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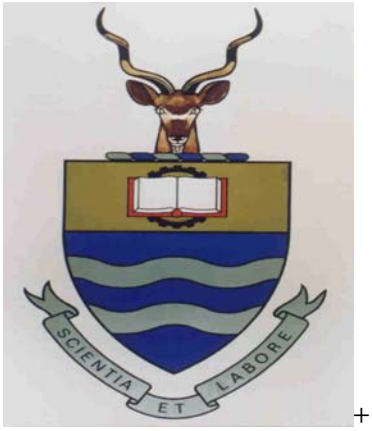

DEVELOPMENT AND APPLICATION OF QUECHERS METHOD FOR EXTRACTION AND ANALYSIS OF POLYCYCLIC AROMATIC HYDROCARBONS (PAHS) IN SOUTH AFRICAN FISH SAMPLES

BY

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A dissertation submitted to the Faculty of Science, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the Degree of Masters of Science

Johannesburg 2014

Declaration

I declare that this thesis is my own unaided work. It is being submitted for the degree of Master of Science in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other university.

_____

(Signature of candidate)

10th day of October, 2014

Supervisor:

Professor Luke Chimuka (WITS, School of Chemistry)

ABSTRACT

Polycyclic Aromatic Hydrocarbons (PAHs) are environmental contaminants which are formed during combustion of organic material such as coal, petroleum compounds and meat. Other sources of PAHs include automobile exhaust, coal tar and crude oil. Natural sources of PAHs include volcanoes, forest fires. Some PAHs are known to be carcinogenic and toxic. They are available to aquatic organisms such as fishes through contaminated sediments and other marine organisms. Due to their toxicity, it is essential to study and monitor PAHs in the environment.

In this study, the QuEChERS extraction method was developed and optimised for the extraction of 5 PAHs (Naphthalene, acenaphthene, phenanthrene, fluoranthene, and pyrene). The optimised parameters were the extraction speed, extraction time, volume of solvent, type of solvent, the mass of salt ($MgSO_4$), the mass of fish used for extraction, mass of sorbent (PSA) and the type of sorbent used. After optimisation, the optimised QuEChERS extraction method was then compared to the Soxhlet extraction method. Firstly by spiking with different concentration and comparing the recoveries. Recoveries obtained where similar, this shows that the QuEChERS method is efficient for extraction of PAHs.

The optimised QuEChERS extraction method and Soxhlet extraction were applied for analysis of PAHs in real fish samples. Fish samples were obtained from two dams, Jericho dam in Mpumalanga and Hartbeespoort dam in Gauteng. The result obtained for both extraction method were compared. The concentration of PAHs obtained for both methods were similar. Naphthalene was not detected in Hartbeespoort dam. The concentration of PAHs in Hartbeespoort and Jericho dam using QuEChERS extraction method range from 0.8 µg/kg to 739 µg/kg and 0.8 µg/kg to 7.4 µg/kg respectively. Acenaphthene and phenanathrene were not detected in all fish sample from Jericho dam, while pyrene was not detected in some of the fish samples from Jericho dam. Application of Soxhlet extraction to real sample gave concentration ranging from 1.9 µg/kg to 908 µg/kg for Hartbeespoort dam and 1.2 µg/kg to 18.7 µg/kg for Jericho dam. High concentration of PAHs was obtained from Hartbeespoort dam.

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Contents

Abstract…….. ii

Acknowledgements……. iii

List of Figures…… vii

List of Tables……. x

List of Abbreviations…… xi

CHAPTER ONE - INTRODUCTION

1.1 General introduction…… 1

CHAPTER TWO -LITERATURE REVIEW

2.1 Characteristics, sources and health effects of PAHs…… 4

2.1.1 Characteristics of PAHs…… 4

2.1.2 Sources and fate……7

2.1.3 Environmental and Health Effect of PAHs……..7

2.1.4 Exposure pathway…… 9

2.1.5 Regulations and limits……..10

2.2 Analytical methods for extraction of PAHs…….. 12

2.2.1 Soxhlet extraction method……12

2.2.2 Microwave assisted extraction method…… 14

2.2.3 Ultrasonic extraction method……... 18

2.2.4 Pressurized fluid extraction (PLE) method…….. 19

2.2.5 Pressurized hot water extraction……...21

2.2.6 Supercritical fluid extraction…… 22

2.2.7 QuEChERS extraction method…….23

2.3 Separation and identification of PAHs…….. 25

2.3.1 High Performance Liquid Chromatography (HPLC) – Fluorescence (FL)……. 25

2.3.2 Gas Chromatography (GC) – Flame Ionization Detector (FID)……28

2.3.3 Gas Chromatography (GC) – Mass Spectroscopy……..... 29

CHAPTER 3 - RESEARCH OBJECTIVES

3.1 General objectives……. 30

3.2 Justification of research …… 30

3.3 Specific objectives……. 30

3.4 Hypothesis and research question……. 31

3.4.1 Hypothesis……. 31

3.4.2 Research Question……. 31

3.4.3 Novelty…….. 31

CHAPTER 4 - RESEARCH METHODOLOGY

4.1 Sampling…… 32

4.2 Chemicals…….. 33

4.3 Instrument…… 34

4.4 Quality assurance…….. 34

4.4.1 Preparation of stock solution…….. 34

4.4.2 Preparation of calibration curve……. 35

4.5 Optimisation of QuEChERS method……. 36

4.5.1 Centrifuge speed……. 36

4.5.2 Centrifuge time…….. 37

4.5.3 Volume of solvent used……. 37

4.5.4 Solvent type…… 37

4.5.5 Mass of fish sample…… 37

4.5.6 Quantity of salt used for extraction…… 38

4.5.7 Quantity of PSA used for extraction…….. 38

4.5.8 Optimisation of sorbent type…….. 38

4.5.8.1 Sorbent used…….. 38

4.5.8.2 Primary Secondary Amine (PSA)……. 38

4.5.8.3 Non imprinted polymers (NIP)……. 38

4.5.8.4 Molecular Imprinted Polymers (MIP)…….. 39

4.5.8.5 Magnetite…….. 39

4.5.8.6 Multi-walled carbon nanotubes…… 39

4.6 Soxhlet extraction method in extraction of PAHs from fish sample. 39

4.7 Application to real sample…… 40

4.7.1 QuEChERS…… 40
4.7.2 Soxhlet……... 40

CHAPTER 5 - RESULT AND DISCUSSION

5.1 QuEChERS experiment…… 41
5.1.1 Optimisation of QuEChERS method…… 41
5.1.2 Centrifuge time…….. 41
5.1.3 Centrifuge speed…… 42
5.1.4 Solvent type…… 43
5.1.5 Mass of fish sample…… 48
5.1.6 Quantity of salt used for extraction…… 49
5.1.7 Quantity of PSA used for extraction…….. 50
5.1.8 Volume of solvent used…….. 52
5.1.9 Type of sorbent used…….. 54

5.2 Comparison of recoveries and reproducibility of QuEChERS and Soxhlet extraction method…… 66

5.3 Application of QuEChERS method and Soxhlet method to fish Sample…… 69

5.3.1 Application of developed methods…….. 69

5.3.2 Application of Soxhlet method to real sample…… 72

5.3.3 Comparison of result obtained from application of QuEChERS and soxhlet method……. 74

5.3.4 Comparison of obtained results with regulating agencies….. 75

CHAPTER 6 - CONCLUSION AND RECOMMENDATION

6.1 Conclusion……. 76
6.2 Recommendation…….. 77
7 REFERENCES…… 78

LIST OF FIGURES

Figure 1 Structures of the sixteen PAHs listed as priority pollutant…….4

Figure 2 A pressurised liquid extraction cell…….……... 20

Figure 3 Comparison of centrifuge time keeping all parameters constant……... 42

Figure 4 Comparison of centrifuge speed keeping all parameters constant……... 43

Figure 5 Influence of solvent type on extraction …… 44

Figure 6 Chromatogram of 1ppm standard solution of PAHs Injected into GC-FID…… 45

Figure 7a Chromatogram of non-spiked fish extract with acetone…..... 45

Figure 7b Chromatogram of non-spiked fish extract with ethylacetate……46

Figure 7c Chromatogram of non-spiked fish extract with acetonitrile……. 46

Figure 8a Chromatogram of spiked extracted with ethylacetate……...47

Figure 8b Chromatogram of spiked fish extracted with acetone……..... 47

Figure 8c Chromatogram of spiked fish extracted with acetonitrile…… 48

Figure 9 Effect of fish mass on extraction……... 49

Figure 10 Varying mass of salt ……..…….. 50

Figure 11.1 Varying mass of PSA…… 51

Figure 11.2a Chromatogram of extract with 50 mg PSA……..... 51

Figure 11.2b Chromatogram of extract with 100 mg PSA……... 52

Figure 11.2c Chromatogram of extract with 150 mg PSA……... 52

Figure 12a Effect of solvent volume on extraction ……..... 53

Figure 12b Result obtained for varying solvent volume…….....53

Figure 13 Chromatogram obtained for 1.0 mg/l standard PAHs solution……..……..... 55

Figure 14 Chromatogram for injection of acetonitrile solvent……. 55

Figure 15a Chromatogram of acetonitrile solvent with PSA……56

Figure 15b Chromatogram of acetonitrile solvent with NIP……..... 56

Figure 15c Chromatogram of acetonitrile solvent with MIP……..... 57

Figure 15d Chromatogram of acetonitrile solvent with magnetite…... 57

Figure 15e Chromatogram of acetonitrile solvent with carbon…….. 58

Figure 16a Chromatogram of blank fish sample with PSA……. 58

Figure 16b Chromatogram of Blank fish sample with NIP……..59

Figure 16c Chromatogram of Blank fish sample with MIP……. 59

Figure16d Chromatogram of blank fish sample with magnetite…….. 60

Figure16e Chromatogram of blank fish sample with carbon……. 60

Figure 17.1a Chromatogram of Spiked fish sample with PSA…….....61

Figure 17.2a Chromatogram of spiked sample with PSA continued……61

Figure 17.1b Chromatogram of spiked sample with NIP……..... 62

Figure 17.2b Chromatogram of spiked sample with NIP 2 continued…… 62

Figure 17.1c Chromatogram of spiked sample with MIP…… 63

Figure 17.2c Chromatogram of spiked sample with MIP continued……... 63

Figure 17.1d Chromatogram of spiked sample with magnetite……... 64

Figure 17.2d Chromatogram of spiked sample with magnetite continued.. 64

Figure 17.1e Chromatogram of spiked sample with multi-walled carbon nano tube……. 65

Figure 17.2e Chromatogram of spiked sample with multi-walled carbon

nano tube continued……65

Figure 18 Comparision of recoveries obtained for different sorbent in QuEChERS extraction method…… 66

Figure 19a Peak area obtained for different spiking concentration for Soxhlet extraction method with GC-FID……67

Figure 19b Recoveries obtained for Soxhlet extraction method with GC-FID….....67

Figure 20a Peak area obtained for different spiking concentration for optimised QuEChERS extraction method with GC-FID……68

Figure 20b Recoveries obtained for QuEChERS extraction method at Optimised conditions with GC-FID……...68

Figure 21 Influence of fish age on bioaccumulation of PAHs…….…….70

Figure 22 Comparison of QuEChERS and Soxhlet results obtained From fish samples from Jericho dam …….……74

Figure 23 Chromatogram of PAHs obtained from QuEChERS extraction method using HPLC-FL……...75

LIST OF TABLES

Table 1 Physical-chemical characteristics of the 16 PAHs…….....6

Table 2 Acceptable PAHs level in soil and water……11

Table 3 Advantages and Disadvantages of MAE……..17

Table 4 Mean concentration of PAHs extracted from soil in (mg/ kg)…….21

Table 5 Examples of QuEChERS extraction method for extraction of PAHs from various samples including fish……. 25

Table 6 Excitation and emission wave length reported for 16 PAHs for HPLC-FL…… 27

Table 7 Concentration of PAHs analysed from different samples using GC-FID……... 29

Table 8 Fish types and sites with length and breadth……..... 33

Table 9 GC-FID calibration curve……..... 35

Table 10 HPLC-FL calibration curve……..... 36

Table 11 Concentration obtained from fish samples from Jericho dam Mpumalanga and Hartebeesport dam Gauteng using QuEChERS method…….. 71

Table 12 Concentration (µg/kg) obtained from fish samples from Jericho dam Mpumalanga and Hartebeesport dam Gauteng using soxhlet extraction method……. 73

LIST OF SYMBOLS AND ABBREVIATIONS

ATSDR	Agency for Toxic Substance and Disease Registry
HPLC-FL	High Performance Liquid Chromatograph-Fluorescence
SPE	Solid Phase Extaction
SFE	Supercritical Fluid Extraction
MIP	Molecular Imprinted Polymers
NIP	Non Imprinted Polymers
WHO	World Health Organisation
QuEChERS	Quick, Easy, Cheap,Effective, Rugged and Safe
NAP	Naphthalene
ACE	Acenaphthene
PHE	Phenanthrene
FLU	Fluoranthene
PYR	Pyrene
POP	Persistant Organic Pollutant
EPA	Environmental Protection Agency
EC	European Commission
SE	Soxhlet Extraction
MAE	Microwave Assisted Extraction
PLE	Pressurised Liquid Extraction
PHWE	Pressurised Hot Water Extraction
SWE	Subcritical Water Extraction

GC-MS	Gas Chromatography-Mass Spectroscopy
GC-FID	Gas Chromatography-Flame Ionization Detector
PSA	Primary Secondary Amine
MSPD	Matrix Solid Phase Dispersion

CHAPTER 1

1.1 INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are neutral, non-polar organic molecules that are made up of two or more aromatic rings arranged in various configuration containing hydrogen and carbon atoms. PAHs may have up to four, five, six or seven member rings. PAHs with only six member rings are known as alternant while those PAHs which have rings other than those with six carbon atoms are known as non-alternant PAHs. Pure PAHs which are manufactured usually exists as colourless, white, pale, yellow or green solid. Most PAHs found naturally occur as a mixture from combustion of organic material at low temperature and high pressure (Douben., 2003). There are more than 100 known (POP) because they can cause health problems when human or animals are exposed to them. Therefore, these 16 PAHs are the most widely studied (ATSDR., 1995). Many PAHs are serious environmental contaminants as they exhibit toxic and hazardous properties because they are carcinogenic and mutagenic (Teranishi et al., 1974). Therefore, US EPA lists sixteen PAHs as “priority pollutants” of the environment (Kubinec et al., 1993).

PAHs in the environment mainly originate from pyrolytic and petrogenic sources (Neff et al., 2005). Pyrolytic sources involve burning of organic substances such as burning of coal to produce creosote and coal tar. When organic substances are heated to a very high temperature they decompose into fragments which combine to form PAHs. Petrogenic sources are from coal and all other forms of fossil fuel and sediments which have been deposited over a long period of time. Other sources of PAHs include natural sources such as volcanoes, forest fires, wax on leaves, cuticles of insects and lipids of microorganisms (Mougin ., 2002)

Coastal and inland waters usually act as receptors for sewage effluents, industrial effluents and urban and rural runoff. As streams, rivers, lakes and ponds are frequently used for portable water supply, contamination of water courses, where waste water which runs off downstream into rivers or streams are been reused without been treated is highly probable. After entering the aquatic

environment, the behaviour and fate of PAHs depend on their physiochemical properties. The solubility of PAHs in water is low and decreases with increasing molecular weight (Manoli et al., 1999). Due to their hydrophobic nature, PAHs in aquatic environment rapidly bind with particles and deposited sediments become their primary reservoirs (Latimer and Zheng., 2003).

In aquatic ecosystems, the hydrophobic PAHs bound preferentially to sediments. As the deposition of sediments continues over time, they act as standard of measurement of contaminant deposition in the environment as well as of general environmental change over time. Contaminated sediments can directly affect bottom dwelling organisms and represent a continuing source for toxic substance in aquatic environments (Kirunthachalm et al., 2005).

In marine environment, PAHs are bioavailable to marine through the food chain, as water borne compounds and from contaminated sediments. As lipophilic compounds they can easily cross lipid membranes and have the potential to bioaccumulate in aquatic organisms (Ramalhosa et al., 2009).

Due to their carcinogenicity, being able to quantify and identify the origin of PAHs in the environment is of great importance to prevent further contamination. To extract PAHs from solid samples, analytical methods that are accurate and reproducible have been developed for the success of this purpose.

Adamo et al., (1996) investigated the bioaccumulation of PAHs in aquatic organisms. Mussels, seabass and shellfish were placed in a tank which contains algae. Equal portions of benzo(a)pyrene and 7,12-dimethyl benzo(a)anthracene were introduced into the tank. Levels of PAHs increased in the mussel and seabass during the period of exposure to a maximum concentration of 30 ng g^{-1}. Benzo(a)anthracene increased more significantly than benzo(a)pyrene. In shellfish, benzo(a)pyrene increased significantly reaching concentration of 50 ng/g. PAHs in muscles of seabass and shell fish was caused by feeding on contaminated mussels.

Matrix Solid Phase Dispersion (MSPD) extraction method was used to extract PAHs from fish muscles analysis was done by high performance chromatography

coupled with fluorescence detector (HPLC-FL). Recoveries obtained were higher than 84% (Pensado et al., 2005). PAHs in soil have been analysed using solid – phase dispersion method (MSPD). The extraction conditions were carefully applied to obtain a highly efficient extraction method with optimal PAH recoveries higher than 94% and relative standard deviations lower than 2% (Pena et al., 2007). Supercritical Fluid Extraction (SFE) and High performance Liquid Chromatography-Florescence (HPLC-FL) have been used to determine the concentration of 10 selected PAHs in barn owls. Recoveries greater than 90% were obtained (Amigos et al., 2002). The Soxhlet extraction method was used to extract PAHs in sediments and fish samples. Analysis was done using GC--MS (Liang et al., 2006). The main disadvantages of these methods are that they are time consuming and use a large amount of solvent. Also some of these methods results in the loss of and degradation of sample during the extraction process (Joa et al., 2009). In order to gather large amount of data in a very short space of time and also to minimize the amount of solvent used, a new method which is known as the quick easy cheap effective rugged and safe method (QuEChERS) has been reported (Kalachova et al., 2011). The QuEChERS method have been used to analyse PAHs in fish with recoveries between 63.5 – 110% (Ramalhosa et al., 2009). The various extraction methods will be discussed more extensively in the literature review.

The research optimized the QuEChERS method for PAHs extraction in fish by varying the following parameters: amount of salt, extraction solvent, centrifuge speed and time. Further, besides using traditional sorbents, multi-walled nanotubes, molecular imprinted polymers (MIP), non-molecular imprinted polymer (NIP) and magnetite were tested as sample clean-up of fish sample. Optimized method were then applied to quantify PAHs in fish samples. The optimized level in fish were compared to international standards such as World Health Organisation (WHO).

CHAPTER 2

2.1 LITERATURE REVIEW

2.1.1 Characteristics of PAHs

Polycyclic aromatic hydrocarbon consists of two or more fused benzene ring as the aromatic fused ring share carbon atoms. Figure 1 shows the structures of the 16 PAHs that are listed as priority environmental pollutant by the EPA (Bojes et al., 2007)

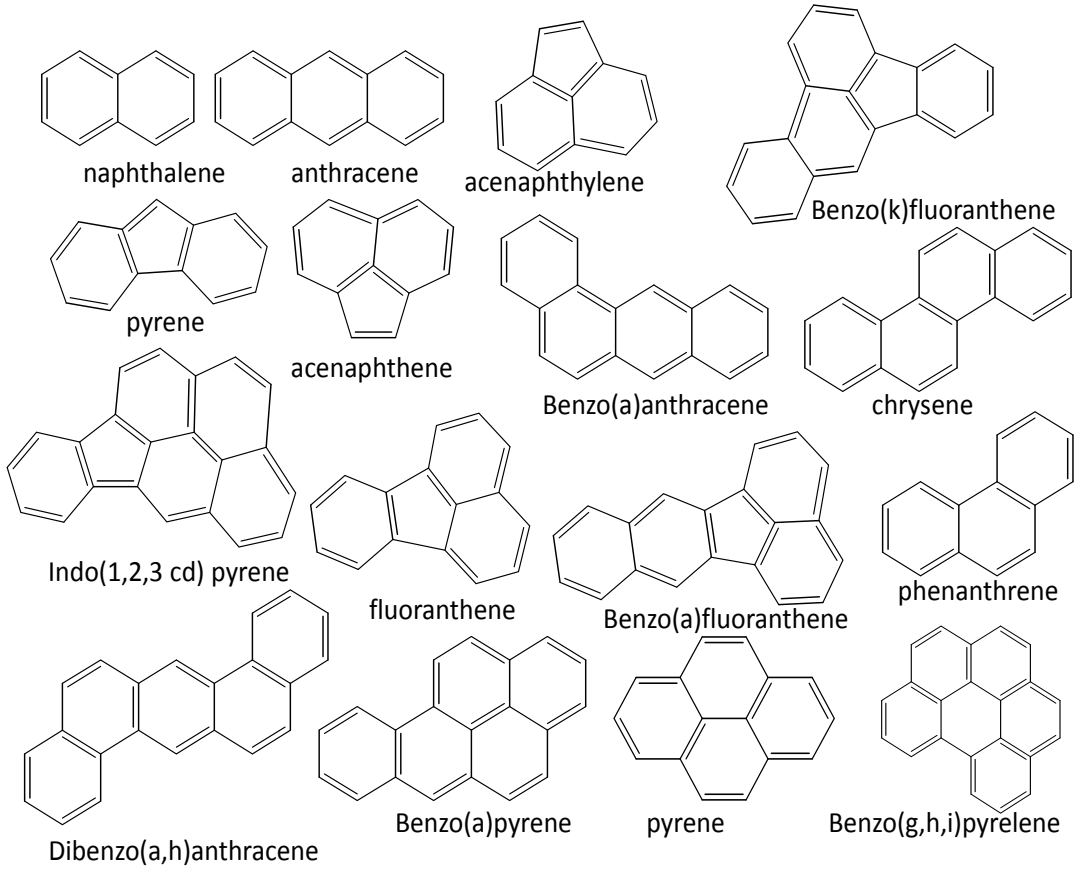

Figure 1: Structures of the sixteen PAHs listed as priority pollutant

PAHs containing six aromatic rings are called small PAHs while those with more than six fused rings are called large PAHs. Large PAHs are found in smaller quantities than the small PAHs due to the kinetic limitation of their production through addition of successive rings and also, many more isomers are possible for larger PAHs so the occurrence of specific structure is smaller. Therefore due to availability of small PAHs, majority of research on PAHs has

been those of up to six member rings. The physical and chemical characteristics of PAHs vary with molecular weight. An increase in molecular weight of PAHs increases the resistance of PAHs to oxidation, reduction, vaporization and solubility of the compound decreases. PAHs possess characteristic UV absorbance spectra with many absorbance bands that are unique for each ring. This is very useful in identification of PAHs. They also fluorescent, when they are excited they emit light of certain frequency. The presence and quantities of these 16 PAHs are often measured in environmental sample because of their carcinogenic and hazardous properties. Table 1 shows the physiochemical characteristics of the 16 PAHs listed as priority pollutant.

Table 1: Physical-chemical characteristics of the 16 PAHs listed as priority PAHs Bojes et al., 2007, Nikolaou et al., 2009.

PAHs	Molecular weight ($gmol^{-1}$)	Boiling point (^oC)	Melting point (^oC)	Log K_{ow}	Vapour pressure at 25^oC Pa x 10^{-5}	No of rings
Naphthalene	128.2	218	80.5	3.37	1040000	2
Acenaphthene	154.2	278	96.2	3.92	30000	3
Acenaphtylene	152.2	265	92	4.00	90000	3
Fluorene	166.2	295	111	4.18	9000	4
Phenanthrene	178.2	339	101	4.57	2000	3
Anthracene	178.2	340	216	4.54	100	3
Fluoranthene	202.3	375	116	5.22	1200	4
Pyrene	202.3	360	156	5.18	60	4
Benz[a]anthracene	228.3	435	160	5.91	2.8	4
Chrysene	228.3	448	255	1.65	0.057	4
Benzo[b]fluoranthene	252.3	481	168	5.80	NA	5
Benzo[k]fluoranthene	252.3	481	217	6.00	0.0052	5
Benzo[a]pyrene	252.3	495	175	6.04	.07	5
Benzo[ghi]perylene	276.3	n/a	277	6.50	0.006	6
Indeno[1,2,3-cd]-pyrene	276.3	536	163	6.58	1.20	6
Dibenz[a,h]-anthracene	278.4	524	267	6.75	0.000037	5

2.1.2 Sources and Fate

PAHs were one of the first atmospheric pollutants to be identified as being carcinogenic (Baek et al., 1991). They are part of the group of organic compounds which have received major attention because of their documented carcinogenicity. These PAHs are ubiquitous in the urban atmosphere and therefore undergo considerable scrutiny (Baek et al., 1991). PAHs belong to the group of persistent organic pollutant (POPs). They are resistant to degradation; they can remain in the environment for a long period and have the potential to cause adverse environmental effects. Some of these PAHs are capable of being dispersed on a global scale (Maliszewska., 1999).

