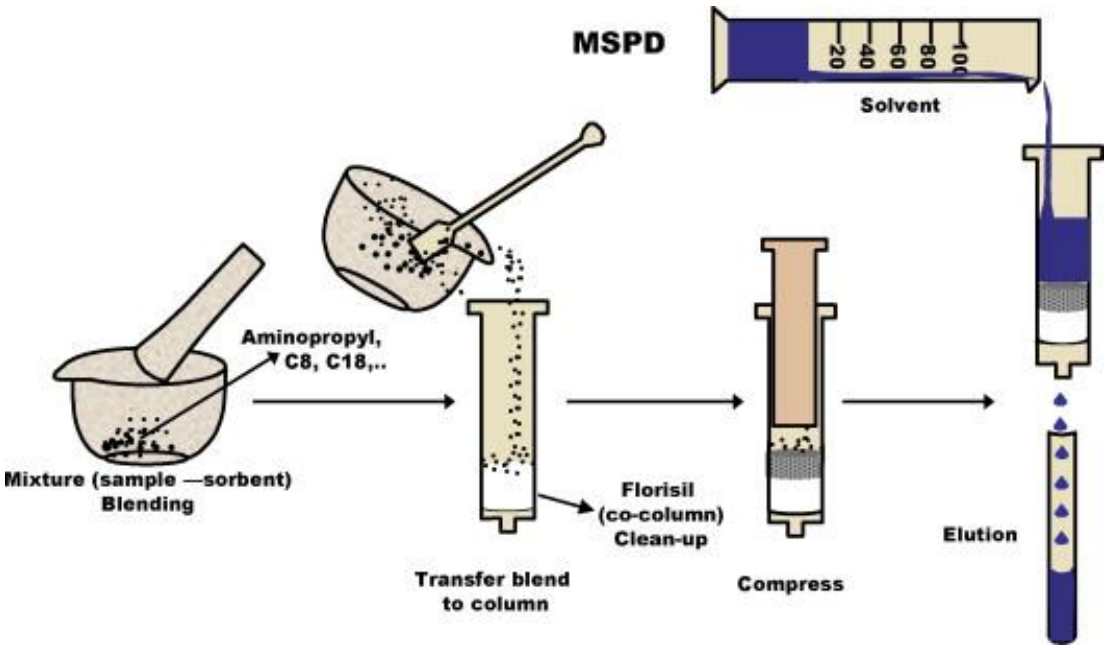
above boiling point. The flow-through extraction mode uses fresh solvent continuously introduced into the sample. This increases the extraction efficiency but also dilutes the extract (Bautz *et al.*, 2002). In comparison to supercritical fluid extraction, pressurized liquid extraction is much faster. The extraction time is approximately 15 minutes per sample. The technique uses less solvent volume (15 - 40 mL) and no filtration is required after extraction. The instrument is automated allowing extraction in an unattended operation. Different sample sizes can be accommodated e.g. 11, 22 and 33 mL vessels are available. The two main disadvantages of this technique are limited selectivity (i.e. further clean-up of the obtained extracts is required) and high capital cost that exceeds that incurred in operating the supercritical fluid extraction and microwave-assisted extraction systems (Fidalgo-Used *et al*., 2007).

Pressurized liquid extraction has been accepted as an official EPA method (Method 3545) for the determination of persistent organic pollutants in a variety of environmental solid sample (www.epa.gov). Many research groups have used this technique for sample preparation in environmental analysis. Weichbrodt *et al*., 2000, used pressurised liquid extraction for extracting organochlorine compounds from cod liver oil and fish fillet. The extraction was performed with ethyl acetate-cyclohexane (1:1 v/v), allowing for direct use of gel-permeation chromatography as a clean-up step without solvent exchange. Suchan *et al*., 2004, compared the pressurized liquid extraction with conventional Soxhlet extraction for extracting organochlorine and polychlorinated biphenyls from fish fillet. They used two different solvent mixtures; hexane-dichloromethane (1:1v/v) and hexane-acetone (4:1v/v). The pressurized liquid extraction results were comparable to those obtained in the Soxhlet method.

2.7.6 Matrix solid-phase dispersion (MSPD)

Matrix solid-phase dispersion (**Scheme 2.1**) was introduced in 1989 as a technique for the disruption and extraction of solid samples (Barker, 1989). Matrix solid-phase dispersion combines aspects of several analytical techniques to disrupt and disperse the components of the sample on and onto a solid support. This creates a chromatographic material that possesses unique character for the extraction of compounds from the dispersed sample (Kristenson *et al*., 2006). In matrix solid-phase dispersion, liquid and semi-solid samples are mixed while solid samples are blended with an appropriate sorbent until a homogenous mixture is obtained. The mixture is then packed into a column from which the analytes of

interest are eluted with a suitable organic solvent. The interfering matrix compounds are selectively retained on the column (Ramos *et al*., 2005). In some instances, the matrix solid-phase dispersion column is coupled on-line with a solid-phase extraction (SPE) column or the solid-phase extraction sorbent is packed in the bottom part of the column to remove interfering components (Barker *et al*., 1989; Ramos *et al*., 2004).



Scheme 2.1: Schematic procedure of matrix solid-phase dispersion (Gilbert-Lopez *et al*., 2009).

The advantages of matrix solid-phase extraction are that it is simple, requires short extraction times and uses less solvents when compared to other techniques. In addition, matrix solid-phase extraction does not require preparation and maintenance of equipment. It offers the possibility of simultaneously performing both extraction and clean-up. MSPD is labour intensive, requires the sample to be ground with solid matrix and packed into a column for extraction. The selectivity of matrix solid-phase dispersion depends on the sorbent-combination used. Most methods reported to date use reverse-phase materials such as C_8- and C_{18}-bonded silica as solid support. The nature of the elution solvent is also important since the target analytes should be efficiently desorbed while the bulk of the remaining matrix components should be retained in the column. Most sorbents have been tested in combination with a large variety of solvents. For instance, Pensado *et al*., 2005, described the performance of matrix solid-phase extraction for the extraction of PAHs from fish (salmon and turbot).

The suitability of different solid supports (Florisil, C_{18} and acidic silica gel) were tested. Their influence on the extraction efficiency of the natural fat content in the samples were also tested. Under optimal conditions, tissue samples were dispersed with C_{18} and anhydrous sodium sulphate and transferred to a solid-phase extraction column cartridge containing florisil and C_{18}. Cartridges were eluted with acetonitrile and the extract were directly analysed. Gomez-Ariza *et al*., 2002, compared matrix solid-phase dispersion to pressurized liquid extraction and Soxhlet for extracting PCBs from shellfish and fish. In matrix solid-phase extraction freeze-dried samples were blended with florisil and the mixture was placed in a glass column containing florisil and eluted with dichloromethane-pentane (15:85v/v). The obtained extracts were clean enough for direct analysis by GC-MS and GC- ECD. Similar advantages were achieved from matrix solid-phase dispersion and pressurized liquid extraction methods. However, the capital costs of matrix solid-phase dispersion was much lower than that of the other two methods.

2.8 Solid phase-extraction (SPE) theory

The principle of solid-phase extraction (SPE) is similar to that of liquid-liquid extraction (LLE) as it involves partitioning of solutes between two phases. However, instead of using two immiscible liquid phases as in liquid-liquid extraction, solid-phase extraction involves partitioning between a liquid (a sample matrix or solvent with analyte) and a solid-phase (the sorbent). This sample extraction technique enables the concentration and purification of analytes from solution by sorption on a solid sorbent and purification of extract after extraction. Solid phase extraction can also be used for desalting, derivatization and class fractionation of compounds.

Solid-phase extraction works like low-pressure liquid or affinity chromatography. A small quantity of stationary phase is packed in the bottom of a syringe or similar type of tube or cartridge. A sample solution is introduced into the cartridge and the solvent is forced through the packed bed. The solvent elute out of the cartridge while the anlytes of interest are retained in the stationary phase. A syringe plunger or a vacuum suction is used to facilitate the elution of the solvent. After a rinsing step (meant to clean-up the stationary phase and the retained anlytes), an appropriate solvent is applied to elute the analytes of interest out of the cartridge into a sample collector.

Various stationary phases are available for the solid-phase extraction apparatus (Baker, 1984). They range from ion-exchange to normal and reverse-phase liquid chromatographic

packing material. The wide spectrum of commercially available stationary phases makes solid-phase extraction method attractive for sample clean-up and fractionation. Many solid-phase extraction devices are available. Examples of such devices are Water Sep-Pak cartridge, Baker-10 SPE columns and Analytichem bond-Elut columns. The commercial solid-phase extraction cartridges have between 1 - 10 mL capacities and are discarded after a single use.

Guidelines for the proper use of Sep-pak solid-phase extraction device were developed by Bidlingmeyer in 1984. Two of the most important factors found to affect sample enrichment were flow rate and recovery of loadability effect. The surface of the solid phase must be solvated so that the cartridge can be properly conditioned. Bidlingmeyer's work can be used to optimize other solid-phase extraction techniques in terms of improving reproducibility, sample recovery and loading.

2.8.1 The mechanism of solid phase extraction

The selection of an appropriate SPE sorbent depends on the mechanism (s) of interaction between the sorbent and the analyte of interest. This in turn is influenced by the hydrophobicity, polarity and ionogenic properties of both the solute and the sorbent (Zwir-Ferenc and Biziuk, 2006). 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). Each sorbent offers a unique mix of these properties which can be applied to a wide variety of extraction challenges. The compounds can be retained by the sorbent in any of the following mechanisms;

- Reverse Phase; involves a polar or a moderately polar sample matrix (mobile phase) and a non-polar stationary phase. In this case the typical analyte of interest is mid to non-polar. Retention of the organic analytes from polar solutions onto these SPE materials is due to the attractive forces between the carbon-hydrogen bonds in the analyte and the functional groups on the sorbent surface (Van der Waal forces). A non-polar solvent which can disrupt the forces between the sorbent and compound is used to elute the compounds from a reversed SPE tube or disk. Some applications of the reverse phase mechanism have been in the analysis of drugs in biological fluids and organic pollutants in environmental samples. Zgorka and Hajnos in 2002, isolated flavones and some phenolic acids in

the medicinal plant (*Scutellaria baicalensis*) by combining solid-phase extraction and reverse phase high performance liquid chromatography.

- Normal Phase; involves a polar analyte, a mid to non-polar matrix and a polar stationary phase. Retention of the analytes is primarily due to the interactions between polar functional groups of the analyte and the polar groups on the sorbent surface (π-π intractions and hydrogen bonding). A compound adsorbed by these interactions is eluted by passing solvent that is more polar than the sample matrix. One example of the application of the normal phase mode is in the clean-up of thiobencarb residues in water and soil samples (Redondo *et al.*, 1994)

- Ion Exchange; in this mode the anionic (negatively charged) compounds are isolated on aliphatic quantenary amine group that is bonded to a silica surface. Cationic (positively charged) compounds are isolated by using the silica with aliphatic sulfonic acid groups that are bonded to the surface. The primary retention mechanism of the compound is based mainly on the electrostatic attraction of the charged functional group in the compound to the charged group that is bonded to the silica surface (Supelco, 1998). Some common applications of the ion exchange mechanism are in the assessment of drugs of abuse in biological fluids and removal of fatty acids in food samples (Lai, *et al*, 1997; Yan He and Liu, 2007).

- Silica-based packings usually have a stable pH range of 2 to 7.5. At pH ranges above or below this range, the bonded phase can be hydrolysed and cleaved off the silica surface or the silica itself can be dissolved. This is the reason the SPE cartridges are only used once and then disposed. This makes the technique expensive.

2.8.2 Solid-phase extraction procedure

Solid phase extraction is achieved through the interaction of three components; the sorbent, the analyte and the solvent. The analyte must be more strongly attracted to the sorbent than to the matrix. The best solid phase extraction mechanism and procedure are defined by the characteristics of the analyte in the sample. A typical solid phase extraction procedure (**Figure 2.2**) involves the following steps;

1. pre-treatment
2. Conditioning the column
3. Column equilibration
4. Loading; introduces the sample into SPE cartridge.
5. Washing; removes interfering components.
6. Elution; elutes the analyte if interest adsorbed to the sorbent.

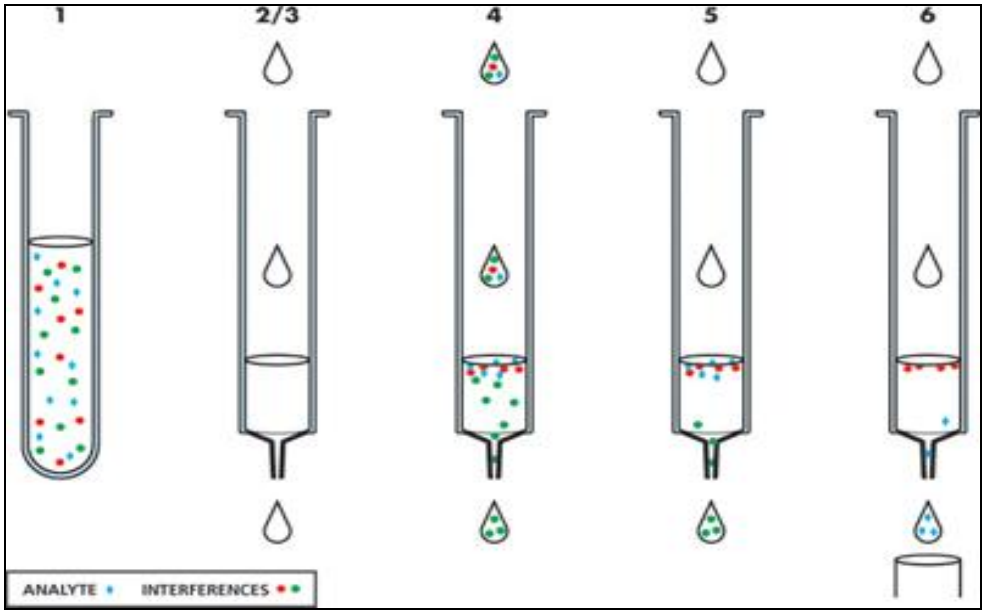


Figure 2.2: A typical solid phase extraction (SPE) procedure (Supelco, 1998).

