

**Lichens as biomonitors for atmospheric polycyclic aromatic hydrocarbons**

by

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Submitted in partial fulfilment of the requirements for the degree

**Magister Scientiae**

In the Faculty of Natural and Agricultural Sciences  
University of Pretoria  
Pretoria

April 2015

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## Declaration

I, Leandri van der Wat, declare that the dissertation, which I hereby submit for the degree Magister Scientiae at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

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## Summary

For the first time in Africa, studies investigating the polycyclic aromatic hydrocarbons (PAHs) levels in lichens (species *Parmotrema austrosinense* (Zalhr.) Hale) was performed successfully using various sample preparation techniques. Sampling campaigns were conducted at 5 different sites across South Africa.

Different sample preparation techniques were explored, and it was found that a quick, easy, cheap, effective, rugged and safe (QuEChERS) technique using *n*-hexane:acetone (1:1, v/v), never before applied to lichens, provided the best recoveries of internal standards (deuterated phenanthrene and pyrene), the highest total peak area for all PAHs of interest and relative standard deviations (%RSDs) comparable with the other preparation techniques explored (ultrasound assisted extraction, Soxhlet and microwave assisted extraction). Statistical analysis confirmed that QuEChERS performed significantly better than the other sample preparation techniques. The optimized sample preparation technique met the study's requirements, since it was a fast method, with good recoveries, using less solvents and minimal energy. It was determined that the internal standard needs a prolonged equilibration time (12 hr) on the lichen matrix for optimal recoveries, a new finding.

Matrix matched standards were prepared and strong matrix effects were found; both strong enhancement (for the lighter PAHs) and strong suppression (for the heavier PAHs) were observed. The use of matrix matched standards were found to be imperative for the accurate determination of PAH concentrations in the samples, described for the first time in this study, since the matrix effects were found to be PAH and lichen specific and are crucial for accurate quantitation. Varying chlorophyll contents of the extracts was observed using fluorescence spectroscopy, despite being prepared in an identical manner and being of the same lichen species. Therefore, the chlorophyll content needs to be given along with determined PAH concentrations in lichens, based on a dried weight basis to allow for comparison between studies.

All the lichen samples contained naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene and benzo[*a*]pyrene. It was seen that a full range of PAHs in terms of mass was extracted and identified, thus *Parmotrema austrosinense* (Zalhr.) Hale was an appropriate choice of lichen species. The order of most impacted sites to least impacted was found to be Pretoria Industrial Area (total PAH concentration 1001 ng.g<sup>-1</sup>) > Daspoort 2 (737 ng.g<sup>-1</sup>) > Daspoort 3 (693 ng.g<sup>-1</sup>) > Bulk urban site (633 ng.g<sup>-1</sup>) > Cathedral Peak (547 ng.g<sup>-1</sup>) > Cape Point Nature Reserve 1 (525 ng.g<sup>-1</sup>) > Cape Point Nature Reserve 2 (514 ng.g<sup>-1</sup>) > Cape Point Nature Reserve 3 (425 ng.g<sup>-1</sup>). The PAH profiles and PAH ring-size profile were used for source diagnostics and was seen to vary according to the types of pollution sources.

## Acknowledgements

I would like to thank my supervisor, Dr Patricia Forbes, for her excellent guidance and support for the duration of my studies. I have enjoyed learning from your expertise and professionalism, and I'm thankful for the freedom you gave me to complete my studies in the manner in which you did.

A word of thanks to SAAQIS and the weather service for the meteorological data provided for the Pretoria West sampling point and for the Cape Point Nature Reserve. I acknowledge the cooperation of SAN Parks, allowing access to the Cape Point Nature Reserve to sample lichens. A special thanks to Ernst Brunke from WeatherSA, who assisted with obtaining the permit.

The financial assistance of the National Research Foundation (NRF) towards this research is hereby acknowledged. Opinions expressed and conclusions arrived at, are those of the author and are not necessarily to be attributed to the NRF.