PAHs originate from different emission sources. These emission sources are either natural or anthropogenic. Anthropogenic sources are caused by humans. They can be by atmospheric discharge which are of either stationary emission origin or non-stationary emission origin. Stationary emission origin are from sources such as petroleum refineries, coal gasification, industrial incinerators, agricultural and refuse burning, generation of heat and electricity. PAHs are formed during incomplete combustion of organic matter such as coal, oil, gases, and other organic substances like tobacco and charbroiled meat and through burning of wood. Non stationary emission sources are from automobile and vehicles. In general, most PAHs are formed when materials burn at low or high temperatures **(**Prabhukumar et al., 2010). PAHs can be introduced to water through discharged from industrial and waste water treatment plant. Water contamination can also occur from oil spillage and leach from soil to surface waters. Other sources of PAHs include natural sources such as volcanoes and forest fires (ATSDR, 1996).

2.1.3 Environmental and Health Effect of PAHs

In 1995, PAHs were included to the hazardous substance. List produced by the Agency for Toxic Substances and Disease Registry (ATSDR) and the Environmental Protection Agency (EPA). US EPA has identified 16 unsubstituted PAHs as priority pollutants hence their distribution in the

environment as potential risks to human health have been the focus of much attention (Manoli and Samara., 1999).

The effects of exposure to PAHs are determined by many factors. These factors include dose, exposure duration, route or pathway, and state of health. PAHs are carcinogenic in both animals and human. The proof that PAHs are carcinogenic in humans can be seen from occupational workers who are exposed to PAHs in processess such as coal production or gasification of coal and oil refinery (ATSDR., 1995). Several PAHs like benz[a]anthracene, indeno[a]pyrene, chrysene, dibenz[a,h]anthracene, and indeno[1,2,3-c,d]pyrene, have caused tumours in laboratory animals when they breathe, ate or have prolonged skin contacts with them (WHO., 2000). Cancer caused by exposure to PAHs occurs mainly in the lungs and skin of people that are exposed to them through breathing or skin contact for a long period (ATSDR., 1995). When a substance containing PAHs particle is swallowed, it can cause the removal of the mucous from the lungs (ATSDR., 1995). Tumour regions have been identified which is related to route of administration. Tumours such as lung tumour have been observed after inhalation, stomach tumour after ingestion and skin tumour after skin contact (ATSDR., 1995).

Mice fed with high level of Benz[a]pyrene during pregnancy had difficulty reproducing and so did their offspring (ATSDR., 1995). The offspring of pregnant mice fed with benzo[a]pyrene also showed other harmful effects, such as birth defects and decreased body weight (ATSDR., 1995). This effect could also occur in humans. Studies have shown that PAHs can cause harmful effects on skin, body fluids and proliferation of tissues such as bone marrow, lymphoid organs, intestinal epithelium and gonads (ATSDR., 1995). They can also cause harmful effect on body's immunity after both short and long term exposure, although these effects have not been reported in people (ATSDR., 1996).

2.1.4 Exposure pathways of PAHs to Humans

Inhalation

PAHs can enter the body through the lungs by breathing contaminated air. Humans are most likely to be exposed to PAHs vapours or PAHs attached to dust and other particles in the air. In homes, PAHs are present in smoke from wood fires and tobacco smoke. Main stream smoke and side stream smoke from tobacco consists of PAHs such as dibenzo(a,g)anthracene, anthracene, benzo(a)pyrene, benzo(a)fluorene and chrysene (ATSDR., 1995). The main stream smoke concentration range from 11 ng to 199 ng cigarette^{-1} and side streams smoke from tobacco has PAHs concentration ranging from 39 ng kg^{-1} to 1224 ng cigarette^{-1} (Nelson., 2001)**.**

Ingestion

Drinking water and swallowing food, soil or dust particles are also routes for these chemicals to enter the body. PAHs contamination of food can be due to the food processing techniques. PAHs may be present in cereals, grains and flour due to the drying technique used for preservation such as heating and smoking. Contamination in unprocessed food like vegetables and fruits is primarily through the atmosphere and soil. Meat processed or pickle foods, and contaminated cow's milk or human breast milk maybe another exposure route for humans. Food grown in contaminated soil or exposed to contaminated air may also contain PAHs. Cooking meat or other food at high temperature may also be a source of PAHs.

Skin contact

PAHs can also enter the body if the skin comes into contact with soil or any substance that contains high levels of PAHs.

Humans may be exposed to PAHs in soil near areas where coal, wood, gasoline, or other products have been burned, or near hazardous waste site such as former gas factory site and wood-preserving facilities such creosote-treated wood products. Coal tar creosote is a by-product of coal tar formed from carbonisation of bitumen during the production of coke or natural gas. Coal tar creosote

consists of about 85% of PAHs such as anthracene, naphthalene and phenanthrene and about 2% to 17% phenolic compounds. Creosote has been used to preserve wood for wood homes and rail road tiles (Sullivian et al., 1993).

2.1.5 Regulation and limitations

Maximum limit have been set for those PAHs listed as carcinogenic, toxic and priority pollutant (ATSDR., 1995). The maximum level of Benzo(a)pyrene specified by European commission (EC) in smoked fish and smoked fish products is 5 µg kg^{-1} wet weight, while maximum levels set for unsmoked fish muscle is 2 µg kg^{-1} wet weight. The maximum limit set for Benzo(a)anthracene, Benzo(b)fluoranthene, chrysene is 30 µg kg^{-1} (EC., 2011). In Czech Republic, the maximum level of Benzo(a)anthracene, Benzo(b)fluoranthene, Benzo(k)fluoranthene, Dibenzo(a,h)anthrancene, Dibenzo(a,h)pyrene, Indeno(1,2,3-cd)pyrene, Dibenzo(a,i)pyrene in fish is 1,0 µg kg^{-1} this limits were set in some European union states before the European commission in 2005 (Wenzel et al., 2006). The Table 2 below also shows some acceptable limits of PAHs in soil and water samples in Spain according to European Union regulations.

Table 2: Acceptable PAHs level in soil and water, (ATSDR.2006 and US-EPA)

	ATSDR		US-EPA
PAH	**Soil (mg kg^{-1})**	**Water (mg ℓ^{-1})**	**Water (mg ℓ^{-1})**
Pyrene	3.0	3.0	
Napthalene	1.0	3.0	
Phenanthrene	3.0	3.0	
Benzo[hgi]perylene	3.0	3.0	
Benzo(a) pyrene	0.3	0.005	0.002
Anthracene	3.0	3.0	
Fluoranthene	3.0	3.0	
Acenaphthene	3.0	3.0	
Acenaphthylene	3.0	3.0	
Benzo (a) anthracene	0.15	0.005	0.001
Benzo (b) fluoranthene	0.3	0.005	0.002
Dibenzo (a) anthracene	0.3	0.005	0.004
Fluorene	3.0	3.0	
Indeno[1,2,3-ghi]pyrene	0.3	0.005	
Indene	-	0.3	
Chrysene			0.002
Benzo(k)fluoranthene			0.002
Dibenz (a,h) anthracene			0.003
Indenol (1,2,3-c,d)pyrene			0.004

2.2 ANALYTICAL METHODS FOR EXTRACTION OF PAHS

2.2.1 Soxhlet extraction(SE) method

The Soxhlet extraction method has been immensely used and it is a standard technique in the extraction of PAHs with nearly 100% recovery (Chemo., 2003). The Soxhlet extraction method is used as a benchmark for several extraction methods (Wang et al., 2006). The procedure involves a solid sample being placed in porous container known as the thimble which is loaded into the main chamber of the Soxhlet extractor. The extractor is placed onto a flask containing the solvent. The solvent is heated to reflux. When the solvent boils, the vapour goes into the condenser where it then condenses and drips back into the thimble. As the solvent floods the thimble, it dissolves the desired compound and drips back into the flask.

Vives et al. (2004) studied PAHs in fish liver in Europe England using Soxhlet extraction method. Livers of the fish were removed and stored until analysis. For extraction, the liver was mixed with activated salt and ground. The mixture was spiked with deuterated PAHs and then Soxhlet extraction was done for 20 hours using hexane and dichloromethane (4:1, v/v). Clean up was done using aluminium oxide chromatographic column with hexane and dichloromethane (1:2, v/v). Recoveries for spiked PAHs were 39% to 117%.

PAHs in soil sample were extracted using the Soxhlet extraction method by Noorsahikin et al. (2009). The soil sample was spiked with 20 ppm for all PAHs. Concentration of PAHs extracted by Soxhlet extraction was compared with that obtained from pressurized liquid extraction of PAHs in soil sample. For HPLC analysis, 2 µl was used. PAHs recovery ranged from 34.3% to 90%. Naphthalene was having the lowest percentage recovery for Soxhlet extraction while percentage recovery for naphthalene with pressurised liquid extraction was 81%.

Chen et al. (1996) extracted 16 PAHs which were classified as organic pollutant in meat sample using Soxhlet extraction method and analysis was done by liquid chromatography. Concentration of PAHs obtained ranged from 2.7 ppb to

55.2 ppb and some PAHs such as acenaphthyne, acenaphthene, fluoranthene and pyrene were not detected.

According to Smith et al. (2006), the Soxhlet method showed relatively effectiveness at extracting heavier molecular PAHs than the lighter molecular. Studies also showed that the amount of PAHs extracted does not depend entirely on the solvent used. The same authors studied the efficiency of Soxhlet extraction using different solvents; dichloromethane, hexane and hexane : acetone (4/1 : v/v). There was an observed general increasing trend in the extracted PAHs in the order DCM > hexane > hexane : acetone (4:1) although not significant. This showed that other factors such as sample preparation and length of time of soxhlet extraction procedure were also contributing factor and these factors might be sufficient enough to overcome solvent polarity. Despite its continued usage, Soxhlet method has some advantages and disadvantages which are as follows (Luque de Castro et al., 1998).

Advantages of Soxhlet extraction method;

- The sample is repeatedly brought in contact with the fresh portion of solvent.
- One batch of solvent can be used and recycled.
- The temperature of the system remains high.
- The basic equipment used is inexpensive.
- Simple methodology is involved which needs little specialized training
- More sample mass can be extracted than most conventional method

Disadvantages of Soxhlet extraction method;

- It is time consuming as reflux has to be done for about 6 to 24 hour (Turlough., 1999).
- Large volume of solvent is used about 150 ml of solvent is used for 10 g of soil sample (Lau et al., 2010). This large volume of solvent is expensive to dispose and can cause environmental problems if not disposed off properly (Luque de Castro et al., 1998).

- Soxhlet extraction method is restricted to solvent selectivity and cannot easily be automated.

In the last decade, there has been an increasing demand for new extraction techniques that are amendable to automation with less extraction time and less solvent consumption (Collin et al., 1996). This prevents pollution in the environment and analytical laboratories and reduce sample preparation cost (Wan et al., 1996). The automated Soxhlet extraction method was improved such that less solvent is used and total extraction time is reduced (Lau et al., 2010). The extraction process commences with the sample in its thimble suspended in boiling solvent. Then at the appropriate time, the thimble is raised and washed with condensed solvent. Collection of sample solvent is done by distillation in the extractor. The total time for extraction is reduced by a factor of 4-10 (Collin et al., 1996). However, traditional Soxhlet extraction method is still being used to date in many routine laboratories (Eskilsson et al., 2000).

2.2.2 Microwave assisted extraction (MAE) method

Microwaves are electromagnetic waves with oscillating magnetic and electric field perpendicular to one another (Mandal et al., 2007). They have frequency range of between 300 MHz to 300 GHz. Microwaves are used for two main purposes, in telecommunication and as a source of energy to generate heat (Letellier et al., 1999). The microwave-assisted extraction (MAE) is the process of using microwave energy to heat solvents in contact with a sample so that analyte can be separated from the sample into the solvent (Eskilsson et al., 2000). This method allows organic or organometallic compounds to be extracted faster and more selectively with recoveries similar or better than convectional extraction methods (Pare et al., 1994). The heating mechanism is based on interaction of the electric field with the molecules of the material, and this is generated in two ways: ionic conduction and dipole rotation. In ionic conduction, heat is generated as the medium opposes the flow of ions. The flow of ions causes collision between molecules and this also generates heat (Letellier et al., 1999).

The microwave assisted method can occur in an opened system or closed system. In the closed system which is also known as focused microwave process, heat

energy is conserved unlike in the conventional method where heat is transferred mainly by conduction and convection. This causes a large amount of energy to be lost to the environment (Letellier et al., 1999). The ability of a substance to absorb microwave energy depends on factors like amount of energy radiated, the temperature of the substance and its dielectric constant. The dielectric constant relates to the ability of the material to store electrical energy. The higher the dielectric constant of a material, the higher the microwave energy that will be absorbed by the material. Selective heating can be done on a system with different chemical characteristic and different dielectric properties which is placed in a uniform environment (Pare et al., 1994). When a substance absorbs microwave energy, it must be able to transform it and pass it on as heat energy. The ability of a solvent to change microwave energy to heat energy can be measured by the loss tangent (*d*) which is the ratio of the dielectric loss ($\mathcal{E}''$) to the dielectric constant ($\mathcal{E}'$) (Stuchly et al., 1983).

$$\text{Tan } \delta = \frac{\varepsilon''}{\varepsilon'} \qquad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (1)$$

Comparison of different solvents shows that polar solvents such as acids are able to absorb microwave energy more than non-polar solvents such as hexane. So such polar solvent get easily heated up when exposed to microwave energy. However, if we compare solvents such as methanol and water, when exposed to microwave energy, water will more easily get heated up than methanol because water is more polar. In terms of heat loss, methanol has the higher ability of converting microwave energy to heat energy.

In order to solve the problems of long extraction time and the use of large amount of solvent, the microwave extraction technique has been used for extraction of polycyclic aromatic hydrocarbons in several samples such as sediments, soil and atmospheric particles (Letellier et al., 1999). The process of extraction of solute from solid matrix occurs through series of steps. During this process, the solvent must penetrate the solid and break down the components of the solid matrix. The solute is transported out of the matrix to the solution. Then, the extract and solid are separated and discharged.

Pena et al., (2006) developed and evaluated the microwave extraction procedure for the extraction of 6 PAHs in fish sample. In this study the influence of temperature, extraction time and solvent volume were optimised. Results showed that temperature, extraction time and the volume of solvents all have an effect on the yield of PAHs.

Optimisation of the focused microwave extraction was done using a standard reference material as a model. The optimised parameters were used in the extraction of PAHs from marine sediments. The results obtained from the standard reference material were compared to those obtained using Soxhlet extraction method. The percentage of PAHs obtained after extraction for both method were similar (71% to 97%). This showed that there is not much difference in the efficiency of both methods to extract PAHs from marine sediments and the focused microwave extraction can be an alternative extraction method to Soxhlet (letellier et al., 1997).

Although microwave extraction procedure has its shortcomings, it has been used effectively in the analytical industry because of the advantages it has over conventional extraction method. The advantages and disadvantages of microwave assisted extraction are summarized in the Table 3 below.

Table 3: Advantages and Disadvantages of MAE

Microwave assisted extraction			
Open vessel system		Closed vessel system	
advantages	disadvantages	advantages	Disadvantages
No risk of explosion	Compared to closed vessel, the time needed for extraction is longer	Less time is used for microwave exposure due to higher temperature	Can cause explosion due to very high pressure
Extra solvent can be added	Ability to analysed less amount of sample compared to closed vessel	No solvent loss	
Any material can be used for container		Small amount of solvent is needed	reagent cannot be increased during procedure
Surplus reagent can readily be dispatched		Exposure to dangerous fumes is avoided	After operation container must be allowed to cool
There is no need for instrument to cool after operation		Many sample to be analysed at the same time.	
Many samples can be analysed at the same time			

2.2.3 Ultrasonic extraction method

Ultrasonic extraction method is also known as ultrasonic assisted extraction method. Ultrasounds are high intensity sound wave of over 20 kHz frequency. Sound wave propagates through a medium by compression and rarefaction. As they propagate through liquid medium, the rarefaction movement causes bubbles to develop in the liquid medium, the bubbles created expand and then collapse vigorously due to the high pressure caused by compression to generate high temperature of about 5000 K and pressure of 1000 atm (Junior et al., 2006). This process is known as cavitation (Junior et al., 2006). Cavitation causes ejection of solvent at high velocity of 100 ms^{-1}. The movement of solvent at a very high speed causes breakdown of particles to smaller sizes, allowing for interaction between extraction solvent and analyte in the sample (Junior et al., 2006). Ultrasonic assisted method is been used in the laboratory for sample preparation and handling (Junior et al., 2006).

The method helps to overcome the problems of conventional soxhlet method of time consumption, large volume of solvent, loss of analyte and sample degradation (Junior et al., 2006). The ultrasonic extraction method need expertise for its effective operation so as to reproduce data (Dean., 2000). In the use of the ultrasonic apparatus important parameters need to be considered as this can affect the effectiveness of extraction of analyte from sample. These parameters are solvent, frequency and intensity of ultrasound, operating temperature and pressure (Capelo et al., 2005).

Ultrasonic assisted extraction method, involves the use of an ultrasonic bath or an ultrasonic probe. There are some problems which can be experienced while using an ultrasonic bath such as the energy that is transported to container is dependent on properties of the bath and most bath do not function at the same frequency and amplitude therefore affecting the reproducibility of extraction procedures. These challenges can be reduced by using a probe. The frequency and amplitude of the probe can be controlled, less time for extraction and energy is released directly into the container (Junior et al., 2006).

Ultrasonic extraction procedure involves placing the sample in a glass beaker, adding sufficient solvent, placing a probe into the beaker and controlling the probe to give the frequency and amplitude that is needed for extraction. Sonication is done for a set time, after sonication, upper part of solvent is collected, filtered and concentrated to less than 10 ml. Then analysis of extract is done (Sun et al., 2006). As discussed earlier, the ultrasonic extraction procedure has its advantages and disadvantages but it is still evolving as a method for sample preparation (Capelo et al., 2005).

2.2.4 Pressurised fluid extraction (PLE) method

Pressurised fluid extraction also known as accelerated solvent extraction or pressurised liquid extraction method has been used for removing analyte from solid since 1955 (Bjorklund et al., 2000). The method requires the use of organic solvent for the determination of PAHs in soil sample (Dean., 2000). It operates with elevated temperature and pressure. The high temperature causes the solvent to reduce its resistance to force thereby increasing the rate of diffusion of the analyte from the sample into the solvent. The pressure increase allows the solvent to access the sample within areas that would not have been attainable under standard conditions (Camel., 2001). During the process of extraction, to ensure that all analytes have been extracted, new solvent is poured into the extraction chamber this produces uneven distribution of solute in the solution thereby increasing further the rate of diffusion (Richter et al., 2006). The process of pressurised liquid extraction can be summarised as follows, sample is placed in the extraction cell and placed vertically upright after which the extraction solvent is introduced into the cell through a solvent tube. The cell is heated to a temperature of between 150 ^{o}C to 200 ^{o}C and pressurised to a set value for about 5 – 10 mins. Solvent is collected in a sample vial and new solvent is poured into the cell to remove any analyte that is left. Lastly, nitrogen gas is used to purge the cell to remove any solvent that is left behind (Bjorklund et al., 2000).

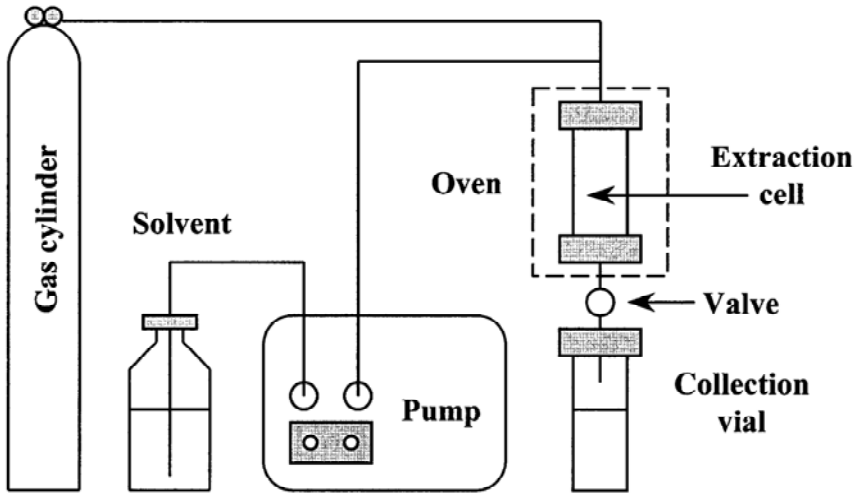

Figure 2: A pressurised liquid extraction cell (Bjorklund et al., 2000)

Figure 2 shows a pressurised liquid extraction cell. Hawthorne (2000) used pressurised liquid extraction process to extract PAHs from solid matrices and compared the extraction efficiency with other method such as SFE, and soxhlet extraction method. Table 3 shows concentration of PAHs obtained for the three extraction method used. He discovered that although the PLE has an advantage of been able to analyse many samples at the same time, time for extraction duration is short and no additional clean-up is necessary.

Table 4: Mean concentration (mg/ kg) of PAHs extracted from soil in using different method (Hawthorne et al., 2000)

PAHs	SOXHLET (6 replicate)	PLE (4 replicates)	SFE (5 replicates)
Naphthalene	ND	53	59
Acenaphthene	58	65	64
Fluorene	134	149	149
Phenanthrene	429	489	502
Anthracene	86	99	101
Fluoranthene	156	166	165
Pyrene	205	239	229
Benz[a]anthracene	80	94	81
Chrysene	89	106	89
Benzo[a]pyrene	58	61	25
Benzo[ghi]perylene	31	31	7
Indeno[1,2,3-cd]-pyrene	20	21	6
Dibenz[a,h]-anthracene	5	5	2

ND – Not determined because of co-eluting interference

2.2.5 Pressurised hot water extraction (PHWE) method

There is a growing need to use less organic solvent in analysis as they are known as pollutant of the environment. This has led to the development of pressurised hot water extraction (Hawthorne et al., 1994). Water is a polar solvent and will definitely dissolve polar molecule. When water is heated to a very high temperature 250 ^{o}C and pressure of 50 bar, it losses it polarity and can dissolve nonpolar substances (Yang et al., 1998). Pressurised hot water extraction is also known as subcritical water extraction (SWE). Water is heated to a very high temperature well above its boiling point at high pressure. These causes the intermolecular force to weaken thereby water becomes less polar because of reduction in permittivity and organic substances will dissolve in it (Lau et al.,

2010). The ability of water to extract non polar compound from sample has been investigated on extraction of PAHs (Yang et al., 1998). It was observed that water is able to extract PAHs at temperature above 200 ^{o}C. By raising the temperature of water, other physical characteristics which affect the efficiency of water has a solute will be controlled such as the resistance of water to flow will reduce allowing organic solute to diffuse faster in water (Yang et al., 1998). Miller et al. (1998) determined the efficiency of water to dissolve organic compounds with change in temperature. The study reported that as temperature of water increases, the solubility of organic compound increases and that higher pressure actually has a negative effect on solubility (Miller et al., 1998).

2.2.6 Super critical fluid extraction (SFE)

Super critical fluid extraction shows a succession of liquid and gas phase in which both part are not distinguishable from one another. The succession of liquid and gas phase exhibit properties which allows them to be able to solubilise any compound. The extraction solvent that is mainly used for SFE is carbon dioxide because of its ability to exhibit supercritical temperature and pressure (Lau et al., 2010). An analytical process must be fast, cost effective, efficient and safe. Super critical fluid extraction method is been used to replace the application of organic solvents in analytical process as they are toxic to the environment (Hawthorne et al., 1990). The ability of supercritical fluid to dissolve and extract organic substance from sample matrix is close to that of organic solvent. Its efficiency and selectivity can be controlled by varying temperature and pressure. At high temperature, polar substances are more effectively extracted than non-polar substance. The method involves sample being weighed into a sample cell and placed in an oven in which temperature has been regulated. Solvent is pumped at a set pressure into the sample cell. As solvent interacts with analyte and extract analyte from solvent the solution of solvent and analyte is collected and analysed (Hawthorne et al., 1990). The super critical fluid extraction is preferred over other extraction method because the solvent used for extraction is non-hazardous and non-reactive. The solvent used is capable of extraction solute over a wide range of temperature and the apparatus is easy to operate. The main setback for this method is that CO_2 it is not able to efficiently extract some solute (Hawthorne

et al., 1994). Hawthorne et al. (1998) extracted PAHs from soil sample using different supercritical solvent and various analytical methods. The result showed that supercritical fluid extraction has higher efficiency in the yield of PAHs when compared to ultra-sonication and soxhlet extraction. The fluid employed for extraction affects recovery yield. The lowest recovery was obtained for CO_2 without a modifier or methanol, while the highest yield was obtain for 5% methanol in dinitrogen oxide (Hawthorne et al., 1998).