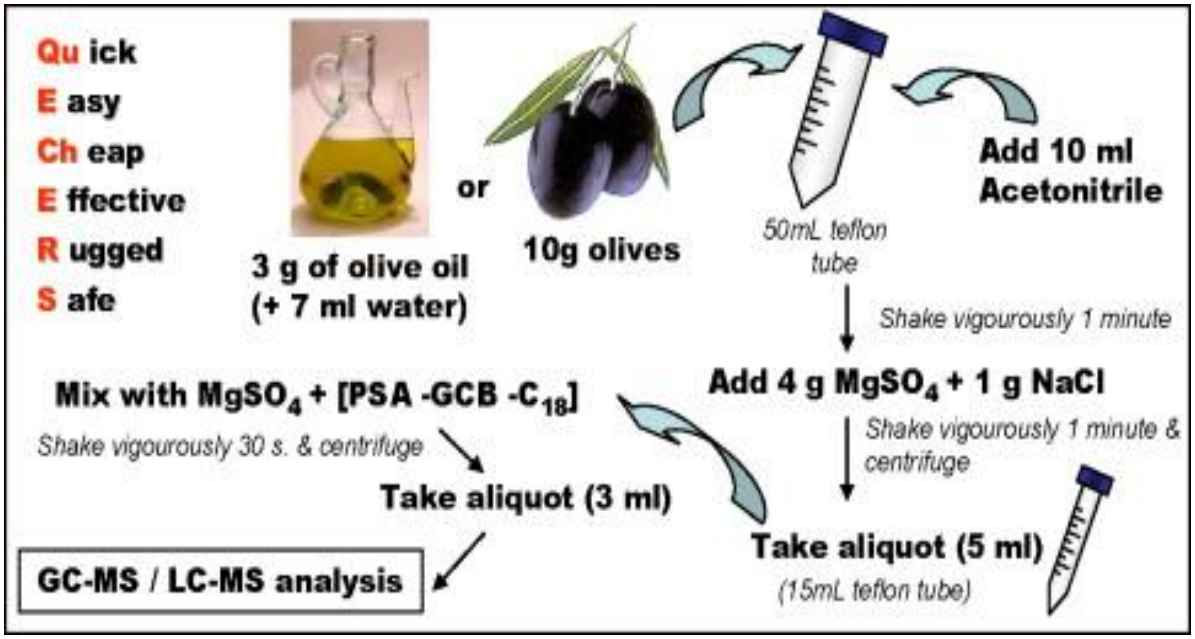
2.8.3 Dispersive solid-phase extraction (d-SPE) / QuEChERS

The setback of solid-phase extraction is that it is laborious, expensive and a multistep procedure. The many steps involved in the method can result in sample contamination, loss of analytes and reduction of sample throughput. An alternative method to solid-phase extraction is the dispersive solid-phase extraction (d-SPE) also referred to as the QuEChERS method (**Scheme 2.1**). Dispersive solid-phase extraction is based on the principles of solid-phase extraction. However, the sorbents are not packed in cartridges, they are directly blended with

the extract and clean-up is simply done by shaking and centrifugation. The interfering compounds are retained in the sorbents and the compounds of interest remain in solution.

QuEChERS is an acronym for quick, easy, cheap, effective, rugged and safe and it is the latest-generation method for the analysis of pesticide residues in food matrices. The technique offers good features for the analysis of polar pesticides. It is a quick and convenient substitute method for LLE as it offers great quality results with less labour-intensive sample preparation steps, low consumption of solvents, less glassware and expensive equipment. It requires very little bench space and reduces the exposure time faced by analytical chemists in most conventional methods. The method also removes delays resulting from equipment downtime due to spare parts and instrument maintenance challenges. The QuEChERS main feature consists of a micro-scale extraction step in which a homogenised sample is extracted by hand-shaking or vortex rotary with an organic solvent (acetonitrile) to give a concentrated extract without the evaporation step. Shaking the sample is preferred over blending due to the fact that (Anatassiades *et al*., (2003):

- During the shaking process the sample does not come into contact with the active metal surfaces of the blender and no heat is generated due to friction. Therefore there is no loss of volatile analytes.
- Cleaning the blender jar/probe between consecutive sample extractions is obviated, so no extra solvent is added to the sample.



Scheme2.2: Schematic procedure of QuEChERS modified for high-fat content vegetable matrices (Gilbert-Lopez *et al*, 2009).

In the extraction step, a mixture of salts usually, anhydrous magnesium sulphate and sodium chloride provides a well-defined phase separation without dilution and use of toxic non-polar organic solvents. The salts are added to the sample to facilitate partitioning of the analytes between the aqueous residue and the solvent. Acetonitrile (MeCN) is the preferred solvent to successfully extract all kinds of pesticides from various food matrices. Use of acetonitrile has shown to provide extraction of the broadest range of organic compounds without co-extraction of large amounts of lipophilic material. Acetonitile is highly compatible with GC-MS and LC-MS applications with less interferences.

For clean-up the extract is blended with C18 and primary secondary amine (PSA) sorbents. Extra salt (anhydrous magnesium sulphate) is introduced to remove any residual water that may have been carried over from the extraction step. PSA is a weak anion exchanger which removes fatty acids, sugars and other matrix co-extractants that form hydrogen bonds. An aliquot of the resulting supernatant is then taken for chromatographic analysis. This method takes a shorter time compared to the traditional SPE and simultaneously eliminates residual water and a lot of polar matrix components.

Chapter 3

Biomarkers in environmental monitoring

3.1 Overview

This chapter gives an overview of uses of biomarkers in the analysis of pollutants in the environment. It discusses acetylcholinesterase (AChE) activity, the biomarker for cholinergic compounds.

3.2 Biomarkers in support of chemical analysis

In the past decades, environmental monitoring programs focused on the measurement of physical and chemical variables and occasionally incorporated biological variables (Lam, 2009). For example, routine water analysis often included the determination of temperature, salinity, dissolved oxygen, nutrients and chemical contaminants. These parameters were perceived to be important indicators of environmental quality and were relatively easy to measure (Lam, 2009). Sediment monitoring involved physico-chemical measurements which included particle size distribution, organic matter and contaminants. Even though such programs gave information on the levels of contamination they did not provide information on the effects of the contaminants on the biological systems (Lam and Gray 2003).

Given that the ultimate goal of environmental monitoring is to protect biological/ecological systems, it is necessary and imperative to study *overall* biological effects of exposure to potentially harmful substances in the environment. This realization has resulted in the incorporation of biological-based analysis to complement chemical analysis.

Bioindicators are animals or plants which accumulate pollutants in their tissues and organs in direct relation to the presence of pollutants in their surroundings. Biomarkers are biochemical or physiological changes that occur in an organism as a result of exposure to pollutants. Biomarkers are recognised as useful tools for the assessment of the pollution impact on aquatic organisms. This is due to their sensitivity, relative ease of application, low cost and specificity to pollution stress (Walker and Livingstone *et al.*, 1982, Huggett *et al.*, 1992, WHO, 1993). Furthermore, biomarkers serve as early warning signals for significant biological effects (Huggett *et al.*, 1992). It is known that the effects of pollutants in biomarkers occur at different levels of organisation i.e. ranging from sub-cellular to whole-

organism and the ecosystem. Sub-organismic (molecular, biochemical and physiological) responses precede those that occur at higher level of biological organisation (**Figure 3.1**).

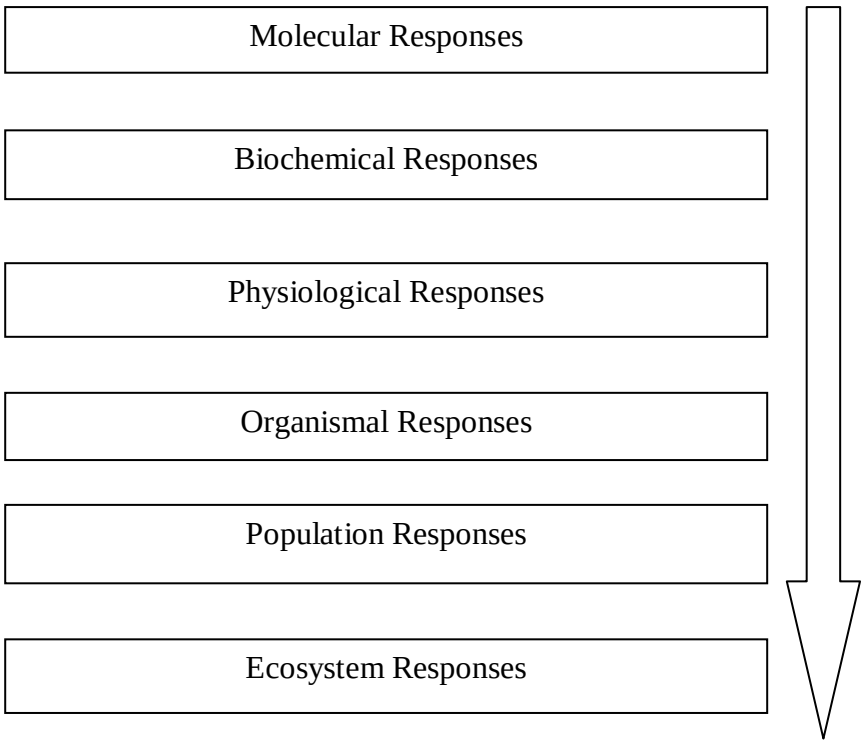


Figure 3.1: Biomarkers and bioindicators at various biological organizational levels (Connell *et al.,* 1999).

3.2.1 Biomarker sensitivity and specificity

Numerous biomarkers have been developed and can respond to toxic stress with differing specificity. Some biomarkers are highly specific and only respond to one kind of chemical or group of chemicals. For example, the enzyme aminolevulinic acid dehydratase (ALAD) is specifically inhibited by lead (Selander and Cramer, 1970). ALAD is sensitive to an extent that the results produced by such studies may be sufficient to replace chemical analysis of the surrounding area. Most biomarkers are less specific and respond to pollution in general. One such biomarker is the mixed function oxygenase (MFO) system which is relatively non-specific. Many organisms posses this detoxification enzyme complex which can be induced by a wide variety of natural and xenobiotic compounds. However, a combination of biomarkers assayed in parallel could elucidate the prevalence of a particular pollutant in the environment. The suitability of various antioxidant paramaters such as glutathione S transferase (GST), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidise

(GPx), glutathione reductase (GR), glutathione (GSH) and lipid peroxidise (LPO) for use as biomarkers has been examined in a variety of organisms. Results using the green-lipped mussel (*Perna viridis*) indicated that the majority of the antioxidant parameters, with exception of gill SOD and LPO were induced by increasing tissue concentrations of polycyclic aromatic hydrocarbons (Cheung *et al.*, 2001).

For organochlorines, GPx is the only enzymatic antioxidant that showed a significant response to polychlorinated biphenyls in tissues (Cheung *et al.*, 2002). The same study also demonstrated a correlation between GST/GSH and chlorinated hydrocarbons. Oxidative stress measured as thiobarbituric acid reactive substances was however correlated with chlorinated pesticide concentration in mussel tissues. No significant correlation was found between tissue concentrations of chlorinated hydrocarbons and other enzymatic antioxidants investigated (Cheung *et al.*, 2002).

One of the limiting factors in the use of biomarkers in monitoring environmental pollution are biotic and abiotic variables which can influence responses induced by pollutants in the biomarkers (Figure 3.2). To be confident that the biomarkers give a true reflection of the pollution status in the environment or organism it is necessary to compare results with those from known healthy organisms or controlled environments.

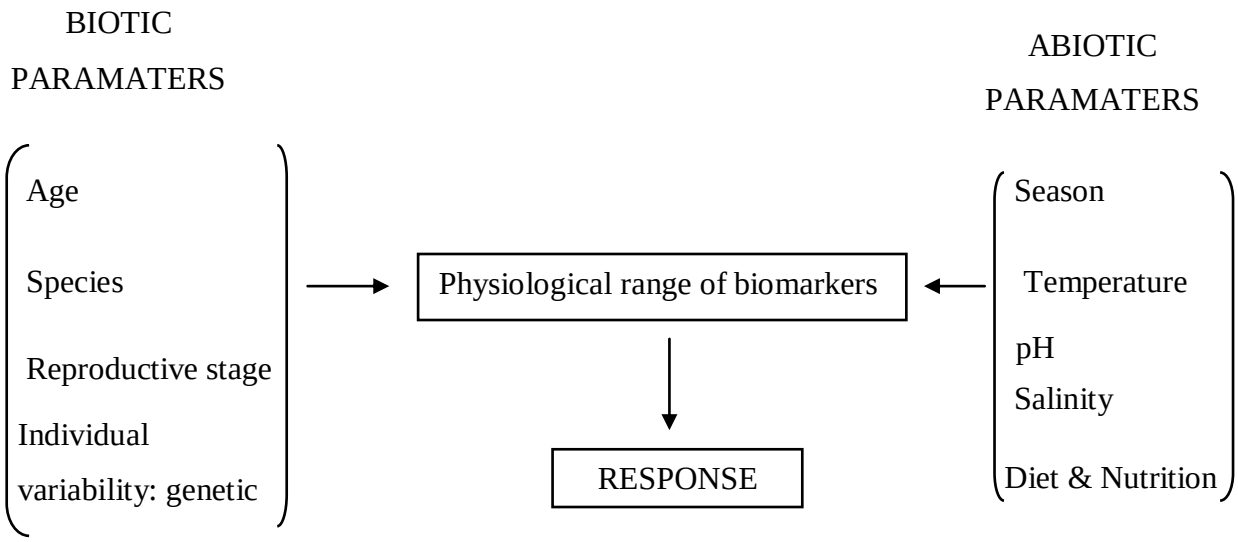


Figure 3.2: Biotic and abiotic paramaters that influence biomarker response to chemical stress (Sanchez and Jean-Marc Porcher, 2009).