Thank you to Jack Cochran, representing the Restek Corporation, for his advice on QuEChERS as well as the supplies, such as the Rxi-PAH column which proved to be very useful. Waters Corporation is also thanked for the technical support and spares for the HPLC. Antoinette Buys at the microscopy unit at the University of Pretoria is thanked for her expertise. Thanks is also given to Wirsam for the fluorescence spectrometer instrumentation and support.

I'd like to thank my colleagues Sifiso Nsibande, Niel Malan and Elize Smit. Yvette Naudé is thanked for her constant assistance and advice regarding the chromatographic techniques used as well as solid phase extraction. Thanks to David Masemula for supplying the gas and liquid nitrogen.

I'd lastly like to thank my friends who've supported me throughout my studies; showing curiosity in what I do and encouragement when needed. A special thanks to Neil, Seline, Nelia, Joshua, Nadine and Jaco who continually offered me liters of coffee and comic relief.

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## Abbreviations

Ace: acenaphthene  
ACN: acetonitrile  
Acy: acenaphthylene  
ANOVA: analysis of variance  
Ant: anthracene  
APHA: American Public Health Association  
ATSDR: Agency for Toxic Substances and Disease Registry  
BaA: benzo[*a*]anthracene  
BaP: benzo[*a*]pyrene  
BbF: benzo[*b*]fluoranthene  
BghiP: benzo[*ghi*]perylene  
CEN: European Committee for Standardization  
Chr: chrysene  
CPS: counts per second  
DAD/V-UV: diode array detector/visible-ultraviolet detector  
DahA: dibenzo[*ah*]anthracene  
DCM: dichloromethane  
DSASE: dynamic sonication assisted solvent extraction  
dSPE: dispersive solid phase extraction  
EDX: energy dispersive X-ray spectroscopy  
EI: electron impact  
FLD: fluorescence detector  
Flu: fluorene  
FluAn: fluoranthene  
GAW: Global Atmospheric Watch  
GC x GC-TOFMS: comprehensive two-dimensional gas chromatography coupled to time-of-flight mass spectrometry  
GC-MS: gas chromatography-mass spectrometry  
GCB: graphitized carbon black  
Hex:ace: *n*-hexane:acetone  
Hex:DCM: *n*-hexane:dichloromethane  
HPLC-DAD: high performance liquid chromatography coupled to a diode array detector  
HPLC: high performance liquid chromatography  
IARC: International Agency for Research on Cancer  
IcdP: indeno[123-*cd*]pyrene  
LC: liquid chromatography  
LOD: limit of detection  
LOQ: limit of quantification  
MAE: microwave assisted extraction  
MIP: molecularly imprinted polymer  
MW: molecular weight  
Nap: naphthalene  
ND: not detected  
NIST: National Institute of Standards and Technology  
PAHs: polycyclic aromatic hydrocarbons  
PCA: principal component analysis  
PCDD/Fs: polychlorinated dibenzo-*p*-dioxins and polychlorinated dibenzofurans

PDMS: polydimethylsiloxane  
PeCDD: pentachlorodibenzo-p-dioxin  
PeCDF: pentachlorodibenzofuran  
Phe-d10: phenanthrene-d10  
Phe: phenanthrene  
PLE: pressurized liquid extraction  
PM: particulate matter  
POPs: persistent organic pollutants  
PSA: primary secondary amine  
PTFE: polytetrafluoroethylene  
PUF: polyurethane foam  
Pyr-d10: pyrene-d10  
Pyr: pyrene  
QuEChERS: Quick, Easy, Cheap, Effective, Rugged, Safe  
RSD: relative standard deviation  
S/N: signal-to-noise  
SAAQIS: South African Air Quality Information System  
SAN Parks: South African National Parks  
SAX: strong anion exchange  
SBME: stir-bar micro extraction  
SEM: scanning electron microscope  
SIM-MS: single ion monitoring mass spectrometry  
SVOCs: semi-volatile organic compounds  
TD - GC-MS: thermal desorption gas chromatography-mass spectrometry  
TDS: thermal desorption system  
TeCDD: tetrachlorodibenzodioxin  
TEF: toxic equivalence factor  
TEQ: toxic equivalence quotient  
TSP: total suspended particulate  
US EPA: United States Environmental Protection Agency  
USAE: ultrasound assisted extraction  
VOCs: volatile organic compounds



