CHAPTER ONE – INTRODUCTION

1.1 Environmental Pollution

Environmental pollution is the introduction of contaminants (physical and biological components) into the environment (e.g. air, water and soil) from man-made waste that causes instability, disorder, harm or discomfort to the ecosystem i.e. physical systems or living organisms to such an extent that normal environmental processes are adversely affected, which comes in the form of chemical substances, or energy such as noise, heat or light. Pollutants, the elements of pollution, can be naturally occurring substances or energies, but are considered contaminants when in excess of natural levels (Wikipedia, 2010a).

There are three major types of environmental pollution which are air, water and soil. Air pollution defines as the release of chemicals and particulates into the atmosphere. Some of the most important air pollutants are sulphur dioxide, nitrogen dioxide, carbon monoxide, ozone, polycyclic aromatic hydrocarbons (PAHs), volatile organic compounds (VOCs) and airborne particulate matter (PM) which is characterized by their micrometre size PM_{10} to $PM_{2.5}$, with radioactive pollutants probably among the most destructive ones (specifically when produces by nuclear explosions). Water pollutants is the release of waste products and contaminants as surface runoff into river drainage systems, leaching into groundwater, liquid spills, wastewater discharges, eutrophication and littering which include insecticides and herbicides, food processing waste, pollutants from livestock operations, VOCs, heavy metals, chemical waste and others. Soil contamination occurs when chemicals are released by spill or underground leakage. Among the most significant soil contaminants are hydrocarbons, heavy metals,

methyl *tert*-butyl ether (MTBE), herbicides, pesticides and chlorinated hydrocarbons (Wikipedia, 2010a).

Other types include radioactive pollution, chemical, invasive species pollution, light, noise, thermal and visual pollution. Radioactive contamination resulted from 20th century activities in atomic physics, such as nuclear power generation and nuclear weapons research, manufacture and deployment. Noise pollution encompasses roadway noise, aircraft noise, industrial noise as well as high-intensity sonar. Light pollution includes light trespass, over-illumination and astronomical interference. Visual pollution which can refer to the presence of overhead power lines, motorway billboards, scarred landforms (as from strip mining), open storage of trash or municipal solid waste. Thermal pollution is a temperature change in natural water bodies caused by human influence, such as use of water as coolant in a power plant (Wikipedia, 2010a).

A large number of environmental pollutants pose significant potential health and environmental risks at varying spatial scales, ranging from highly localized to regional or even global. Air pollution is a major environmental risk to health and is estimated to cause approximately two million premature deaths worldwide per year (WHO, 2010). Sulphur dioxide (SO_2) can affect the respiratory system and the functions of the lungs (aggravation of asthma and chronic bronchitis), and causes irritation of the eyes. When SO_2 combines with water, it forms sulphuric acid; this is the main component of acid rain which is a cause of deforestation and lowers the pH value of soils (WHO, 2010). The emission of greenhouse gases (carbon dioxide, methane, nitrous oxide, ozone) leads to global warming which affects the ecosystem in many ways (Wikipedia, 2010a). Drinking water can become contaminated by untreated sewage, rashes and skin irritations occur due to oil spills, while excessive noise can induces hearing loss, high

blood pressure, stress, and sleep disturbance. Mercury has been linked to developmental deficits in children and neurological symptoms. Chemical and radioactive substances can cause cancer as well as birth defects (Wikipedia, 2010a). Figure 1.1 shows an overview of the effects of some pollutants on human health.

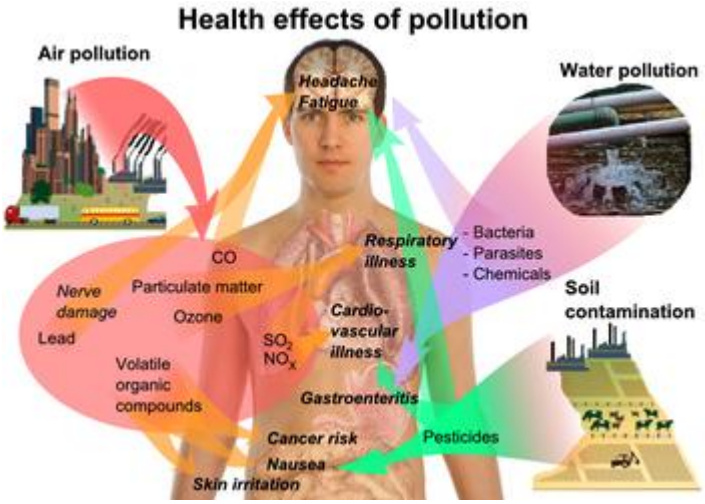


Figure 1.1: Overview of main health effects on humans from some common types of pollution (Wikipedia, 2010a)

From lead in the soil to toxins in the water and radioactive fallout in the air, the Blacksmith Institute has created a list of the world's worst ecological disaster areas. In 2007, Time.com (a division of Time Magazine) (Time.com, 2010) reported the group released its second annual list of the world's 10 most polluted places, anonymous cities like Sumgayit or Sukinda in India, where 2.6 million people are potentially exposed to toxic heavy metals. Blacksmith estimates that more than 12 million people live in the cities on its top 10 list, and a recent World Health Organization (WHO) report showed that environmental factors like bad water and air pollution accounted for 20% of the global burden of disease. The 10 most polluted places are listed in Table 1.1.

Table 1.1: The 10 world's most polluted places based on report by Blacksmith Institute

Place	Number of people potentially affected	Type of pollutant	Source of pollution
1) Lifen, China	3,000,000	Coal and particulates	Automobile and industrial emissions
2) Tianying, China	140,000	Lead and other heavy metals	Mining and processing
3) Sukinda, India	2,600,000	Hexavalent chromium and other metals	Chromite mines and other processing
4) Vapi, India	71,000	Chemicals and heavy metals	Industrial estates
5) La Oroya, Peru	35,000	Lead, copper, zinc and sulphur dioxide	Heavy metal mining and processes
6) Dzerzhinsk, Russia	300,000	Chemicals and toxic byproducts, including sarin and VX gas	Cold war-era chemical weapons manufacturing
7) Norilsk, Russia	134,000	Air pollution – particulates, sulphur dioxide, heavy metals, phenols	Major nickel and metal mining and processing
8) Chernobyl, Ukraine	Initially estimated at 5.5 million, currently disputed	Radiation	Nuclear meltdown
9) Sumgayit, Azerbaijan	275,000	Organic chemicals, oil and heavy metals	Petrochemical and industrial complexes
10) Kabwe, Zambia	255,000	Lead and cadmium	Lead mining and processing

(Source: Times.com, 2010)

Most of the organics, especially their halogen-derivatives, do not undergo rapid biodegradation and can remain in the environment for a long time. This is the main reason why the number of organic compounds found in soils, stream, lake and marine sediments, water, plants and animals is constantly growing (Migaszewski, et al., 2002).

1.2 Environmental Monitoring

To protect the environment from the adverse effects of pollution, many nations worldwide have enacted legislation to regulate various types of pollution as well as to mitigate the adverse effects of pollution. The United States Environmental Protection Agency (U.S. EPA) established threshold standards for air pollutants to protect human health on January 1, 1970 (Wikipedia, 2010b). Some scientists have said that the concentrations which most of these levels indicate are far too high and the exposure of people should be less. In 1999, the U.S. EPA replaced the Pollution Standards Index (PSI) with the Air Quality Index (AQI) to incorporate new $PM_{2.5}$ and Ozone standards. The United States Congress passed the Clean Air Act in 1963 to legislate the reduction of smog and atmospheric pollution in general. That legislation has subsequently been amended and extended in 1966, 1970, 1977, and 1990. Passage of the Clean Water Act amendments of 1977 required strict permitting for any contaminant discharge to navigable waters, and also required the use of best management practices for a wide range of other water discharges including thermal pollution. Passage of Noise Control Act established mechanisms of setting emission standards for virtually every source of noise including motor vehicles, aircraft, certain types of equipments and major appliances (Wikipedia, 2010b).

Thus there is increasing interest in monitoring the levels and dispersal of contaminants into the environment, especially the atmosphere. Such monitoring programmes implicitly involve sampling the atmosphere. The worldwide occurrence and lack of controlled use of these toxic compounds in many countries suggests that global budgets should be developed to assess human exposure, and to determine the routes by which these compounds are removed from the atmosphere (Wikipedia, 2010b).

Assessment of air quality is a very complex problem because field analysis which includes sampling and development of appropriate analytical methodology is required as well as identification and monitoring of possible sources of pollution and critical emissions. In addition, economic aspects have to be integrated in the monitoring programme (Blasco, et al., 2008).

1.3 Biomonitors

The use of pollution biomonitors is an advantageous air pollution-assessment technique complementary to conventional environmental air analysis which provides additional and useful information about the area (Blasco, et al., 2008). Pollution biomonitors also enables easier sampling, even in remote areas and areas with difficult access, because technology for sampling is not necessary, and they are also independent from power sources (Wittig, 1993). It is also a rapid and cost-effective method without the need to use expensive air samplers and analysing equipments. Furthermore, the sample treatment and analysis steps in the laboratory are facilitated, making possible the simultaneous determination of several pollutants in the same matrix.

Many researchers have used pollutant concentrations in plants to qualitatively indicate atmospheric contamination levels. Biomonitors by using plant species integrates contamination over time, and vegetation samples are much easier to collect than air samples, especially in remote locations. The plant material is samples in the field and treated with chemical residue analysis in the laboratory. Plants have been used to identify point sources of organic pollutants, to determine regional contamination within cities, countries, and continents, and to determine the global contamination of organic pollutants (Simonich & Hites, 1995).

The relative amounts of different pollutants collected by air sampler are influenced by both the collection procedures employed and the weather conditions before and during the sampling periods. Plants in remote areas tend to reflect integrated, background levels of trace contaminants originating from long-range transport, while particulate samples tend to reflect pollution directly emitted in the area during (and shortly before) the sampling period. A further difference is that concentrations of substances present in atmospheric phases collected by air samplers can be directly related to unit volumes of air, but concentrations found in plants can only be qualitatively related to the surrounding air masses (Blasco, et al., 2006).

Plants can be used to qualitatively indicate atmospheric contamination levels as long as the mechanism of accumulation is considered. Many recent studies have used plants which bioaccumulates pollutants for monitoring studies. Studies on the accumulation of pollutants by plants can be divided into the following three areas: (a) the mechanism of atmospheric uptake by plants, (b) the use of plants as an indicator of contamination levels, and (c) the importance of plants as a pollutant sink (Simonich & Hites, 1995).

There are several pathways through which organic pollutants enter plants (Simonich & Hites, 1995). The pollutant may enter the plant by partitioning from contaminated soil to the roots and be translocated in the plant by the xylem. (The xylem transports water from the roots to the leaves by transpiration). Organic pollutants may also enter plants from the atmosphere by gas-phase and particle-phase deposition onto the waxy cuticle of the leaves or by uptake through the stomata and be translocated by the phloem. (The phloem transports photosynthatates to the roots and to other plant tissues). Studies indicated that gaseous deposition from the air to the waxy cuticle is a major uptake process for lipophilic organic contaminants. Plant species are often covered by a lipid-

rich cuticle (consists mainly of long-chain esters, polyesters, and paraffins) (Kylin, et al., 1994) and has been shown to accumulate lipophilic persistent organic pollutants (POPs). Hence the concentrations of target compounds measures in biomonitors may represent the integrated concentrations of those chemicals in surrounding air over time.

The mechanism of plants uptake of atmospheric pollutants is governed by the functions of (a) chemical and physical properties of pollutant such as their molecular weights, lipophilicity, aqueous solubilities, and vapour pressures which controls the vapour-particle partitioning and Henry's law constant; (b) environmental conditions such as the ambient temperature and the organic content of the soil and; (c) plant species and structure, which control the surface area and lipids available for accumulation (Simonich & Hites, 1995). Semi-volatile organic compounds (SOCs) preferentially move to the earth's surface at lower ambient temperatures and to the atmosphere at higher ambient temperatures (Simonich & Hites, 1994a). For this reason, higher atmospheric SOC concentrations are observed during hot seasons. For most species examined, however, many controlled exposure experiments and field experiments have shown that the uptake of lipophilic organic pollutants is not a significant pathway of accumulation. In general, lipophilic pollutants are not translocated within the plant, and metabolism is not significant. Uptake from contaminated soil was also investigated and was found to be negligible (Simonich & Hites, 1995).

It is useful to distinguish between bioindicators and bioaccumulator plants. To prevent confusion about terminology, which is not always uniform, the term 'bioindicator' is used in this work to mean an organism (part of an organism or a community of organisms) that contains information on the quality of its environment. A 'biomonitor', on the other hand, is an organism (part of an organism or a community of organisms)

that quantifies the quality of its environment. In the context of both bioindication and biomonitoring an organism reacts to changing conditions in its environment – for instance the effect of pollutants – with a measurable change in its way of life in terms of morphology and/or physiology of its metabolism. Passive bioindicators/biomonitors are organisms already present in the area to be studied. Active bioindicators/biomonitors are organisms that are exposed in an area for a defined period (Siebert, et al., 1996).

To better access the state of natural ecosystems, various effective bioindicators (biomarkers) have been used. These include mosses and lichens, which are commonly regarded as the best bioindicators of air quality because they can accumulate and concentrate a variety of pollutants in their tissues to a far greater level than is necessary for their physiological needs (Migaszewski, et al., 2009) and also do not shed plant parts during growth as readily as vascular plants.

A lot of extensive studies have been done on different vegetation species to monitor atmospheric metals and organic pollutants such as mosses, lichens and pine needles. For mosses, a variety of species have been used extensively, such as *Hypnum cupressiforme* (Thomas, 1986; Holoubek, et al., 2000; Loppi & Bonini, 2000; Fernandez, et al, 2000; Otvos, et al., 2004); *Pleurozium schreberi* (Brid.) Mitt. (Ceburnis, et al., 1997; Wenzel, et al., 1998; Tremper, et al., 2004; Galuszka, 2007; Migaszewski, et al., 2009;); *Hylocomium splendens* (Hedw.) B.S.G. (Ceburnis, et al., 1997; Migaszewski, et al. 2002, 2009; Zechmeister; et al., 2006; Galuszka, 2007; Ratola, et al., 2008); *Tortula muralis* (Hedw.) (Gerdol, et al., 2002); *Sphagnum* spp. (Wegener, et al., 1992; Ceburnis, et al., 1997); *Bryum radiculosum* (Brid.) (Schintu, et al., 2005); *Rhytidiadelphus squarrosus* (Tremper, et al., 2004); *Scleropodium purum* (Hedw.) (Fernandez, et al., 2000, 2002); *Bryum argenteum* (Aceto, et al., 2003; Borghini, et al.,

2005); and *Isopterygium minutirameum* (Lim, et al., 2006). As for lichens, *Parmelia sulcata* (Loppi & Bonini, 2000; Blasco, et al., 2006, 2007), *Xanthoria parietina* (Domeno, et al., 2006), *Hypogymnia physodes* (Migaszewski, et al., 2002) and *Evernia prunastri* (Blasco, et al. 2008) are the few to mention. Pine needles such as *Pinus sylvestris* L. (Tremolada, et al., 1996; Wenzel, et al., 1998; Holoubek, et al., 2000; Migaszewski, et al., 2002;), *Pinus pinea* L. (Ratola, et al., 2006, 2008, 2009), *Pinus strobes* (Simonich & Hites, 1994a, b; Wagrowski & Hites, 1997; Lang, et al., 2000), *Pinus nigra* (Piccardo, et al., 2005), and *Pinus pinaster* Ait. (Piccardo, et al, 2005; Ratola, et al., 2009) have also been used as biomonitors for various pollution studies. These species are commonly found in different parts of the world.

1.4 Mosses as Biomonitors

Mosses, which belong to the Bryophyte division, are effective biomonitors as they take up nutrients and pollutants directly from the atmosphere and other substances also from air since they have no root system (Lim, et al., 2006), so those compounds which are not degraded are accumulated in their tissues. Mosses have a relatively large surface area and with the presence of a thin cuticle allow a more direct interaction with the outer environment in respect to superior plants, and also a penchant to absorb atmospheric pollutants from the atmosphere (Wegener, et al., 1992). Their physiological enables active uptake of water, nutrients and pollutants from the air. These properties make them ideal for evaluating pollutant levels in the air over a monitored period of time. Mosses have a widespread geographical distribution with a tendency to grow all around the year (evergreen plants), and is able to survive in dry weather conditions and in highly polluted zones. These reasons enable the evaluation of the deposition and/or uptake or airborne elements and organic compounds, including a large number of

pollutants. Moreover, mosses can accumulate, concentrate and retain toxic substances that may be present in low concentrations in the local environment (Migaszewski, et al., 2009). This feature of bioaccumulation offers the possibility of reflecting plant geochemical anomalies of the geochemical environment, as well as the ability to record simultaneously a large number of airborne constituents. It is usually assumed that the elements acquired by mosses represent some fraction of the elements present in their immediate environment.

The use of epiphytic mosses as biomonitors is a very appropriate method for determining total levels of atmospheric deposition of contaminants. An epiphyte is a plant that grows upon another plant (such as a tree) non-parasitically or sometimes upon some other object (such as a building or a telegraph wire), derives its moisture and nutrients from the air and rain and sometimes from debris accumulating around it, and is found in the temperate zone and in the tropics (Wikipedia, 2010c). The technique, whereby analysis of the contents of contaminants in mosses is carried out, is known as passive biomonitoring and was first used at the end of the 1960s (Ruhling & Tyler, 1968). Since then the method has been developed and standardized using species native to the areas under study (Fernendez, et al., 2000; Otvos, et al., 2004). Passive monitoring of air pollution is done using naturally occurring mosses grown in a field from tree-trunks or ground surfaces. Pollution in mosses can be monitored over a long period of time in a large scale area.

Moss transplants have also been used as active biomonitors (Fernendez, et al., 2000; Wegener, et al., 1992; Tremper, et al., 2004), often because of the absence of native species in the study area. The transplants are usually carried out by using so-called moss bags which are in the form of spherical bags. In active biomonitoring, plants that have

grown under standard conditions were exposed to pollution for a limited time (usually about several weeks). The selection of the locations is not restricted by the condition of natural occurrence of mosses. Moreover, the time of exposure can be well-defined. This will give an assessment of pollution in urban or industrialised area of the atmosphere.

1.4.1 Heavy Metals

To date, most studies on the accumulation of air pollutants by mosses have focused on their uptake of heavy metals (Loppi & Bonini, 2003; Schintu, et al., 2005; Thomas, 1986; Aceto, et al., 2003; Galuszka, 2007). Metals are one group of pollutants that are frequently monitored with mosses as mosses have the ability to accumulate metals to high concentrations and thus facilitate analysis (Tremper, et al., 2004). Due to their large surface area, cation-exchange properties, and the fact that they derive their water and nutrients almost exclusively from air, mosses are excellent monitors for the deposition of metals from air. There is a big variety of sources that contribute to metals in the atmosphere, such as combustion processes, metal industries and mining. Traffic is a major source of metal pollution, especially in urban areas. Metals associated with traffic emissions are for example zinc, lead and copper. These metals have various sources, such as brake wear and brake lining and tyres (Tremper, et al., 2004).

1.4.2 Organic Compounds

The first applications of mosses as monitors of the deposition of organic micro-pollutants were made in the early 1980s (Wegener, et al., 1992). Wegener, et al. (2006), Lim, et al. (2006) and Borghini, et al. (2005) extended the use of moss from the monitoring of heavy metals to test for the presence of organic pollutants in the

atmosphere, such as organochlorine pesticides (OCPs) and polychlorobiphenyls (PCBs). Some researchers also included a few polycyclic aromatic hydrocarbon (PAHs) compounds into their studies (Holoubek, et al., 2000; Galuszka, 2007; Zechmeister, et al., 2006; Migaszewski, et al., 2009; Wegener, et al., 1992; Thomas, 1986; Gerdol, et al., 2002; Otvos, et al., 2004). It can be argued that the same properties make mosses suitable as monitors not only for air deposition of heavy metals but also for organic pollutants as well. In this case, cation-exchange properties are not of importance, but instead the capacity to absorb large organic molecules is the key parameter.

1.4.2.1 Organochlorine Compounds

OCPs and PCBs belong to a group of organic compounds containing carbon-chlorine bonds, which confer a high degree of chemical stability and flame resistance, particularly among the more highly chlorinated species (Lim, et al., 2006). Their persistent and semi-volatile properties result in long range transport in air or water to remote regions including Antarctica (Borghini, et al., 2005). OCPs have been used effectively in controlling insect disease vectors and agricultural pests since World War II, but many have since been banned or severely controlled following adverse impacts upon non-target organisms. PCBs are a class of compounds in which 1-10 chlorine atoms are attached to a biphenyl. PCBs were first produced around 1930 and were used in a wide number of applications ranging from dielectrics and transformers to plasticisers in paint and ink solvents in carbonless copy paper. Although now subject to a worldwide ban, PCBs are still present in the environment due to insecure disposal practices in their intrinsic recalcitrance (Lim, et al., 2006). Holoubek, et al. (2000) and Lim, et al. (2006) have reported the presence of some OCPs and PCBs at detectable levels in Czech Republic and Singapore respectively using mosses as biomonitors.

1.4.2.2 Polycyclic Aromatic Hydrocarbons (PAHs)

PAHs are a group of ubiquitous organic compounds that is considered as one of the most important environmental contaminants. These compounds are composed of no elements other than carbon and hydrogen atoms, characterised by the presence of two or more fused aromatic rings (ADEME, 2009). This family includes over 100 substances and their physical and chemical characteristics vary with their molecular weight. PAHs are hydrophobic compounds which are poorly soluble in water (solubility in clear tap water of about 0.001 µg/l) (Dugay, et al., 2002). PAHs resistance to oxidation, reduction, and vaporisation, increase with molecular weight, whereas their aqueous solubility decreases. The significance of these xenobiotics as pollutants is of great concern due to their potential toxicity (carcinogenic, mutagenic, terotogenic, neurotoxic and/or immunosuppressive properties) and persistence in the environment (Galuszka, 2007). They may be eliminated or transformed to even more toxic compounds by chemical reactions such as sulfonation, nitration or photo-oxidation (Marce & Borrull, 2000). For instance, in some conditions, traces of nitric acid can transform some PAHs into nitro-PAHs. Considering the great risk that they may pose to the environment and are a widespread threat, PAHs are a subject of environmental quality standards.

As a result of their widespread presence and inclusion in European Union (EU) Directives (Martinez, et al., 2004), PAHs are generally introduced in monitoring programmes. In this sense, in 1976, PAHs were already included in Directive 76/464/CEE. In the year 2000, PAHs remain legislated in the New Framework Water Directive (2000/60/CE) which includes all those compounds with demonstrated carcinogenic, mutagenic, esteroidogenic of affecting endocrine functions of the organisms. Directives 75/440/CE and 80/778/CEE indicate maximum residue levels of

0.2 µg/L in surface water directed to produce drinking water for human consumption. Sixteen PAHs are included among the POPs designated in the "Convention on Long-range Transboundary Air Pollution" adopted by the United Nations Economic Commission for Europe.

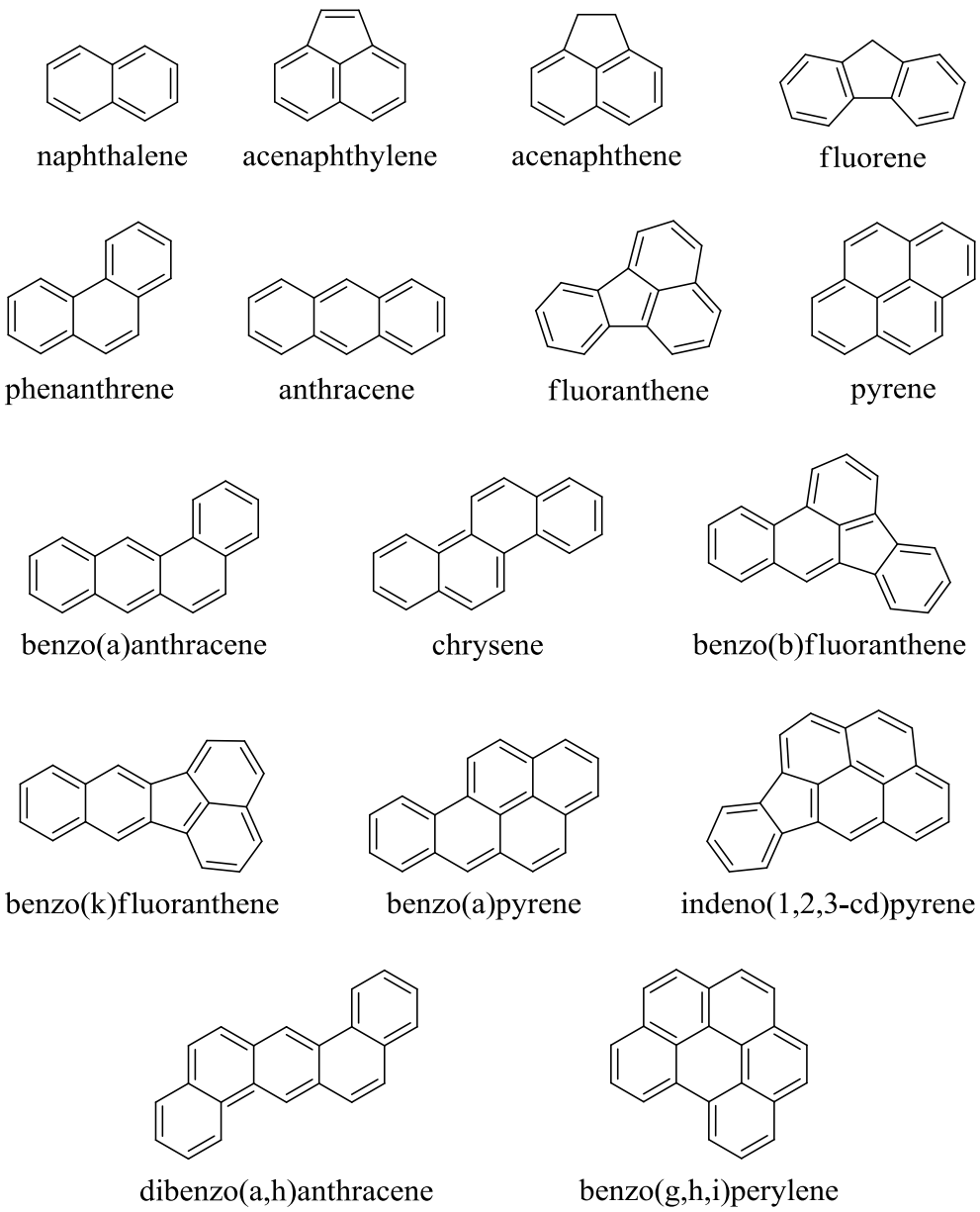


Figure 1.2: Chemical structure of the 16 PAHs considered as priority pollutants by the U.S. EPA

PAHs were also identified as high-priority chemicals for Region VII (Central and North East Asia) in the "Regionally-based Assessment of Persistent Toxic Substances" jointly

conducted by the United Nations Environment Programme and Global Environmental Fund (Blasco, et al., 2006). The U.S. Environmental Protection Agency (U.S. EPA) has also identified sixteen PAHs as Constant Decree priority pollutants for regulatory purposes (U.S. EPA, 2007). Figure 1.2 shows the structure of the 16 PAHs considered as priority pollutants by the U.S. EPA.