3.3 Acetylcholinesterase activity (AChE)

The enzyme acetylcholinesterase represents a biomarker of neurotoxicity widely used for the identification of exposure to anti-cholinesterase chemicals (Walker, 2001). These neurotoxic compounds exert their toxicity by blocking the backbone of acetylcholine (ACh) by the enzyme acetylcholinesterase (AChE). Acetylcholine is the primary neurotransmitter in the sensory and neuromuscular system in most species. The activity of this system is important to normal behaviour and muscular function and represents a prime target on which some toxicants exert a detrimental effect. When acetylcholinesterase is inactivated by pollutants such as organochlorine, organophosphorus and carbamates pesticides the concentration of acetylcholine in the nerve junction remains high in comparison with the unaffected organism (Bocquene *et al*., 1990, Galgani and Bocquene, 1990, 2000, Galgani *et al*., 1992, Escartin and Porte, 1997, Narbonne *et al*., 1999). The inhibition of acetylcholinesterase results in a build up of acetylcholine causing a continuous and excessive stimulation of the nerve or muscle fibres which leads to tetany, paralysis and eventually death.

Measurement of acetylcholinesterase activity in aquatic organisms including fish has already been used as a biomarker of the effects of neurotoxic contaminants (Habig and Di Giulio, 1988; Galgani *et al.,* 1992; Sole *et al.,* 2000). Fish are used extensively as bioindicators for environmental monitoring because they take up contaminants directly from water and diet (Lanfranchi *et al.,* 2006). Generally, the ability of fish to metabolize organochlorine compounds is moderate therefore contamination loading in fish is well reflective of the state of pollution in the surrounding environments (Fisk *et al.,* 1998; Lanfranchi *et al.,* 2006).

Acetylcholinesterase activity is determined by a method popularly known as the Ellman's method named after George Ellman who developed the method in 1961 (Ellman *et al.,* 1961). The esterase activity is measured by providing an artificial substrate, actylthiocholine iodide (ATCI). Thiocholine is released as the substrate (ATCI) is cleaved by the enzyme (AChE). The released thiocholine then reacts with 5, 5′- dithiobis-2-nitrobenzoic acid (DTBN) which is reduced to thionitrobenzoic acid (yellow in colour).

These coupled reactions are presented by the following equations;

Acetylthiocholine iodide $\xrightarrow{\text{AChE}}$ thiocholine + acetate (3.1)

Thiocholine + dithiobisnitrobenzoate acid $\longrightarrow$ thionitronenzoic acid (3.2)

This yellow coloured anion strongly absorbs at a wavelength of 412 nm and it is. Its detected using a UV spectrophotometer. The activity of the enzyme is generally expressed as a rate: the concentration of the substrate (in moles) which is hydrolyzed by a known concentration of the enzyme per unit time. In this study, it is the concentration of ATCI which is broken down by acetylcholinesterase per min.The enzyme activity was calculated using **eqn. 3.3**.;

$$R = 5.75 \times 10^{-4} \times A/Co \qquad (3.3)$$

Where,

R = rate in moles of substrate hydrolyzed / minute per gram of tissue

A = change in absorbance per minute

Co = original concentration of the tissue (mg / mL)

Chapter 4

Experimental

4.1 Supplies

4.1.1 Standards, solvents and chemicals

The standards o, p′-DDT (98%), p, p′-DDE (99.5%), Aldrin (98.8%), and Dieldrin (99.0%) suitable for use with all EPA methods were obtained from ChemService (PA, USA). The solvents n-hexane, acetone and acetonitrile were of HPLC grade and were purchased from Merck (Darmstadt, Germany). Acetylthiocholine iodide (ATCI) and 5, 5′-dithiobisnitrobenzoic acid (DTNB) were obtained from Sigma-Aldrich (Germany), while K_2HPO_4, NaH_2PO_4, $NaHCO_3$ and NaOH all chemically pure were purchased from Merck (Darmstadt, Germany).

4.1.2 Standard stock solutions

Stock solutions of the individual standards were prepared in n-hexane at concentrations of 1000 mgL^{-1}. Working standards were prepared freshly every 48 h by making appropriate dilutions.

4.1.3 0.1 M phosphate buffer solution

Solution A; 5.22 g of K_2HPO_4 and 4.68 g of NaH_2PO_4 were dissolved in 150 mL of distilled water. Solution B; 6.2 g NaOH was dissolved in 150 mL of distilled water. The above solutions were mixed, the pH adjusted to 8 and the final volume was made to 300 ml.

4.1.4 5, 5′- dithiobis-2-nitrobenzoic acid (DTNB) reagent

39.6 mg of dithiobisnitrobenzoic acid and 15 mg of $NaHCO_3$ were dissolved in 10 mL of 0.1 M phosphate buffer.

4.1.5 Acetylthiocholine iodide (ATCI) substrate

21.67 mg of acetylthiocholine iodide was dissolved in 1 mL of distilled water.

4.2. Sample site

The samples were collected from two sites, the upper and lower regions of the delta (**Figure 4.1**). The upper delta is towards the Namibian border where the Okavango River enters into Botswana and the lower delta is at the periphery of the town of Maun. The site marked M is Samuchima in the upper region of the delta and Boro is marked T, located at the lower end of the delta.

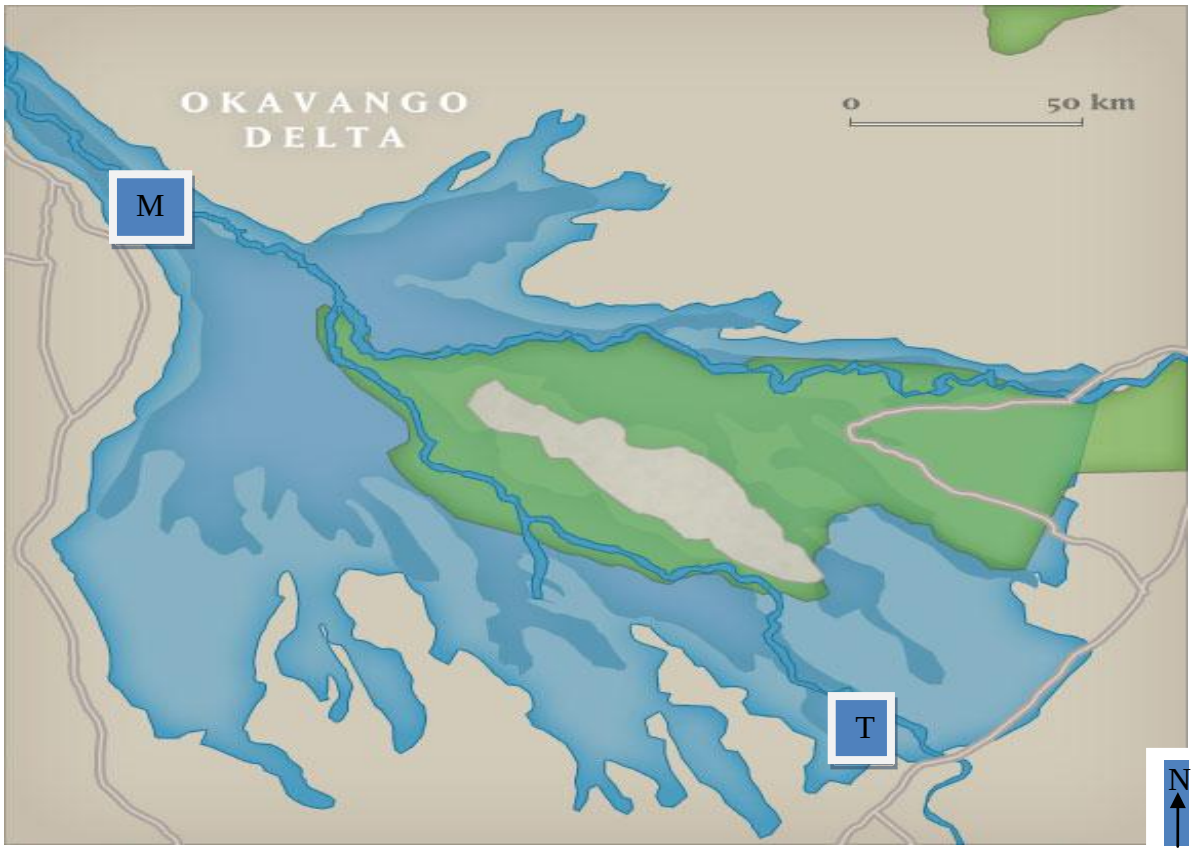


Figure 4.1: Map of the Okavango Delta showing the sampling sites. (M = Samuchima and T = Boro).

4.3 Sampling

Fishing nets were cast late in the afternoons and the catch was retrieved the following mornings. Four fish species; *Clarias ngamensis, Oreochromis andersonni, Hydrocynus vittatus and Tilapia rendalli* **(Figure 4.2)** were selected at random from the catch. These fish species are commonly consumed by the riparian communities. Fish identification, mass and fish total length were recorded before portioning the samples. The brain for enzyme activity studies, the otoliths (sagittae) for determining the age of the fish and the fillet for pesticide analysis were retrieved from each fish species. The brain and the fillet were wrapped in clean tin foil and kept on ice. The otoliths were washed with distilled water and kept in glass vials. In the laboratory the brain and fillets were kept frozen at -20 °C while the otoliths were kept at room temperature. In preparation for chemical analysis the fillets were freeze dried for 72 hours. The dried fillets were ground into free flowing powder using mortar and pestle. The powders were stored in pre-cleaned glass bottles and kept in a dessicator to avoid moisture being absorbed by the powdered samples.

(a)

(b)

(c)

(d)

Figure 4.2: Photographs of the fish specimen (a) *Hydrocynus vittatus* (tigerfish), (b) *Clarias ngamensis* (blunt-tooth catfish), (c) *Oreochromis andersonni* (three spot tilapia) and (d) *Tilapia rendalli* (red-breasted tilapia).

4.4 Determination of fish age

The age of the fish samples was estimated by counting the annual growth rings on the otoliths. One otolith was chosen from each pair and mounted on clear casting resin. Once set, the resin rod was sectioned transversely using a double-bladed diamond-edge saw through the nucleus producing otolith sections of approximately 0.15 mm. The otolith sections were then mounted on glass slides using DPX mountant. The slides were viewed under a dissecting microscope at 12 × using transmitted light. Age estimates were obtained by counting the number of opaque bands from the nucleus to the margin.

4.5 Determination of lipid content

The extracts for determining lipid content were extracted by the QuEChERS method. The extracts were not carried through to the clean-up stage as this would remove the lipids. The resulting extracts were pre-concentrated to approximately 10 mL. Then 1 mL of the concentrate was pipetted into a pre-weighed clean aluminium drying pan and dried in the oven at 100 °C for 5 minutes. The extracts were allowed to cool and re-weighed. Percent lipid content was calculated using **eqn. 4.1**;

$$\% \text{ Lipid} = (1 - \text{mass of residual lipid})\ 100 \qquad (4.1)$$

4.6 Preparation of brain tissue for enzyme activity assay

The brain tissues were allowed to thaw at room temperature. 30 mg of the brain tissue was homogenised in 1 ml of 0.1 M phosphate buffer pH 8 and kept on ice. The homogenate was then centrifuged at 3000 rpm for 15 min and the resulting supernatant was kept on ice for enzyme activity assay.