# Chapter 1: Introduction

## 1.1 Problem Statement

The backbone of a thriving economy is heavily dependent on infrastructure, including transportation, to support industries. In South Africa, the increase in industrial production as well as an established transport industry (Sichei et al., 2005) means that despite economic growth, other considerations such as the impact of these industries, on the environment need to be accounted for to understand the price at which this growth comes. The increasing movement of citizens living in rural areas towards the densely populated urban areas impacts on the local air quality as well, as a result of increased transport load and an increase in emissions due to cooking and household heating (Diamond et al., 2001; Fromme et al., 2004).

In the past, various air pollutants, for example carbon monoxide (Röllin et al., 2004), particulate matter (PM), sulphur dioxide and NO<sub>x</sub> emissions have been monitored as an indication of air quality in South Africa (Gwaze et al., 2007; Nzotungicimpaye et al., 2014; Thambiran & Diab, 2011) which has resulted from the implementation of air quality legislation. The Air Quality Act (Act No. 39 of 2004) introduced local air quality management plans in cities that have set targets for emission reductions (National Environment Management, 2005). This act however, does not include polycyclic aromatic hydrocarbons (PAHs), which are of growing concern due to their persistence and potential human toxicity.

PAHs fall into a category of compounds called semi-volatile organic compounds (SVOCs) as their vapour pressure falls within the range of  $1.0132 \times 10^{-4}$  –  $1.0132 \times 10^{-12}$  bar (Yamasaki et al., 1982). PAHs are found either in the gaseous phase or bound to particulates in the atmosphere. Typically the 2- and 3- ring PAHs are found in the gaseous phase, the 4- ring PAHs are a mixture of both phases, and the heavier 5- and 6-ring PAHs are predominantly found bound to particulates (Pankow & Bidleman, 1992). It has been reported that human exposure to 5-/6-ring PAHs have potentially worse effects, hence the investigation into the

presence of the full range of PAHs in our atmosphere is critical to human and ecological wellbeing (Ravindra et al., 2001).

Many studies have been undertaken to identify and quantify PAHs in the environment in South Africa, including studies on soils, sediments and water samples (Das et al., 2008; Havenga & Rohwer, 2000; Moja et al., 2013; Nieuwoudt et al., 2011; Quinn et al., 2009), however fewer studies on PAHs in air have been conducted (Forbes & Rohwer, 2009; 2010). These do not necessarily reflect the integrated PAH profile of the atmosphere over a number of years. This is due to the fact that soils act as sinks, collecting PAHs as a result of deposition including oil spills and domestic fires and biomass burning (Wilcke, 2007; Wild & Jones, 1995; Yunker et al., 2002). As a result of the lack of transformations occurring in soils, the concentration collected therein is often representative of the PAH profile emitted from the pollution source (Nieuwoudt et al., 2011) but not necessarily an accurate description of the atmospheric air quality with regards to PAHs, since sources of PAHs in soils include the wear of asphalt pavement surfaces, car tyres and rain runoff from roads and parking lots (Gryniewicz et al., 2002; Halsall et al., 1994; Harrison et al., 1996). There is therefore a need for a study on ambient air quality with particular focus on PAHs in South Africa, where the profile of PAHs in the atmosphere over an integrated period of time can be explored. This therefore supports the use of biomonitors, such as lichens, to act as sampling devices for PAHs (Blasco et al., 2008).