PAHs are emitted into the atmosphere from both natural and anthropogenic sources (Simonich & Hites, 1995). Natural (e.g. volcanic eruptions, natural forest fires and biosynthesis) and anthropogenic sources (include vehicle emissions, domestic heating, industrial processes, electric power production, and waste incineration) account for their diffusion in the environment, as a consequence of atmospheric transport, deposition and dispersion in the environment (Ratola, et al., 2006; Blasco, et al., 2006). In urban settings, motor vehicle exhausts are considered to be a dominant source of these pollutants.

PAHs occur in both gaseous forms and as adsorbents on particles in the atmosphere, with their partitioning between these phases depending, *inter alia*, on the volatility of the PAH species. Non-volatile PAHs (highly condensed molecules with four or more rings) tend to be largely particle-bound, whereas volatile PAHs (smaller molecules) mainly remain in the gas phase. PAHs tend to persist for relatively long periods in the environment due to their comparatively stable molecular structure and slow rates of photochemical deposition and biodegradation (Blasco, et al., 2006). For these reasons concentrations of PAHs in mosses could provide relatively stable indicators of pollution levels in their respective environments.

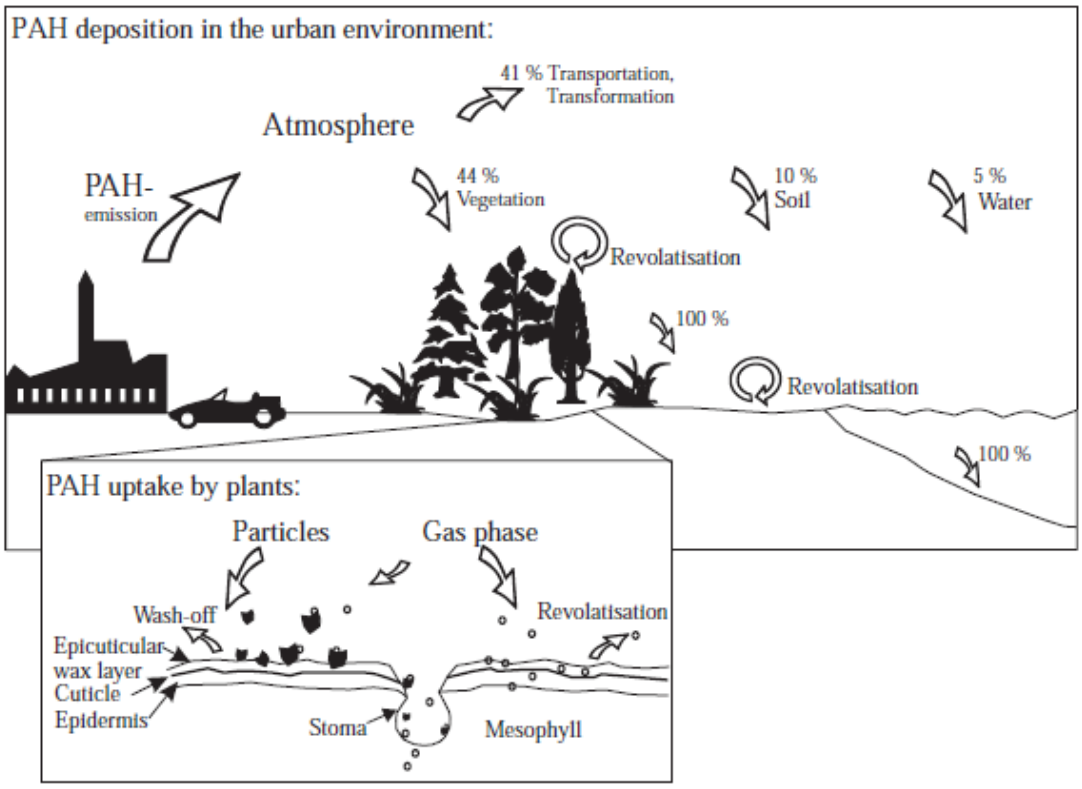


Figure 1.3: Generalized model of PAH transportation and deposition. Vegetation acts as an important sink for lipophilic organic pollutants, scavenging about 41% of the total PAH emission in urban and 4% in rural environments. The inset illustrates PAH accumulation modes on plants emphasizing differences in uptake of particle-bound and gaseous pollutants (Lehndorff & Schwark, 2004).

Because of the wide variety of forms that these substances can present, airborne PAHs are largely in air, water, aerosols, soils, sediments and biota. Levels up to 800 mg/kg were found in surface sediments in Finland and from 10 to 144 mg/kg in deeper layers, and mussels were capable to bioaccumulate such compounds, being the levels up to 32 mg/kg in finish lakes (Martinez, et al., 2004). Although PAHs are distributed in the whole environment, the major route of exposure for humans is the atmosphere. In addition, these compounds are lipophilic and may accumulate in vegetation, an environmental fate that could indirectly cause human exposure through food consumption.

Mosses may take up PAHs from the atmosphere or soils (low molecular weight compounds are volatile and may evaporate from soil). Although environmental PAH are usually in trace amounts, they have a strong tendency to bioaccumulate in organic plant tissues due to their lipophilic character and electrochemical stability. However, identifying the sources of PAHs in moss tissues is not straightforward because, as mentioned above, once PAHs are emitted they redistribute between the gas and particle phases and are removed by oxidative and photolytic reactions and by both dry and wet deposition (Blasco, et al., 2006). Deposited PAHs can also be re-emitted (through volatilisation) if the temperature increases. There is no evidence for PAH biosynthesis by mosses (Galuszka, 2007), however the formation of PAHs by vascular plants have been reported (e.g. Krauss, 2005).

To mitigate the adverse health and environmental effects of PAHs it is essential to identify their sources, which in turn requires the identification and quantification of PAHs present in the affected area. Previous studies (Zechmeister, et al., 2006; Viskari, et al., 1997) have found that mosses can accumulate considerable concentrations of PAHs, and that their PAH concentrations may be directly correlated with levels of vehicular traffic in the surrounding area. To evaluate the contribution of traffic to the PAH load in remote areas, air can be sampled by the roadside site, where the contribution of traffic is likely to be strongest, and the PAH profiles and concentrations found in the samples can be compared with those collected from other areas. To date, most studies have only been completed in the western hemisphere (e.g. northern Europe, north-eastern United States), with only one study reported for Asia (Singapore) (Lim, et al., 2006). There is a need to employ alternate moss species for monitoring studies, especially on a local scale. For these reasons, the development of an effective analytical process of PAHs from any matrix is very important.

1.5 Objective of Study

Since PAHs is one of the main concern of air pollution, scientists have been finding new ways to develop methods which are faster, economical, and more reliable in determining the level of sixteen priority PAHs in the atmosphere. Mosses have been studied and found to be effective biomonitors as they take up nutrients and pollutants directly from the atmosphere and this make them ideal in evaluating the amount of PAHs in the atmosphere.

The objective of this study is to determine the suitability and effectiveness of using the moss species *Hyophila involuta* which is commonly found in Peninsular Malaysia as a biomonitor by using the optimised headspace SPME-GC-FID method to evaluate and make comparison between the level of sixteen PAHs from an uncontaminated area with five other contaminated areas.

In this work, the headspace SPME extraction method for the determination of PAHs from uncontaminated moss samples (which was collected from Fraser's Hill, Pahang) was first developed and optimised followed by the analysis using GC-FID instrument. Factors affecting the method such as the extraction mode and effects of the addition of chemical modifiers (e.g. salt solution) were evaluated, and the precision, linearity and detection limits were validated. Then the optimised and validated method was applied to real contaminated moss samples which were collected throughout the Klang Valley to determine the level of PAHs in the atmosphere at selected stations.

CHAPTER TWO – LITERATURE REVIEW

2.1 Sample Preparation

The extraction of PAHs from the multiple solid matrices found in the environment is a difficult step which has been hardly investigated and innovated (Domeño, et al., 2006). In general, most organic pollutants of interest in environmental samples have to be extracted and enriched before instrumental determination. The amount of sample preparation needed depends on the sample matrix and the properties and level of analyte to be determined. The typical steps within sample preparation include sampling/homogenisation, extraction, clean-up and concentration followed by the final analysis. Another step that can be included at several points is derivatisation. For the determination of trace organics, the final analysis is invariably achieved using a powerful separation technique, typically chromatographic, combined with an appropriate detector (Ridgway, et al., 2007).

The sample preparation must be tailored to the final analysis, considering the instrumentation to be used and the degree of accuracy required, whether quantitative or qualitative. Sample preparation is generally matrix and analyte dependent. For quantitative analysis, consideration must also be given to the most appropriate preparation of calibration standards. In some cases matrix matched standards or the method of standard additions may be necessary. The use of a suitable internal standard should also be considered. Any technique, whether it is a manual or automated process, must be robust and reproducible. Quality control and safety in environmental analyses demand reliable methodologies that are both rapid and easily transferable (Ridgway, et al., 2007).

The impact of matrix effects on detection systems, such as ionisation efficiency, detector noise and ultimately on limits of detection and on quantification, as a direct result of the extent of sample preparation applied must always be considered (Ridgway, et al., 2007). The analysis of mosses requires effective extraction and clean-up procedures to retain the target compounds and eliminate matrix interferences. The main reason is the complicated nature of mosses (plant matrices), which contain large fractions of structures abundant in lipids (different organic compounds such as alcohols, pigments, essential oil, fatty acids, chlorophylls, alkanes, phenols, and esters) and are comparable to many lipophilic organic pollutants (including PAHs) in terms of their physicochemical properties, e.g. solubility, molecule size. This could introduce errors in the analysis since co-extraction and co-elution of plant lipids (matrix peaks) and target pollutants (analyte peaks) yield interferences and peak overlapping during chromatographic separation, which consequently lead to faulty interpretation of analyses values (Ratola, et al, 2008). This would also significantly reduce the performance of gas chromatography-mass spectrometry (GC-MS) due to their accumulation in the injection port, column, and ionisation source.

The added benefit is that the cleaner the sample, the better the chromatography and generally the less time spent on instrument maintenance. More importantly, the enhanced sensitivity (less noise) in the detection step and reduction of interfering compounds facilitates unequivocal identification and confirmation, all of which are particularly important when investigating the presence or absence of low concentration of a contaminant in complex sample matrices (Ridgway, et al., 2007). For these reasons and considering the low concentration levels of PAHs in the environment (usually in ng/g levels), several approaches are continuously being attempted in search of faster, cleaner and more reliable analytical methodologies for effective extraction, enrichment

and clean-up procedures in order to be able to quantitatively extract the analytes from mosses prior to the final chromatographic analysis (Blasco, et al., 2007).

2.1.1 Conventional Extraction Methods

The analyses of trace PAHs from solid matrices have been accomplished by many different extraction techniques. Traditional extraction techniques, like Soxhlet (Wegener, et al., 1992; Termolada, et al., 1996; Holoubek, et al., 2000; Martinez, et al., 2004; Domeno, et al., 2006; Lim, et al., 2006; Ratola, et al., 2006, 2009; Zechmeister, et al., 2006; Migaszewski, et al., 2009) and ultrasonic extraction (USE) (Simonich & Hites, 1994; Kipopoulou, A.M., et al., 1999; Dugay, et a., 2002; Gerdol, et al., 2002; Martinez, et al., 2004; Otvos, et al., 2004; Piccardo, et al., 2005; Domeno, et al., 2006; Ratola, et al., 2006) have and is still being widely used among many researchers.

Soxhlet extraction, a continuous solvent extraction method, is the standard technique used in most U.S. EPA methods. The technique is used for the isolation and enrichment of analytes of medium and low volatility and thermal stability (Romanik, et al., 2007). Due to the temperatures involved, Soxhlet extraction can degrade thermally labile compounds. The extraction time required is often long, typically 1-6 hours, and a significant volume of organic solvent (50-200 ml for a 10 g sample) is required (Ridgway, et al., 2007).

In USE, ultrasounds are waves with frequencies ranging from 16 kHz to 1 GHz, inaudible to humans. Ultrasonic vibrations are the source of energy facilitating the release of some analytes from the sample matrix (Romanik, et al., 2007). Several extractions can be performed simultaneously in USE and as no specialised laboratory

equipment is required, the technique is relatively inexpensive (Ridgway, et al., 2007). The extraction is carried out at room temperature, which makes it suitable for the extraction of thermally labile analytes. The need for separation of the extract from the sample following the extraction is a disadvantage of this technique (Romanik, et al., 2007).

The extractant solvents such as dichloromethane (Simonich & Hites, 1994, 1997; Kipopoulou, et al., 1999; Holoubek, et al., 2000; Piccardo, et al., 2005; Domeno, et al., 2006; Lim, et al., 2006; Migaszewski, et al., 2009), toluene (Dugay, et al., 2002) or n-hexane (Termolada, et al., 1996; Wenzel, et al., 1998; Gerdol, et al., 2002; Zechmeister, et al., 2006; Blasco, et al., 2008) or a mixture of solvent, for example, hexane-dichloromethane (Martinez, et al., 2004; Ratola, et al., 2006, 2009) and hexane-acetone (Otvos, et al., 2004) are still being used up to today. Although these conventional extraction methods have been applied successfully giving efficient and precise results, they have the inherent disadvantages of being rather labour-intensive, cumbersome, time-consuming, multi-step procedures prone to loss of analytes, requiring large volumes of hazardous solvents, highly expensive with respect to the disposal of waste solvents and the inclusion of supplementary clean-up steps.

2.1.2 Modern Extraction Methods

Modern trends in analytical chemistry are towards the simplification of sample preparation. For the past few years, new analytical methods for the extraction of PAHs from environment solid matrices have been developed, such as supercritical fluid extraction (SFE) (Lang, et al., 2000), subcritical water extraction (SWE) (Hageman, et al., 1996), pressurised liquid extraction (PLE) (Ratola, et al., 2006; Wenzel, et al.,

1998), microwave-assisted extraction (MAE) (Barnabas, et al., 1995; Ratola, et al., 2009), direct sonication-assisted solvent extraction (DSASE) (Domeño, et al., 2006) and solid-phase extraction (SPE). Selection of an appropriate extraction technique entails consideration of not only the recovery but also the cost, time of extraction, and the volume of solvent used. A comparison of the previously described extraction techniques for the isolation of groups of components from plant material is shown in Table 2.1 (Romanik, et al., 2007). These methods were developed to improve automation, provide higher extraction efficiency, better selectivity, shorter extraction times, less sample clean-up, smaller amounts of samples are used, and minimal or no hazardous solvents are required.

Table 2.1: Comparison of various liquid-solid extraction techniques used in the analysis of plant metabolites

Extraction	Soxhlet	USE	PLE	MAE	SFE
Cost	Low	Low	High	Medium	High
Extraction time	6-48 hr	< 30 min	< 30 min	< 30 min	< 60 min
Solvent used (ml)	200-600	< 50	< 100	< 40	< 10

(Source: Romanik, et al., 2007)

For SFE, the most commonly used extracting agent is carbon dioxide (CO_2), because of its low cost, low toxicity, and favourable critical parameters (T_c = 31.1 ºC, P_c = 74.8 atm). CO_2 as a non polar substance is capable of dissolving non-polar or moderately polar compounds. A mixture of CO_2 with modifiers (polar organic solvents) is used for the extraction of polar substances. The modifiers increase the solubility of analytes, preventing them from adsorption on the active sites of sample matrix. SFE is relatively efficient even for materials with compact and hardly accessible structure. It is especially well suited for the isolation of substances of low and medium polarity and high volatility (Romanik, et al., 2007).

SWE takes advantage of the lowered dielectric constant of water observed at temperatures and pressures somewhat below the critical point (condensed phase between 100 ºC and the critical point) to extract organic contaminants from samples (Hageman, et al., 1996).

PLE, also known as accelerated solvent extraction (ASE), makes use of the same solvents as do other extraction techniques, but at an increased pressure (ca. 100-140 atm) and at an elevated temperature (50-200 ºC). The design of the extractor, capable of withstanding high pressures, allows the extraction temperature to be raised above the boiling point of the solvent used. The high pressure allows maintaining the solvent in a liquid state at a high temperature. Under these conditions, the solvent has properties favouring the extraction process, such as low viscosity, high diffusion coefficients, and high solvent strength. This result in good kinetics of dissolution processes and favours desorption of analytes from cellular wall or organelles. The disadvantage of ASE is the high cost of the equipment (Romanik, et al., 2007).

MAE is based on absorption of microwave energy by molecules of polar chemical compounds (Romanik, et al., 2007). The microwave energy is a non-ionizing radiation that causes molecular motion by migration of ions and rotation of dipoles, but does not increase changes in molecular structure (Barnabas, et al., 1995). The energy absorbed is proportional to the dielectric constant of the medium, resulting in rotation of dipoles in an electric field (usually 2.45 GHz), and is carried out at a temperature from 150-190 ºC (Romanik, et al., 2007). MAE agitates and heats the sample during extraction, and the analytes are released into a cooler solvent. As non-polar solvents (hexane and toluene) do not absorb microwave energy, at least some polar solvent (e.g. water) must be used (Ridgway, et al., 2007).

DSASE is a dynamic extraction technique, which is a flow system that continuously supplies fresh solvent to the extraction vessel with. This approach may be considered as if adsorbed analytes are continuously forced to partition into the extraction solvent. Another feature is that the analytes are transferred out of the extraction vessel system as soon as they are extracted. This can be especially important to avoid degradation of the analytes due to sonication and when thermo-labile analytes are extracted at high temperatures and pressures (Domeño, et al., 2006).

Solid-phase extraction (SPE) is a sample treatment technique which passes a liquid sample through a sorbent (Marcé & Borrull, 2000). SPE is a popular technique that is used both to pre-concentrate components and to clean up matrices for sample analysis. The pre-packed cartridges provide users of SPE with a variety of stationary phases to selectively separate and concentrate analytes for detection (Blasco, et al., 2007). Both the analytes to be determined or the interferences of the samples are retained on the sorbent by different mechanism. In the first case, the analytes are eluted in a small volume of a solvent and so, the analytes are concentrated; in the second case the function of SPE is to clean the sample. The first case is mainly used for liquid samples and the second for solids, gases or liquids, usually after another sample-treatment technique. So, SPE is extremely versatile in the sense that it can be applied to a wide range of samples (Marcé & Borrull, 2000). SPE is used alone or in combination with other techniques. For instance, for determining PAHs in solid samples, SPE has been used after Soxhlet extraction (Simonich & Hites, 1994; Ratola, et al., 2006), USE (Wagrowski & Hites, 1997; Ratola, et al., 2009) or DSASE (Blasco, et al., 2008). SPE has a number of advantages, including the ability to isolate and enrich both volatile and non-volatile analytes, long storage time of adsorbed analytes, elimination of emulsion formation (common in liquid-liquid extraction) or foaming (common in gas-liquid

extraction (Romanik, et al., 2007). However, disadvantages include significant background interferences, as well as plugging and poor reproducibility between cartridges.

2.2 Solid-Phase Microextraction

Solid-phase microextraction (SPME), the technique invented by Pawliszyn and co-workers in 1989 (Arthur & Pawliszyn, 1990), is a new alternative to classical extraction methods and a growing sample preparation technique. Since its introduction, SPME has attracted increasing attention in the field of environmental analysis for its simplicity, efficiency, ease of automation, suitability for field and on-line applications due to its portability, and good analytical performance e.g. linear results over wide concentrations of analytes, good precision, accuracy and low detection limit for the sampling of a variety of volatile and semivolatile organic substances (Lord & Pawliszyn, 1997). This is because it allows sampling, extraction, concentration and sample introduction to be carried out in a single step (Zhang, et al., 1994), and thus reduces solvent usage and exposure, disposal costs, preparation time, and also eliminates the need of complicated apparatus. Other advantages of this technique include the requirement of only small amounts of sample, low cost and under most conditions, a single SPME fiber is being capable of performing between 50 and 100 extractions (King, et al., 2004). The number of extractions that can be performed is governed by the care given and the nature of the components in the samples being analyzed.

2.2.1 SPME Device and Fibers

Figure 2.1 shows the outline and schematic diagram of a commercial SPME unit. A 1 cm fine rod (fused silica or metal) coated with a polymer, is attached to a metal rod, and both are protected by a metal sheath that covers the fiber when it is not in use. This assembly is installed in a holder that resembles a modified microliter syringe. The plunger moves the fused silica fiber into and out of a hollow needle (Wercinski, 1999).

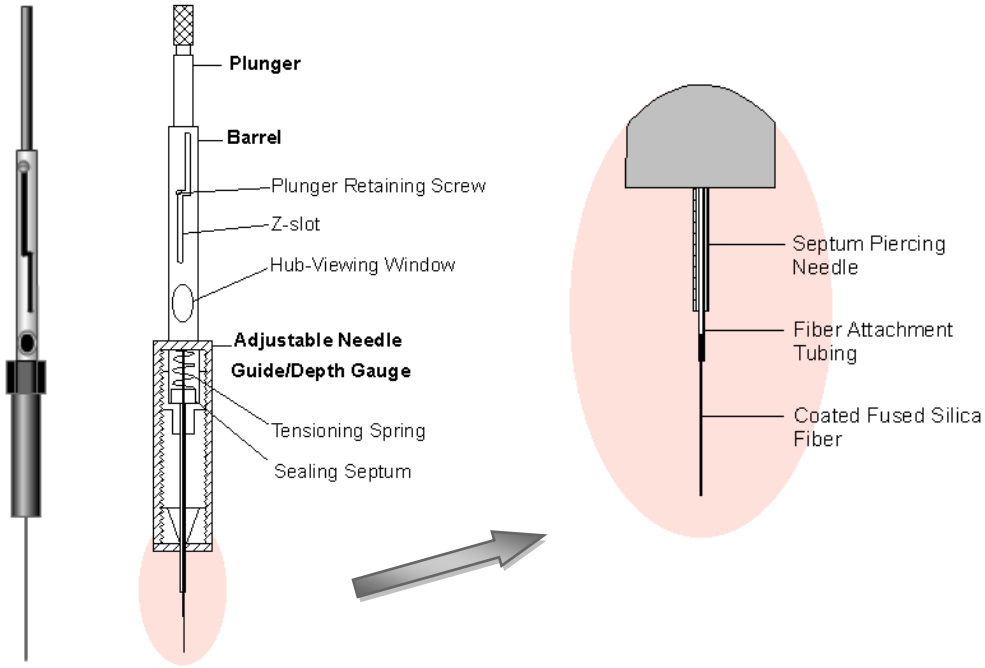


Figure 2.1: Schematic diagram of a commercial SPME device
(Source: Sigma-Aldrich/Supelco)

Sigma-Aldrich/Supelco manufactures the SPME fibers and holders, which is given the license for the patented technology, and they are continually improving the fiber technology. Some commercially available fibers for GC injection (unless stated otherwise) are summarised in Table 2.2.

Table 2.2: Some commercially available SPME fibers in the market

Phase (film thickness / coating stability)	Applications
Absorption Fiber Polydimethylsiloxane (PDMS) • 7 µm / bonded • 30 µm / non-bonded • 100 µm / non-bonded	Non-polar phase (for many semi-polar compounds: aromatics, esters, many pesticides). 100 µm used for relatively volatile compounds; the thinner phases are for non-polar and semi-polar compounds of low volatility.
Absorption Fiber Polyacrylate (PA) • 85 µm / partially crosslinked	Polar compounds such as phenols, esters.
Polar and Adsorption Fiber Carbowax/divinylbenzene (CW/DVB) • 65 µm / partially crosslinked • 70 µm StableFlex / highly crosslinked	More polar than polyacrylate, for alcohols.
Adsorption Fiber PDMS/divinlybenzene (PDMS/DVB) • 60 µm / partially crosslinked • 65 µm / partially crosslinked • 65 µm StableFlex / highly crosslinked	Moderately polar, for amines. 60 µm – general purpose (for HPLC only)
Adsorption Fiber PDMS/Carboxen (PDMS/CAR) • 75 µm / partially crosslinked • 85 µm StableFlex / highly crosslinked	Ideal for gases and highly volatile compounds including vinyl chloride, sulphur gases.
Polar and Adsorption Fiber CW/templated resin (CW/TPR) • 50 µm / partially crosslinked	Surfactants (for HPLC only)
Adsorption Fiber DVB/CAR/PDMS • 50/30 µm StableFlex / highly crosslinked	Ideal for broad range of polarities, good for C3 – C20 range

(Source: Sigma-Aldrich/Supelco)

The fiber coating's ability to crosslink and bond determines its stability. Three classifications are used to describe the coating stability: non-bonded, crosslinked, and bonded. Non-bonded phases are stabilized, but do not contain any crosslinking agents. These phases are not solvent-resistant and tend to swell in organic solvents; however we have determined that they can withstand some polar organic solvents (methanol and

acetonitrile). Finally, non-bonded fiber coatings have less thermal stability than bonded fiber coatings (Wercinski, 1999).