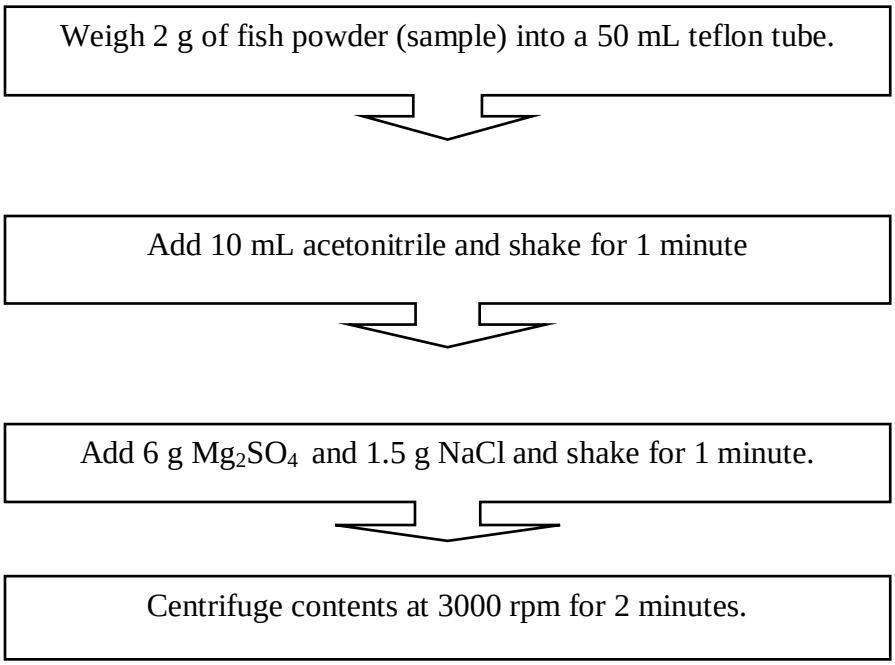
4.6.1 Acetylcholinesterase (AChE) Activity Assay

2.6 mL of 0.1 M phosphate buffer was pipetted into a cuvette into which 400 µl of the brain supernatant was added. 100 µl DTNB (reagent) was added to the above contents and the cuvette was placed on the UV spectrophotometer and the instrument was zeroed. The reaction was started by adding 20 µl of ATCI (substrate) and the contents were quickly mixed. The absorbance was recorded at 412 nm over a period of 10 min at 1 min intervals.

4.7 Extraction and clean-up procedures

4.7.1 Extraction

2 g of ground fish fillet was weighed into a 50 mL teflon tube (n = 3). 10 mL of acetonitrile was added into the tube and the contents were hand shaken for one minute. 6 g $MgSO_4$ and 1.5 g NaCl (pre-weighed non-buffered Agilent sampliQ QuEChERS kit P/N 5982-5555) were added into the tube and the contents were further shaken for one minute. The contents were then centrifuged at 3000 rpm for two minutes. The procedure is summarised in the **Scheme 4.1.**

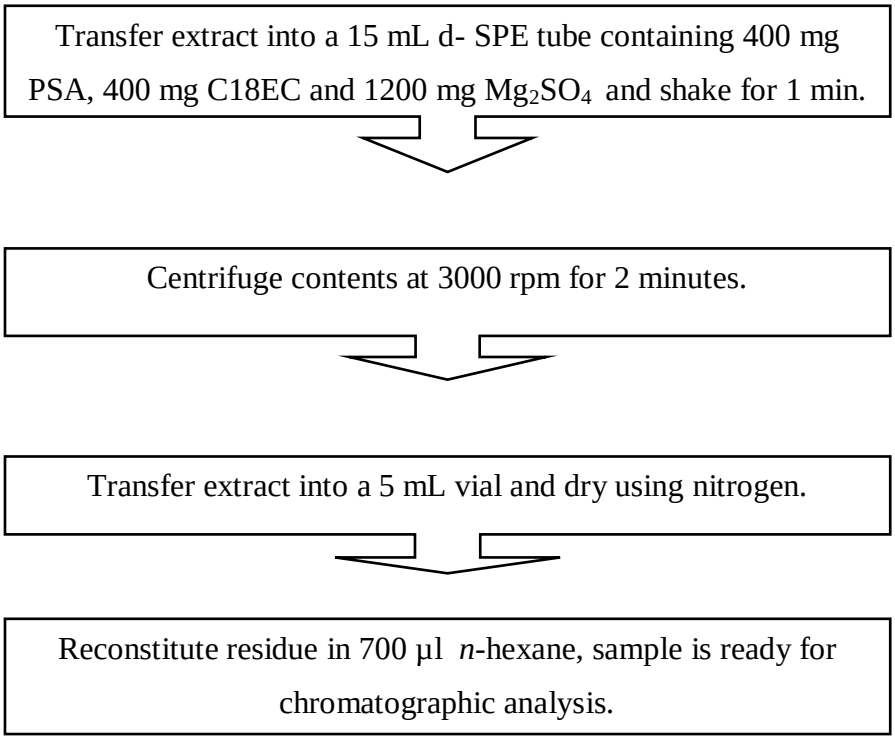


Scheme 4.1: QuEChERS extraction Flow chart.

4.7.2 Sample Clean-up (d-SPE)

The resulting supernatant from **scheme 4.1** above was transferred into a 15 mL d-SPE tube (Agilent SampliQ QuEChERS kit P/N 5982 - 5158) containing the sorbents 400 mg PSA, 400 mg C18 EC and 1200 mg $MgSO_4$. Blending was done by shaking the contents for one minute. The contents were then centrifuged at 3000 rpm for a period of two minutes. The supernatant was decanted into a separate vial and concentrated by blowing a stream of

nitrogen through the extract. The dry extract was reconstituted in 700 µl *n*-hexane and taken for chromatographic analysis. **Scheme 4.2** below gives a summary of the clean-up procedure.



Scheme 4.2: Dispersive-SPE flow chart for the claen-up experiment

4.8 Instrumental analysis

An Agilent 6820 gas chromatograph equipped with a micro Electron Capture Detector (µECD) was used in the analysis of the compounds. The separation was performed on a fused capillary column, 30 m × 0.32 mm ID., 0.25 µm film thickness 5% phenyl and 95% dimethylpolysiloxane (HP-5). The injector and detector temperatures were set at 250 °C and 300 °C respectively. Helium was used as carrier gas and it was kept at a flow rate of 1 mL/min. The head pressure was set at 15 psi. Nitrogen was the make up gas and its flow rate was set at 60 mL/min. 1µl of sample was injected in splitless mode and the purge time was 0.75 min. The temperature programme for separating the compounds is given in **Table 4.1.**

Table 4.1 Temperature programme for HP-5 capillary column

Ramp (°C/min)	Temp. (°C)	Hold time (min)
-	50	1
30	180	1
10	250	2
30	280	2

The analytes were confirmed on a 6890N gas chromatograph equipped with a 7683B autosampler manufactured by Agilent Technologies (Shangai, China) connected to a GCT Premier Time-of-flight mass spectrometer manufactured by Waters (Manchester, England). The conditions and temperature programme employed for the GC-ECD was used in the GC-MS system. The injector and transfer line temperatures were both maintained at 250 °C while the ion source was kept at 220 °C. The electron impact (EI) source in the positive mode was operated at 70 eV and the mass spectra were acquired in the 50 to 500 m/z range. The solvent delay time was set to 3.8 minutes. The mass spectra were compared to the NIST/EPA Mass Spectral Library - 2002 Version 200a (Newfield NY, USA).

4.9 Method validation

4.9.1 Linearity, Limits of detection and Limit of Quantification

The linearity of the detector response to the analytes was examined by injection of the standards at five concentrations 2, 4, 6, 8 and 10 µg/L. Calibration graphs for the individual pesticides were constructed by plotting the peak area versus the concentration of the standard. The concentrations of the analytes were calculated from extrapolations done on these graphs. The limits of detection (LOD) and limit of quantification (LOQ) of the compounds were estimated from the concentration of the pesticide standards required to give a signal-to-noise ratio of 3 and 10 respectively. Repeatability experiments were performed by injecting a spiked sample (free of the analyte of interest) with a standard mixed solution (2, 5 and 10 µg/L) three times.

4.9.2 Recovery experiments

Recovery experiments were conducted in order to evaluate the extraction efficiency of the QuEChERS method in extracting the four pesticides residues from the fish samples. In the absence of certified reference material (CRM) for fish or similar matrices, the method's efficiency was evaluated on the basis of recoveries obtained from fortified samples. A fish sample (*Clarias ngamensis*) obtained from a reference site (control) was used in the recovery experiments. The sample was previously analysed and found to be free of the compounds under investigation. The samples were fortified with 200 µl of the spiking mix solution at three levels (2, 5 and 10 ng/g). The spiked samples were allowed to stand for 1 hour at room temperature before the extraction was performed. This was meant to allow sufficient contact between the spiked compounds and the fish powder.

Chapter 5

Results and Discussion

5.1 Optimization of the extraction conditions

5.1.1 Mass of sample

To maintain low solvent usage, 10 mL of the extraction solvent was used in all the extraction experiments. This volume was used in the original QuEChERS method by Anastassiades *et al*., 2003 for the extraction of pesticides in fruits and vegetables, Ramalhosa et al., 2009 for the extraction of PAHs in fish tissue and Rawn *et al*., 2010 for the extraction of pyrethrins and pyrthroids in fish tissue. The mass of the sample used was optimized by extracting 0.5, 1.0, 1.5, and 2.0 g of the blank fish sample spiked with a DDT standard at 2 µg/L. Above 2.0 g all the solvent was absorbed by the sample and the salts. The optimum extraction was achieved at 2.0 g. The peak area almost remained unchanged between the masses 0.5 and 1.5 (**Figure 5.1**).

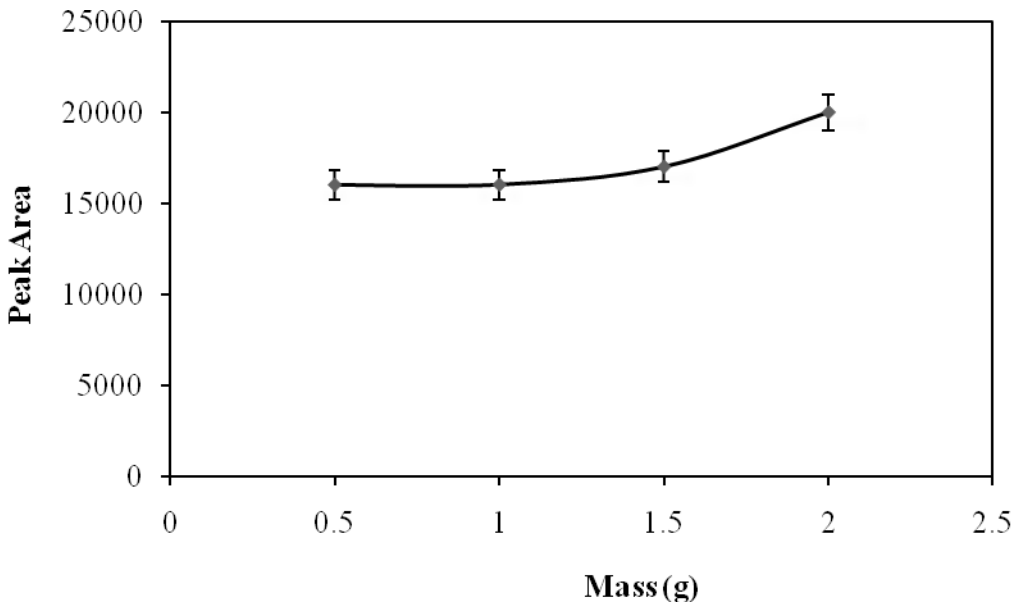


Figure 5.1: Peak area response to sample mass

5.1.2 Shaking time

Ideally long stirring and shaking times during the extraction process enhance the extraction of the analytes from the sample matrix. To avoid unnecessary prolonged extraction times the shaking time was optimised by shaking the sample at 1,2,3,4 and 5 minutes. 2.0 g of sample was spiked with 5µg/L of a DDT standard. Toe optimum shaking time was at 1 minute since the peak area response remained unchanged with the increase in time (**Figure 5.2**).

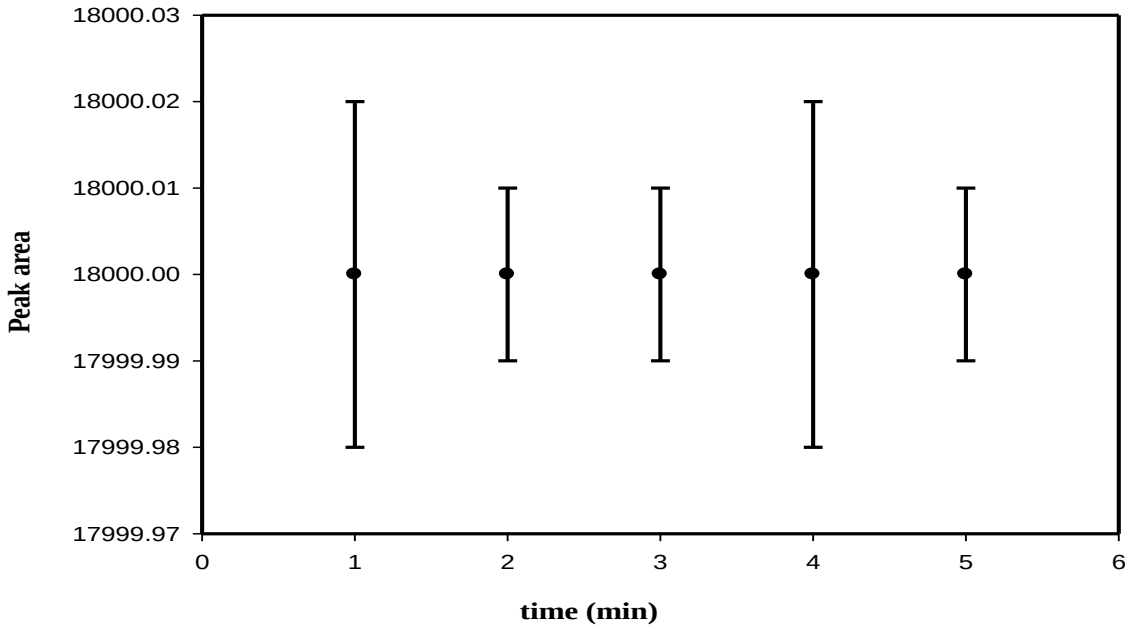


Figure 5.2: A plot of peak area response of DDT versus shaking time

5.2 Method validation

Table 5-1 shows the regression equations, correlation coefficients, limits of detection and quantification for the four organochlorine pesticides. The generated calibration graphs were linear and gave correlation coefficients higher than 0.99. The limits of detection and limit of quantification were in the range 0.12 – 0.83 µg/L and 0.04 – 0.25 µg/L respectively.