Lichens are able to accumulate pollutants in the air and due to their longevity and slow growth rate, are able to reflect an integrated exposure over time (Blasco et al., 2006), and therefore fill a gap in the understanding of atmospheric air quality that other passive sampling techniques cannot accomplish.

The quantification of PAHs in the environment is generally broken up into two stages: sample preparation and analysis by either high performance liquid chromatography (HPLC)-fluorescence detection or gas chromatography-mass spectrometry (GC-MS) (Blasco et al., 2008; Shukla & Upreti, 2013). Advances in the sensitivity of analyses has created new opportunities for an improvement in

sample preparation techniques which extract the analytes of interest in a representative and repeatable manner. The use of lichens as biomonitors for atmospheric PAHs has been successfully applied using a range of sample preparation techniques (Augusto et al., 2009; Blasco et al., 2006; Guidotti et al., 2003), however the application of newer sample preparation techniques using less solvent with lower energy consumption and quicker extraction times can be developed. In addition, no studies where the matrix effects incurred when using lichens as biomonitors for atmospheric PAHs have been documented and a deeper understanding of the interaction between the lichen matrix and the extracted PAHs is needed in order to confidently draw conclusions about the concentration of PAHs accumulated in the lichen samples.

## **1.2 Aim and objectives of the study**

The study was undertaken to explore the use of lichens as biomonitors for organic air pollutants with a particular focus on polycyclic aromatic hydrocarbons (PAHs) in South Africa. First and foremost, a quick, efficient and reproducible method for the extraction of PAHs from lichen matrices needed to be developed. This was to be compared to traditional sample extraction methods with respect to extraction efficiency, recoveries and repeatabilities (Augusto et al., 2009; Blasco et al., 2006; 2008; Shukla & Upreti, 2009; 2012). Once the best sample preparation technique was ascertained, samples of interest from different sampling sites in South Africa were extracted using this technique and then analysed using chromatographic techniques so that the concentration of PAHs as well as the PAH profiles could be determined. The profile of identified PAHs from lichen samples was then used as an indication of the air quality. PAH ratios in samples were used for pollution source diagnostics so that the major contributors towards atmospheric PAHs could be elucidated (Ravindra et al., 2008).

The objectives of this study were therefore:

- To use established sample preparation techniques to extract PAHs from a bulk lichen sample

- To develop new sample preparation techniques for extracting PAHs from lichens, including microwave-assisted extraction, QuEChERS and thermal desorption on the same bulk lichen sample
- To compare recovery, repeatability and sensitivity of new, as well as established, sample extraction methods
- To statistically compare the results from different sample extraction methods to establish whether there are meaningful differences between results using different sample preparation techniques
- To study the possible influence of the matrix on the quantification of PAHs and to determine the extent of the matrix effect, if observed
- To identify and quantify the PAHs in each extract using optimized chromatographic techniques coupled to mass spectrometry or diode-array detection
- To use the principles of multi-source fingerprinting based on PAH diagnostic ratios (Augusto et al., 2009) to identify pollution sources contributing towards the air quality at the sampling sites
- To determine whether the species *Parmotrema austrosinense* (Zahlbr.) Hale is able to reflect differences in atmospheric PAH levels between non-polluted and industrial sites

### **1.3 Justification for the study**

Numerous studies have been conducted to assess atmospheric metal pollution utilizing lichens and tree bark as biomonitors (Binning & Baird, 2001; Forbes et al., 2009; Mandiwana et al., 2006; Rosental et al., 1986; True et al., 2012). A number of studies have also been undertaken in South Africa which have investigated the presence of PAHs in the environment with particular focus on aqueous samples (Havenga & Rohwer, 2000), soil and sediment samples (Das et al., 2008; Nieuwoudt et al., 2011; Quinn et al., 2009), and air (Forbes & Rohwer, 2009; 2010), but no studies utilizing lichens for the purpose of monitoring atmospheric PAH profiles in South Africa have been recorded.