2.2.7 QuEChERS extraction method

Over the years, there has been a lot of focus on improving sample extraction procedures. In the mid-nineties there was a need to limit the labour intensity and the amount of organic solvent used for extraction so as to reduce toxicity and environmental pollution. This resulted in the development of many extraction techniques. Extraction techniques have emerged to improve extraction quality and efficiency and to overcome the short comings of previously developed analytical methods (Anastassiades et al., 2003). An extraction procedure must be efficient, safe, cheap and easy to use. The QuEChERS method was introduced in 2003(Anastassiades et al., 2003). It is a simple, easy, fast and cheap analytical method (Omar et al., 2013). The QuEChERS method reduces the complication of analytical process by using less solvent. It also reduces sample preparation time, uses less glassware so that clean up after extraction becomes easy, and it is very efficient for extraction of analyte from sample (Anastassiades et al., 2003). The QuEChERS method has been used in the extraction of several compounds such as pesticides and PAHs from solid samples (Kalachova et al., 2011).

The QuEChERS procedure involves two stages: firstly the extraction stage which involve the mixing of sample and extraction solvent. There after the partition of liquid phase by using salts and lastly the clean-up stage.

Kao et al. (2012) analysed the 16 PAHs in chicken and duck, included in their study was the effect of marinating and frying on accumulation of PAHs in meat using QuEChERS method. For analysis, marinated, fried and untreated meats were ground, mixed with 10 ml deionised water. The mixture was centrifuged for 1 min and then 10 ml of acetonitrile was added and shaken vigorously for 1 min.

For partitioning 6 g of $MgSO_4$ was mixed with 1.5 g of NaOAc and added to mixture after which centrifuge was done for 5 min at 4000 rpm. After centrifuge, 6 ml was collected and 400 mg of PSA, with 1200 mg of $MgSO_4$ and 400 mg of C_{18} was added. Mixture was centrifuged for 5 min and 4000 rpm. Analysis was done using GC-MS. Recoveries were between 68% - 117%.

Ramalhosa et al. (2009) used the QuEChERS method to analyse PAHs in fish sample. Recoveries were 63.5 - 110%. 16 PAHs were analysed. 50 ml Teflon tube was used for extraction, for partitioning, 6 g of $MgSO_4$ and 1. 5 g of NaOAc. 300 mg PSA, 150 mg C 18 and 900 mg $MgSO_4$ was used for clean-up in 15 ml Teflon tube. Analysis was done using Liquid Chromatography (LC) - Fluorescence Detector (FLD). The table 5 below shows the application of a summarised form of the developed QuEChERS method to PAHs extraction in various solid samples including fish sample.

Table 5: Example of QuEChERS extraction methods for extraction of PAHs from various samples including fish.

Number of PAHs analysed	Sample type	Separation and detection technique	Detection limit	Recovery	references
32	Fish and shrimps	GC-MS	0.05-0.25 µg /kg	73-109%	Kalachova et al., 2011
20	Seafood (fish, oyster, crab and shrimps)	GC-MS/MS	0.3-25 ng/ kg	71-130%	Johnson., 2012
33	Fat smoked salmon	GC-MS	1-5 pg/ L	35-87%	Forsberg et al., 2011
16	Rice	GC-MS	1-5 µg/kg	70-106%	Escarrone et al., 2013
16	Tea	GC-MS	0.05-0.2 µg kg^{-1}	74-98%	Drabova et al., 2012

2.3 Separation and Identification of PAHs

2.3.1 High Performance Liquid Chromatography (HPLC) – Fluorescence (FL)

PAHs contains strong double bond which are made from sigma and pi bonds the bonds are caused by overlap of p orbital causing the molecule to have free electrons which are able to gain energy when radiated with light of certain energy. This causes the electron to move from a lower energy level to a higher energy level. As they move back to their original position the give off light of specific frequency. This causes PAHs to fluorescence at particular wavelength and this property is used to analyse the quantity of PAHs in fish (Johnson et al., 1989). Sample which contains many different types of PAHs can be analysed without

having to separate the PAHs into different compounds (Beyer et al, 2010). The need for analysing the fluorescence of polycyclic aromatic compound in a fast and cost effective way led Krahn et al. (1984) to introduce the HPLC in the early nineteen eighties for the analysis of PAHs in fish bile (Lin et al., 1996).

The HPLC -FL consists of the HPLC equipment and a florescence detector which is programmed at different excitation and emission wavelength to accommodate the different absorption energy for the PAHs. The sample is injected into the HPLC instrument, the pump regulates the flow of the mobile phase and PAH compounds are separated as sample flows through the column. The xenon lamp is directed through the sample as they are eluted. As separate compounds are eluted, they absorb energy and fluoresce. The fluorescence is detected by the fluorescence detector. Table 6 shows different excitation and emission wave length reported for 16 PAHs using HPLC-FL.

Table 6: Excitation and emission wave length reported for 16 PAHs for HPLC-FL

PAHs	Excitation wavelength	Emission wavelength	references
Naphthalene	315	260	Ramalhosa et al., (2009)
Acenaphthene	315	260	Ramalhosa et al., (2009)
Fluorene	315	260	Ramalhosa et al., (2009)
Phenanthrene	300	408	Karl et al., (1995)
Anthracene	300	408	Karl et al., (1995)
Fluoranthene	300	408	Karl et al., (1995)
Pyrene	300	408	Karl et al., (1995)
Benz[a]anthracene	300	408	Karl et al., (1995)
Chrysene	300	408	Karl et al., (1995)
Benzo[a]pyrene	300	408	Karl et al., (1995)
Benzo[ghi]perylene	300	408	Karl et al., (1995)
Indeno[1,2,3-cd]-pyrene	300	408	Karl et al., (1995)
Dibenz[a,h]-anthracene	300	408	Karl et al., (1995)
Benzo(b)fluoranthene	300	408	Karl et al., (1995)
Benzo(k)fluoranthene	300	408	Karl et al., (1995)
pyrylene	300	408	Karl et al. (1996)

2.3.2 Gas Chromatography (GC) - Flame Ionization Detector (FID)

The GC-FID was first reported in the mid-nineties and it is commonly used analytical method for organic compounds. It is a dependable method and does not respond to waste material in the carrier gas (Pacchiarotta et al., 2010). The GC-FID consists of a gas chromatography coupled with a flame ionization detector. The gas chromatography is used to separate compound that do not decompose when they vaporise. It consists of carrier gases which are inert gas such as helium and argon and also nitrogen gas. Mixture is separated into different component as it vaporises and interacts with the column which is placed in an oven at a set temperature. After separation, organic compounds are ionised in hydrogen and air flame. The ions generated produce current which is measured and integrated. The GC- FID has the advantage of rate of flow of mobile phase does not affect detector response and gases that do not undergo combustion does not affect detector response. It can easily be operated and it does not wear easily. It has a disadvantage that compounds can undergo thermal degradation (Skoog et al., 2007). The sensitivity of GC-FID is not very sensitive for PAHs as HPLC-FL or GC-MS, this is because ionisation of PAHs in FID is not as easy as non-aromatic hydrocarbons. Analysis of PAHs in fish, sediments and water was done by (Nasr et al., 2010) using GC-FID and the recorded recoveries were 80% to 90%. PAHs higher mass were the most abundant. Higher concentration of PAHs was found in sediments and fish than in water. This is because PAHs degrade faster in water because of the presence of oxygen than in sediments. In fish, PAHs are deposited into fat which makes PAHs to accumulate in them as they are lipophilic. The concentration of PAHs obtained from different sites for water, fish and sediments are given below in table 7.

Table 7: Concentration of PAHs analysed from different samples using GC-FID (Nasr et al., 2010)

Samples	**Concentration**
Water	226.9 -1492.2 ng L^{-1}
Fish	371.68-2019 ng g^{-1}
Sediments	1197-2701 ng g^{-1}

2.3.3 Gas Chromatography (GC) – Mass Spectroscopy (MS)

The GC-MS consist of a gas chromatography for separation of sample coupled with the mass spectrometer for sample analysis and sample identification and quantification. The gas chromatography mass spectroscopy analytical method was used in the late nineteenth century (Capriotti et al., 2011).

The mass spectrometer is one of the most reliable instruments used in the analysis of organic compounds. It consists of a sample inlet, through which solid or liquid samples are introduced into the instrument. The sample is vapourised and converted to ion. The ions are directed to the mass analyzer which processes them as electrical beam. Degradation of sample can occur and decomposed sample can still be analysed (Skoog et al., 2007). Kao et al. (2012) used GC-MS for the determination of 16 PAHs in poultry meat. Recoveries of PAHs obtained were above 85%.

CHAPTER 3

3 RESEARCH OBJECTIVES

3.1 General Objectives

- To develop the QuEChERS method in extraction of PAHs in fish.
- To evaluate the extent of PAH distribution in fish dams in Jericho dam Mpumalanga and Hartebeesport dam Gauteng province.
- To compare the developed extraction method with other traditional methods.

3.2 Justification of Research

Many PAHs are serious environmental contaminant because of their carcinogenicity, mutagenicity and toxicity. Due to their many sources, PAHs are wide spread in the environment. In areas such as Mpumalanga where there are a lot of mine and extensive burning of forests, there is little information available on the distribution of PAHs in aquatic species such as fish. The same is true for Gauteng province. Further, very little studies have been reported on the use of molecular imprinted polymers and multi-walled carbon nano tubes as sorbents in QuEChERS method.

3.3 Specific Objectives

- To optimize the various parameters that affect the QuEChERS method of extraction of PAHs from fish.
- To test other cleaning sorbents based on concentration of PAHs in fish using QuEChERS method.
- To identify and quantify PAHs that may be present in fish samples from Hartebeesport dam in Gauteng and Jericho dam in Mpumalanga.
- To compare developed QuEChERS extraction method to Soxhlet extraction in terms of time taken, selectivity, and amount of solvent used.
- To study accumulation of PAHs in fish by determining the concentration of PAHs in different ages of same fish type.

3.4 Hypothesis and research question

3.4.1 Hypothesis

- The dams in this environment are contaminated with PAHs due to excessive burning of wood, burning of petroleum products and coal mining in surrounding area.
- The fish in these environment are also contaminated with PAHs due to bioconcentration and bioaccumulation behavior of PAHs

3.4.2 Research Question

- Are the fish found in the two dams contaminated with PAHs?
- Can the selectivity in QuEChERS method be improved by using different cleaning sorbents?

3.4.3 Novelty

This research is novel because it attempted to use different sorbents as clean-up in QuEChERS methods. Further, very little studies have been reported on this.

CHAPTER 4

4 RESEARCH METHODOLOGY

4.1 Sampling

Carp fish samples were obtained from Hartbeesport dam in Gauteng and Jericho dam in Mpumalanga. Targeted sites were sites with high concentration of mining industries and sites where forest burning is predominant. Fish were obtained from fishermen at the two dams. These were bought in June/July 2011. Table 8 shows the sites and types of fish obtained. Samples that were of interest were fish that inhabit muddy sites of the dam, such as the catfish and fish that were found far into the middle of the dam such as the carps. Efforts were made to obtain the same type of fish but in different location in the dam.

Individual fish sample were bought still alive and fresh. The length and breadth of fish samples were measured, thereafter fish were wrapped in aluminium foil. Each wrap was labelled, placed in a plastic bag, sealed and kept in a freezer for 2 days until analysed. The aluminium foil was soaked in methanol before use and allowed to dry. This is to preserve the fish sample and avoid the loss of PAHs and also to avoid the growth of unwanted micro- organisms.

Table 8: Fish types and sites with length and breadth

SITE	Fish type	Label	Length (CM)	Breadth (CM)
Hartbeesport dam	Carps fish	HPB1	21	7
Hartbeesport dam	Carps fish	HPB2	22.4	7.6
Hartbeesport dam	Carps fish	HPB3	24.5	8.6
Hartbeesport dam	Carps fish	HPB4	35.5	12.5
Jericho dam	Carps fish	A1	28.4	10.6
Jericho dam	Carps fish	J1	51	34
Jericho dam	Carps fish	J2	52	36
Jericho dam	Carps fish	J3	50	24
Jericho dam	Carps fish	J4	52	35

4.2 Chemicals

All PAHs used, namely naphthalene (99.9%), acenaphthene (99.9%), phenanthrene (99.9%), fluoranthene (99.9%) and pyrene (99.9 %) were purchased from Sigma-Aldrich (Steinhein, USA). Acetone (99%) and Methanol (99 %) purchased from Fischer Scientific (Loughborough, UK) while acetonitrile (99.9%), purity was obtained from Sigma-Aldrich (Steinhein, USA). Hexane (99.5%), ethyl acetate (99%) and dichloromethane (99.5%) were purchased from Fluka (Steinhein, USA). Magnesium sulphate was obtained from Sigma- Aldrich (Steinhein, USA) and sodium chloride analytical grade were obtained from Merck KGaA (Darmstadt, Germany). For clean- up, primary secondary amine (PSA) bonded with Silica 100 g bulk was purchased from Sulpeco (Bellefonte, PA, USA). Multiwalled carbon nano tubes were donated by the material science research group in the department. MIP and NIP were donated by MIP Technology (Lund, Sweden) now part of Biotage (Uppsala, Sweden).

4.3 Instruments

The centrifuge used was a S-8 centrifuge obtained from Boeco (Hamburg, Germany). GC-FID Agilent Technologies, (7890A) with a WCOT fused silica capillary column (30 x 0.25 mm ID, 0.25 m film thickness) and Chemstation software was used for analysis. GC parameters are as follows: Oven 40 ^{o}C (5 min), 10 ^{o}C min^{-1} to 179 (2min), 9 ^{o}C min^{-1} to 300 ^{o}C (10 min). This temperature was adequate to separate all the PAHs to be analysed. For evaporation of solvent, rotavapor R11 from Labotec (Flawil, Switzerland) was used. A 460 Elma ultrasonic bath (Elma, Germany) was used for removal of bubbles from mobile phase and for dissolving PAH standards. HPLC containing water pump was obtained from (Milford, Massachusetts). The pump's flow rate was set at 1.3 ml min^{-1}. This was connected to RF- 10AxL Shimadzu fluorescence detector (Kyoto, Japan) and clarity software were purchased from Prodohradska, (Prague, Czech). Excitation and emission wavelength for HPLC- FL was set as follows for the PAHs. naphthalene 280 nm and 490 nm, phenanthrene 225 nm and 460 nm, pyrene 333 nm and 390 nm, acenaphthene 280 nm and 490 nm and fluoranthene 290 nm and 320 nm respectively. A 5 µl and 100 µl syringes obtained from (SUPELCO Analytical, Pennsylvania, USA) were used for GC and HPLC injections.

4.4 Quality assurance

4.4.1 Preparation of stock solution

A stock solution of 1000 mg L^{-1} of mixtures of the five PAHs used; naphthalene, phenanthrene, acenaphthene, fluoranthene and pyrene was prepared in a 250 ml volumetric flask by weighing 250 mg of PAH each into the flask and there after filling the volumetric flask to mark with methanol. A 10 mg L^{-1} stock solution was prepared from the 1000 mg L^{-1} by withdrawing 100 µl from the 1000 mg L^{-1} into a 10 ml volumetric flask, thereafter the flask was filled to mark with methanol. The 10 mg L^{-1} standard was used to prepare the standard for both calibration curves.

4.4.2 Preparation of calibration curve

A 10 mg L^{-1} standard solution was prepared from a 1000 mg L^{-1} stock solution which was prepared earlier. A standard solution of PAHs of different concentration ranging from 0.2 mg L^{-1} to 1.0 mg L^{-1} was prepared and used for determination of calibration curve. Five concentrations of standards were prepared in a 5 ml volumetric flask. Preparation of the standards for GC- FID was done by drawing 100, 200, 300, 400 and 500 µl of 10 mg L^{-1} of stock into the 5 different 5 ml volumetric flask each and thereafter, the flasks were filled to mark with methanol. Three range of concentration of standards for calibration curve for HPLC were prepared from the same stock solution for GC- FID. Concentration of standard was from 0.025 to 0.075 mg L^{-1}. Standard preparation was done by preparing 12.5, 25.0 and 37.5 µl 10 mg L^{-1} in a 5 ml volumetric flask and filling up to mark. The calibration curves were best fitted to linear as shown as results are summarized in Tables 9 and 10.

Table 9: GC-FID calibration curve

PAH	**r^2**	**Slope**	**intercept**	**detection limit (mg/l)**
Napthalene	0.9951	42.40	0.500	0.10
Acenaphthene	0.9960	52.01	0.443	0.10
Phenanthrene	0.9956	51.54	0.005	0.10
Fluoranthene	0.9951	54.54	0.549	0.15
Pyrene	0.9922	55.04	0.862	0.10

Note: detection limit is for direct injection

Table 10: HPLC-FL calibration curve

PAH	r^2	Slope	intercept	detection limit (µg/kg)
Napthalene	0.9585	35208	3972.1	0.2
Acenaphthene	0.9998	21062	3548.6	1.1
Phenanthrene	1.0000	7902	1843.3	1.1
Pyrene	0.9954	3402	513.7	0.7
fluoranthene	1.0000	207219	131.99	0.2

4.5 Optimization of QuEChERS method

Hake fish samples were obtained from shops. Samples were eviscerated and filleted. Fish muscles were homogenized using a mortar and stored in a foil wrap and kept frozen until analysis. The QuEChERS extraction method was done using the procedure reported by Ramalhosa et al., (2009). 2.5 g of fish sample was weighed in a 25 ml Teflon tube and spiked with 65 µl (0.13 mg kg^{-1}) of 10 mg L^{-1} standard of the five PAHs. The spiked sample was allowed to stand for 30 min and then 8 ml of acetonitrile was added and shaken vigorously for 1 min. This was followed by the addition of 2 g of $MgSO_4$ and 0.5 g of NaCl salt into the tube and the mixture was shaken vigorously for 1 min and then placed in the centrifuge. After centrifuge, 5.5 ml of sample was collected and primary secondary amine (PSA) was added for clean- up. The sample was centrifuge for 5 min and filtered using a 0.45 µm PTFE and injected in the GC/MS for analysis. For optimization procedure, parameters that can affect extraction such as the speed of centrifuge, time of centrifuge and quantity of salt, volume of solvent, type of solvent, fish mass, salt mass and type of sorbent used were varied.

4.5.1 Centrifuge speed

During optimization of centrifuge speed, 2.5 g fish sample was placed in a 25 ml Teflon tube and spiked with 0.13 mg kg^{-1} of PAHs. 8 ml of acetonitrile was then added and shaken vigorously. After shaking for about 1 min, 2 g of $MgSO_4$ and 0.5 g of NaCl was added and sample was place in the centrifuge. Speed for centrifuge was varied from 3400 to 6000 rpm and the time was kept at 10 min. Extracts were obtained for each speed, cleaned, filtered and analysed as described earlier.

4.5.2 Centrifuge time

The procedure for spiking in 4.5.1 was repeated and centrifuge speed was kept constant at 5000 rpm as this speed was found to be best for extraction. Extraction time was varied from 10 min to 30 min. The purpose for varying extraction time is to observe the effect of the length of time sample interact with solvent on extraction efficiency. The sample was cleaned in the usual way and the extract obtained and analysed in the same way as stated earlier.

4.5.3 Volume of solvent used

Volumes used for optimization were varied from 6 ml, 8 ml and 10 ml respectively. Using optimized condition for centrifuge speed and time, the volume of solvent was varied. Sample clean up was done as reported earlier

4.5.4 Solvent type

For optimization of solvent, three types of solvents were used as these solvent have been reported for the extraction of PAHs (Forsberg et al., 2011). The solvents used were ethylacetate, acetone and acetonitrile. Optimized conditions for previously optimized parameters were used and for solvent volume, 10 ml of solvent was used as this gave better recovery. Clean up was done using method reported earlier.

4.5.5 Mass of fish sample

The mass of fish sample used was varied to get the optimal mass. For optimization, fish mass were 0.5 g, 1.0 g, 2.5 g. Extraction was done using optimized parameters. Clean up was done as reported earlier.

4.5.6 Quantity of salt used for extraction

The amount of salt used for extraction was varied while keeping centrifuge speed of 5000 rpm and extraction time of 10 min constant. The amount of magnesium salt varied from 1 to 2.5 g keeping sodium salt constant at 0.5 g. The $MgSO_4$ ensures a phase separation between organic solvent and water.

4.5.7 Quantity of PSA used for extraction

Cleaning sorbent was optimized by varying the mass of PSA used. All other optimized condition were kept constant for extraction procedure. During clean up, mass of PSA was varied using 50 mg, 100 mg and 150 mg respectively.

4.5.8 Optimization of sorbent type

4.5.8.1 Sorbent used

Sorbents are solid phases used to remove unwanted matrix from sample. PSA have been used widely in the recovery of PAHs from sample matrix. Other sorbents that were used are molecular imprinted polymers (MIP), non-imprinted polymers (NIP), magnetite and multi-walled carbon nanotubes. MIP is known to adsorb specific molecules (Southard et al., 2006). Magnetite has been used as sorbent in removal of arsenic in water (Mayo et al., 2007). Varying the types of solvent is to explore if there can be more effective sorbent other than PSA.

4.5.8.2 Primary Secondary Amine (PSA)

Fish sample was treated using optimized conditions as previously reported. For clean- up, the optimized condition was used, using PSA in silica gel.

4.5.8.3 Non Imprinted Polymer (NIP)

Nip was initially washed several times with methanol, thereafter dried in the oven for 24 hours and then used as cleaning sorbent. Fish sample were treated using optimized condition as reported previously. For clean up, 150 mg of NIP and 150 mg of $MgSO_4$ were added to aliquot of sample. The mixture was shaken vigorously for 1 min and placed in the centrifuge at 5000 rpm for 5 min. After centrifuge, mixture was filtered and analysed.

4.5.8.4 Molecular Imprinted Polymer (MIP)

MIP was initially washed several times with methanol, thereafter dried in the oven for 24 hours. For extraction, the fish sample was treated using optimized conditions as previously reported. After extraction, 150 mg of MIP and 150 mg of $MgSO_4$ were added to aliquot of sample, shaken vigorously and centrifuged at 5000 rpm for 5 min. The Sample was filtered using a 0.45 µm PTFE filter and 1 µl was injected into the GC for analysis.

4.5.8.5 Magnetite

Magnetite was initially washed several times with methanol, thereafter dried in the oven for 24 hours Fish sample was treated using optimized conditions as previously reported. After extraction, 150 mg of magnetite and 150 mg of $MgSO_4$ was added to aliquot of sample, shaken vigorously and centrifuged at 5000 rpm for 5 min. Sample was filtered using a 0.45µm PTFE filter and 1µL was injected into the GC for analysis

4.5.8.6 Multi-walled carbon nanotubes

Carbon nanotubes was initially washed several times with methanol, thereafter dried in the oven for 24 hours. For extraction, the fish sample was treated using optimized conditions as previously reported. After extraction, 150 mg of carbon and 150 mg of $MgSO_4$ was added to aliquot of sample, shaken vigorously and centrifuged at 5000 rpm for 5 min. Sample was filtered using a 0.45 µm PTFE filter and 1µL was injected into the GC for analysis.

4.6 Soxhlet extraction method in extraction of PAHs from fish sample

Fish sample was Soxhlet extracted using the method previously reported (US EPA, 1996C). 15 g of fish sample were weighed in triplicate and spiked with different concentration of PAHs ranging from 0.5, 1.0 and 1.5 µg/g. Spiked fish sample were placed in a Soxhlet thimble and mixed with 15 g of $MgSO_4$. Fish samples were extracted using 200 ml of dichloromethane and acetone in ratio 1: 1 for 20 hours at a temperature of 35^oC. After extraction, the solvent was evaporated to 5 mL and was made up to 10 mL with hexane and then further evaporated under nitrogen to 2 ml. The extract was passed through a column of silica consisting of 3 g of silica and 2 g of Na_2SO_4 which had been previously dried. 10 mL of dichloromethane and hexane ratio 1:4 was used to wash the column and the mixture was evaporated to dryness under a gentle steam of nitrogen and then 3 mL of acetonitrile was added. For analysis, 1 µL of extract was injected into the GC.

4.7 Application to real sample

4.7.1 QuEChERS

Fish samples were prepared as earlier reported. 2.5 g of fish sample was placed in a 25 ml Teflon tube, 10 ml of acetonitrile added and sample shaken vigorously for 1 min. A mixture of 2.5 g of $MgSO_4$ and NaCl was added and shaken vigorously for 1 minute, then the mixture was centrifuged for 10 minutes at 5000 rpm. After centrifuge, 7.9 ml of aliquot was collected into a 25 ml Teflon tube. 150 mg of PSA and 150 mg of $MgSO_4$ were added to the sample. Sample was shaken vigorously for 1 min and then placed in a centrifuge for 5 min at 5000 rpm. After centrifuge, sample is filtered using a 0.45µm PTFE filtered. Sample was analysed using GC- FID.

4.7.2 Soxhlet

The Soxhlet extraction method was also applied to real fish sample. The method used has been described already under section 4.6.

CHAPTER 5

RESULT AND DISCUSSION

5.1 QuEChERS experiment

5.1.1 Optimisation of QuEChERS method

In order to get best recovery of the QuEChERS method, various parameters were optimised. Results of the various optimised parameters are given and discussed below.