Table 5 -1 Analytical parameters for the organochlorine pesticides.

Pesticide	Equation	R^2	LOQ	LOD
dieldrin	y = 605.77x + 69.14	0.9983	0.06	0.22
DDE	y = 840.49x + 42	0.9990	0.13	0.42
DDT	y = 1113.9x	0.9991	0.25	0.83
aldrin	y = 3391.7x + 315.48	0.9993	0.04	0.12

The mean recoveries at the three fortification levels were greater than 75% in all cases with relative standard deviation of less than 13% (**Table 5.2**). The mean recoveries of the fortified samples at 10 ng/g level ranged from 76 to 96%. At 5 ng/g level of fortification the recoveries were between 78 and 89%. The lower level of fortification (2 ng/g) gave recoveries between 81 and 94%. The recovery values seemed not to be related to the spiking levels.

Table 5-2 Recoveries and percent RSD values for the four organochlorine pesticides.

	Fortification level (ng/g) (n = 3)		
	2	5	10
Pesticide	% Recovery	% Recovery	% Recovery
Aldrin	81 (6.8)	80 (7.5)	76 (9.7)
Dieldrin	94 (4.3)	86 (12.2)	88 (6.8)
DDT	90 (7.6)	89 (9.0)	96 (8.3)
DDE	81 (9.3)	78 (3.9)	82 (5.6)

In all the three fortification levels, the recoveries of aldrin and DDE were lower than that of DDT and dieldrin. DDE and aldrin are high lipophilic pesticides. Highly lipophilic pesticides tend to give lower recoveries in the QuEChERS method applied to matrices with a higher quantity of fat (Cunha *et al.*, 2007, Lehotay *et al.*, 2005 and Li *et al.*, 2007). This happens because the fat is practically not dissolved in acetonitrile and thus form an additional layer. This leads to the partitioning of lipophilic pesticides between acetonitrile and fat layers, an extent which depends mainly on the analyte lipophilicity and the amount of fat in the matrix. In this study this seemed not too significant because of the small sample to solvent ratio (2 g of sample to 10 mL of acetonitrile).

Concerning these recovery results, it should be borne in mind that in spiked test samples the introduced analyte is not held strongly as in the natural matrix and is likely to be released more readily compared to that in the natural material. So there is a possibility that recoveries obtained this way may be unrealistically high. As such these result need to be confirmed by conducting recovery experiments on certified reference material (CRM) most probably of natural origin.

5-3 Sample biometric data

The length and mass of the fish samples investigated in this study ranged between 32.1- 41.5 cm and 486 – 704 g respectively (**Table 5.3**).

Table 5.3 Sample biometric data.

Sampling site	Fish species	Length (cm)	Mass (g)	n
Upper delta	*H. vittatus*	39.2 - 41.2	522 - 703	5
	O. andersonni	34.7 - 40.3	498 - 704	5
	T. rendalli	35.7 - 40.7	502 - 699	5
	C. ngamensis	38.4 - 41.5	518 - 704	5
Lower delta	*O. andersonni*	32.1 - 40.0	486 - 698	5
	T. rendalli	32.8 - 40.0	487 - 696	5
	C. ngamensis	35.8 - 40.8	503 - 700	5
Reference site	*C. ngamensis*	33.6 - 37.0	487 - 510	5

The set chromatographic conditions gave a clear separation of the compounds (**Figure 5.3**). The retention times of the compounds ranged between 13.7 and 17.8 minutes. Peak or compound identifications were conducted by comparing the retention times of the standards to those obtained from the samples. Three organochlorine pesticides residues viz, aldrin, p, p′- DDE and dieldrin were detected in all the four fish species investigated. Figure 5.4 shows a chromatogram of one of the fish species (*T. rendalli*), the peaks a, b and c are aldrin, p, p′-DDE and dieldrin respectively. To verify the results, samples were spiked with 10 µg/L of a standard mix solution of aldrin, p, p′-DDE and dieldrin. The signals of the peaks corresponding to the compounds under investigation increased significantly. An example is given on **Figure 5.5**.

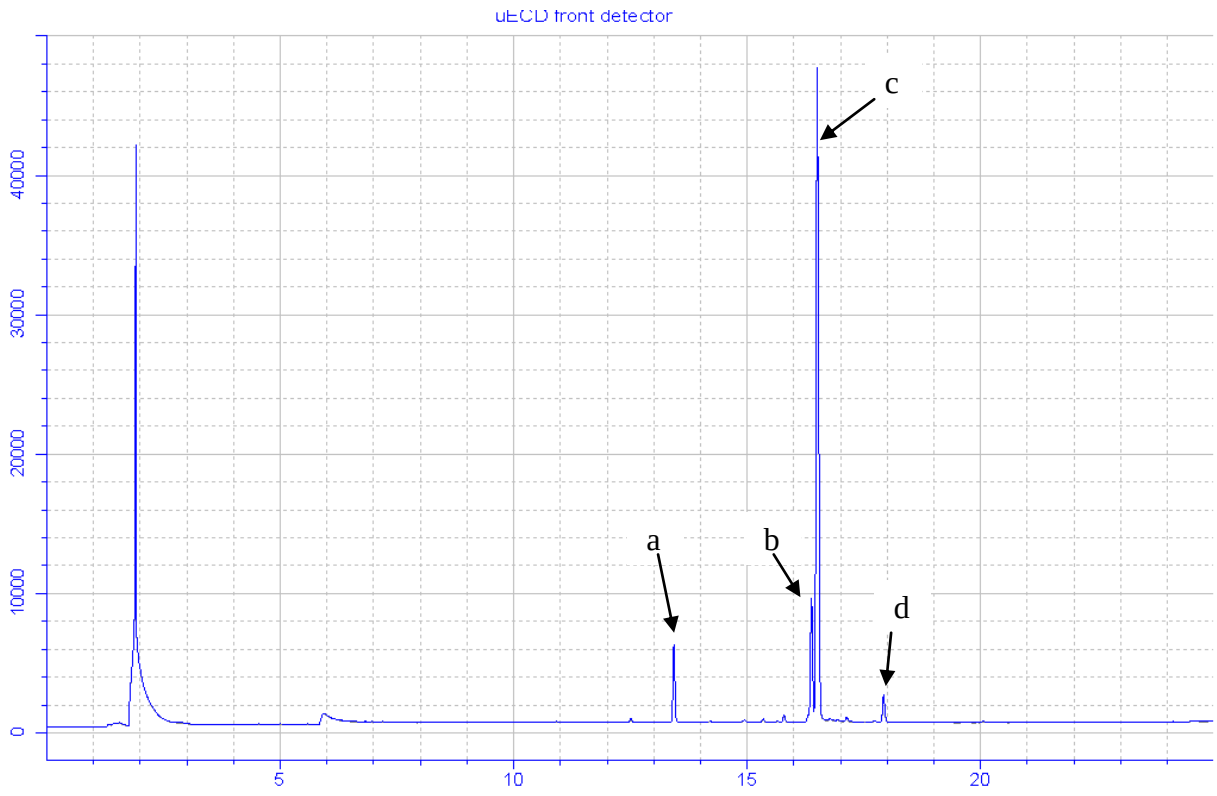


Figure 5.3: Elution order and retention times of the pesticides separated from a mixed standard solution of concentration range 2 - 10 µg/L. a = aldrin, b = p, p′-DDE, c = dieldrin and d = o, p′- DDT.

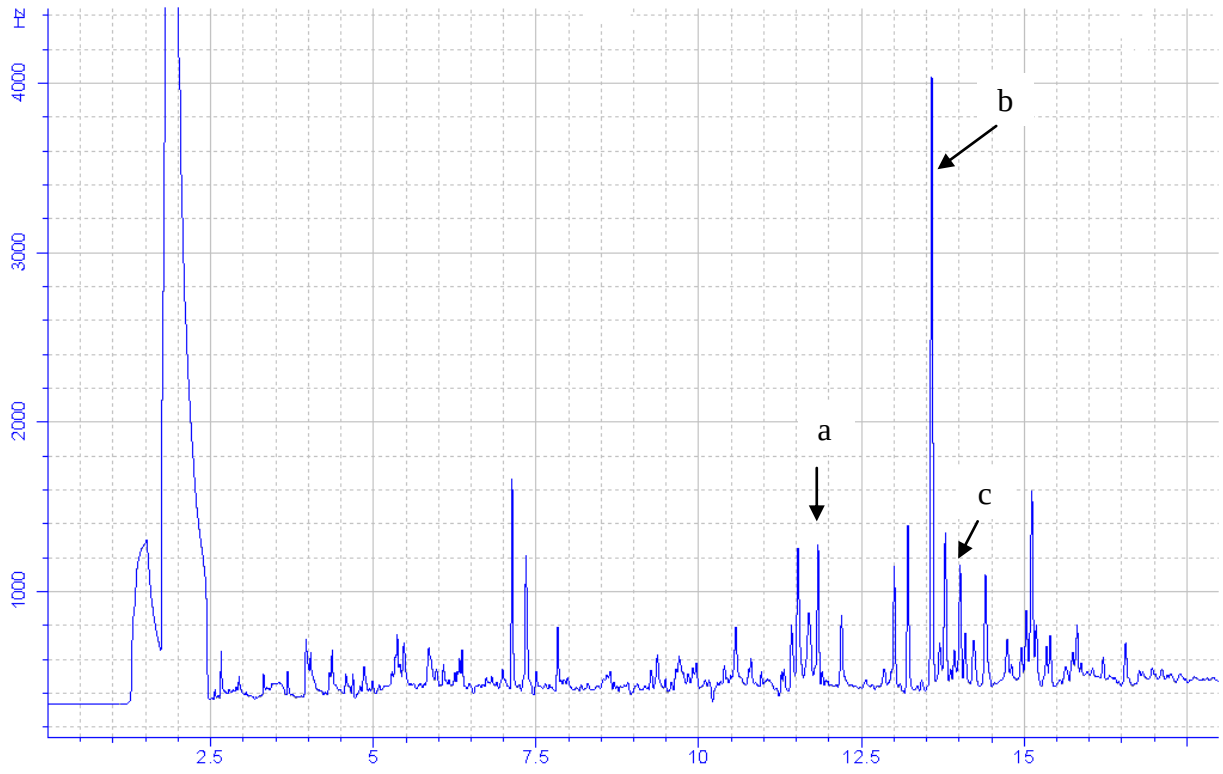


Figure 5.4: A chromatogram of a *C. ngamensis* sample. A = aldrin, b = p, p′-DDE and c = dieldrin.

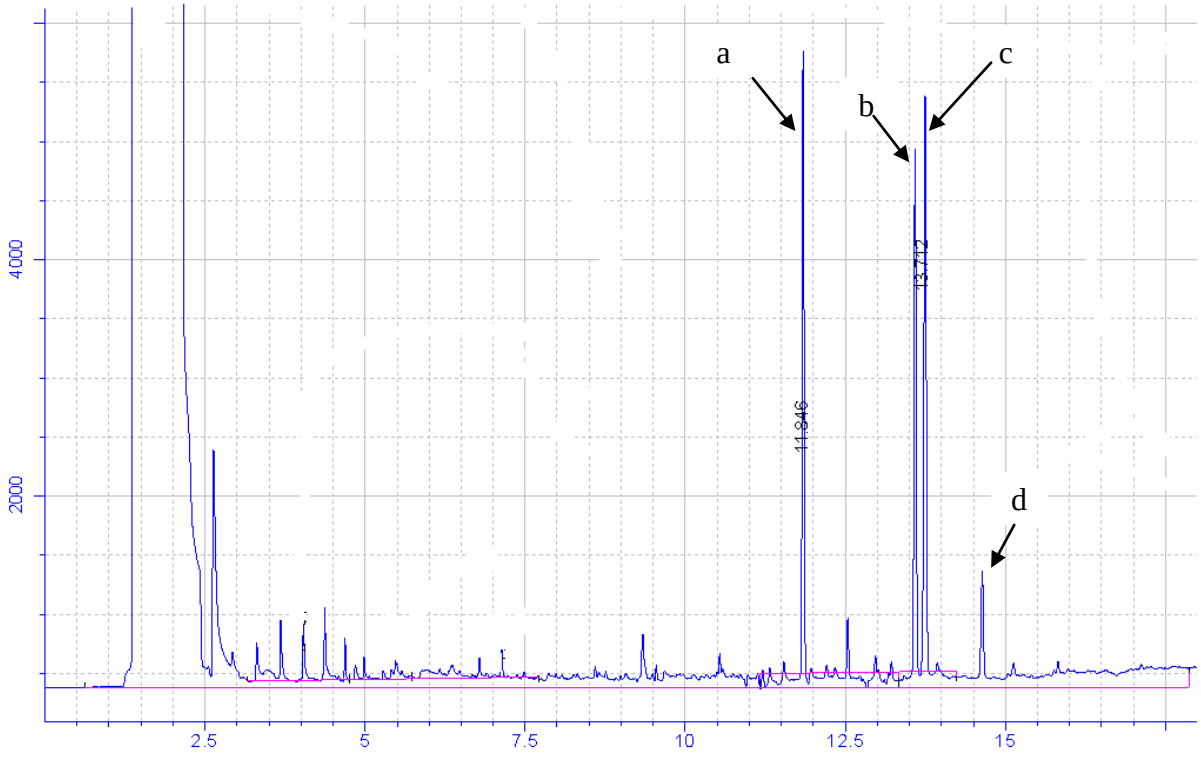


Figure 5.5: A chromatogram of *O. andersonni* (three spotted tilapia) sample spiked with 10 µg/L of mixed standard solution. A = aldrin, b = p, p′-DDE, c = dieldrin and d = o, p′-DDT.