Partially crosslinked fiber coatings contain crosslinking agents (e.g. vinyl groups). The phase crosslinks with itself producing a more stable coating; however, the coating does not tend to bong to the fused silica. Partially crosslinked fiber coatings are more solvent resistant than bonded coatings and have better thermal stability. Most of these fibers can be exposed to a variety of solvents, but special care must be taken to prevent the fiber coating from being stripped off the fused silica core due to swelling (Wercinski, 1999).

Bonded fiber coatings, like partially crosslinked coatings, contain crosslinking agents. The difference is that the coating is crosslinked not only to itself, but also to the fused silica. These coatings are very resistant to most organic solvents and have good stability; furthermore, they can be rinsed in organic solvent with minimal swelling. These coatings tend to be thinner (Wercinski, 1999).

Table 2.3: Difference between adsorbent and absorbent fibers

Adsorbent (particle) fibers	Absorbent (film) fibers
Physically traps or chemically reacts bonds with analytes • porous material • high surface area	Analytes are extracted by partitioning • liquid phase • retains by thickness of coating
Analytes may compete for sites	Analytes do not compete for sites
Fibers have limited capacity	Fibers can have high capacity

(Source: Sigma-Aldrich/Supelco)

Fiber coatings are generally classified by polarity and film thickness. As shown in Table 2.2, three poly(dimethylsiloxane) (PDMS) fibers are available with film thickness of 100 µm, 30 µm and 7 µm. A thicker coating extracts more of a given analyte, but the

extraction time is longer than for thinner coating. Different types of sorbents will extract different groups of analytes; therefore, many different fiber coatings have been developed. Similar to selecting an analytical GC column where "like dissolves like", a fiber is chosen based on its selectivity for certain target analytes and their volatility ranges. Non-polar coatings such as PDMS retain hydrocarbons very well. In contrast, polar fiber coatings (e.g. polyacrylate and carbowax) extract polar compounds such as phenols and carboxylic acid very effectively. The affinity of the fiber coating for target analytes is crucial in SPME sampling because both the matrix and fiber coating are competing for analytes. For example, a polar coating chosen to extract polar compounds from water must have a stronger affinity for the analytes than water in order for them to be extracted (Wercinski, 1999).

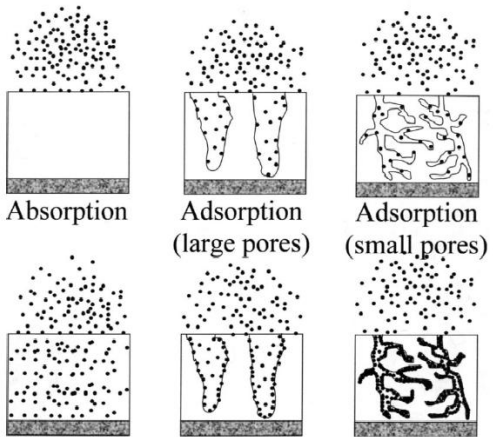


Figure 2.2: Schematic representation of absorptive vs. adsorptive extraction, and adsorption in small vs. large pores (Lord & Pawliszyn, 2000)

There is a substantial difference in performance between the liquid and solid coatings (Figure 2.2 and Table 2.3). In the case of liquid coatings (absorption), the analytes partition onto the extraction phase where the molecules are solvated by the coating molecules. The diffusion coefficient in the liquid coating allows the molecules to penetrate the whole volume of the coating within a reasonable extraction time, if the coating is thin. When the mass of absorbent is doubled, twice as much analytes can be

absorbed. In the case of solid sorbents (adsorption), the coatings have a well defined crystalline structure, which if dense, substantially reduces the diffusion coefficients within the structure. The total amount of analytes that adsorb onto the surface of solid is proportional to the total surface are of the solid and not its volume or mass. When the surface coverage becomes significant, the extraction amount is no longer proportional to the concentration of the analytes. Where more than one compound is present, the adsorption process is competitive. Therefore extraction occurs only on the surface of the coating (Yu, et al., 1999; Lord & Pawliszyn, 2000).

2.2.2 Principles of SPME

In SPME, a small amount of extracting phase associated with a solid support is placed in contact with the sample matrix for a predetermined amount of time. If the time is long enough, a concentration equilibrium is established between the sample matrix and the extraction phase. When equilibrium conditions are reached, then exposing the fiber for a longer time does not accumulate more analytes (Lord & Pawliszyn, 2000).

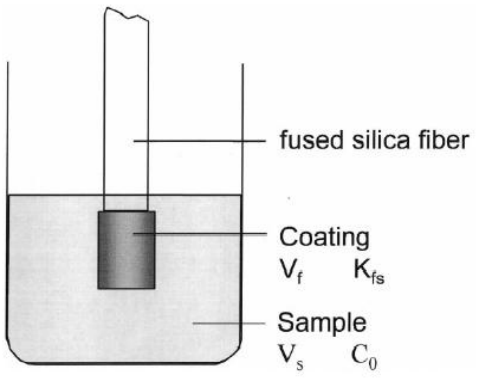


Figure 2.3: Microextraction with SPME. V_f, volume of fiber coating; K_{fs}, fiber/sample partition coefficient; V_s, volume of sample; C_o, initial concentration of analyte in the sample (Lord & Pawliszyn, 2000).

The principle behind SPME is the partitioning of analytes between the sample matrix and the extraction medium (Zhang, et al., 1994). A more traditional approach to SPME involves liquid polymeric coated fibers. The transport of analytes from the matrix into the coating begins as soon as the coated fiber has been places in contact with the sample (Figure 2.3). The extraction is considered to be complete when the analyte concentration has reached distribution equilibrium between the sample matrix and the fiber coating. In practise, this means that once equilibrium is reached, the extracted amount is constant within the limits of experimental error and it is independent of further increase of extraction time. Moreover, this also means that there is a direct proportional relationship between sample concentration and the amount of analyte extracted. This is the basis for analyte quantification (Lord & Pawliszyn, 2000).

The amount of analyte absorbed by the coating at equilibrium is directly related to its concentrations in the sample:

$$n = \frac{K_{fs} V_f V_s C_o}{K_{fs} V_f + V_s} \qquad \text{..... Equation (1)}$$

where n is the mass of an analyte absorbed by the coating; K_{fs} is the partition coefficient of the analyte between the coating and the sample matrix; V_f and V_s are the volumes of the coating and the sample, respectively; and C_o is the initial concentration of the analyte in the sample. Equation (1) clearly indicates the linear relationship between the amount of analytes absorbed by the fiber coating and the initial concentration of these analytes in a sample (Zhang, et al., 1994). This is true when the fiber and the sample reach equilibrium or before equilibrium, as long as you carefully control the sampling parameters.

Because the coatings used in SPME have strong affinities for organic compounds, K_{fs} values for targeted analytes are quite large, which means that SPME has a very high

concentrating effect and leads to good sensitivity. In many cases, however, K_{fs} values are not large enough to exhaustively extract most analytes in the matrix. Instead, SPME, like static headspace analysis, is an equilibrium sampling method and, through proper calibration, can be used to accurately determine the concentration of targeted analytes in as sample matrix. As Equation (1) indicates, if Vs is very large ($Vs >> K_{fs}V_f$), the amount of analyte extracted by the fiber coating is not related to the sample volume:

$$n = K_{fs}V_f C_o \quad \text{..... Equation (2)}$$

This feature, combined with its simple geometry, makes SPME ideally suited for field sampling and analysis (Zhang, et al., 1994). In Equation (2), the amount of extracted analyte is independent of the volume of the sample. In practise, there is no need to collect a defined sample prior to analysis as the fiber can be exposed directly to the ambient air, lake, river, etc. The amount of extracted analyte will correspond directly to its concentration in the matrix, without being dependent on the sample volume (Lord & Pawliszyn, 2000).

The method of analysis for solid sorbent coatings is analogous for low analyte concentration, since the total surface area available for adsorption is proportional to the coating volume if we assume constant porosity of the sorbent. For high analyte concentrations, saturation of the surface can occur. Similarly, high concentration of a competitive interference compound can displace the target analyte from the surface of the sorbent. Although the objective of SPME is never an exhaustive extraction and is mainly an equilibrium extraction technique, it has the ability to perform exhaustive extraction. If the coating/matrix partition coefficient, K_{fs} is very large ($K_{fs}V_f >> V_s$), the amount of analyte absorbed by the coating is Equation (3) and exhaustive extraction is achieved (Zhang, et al., 1994).

$$n = C_o V_s \quad \text{..... Equation (3)}$$

2.2.3 Modes of Operation

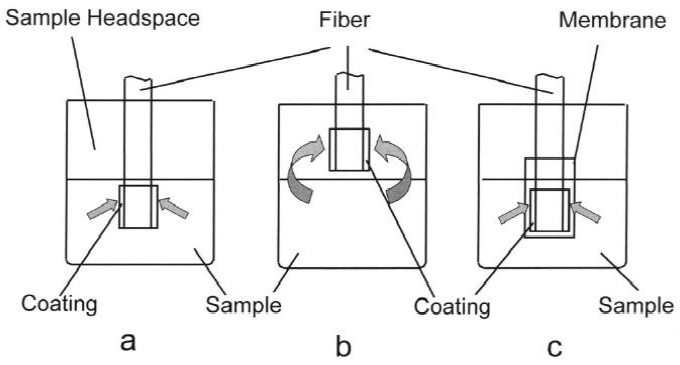


Figure 2.4: Modes of SPME operation: (a) direct extraction, (b) headspace SPME, (c) membrane-protected SPME (Lord & Pawliszyn, 2000)

Three basic types of extractions can be performed using SPME: direct extraction, headspace configuration, and a membrane protection approach (Lord & Pawliszyn, 2000). Aqueous samples can be studied by immersing the fibers directly into the solution, while particulate and solid samples (e.g. sediment, sludge and blood) as well as aqueous samples can be extracted by exposing fibers to the headspace above the samples. Figure 2.4 illustrates the differences among these modes. In the direction extraction mode (Fig. 2.4a), the coated fiber is inserted into the sample and the analytes partition between the sample solution and the fiber, where the analytes are transported directly from the sample matrix to the extracting phase. To facilitate rapid extraction, some level of agitation is required to transport analytes from the bulk of the solution to the vicinity of the fiber. For gaseous samples, natural convection of air is sufficient to facilitate rapid equilibration. For aqueous matrices, more efficient agitation techniques are required (e.g. stirring or sonication). These conditions are necessary to reduce the effect caused by the "depletion zone" produced close to the fiber as a result of fluid shielding and slow diffusion coefficients of analytes in liquids matrices (Lord & Pawliszyn, 2000).

Figure 2.4c shows the principle of indirect SPME extraction through a membrane. The main purpose of the membrane barrier is to protect the fiber against damage, similar to the use of headspace SPME when very dirty samples are analysed. However, membrane protection is advantageous for determination of analytes having volatilities too low for the headspace approach. In addition, a membrane made from appropriate material can add a certain degree of selectivity to the extraction process. The kinetics of membrane extraction are substantially slower than for direct extraction though, because the analytes must diffuse through the membrane before they can reach the coating. The use of thin membranes and increased extraction temperatures will result in faster extraction times. The thicker membranes can be used to slow down the mass transfer through the membrane resulting in the slower extraction time (Lord & Pawliszyn, 2000).

2.3 Chromatographic Analysis

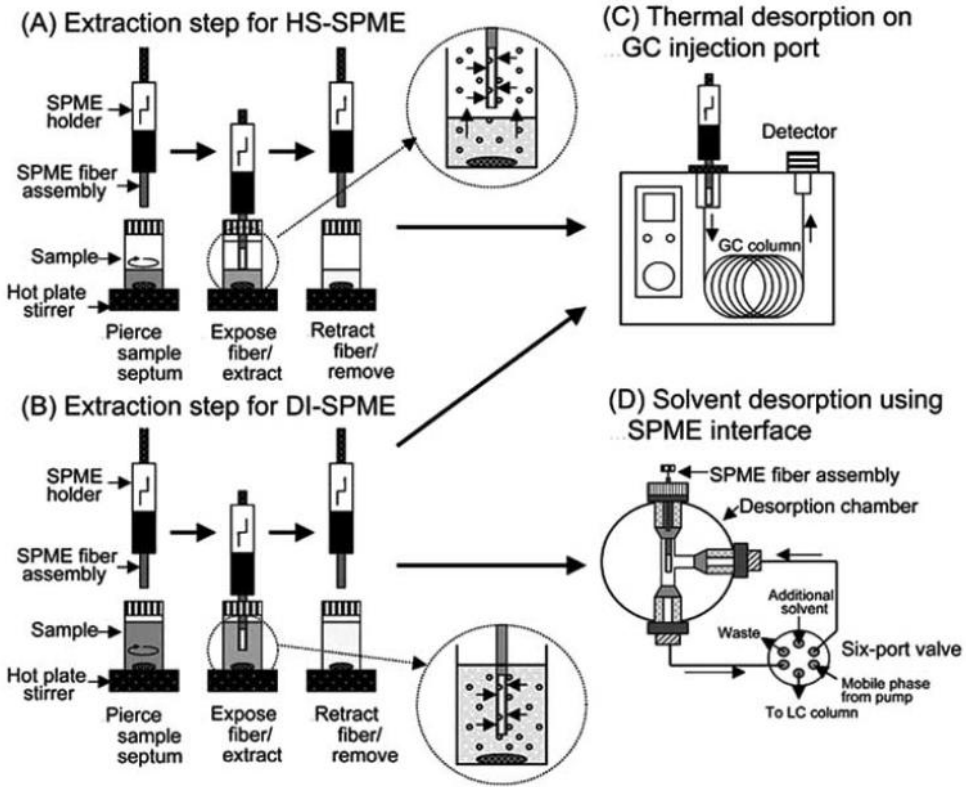


Figure 2.5: SPME procedure by headspace and immersion fiber, and desorption systems for GC and HPLC analysis (Kataoka, et al., 2000)

Numerous reviews have been published dealing with the identification and quantification techniques of PAHs. Chromatographic techniques are often used to determine compounds because they can determine individual concentrations. PAHs can be determined by high performance liquid chromatography (HPLC) (Kiss, et al., 1996; Manoli & Samara, 1999), gas chromatography (GC) (Manoli & Samara, 1997), supercritical fluid chromatography (SFC) (Bernal, et al., 1997; Manoli & Samara, 1997), and capillary electrophoresis (CE) (Szolar, et al., 1995; Nguyen & Luong, 1997). The two most used techniques for PAHs determination are GC and HPLC. GC and to a lesser extend HPLC are commonly used for PAH determination in environmental and food samples. Flame ionization detector (FID) (Ghiasvand, et al., 2006; Lord & Pawliszyn, 1997; Zuazagoitia, et al., 2009) and mass spectrometry (MS) (Barnabas, et al., 1995; Wenzel, et al., 1998; Ratola, et al., 2006; Blasco, et al., 2008), followed by ultraviolet (UV) (Bernal, et al., 1996) and fluorescence detection (FLD) (Kiss, et al., 1996) are the most commonly used detectors for GC and HPLC, respectively.

SPME has been routinely used in combination with GC-FID and GC-MS and successfully applied to PAHs from environmental (Ghiasvand, et al., 2005; Zuazagoitia, et al., 2009), biological (Lord & Pawliszyn, 1997; Yu, et al., 1999) and food samples (Guillen & Sopelana, 2005). SPME produced relatively clean and concentrated extracts, and therefore is ideal and suitable for MS applications. Figure 2.5 summarizes the SPME extraction and desorption procedures for the GC and HPLC systems.

2.3.1 Gas Chromatography coupled to Flame Ionization Detector (GC-FID)

Gas chromatography (GC), specifically gas-liquid chromatography, involves separating and analysing compounds that can be vaporised without decomposition. In GC, the

mobile phase is a carrier gas, usually an inert gas or an unreactive gas. The stationary phase is a microscopic layer of liquid or polymer on an inert solid support, inside a piece of glass or metal tubing called column (Wikipedia, 2010d).

2.3.1.1 Gas Chromatography Analysis

In a GC analysis, a known volume gaseous of liquid analyte is injected into the injector. The sample is transported through the column by the flow of the carrier gas. The gaseous compounds being analysed interact (adsorption) with the walls of the column, which is coated with different stationary phases. This causes each compound to pass through the column at different rates depending on their various chemical and physical properties and their interaction with the specific column. Each compound eluates at a different time, which is known as the retention time of that specific compound. As the compounds exit the end of the column, they are identified (qualitatively) electronically by a detector and the amount of that compound can be determined (quantitatively). The comparison of retention times is what gives GC its analytical usefulness. Other parameters that can be used to alter the order or retention time are the carrier gas flow rate and the oven temperature (Wikipedia, 2010d).

2.3.1.2 Instrumental Components

Figure 2.6 shows a schematic diagram of all the components in a gas chromatograph. The carrier gas must be chemically inert. Commonly used gases include nitrogen, helium, argon, and carbon dioxide. The choice of carrier gas is often dependent upon the type of detector which is used. The carrier gas system also contains a molecular sieve to remove water and other impurities (Biosciences, 2010).

The injector can be used in one of two modes; split or splitless (Figure 2.7). The injector contains a heated chamber containing a glass liner into which the sample is injected through the septum. The carrier gas enters the chamber and can leave by three routes (when the injector is in split mode). The sample vapourises to form a mixture of carrier gas, vapourised solvent and vapourised solutes. A proportion of this mixture passes onto the column, but most exits through the split outlet. The septum purge outlet prevents septum bleed components from entering the column (Biosciences, 2010).

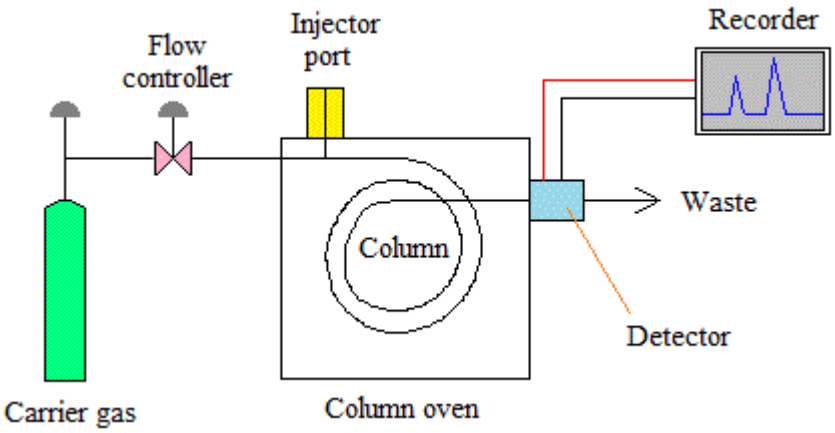


Figure 2.6: Schematic diagram of a gas chromatograph (Biosciences, 2010)

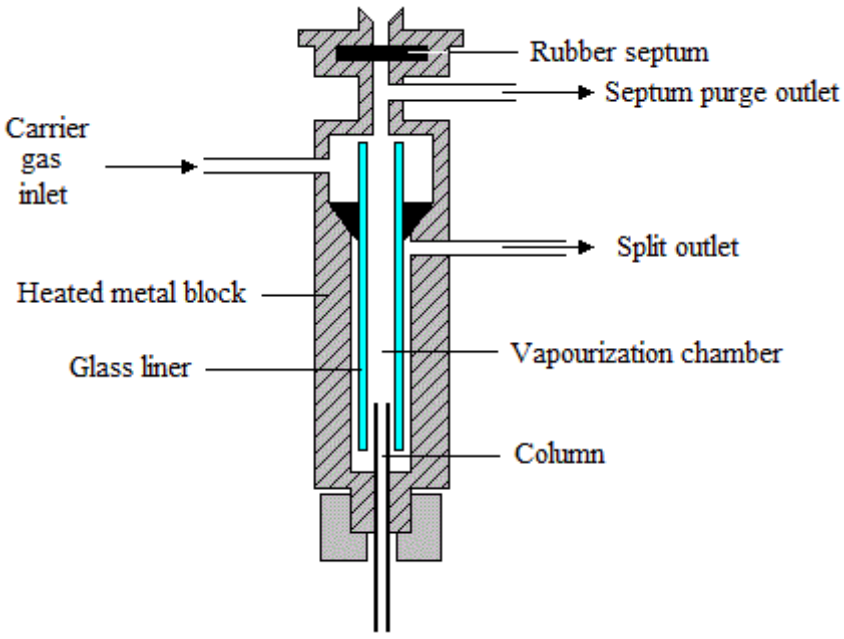


Figure 2.7: A cross-section of a split/splitless injection system (Biosciences, 2010)

There are two general types of column, packed and capillary (also known as open tubular). Packed columns contain a finely divided, inert, solid support material (commonly based on diatomaceous earth) coated with liquid stationary phase. Most packed columns are 1.5-10 m in length and have an internal diameter of 2-4 mm. Capillary columns have an internal diameter of a few tenths of a millimeter. They can be one of two types; wall-coated open tubular (WCOT) or support-coated open tubular (SCOT). Wall-coated columns consist of a capillary tube whose walls are coated with liquid stationary phase. In support-coated columns, the inner wall of the capillary is lined with a thin layer of support material such as diatomaceous earth, onto which the stationary phase has been adsorbed. SCOT columns are generally less efficient than WCOT columns. Both types of capillary column are more efficient than packed columns (Biosciences, 2010).

There are many types of detectors which can be used in gas chromatography. Different detectors will give different types of selectivity. A non-selective detector responds to all compounds except the carrier gas, a selective detector responds to a range of compounds with a common physical or chemical property and a specific detector responds to a single chemical compound (Biosciences, 2010).

The detector being used in this work is the flame ionization detector (Figure 2.8). The effluent from the column is mixed with hydrogen and air, and ignited. Organic compounds burning in the flame produce ions and electrons which can conduct electricity through the flame. A large electrical potential is applied at the burner tip, and a collector electrode is located above the flame. The current resulting from the pyrolysis of any organic compounds is measured. FIDs are mass sensitive rather than concentration sensitive; this gives the advantage that changes in mobile phase flow rate

do not affect the detector's response. The FID is a useful general detector for the analysis of organic compounds; it has high sensitivity, a large linear response range, and low noise. It is also robust and easy to use, but unfortunately, it destroys the sample (Biosciences, 2010).

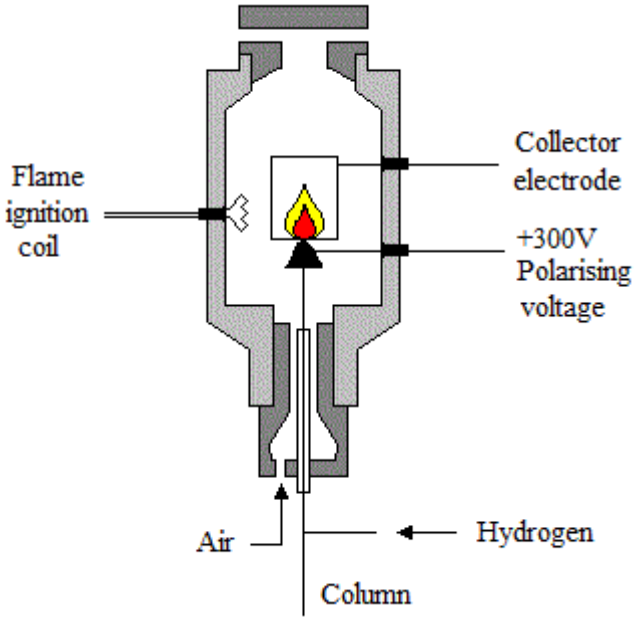


Figure 2.8: Schematic diagram of a flame ionization detector (Biosciences, 2010)

2.3.2 High Performance Liquid Chromatography (HPLC)

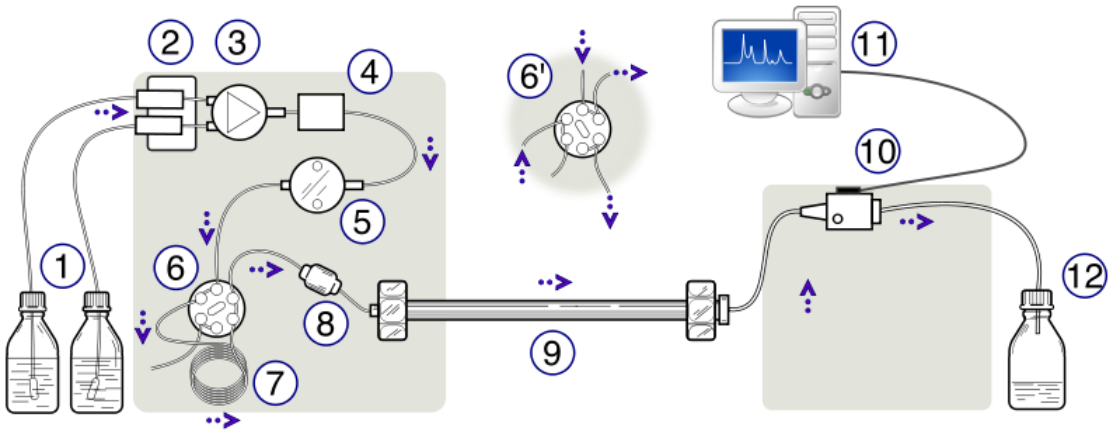


Figure 2.9: Schematic representation of a HPLC unit; (1) Solvent reservoirs, (2) Solvent degasser, (3) Gradient valve, (4) Mixing vessel for delivery of the mobile phase, (5) High-pressure pump, (6) Switching valve in "inject position", (6') Switching valve in "load position", (7) Sample injection loop, (8) Pre-column (guard column), (9) Analytical column, (10) Detector (i.e. IR, UV), (11) Data acquisition, (12) Waste or fraction collector (Wikipedia, 2010e).