5.1.2 Centrifuge time

Figure 3 shows the results obtained when centrifuge time was varied keeping all other parameters constant. The result shows that recovery of PAHs reached maximum at extraction time of 10 min. Increasing the centrifuge time from 10 to 30 min did not improve the recovery. Therefore 10 min was chosen as the optimal time for centrifuge. Ramalhosa et al., (2009) studied recovery of PAHs from fish sample using ultrasonic bath by increasing extraction time from 3 to 20 min. Centrifuge time was also investigated by Keegan et al., (2009) and 10 mins was found to be optimum. The optimum time of 10 min found in this study is therefore consistent with other previous studies. The shorter centrifuge time used compared to that of the ultrasonic bath also indicates that extraction using the centrifuge method is less time consuming and therefore more economical.

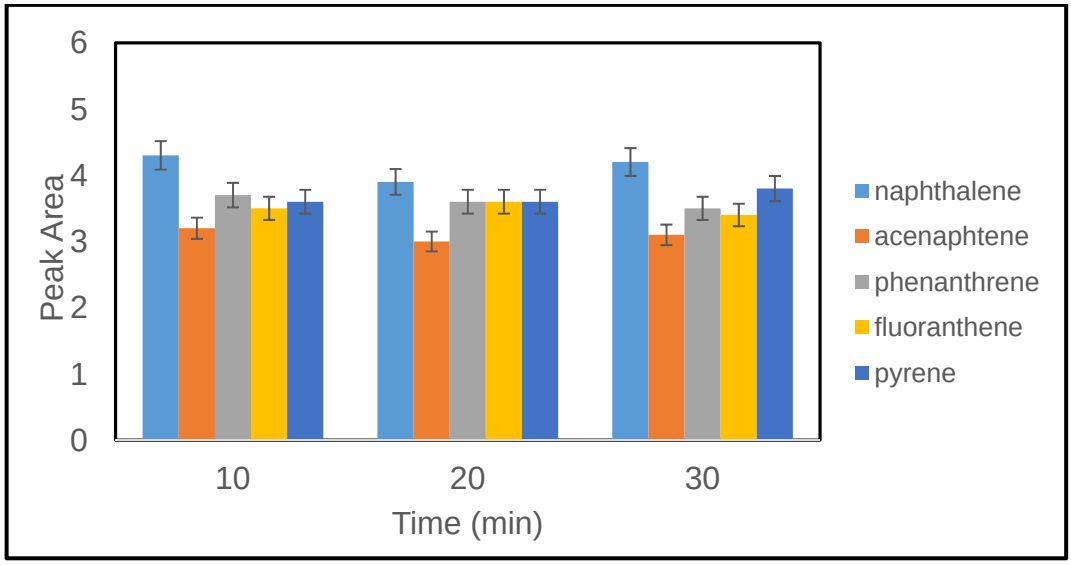

Figure 3: Comparison of centrifuge time keeping parameters such as solvent volume at 6 ml, centrifuge speed at 3400 rpm, mass of fish and mass of salt constant.

5.1.3 Centrifuge speed

Figure 4 shows the result obtained when centrifuge speed was varied keeping all other parameters constant. There is not much variation in recovery as centrifuge speed is increased from 3400 to 5000 rpm. Rodrigues et al., (2012) applied centrifuge speed of 5000 rpm for QuEChERS extraction of pesticides. Martin et al. (2010) and Lopes et al. (2012) also used centrifuge speed of 5000 rpm. This shows that 5000 rpm is the most commonly used. There was a decline in recovery as centrifuge speed increases to 6000 rpm. The reason for this is not known but it might be related to the dissolution process. At centrifuge speed of 5000 rpm, maximum recovery of PAHs was obtained. This was chosen as optimal centrifuge speed. Centrifuging allows the solvent to be more in contact with the sample therefore making it to be more effective in dissolution of analyte (Kouzayha et al., 2011). The centrifuge speed can reduce the time needed for extraction (Dongshun et al., 2001).

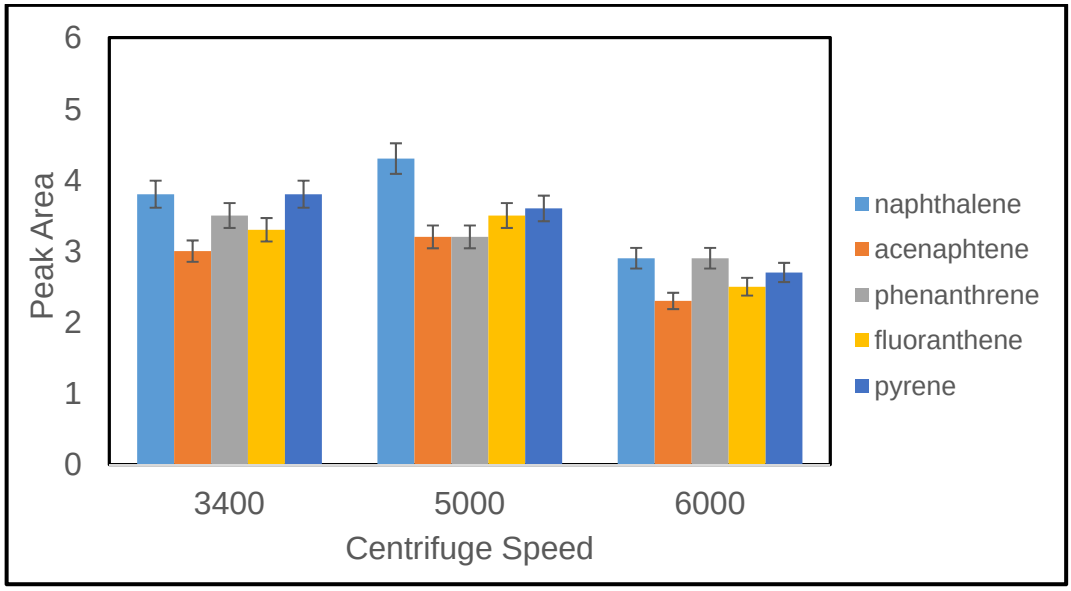

Figure 4: Comparison of centrifuge speed keeping all parameters such as solvent volume at 6 ml and centrifuge time at optimized condition and other parameters mass of fish and mass of salt constant.

5.1.4 Solvent type

The solvent type is very important when the QuEChERS method is developed. The solvent must be able to extract target analyte thus solvent polarity should be taken into consideration. Further, the solvent must be less expensive, compatible with analytical instruments and environmentally friendly (Anastassides et al., 2003). Acetonitrile and ethyl acetate have been widely used to extract polar to non-polar analytes (Carabias-Martinez et al., 2005).

A variety of solvents such as acetonitrile, acetone and ethyl acetate were tested. The results obtained are shown in Figure 5. The results indicate that there was no major differences in the peak areas obtained from extraction using different solvents. Pyrene and fluoranthene seem to have slightly extracted slightly more using acetonitrile. Those slight difference, reflect the type of intermolecular interaction that takes place between the PAHs and extraction solvent. PAHs are non-polar with mostly hydrophobic interactions through pi bonds being involved. This may explain why acetonitrile also with pi bonds and linear in geometry gave slightly better extractions. The geometry of the solvent should allow maximum interactions with the analytes besides its polarity.

Another important factor to take in choosing the right solvent is the selectivity Figures 6-8 shows chromatograms of extracted blank solution and those of spiked counter parts. From the obtained chromatograms, it shows that ethyl acetate extracts were worst in selectivity. Acetone and acetonitrile gave slightly better selectivity therefore, taking both recovery and selectivity into account, acetonitrile solvent was taken as optimum solvent. As observed acetonitrile is most selective and gives a cleaner chromatogram. Acetonitrile is one of the most selective solvent and it has more advantage over most solvent used in the QuEChERS method (Anastassiades et al, 2003). Acetonitrile separates more easily from water than other solvents used in the QuEChERS method in the presence of salts. This gives a good phase separation which prevents interaction of polar matrix (Diez et al., 2006). Therefore, acetonitrile was chosen as the optimal solvent for extraction.

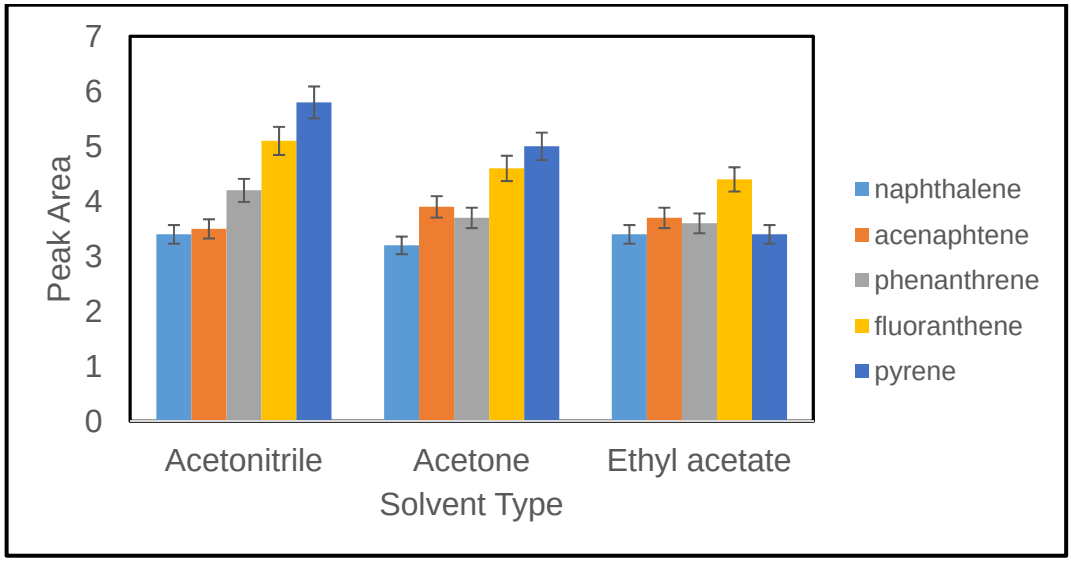

Figure 5: Influence of solvent type on extraction

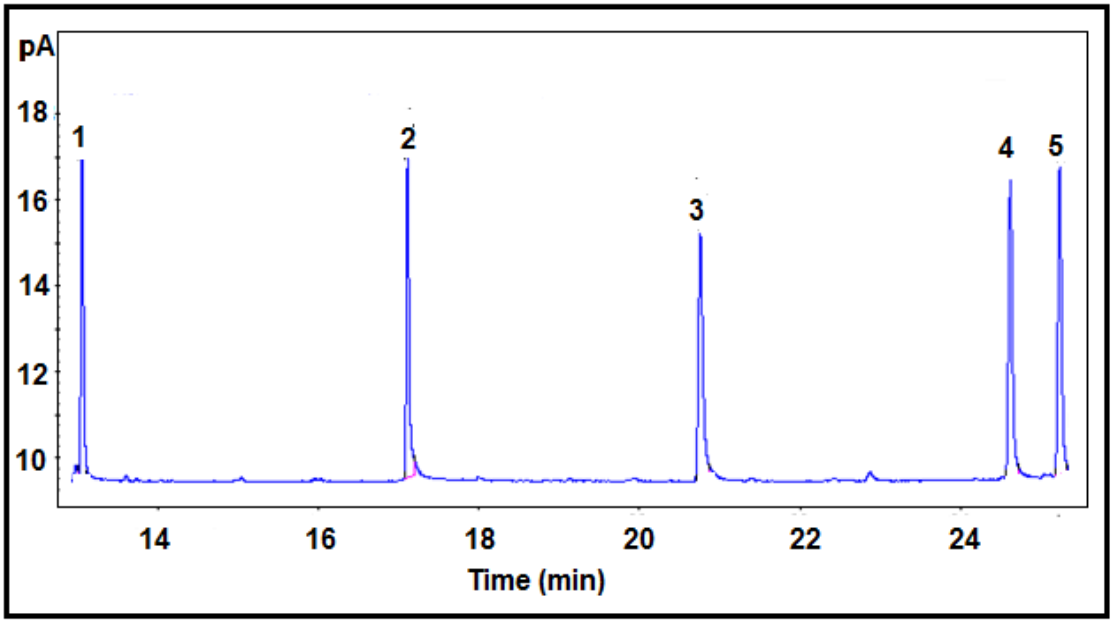

Figure 6: Chromatogram of 1ppm standard solution of PAHs: 1- naphthalene, 2- acenaphthene, 3-phananthrene, 4-fluoranthene and 5- pyrene injected into GC-FID.

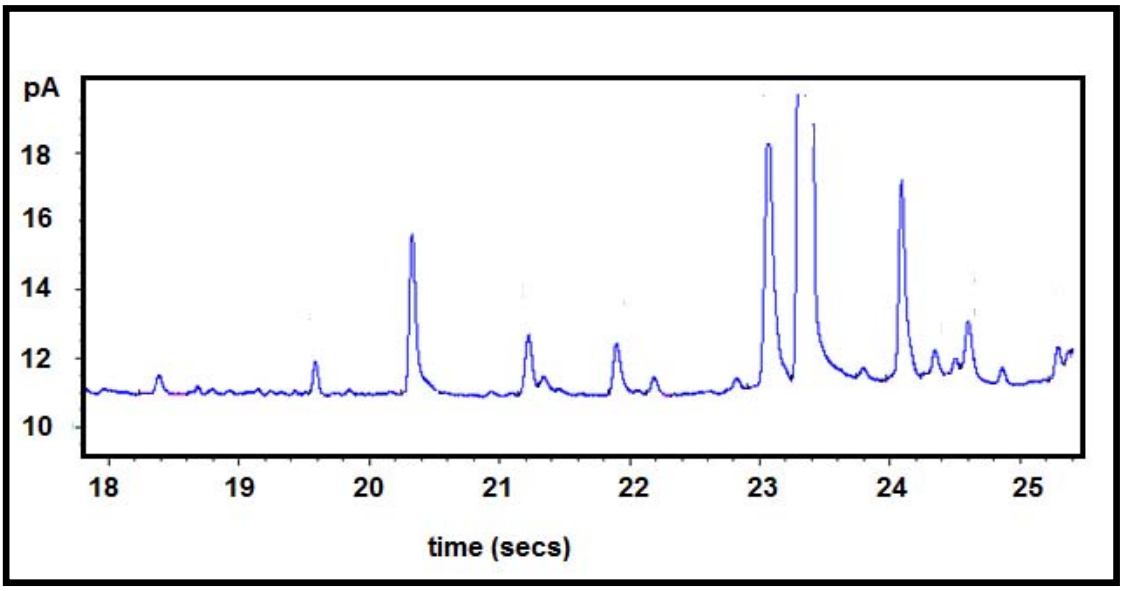

Figure 7a: Chromatogram of non-spiked fish sample extracted with acetone. Injected in GC-FID.

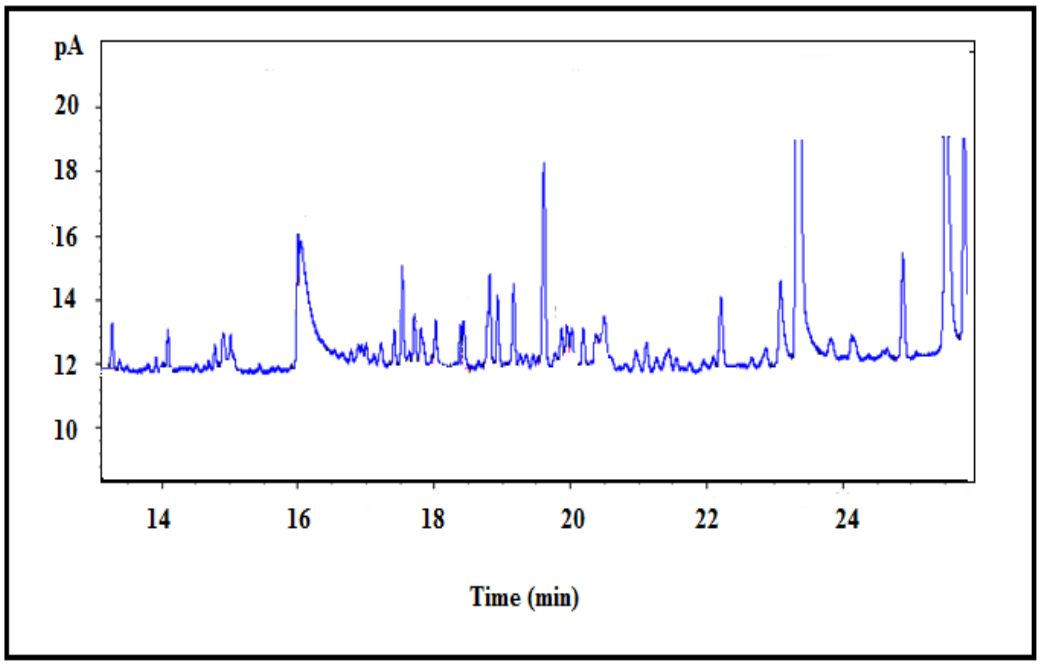

Figure 7b: Chromatogram of non-spiked fish sample extracted with ethylacetate. Injected in GC-FID.

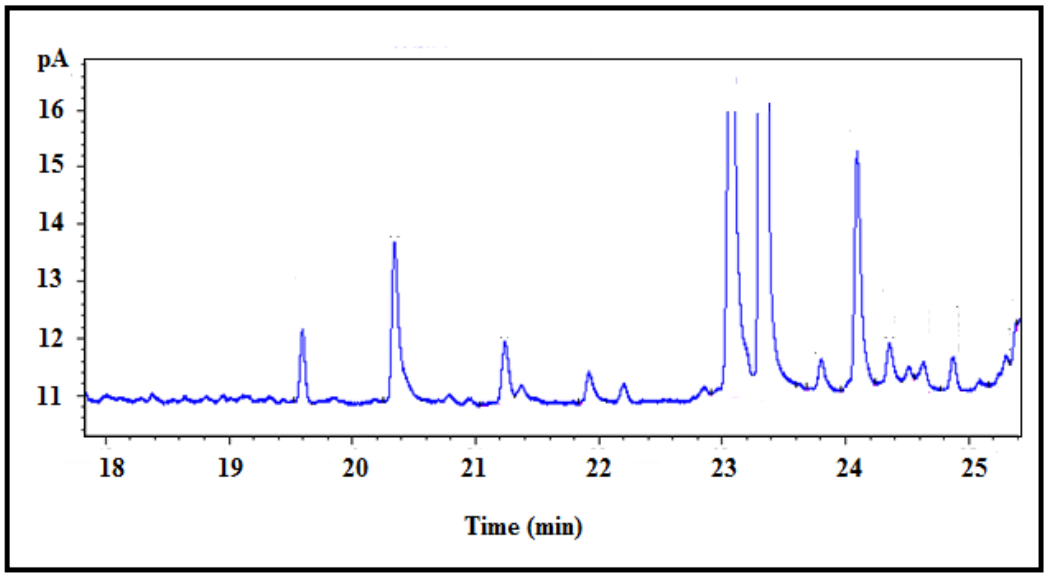

Figure 7c: Chromatogram non-spiked fish sample extracted with acetonitrile. Injected in GC-FID

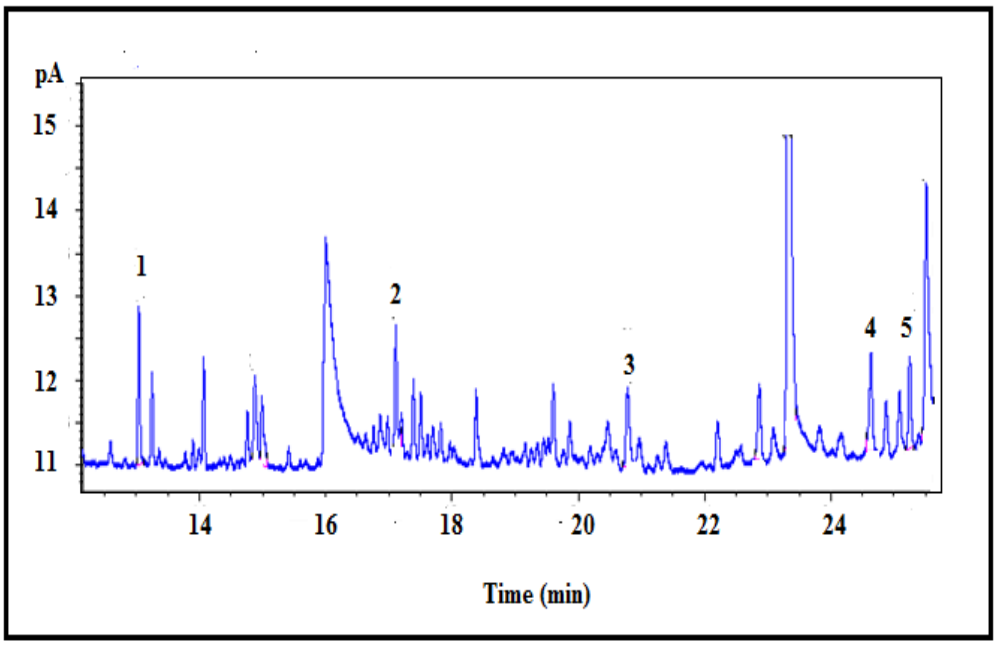

Figure 8a: Chromatogram of spiked fish sample extracted with ethylacetate and injected into the GC-FID. Sample was spiked with 0.13 mg kg^{-1} concentration of PAH

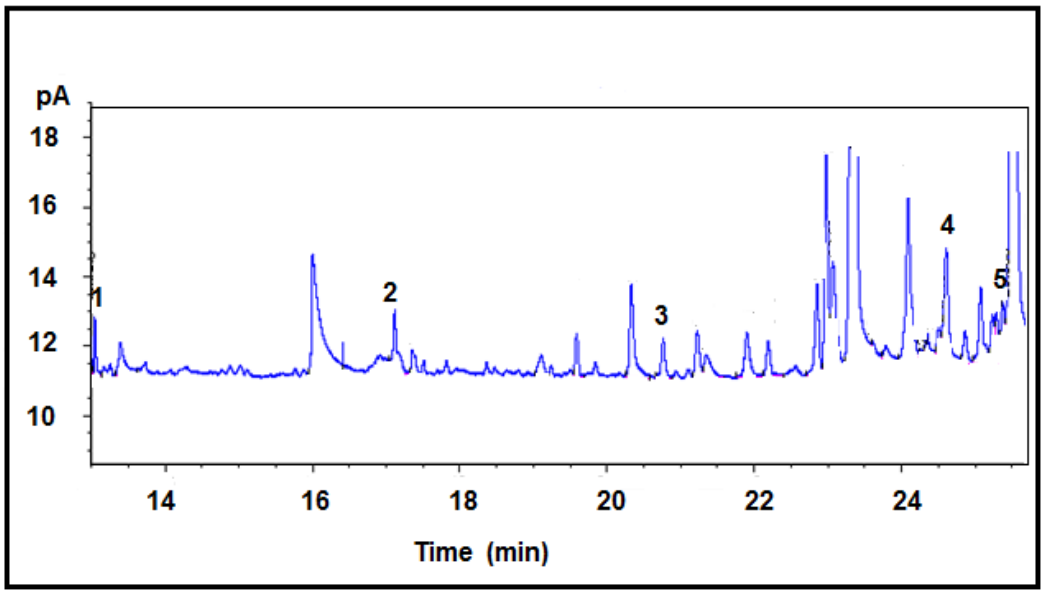

Figure 8b: Chromatogram of spiked fish sample extracted with acetone and injected into the GC-FID. Sample was spiked with 0.13 mg kg^{-1} concentration of PAHS

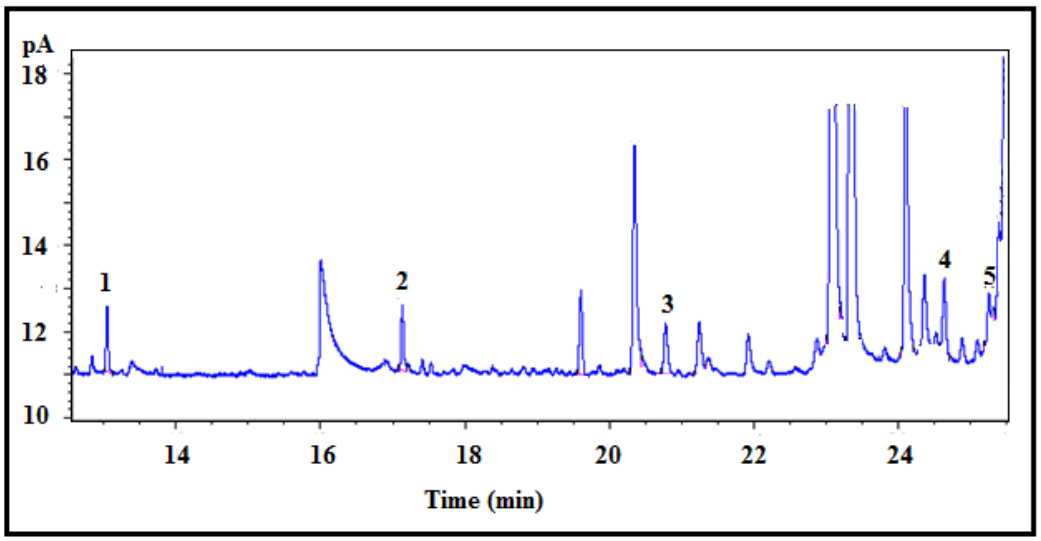

Figure 8c: Chromatogram of spiked fish sample extracted with acetonitrile and injected into the GC-FID. Sample was spiked with 0.13 mg kg^{-1} concentration of PAHS

5.1.5 Optimisation of mass of fish sample

Figure 9 shows the result obtained when the mass of fish sample was varied while keeping all other parameters constant. There was slight variation in the peak areas obtained. Overall 1.0 and 2.5 g gave slightly higher peak areas than 0.5 g. Homem et al. (2013) investigated the mass solvent ratio and observed that ratio lower than 0.17 gave very low recoveries. In this study, volume was kept at 10 ml and fish mass varied from 0.5 g, 1.0 g and 2.5 g. This gave a mass to solvent ratio of 0.05, 0.1 and 0.25 respectively. This might explain why the 0.5 g mass fish sample gave a lowest recovery compare to 1.0 g and 2.5 g. Therefore, 2.5 g was used has optimum fish mass as it gave a slightly higher peak area.