Confirmatory analysis by gas chromatography-mass spectrometry indicated the presence of aldrin, p, p′-DDE and dieldrin in the samples as recorded with GC-ECD. **Figure 5.6** shows an ion chromatogram of one of the fish species (*T. rendalli*). **Figures 5.7, 5.8** and **5.9** show the electron impact (EI) spectra of the compounds; aldrin, p, p-DDE and dieldrin detected in *T. rendalli*.

Table 5.4 gives a summary of the organochlorine pesticide residues confirmed by gas chromatography-mass spectrometry.Interestingly, chlordane epoxide and isodrin were detected in *O. andersonni*. Traces of isodrin were also detected in *C. ngamensis.*These compounds were not detected in the other two species i.e. *H. vittatus* and *T. rendalli*.

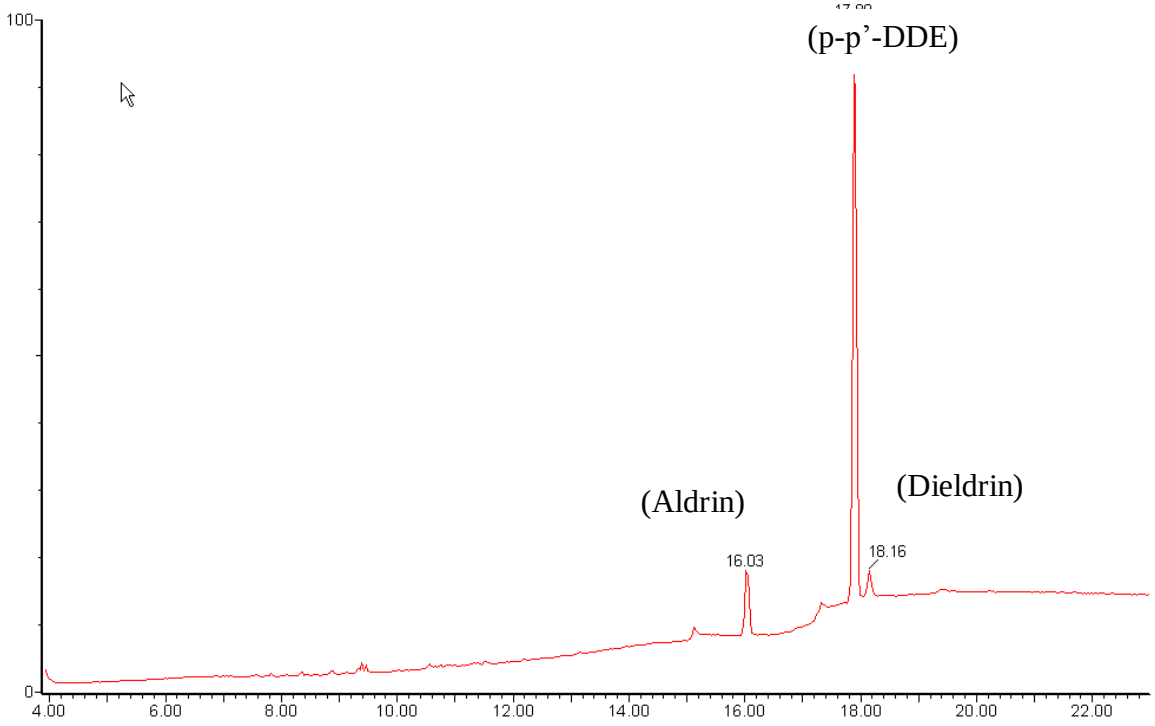


Figure 5.6: An ion chromatogram of *T. rendalli* showing the compounds aldrin, p, p-DDE and dieldrin .

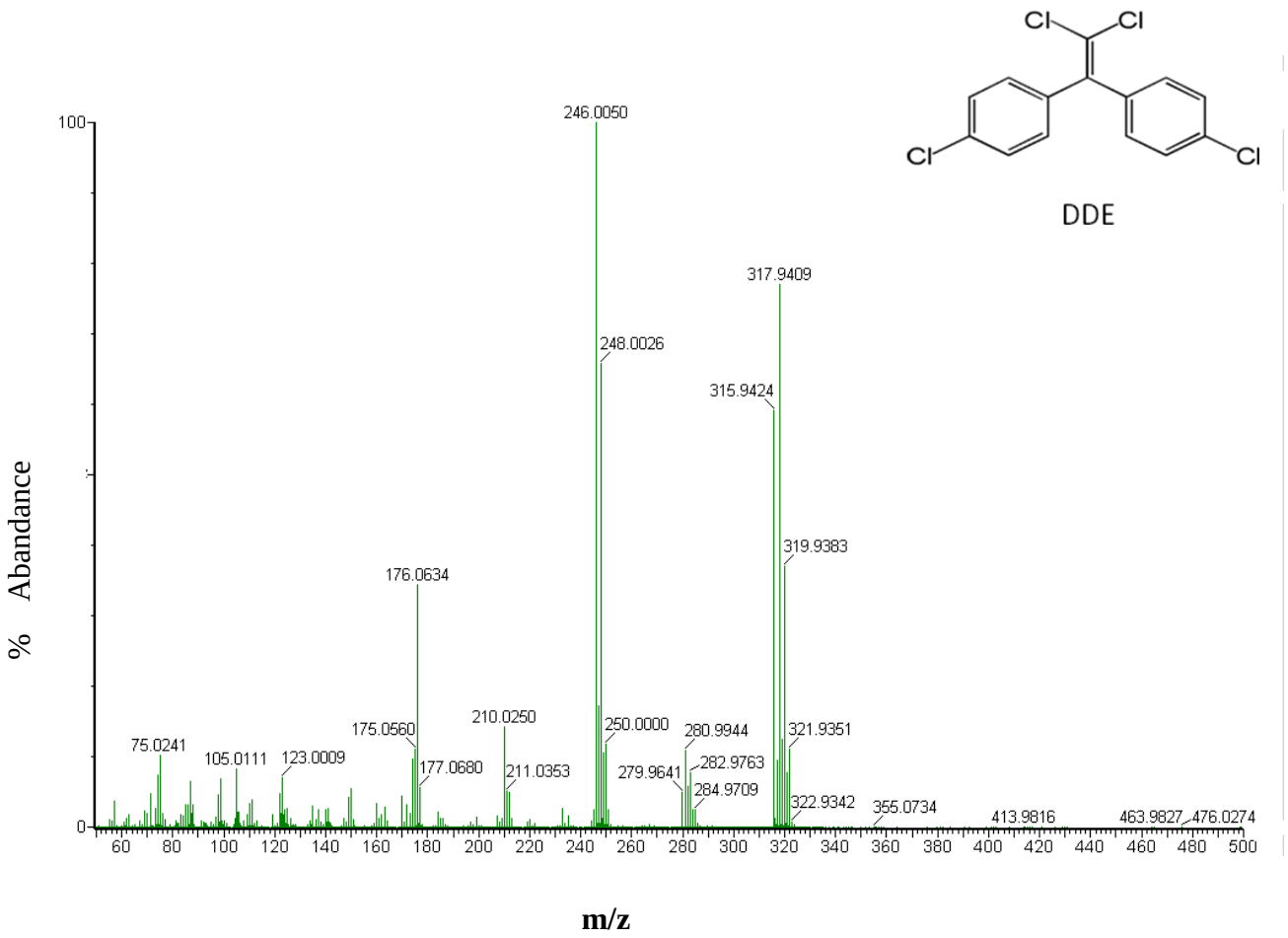
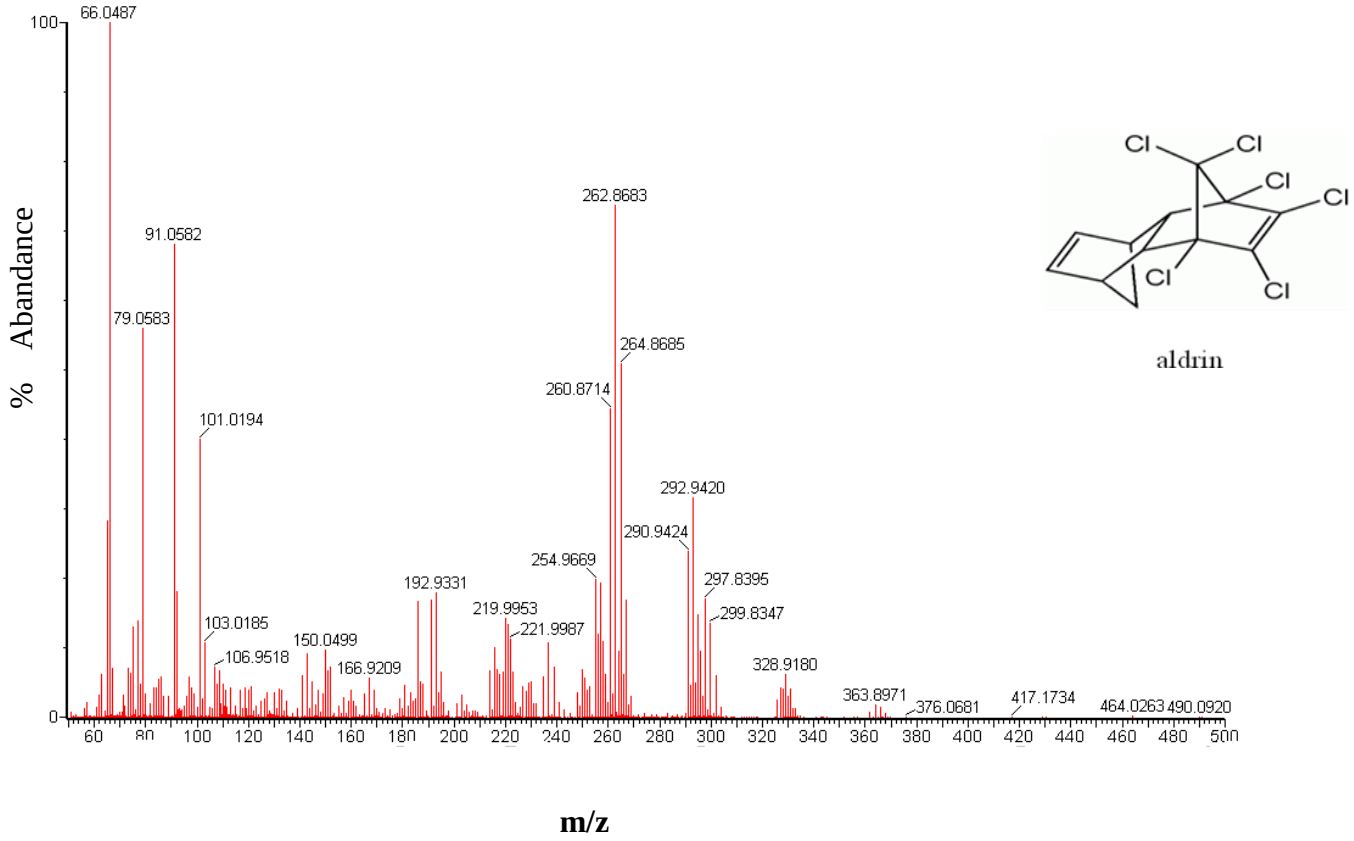