High performance liquid chromatography (or high pressure liquid chromatography, HPLC) is a form of column chromatography to separate, identify, and quantify compounds based on their idiosyncratic polarities and interactions with the column's stationary phase. HPLC utilizes different types of stationary phase (typically, hydrophobic saturated carbon chains), a pump that moves the mobile phase(s) and analytes through the column, and a detector that provides a characteristic retention time for the analyte (Wikipedia, 2010e).

The principle behind the separation in HPLC is similar with GC, except that the mobile phase is liquid (solvent) instead of gas, and uses a pump to create high pressure to propel the mobile phase and analytes through the densely packed column with isocratic or gradient elution instead of an oven with temperature programming. Common solvents used include any miscible combination of water or various organic liquids (the most common are methanol and acetonitrile). Water may contain buffers or salts to assist in the separation of the analyte components, or compounds such as trifluoroacetic acid which acts as an ion pairing agent. A normal gradient for reversed phase chromatography might start at 5% methanol and progress linearly to 50% methanol over 25 minutes; the gradient chosen depends on how hydrophobic the analyte is. The gradient separates the analyte mixtures as a function of the affinity of the analyte for the current mobile phase composition relative to the stationary phase. Analyte retention time varies depending on the strength of its interactions with the stationary phase, the ratio/composition of solvent(s) used, and the flow rate of the mobile phase (Wikipedia, 2010e).

2.4 Analytical Method Validation

Method validation is the process of proving that an analytical method employed for a specific test is acceptable for its intended purpose (Green, 1996; Labcompliance, 2010). Results from method validation can be used to judge the quality, reliability and consistency of analytical results; it is an integral part of any good analytical practise. Analytical methods need to be validated or revalidated when it is necessary to verify that its performance parameters are adequate for use for a particular problem (Eurachem, 1998). For example: (a) new method developed for a particular problem; (b) established method revised to incorporate improvements or extended to a new problem; (c) when quality control indicates an established method is changing with time; (d) established method used in a different laboratory, or with a different analysts or different instrumentation; and (e) to demonstrate the equivalence between two methods, e.g. new method and a standard.

In general, validated methods must include studies on specificity, linearity, accuracy, precision, range, detection limit, quantitation limit, and robustness. Although not all of the validation characteristics are needed for all types of tests, typical validation characteristics are (U.S. FDA, 2000):

i. Accuracy – the closeness of the measured value to the true value for the sample;
ii. Precision – the amount of scatter in the results obtained from multiple analyses of a homogeneous sample;
iii. Specificity – the ability of the method to accurately measure the analyte response in the presence of all potential sample components;

iv. Limit of detection – the lowest analyte concentration that produces a response detectable above the noise level of the system, typically, three times the noise level;

v. Limit of quantitation – the lowest level of analyte that can be accurately and precisely measured, or calculated as the analyte concentration that gives S/N = 10;

vi. Linearity and Range – Linearity verifies that the sample solutions are in a concentration range where analyte response is linearly proportional to concentration; and Range is the concentration interval over which acceptable accuracy, linearity, and precision are obtained. In practice, the range is determined using data from the linearity and accuracy studies;

vii. Robustness – the ability to remain unaffected by small changes in parameters such as percent organic content and pH of the mobile phase, buffer concentration, temperature, and injection volume.

Although a thorough validation cannot rule out all potential problems, the process of method development and validation should address the most common ones. Examples of typical problems that can be minimized or avoided are particular type of column that no longer produces the separation needed because the supplier of the column has changed the manufacturing process; an assay method that is transferred to a second laboratory where they are unable to achieve the same detection limit; and a quality assurance audit of a validation report that finds no documentation on how the method was performed during the validation.

CHAPTER THREE – MATERIALS AND METHODOLOGY

3.1 Reagents and Chemicals

All 16 PAHs (naphthalene, acenaphtylene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzo(a)anthracene, chrysene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(a)pyrene, indeno(1,2,3-cd)pyrene, dibenzo(a,h)anthracene, benzo(g,h,i)perylene) solid standards were purchased from Supelco Inc. (Bellefonte, PA, USA). The stock standard solutions of PAHs were dissolved in acetone and working PAH standard solutions were freshly prepared before use by diluting in acetone. All PAH solutions were stored in the dark at 4°C to limit the degradation of the light-sensitive compounds. Acetone (A.C.S grade) was purchased from Fisher Scientific, florisil (100-200 mesh) from Sigma-Aldrich (Deisenhofen, Germany), and anhydrous sodium sulfate from Merck (Darmstadt, Germany). Both dichloromethane and n-hexane were from Merck and were of GC grade (SupraSolv). Nitrogen gas (purity grade 99.9995%), compressed air, purified hydrogen (99.999%) and purified helium (99.999%) were supplied by Mox Gases.

3.2 Equipments

Three different commercially-available SPME coated fibers of 100 µm polydimethylsiloxane (PDMS), 85 µm polyacrylate (PA), and 65 µm polydimethylsiloxane-divinylbenzene, (PDMS-DVB), SPME holder, glass vials (15 ml) and PTFE-silicone septa were purchased from Supelco Inc. The fibers were conditioned prior to use under nitrogen by heating in the injection port of the GC according to the manufacturer’s recommendations (Table 3.1). All samples were extracted from 15 ml

clear glass vials with PTFE-silicone septa and open-hold phenolic caps. To prevent evaporation of the analytes from the moss samples, the vials were immediately sealed with hole-caps and PTFE/silicone septa. Samples were heated using a hot plate stirrer (Harmony, LMS) for the SPME extraction.

Table 3.1: Conditioning temperatures and time for three SPME fiber coatings

Fiber Coating	Conditioning Temperature (ºC)	Conditioning Time (Hrs)
100 µm PDMS	250	0.5
85 µm PA	280	1.0
65 µm PDMS-DVB	250	0.5

3.3 Gas Chromatographic Analysis

Table 3.2: Experimental GC-FID conditions for SPME extraction

GC-FID	Conditions
Flow control mode/rate	Linear velocity; 32.8 cm/sec
Oven temperature	80 ºC (5 min hold time) 180 ºC (20 ºC/min) 250 ºC (5 ºC/min) 270 ºC (4 ºC/min) (2 min hold time) 320 ºC (8 ºC/min) (6 min hold time)
Carrier gas (total flow)	He, 2.3 ml/min
Column flow	1.33 ml/min
Make-up gas	He, 30 ml/min
Compressed air	400 ml/min
Hydrogen	40 ml/min

All gas chromatographic analyses were performed on a Shimadzu GC-2010 system coupled to a flame ionization detection (FID) system (Figure 3.1). Data acquisition and processing were carried out on a DELL computer running the software programme GCsolution, version 2.30 (Fisher Scientific) linked to the GC system. The separation was achieved with a 30 m × 0.25 mm i.d. × 0.25 µm film thickness BPX-5 capillary column (SGE Analytical Science) coated with 5% phenyl polysilphenylene-siloxane.

The oven temperature was programmed from 80 °C (holding time 5 min) to 180 °C at 20 °C/min to 250 °C at 5 °C/min and then to 270 °C at 4 °C/min (holding time 2 min) and finally to 320 °C at 8 °C/min, keeping the final temperature for 6 min. The total runtime was 43.25 min. Injection was performed in the splitless mode, with the split-splitless purge valve opened 1 min after injection. The flow control mode is set to linear velocity with a rate of 32.8 cm/sec. Helium was chosen as the carrier gas with a total flow-rate of 2.3 ml/min. The column flow was 1.33 ml/min. The injection port and detector temperature were 275 °C and 350 °C, respectively, with gas flows of hydrogen, compressed air and helium (make-up gas) set at 40, 400 and 30 ml/min, respectively. Table 3.2 summarizes the GC-FID experimental conditions used for the SPME method for the determination of PAHs in mosses.

Figure 3.1: The GC-FID of Shimadzu model GC-2010

3.4 Study Area and Sampling

Figure 3.2 (a): The moss species, *Hyophila involuta*; (b) A representative of moss collected in Chemistry Department, Faculty of Science, University of Malaya

There is a need to employ alternative moss species for monitoring studies in tropical countries where the climate is hot and humid such as Malaysia since to our knowledge no moss species have been used for such a study using SPME method. A preliminary area was surveyed in order to locate appropriate sites for moss sampling in pristine and polluted areas. Passive monitoring were being used in our studies, naturally occurring mosses were collected from ground surfaces.

The species chosen for this study was the moss *Hyophila involuta* (Figure 3.2(a)), as this species of moss is easily found and grows prevalently and abundantly throughout Malaysia. Mosses of this species were collected from Fraser's Hill in Pahang, which was chosen as the reference sample. Fraser's Hill experiences low levels of air pollution since its surroundings are covered with forests and human activities are limited to recreational only. For mosses as representatives from polluted areas, mosses were collected around the Klang Valley area: (i) Livillas Condominium, Section 16, Petaling Jaya (PJ); (ii) Jalan 17/21, Section 17, PJ; (iii) Jalan Universiti, PJ; and Kuala Lumpur (KL): (i) Chemistry Department, Faculty of Science, University of Malaya (UM) (Figure 3.2 (b)); (ii) Jalan Pudu, KL city centre. The samples were collected using a metal spatula to remove the upper moss layers from the ground. The samples were kept in glass bottles, and transported back to the laboratory to be stored in the dark at 4°C.

3.5 Sample Preparation

In the laboratory, the mosses were detached from the brown dead lower part of the moss cushion, and gently cleaned from other materials like soil and dirt using metal forceps. The green part of the mosses was partially oven-dried between 30-50 °C for 72 hours, and stored in a desiccator until further analysis.

3.6 Headspace SPME Extraction Method

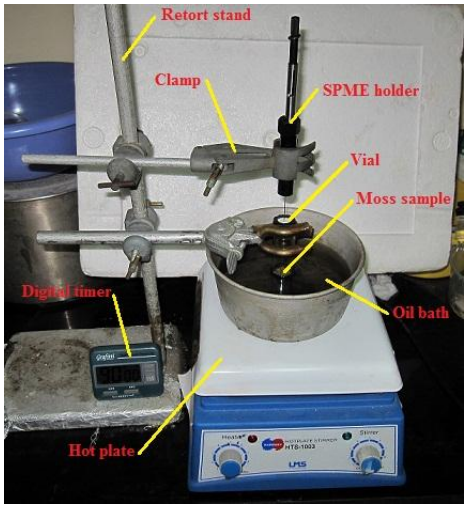


Figure 3.3: Headspace SPME procedure for the extraction of moss samples

Figure 3.3 shows the set-up of the headspace SPME procedure for the extraction of PAHs from moss samples. Sample vial was prepared by first weighing 0.25 g of dried moss sample into an empty vial followed by the addition of 250 µl of 5% (w/v) sodium chloride (NaCl) salt solution which was then sealed with a phenolic screw cap and PTFE-coated silicone septa. The SPME fiber was inserted into the headspace by piercing the septum with the needle of the SPME holder and then lowered by depressing the plunger to expose the 100 µm PDMS fiber above the sample (refer to Figure 3.4(1)). Care was taken to ensure that the fiber did not contact the sample during the analysis. The sample vial was heated at 150 °C (± 5 °C) by supporting it with a clamp in an oil bath which was heated on a hot plate. The PAHs were then extracted in the headspace where the exposed fiber then absorbed the PAHs (Figure 3.4(2)). After equilibrium of 90 min, the fiber was retracted into the needle of the holder (Figure 3.4(3)), and immediately transferred into the GC injector port at 275 °C. The fiber was exposed for 15 min and the PAHs were desorbed thermally into the hot injector port (Figure 3.4(4)). Reinserting the fiber after the run did not show any carry over. The extracted PAHs were separated along the GC capillary column to be detected by the FID detector.

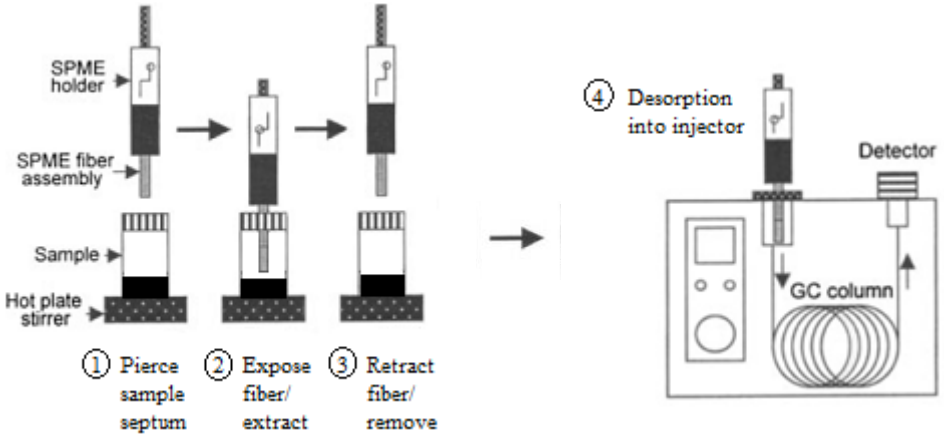


Figure 3.4: Headspace SPME procedure followed by thermal desorption on GC injector port

The results were interpreted by applying the peak area values versus concentration based on their respective retention times and peaks of the individual PAHs in the chromatograms. All samples were done in triplicate.

3.7 Optimization of Headspace SPME Extraction Method

Before the HS SPME extraction can be carried out in our studies, the method is required to be optimized to achieve the best extraction efficiency of PAHs from moss samples. Different parameters affecting the extraction were studied, such as fiber coating selection, extraction temperature and time, desorption conditions, and the effect of salt and water addition.

For our preliminary studies, all experimental steps for the optimization method are the same with the headspace SPME extraction method as above except where mentioned. All moss samples (from Fraser's Hill) tested were spiked to 5000 ng/g of PAH standard solutions.

3.7.1 Fiber Selection

Three types of SPME fiber coatings were compared for the best extraction efficiency of the sixteen PAHs from moss samples. The three commercially available fibers used in this study were 100 µm PDMS (liquid and non-polar), medium polarity 65 µm PDMS-DVB (porous spheres where DVB microspheres are immobilized by PDMS) and 85 µm PA (polar) fibers (Vinas, et al., 2007). These fiber coatings were chosen in order to compare the effect of different polarity of the stationary phases towards the extraction of PAHs.

At this stage, extraction time and temperature was set at 90 min and 150 ºC respectively, in order to assure that either the equilibrium could be established between the headspace and the coating or a large amount of compounds would be extracted into the fiber coating. No addition of water or salt solution were carried out, and desorption time was set to 15 min.

3.7.2 Extraction Temperature

The influence of the extraction temperature was tested at three different temperatures at 110, 130 and 150 ºC to observe the behaviour of PAHs in order to identify the conditions allowing a better uptake from moss samples. The extraction time of 90 min, desorption temperature and time of 275 ºC and 15 min were chosen respectively. There was no addition of water or salt solution into the samples and the fiber used for the headspace SPME extraction method was 100 µm PDMS.

3.7.3 Extraction Time

The effect of the amount of PAHs absorbed was studied by exposing the fiber to the headspace above the spiked moss samples in a vial for 30, 60 and 90 min. The temperature of adsorption was set to the highest temperature at 150 ºC throughout the extraction process and water or salt solution was not added. The desorption temperature and time were fixed at 275 ºC and 15 min respectively, and the fiber used was 100 µm PDMS. The optimization of the extraction time was done to determine the time needed to reach equilibrium or an amount high enough to obtain a proper quantification for analysis.

3.7.4 Desorption Conditions

Desorption conditions for the headspace SPME extraction method were set for desorption temperature at 275 ºC and desorption time at 15 min. Since the recommended operating temperature of the 100 µm PDMS fiber is in the range of 200-280 ºC, the desorption temperature chosen is 275 ºC, 5 ºC below the maximum temperature. This is to prolong the life of the fiber and ensure that the fiber can perform at its optimum condition.

The desorption time of 15 min were chosen base on previous studies (King, et al., 2003; Waidyanatha, et al., 2003) being done and results were sufficient for the complete desorption of PAHs from the PDMS fiber. Moreover, reinserting the fiber into the GC injector after the run did not show any obvious carry-over.

3.7.5 Salt and Water Addition

The addition of water or salt solution can improve the extraction of analytes in the SPME extraction method. A 100 µm PDMS fiber was used with extraction temperature and time profiles set to 150 ºC and 90 min. The desorption temperature and time used were 275 ºC and 15 min. A volume of 100, 250 and 500 µl of water or 5% (w/v) salt solution were added into the moss respectively to increase the salting out effect of PAHs into the headspace from the moss samples.

3.8 Quality Assurance and Quality Control

The HS SPME method has been checked for linearity, accuracy and precision and to determine the limit of detection (LOD) and limit of quantitation (LOQ) of PAHs by applying the standard addition method. To evaluate the linearity, a seven-level calibration study was performed using 0.25 g of dried moss samples that were spiked with a series of standard solutions containing all 16 PAHs, with increasing concentrations to give 0, 150, 220, 500, 1000, 2000 and 2600 ng/g. For each level, two replicate extractions were performed at optimal conditions. The linearity of the method was evaluated by regressing PAH peak areas, separately, versus the analyte concentration for standard fortified moss samples after the optimized SPME procedure was applied to all standards. Prior to extraction, the recovery and repeatability studies was investigated on fortified moss samples obtained by spiking a mixture of PAHs into moss samples to three different final concentration of the standards (high, mid and low concentrations at 2600, 2000, and 150 ng/g). Each concentration was analyzed in triplicate. Five replicates were analyzed for the determination of LOD and LOQ for all 16 PAHs.

CHAPTER FOUR – RESULTS AND DISCUSSION

In order to investigate the performance of the SPME technique for the analysis of PAHs, preliminary studies were carried out by performing experiments under conditions providing the highest extraction efficiency of PAHs from spiked moss samples. Since HS SPME efficiency results from a very complex process and potentially affected by a large number of factors, the optimisation of the experimental conditions represent a critical step in the development of a SPME method. Attention to seemingly minor details can often contribute greatly to the success of the PAHs analysis.

4.1 Optimization of SPME Parameters

SPME method development typically involves a number of stages. Consideration is given to such areas as fiber coating selection, extraction temperature and time, desorption conditions, addition of modifiers and detection. In the following discussions, the areas given consideration in the current method development are presented, and factors influencing the decisions made are explained. Remember that in SPME neither complete extraction of analytes nor full equilibrium is necessary, but consistent sampling time and temperature are critical.

In this work, we have evaluated several factors affecting analyte recovery and apply the findings to the optimization of PAH analysis in mosses, and the method was validated using naturally contaminated moss samples which were collected at five locations in the vicinity of Klang Valley in Malaysia.

4.1.1 Extraction Mode

Liquid sampling involves immersing the SPME fiber directly into the liquid phase of a sample, whereupon trace components are enriched on the fiber's surface. This technique is suitable only for relatively clean samples that do not contain large amounts of non-volatile components and interfering contaminants, as these may be adsorbed to the fiber surface as well. On the other hand, direct sampling may not work well when sampling analytes from wastewater samples with grease or oil, or more complex samples that contain solid or high molecular weight materials such as soil or sludge. Sampling from the headspace above the sample is necessary in these cases (Wercinski, 1999).

In headspace SPME, three phases (coating, headspace, and matrix) are involved, and the chemical potential difference of analytes among the three phases is the driving force that moves analytes from their matrix to the fiber coating (Zhang, et al, 1994). Headspace sampling also conveniently eliminates concerns of absorbing non-volatile, high K_{fs} components and reduces the matrix effect. In addition to that, the equilibrium concentration is independent of the fiber location in the sample/headspace system (Wercinski, 1999).

In our study, the headspace extraction was selected for method development in comparison to the direct-immersion SPME method for the analysis of PAHs in mosses for the following reasons. Firstly, the aim was to directly extract the PAHs into the headspace and into the fiber without under-going any other extraction or clean-up steps. Secondly, by isolating the fiber coating from direct contact with the sample matrix, adverse effects due to harsh sample conditions could be minimized, and this could maximize the number of extractions before the fiber needs to be changed, which means

that the lifetime of the fiber can be prolong. Equilibrium is attained more rapidly in headspace SPME than in direct immersion, because the analytes can diffuse more rapidly to the coating on the fiber.

4.1.2 Fiber Selection

When selecting the appropriate fiber for extracting a sample, the physical characteristics of the analytes of interest and detection levels must be considered. These factors are molecular weight, boiling point, vapor pressure, polarity, functional group, concentration range and detector type. These factors need to be matched with the variables in fiber coatings, such as film thickness, polarity and porosity. The thicker the coating, the more the analyte is absorbed by the coating, and vice versa; the thickness of the coating will also affect the detection limit of the SPME method. Most PAHs, and especially those compounds with greater than four rings, have high distribution constants (K_{fs}) which result in high sensitivity. Their non-polar character makes them ideal for extraction using a PDMS stationary phase. Thicker fiber coatings have value where extreme sensitivity is required and the sample carryover problem can be solved, possibly by increasing GC desorption time and injector temperature. Thicker coatings also result in longer extraction time (Wercinski, 1999).

In SPME, equilibrium is established among the concentrations of an analyte in the sample, in the headspace above the sample, and in the polymer coating on the fused silica fiber. The amount of analyte adsorbed by the fiber depends on the thickness of the polymer coating and on the distribution constant for the analyte. Extraction time is determined by the length of time required to obtain precise extractions for the analytes with the highest distribution constants. The distribution constant generally increases

with increasing molecular weight and boiling point of the analyte. Selectivity can be altered by changing the type of polymer coating on the fiber, or the coating thickness, to match the characteristics of the analytes of interest. In general, volatile compounds require a thick coating, and a thin coating for semi-volatile analytes (Supelco, 2010).

Table 4.1: The retention time and some physicochemical properties (boiling point, molecular weight and number of rings) of the sixteen PAHs

Compound	Retention time (min)	Boiling point (°C)*	Molecular weight (g/mol)*	Number of rings
Naphthalene	8.65	218	128.19	2
Acenaphthylene	11.22	270	152.21	3
Acenaphthene	11.50	279	154.21	3
Fluorene	12.50	294	166.23	3
Phenanthrene	14.90	338	178.24	3
Anthracene	15.05	340	178.24	3
Fluoranthene	18.97	383	202.26	4
Pyrene	19.87	393	202.26	4
Benzo(a)anthracene	25.03	-	228.30	4
Chrysene	25.20	441	228.30	4
Benzo(b)fluoranthene	30.02	481	252.32	5
Benzo(k)fluoranthene	30.17	481	252.32	5
Benzo(a)pyrene	31.75	496	252.32	5
Indeno(123-cd)pyrene	36.08	-	276.34	6
Dibenzo(ah)antracene	36.16	-	278.36	5
Benzo(ghi)perylene	36.90	-	276.34	6

*(Source: Bjørseth, 1983)

PAHs are generally classified as non-polar compounds with low water solubility. Among the sixteen PAHs, these compounds have different physiochemical properties (Table 4.1) and showed different sorption behaviours in the polymeric organic phases of the fibers. Different fibers have different selectivities and sensitivities to target analytes, so the fiber must first be evaluated and then one selected for future studies. Taking into

account the findings of several authors (Garcia-Falcon, et al., 2004; Guillen & Sapelana, 2005; Fernandez-Gonzalez, et al., 2007; Vinas, et al., 2007; Zuazagoitia, et al., 2009), it seems clear that fiber coatings of PA, PDMS and PDMS-DVB were mostly being used for the study of PAHs in various matrices. The most widely used has been the 100 µm thickness PDMS. Nevertheless, 65 µm PDMS-DVB and 85 µm PA coating and have also given good results. Therefore the behaviour of these three fibers were compared in order to choose which fiber coating gives the best PAH extraction efficiency.

From the chromatograms of Figure 4.1(a) and 4.1(b), all fibers gave satisfactory peak area and the highest peak area was obtained for the lower molecular mass PAHs. It can be seen that PA fiber showed an increase in the peak area for the two- and three-rings PAHs (naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, anthracene) relative to the PDMS-DVB and PDMS fiber. This means that the PA fiber could easily improve the extraction efficiency of the low-molecular-mass PAHs, and this matches well with the results of Doong, et al. (2000) and Guillen and Sopelana (2005) because, according to these authors, PA enhances the extraction efficiency of PAH with two and three rings. For the four-membered ring PAHs (pyrene, benzo(a)anthracene), the PDMS-DVB demonstrated to have better adsorption characteristic due to the bipolar nature of these PAHs. On the other hand, the PDMS fiber showed to have higher affinities towards the five- and six-ring PAHs. The readily partitioning of the high-molecular-mass PAHs is favoured into a more non-polar fiber coating in comparison to a polar fiber. After investigation, the extraction efficiency of the PAHs is dependent on the polarity of the polymeric phase of the fiber coating and the number of fused-benzene rings of the PAHs. The 100 µm PDMS fiber was the best of the three coatings after considering the detection sensitivity of the sixteen PAHs, especially the five and six-ring PAHs and therefore was chosen for further optimization of the procedure.