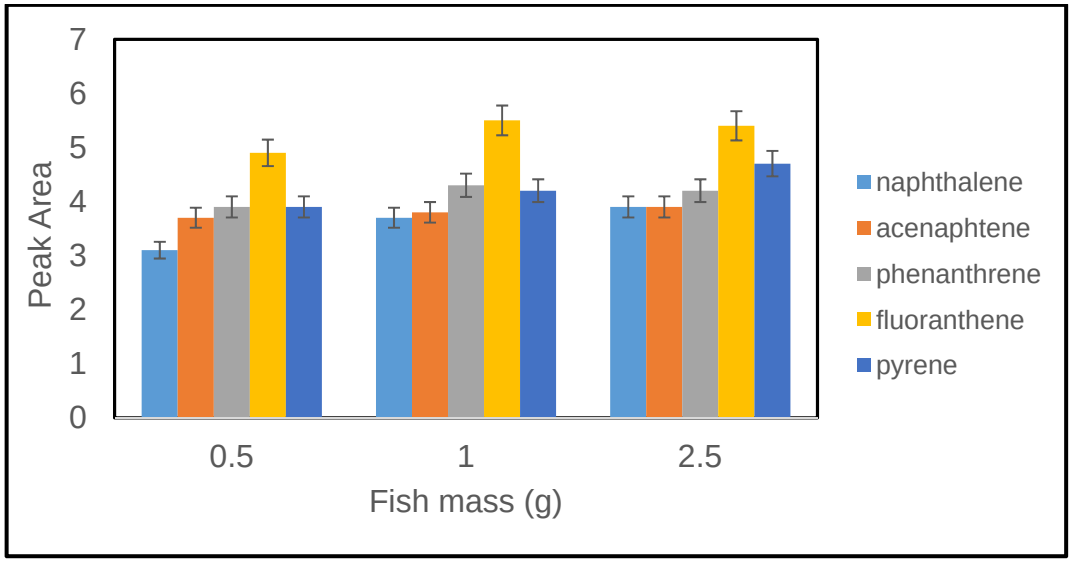

Figure 9: Effect of fish mass on extraction

Quantity of salt used for extraction

Figure 10 shows the result obtained when mass of salt was varied and all other parameters were kept constant. There was a remarkable increase in recovery of naphthalene, acenaphthene and phenanthrene with the use of 2 g of $MgSO_4$. Increase in salt allows greater phase separation but high salt level can also affect the effectiveness of the extraction system (Coelho et al., 2008). The right combination of quantity of salt used in phase separation is important as it regulates the polarity of the mixture. $MgSO_4$ removes a lot of water and the reaction is exothermic which can improve the process of extraction. (Anastassiades et al, 2003). Addition of salt increases the temperature of the system, lowers activation energy and also decrease the viscosity of the solvent thereby increasing solvent matrix interaction (Richter et al., 1996). The result shows that the optimal mass of salt is 2 g as there is not much variation between the 2 g mass and the 2.5 g mass of salt.

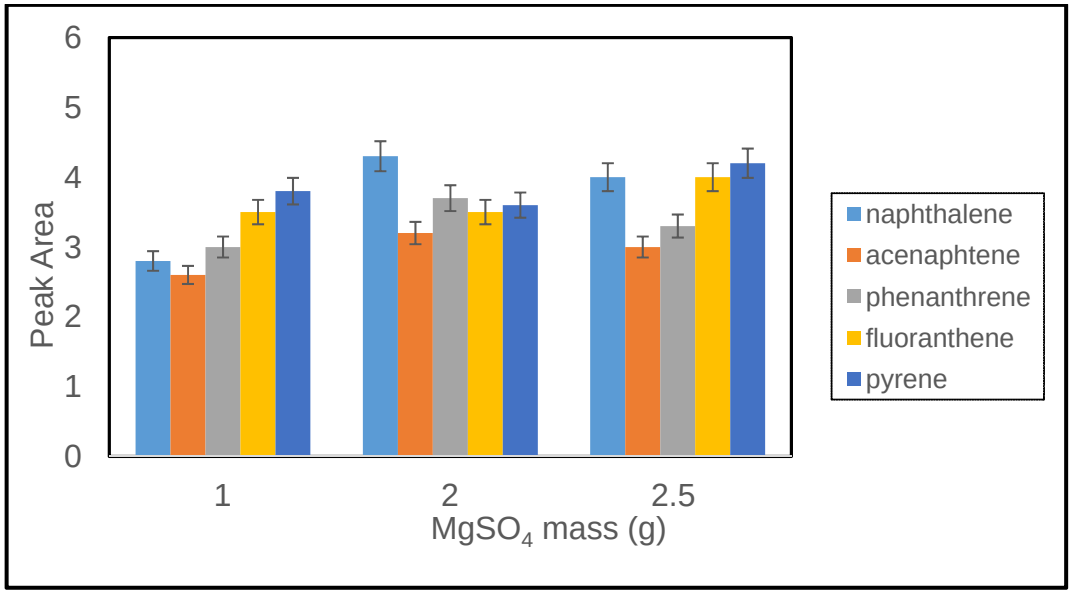

Figure 10: Varying mass of salt and keeping all optimized parameter constant and using 150 mg mass of PSA

5.1.6 Quantity of PSA used for extraction

Figure 11.1 show the result obtained in terms of peak area. There was not much difference in the peak area but in selectivity. Homem et al., (2013) analysed the effect of PSA on extraction by using different masses of PSA of 0, 30 and 60 mg. 60 mg PSA gave optimal result in terms of the selectivity of extraction. Fernandez et al., (2011) used 150 mg of PSA in the clean-up procedure in the application of QuEChERS extraction method. The sample matrix can cause variety of damage to equipment. It can also affect recovery. PSA has been widely used as a cleaning sorbent. Figure 11.2a- 11.2c shows the result obtained for the optimisation of mass of PSA. The chromatogram obtained for the 150 mg PSA was the cleanest. Figure 11.2c shows a significant reduction in base line noise and also better peak separation compared to figure 11.2a and 11.2b. Conclusively there was a reduction in base line noise as the amount of PSA increased. From the result obtained in the experiment, 150 mg PSA was chosen as the optimum mass.

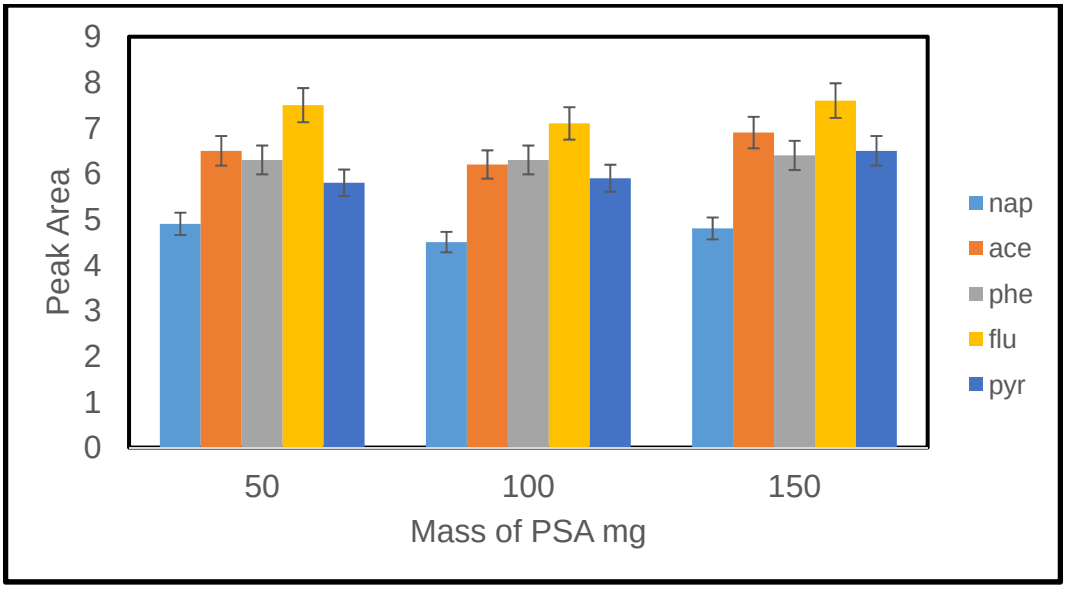

Figure 11.1: Varying mass of PSA and keeping all other parameters constant

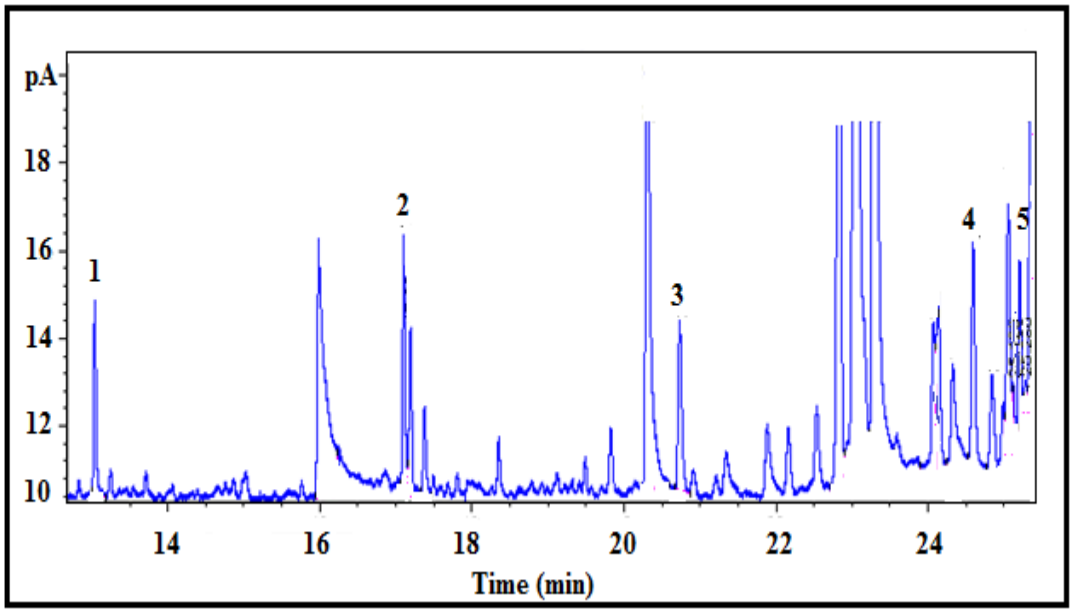

Figure 11.2a: Chromatogram of extract with 50 mg PSA keeping all other parameters constant.

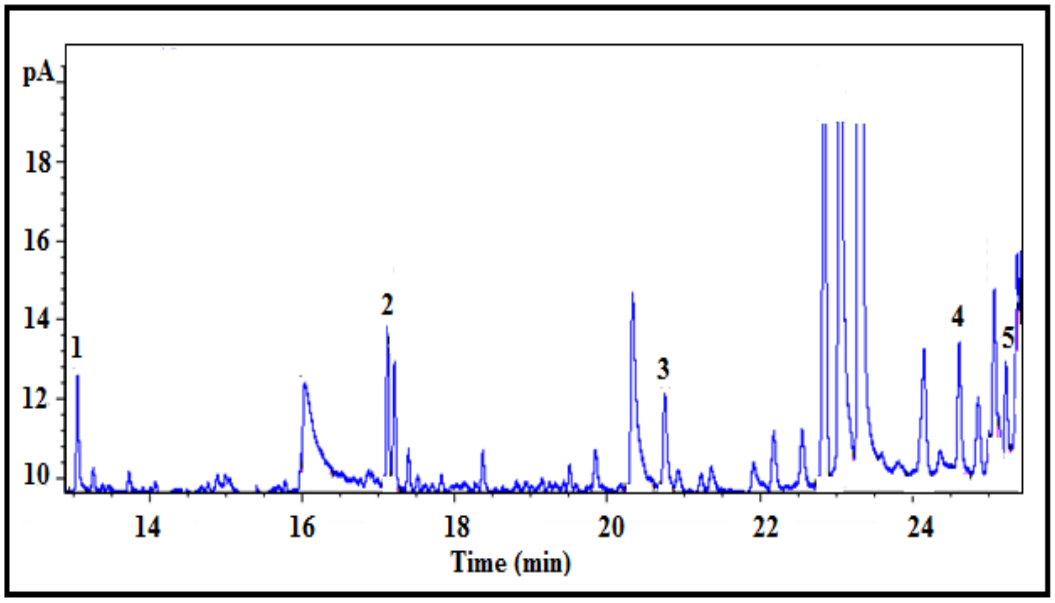

Figure 11.2b: Chromatogram of extract with 100 mg PSA keeping all other parameters constant.

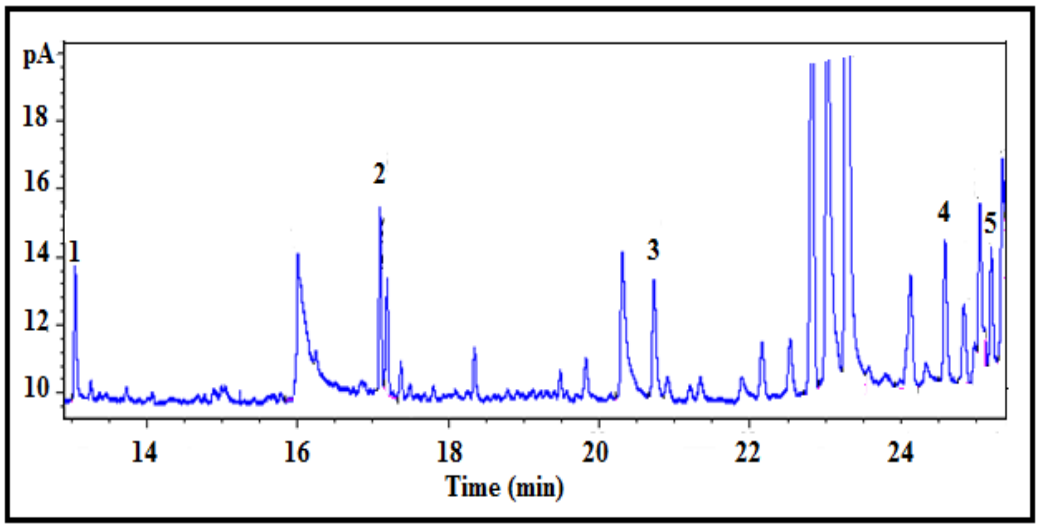

Figure 11.2c: Chromatogram of extract with 150 mg PSA keeping all other parameters constant.

5.1.7 Volume of solvent used

In the QuEChERS extraction method the volume of solvent used range between 2 ml to 10 ml depending on the mass of sample. The solvent volume can play an essential role in recovery obtained. The volume of solvent used must be sufficient

to completely immerse the sample for maximum solvent and analyte interaction. Figure 12a shows the peak area obtained for different acetonitrile volume while other parameters were kept constant. There was a slight decrease in amount extracted with increase in volume of solvent. Decrease was more pronounced with 10 ml. However, it can be noted be noted that from recovery, 6 and 8 ml gave recoveries that are more that are more than 100%. Homem et al., (2013) studied the effect of sample mass to solvent ratio on recovery. Optimum ratio obtained was 0.17. In this study, the volume used gave sample mass to volume of 0.41, 0.31, and 0.25 respectively. Closest to that of 0.17 was 0.25 which was the 10 ml. Satisfactory recovery was obtained for 10 ml volume which range from 95 to 104 % as noted above, while for the 6 ml and 8 ml volume recovery of fluoranthene was 130 % while in the 6 ml volume recovery of phenanthrene exceeded 120%. These recoveries are not acceptable according to European directives 96/46/EC. Larger recoveries may be due to matrix effect (Homem et al., 2013, Rodrigues et al 2012). 10 ml volume was used as optimal volume since recoveries obtained are within acceptable limit.

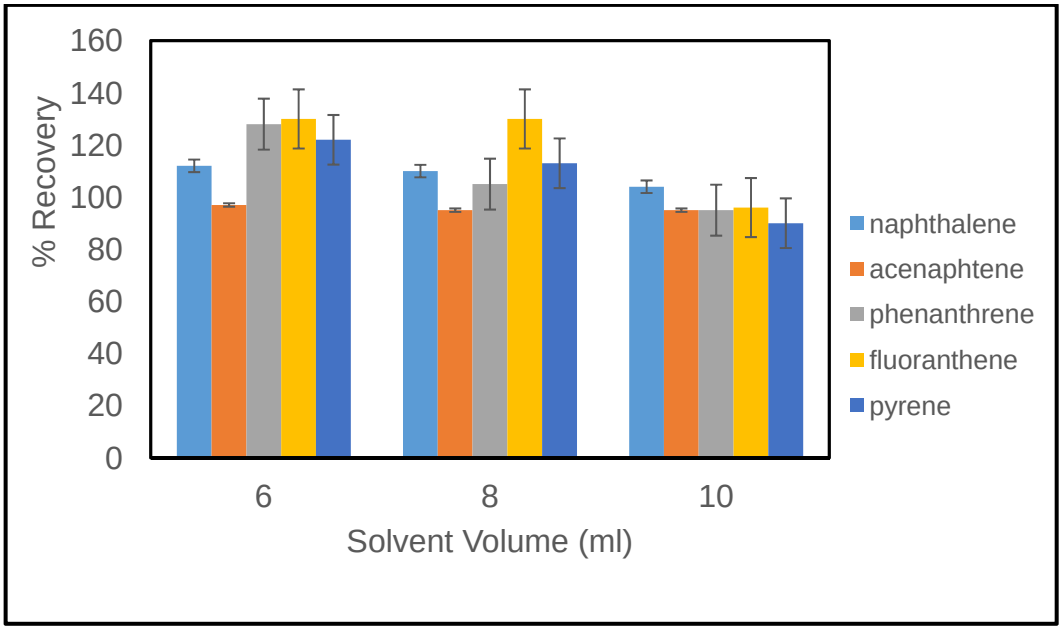

Figure 12a: Result obtained for varying volume of acetonitrile keeping other parameters constant

5.1.8 Type of sorbent used

The use of variety of cleaning sorbent gives cleaner sample (Lehotay et al., 2010). The sorbent used for extraction is very important, it can have various effect on recovery and selectivity. In this experiment, different combination of cleaning sorbents was applied in the extraction of fish sample. The effects of sorbent type were evaluated by analysing parameters such as recoveries and selectivity. Figure 13 shows the expected peaks for PAHs. Figure 14 shows the chromatogram obtained when 1uL of acetonitrile solvent was injected. This is done for quality assurance Injection of solvent washings from various sorbents (figures 15a-15e) shows that magnetite gave less selectivity while other sorbents were similar. Chromatograms of injection blank extracts from various sorbents also did not differ much in selectivity. Figure 16a-16e shows the chromatograms of non-spiked fish with different sorbent. The chromatogram is used for quality assurance. Figure 17.1a to 17.2e shows the chromatogram obtained for spiked fish using different types of solvent. However, for selectivity of spiked extracts, target PAHs were easily identified with PSA and multi-walled carbon nanotubes as sorbents.

Figure 18 compares the recoveries from various sorbents. There was not much difference in the recovery, PSA was therefore still taken as sorbent of choice. Molecular imprinted polymers (MIP) ideally should give best selectivity and high recovery because the extraction is based on specific interaction between the PAHs and polymers. In this case, improved selectivity was not observed, the reason could be due to the presence of water that reduced (MIP) performance.

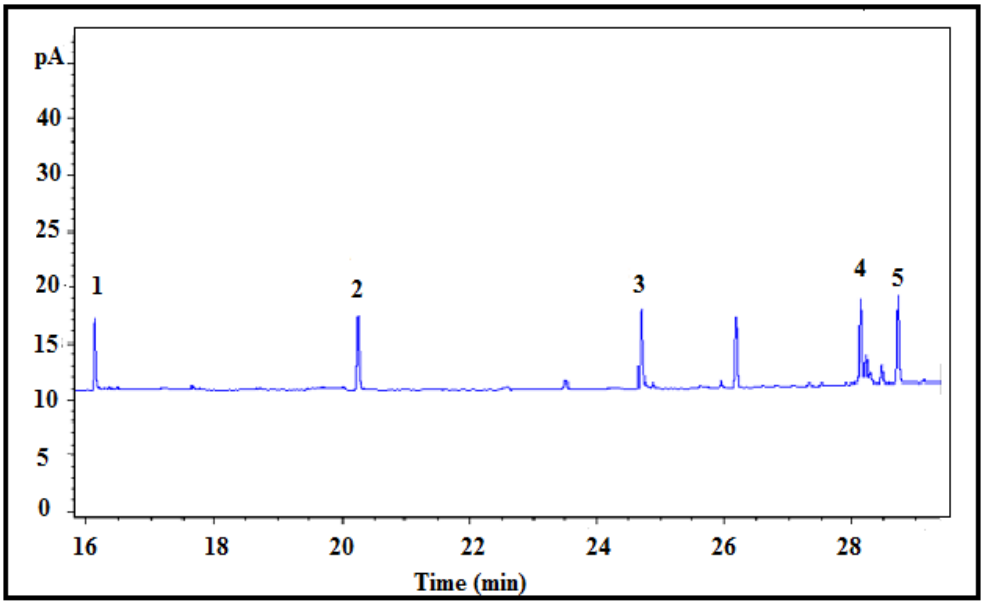

Figure 13: Chromatogram obtained for 1.0 mg/l standard PAHs solution: 1- naphthalene, 2- acenaphtene, 3- phenathrene, 4 - fluoranthene, 5- pyrene.

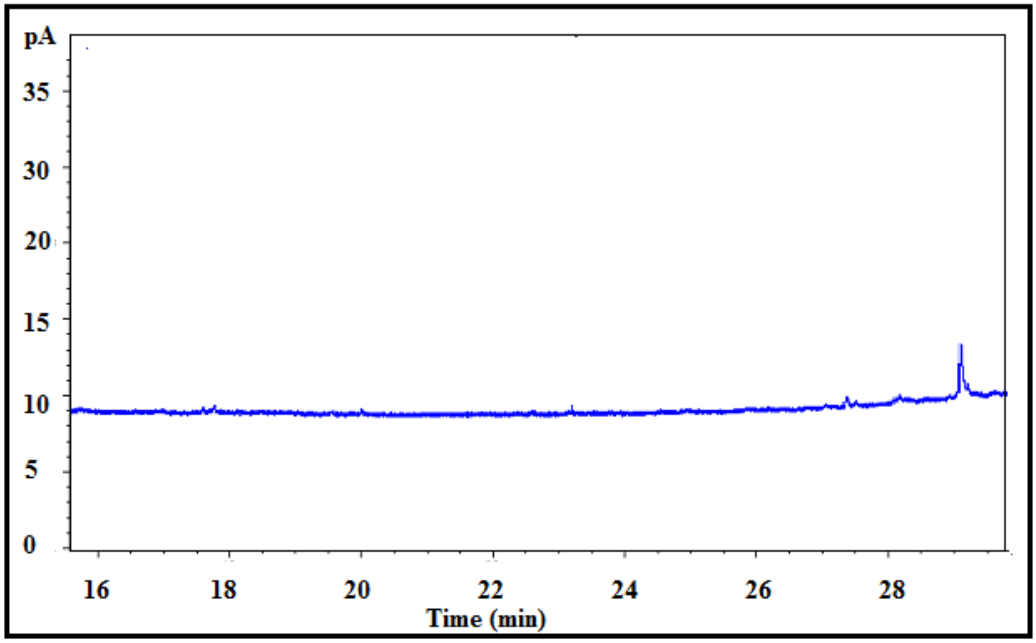

Figure 14 : Chromatogram for injection of acetonitrile solvent.