In addition to contributing to an understanding of the levels of PAHs in various environmental media in South Africa, this work contributes to the current field of

*'lichens as biomonitors for atmospheric PAHs'* via the implementation of new sample preparation methods which have the potential to save time, reduce the cost of extraction and solvent wastage, as well as improve extraction efficiencies. In addition, the study on the lichen matrix effects presented in this work provides a clearer understanding of how the analytes' response may be suppressed or enhanced during analysis as a result of interactions with the matrix.

#### **1.4 Dissertation Outline**

This dissertation will cover a study on the sample preparation techniques used to extract PAHs from lichens and compare the different techniques, some of which are established and two techniques which are novel for this application. These methods are discussed in Chapter 2, as well as the background for using lichens as biomonitors for organic air pollutants with a specific focus on PAHs. The third chapter discusses the bulk of the work done in this study: the sample extraction procedure development. The largest portion of my time was spent on the testing of existing sample preparation techniques and these are detailed in Chapter 3 along with the analytical methods employed. The results and discussion thereof follow in Chapter 4, including microscopy micrographs, selected chromatograms, calibration curves, matrix effect considerations and the results from the sampling points of interest, namely the Cape Point Nature Reserve, the Drakensberg, Pretoria Industrial area and the Daspoort Tunnel in Pretoria. The final part of this dissertation makes recommendations towards future work and draws conclusions from the results and findings of this study. Appendices are attached for reference including chromatograms, calculations, images from sampling sites and information on reference materials and sorbents used in the sample preparation techniques as well as research outputs from this work.

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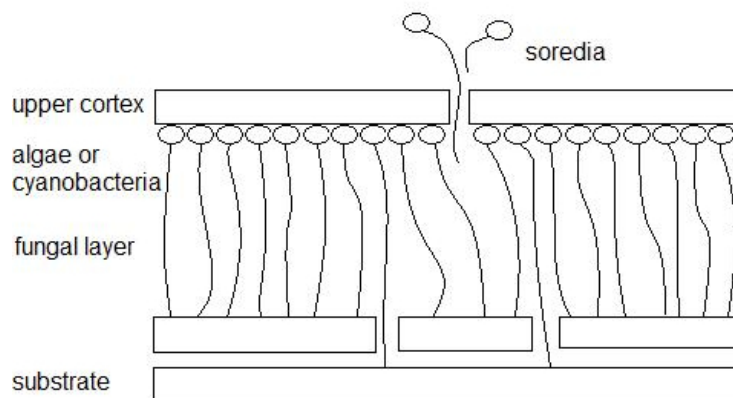


## Chapter 2: Literature Review

### 2.1 The use of lichens in pollution studies

Lichens are symbiotic organisms found on trees, rocks, in soils and even on weevils and giant Galapagos turtles (Hale, 1974). They are perennial, resilient and are able to live for many years in extreme conditions – being found in locations from the icy Himalayas to deserts (Bergamaschi, 2004; Maphangwa, 2012). The use of lichens as biomonitors comes as a result of their ability to respond to air pollutants at different levels, their slow growth rate, longevity and their ability to indicate the presence and concentrations of these pollutants (Nimis et al., 1993; Sloof et al., 1988).

The lichen thallus is a complex symbiotic vegetative lower plant composed of two organisms: a fungus and an algae or cyanobacteria. The cell wall consists of a multilaminar and a granular layer. Adhering to the outside of the cells is a fibrous polysaccharide layer. The lichens' hyphae can either orientate randomly or regularly in a parallel manner – these two types form the basic structure of the layers. The cortex (outer layer) of the lichen serves as a regulator for gas exchange and protective support of the lichen and it is in this layer that small gaps are found, allowing the soredia (the reproductive structures of lichens) to pass into the atmosphere. This layer is covered by an epicortex, which is a porous, non-cellular polysaccharide. It is believed that the porous nature of the epicortex is what enables efficient gas exchange (Hale, 1976). It has been found that lichens produce many compounds, generally referred to as lichen substances, such as xanthenes, anthraquinones, pulvinic acid derivatives, depsides, numerous depsidones, usnic acids, dibenzofuranes and aliphatic acids (Honda et al., 2010), of which many are believed to be unique to the lichen symbiosis (Rundel, 1978). Figure 2.1 shows a simplified illustration of a cross section of foliose lichens.