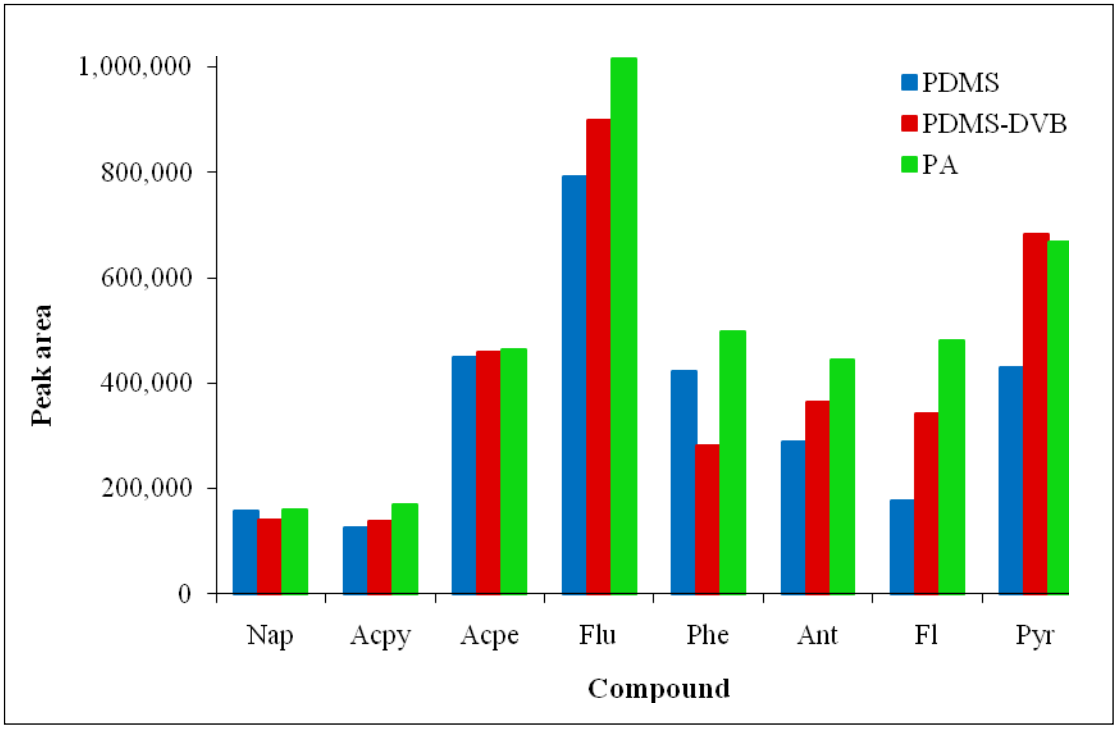


Figure 4.1(a): Comparison of three SPME fibers for the extraction of 8 PAHs from mosses (Nap: naphthalene; Acpy: acenaphthylene; Acpe: acenaphthene; Flu: fluorene; Phe: phenanthrene; Ant: anthracene; Fl: fluoranthene; Pyr: pyrene)

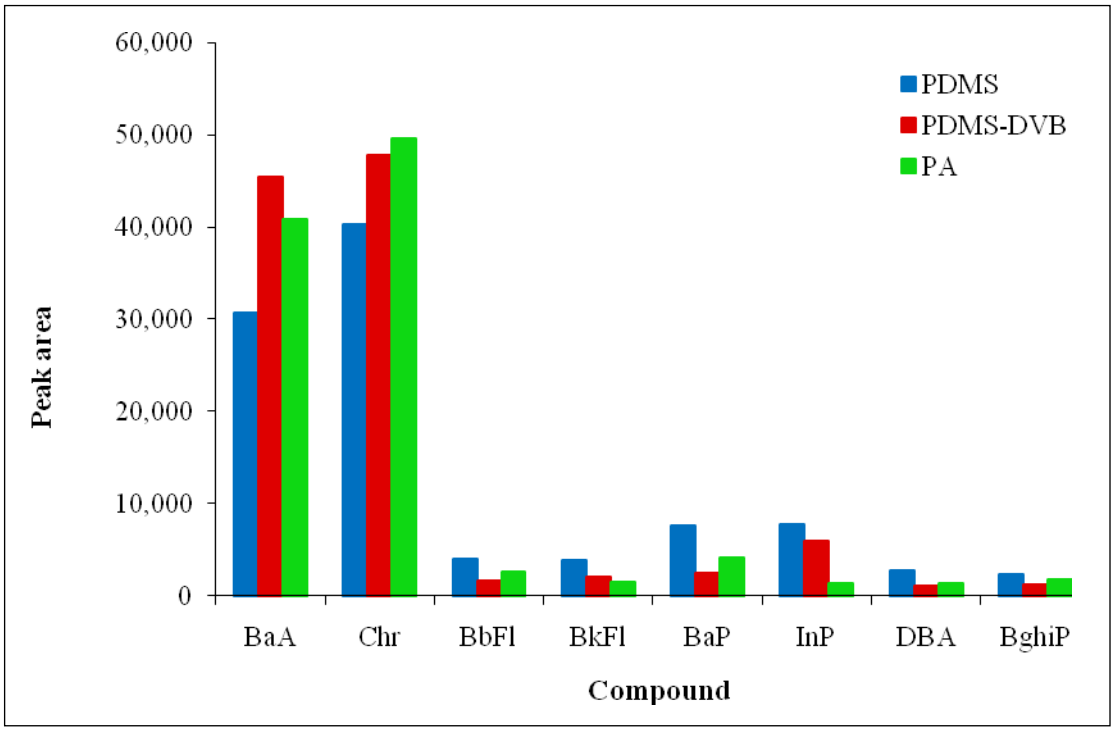


Figure 4.1(b): Comparison of three SPME fibers for the extraction of 8 PAHs from mosses (BaA: benzo(a)anthracene; Chr: chrysene; BbFl: benzo(b)fluoranthene; BkFl: benzo(k)fluoranthene; BaP: benzo(a)pyrene; InP: indeno(123-cd)pyrene; DBA: dibenzo(ah)anthracene; BghiP: benzo(ghi)perylene)

4.1.3 Extraction Temperature

The other parameter that is very important to optimise in SPME extraction is the extraction temperature. At elevated temperatures native analytes can effectively dissociate from the matrix and move into the headspace for rapid extraction by the fiber coatings. The less volatile compounds (e.g. PAHs) or compounds which have strong chemical interactions with the moss can be desorbed by heating the sample to an elevated temperature and then be sampled by the headspace SPME method. Thermal desorption of organic compounds from the contaminated moss is quite effective for analysing this difficult category of samples since high temperature can overcome the energy barrier of the chemisorptions of analytes on moss.

Generally, increasing the sample temperature will increase the sensitivity for the higher boiling components, but decrease the sensitivity for the lower boiling components (Wercinski, 1999). This means that increasing the sample temperature creates increased partition coefficients and decreased the coating/sample partition coefficient, resulting in a diminution in the equilibrium amount of analyte extracted. In headspace SPME, an increase in the sample temperature increases the analyte concentration in the headspace, thereby providing faster extractions. Additionally, the higher temperature increases the extracted amount for high molecular weight compounds. However, the temperature increase has an adverse effect for more volatile components. This can be seen for naphthalene and acenaphthylene which resulted in lower extraction efficiencies compared with the other higher rings PAHs in Figure 4.2(a) and 4.2(b) and because of their higher volatility which contributes to the significant reduction of the partition coefficients at the same temperature of 110-150 ºC.

The results for most PAHs showed an increase in the peak area when the temperature increased from 110 °C to 130 °C, and finally to 150 ºC. Theoretically, the mass-transfer process of the analytes from the matrix to the fiber will improve at elevated temperatures, and therefore increasing the diffusion coefficients of the analytes into the headspace, especially for the semi-volatile PAHs (high-molecular-mass PAHs). On the other hand, it can be observed from Figure 4.2(a) and 4.2(b), the compounds with lower boiling points (naphthalene and acenaphthylene) exhibited a small decrease in the extraction efficiency when the temperature is raised from 130 °C to 150 ºC. Since the adsorption of analytes by the fiber coating is an exothermic process, the partition coefficients decreased with the increased in temperature. This resulted in a decrease in the affinity of the coating for absorption, especially for lower boiling point PAHs and the sensitivity would be decreased. Therefore the extraction capacity is a compromise between these two different parameters. Temperature higher than 150 ºC was not considered as an option because of the extreme heat which is not suitable for working conditions and also maintaining a constant temperature in the oil bath over the extraction time is difficult. Taking into account all these findings, it was decided to keep the extraction temperature at 150 ºC.

Semi-volatiles (e.g. PAHs) in dirty matrices were more difficult to determine with SPME, and so required heating to drive the analytes into the headspace. Heating also altered the partitioning of the analyte between the headspace and the fiber to favour the headspace. When sampling at elevated temperatures, it was particularly important to use vials that will not leak and lose analyte with the increased pressure that generated.

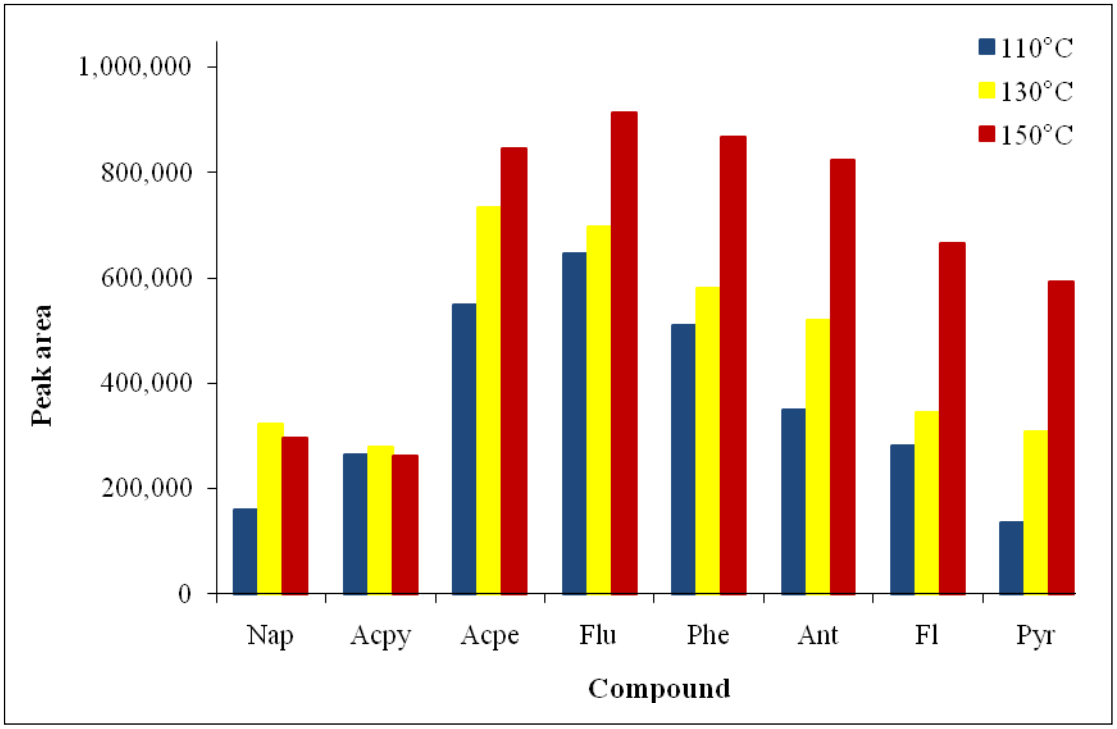


Figure 4.2(a): Comparison of three extraction temperatures for the determination of 8 PAHs from mosses (Nap; Acpy; Acpe; Flu; Phe; Ant; Fl; Pyr)

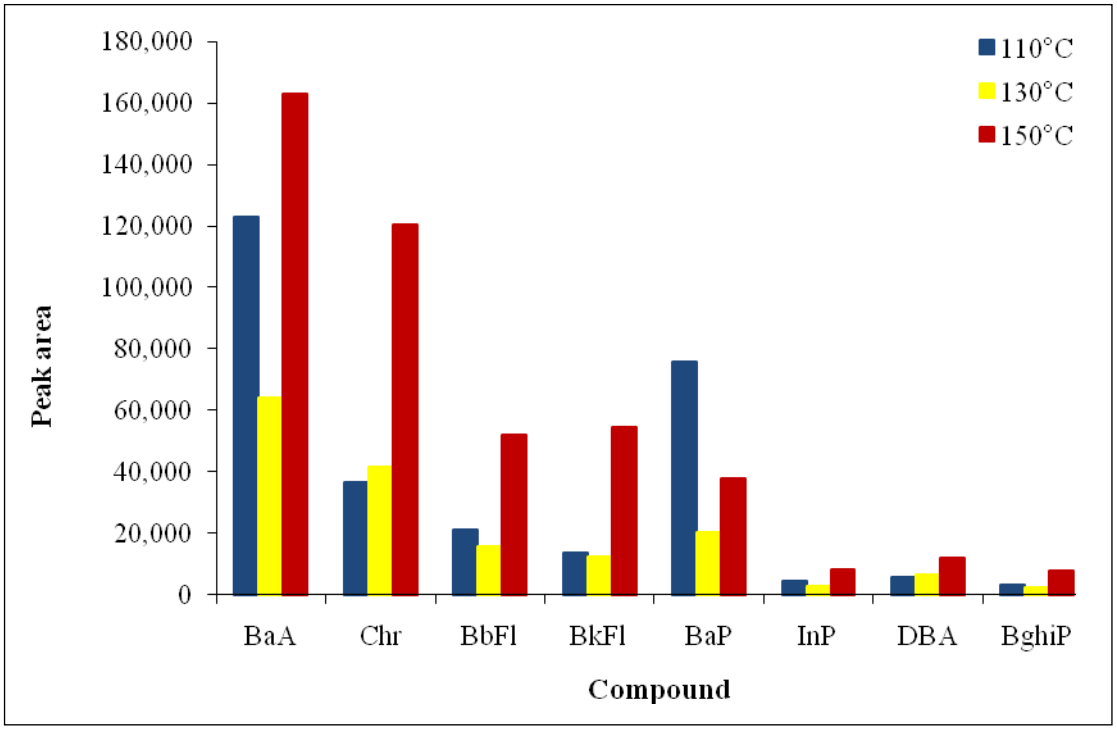


Figure 4.2(b): Comparison of three extraction temperatures for the determination of 8 PAHs from mosses (BaA; Chr; BbFl; BkFl; BaP; InP; DBA; BghiP)

4.1.4 Extraction Time

When developing conditions for SPME sampling, one should plot detector response versus sampling time for each analyte in the sample. The data will show that highly volatile analytes will reach a plateau in 15 minutes or less, indicating equilibrium. Compounds of lower volatility will show a steadily increasing response with time. For many volatile and semi-volatile compounds, equilibrium is usually not achieved in a reasonable time (even with sampling mixing) (Wercinski, 1999). Although it is not necessary to reach extraction equilibrium for all PAH, the extraction time should be sufficient to permit the detection of the PAH with higher molecular weights.

Headspace SPME sampling time is dependent on the kinetics of mass transport in which the analytes move from the liquid or solid phase to the headspace and finally to the fiber coating. As the fiber coating absorbs organic analytes, the analytes vaporised and undergo a series of transport processes from the matrix to gas phase, and eventually to the coating, until the system reaches equilibrium. For semi-volatile compounds (e.g. PAHs), the low volatility and relatively large molecular size may slow the mass transfer from the matrix to the headspace, consequently resulting in a longer extraction time (Wercinski, 1999). Volatile analytes usually are extracted faster than semi-volatile ones, owing to higher diffusion coefficients. The equilibration time is usually shorter for analytes of lower sample/coating partition coefficients, since less analyte has to get to the coating through the stationary aqueous layer to reach equilibrium. When sampling from headspace, analytes that have small Henry's constants (are well soluble in water) tend to equilibrate more slowly, since at any given moment only a limited number of analyte molecules can be transported through the headspace which acts as a bottleneck. Temperature increases the diffusion coefficients of the analyte molecules and decreases

the analyte/coating partition coefficients; hence equilibration times are shorter at elevated temperatures. On the other hand, increasing the temperature adversely affects sensitivity and complicates the experimental setup.

The extraction time profiles are shown in Figure 4.3(a) and 4.3(b) for the sixteen PAHs using the HS SPME method. For most cases, the peak areas were generally more pronounced for PAHs of higher-molecular-mass. For naphthalene, acenaphthylene, acenaphthene and fluorene, 30 min of extraction time gave the highest peak areas; while phenanthrene reached equilibrium in 60 min. For the higher-molecular-mass PAHs (anthracene onwards), 90 min of adsorption time was needed for maximum adsorption of the compounds. The more volatile PAHs (lower-molecular-mass) equilibrated faster into the headspace compared to the less volatile PAHs since the less volatile PAHs have lower partition coefficients. Longer exposure time of the fiber increased the equilibration process of the less volatile PAHs between the matrix and the headspace, and therefore increased the competition of the more volatile PAHs towards the affinity of the fiber. This has decreased the amount of more volatile PAHs being adsorbed to the fiber. Extraction time longer than 90 min were not studied in order to avoid the excessive extension of the analysis time. Therefore, a 90 min extraction time was adopted, even though the high-ring PAHs had not reach the equilibrium at this time, but the analytical sensitivity was sufficient.

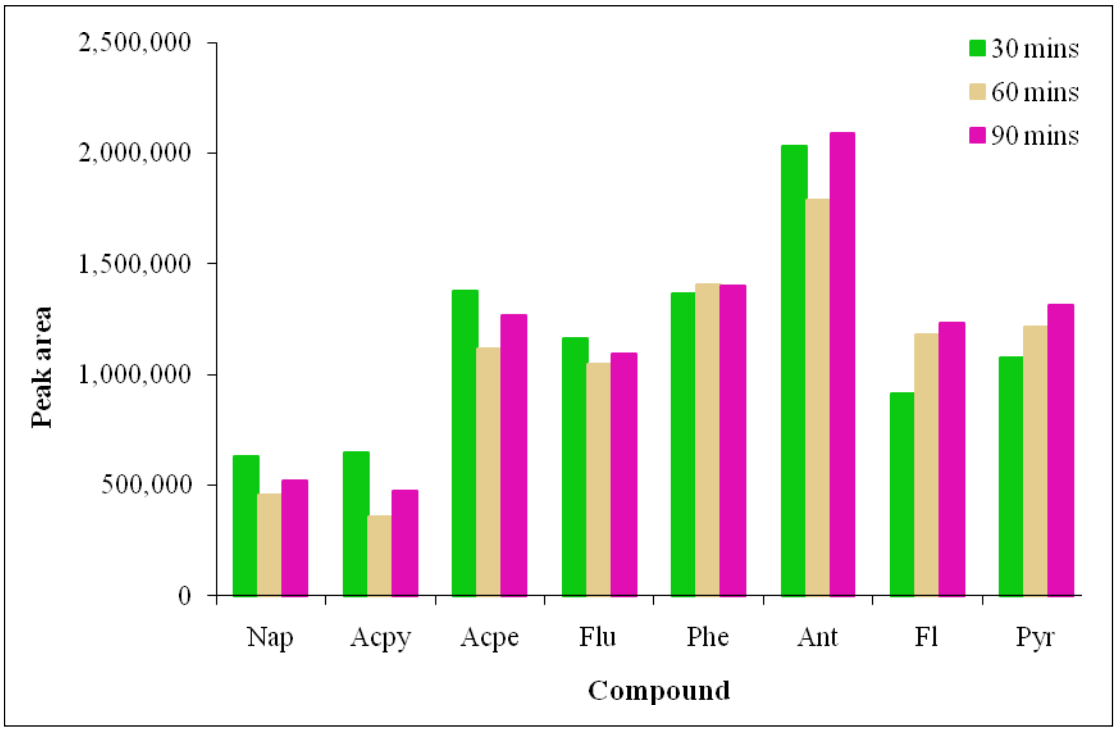


Figure 4.3(a): Comparison of three extraction times for the determination of 8 PAHs from mosses (Nap; Acpy; Acpe; Flu; Phe; Ant; Fl; Pyr)

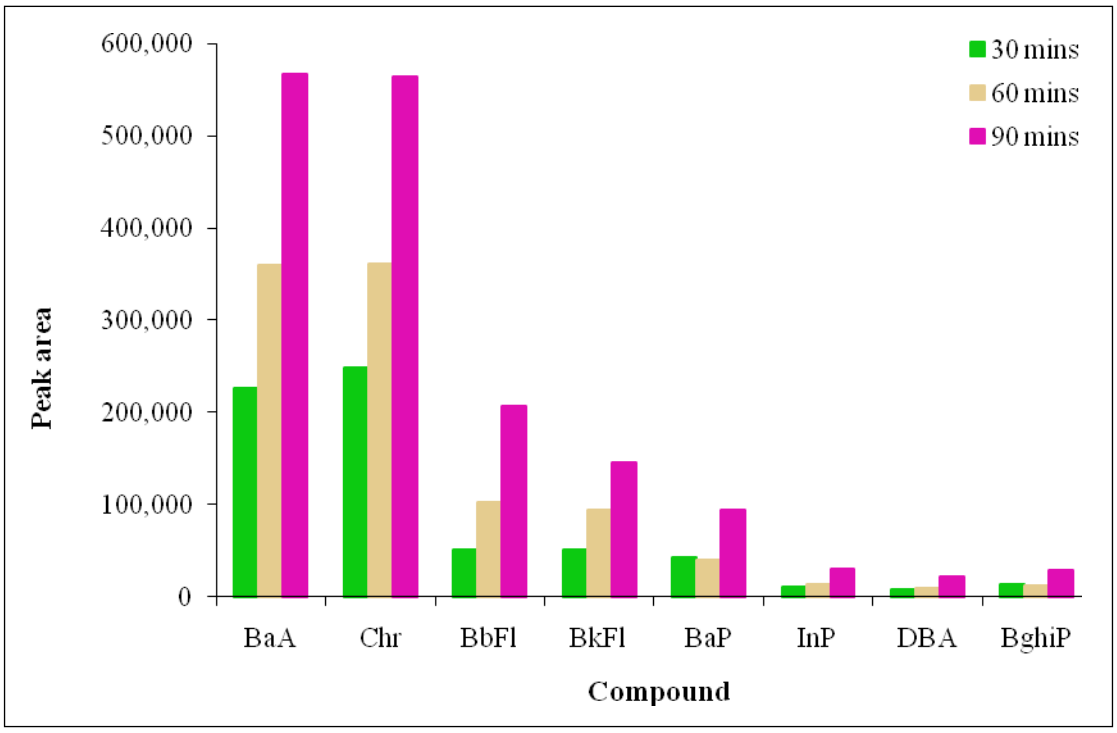


Figure 4.3(b): Comparison of three extraction times for the determination of 8 PAHs from mosses (BaA; Chr; BbFl; BkFl; BaP; InP; DBA; BghiP)

4.1.5 Desorption Conditions

Desorption, which is closely related to the efficiency of the chromatographic separation and the precision of quantitation, has a substantial influence on the quality of data obtained. As the temperature increases, the coating/gas partition coefficients decrease and the fiber coating's ability to retain analytes quickly diminishes (Wercinski, 1999). The desorption time is also an important parameter to ensure that PAHs are completely desorbed from the fiber to reach the highest sensitivity and to avoid carry-over, but must also consider the stability and lifetime of the fiber.

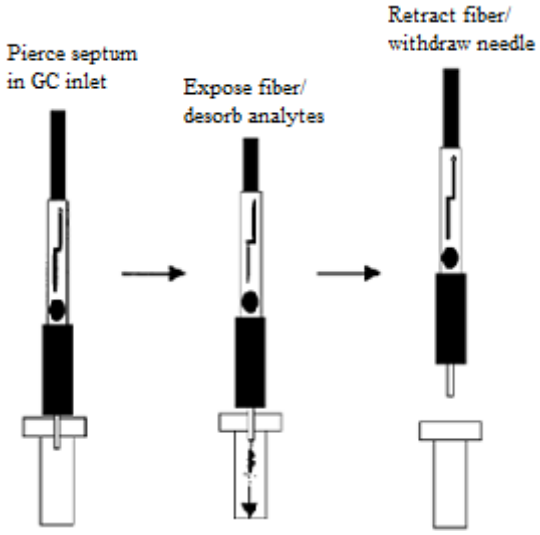


Figure 4.4: Desorption procedure on GC injector port

During the desorption process, the analyte diffuses from the coating into the stream of carrier gas in the injection port of the GC instrument (Figure 4.4). Desorption of an analyte from a fiber depends on the boiling point of the analyte, the thickness of the coating on the fiber, and the temperature of the injection port (Supelco, 2010). The more volatile the compounds, the faster the desorption. A lower temperature may cause unnecessarily slow desorption while an excessively high temperature may destroy the coating, cause increased fiber background levels and may lead to accelerated coating deterioration.

The manufacturer suggested the maximum temperature of the PDMS fiber to be 280 ºC and the recommended operating temperature is between 200-280 ºC, and therefore in this study, the desorption temperature of 275 ºC was chosen. The fiber was used more than 100 times in our studies. No other optimum desorption temperature studies were carried out. A desorption time of 15 min was sufficient to desorb all PAHs quantitatively from the 100 µm PDMS fiber (King, et al., 2003; Waidyanatha, et al., 2003). When chromatographic analysis was completed, the fiber was immediately desorbed again for one or two more minutes at 275 ºC to ascertain that no carry-over occurs; no peaks were registered. Hence, 15 min was used as the optimal desorption time for all of the experiments.