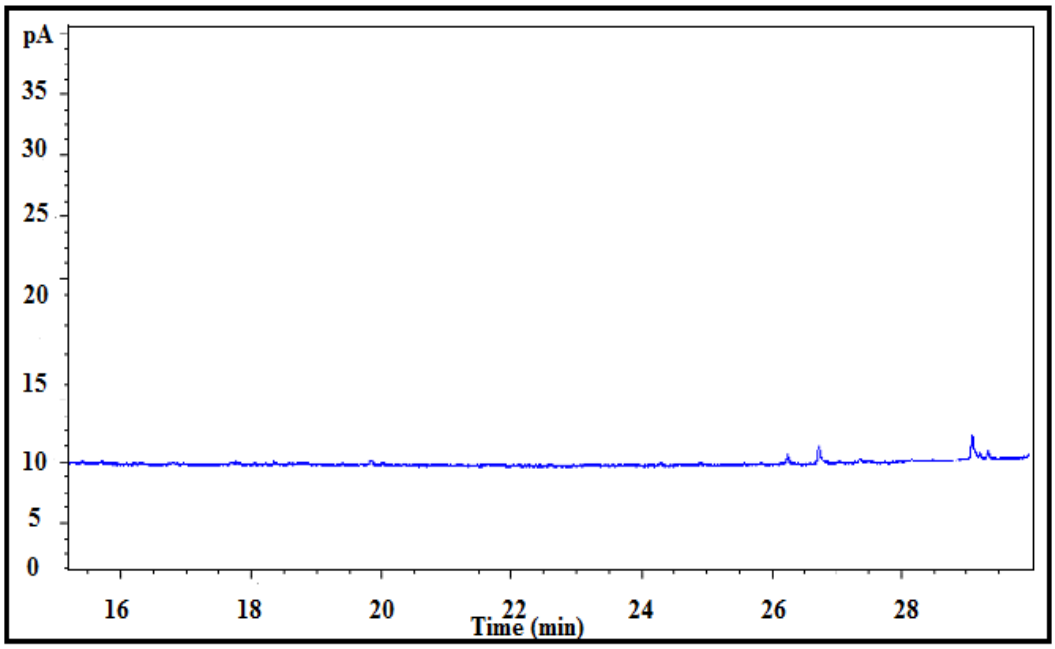

Figure 15a: Chromatogram of acetonitrile solvent with PSA

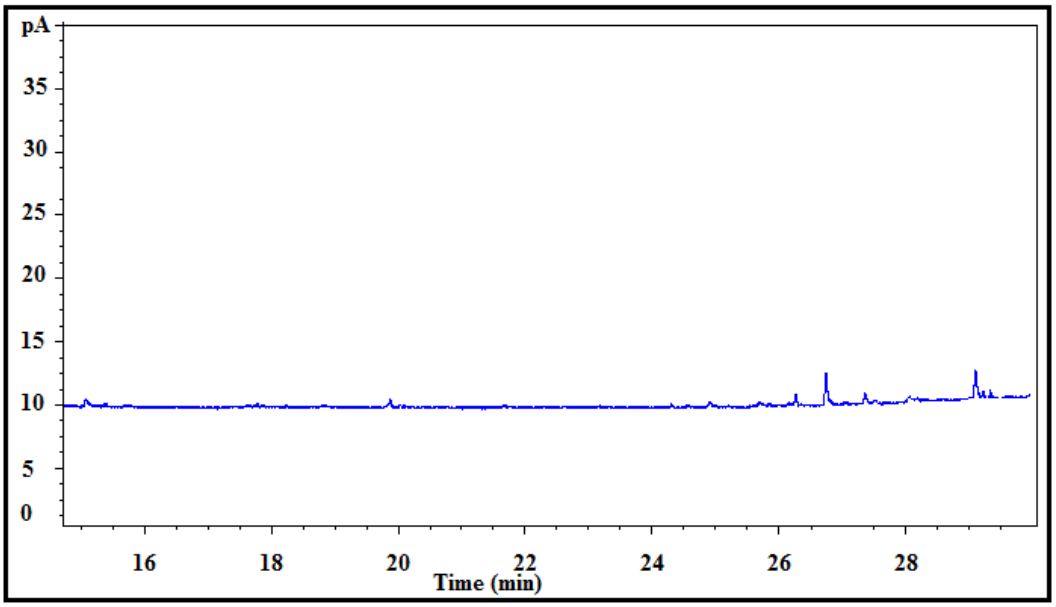

Figure 15b: Chromatogram of acetonitrile solvent with NIP

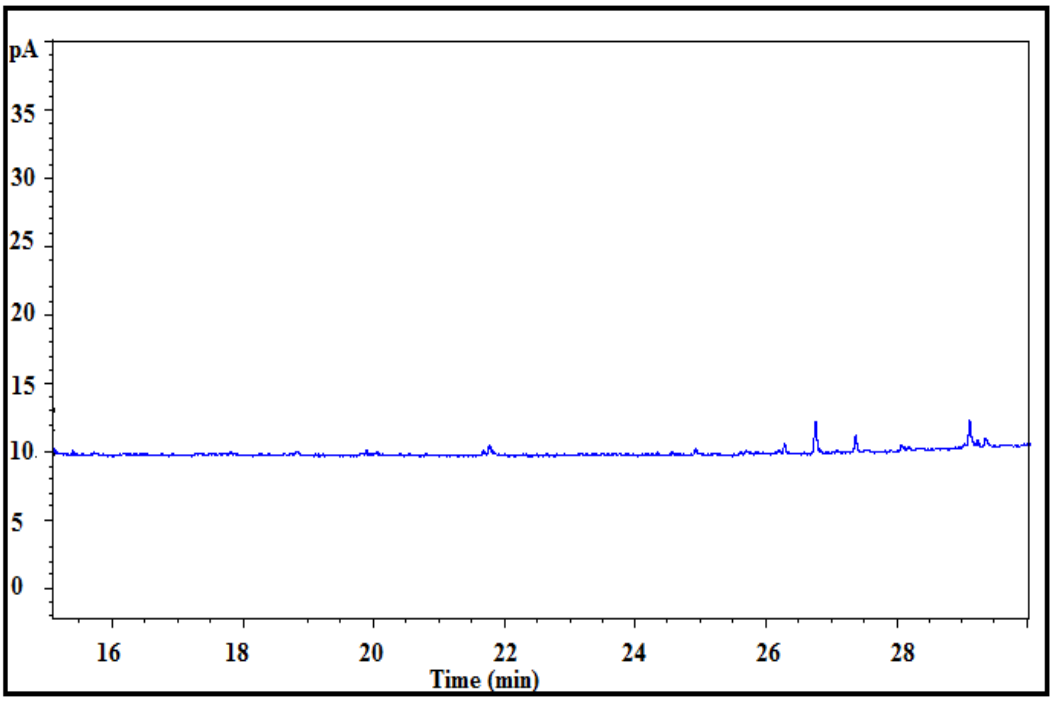

Figure 15c: Chromatogram of acetonitrile solvent with MIP

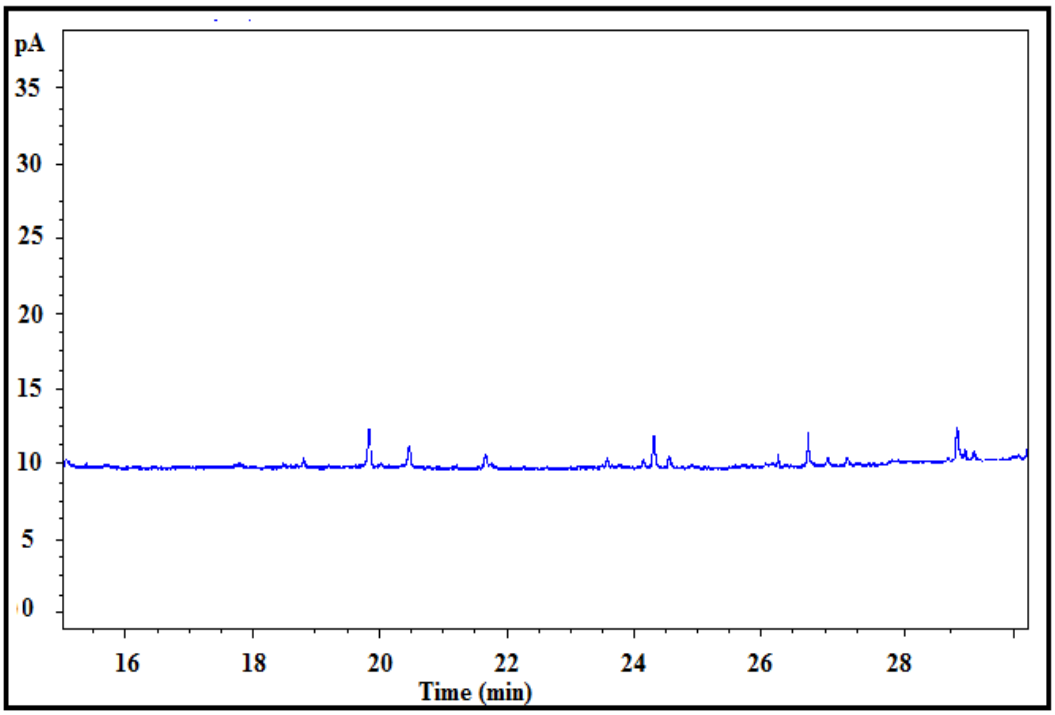

Figure 15d: Chromatogram of acetonitrile solvent with MAGNETITE

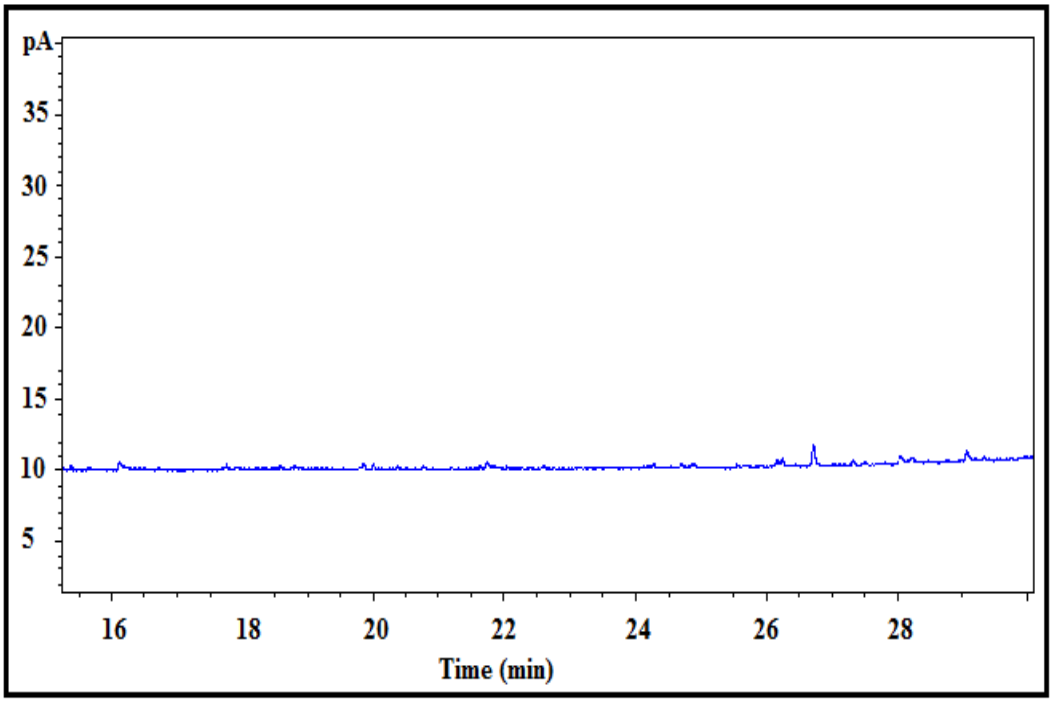

Figure 15e: Chromatogram of acetonitrile solvent with CARBON

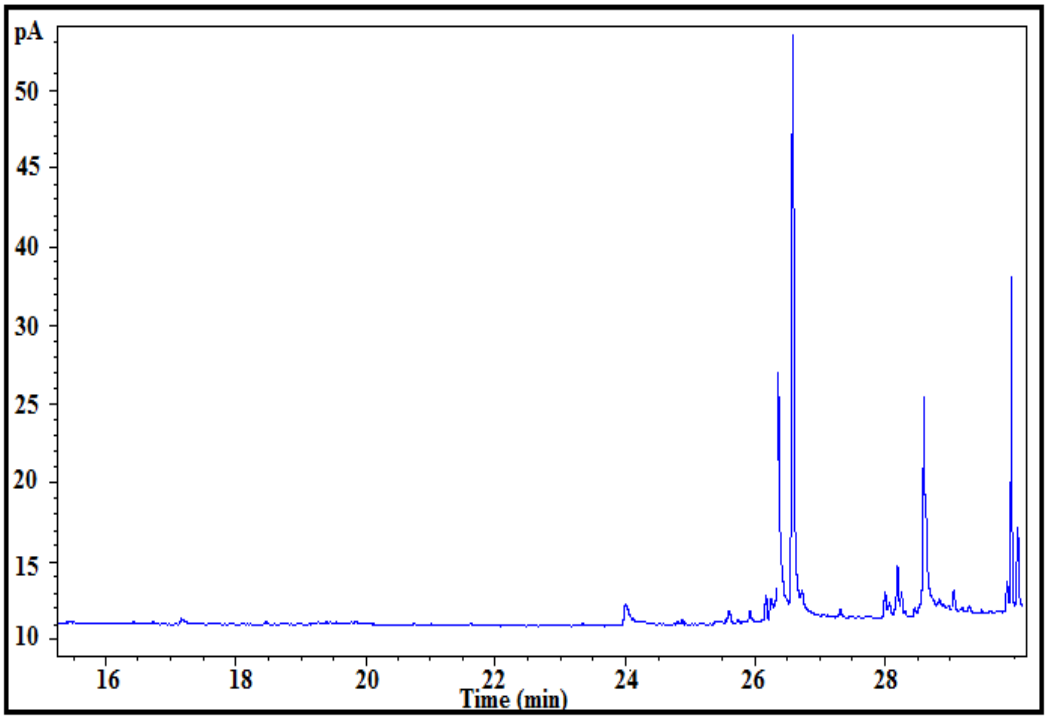

Figure 16a: Chromatogram of blank fish sample with PSA

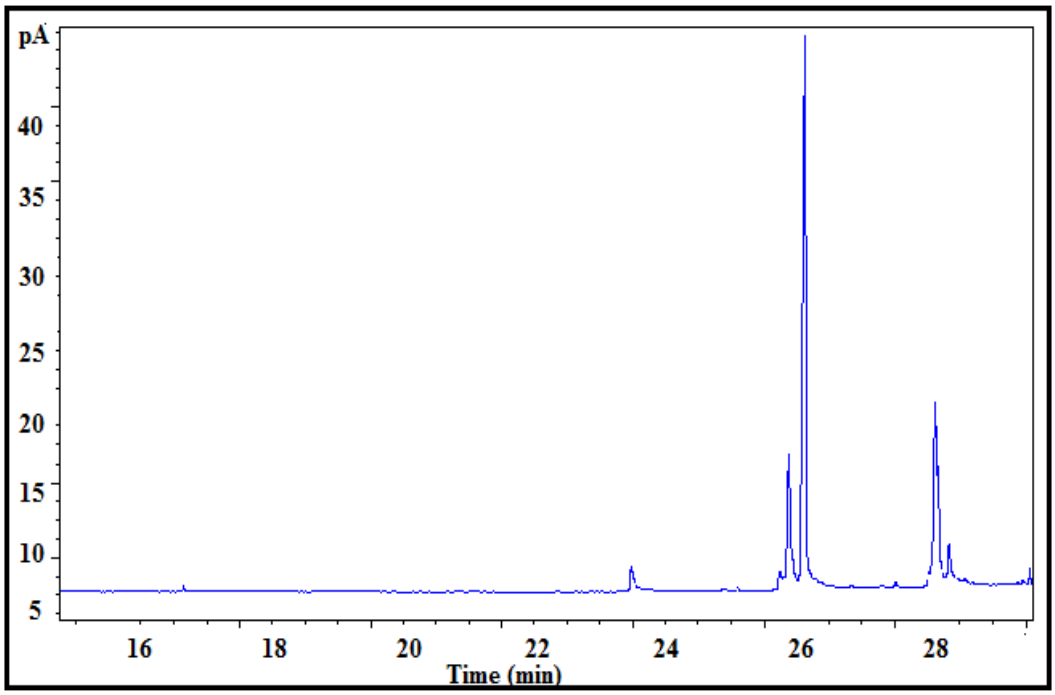

Figure 16b: Chromatogram of Blank fish sample with NIP

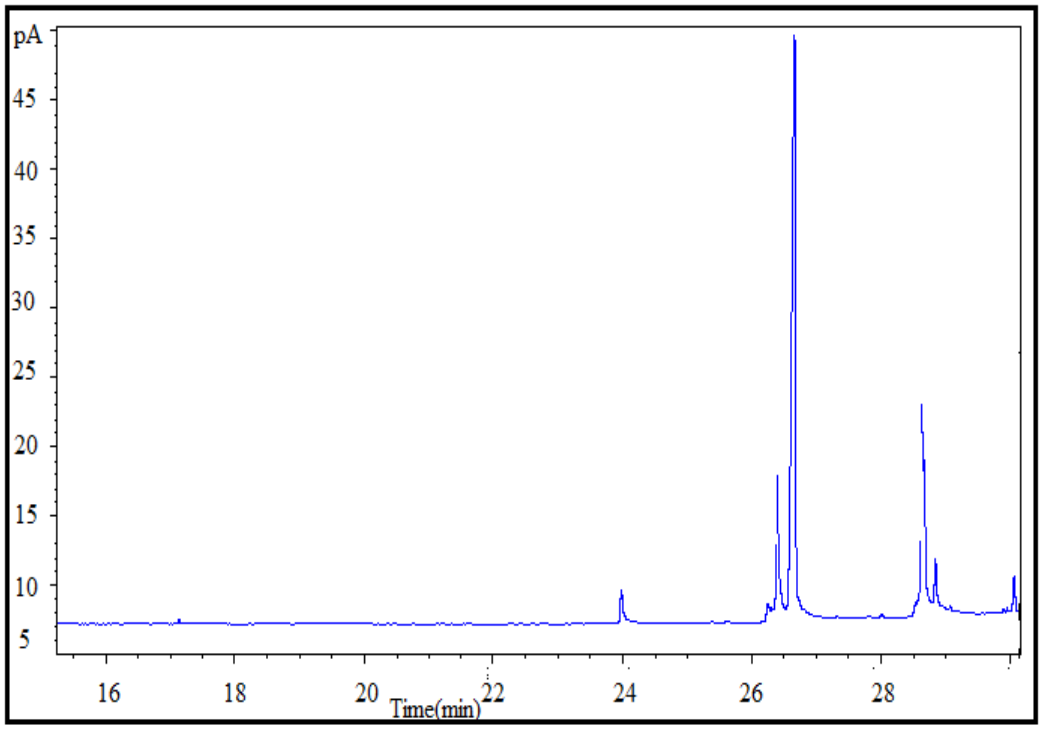

Figure 16c: Chromatogram of Blank fish sample with MIP

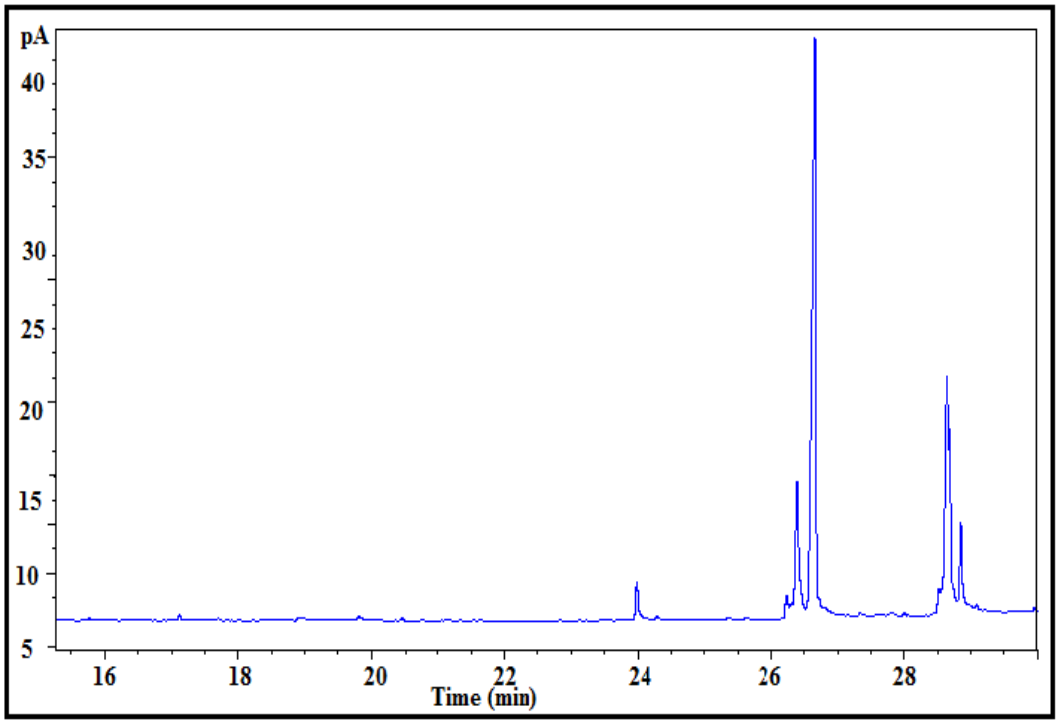

Figure16d: Chromatogram of blank fish sample with magnetite

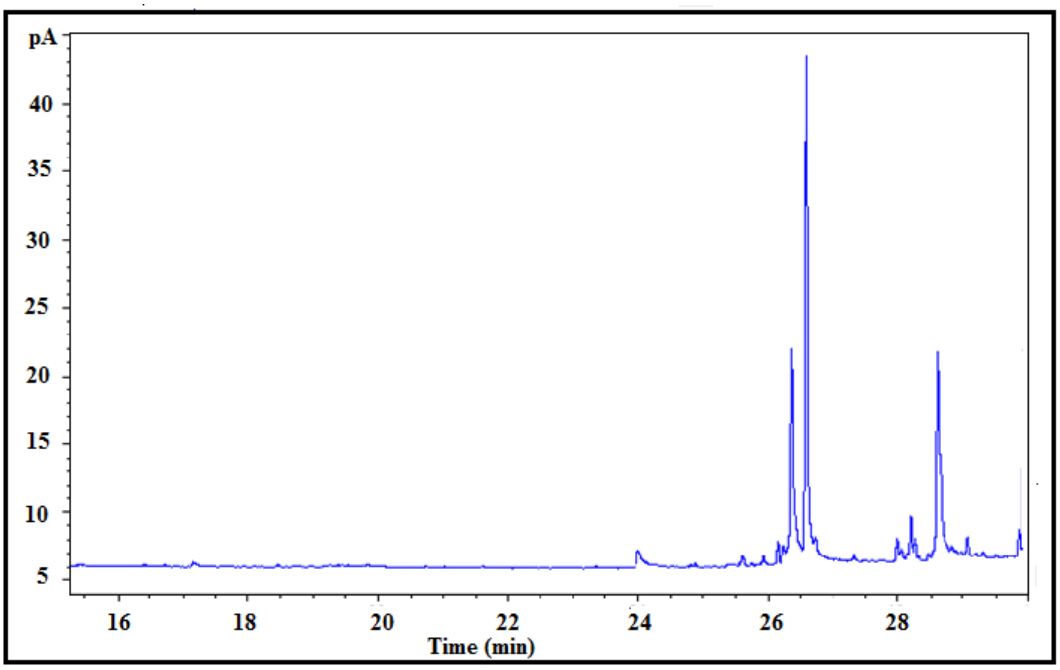

Figure16e: Chromatogram of blank fish sample with carbon

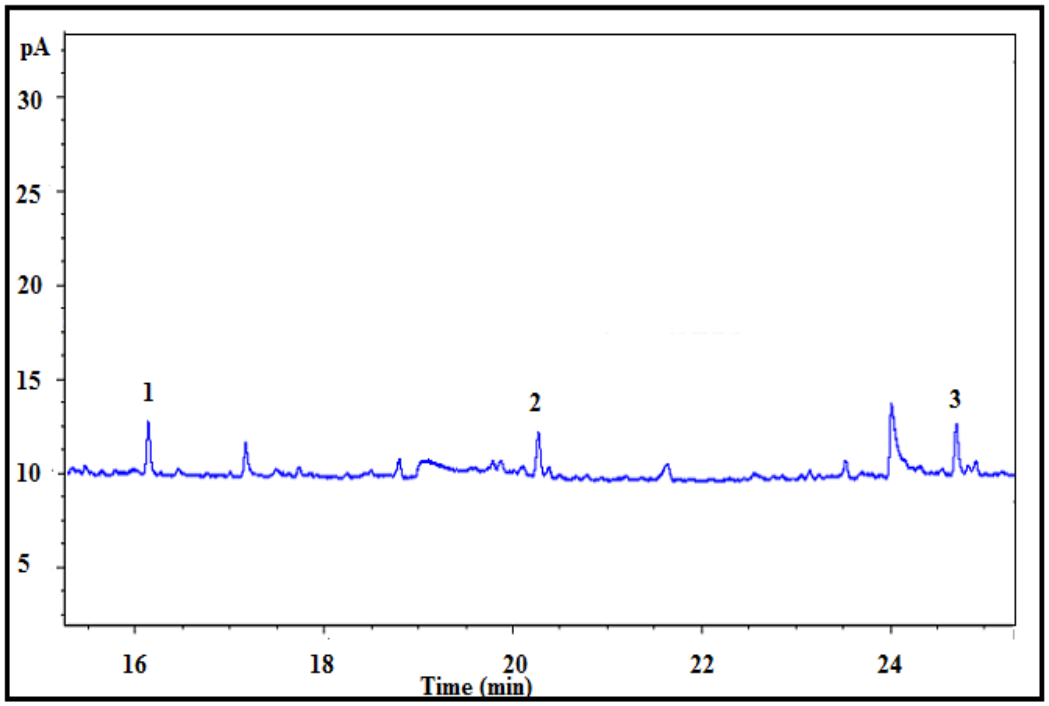

Figure 17.1a: Chromatogram of spiked fish sample with PSA

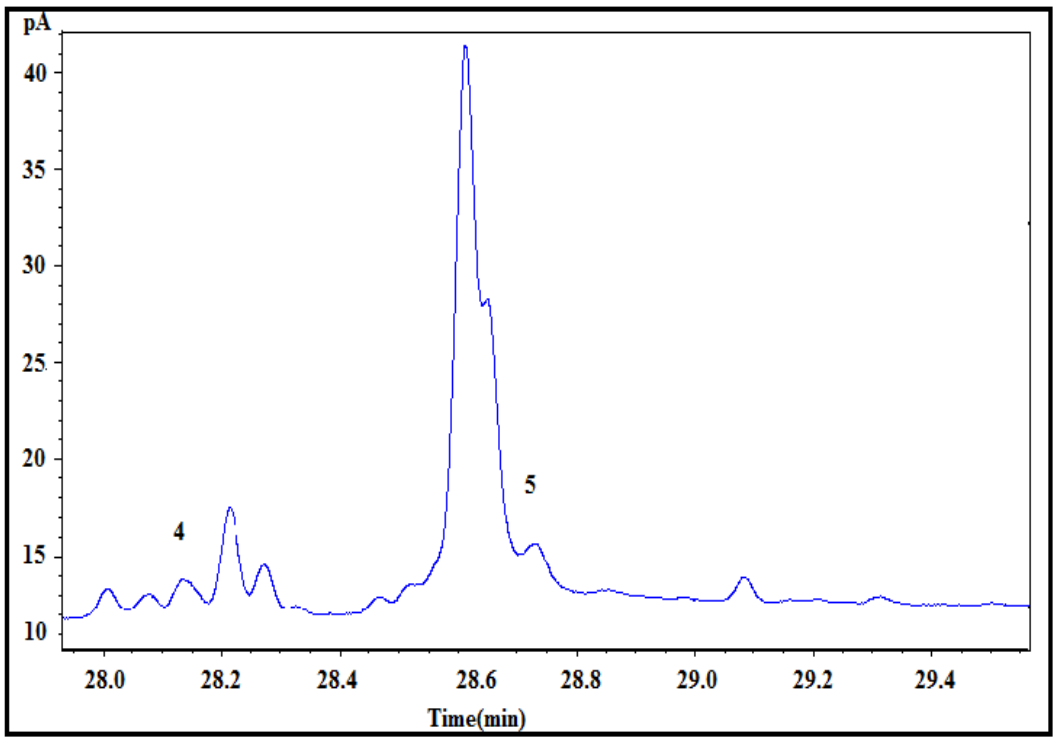

Figure 17.2a: Chromatogram of spiked sample with PSA continued

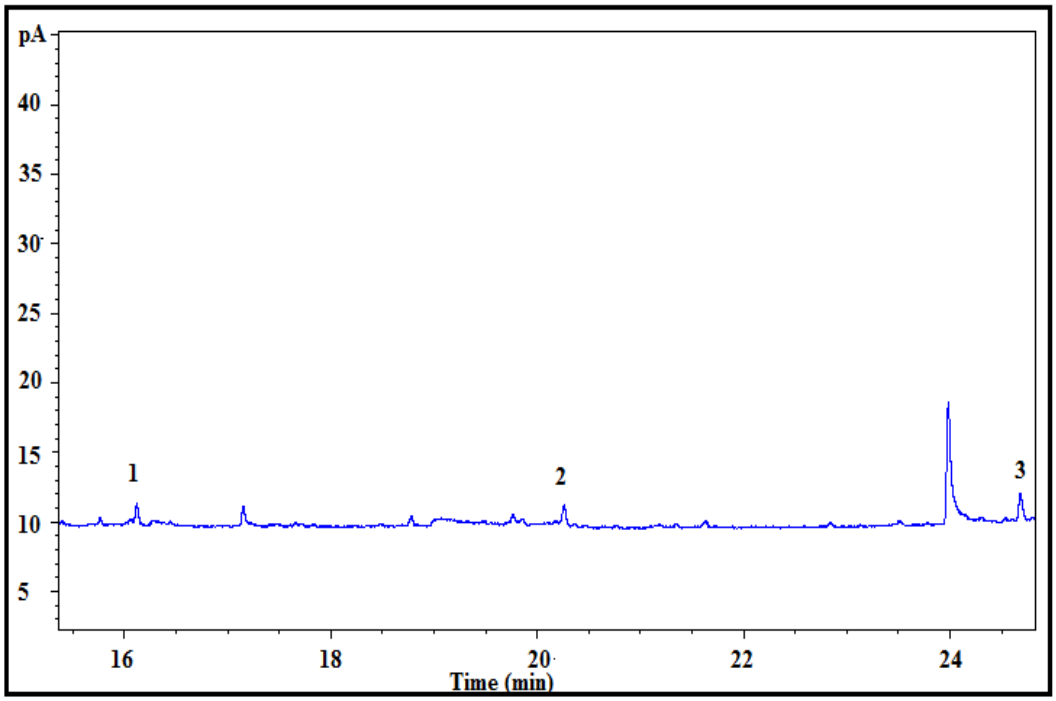

Figure 17.1b: Chromatogram of spiked sample with NIP

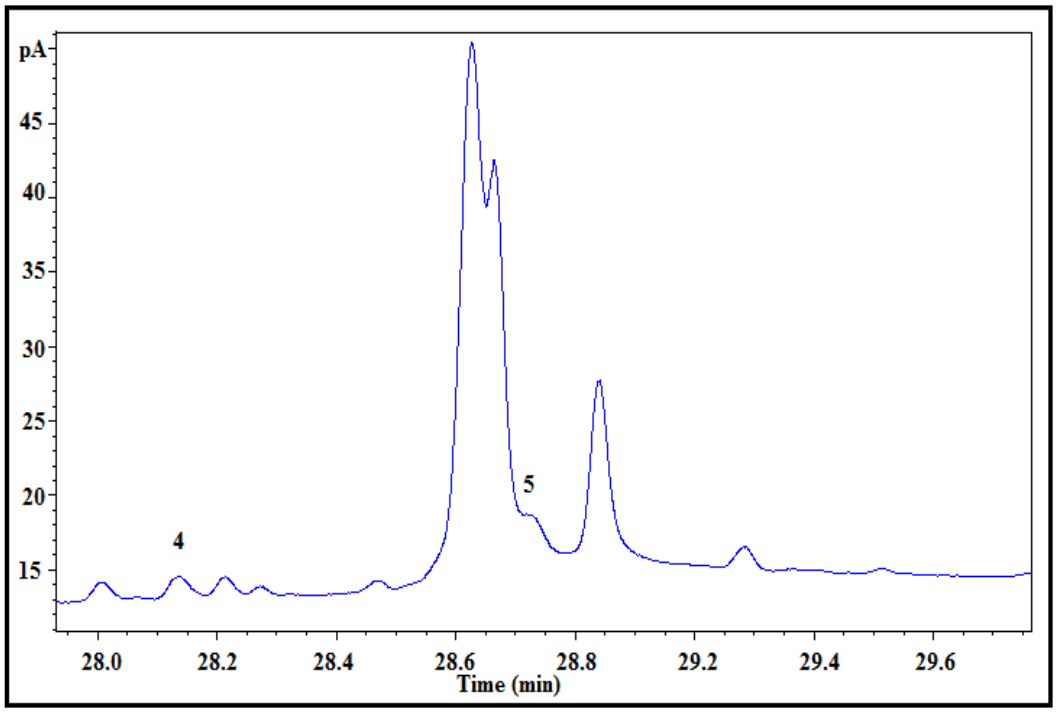

Figure 17.2b: Chromatogram of spiked sample with NIP 2 continued

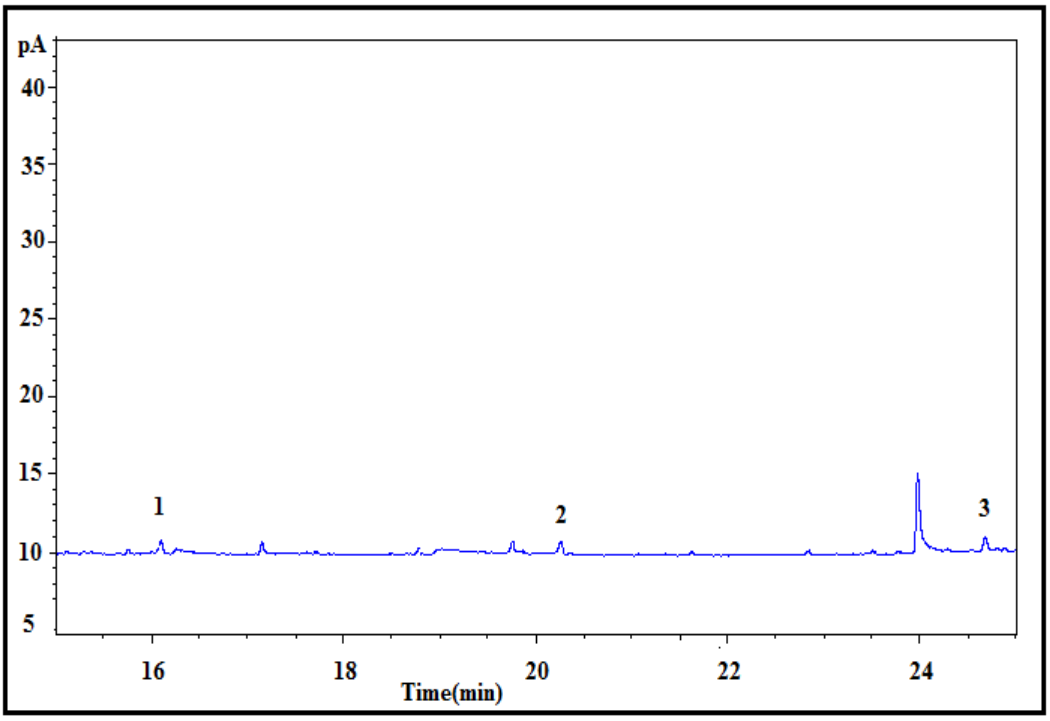

Figure 17.1c: Chromatogram of spiked sample with MIP.

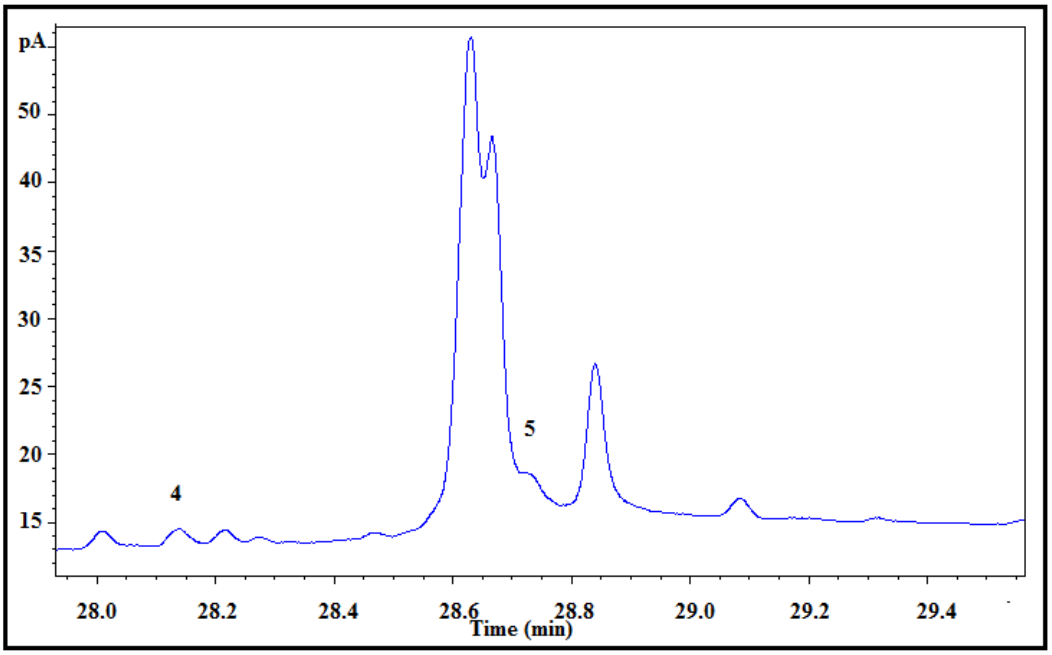

Figure 17.2c: Chromatogram of spiked sample with MIP continued.

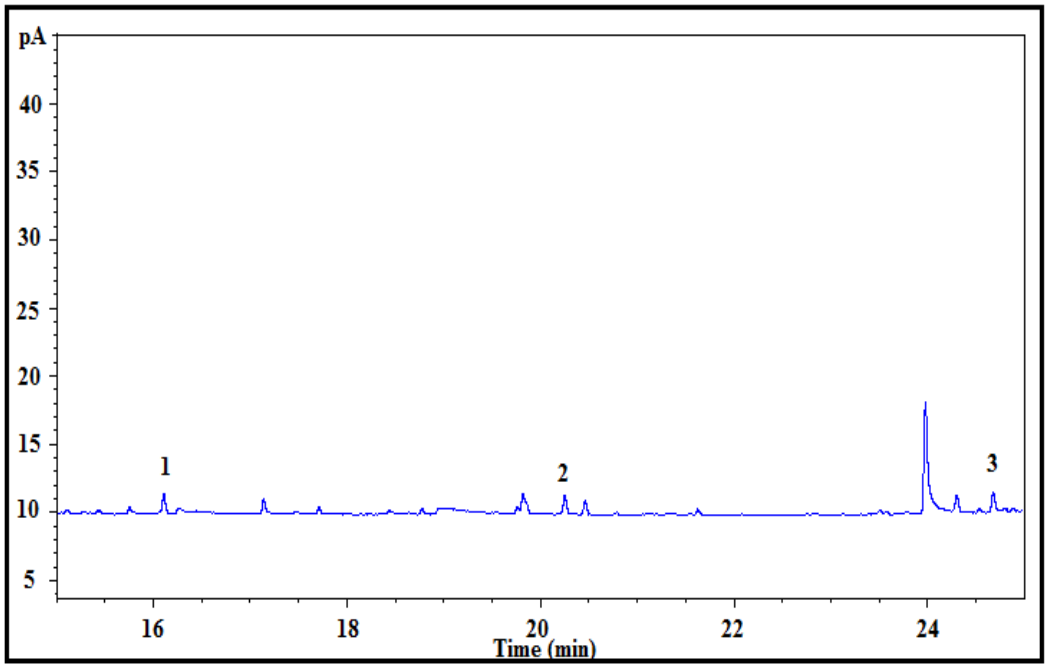

Figure 17.1d: Chromatogram of spiked sample with magnetite.

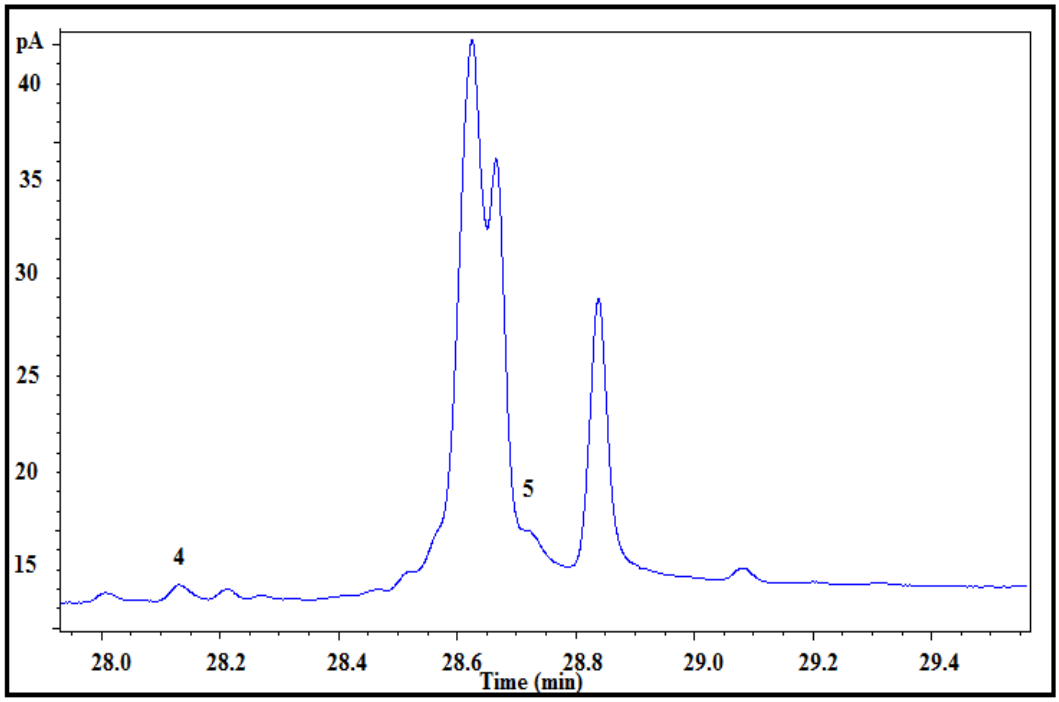

Figure 17.2d: Chromatogram of spiked sample with magnetite continued.