Figure 5.7: EI mass spectrum of p, p′-DDE



5.8: EI mass spectrum of aldrin

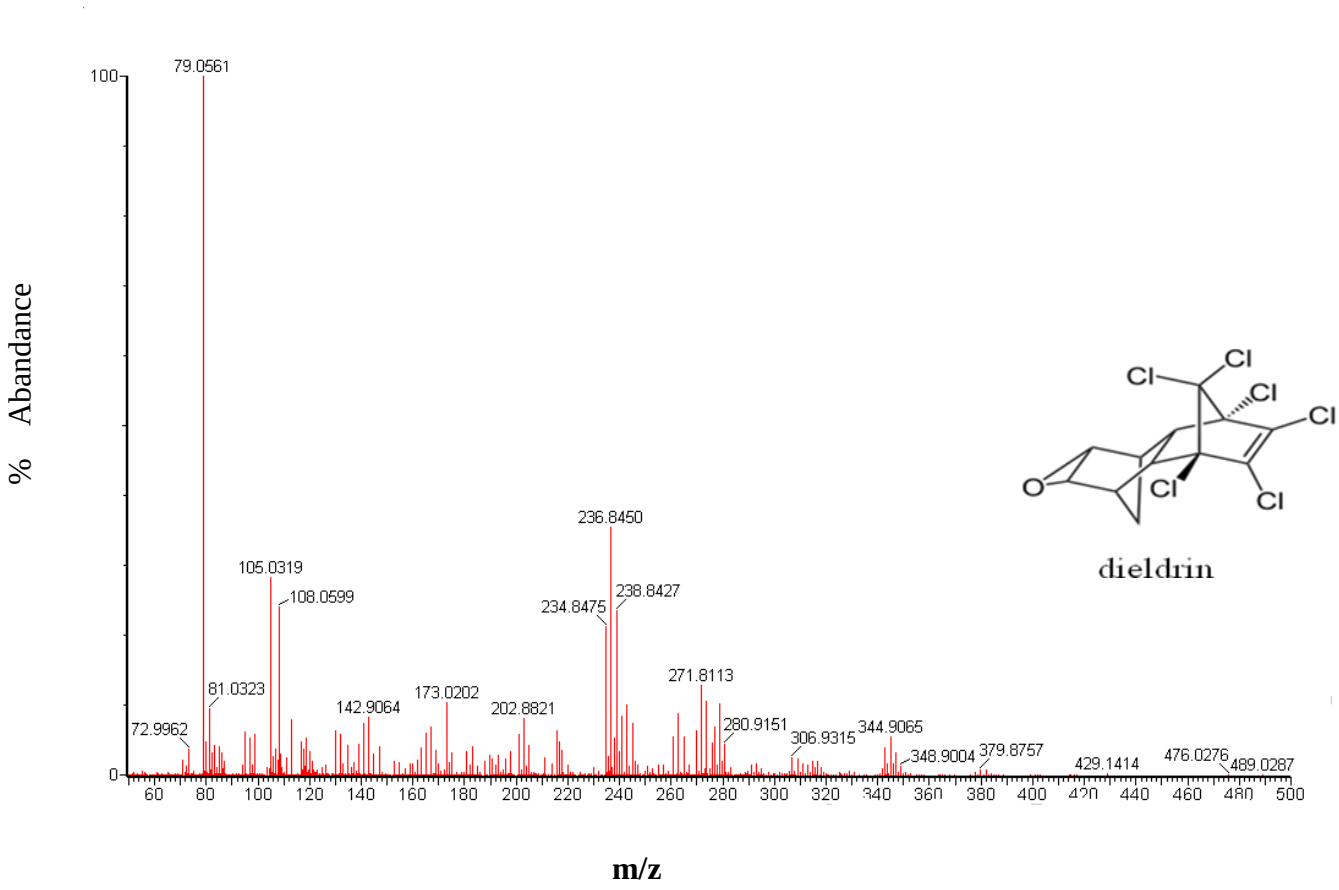


Figure 5.9: EI mass spectrum of dieldrin

Table 5.4 Organochlorine pesticide residues confirmed by GC-MS

Fish Species	Compound	Retention time
T. rendalli	Aldrin	16.04
	p,p′-DDE	17.89
	Dieldrin	18.15
O. andersonni	Aldrin	16.03
	Chlordane epoxide	16.32
	Isodrin	17.66
	p,p′-DDE	17.88
	Dieldrin	18.14
C. ngamensis	Aldrin	16.03
	Isodrin	16.65
	p,p′-DDE	17.88
	Dieldrin	18.15
H. vittatus	Aldrin	16.02
	p,p′-DDE	17.88
	Dieldrin	18.14

5.4 Concentration of the pesticide residues in the fish tissues

Of the four pesticides analysed DDE, aldrin and dieldrin were detected in the samples from both the lower and upper regions of the delta. DDT was below the detection level limit. The level of the pesticide residues in the samples from the upper and the lower regions of the delta were the same. The explanation to this scenario is that the contaminants arise from the same source and one perfect reason is that fish swim all over the shore and get carried by moving water. Fish harvested from the lower part of the delta are likely to have come from the upper part of the delta. There is no control of fish movement in the wild and as such one can never be certain of the fish's original location.

Table 5.5, shows individual pesticide residues and their respective mean concentrations in the analysed samples. Margin of errors are standard deviation based on triplicate determination of each pesticide.

Table 5.5 Mean concentration of the individual pesticide residues in the fish samples (ng/per dry weight)

	Fish Species							
	O. andersonii		*C. ngamensis*		*T. rendalli*		*H. vittatus*	
	A	**B**	**A**	**B**	**A**	**B**	**A**	**B**
Aldrin	0.04 ± (1.78)	0.04 ± (0.69)	0.07 ± (0.50)	0.07 ± (0.28)	0.04 ± (0.96)	0.04 ± (0.69)	0.03 ± (0.54)	ns
Dieldrin	0.07 ± (0.66)	0.07 ± (0.67)	0.08 ± (1.34)	0.08 ± (0.46)	0.07 ± (0.93)	0.07 ± (1.11)	0.05 ± (0.70)	ns
DDE	0.29 ± (1.91)	0.29 ± (2.25)	0.33 ± (0.85)	0.33 ± (0.49)	0.28 ± (1.46)	0.28 ± (0.64)	0.24 ± (0.80)	ns
DDT	nd	nd	nd	nd	nd	nd	nd	ns

A = Upper delta (**Samuchima**) and **B** = Lower delta (**Boro)**, nd = not determined. n = 3. ns = no samples.

The concentrations of the three organochlorine pesticide residues detected in this study fell below the Australian Maximum Residue Limits (MRL) of 50 to 1000 ng/g for fresh water fish (Austrialian MRL, 2009). The Maximum Residue Limits (MRL) is the maximum amount of the pesticide residue which is found in food substances that will not cause any health effect or hazard (Gerken *et al.,* 2001). The concentration of the pesticide residues ranged from 0.03 to 0.33 ng/g. The highest concentration of 0.33 ng/g was DDE measured in *Clarias ngamensis* sampled from both the lower and the upper delta. The lowest concentration of 0.03 ng/g was aldrin recorded in *Hydrocynus vittatus* from the upper region of the delta. The detection of DDE in the samples is an indication of photochemical degradation of p, p′ DDT from past use. The parent DDT was not detected in all the four species most probably due to its breakdown into the stable metabolites DDE and DDD as suggested above. The other reason could be that there is no recent input of DDT in the delta. These results are in agreement with those of Mbongwe *et al*., 2003, who reported DDE metabolite being the most abundant fraction of total DDT recorded in fish of the Okavango Delta. The average concentration of total DDT and its metabolites was 18.76 ng/ g wet weight in fish. The source of the traces of aldrin and dieldrin found in the fish could be from past use of these pesticides in horticulture projects in the fringes of the delta. Figure 5.10 shows the distribution of p,p′ DDE in the fish samples.

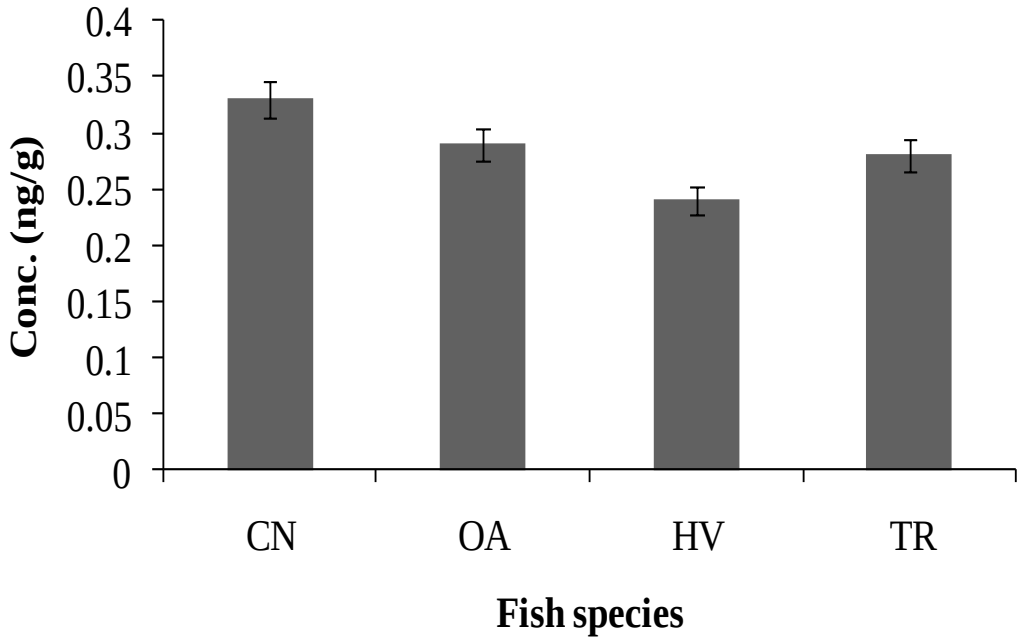


Figure 5.10: Distribution of DDE in the fish samples (CN = *Clarias ngamensis*, OA = *O. andersonni*, HV = *H. vittatus* and TR = *T. rendalli*).

As observed in figure 5.10, the concentration of DDE is high in *Clarias ngamenis* compared to the other three species. The concentrations recorded in the fish species are 0.33. 0.29, 0.28 and 0.24 ng/g for *C. ngamensis, T. rendalli, O. andersonii and H. vittatus* respectively. The explanation to a higher concentration of DDE in *Clarias ngamensis* could be associated with lipid content. The *Clarias ngamensis* species has more lipid compared to the other species (**Table 5-6**).

Table 5.6 Lipid content (% per dry weight) and fish age estimates (years)

Sampling site	Species	% lipid	Age (years)
Upper delta	*H. vittatus*	1.24	4 - 6
	O. andersonni	2.14	6 - 7
	T. rendalli	2.19	4 - 5
	C. ngamensis	4.46	6 - 8
Lower delta	*O. andersonni*	2.22	3 - 7
	T. rendalli	2.13	5 - 8
	C. ngamensis	4.50	5 - 8
Reference site	*C. ngamensis*	3.96	3 - 4

Organochlorine pesticides are soluble in lipid; therefore higher levels of pesticides are expected in fish species with high percentage of fat content. Differences between organs and tissues within an individual have been reported e.g. high concentrations were recorded in abdominal fat (Heath, 1992., Monod and Keck, 1982). Higher concentrations were also reported in whole fish in comparison with fillets because whole fish samples contained more fat than fillet alone (Baumann and Whittle, 1988). Notably, the concentration of DDE in *O. andersonni* and *T. rendalli* were the same, this corresponded with the fat content in these species. The fat content in these two fish species was almost the same (2.14 and 2.19% in *O. andersonni* and *T. rendalli* respectively). This is in order because these fish species belong to the same class; they are both herbivores and have the same feeding habit. **Figure 5.11** shows the distribution of aldrin and dieldrin in the samples.

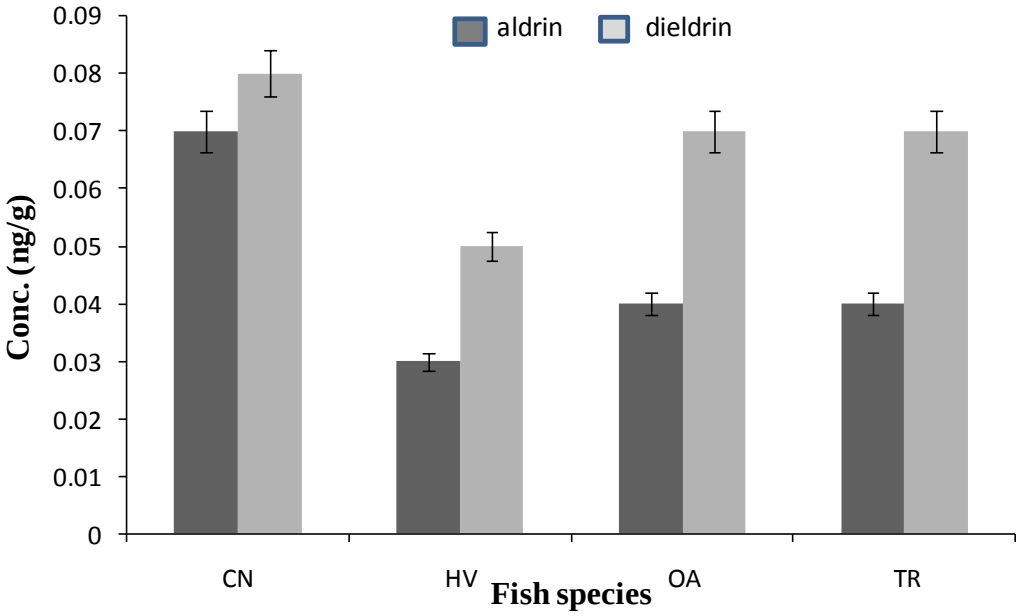
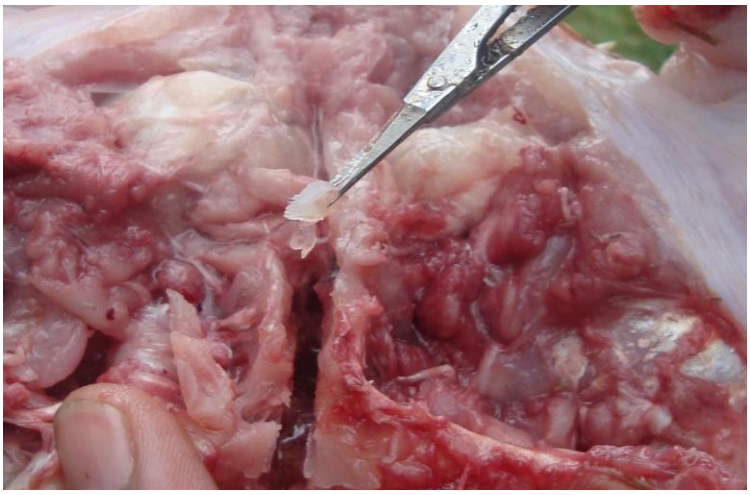


Figure 5.11: Distribution of aldrin and dieldrin in the fish samples. *(*CN = *C. ngamensis*, HV = *H. vittatus*, OA = *O. andersonni* and TR = *T. rendalli)*.