4.1.6 Salt and Water Addition

Additives (small amount of appropriate compounds added to the matrix or extracting phase to enhance the extraction of analytes) or chemical modifiers are also an alternative method to improve extraction efficiency. The modifiers included salts, water and organic solvents such as methanol. Various additives affect analytes in matrices differently, so that polar analytes are more affected by the presence of salt in the sample and non-polar analytes might be more affected by a fatty or oily matrix (Wercinski, 1999). The applications have been found to be useful for analysis of PAHs.

Water had proven to be a very effective additive to facilitate the release of analytes from the matrix and it is often used to accelerate extraction. It can also be used in combination with high temperature extractions to remove and dissolve even very non-polar analytes (e.g. PAHs). This is possible because the dielectric constant of water decreases rapidly with temperature increase. This property has been used in the SPME

method of liquids and solids (Pawliszyn, 1997). Another common technique to enhance extraction of organics from sample matrix is salting. Salting out effect can increase or decrease the amount extracted, depending on the compounds and salt concentration. In general, the salting effect increases with increase of compound polarity.

From Figure 4.5(a) and 4.5(b), both show that the addition of distilled water or salt solution (5% w/v) has a significant effect on the extraction efficiency of PAHs, especially for anthracene, fluorene, benzo(a)anthracene, chrysene, benzo(b)fluoranthene, benzo(k)fluoranthene and benzo(a)pyrene. No addition of either water or salt solution also showed to have good influence on the amount of PAHs extracted. Comparing between the addition of salt and water, the salting out effect seems to have a greater influence on the amounts of PAHs extracted. This concludes that the addition of a displacing agent can help to displace the PAHs from the surface of mosses followed by extraction by headspace SPME, and this has shown improvement in the results compared to the extraction of mosses without any water or salt addition.

On the other hand, spiking with higher amounts of water is not a reasonable solution; its evaporation at 150 ºC causes a high pressure in the extraction vial and increases the risk of leaking, especially for lower boiling point compounds such as naphthalene, acenaphthylene and fluoranthene as can be seen in Figure 4.5(a) and 4.5(b). Therefore the optimal condition being used is by the addition of 250 µl of 5% (w/v) NaCl solution into the moss samples.

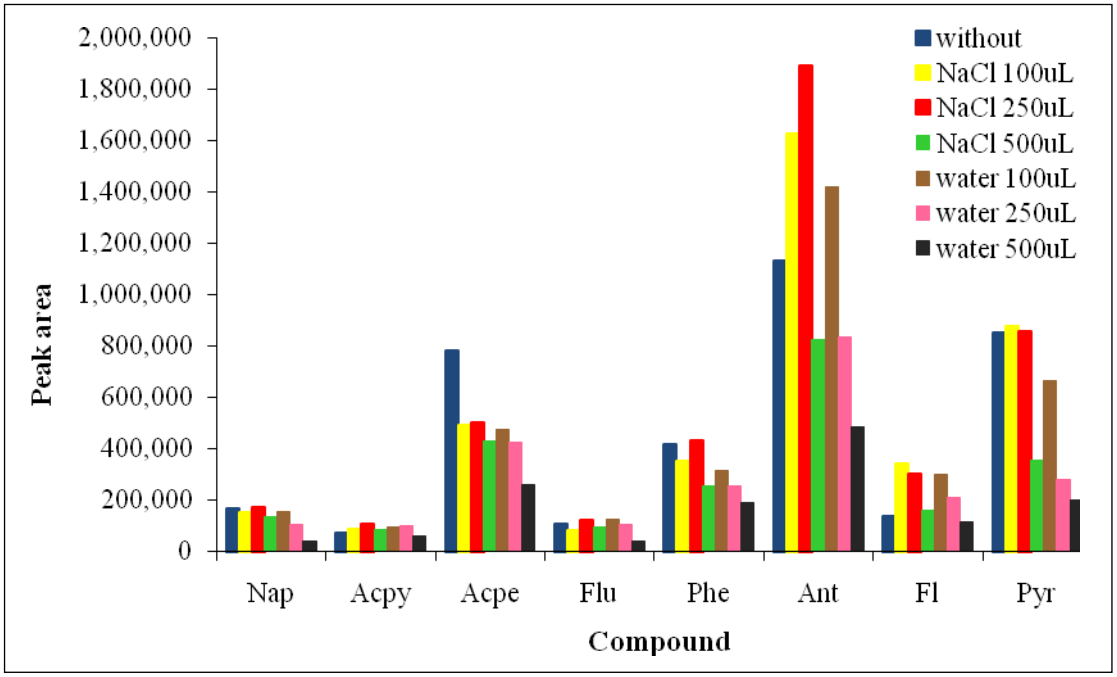


Figure 4.5(a): Comparison of non-addition, addition of water or 5% (w/v) salt solution of different volumes for the determination of 8 PAHs from mosses (Nap; Acpy; Acpe; Flu; Phe; Ant; Fl; Pyr)

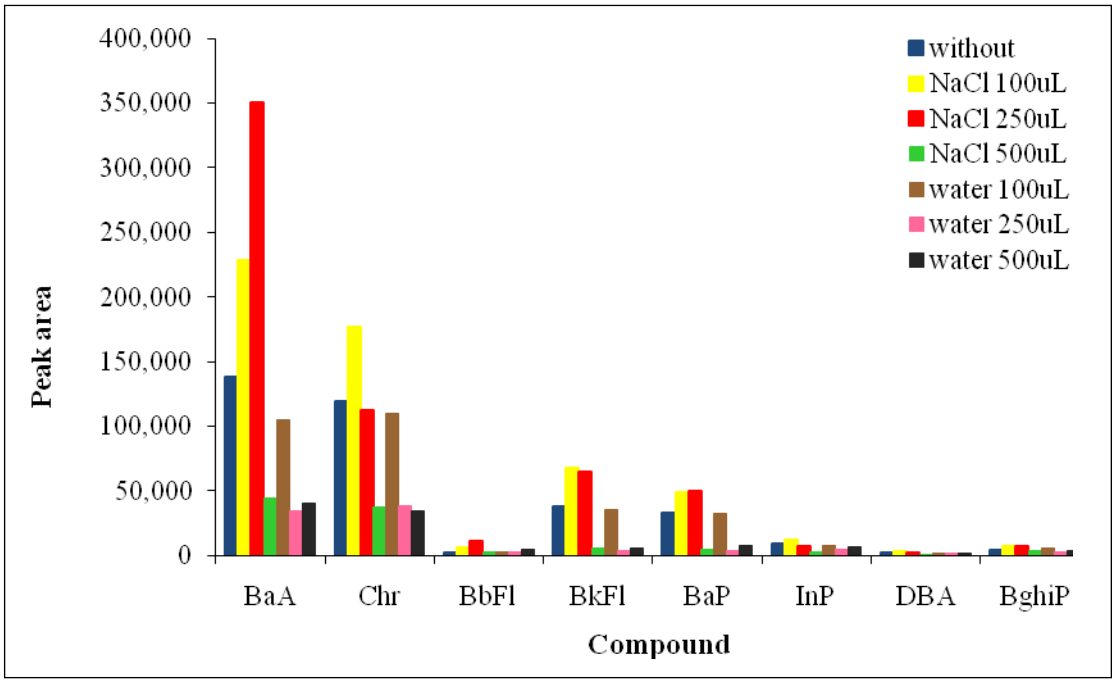


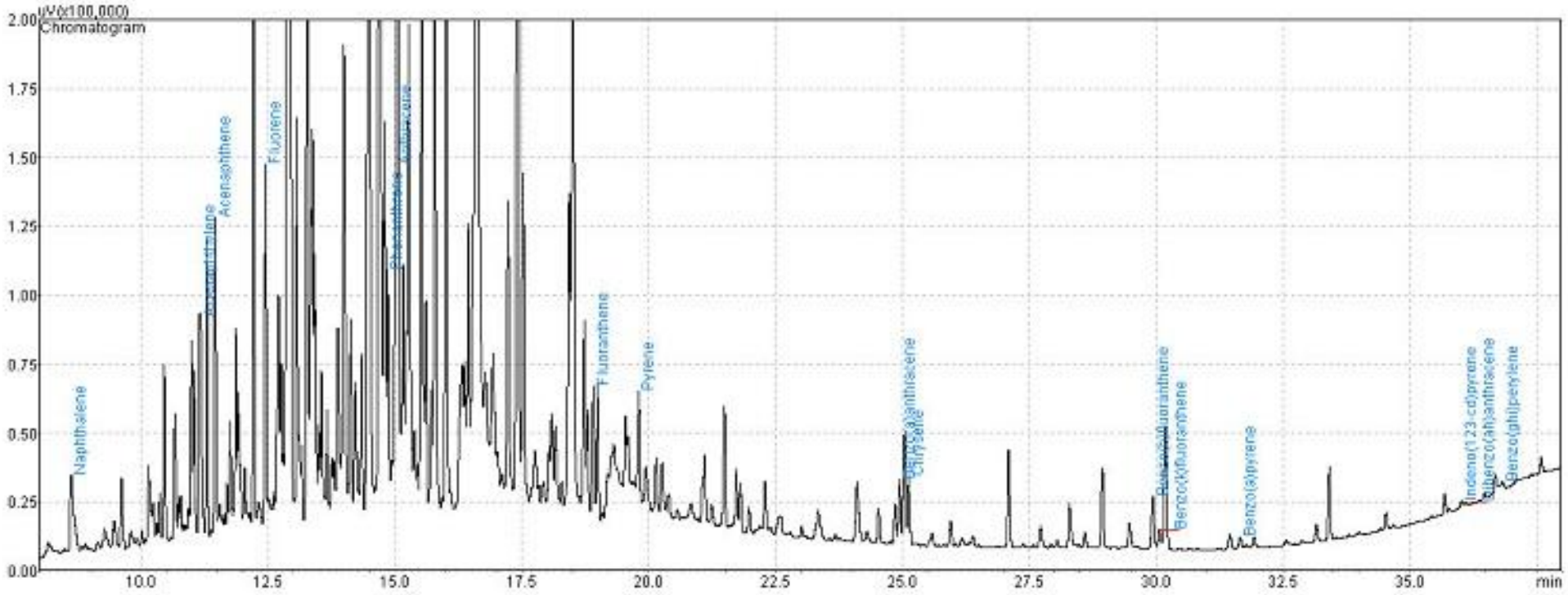
Figure 4.5(b): Comparison of non-addition, addition of water or 5% (w/v) salt solution of different volumes for the determination of 8 PAHs from mosses (BaA; Chr; BbFl; BkFl; BaP; InP; DBA; BghiP)

4.1.7 Matrix Effect

Sample matrix clearly has an important influence on the extraction efficiency of the analytes of interest. In complex matrices such as mosses, when some of the matrix components (e.g. suspended matter and other interfering compounds) are liable to compete with the fiber in the extraction process (matrix absorbs analytes more strongly than the fiber) or to modify the properties of the coating (e.g. surfactant), the partition coefficients are low and therefore sensitivity is poor (Fernandez-Gonzalez, et al., 2007). In addition, it is rather difficult to find the same kind of matrix, and the matrix effect normally appears. Therefore, quantitation methods that take the matrix into account should be used, which in this study the standard addition calibration procedure by using spiked moss samples, has been applied. In this method, the target analytes were assumed to behave similarly to spikes during the extraction. Quantitation is performed by comparing extracted amounts of native and spiked analytes in increasing concentrations.

Hence, the quantitative analyses of PAHs in real moss samples were pursued using the standard addition method, under the following optimal experimental conditions: extraction temperature of 150 ºC, extraction time of 90 min, desorption time and temperature of 15 min and 275 ºC respectively, with the addition of 250 µl of 5% (w/v) NaCl solution. Figure 4.6 shows a representative chromatogram of the quantitative extraction of 150 ng/g of PAHs in moss sample.

Figure 4.6: Chromatogram of extracted PAHs from spiked moss sample using the HS SPME method (extraction temperature, 150 ºC; extraction time, 90 min; desorption time, 15 min; desorption temperature, 275 ºC; 250 µl of 5% (w/v) NaCl solution)



4.2 Method Validation

The fitness for purpose of the HS SPME technique for the determination of PAHs in mosses was then investigated in a series of quality control experiments which comprises of linearity, precision, accuracy, limit of detection and limit of quantitation.

4.2.1 Linearity

Calibration of SPME is usually carried out by a standard addition method in solid samples. In standard addition, known quantities of analyte are added to the unknown, and the increased signal lets us deduce how much analyte was in the original unknown. However, in complex matrix samples, such as mosses, it is rather difficult to find the same kind of matrix, and the matrix effect normally appears. For this reason, spiked moss was used for the application of experimental design and optimization of the extraction parameters of PAHs.

When sample concentrations become too high, saturation occurs, and uptake rates are no longer linear. The concentration of an analyte is determined by its linear relationship with the amount of the analyte extracted by the fiber coating instead of the total extraction of the analyte. Table 4.2 illustrates the regression equation, regression coefficient (R^2) and linear dynamic range (LDR) for 16 PAHs, while Figure 4.7 to Figure 4.22 shows the linear calibration curves for each of the 16 PAHs between 0-2600 ng/g in moss samples.

The linearity of the method was tested by GC-FID by extracting non-spiked and spiked moss samples, with increasing concentrations of 16 PAHs, over a range between 0-2600 ng/g. All the seven-point calibration curves exhibited good linearity in the dynamic ranges, with regression coefficients ranging between 0.9721 and 0.9974, except for benzo(ghi)perylene with 0.9603, which is slightly poor. Low concentration of five- and six-rings PAHs may be attributed to the low water solubilities and long equilibrium times of these compounds (Doong, et al., 2000).

Table 4.2: Regression equation, regression coefficient (R^2) and linear dynamic range (LDR) for 16 PAHs in moss samples analysed by the proposed HS SPME method

Compound	Regression equation	R^2	LDR[a] (ng/g)
Naphthalene	y = 29.469x + 40267	0.9934	0.054 – 2600
Acenaphthylene	y = 43.141x + 13439	0.9893	0.012 – 2600
Acenaphthene	y = 83.528x + 5776.6	0.9834	0.004 – 2600
Fluorene	y = 88.468x + 23688	0.9933	0.008 – 2600
Phenanthrene	y = 60.253x + 41989	0.9834	0.020 – 2600
Anthracene	y = 79.078x + 46962	0.9799	0.005 – 2600
Fluoranthene	y = 50.358x + 998.71	0.9825	0.010 – 2600
Pyrene	y = 83.321x + 50067	0.9925	0.010 – 2600
Benzo(a)anthracene	y = 23.027x – 927.55	0.9964	0.012 – 2600
Chrysene	y = 22.444x + 7341.3	0.9974	0.050 – 2600
Benzo(b)fluoranthene	y = 11.015x + 5311.7	0.9788	0.218 – 2600
Benzo(k)fluoranthene	y = 10.244x – 684.43	0.9809	0.039 – 2600
Benzo(a)pyrene	y = 3.6714x – 121.53	0.9891	0.331 – 2600
Indeno(123-cd)pyrene	y = 1.5973x – 144.38	0.9721	0.192 – 2600
Dibenzo(ah)antracene	y = 0.7069x + 37.581	0.9803	0.644 – 2600
Benzo(ghi)perylene	y = 2.2661x – 114.09	0.9603	0.185 – 2600