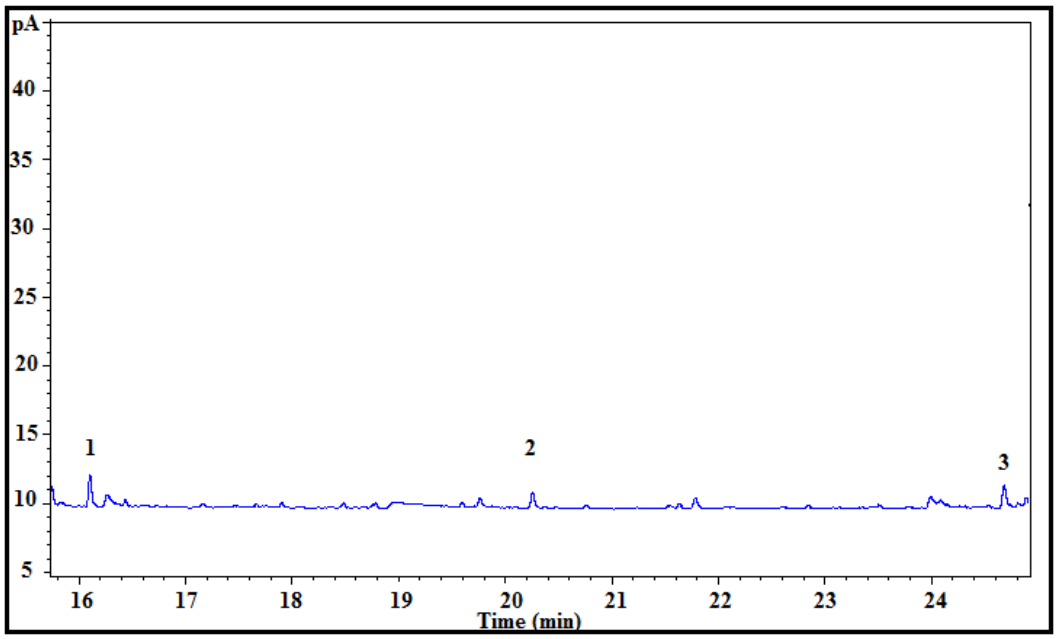

Figure 17.1e: Chromatogram of spiked sample with multi-walled carbon nano tube

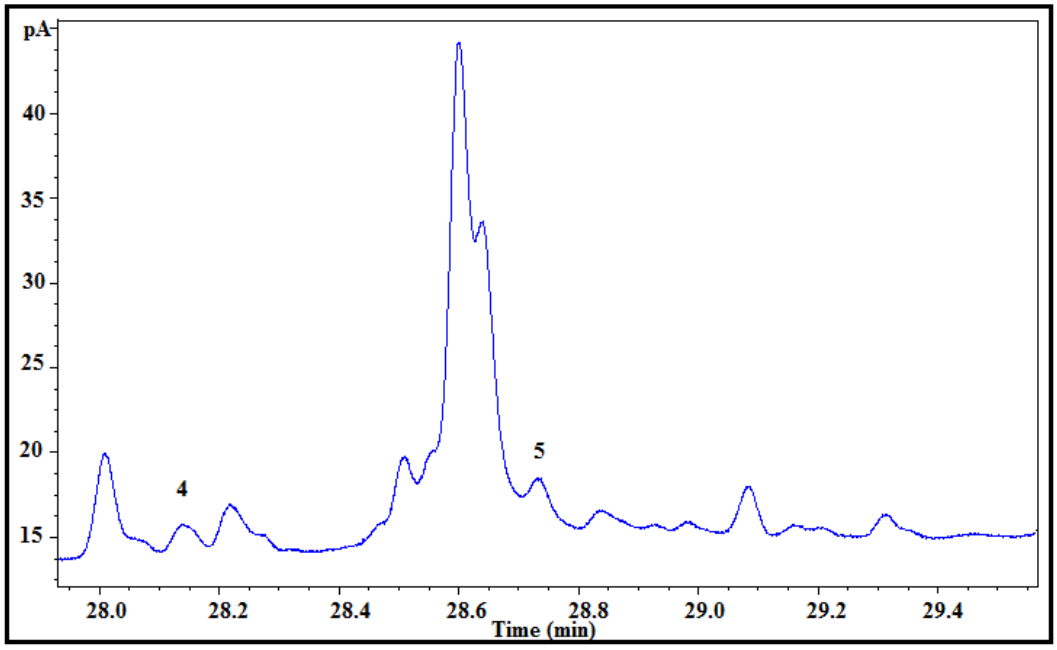

Figure 17.2e: Chromatogram of spiked sample with multi-walled carbon nano tube continued

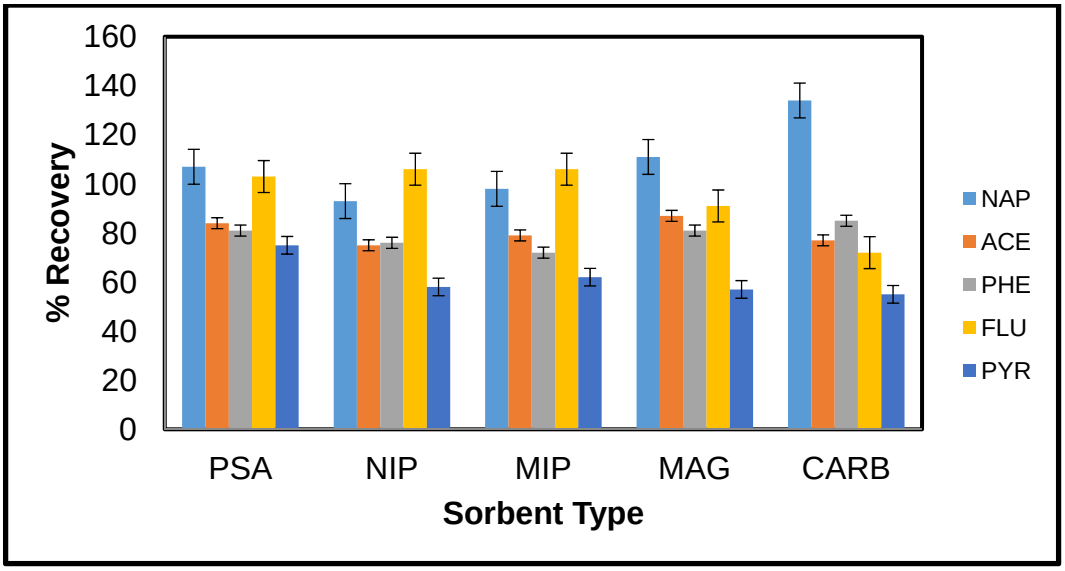

Figure 18: Comparision of recoveries obtained for different sorbent in QuEChERS extraction method

5.2 Comparison of recoveries and reproducibility of QuEChERS and Soxhlet extraction method

Figure 19a shows the peak area obtained for different spiking concentration for Soxhlet extraction method. As the spiking concentration increases, the peak area increases. This trend is expected for validation of the method and the quantification of PAHs in real sample. Figure 19b shows the percentage recovery obtained. As expected and observed the recovery obtained is independent on the sample concentration. Figure 20a and 20b shows the peak area obtained for optimised QuEChERS extraction method and recoveries respectively. As spiking concentration increases, the peak area increases and recovery obtained is independent of the spiking concentration. This trend is expected for validates and its application to real sample. Recoveries obtained for QuEChERS extraction method at different concentrations were from 80% to 140%, while those obtained for Soxhlet extraction method were from 60% to 100%. Vives et al., (2002) used Soxhlet extraction method to analyse PAHs in fish liver, recoveries obtained was 77.5%-99%. Kalachova et al., (2011) used the QuEChERS method in the analysis of PAHs in fish and shrimps, recoveries obtained were between 76-120%. QuEChERS method is known to give a high recovery. Although QuEChERS method gave a higher recovery, both method a suitable for PAHs extraction.