The concentration of dieldrin was slightly higher than that of aldrin in all the species. This could be an indication that there is more dieldrin in the environment compared to aldrin. This trend is supported by the fact that aldrin photolysis to dieldrin in the environment. These results are in agreement with those of the United States Department of Health and Human services (USDHHS), 1993 who reported that aldrin is readily and rapidly converted into dieldrin in plant and animal tissues. This is so because dieldrin is extremely non-polar and therefore has a strong tendency to adsorb tightly to lipids such as animal fat and plant waxes. It is for this reason that dieldrin bioconcentrates and biomagnifies through the terrestrial and aquatic food webs. Matsumura 1985, pointed out dieldrin is one of the most persistent chemicals known. He also reported that dieldrin's bioaccumulation in animal tissue is due to its resistance to degradation and biologic metabolism. Similar to DDT and its metabolites, dieldrin is not easily metabolised in water and has limited capacity of being digested and excreted from the body. It is, however, easily absorbed and transported throughout the blood of vertebrates and hemolymph of invertebrates. The pattern of these two contaminants (aldrin and dieldrin) is the same as that of DDE. In decreasing order the pattern was *C. ngamensis, O. andersonni* and *T. rendalli, H. vittatus*. This pattern is still related to the levels of fat content in the species.

(a)



(b)

Figure 5.12: Photograph of otoliths (the sagittae) of *Hydrocynus vittatus sp.* (a) The otoliths are retrieved from the skull of the fish. (b) The otoliths set in a casting resisn rod ready for sectioning.

Figure 5.13: Photomicrograph of a cross sectioned otolith from an 8 - year old *C. ngamensis.*

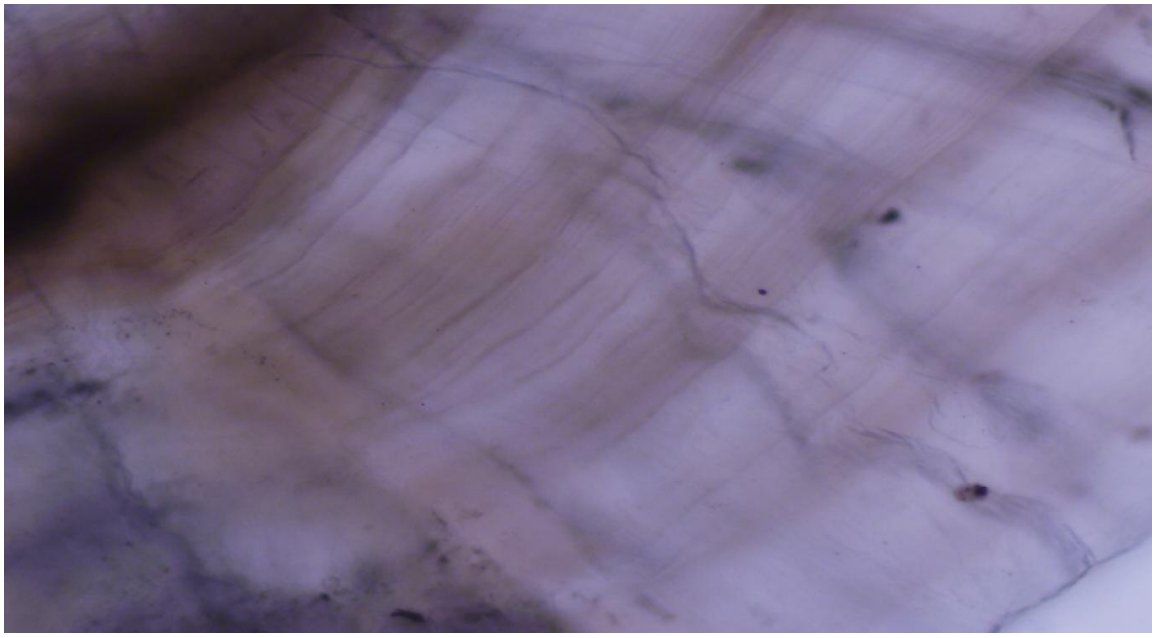


Figure 5.14: Photomicrograph of a cross sectioned otolith from a 4 - year old *O. andersonni.*

The otoliths from all the species yielded interpretable age estimates. The sectioned otoliths for all the species showed clear growth zones, examples are given in **Figures** 5.13 and 5.14 above. Age estimation ranged from 4 to 6 years for *Hydrocynus vittatus*, 3 to 7 years for *Oreochromis andersonni*, 4 to 8 years for *Tilapia rendalli* and 3 to 8 years for *Clarias ngamensis.*

5.5 AChE inhibition studies

Acetylcholinesterase activity is usually high in fish living in unpolluted environments and decreases in polluted areas. Kirby *et al*., 2000 indicated that there was cholinesterase inhibition in flounder tissues in samples from a number of contaminated English estuaries compared to those from a control estuary. Galgani *et al.*, 1992 demonstrated that acetylcholinesterase activity in the muscles of North Sea dab (*Limanda limanda*) varied according to the contamination gradient and was higher in less polluted waters. Bocquene *et al*., 1995 indicated high inhibitory activities of several organophosphate pesticides (OP) and carbamates (CB) compounds on acetylcholinesterase extracts of marine organisms including fish.

Figure 5.15 shows a time graph of acetylcholinesterase activity assayed in the fish brain tissue. The graph starts off exponentially and then levels off as the enzyme becomes limiting (all used up). The slope of the linear portion is used in the calculation of the enzyme activity.

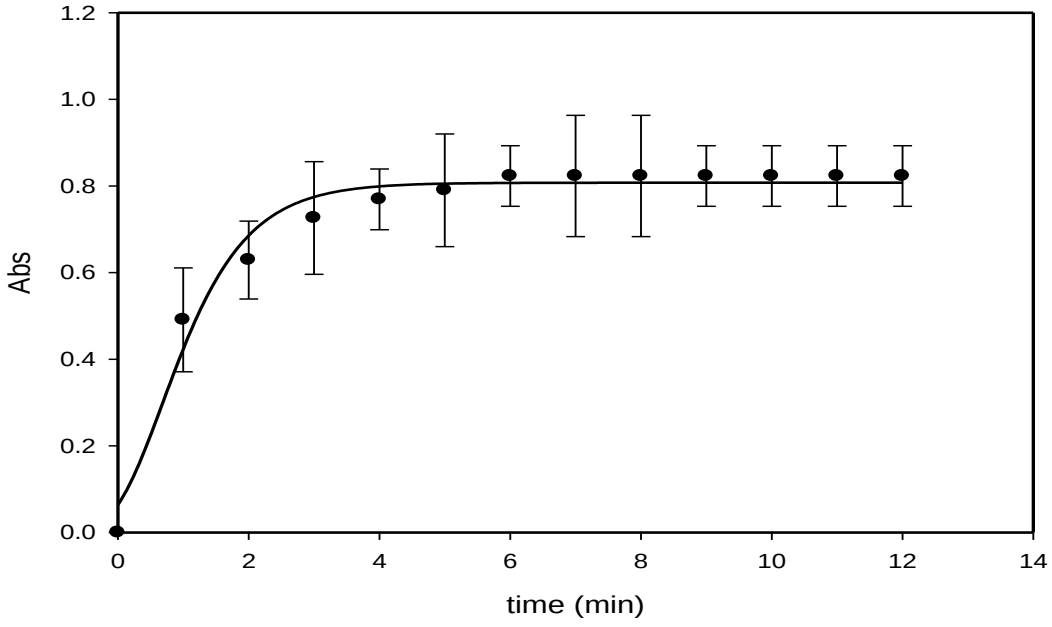


Figure 5.15: Acetylcholinesterase activity in brain tissue of a *Clarias ngamensis* sample from the Okavango Delta.

The acetylcholinesterase activity recorded in *C. ngamensis* brain samples collected from a reference site and from the delta are given in **Table 5.7**. The mean AChE activity of fish samples from both the reference site and that from the delta were 12.31 µmole of ATCI hydrolysed/min/g of brain tissue.

T**able 5.7** Acetylcholinesterase activity (µmol AChE hydrolysed/min/g) in brain tissue of *C. ngamensis* samples from the reference site and the delta.

Sampling Region	AChE activity (µmol/ hydrolysed/min/g
Okavango Delta	12.31 (1.27)
Reference Site	12.31 (1.42)

The enzyme activity was the same in the samples from the two sites suggesting no notable inhibitory effect on the enzyme in the tested species. This means that the level of pesticide residues recorded in the fish is low to induce inhibitory effect in fish. If there was any inhibition, samples from the delta would have given values lower than those from the reference site.

The results would have been more comprehensive if other species (*O. andersonni, T. rendalli and H. vittatus* were assayed for enzyme activity as well. Clearly different species respond to pollutants differently. Unfortunately these species could not be obtained from the reference site. The study should also have taken into account the seasonal influence not only on enzyme activity but also on pollutant accumulation by the organisms. Time allowing, samples should have been collected in winter, summer and in autumn. Contaminants tend to be diluted during the rainy season and become concentrated during the hot summer months. Abdel-Halim *et al*., 2006, reported lower activity of cholinesterase in brain and liver of tilapia fish samples collected from New Damietta drainage canal in winter compared to those collected in spring and autumn. This difference was related to changes in water temperature.

The contribution of age to both enzyme activity and pesticide residue accumulation by the fish was difficult to be pointed in this study. The age of the fish was diverse, ranging from 3 to 8 years. The age range of *Clarias ngamensis* from the delta was 5 – 8 years while the samples from the reference site had an age range of 3 – 4 years. It would have been ideal had

it been possible to obtain fish of the same age and this would bring in a fair platform of comparison.

The QuEChERS method in this study gave good recoveries of the compounds investigated, a clear indication that the method is applicable for the determination of chlorinated pesticides in fish matrices. This is supported by studies by other researchers who applied the method on various other matrices especially fruits and vegetables. Recovery studies on organochlorine pesticides investigated on apples and cucumber by Anastassiades *et al* 2004 ranged between 70 - 110% with relative standard deviation falling between 1 - 15%. The technique has also been proven successful in the extraction of pesticides from a variety of other fruits and vegetables. Two research groups, Schenck *et al* 2004 and Cunha *et al* 2007 investigated pesticide residues in olives and olive oil. Diez *et al* 2006 carried out similar investigation in barley. Though good results were obtained for most pesticide residues investigated in fruits and vegetables there were exceptions with pH-dependent pesticides. In non-acidic matrices such as lettuce, pesticides sensitive to basic media like captan, folpet, dichlorofluanid and chlorothalonil were degraded. This challenge was overcome during the extraction process by addition of 0.1% solution of acetic or formic acid (Lehotay et al., 2005)

The method has been extended to other compounds other than pesticides. Fagerquist *et al* 2005 investigated the dispersive solid-phase extraction technique for the clean-up of beta-lactam antibiotics in bovine kidney tissues and Mcmullen *et al* 2004 determined ivermectin in salmon and antioxidants in pet food using the QuEChERS method.

A few studies have been reported on the application of the QuEChERS method to fish samples. Ramalhosa *et al* 2009 evaluated QuEChERS method for the extraction of 16 PAHs in fish samples. The targeted compounds were successfully extracted from the SRM 2977 with recoveries ranging from 63.5 - 110.0% and coefficients of variation of less than 8%. Recently, Rawn *et al*, 2010 successfully applied the QuEChERS method to determine natural pyrethrins and pyrethroids insecticides in fin and non-fin fish tissues.

Chapter 6

Conclusion and Perspectives

The QuEChERS method, which has been tested extensively in produce, was optimised for the extraction of four organochlorine pesticide residues (OCPs) in fish samples. The optimum extraction conditions were 2 g sample powder, 10 mL acetonitrile and 1 minute shaking time. The mean recoveries obtained from fortified samples of the method at spiking levels of 2, 5 and 10 ng/g were 81 - 94%, 76 - 90% and 76 - 96% respectively. The coefficient of variation of all the pesticides was below 13%. These results demonstrated that the QuEChERS method is applicable for the determination of organochlorine pesticide residues in fish matrices.

The chemical analyses results showed the presence of aldrin, dieldrin and p, p′-DDE in the fish tissue. DDT was below the detection limit. The concentration of the pesticides ranged from 0.03 to 0.33 ng/g with the highest 0.33 ng/g recorded for DDE in *C. ngamensis* species. However, these levels were lower than the US-EPA 0.1 µg/g allowable limit in edible fish and the Australia Maximum Residue Limit (MRL) of 50 – 1000 ng/g in fresh water fish. These results indicated that the fish from this aquatic ecosystem are safe for human consumption. However, a monitoring programme is recommended as the situation may change depending on use of the pesticides in the fringes of the delta.

The acetylcholinesterase activity experiments conducted on *C. ngamensis* brain tissue in samples obtained from the delta and a reference site gave the same result. This shows that the levels of pesticide residues in them are low to induce inhibition of the enzyme. However, for more comprehensive results, enzyme assays needed to be conducted in all the species studied. The influence of seasonal changes to pollutant accumulation and response by biota should have been accounted for. Samples should have been collected seasonally. In addition to species, samples needed to be sorted and grouped according to age and gender.

Future studies in this subject should cater for abiotic factors such as seasonal changes. Samples should be collected in winter and summer. Furthermore, certified reference material (CRM) should be used in recovery experiments. The level of pesticides at high trophic level such as in human breast milk and crocodile blood need to be assessed.

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