[a] n = 7 in duplicate determinations

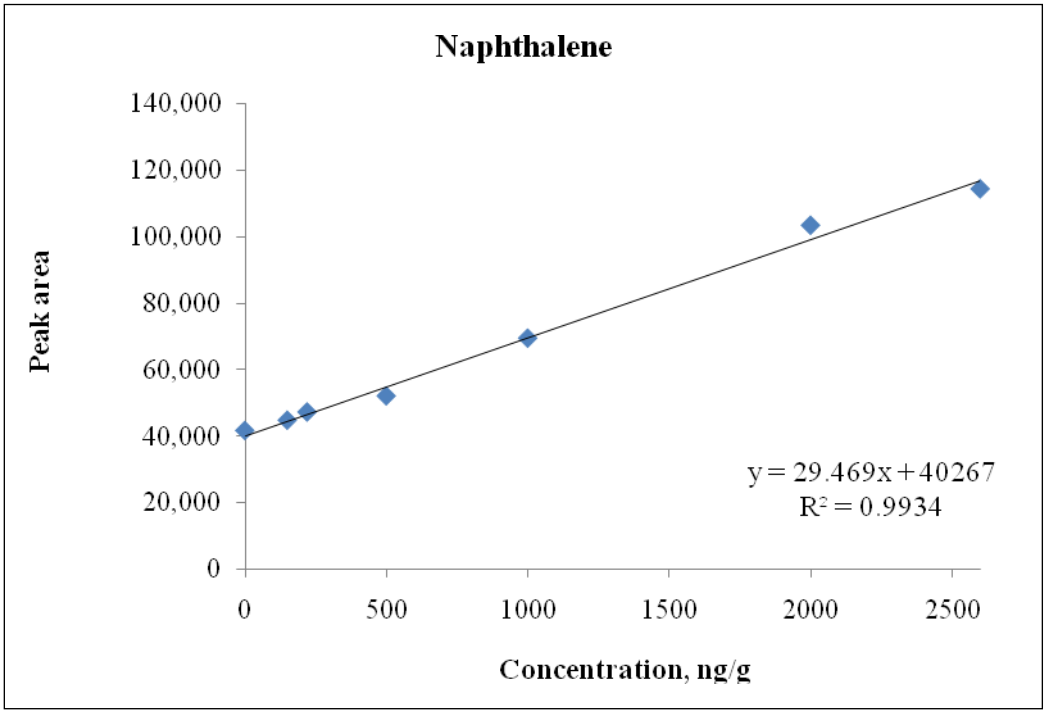


Figure 4.7: Calibration curve for naphthalene

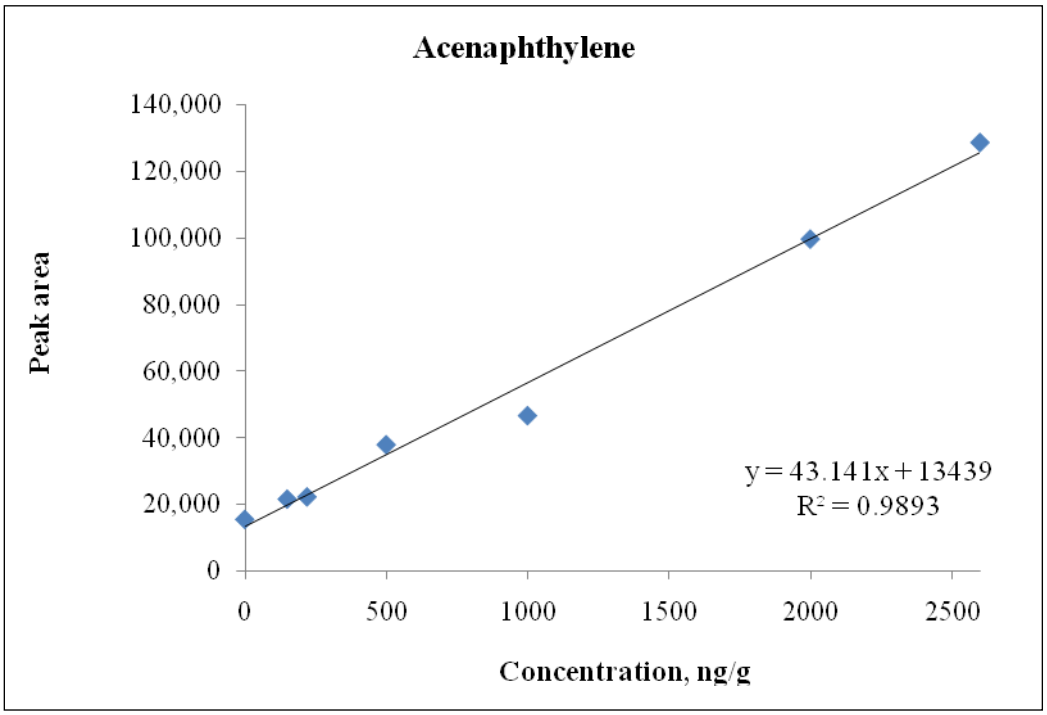


Figure 4.8: Calibration curve for acenaphthylene

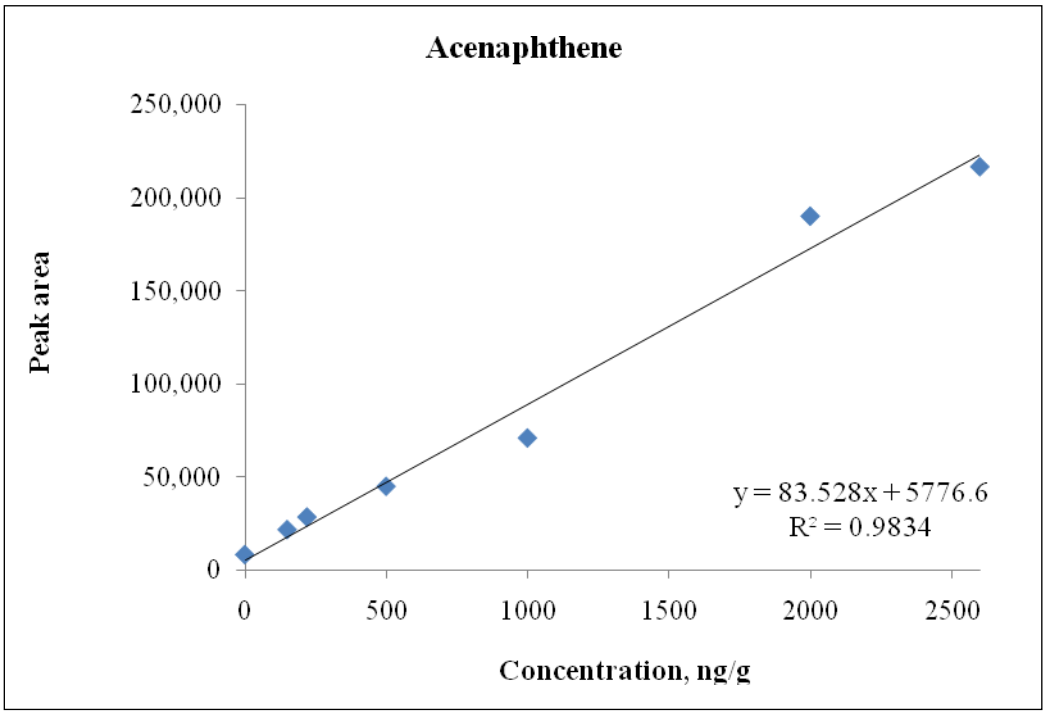


Figure 4.9: Calibration curve for acenaphthene

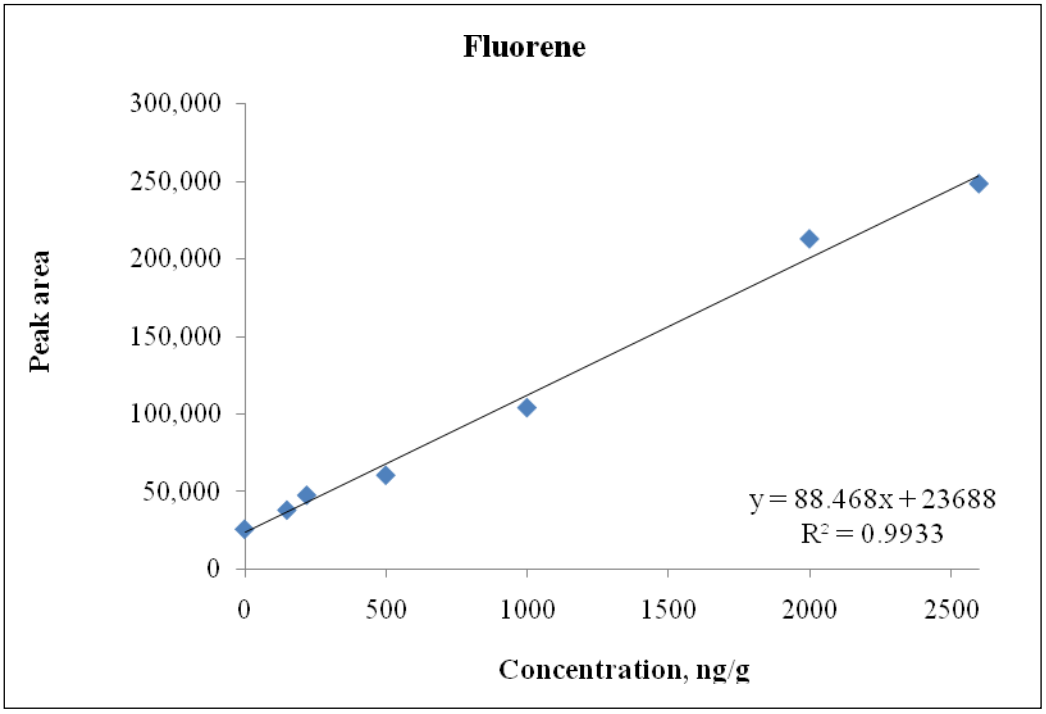


Figure 4.10: Calibration curve for fluorene

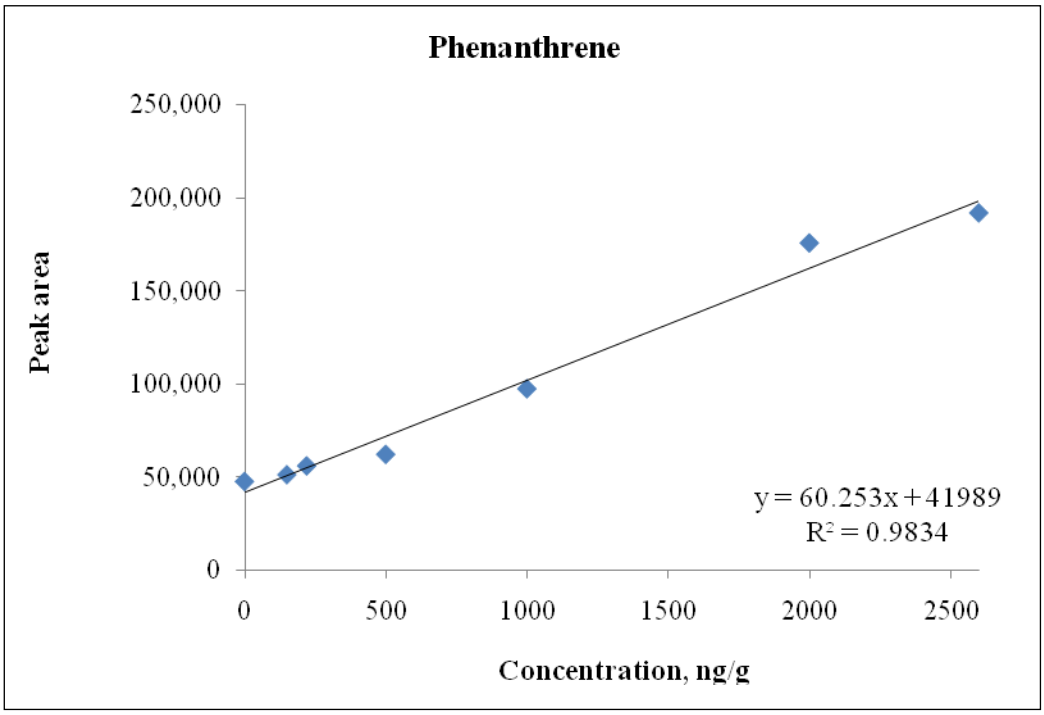


Figure 4.11: Calibration curve for phenanthrene

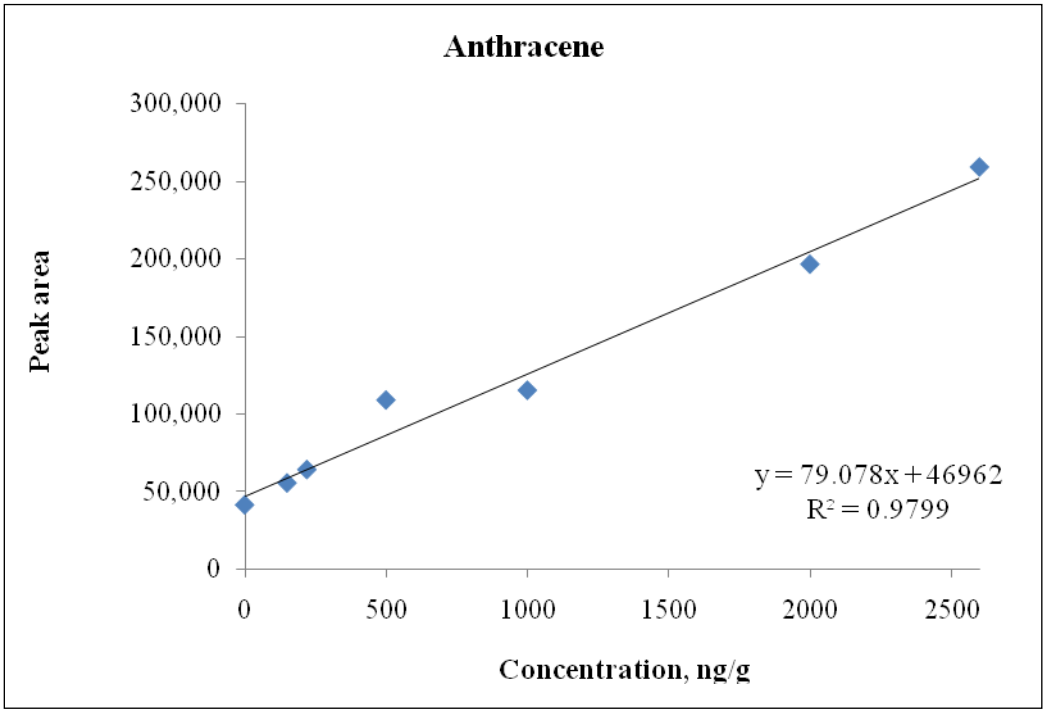


Figure 4.12: Calibration curve for anthracene

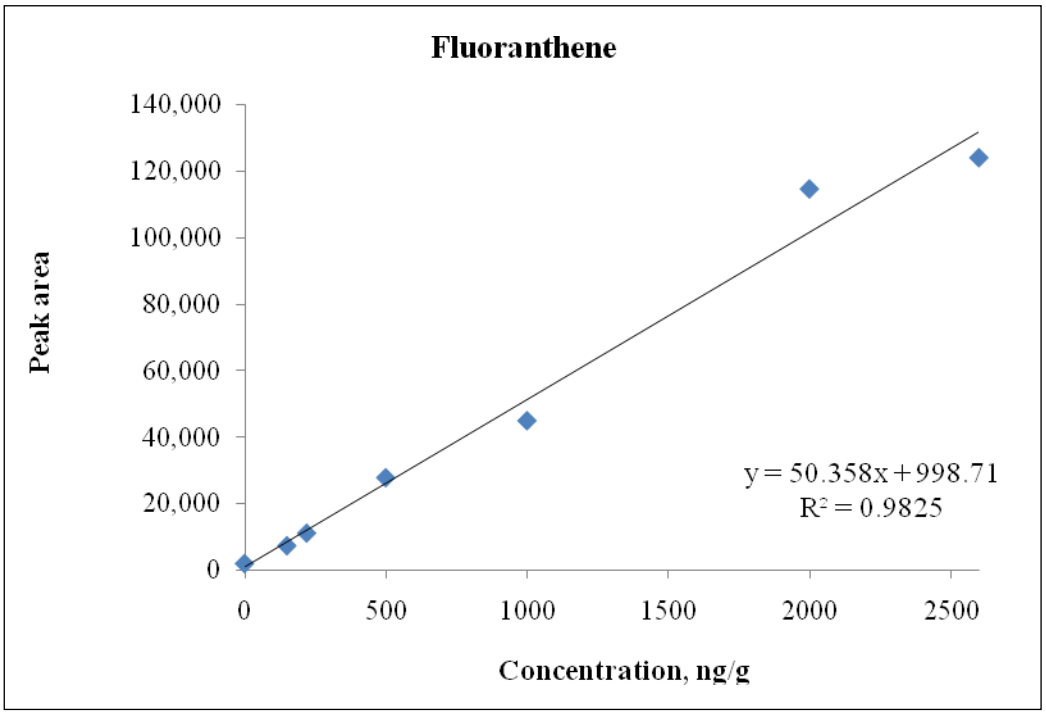


Figure 4.13: Calibration curve for fluoranthene

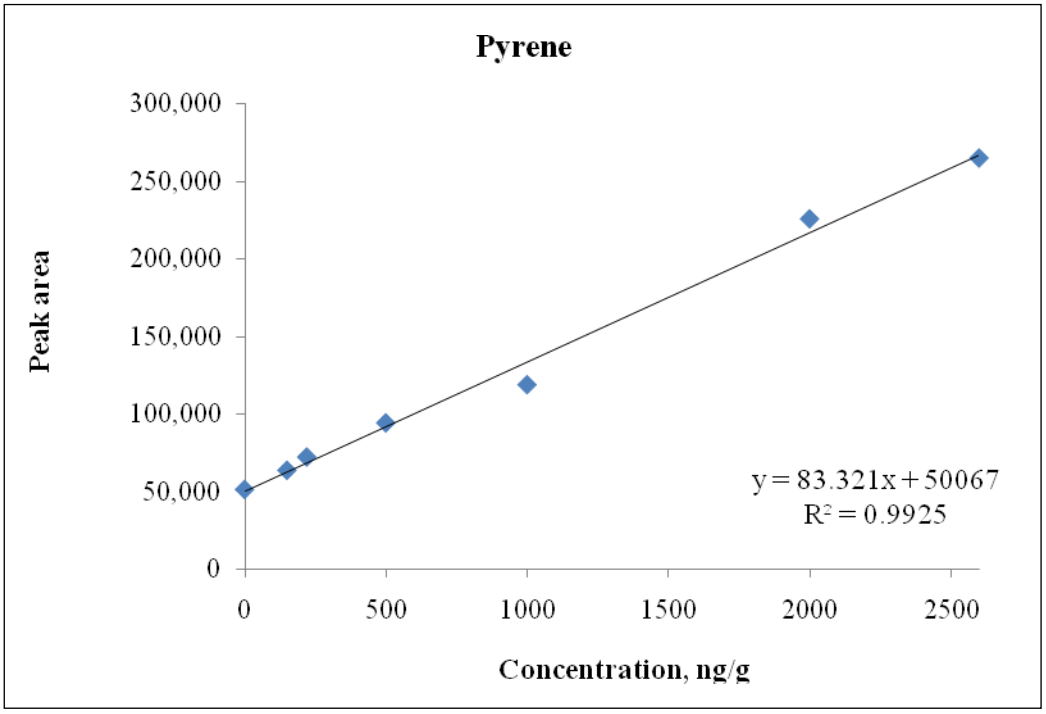


Figure 4.14: Calibration curve for pyrene

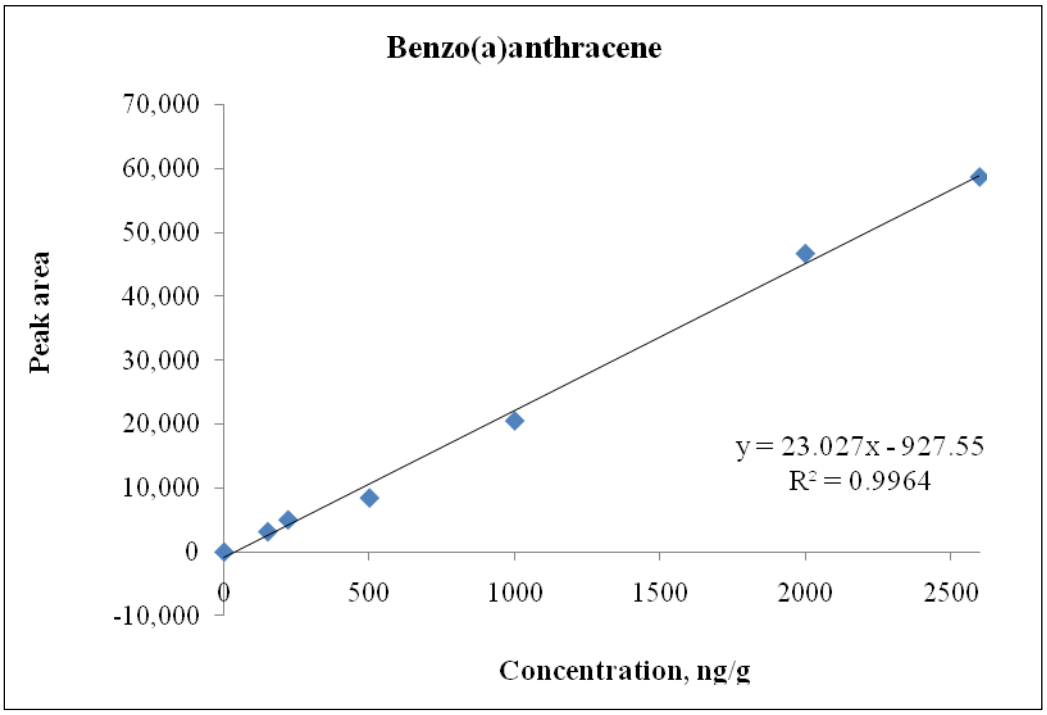


Figure 4.15: Calibration curve for benzo(a)anthracene

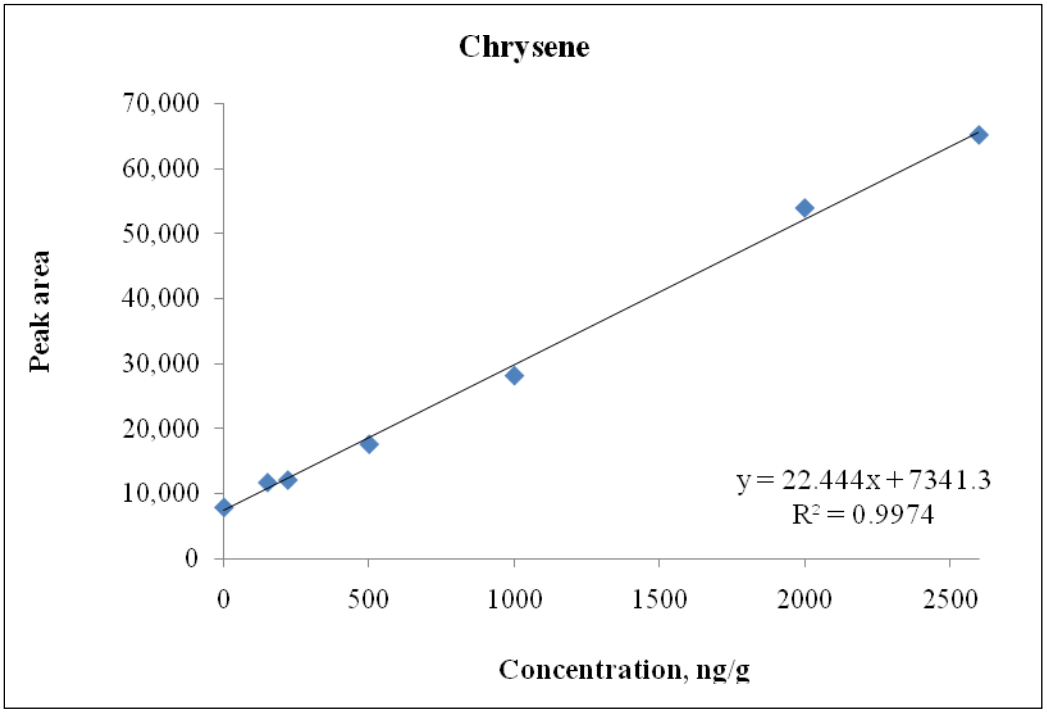


Figure 4.16: Calibration curve for chrysene

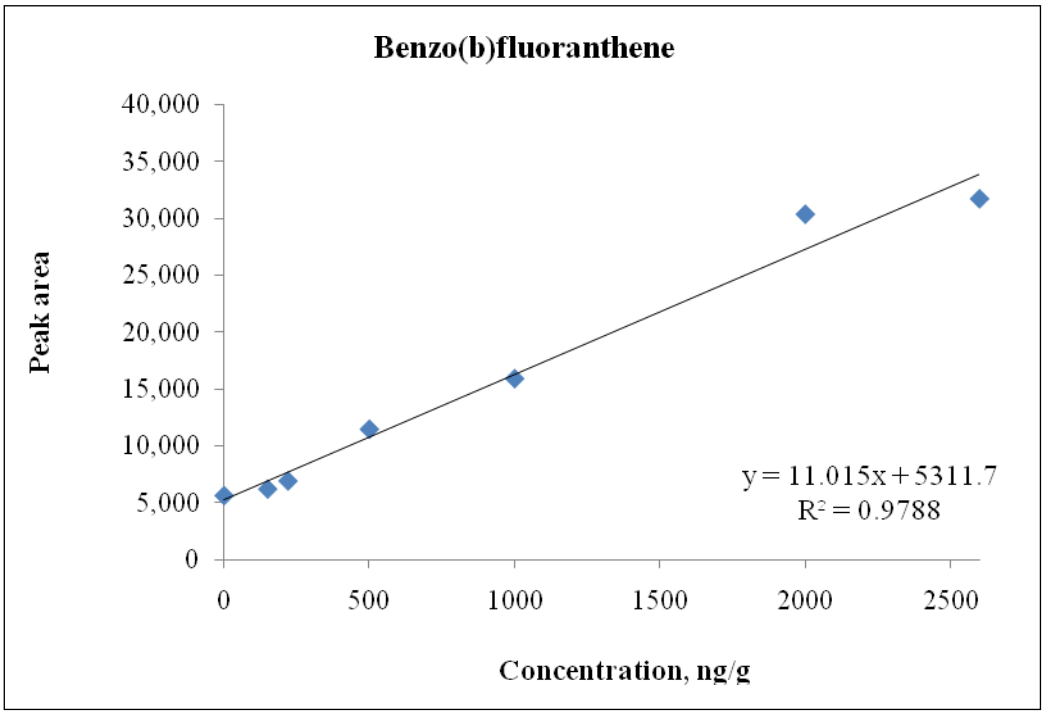


Figure 4.17: Calibration curve for benzo(b)fluoranthene

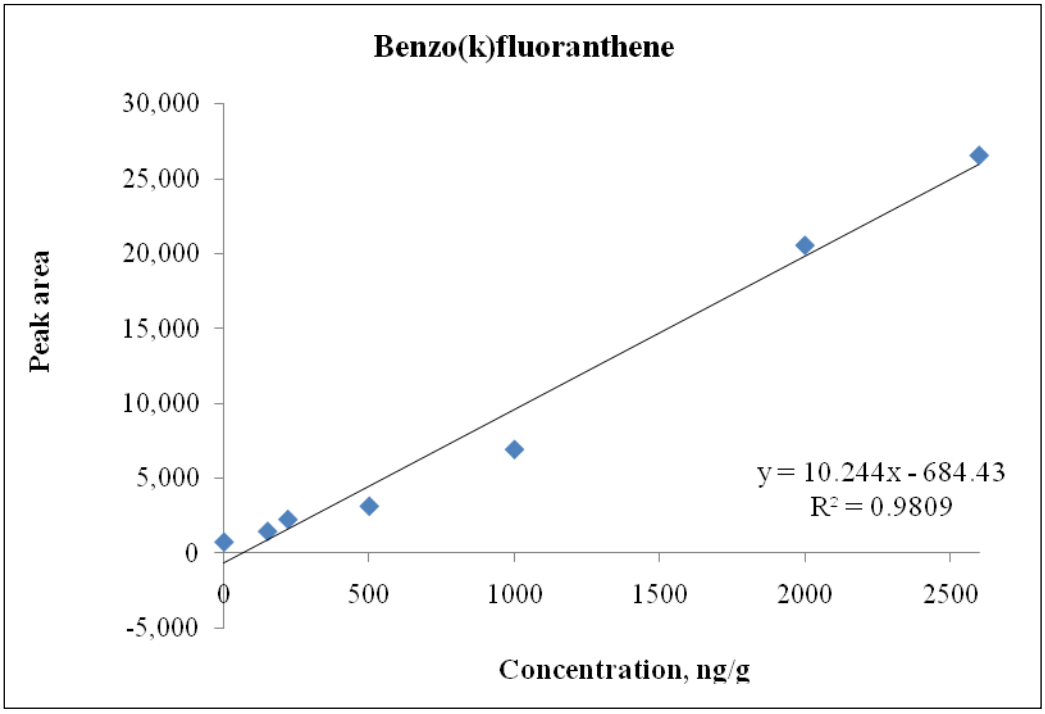


Figure 4.18: Calibration curve for benzo(k)fluoranthene

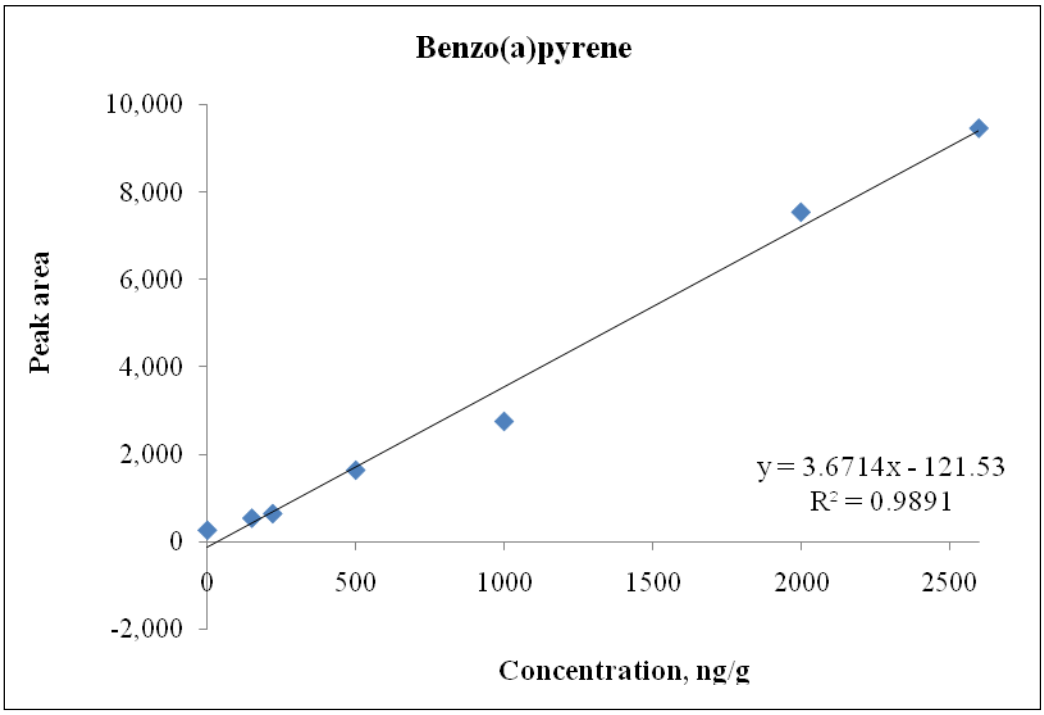


Figure 4.19: Calibration curve for benzo(a)pyrene

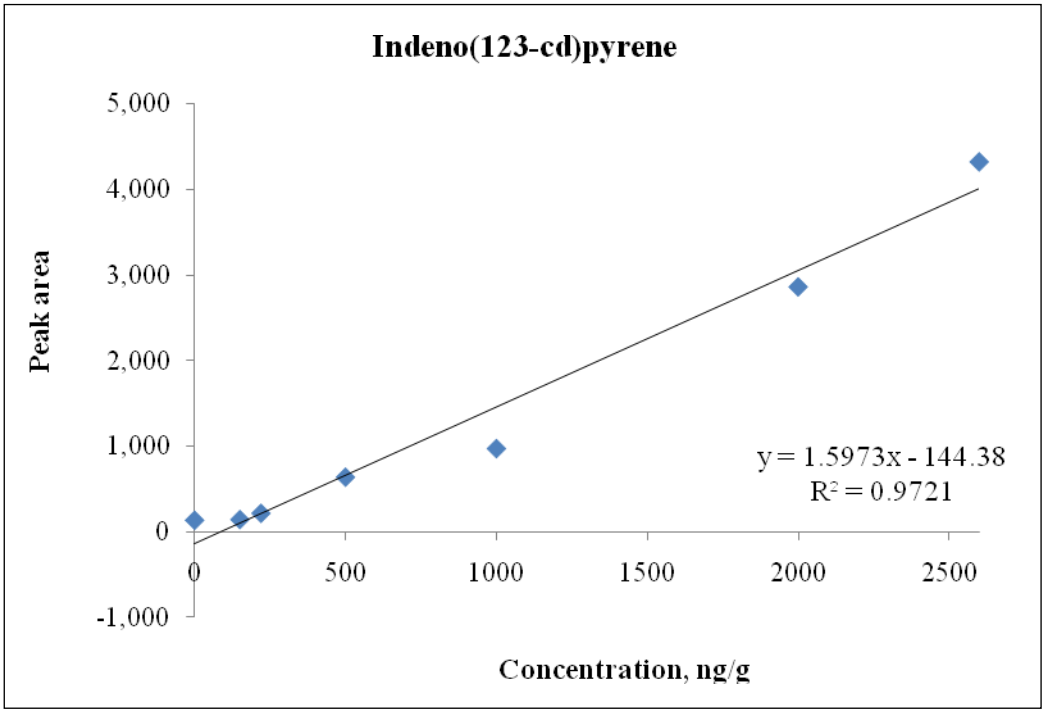


Figure 4.20: Calibration curve for indeno(123-cd)pyrene

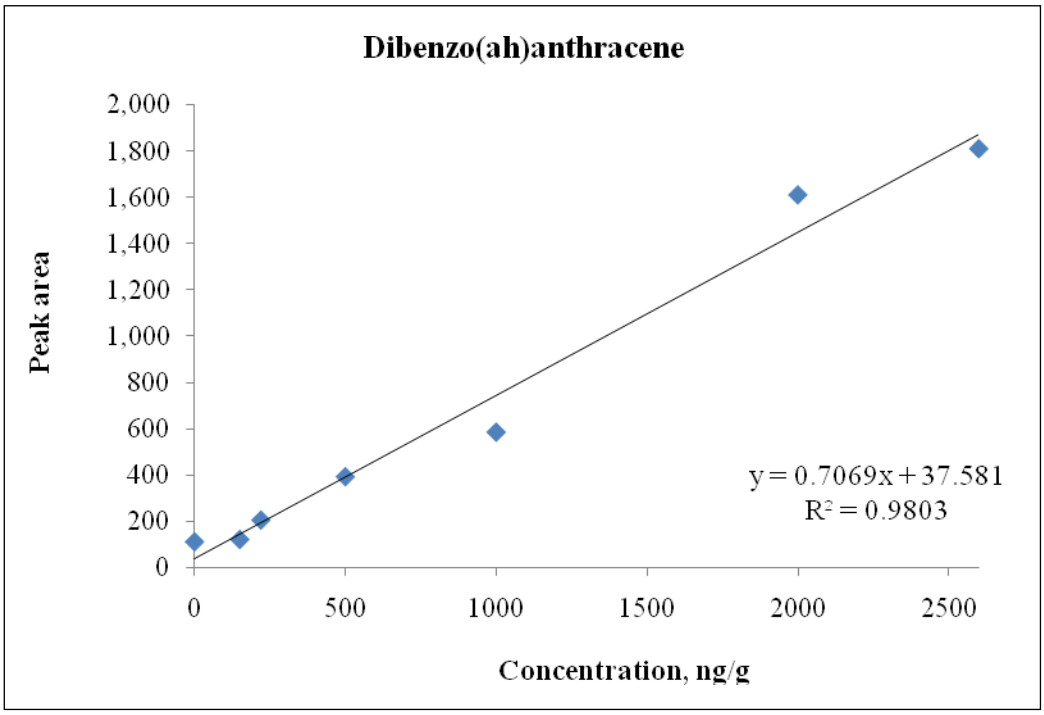


Figure 4.21: Calibration curve for dibenzo(ah)anthracene

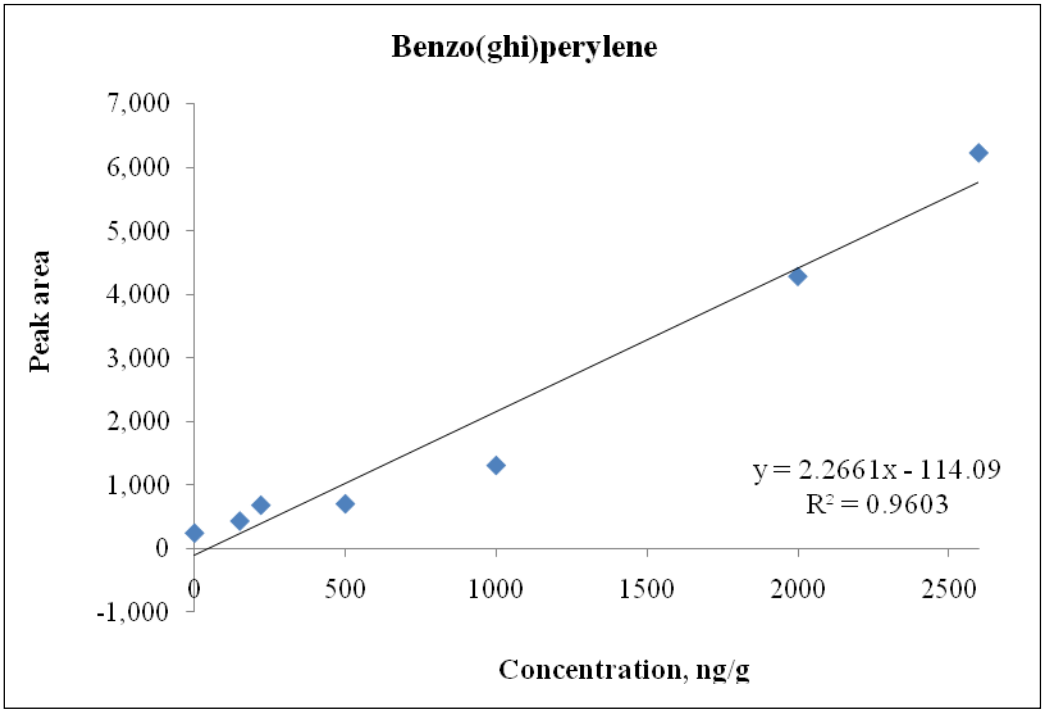


Figure 4.22: Calibration curve for benzo(ghi)perylene

4.2.2 Precision

Precision is the closeness of agreement between independent results obtained under stipulated conditions. It is usually specified in terms of standard deviation or relative standard deviation, which is also called the coefficient of variation (CV) (Thompson, et al., 2002). The precision of the proposed method was characterised in terms of relative standard deviation (R.S.D. %) value by performing a minimum of nine determinations covering the specific range for the procedure (ICH, 1996), where in this study three replicates at three different concentration levels of moss samples were spiked with all the studied PAHs at 150 ng/g (low concentration), 2000 ng/g (medium concentration) and 2600 ng/g (high concentration).

The standard deviation is calculated as:

$$\text{Standard deviation, s} = \sqrt{\sum_{i=1}^{n} \frac{(yi - m)^2}{n-1}}$$

where s = standard deviation

i^{th} = value of the i^{th} measured concentration

m = mean value of measured concentrations

n = number of replicates

The percent R.S.D. is calculated as:

$$\%\ \text{R.S.D.} = \frac{s}{m} \times 100\%$$

where s = standard deviation

m = mean of replicate measurements

Table 4.3: Relative standard deviation (R.S.D. %) of 16 PAHs from spiked moss samples with different concentrations (150, 2000 and 2600 ng/g) using the proposed HS SPME method

Compound	R. S. D.[a] (%)		
	150 ng/g	2000 ng/g	2600 ng/g
Naphthalene	5	13	16
Acenaphthylene	13	20	34
Acenaphthene	15	7	12
Fluorene	10	3	23
Phenanthrene	16	11	23
Anthracene	4	14	19
Fluoranthene	24	22	23
Pyrene	2	4	32
Benzo(a)anthracene	19	18	29
Chrysene	20	2	24
Benzo(b)fluoranthene	42	15	6
Benzo(k)fluoranthene	27	44	17
Benzo(a)pyrene	46	14	47
Indeno(123-cd)pyrene	23	16	69
Dibenzo(ah)antracene	32	12	37
Benzo(ghi)perylene	8	28	24

[a] Calculated using nine determinations, three different concentrations, three replicates each

In general, the higher R.S.D. values were determined for compounds having higher molecular mass (Doong, et al., 2000). As can be seen from Table 4.3, the overall results obtained ranged between 2 to 37% R.S.D., which should be satisfactory (considering the plant matrix) for determining PAHs in environmental analysis of complicated matrix samples using headspace SPME method, considering the low concentration of PAHs in the solid matrices and no further pre-treatment of the samples.

The exceptions were for benzo(a)fluoranthene (42% at 150 ng/g), benzo(k)fluoranthene (44% at 2000 ng/g), benzo(a)pyrene (46% at 150 ng/g; 47% at 2600 ng/g), and indeno(1,2,3-cd)pyrene (69% at 2600 ng/g) which were quite high. The very poor precision found for the higher boiling PAHs is probably due to insufficient extraction time and inconsistent extraction temperature (150 ± 5 °C) of the moss samples.

4.2.3 Accuracy

The accuracy of an analytical method describes the closeness of mean test results obtained by the method to the true value (concentration) of the analyte. A minimum of three concentrations in the range of expected concentrations is recommended (U.S. FDA, 2001). Accuracy should be assessed using a minimum of nine determinations over a minimum of three concentration levels covering the specified range (e.g., three concentrations of three replicates each of the total analytical procedure) (ICH, 1996). The percent recovery of an analyte that is added to a blank test sample is a related measurement that compares the amount found by analysis to the amount added to the sample. At relatively high concentrations, analytical recoveries are expected to approach 100%. At lower concentrations and particularly with methods involving a number of steps including extraction, isolation, purification and concentration, recoveries are often lower.

The accuracy (as percent recovery) was tested to investigate the effect of the real sample matrix by comparing the analytical results for spiked moss samples with PAHs at three concentration levels at 150 ng/g (low concentration), 2000 ng/g (medium concentration) and 2600 ng/g (high concentration) with unspiked samples to be analysed with the HS SPME sampling method.

The % recovery of a known amount of target analyte is calculated as (Eurachem, 1998):

$$\%\, R \;=\; \frac{C_1 - C_2}{C_3} \;\times\; 100\%$$

where C_1 = concentration determined in fortified sample

C_2 = concentration determined in unfortified sample

C_3 = concentration of fortification

Table 4.4: Percentage recoveries (% *R*) of 16 PAHs from spiked moss samples with different concentrations (150, 2000 and 2600 ng/g) using the proposed HS SPME method

Compound	Recovery[a] (%)		
	150 ng/g	2000 ng/g	2600 ng/g
Naphthalene	88	93	108
Acenaphthylene	80	113	105
Acenaphthene	92	104	101
Fluorene	111	104	111
Phenanthrene	81	117	113
Anthracene	116	109	106
Fluoranthene	85	109	107
Pyrene	113	103	84
Benzo(a)anthracene	102	106	118
Chrysene	116	103	116
Benzo(b)fluoranthene	101	119	87
Benzo(k)fluoranthene	67	96	99
Benzo(a)pyrene	82	108	80
Indeno(123-cd)pyrene	23	89	90
Dibenzo(ah)antracene	35	109	118
Benzo(ghi)perylene	62	85	93

[a] Calculated using nine determinations, three different concentrations, three replicates each

The recoveries of the PAHs varied from 80 to 119% as listed in Table 4.4, which is in the acceptable range within ± 20% of the expected 100% value, except for benzo(k)fluoranthene (67%) and benzo(ghi)perylene (62%) which have lower recoveries, while for indeno(1,2,3-cd)pyrene (23%) and dibenzo(ghi)perylene (35%) have extremely low recoveries. The low recoveries can be explained by the combination of the spiked moss samples at low concentration (150 ng/g) and the low volatility of high-molecular-mass PAHs which decreases the extraction efficiency for the said analytes.

4.2.4 Limit of Detection / Limit of Quantitation

In broad terms, the detection limit (limit of detection, LOD) is the smallest amount or concentration of analyte in the test sample that can be reliably distinguished from zero (Thompson, et al., 2002). Or in another term, LOD is defined as the lowest amount of analyte in the sample that can be detected, but not necessarily quantitated as an exact value (APVMA, 2004), and yielding a signal-to-noise ratio of three.

The LOD may be determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level (lowest calibration standard) at which the analyte can be reliably detected. The lowest calibration standard which produces a peak response corresponding to the analyte should be measured *n* times (normally 6-10) (APVMA, 2004).

LOD is expressed as:

$$LOD = 3\sigma$$

where σ = standard deviation of responses

Limit of quantitation (LOQ) is defined as the minimum concentration of analyte that could be measured with precision and accuracy giving a signal-to-noise ratio of ten. The limit of quantitation is the lowest amount of the analyte in the sample that can be quantitatively determined with defined precision under the stated experimental conditions (APVMA, 2004).

The LOQ is mathematically defined as equal to ten times the standard deviation of the results for a series of replicated used to determine a justifiable limit of detection (LCP, 1996). LOQ are matrix, method and analyte specific. The solution should be injected and analysed *n* times (normally 6-10) (APVMA, 2004).

LOD is expressed as:

$$LOQ = 10\,\sigma$$

where σ = standard deviations of responses

Table 4.5 illustrates the experimental results for the determination of LOD using six determinations. The lowest LOD was 1.3 pg/g for acenaphthene, while dibenzo(ah)anthracene exhibited the highest LOD, at 193.1 pg/g. However, high detection limits for high-ring PAHs were observed to be in between 11.6–193.1 pg/g (chrysene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(a)pyrene, indeno(123-cd)pyrene, dibenzo(ah)anthracene, benzo(ghi)perylene) and also including naphthalene (16.3 pg/g), which is a low-ring PAH. The lowest LOQ was 4.2 pg/g for acenaphthene and highest for dibenzo(ah)anthracene at 643.6 pg/g.

Table 4.5: Limit of detection and limit of quantitation of 16 PAHs from spiked moss samples spiked (150 ng/g) using the proposed HS SPME method

Compound	LOD[a] (pg/g)	LOQ[a] (pg/g)
Naphthalene	16.3	54.3
Acenaphthylene	3.7	12.4
Acenaphthene	1.3	4.2
Fluorene	2.5	8.2
Phenanthrene	6.1	20.2
Anthracene	1.5	4.9
Fluoranthene	3.0	9.8
Pyrene	3.1	10.2
Benzo(a)anthracene	3.7	12.4
Chrysene	14.9	49.8
Benzo(b)fluoranthene	65.3	217.6
Benzo(k)fluoranthene	11.6	38.6
Benzo(a)pyrene	99.3	330.9
Indeno(123-cd)pyrene	57.6	192.0
Dibenzo(ah)antracene	193.1	643.6
Benzo(ghi)perylene	55.4	184.6

[a] n = 6 determinations

4.3 Application to Real Moss Samples

The final phase of this study included an evaluation of the proposed method with contaminated moss samples collected throughout the year from 2006 to 2009 within the vicinity of Klang Valley area. The five moss samples were collected:

- Along the main road in front of Chemistry Department of Faculty of Science, University of Malaya (slightly high concentration of PAHs)
- In Livillas Condominium, Section 16, Petaling Jaya (high concentration of PAHs)
- Along Jalan 17/21, Section 17, Petaling Jaya (high concentration of PAHs)
- Along Jalan Universiti, Petaling Jaya (high concentration of PAHs)
- Along Jalan Pudu, Kuala Lumpur (very high concentration of PAHs)

These samples represent moderate and extreme samples of moss types that could be encountered during environmental investigations.

The concentrations of PAHs measured in *Hyophila involuta* collected from each site are reported in Table 4.6. The results derived from the determinations of PAHs in mosses using the proposed HS SPME method under optimal experimental conditions show considerable variability in concentrations within the vicinity of Klang Valley. It was revealed that the total PAHs content (∑PAHs) from the less contaminated area (Fraser's Hill) is 1080 ng/g, while the ∑PAHs in contaminated mosses varied from 8186 to 14013 ng/g, which is around ten to fifteen times higher than mosses from Fraser's Hill. The ∑PAHs in mosses from Chemistry Department, Livillas Condominium, Jalan 17/21, and Jalan Universiti were found to be 8167, 10027, 10555, and 10814 ng/g respectively. The highest concentration of ∑PAHs was recorded in the mosses along Jalan Pudu, noted to be 14013 ng/g.

Table 4.6: The comparison of concentrations (ng/g) of 16 PAHs found in six moss samples from different locations in Peninsular Malaysia (one sample collected from Fraser's Hill, Pahang; and five samples collected around the vicinity of Klang Valley)

Compound	Location (ng/g)[a]					
	Fraser's Hill	Chemistry Dept	Livillas Condo	Jln 17/21	Jln Universiti	Jalan Pudu
Naphthalene	47	529	1,492	1,497	2,010	931
Acenaphthylene	42	228	1,493	269	2,043	2,099
Acenaphthene	53	23	1,097	501	809	242
Fluorene	23	377	1,879	1,329	n.q.	919
Phenanthrene	6	217	889	651	1,958	1,070
Anthracene	n.d.[b]	236	707	100	1,088	1,144
Fluoranthene	24	n.q.[c]	1,633	1,346	2,179	n.q.
Pyrene	n.d.	1,294	n.d.	566	95	905
Benzo(a)anthracene	41	48	81	40	78	40
Chrysene	36	n.d.	n.d.	41	1	n.d.
Benzo(b)fluoranthene	100	n.d.	n.d.	n.d.	n.d.	247