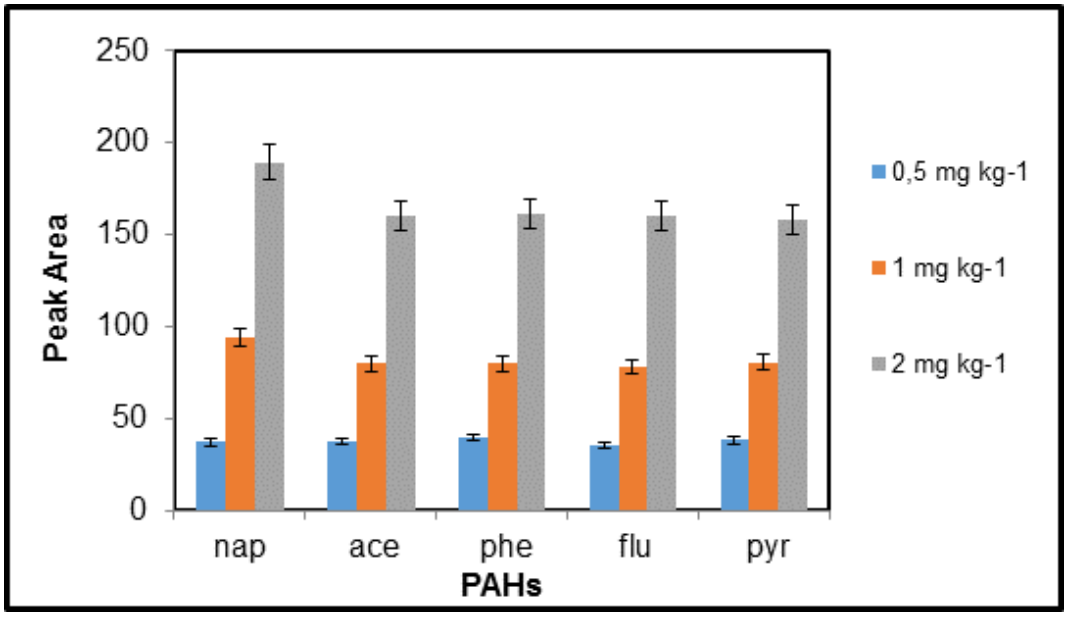

Figure 19a:Peak area obtained for different spiking concentration for Soxhlet extraction method with GC-FID

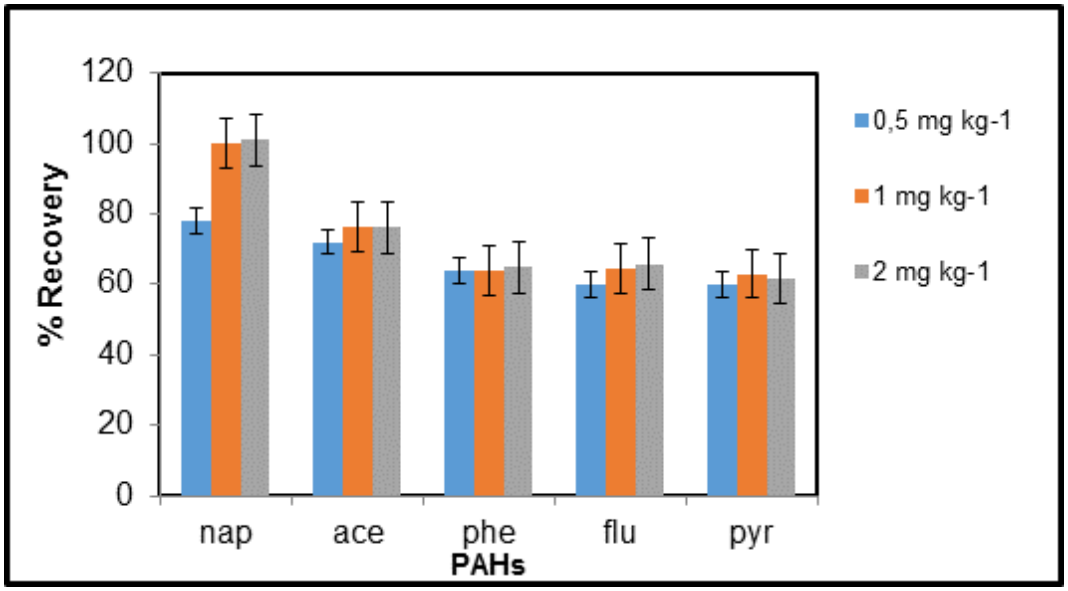

Figure 19b: Recoveries obtained for Soxhlet extraction method with GC-FID

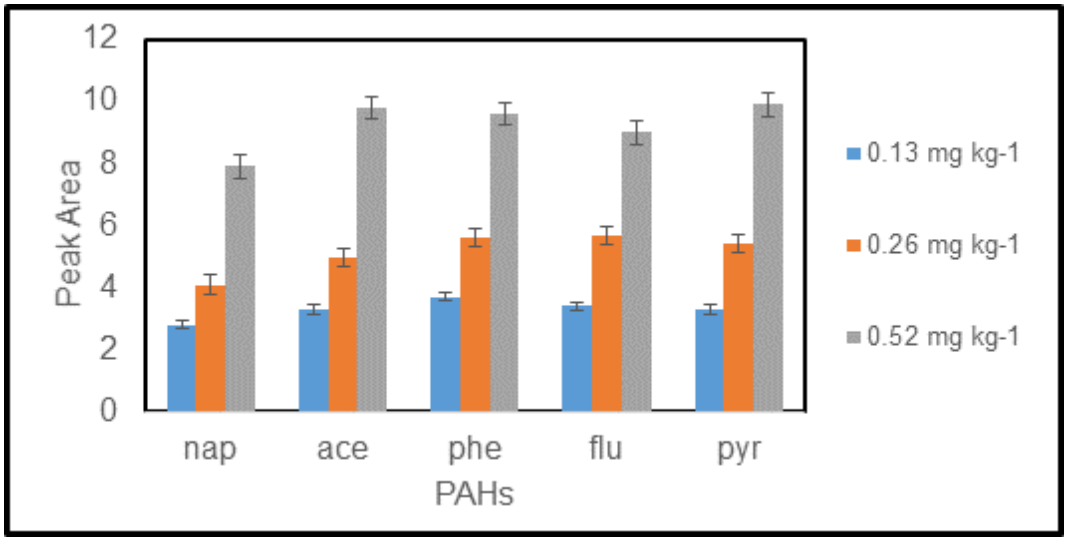

Figure 20a: Peak area obtained for different spiking concentration for optimised QuEChERS extraction method with GC-FID

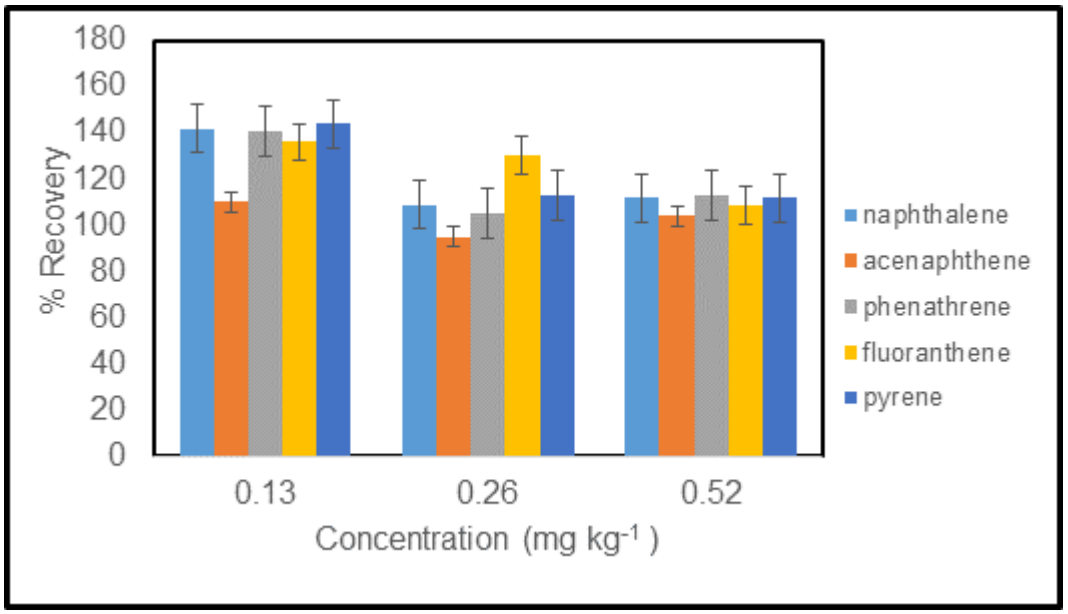

Figure 20b: Recoveries obtained for QuEChERS extraction method at optimised conditions with GC-FID

5.3 Application of QuEChERS method and Soxhlet method to fish sample

5.3.1 Application of developed QuEChERS methods

The developed QuEChERS method was applied to real fish samples obtained from Jericho dam Amsterdam Mpumalanga and Hartbeespoort dam Gauteng province. Table 11 shows the concentration levels of PAHs obtained in fish samples. From the data obtained, acenaphthene and phenanthrene were not detected in fishes from Jericho dam. This might be due to the fact that the concentration level for these PAHs were very low as the mass of fish sample used in the QuEChERS method is very small. The highest concentration of PAHs discovered in Jericho dam was that of fluoranthene which range from 0.8 to 7.4 μg kg^{-1}. Fluoranthene and Naphthalene were discovered in all fish samples taken from Jericho dam. Concentration of naphthalene range from 1.6 to 1.8 μg kg^{-1}. Pyrene was detected in J1 and J2 with concentration of 1.2 and 1.1 μg kg^{-1} respectively. Pyrene was not detected in J3 and J4.

The data obtained for PAHs concentration in Hartbeespoort dam showed that Naphthalene was not discovered in fish sample from the dam, phenanthrene has highest concentration of PAHs ranging from 641.4 to 739 μg kg^{-1}. Acenaphthene has concentration ranging from 37.7 to 50.3 μg kg^{-1}, fluoranthene and pyrene have the lowest concentration of 0.91 to 6.11 μg kg^{-1} and 1.5 to 2.0 μg kg^{-1} respectively.

Figure 21 shows a plot of concentration of PAHs against fish length for pyrene and acenaphthene from the fish samples obtained from Hartbeespoort dam. The concentration of pyrene do not show a significant increase as the fish length increases, but the concentration of acenaphthene increased significantly as the fish length increases. It can therefore be deduced that concentration of PAHs depends on the size of fish. It can also be deduced from the data that PAHs accumulation does not depend only on fish size, but it can also depend on the contamination level of site, as fish samples from Hartbeesport dam has higher PAHs concentration in some fish samples which were obviously smaller in size than those fish samples obtained from Jericho dam.

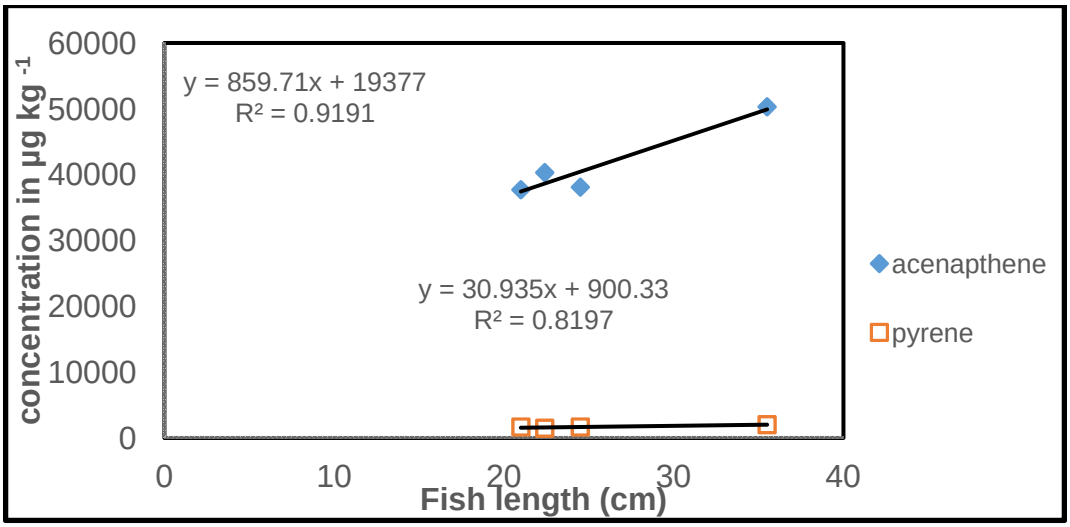

Figure 21: Influence of fish age on bioaccumulation of PAHs.

Table 11: Concentration (μg kg^{-1}) obtained from fish samples from Jericho dam Mpumalanga and Hartbeespoort dam Gauteng using QuEChERS method with HPLC-FL

Sample site	Fish length	Fish breadth	PAHs				
			Nap	Ace	Phe	Flu	Pyr
J1	50	34	1.6 (0.03)	nd	nd	1.4 (0.13)	1.2 (0.09)
J2	51	34	1.8(0.01)	nd	nd	2.2 (0.26)	1.1(0.005)
J3	52	36	1.8(0.006)	nd	nd	7.4 (0.03)	nd
J4	45	24	1.7 (0)	nd	nd	0.8 (0.02)	nd
HPB 1	21	7.0	nd	37.7	683.6(0.92)	0.91(13.24)	1.7 (3.87)
HPB 2	22.4	7.6	nd	40.3	739 (0.50)	4.7 (11.3)	1.5(7.97)
HPB 3	24.5	8.6	nd	38.1	717.2(4.93)	3.72 (8.65)	1.7 (5.28)
HPB 4	35.5	12.5	nd	50.3	641.4(1.15)	6.11(10.54)	2.0 (2.18)

*Number of replicate is 3, the numbers in brackets shows the standard deviation, J means Jericho dam, HPB means Hartbeespoort dam. nd means not detected.

5.3.2 Application of Soxhlet method to real sample

Table 12 shows the concentration levels of PAHs obtained by Soxhlet extraction method from same fish samples taken from Jericho dam in Amsterdam Mpumalanga and Hartbeespoort dam Gauteng. The same trend was obtained as in QuEChERS extraction method. Concentration of fluoranthene was the highest for fish samples obtained from Jericho dam it ranged from 14.4 to 18.7 µg kg^{-1}. This is followed by naphthalene with concentration ranging from 4.8 to 7.6 µg kg^{-1}. Acenaphthene has the lowest concentration of 1.2 to 1.4 µg kg^{-1}. Phenathrene which is not as low as acenaphthene has concentration from 2.2 to 2.4 µg kg^{-1}. Pyrene was not detected in fish J 2 and J 4. Phenathrene and Acenaphthene were not detected in fish J4. The concentration of fluoranthene is highest in fish J2. Just as in the QuEChERS method, there is an indication that the oldest fish has the highest concentration of fluoranthene. The PAHs concentration obtained in Hartbeespoort dam were as follows, phenathrene 838.5 to 908 µg/kg, acenaphthene 38.8 to 55.2 µg kg^{-1}, fluoranthene 1.91 to 6.11 µg kg^{-1}, pyrene 4.5 to 7.8 µg kg^{-1}, napthalene was not detected. These values do not differ much from the QuEChERs method this is discussed in more details under 5.3.3

Table 12: Concentration (μg kg^{-1}) obtained from fish samples from Jericho dam Mpumalanga and Hartebeesport dam Gauteng using soxhlet extraction method with HPLC-FL

Sample site	Fish length	Fish breadth	PAHs				
			Nap	Ace	Phe	Flu	Pyr
J1	50	34	4.8 (0.03)	1.3	2.4	14.4 (0.13)	1.2 (0.09)
J2	51	34	6.8 (0.01)	1.2	2.4	16.7 (0.26)	nd
J3	52	36	7.6(0.006)	1.4	2.2	18.7 (0.03)	1.8
J4	45	24	6.4 (0)	nd	nd	16.7 (0.02)	nd
HPB 1	21	7.0	nd	38.8(0.58)	838.5(0.97)	1.91(2.17)	5.2(0.07)
HPB 2	22.4	7.6	nd	43.2(2.27)	890(7.17)	9.4(0.15)	4.5(0.38)
HPB 3	24.5	8.6	nd	46.3(0.37)	908.5(0.73)	6.11(0.008)	6.2(0.95)
HPB 4	35.5	12.5	nd	55.2(2.37)	902.6(3.76)	6.11(1.07)	7.8(4.07)

*Number of replicate is 3, the numbers in brackets shows the standard deviation, J means Jericho dam, HPB means Hartbeespoort dam. nd means not detected

5.3.3 Comparison of result obtained from application of QuEChERS and Soxhlet method

Figure 23 shows chromatogram obtained using HPLC-FL and Figure 22 shows a correlation plot of the QuEChERS and the Soxhlet method. The correlation value obtained is above 0.9, this shows that both methods are suitable for PAHs extraction in fish sample.

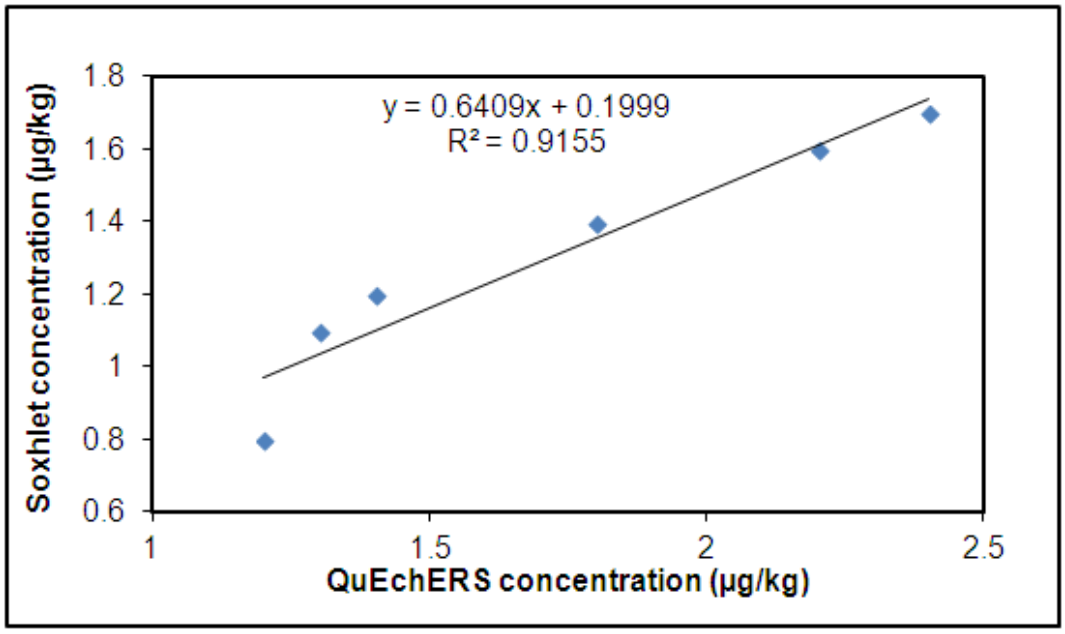

Figure 22: Comparison of QuEChERS and Soxhlet results obtained from fish samples from Jericho dam.

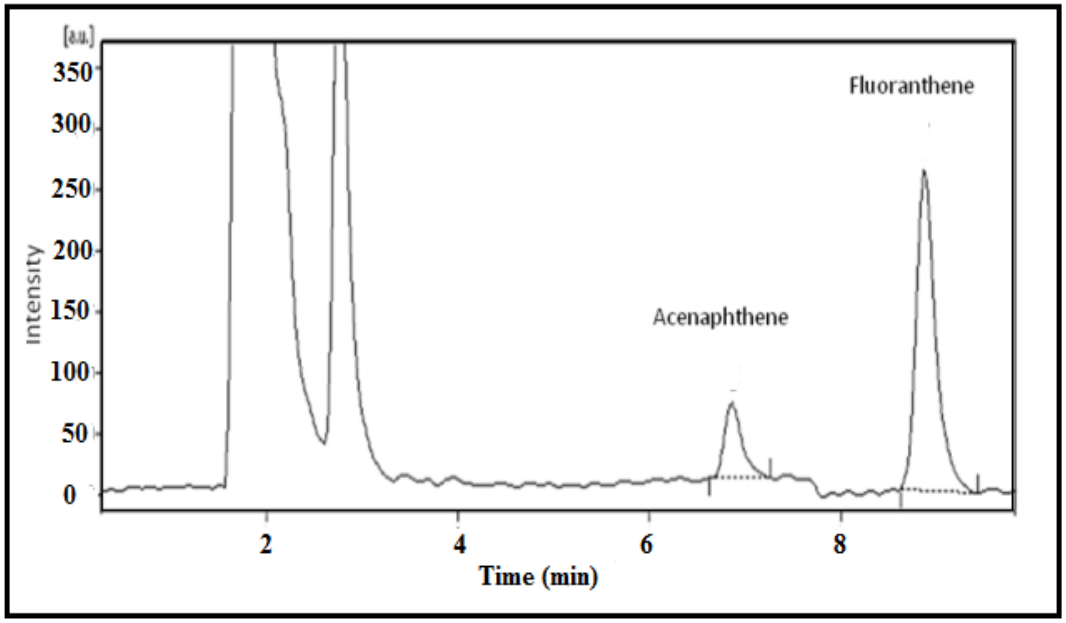

Figure 23: Chromatogram of PAHs obtained from QuEChERS extraction method.

5.3.4 Comparison of obtained results with regulating agencies

The regulation limits for the PAHs studied are not available although there are information on acceptable limits for others by the EU such as benzo(a)pyrene which is 5 µg $kg^{-1}$for smoked fish and 2 µg kg^{-1}. In this study, the minimum PAHs obtained was 0.8 µg kg^{-1}fluoranthene and the maximum was 909 µg $kg^{-1}$for acenaphthene. The 0.8 µg kg^{-1} is within acceptable taking the value for smoked fish, while that of acenaphthene is well above the acceptable limit.

CHAPTER 6

6.1 Conclusion

In any analytical process, the extraction method is very important. The extraction method must work at its optimal in the extraction of analyte from sample. In this work a relatively new method the QuEChERS has been applied in the extraction of PAHs. The QuEChERS method was optimised, this is very necessary when applying any analytical method as parameters such time of extraction, extraction speed, sample mass, solvent volume can all affect extraction efficiency. The optimised conditions were then applied in the extraction PAHs to real sample. From the result obtained the QuEChERS method has proven to be very effective in the extraction of PAHs from fish muscle. This method has also shown to utilise very minimal amount of solvent and it also use less time. Using the QuEChERS method, about 20 samples can be analysed in less than an hour. Clean up after extraction was very easy because minimal amount of instruments were used. The QuEChERS method was also compared to the Soxhlet method. The method was applied to real sample using optimised method from literature. The soxhlet method was effective in extraction of analyte from sample. Some PAHs were recovered using the Soxhlet extraction method while they were not recovered when the QuEChERS method was used. This could have been due to the amount of sample used as more sample is used in the Soxhlet method. The Soxhlet method is more time consuming than the QuEChERS method as a single extraction will take about 20 hours for the Soxhlet method while the QuEChERS method will take about 20 min. More apparatus are needed for the soxhlet extraction method. The volume of solvent used for Soxhlet extraction method is about 20 times that of the QuEChERS method. Only 10 ml of solvent was used for QuEChERS extraction while about 200 ml was used for Soxhlet extraction method. In the Soxhlet extraction method, a change of solvent is required this could also lead to loss of analyte and sometimes very challenging. The rotor vapour is used for evaporation if not carefully handled, some of the solvent with analyte with solvent is sucked up the rotor vapour. The work shows that both the QuEChERS method and the Soxhlet method are both effective in the extraction of PAHs from fish sample.

6.2 Recommendation and future work

Fish sample from more dams and rivers should be analysed using optimised QuEChERS method as the method has proven to be effective and efficient. This will help to acquire data on PAHs contamination of various dams and rivers across South Africa. More so, the method can analyse many samples over a short period of time. That means more samples can be analysed using this method.

PAHs can be extracted from other part of the fish such as the liver and the gall bladder other than the fish muscle using the optimised QuEChERS method. According to previous studies, the amount of PAHs in liver is about 100 times greater than that of fish muscle (Varanasi et al 1991). This can actually enable the determination of level of PAHs concentration as this can affect other organism apart from humans which consume the fish with the liver.

The QuEChERS extraction method can be compared to other extraction method such as the microwave assisted method (MAE), pressurised fluid extraction (PLE), ultrasonic extraction method in the extraction of PAHs from fish sample. This will enable more insight into the efficiency and effectiveness of the QuEChERS method to extract PAHs from fish samples compared with other methods.

Further work should be done to include analysing the heavier PAHs using QuEChERS extraction method as these PAHs are most toxic. This analysis could not be included in this study because there is no gradient elution HPLC system that can allow separation of as many compounds as possible.

7 References

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