Benzo(k)fluoranthene	122	158	225	142	154	203
Benzo(a)pyrene	109	1,524	138	1,001	206	1,597
Indeno(123-cd)pyrene	158	1,284	168	1,831	90	1,602
Dibenzo(ah)antracene	172	598	84	531	53	1,989
Benzo(ghi)perylene	147	1,651	141	710	50	1,025
Total PAHs, ∑PAHs	1,080	8,186	10,027	10,555	10,814	14,013

[a] $n = 3$ determinations; [b] n.d., not detected; [c] n.q., not quantified

Five- and six-rings PAHs (benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(a)pyrene, indeno(123-cd)pyrene, dibenzo(ah)anthracene, benzo(ghi)perylene) dominated the PAHs burden of samples from the reference sample (Fraser's Hill). It is interesting to note that Fraser's Hill lie within relatively pristine areas, for example, the site is situated within the large forest in Pahang, and is surprising to find slightly high concentrations of high molecular weight PAHs (six-rings) in the moss sampled at the less contaminated site of Fraser's Hill.

Naphthalene, acenaphthylene, acenaphthene, fluorene and fluoranthene dominated in Livillas Condominium, while naphthalene, fluorene, fluoranthene, benzo(a)pyrene and indeno(123-cd)pyrene dominated in Jalan 17/21 with concentrations of PAH above 1,000 ng/g at both sites. In contrast, Chemistry Department and Jalan Pudu showed extremely high concentration of fluoranthene which were not quantified. Because of its relatively high environmental levels and high level of toxicity in larger health impact than any other PAH identified in the environment, the presence of benzo(a)pyrene at some sites is of concern as it is considered the most common carcinogenic PAH.

Among the individual PAHs, chrysene and benzo(b)fluoranthene were usually not identified at most of the sampling sites of the studied locations. These compounds had either higher reactivity in the atmosphere or no emission from the source (Otvos et al., 2004). The high vehicle pollution sources along Jalan Pudu are located in a very heavily polluted region of KL and here PAH contamination of moss samples is more 'diffusive'. A study of the spatial distribution and mixture of PAHs in moss samples across the Klang Valley showed that naphthalene (6459 ng/g) is the most prevalent (dominant PAH species) compound at all five contaminated sites, whereas acenaphthylene (6132 ng/g) was subordinate.

Low molecular weight PAHs in our study are mostly in the gaseous form and can travel a long distance away from the road. As for the PAHs, accumulation patterns of naphthalene differ from those of the other substances maybe because naphthalene is hardly ever bound to particles and distributed mainly in the gaseous phase (Zechmeister, et al., 2006). Acenaphthene is also mainly represented in the gaseous phase, but in contrast to naphthalene, much smaller quantities are emitted by road traffic. Another member fluorene, has an intermediate position among the substances mainly represented in the gaseous and particle phase.

Although PAHs are also emitted by natural sources, such as forest fires, their occurrence in the atmosphere is prevailingly the result of anthropogenic activities (Gerdol, et al., 2002). The high concentrations and full spectrum of PAHs in moss samples suggest the presence of emissions coming from various sources including vehicle particle- and vapour-phase emissions. A relatively dense transportation system further contributes to PAH emissions from gasoline- and/or diesel-powered vehicles. According to Migaszewski et al. (2009), automobile emissions contain more benzo(ghi)perylene and pyrene. Since combustion technology has considerably improved in recent years, vehicle exhaust is generally recognised as the principal source of PAHs in the urban atmosphere. Heavy vehicles equipped with diesel engines operating at high speed and, secondly, cars with defective catalyst have been found to play the major role as PAH emitters. The PAHs associated with small size particles are rather refractory to photochemical degradation, as well as to evaporative or oxidative reactions with gaseous pollutants. In this form, PAHs may undergo long-range transport even to remote regions.

It is interesting to compare concentrations of aromatic hydrocarbons with different numbers of benzene rings (Table 4.7). In the examined moss samples, three- and five-rings aromatic hydrocarbons prevail upon their six- and four- equivalents. Three-ring PAHs predominate in moss samples from Livillas Condominium, Jalan Universiti and Jalan Pudu (6065, 5898 and 5474 ng/g respectively) with the highest total PAHs of 21492 ng/g. Five-ring compounds reveal the second highest concentrations especially in Jalan Pudu (4036 ng/g), whereas their four-ring equivalents of ∑PAHs are nearly similar (8448 ng/g) to the six-ring compounds (8857 ng/g). This concludes that the ring sequence of aromatic hydrocarbons in mosses is three > five > six > four.

Table 4.7: Total concentrations of aromatic hydrocarbons with different numbers of benzene rings in moss samples

Location	Number of benzene rings (ng/g)[a]			
	Three	Four	Five	Six
Fraser's Hill	124	101	503	305
Livillas Condo	6065	1714	447	309
Jalan 17/21	2850	1993	1674	2541
Chemistry Dept	1081	1342	2280	2935
Jalan Universiti	5898	2353	413	140
Jalan Pudu	5474	945	4036	2627
Total PAHs	21492	8448	9353	8857

[a] $n = 3$ determinations

4.4 Conclusion

Atmospheric pollution is an important concern for the human health and to the environment. The development of new methods appears to be promising. From the results obtained the combination of HS SPME method with GC-FID for the analysis of mosses *Hyophila involuta* showed to be a good method for the determination of PAHs. The SPME procedure is solvent-free, fast, and simple to be carried out. This study mainly investigated the possibility of applying the HS SPME technique in the extraction of PAHs from moss samples. By using this technique, the sampling, extraction, concentration and sample introduction can be carried out in one step.

The various plant species are ideal biomonitors (especially mosses). They play an important role in the global cycling of pollutants (organic and inorganic) since they cover over 80% of the Earth's land surface, the surface area of plants is generally much greater than the area of the ground they cover, and the vegetation has a high lipid fraction which is likely to accumulate lipophilic persistent compounds (e.g. PAHs). The moss sampling procedure is fast and inexpensive; moreover, *Hyophila involuta* is particularly suitable for passive sampling because of its widespread distribution in Peninsular Malaysia and also because they depend entirely on the atmosphere for delivery of nutrients and lack both cuticle and internal transport mechanisms.

Vegetation is the link between the atmosphere and the human food supply. This it is important to understand those processes by which pollutants enter this environmental compartment. Because of the complexity of the plant matrix, the use of modern extraction, cleanup and analysis methods has been necessary to advance in this field. Even though only limited sampling sites were used for this study and the analytical

results may not be representative for other areas, they show that vegetation can be used as an indicator of regional contamination levels. The findings obtained open up new possibilities for the estimation of regional and global contamination levels in pollution monitoring, their ecotoxicological relevance and the assessment of the importance of vegetation as a pollutant sink.

The proposed method permitted to detect all sixteen PAHs at different concentrations and PAH patterns were observed accordingly to the sampling site, thus providing the suitability of the approach to distinguish trace concentrations of PAH from pristine to polluted areas. The results obtained from real contaminated moss samples showed low to moderate level pollution prevailing in the Klang Valley, and analytical measurement of the sixteen PAHs in samples of *Hyophila involuta* collected at the surveyed area. PAH compounds are usually released into the atmosphere due to incomplete combustion where mobile sources are one of the major contributors of PAHs in the urban atmosphere. Distribution of PAHs in atmospheric aerosol particles in different regions of Klang Valley also suggests the influence of local industry and road traffic. Long-range transport rather than local sources may thus be mostly responsible for spatial distribution of PAHs in our study. The results of this study can be interpreted in terms of regional use, and the collection of mosses can be readily and repeatedly done from a given location. In the case of PAHs, mosses reflect more the difference between background locality and polluted areas, suggestive of continuing/ongoing sources of these compounds.

The results of the study also indicate that mosses, and more specifically the species *Hyophila involuta*, are potentially useful indicators of atmospheric pollution by PAHs. Mosses absorb pollutants throughout their lifetime, and their PAH profiles indicate both

the origin and proximity of the contamination source(s). This work emphasizes the real possibility of using mosses of the species *Hyophila involuta* as indicators of pollution by PAHs. In fact, PAH content determined in this species can be considered as indicative of the grade of environmental pollution after evaluating PAH distribution in mosses collected on the basis of considerations on vehicular traffic in the sites studied. Therefore it can be concluded that the air quality in natural ecosystems can be assessed by quantitative analysis of PAHs in mosses which behave as good biomonitors of such compounds in ambient air.

In this work, the proposed HS SPME followed by GC-FID method was successfully developed and applied for the determination of sixteen priority PAHs in moss samples. There are several factors that can influence extraction efficiency but to varying degrees. The PDMS fiber has demonstrated to be the most appropriate for the analysis of PAHs studied with a wide range of polarities. Chemical modifiers such as sodium chloride salt and water have a small influence on the efficiency of extraction while fiber exposure time and the application of sample heating are critical to the amount of PAHs extracted from mosses. The optimal operating conditions for the proposed headspace SPME in moss samples were using the standard addition method with 100 µm PDMS fiber under the following conditions: extraction temperature of 150 ºC, extraction time of 90 min, desorption time and temperature of 15 min and 275 ºC respectively, with the addition of 250 µl of 5% (w/v) NaCl salt solution.

Chemistry Department (8167 ng/g) showed to have the lowest concentration of total PAHs within the Klang Valley area, while Jalan Pudu (14013 ng/g) is the most polluted location in this study. The ∑PAHs found in mosses at Livillas Condominiun, Jalan 17/21, and Jalan Universiti were 10027, 10555, and 10814 ng/g respectively. Three-ring

hydrocarbons (21492 ng/g) constitute the largest proportion of all aromatic hydrocarbon groups in the moss species examined. Five-ring hydrocarbons (9353 ng/g) reveal the second highest concentration and prevail distinctly upon their four- (8448 ng/g) and six-ring (8857 ng/g) equivalents. The concentrations of PAHs differ from site to site.

The proposed method offers significant analytical performance, very good sensitivity and reasonable precision. It is a powerful method for the direct quantitative analysis of PAHs in complex environmental samples such as mosses. The method employed is simple enough to be easily utilized for rapid screening, while method accuracy, precision, and detection limits are sufficient that it could also be used for quantitative analysis. Moss samples collected nearby roadsides with heavy traffic flow were analysed using the proposed method to determine the amount of PAH concentrations. In the future, this technique can be applied to the real-time monitoring of environmental monitoring of pollution.

However, as it is easy, rapid and considers the complexity and the impact of the plant matrix, it is also of interest for scientists working in several important fields of research: environmental, ecotoxicological, human health, etc. It could also be applied to the detection of PAHs in other plant biota, enlarged to crop plants, wild plants or algae. When grazing animals or humans consume such plant materials, its application may be extended to human health-related studies. It is important to determine the PAH levels in the atmosphere and vegetation to evaluate public health risks.