**Figure 2.1: Cross section through a foliose lichen**

Lichens have the unusual capability towards the uptake of ions and substrates at concentrations beyond their needs (Bačkor & Loppi, 2009). Metal ions are typically absorbed in a passive, extracellular manner and are bound reversibly by an ion exchange mechanism (Garty, 2001). It has been found that lichens are able to bind cadmium, lead, tin and zinc at higher concentrations than higher plants, even mosses (Tuominen & Jaakkola, 1974). It has been suggested that these trace elements are absorbed and stored by particulate entrapment as well as passive and active intracellular uptake in addition to ion exchange (Richardson & Nieboer, 1981; Tyler, 1989). There are many factors that determine the absorption and release processes in the lichens, namely the chemical nature of the compound, the presence and influence of other compounds, the size of the particles to be absorbed, and the chemical composition of the particles (Sloof, 1995).

The absorption of atmospheric pollutants by lichens has consequently been a field of research interest for many years, including the investigation into the uptake of sulphur dioxide by a number of researchers (Hawksworth & Rose, 1970; Rogers, 1977). It is understood that lichens absorb particle bound pollutants (metals and organic air pollutants) by either wet or dry deposition (Garty, 2001). The absence of a cuticular wax layer on lichens means that they

are able to absorb pollutants much more easily than other higher plants (Augusto et al., 2010).

One of the earlier uses of lichens as biomonitors of air pollution was by Sloof and Wolterbeek (1991) who studied the concentration of pollutants in lichens and compared it qualitatively with the atmospheric concentrations of suspended and deposited particulate matter. Later, elemental analyses were performed on lichens and successfully related the determined concentrations with atmospheric concentrations (including cobalt, scandium and zinc) (Sloof, 1995). In addition, a  $^{137}\text{Cs}$  study after the Chernobyl accident on both lichens and wet and dry depositions showed good correlations with dispersion model data sets (Sloof & Wolterbeek, 1992). It was consequently shown by Sloof (1995) that elemental concentrations within lichens appear to equilibrate with the surrounding atmospheric concentration levels, which proved that lichens are suitable candidates for the biomonitoring of air pollution.

The absorption of lead by lichens has been extensively studied due to the toxic nature of the heavy metal. An early application in this regard was a study that used lichens as biomonitors to track the lead emissions from automobiles along highways (Garty et al., 1977). Over the years, lichen biomonitoring research has been conducted into a range of inorganic analytes including mercury, most transition metals, radionuclides, fluoride, sulphur, nitrogen and acid rain, which all accumulate in the lichen thallus (Forbes et al., 2009; Garty, 1993; Gombert et al., 2003; Jeran et al., 2007; Puckett, 1988; True et al., 2012). For reviews on the progress made into biomonitoring of metal pollution using lichens, the reader is referred to references (Garty, 2001) and (Conti & Cecchetti, 2001).

More recently, the use of lichens as biomonitors for organic air pollutants has been investigated as reviewed (van der Wat & Forbes, 2015). Although lichens do not have a waxy cuticle or stomata, they do, however, produce and release lipid metabolites onto their surfaces, which are suspected of behaving in a similar manner as the cuticle, does in plants (Oksanen, 2006). The lipophilic nature of the surface of lichens then readily attracts lipophilic compounds, such

as atmospheric semi-volatile organic compounds (SVOCs), and facilitates their uptake, where their incorporation into cells would be metabolically controlled (Branquinho et al., 1999; Branquinho, 2001).

Organic air pollutants are typically hydrophobic and this means that uptake tends toward dry deposition (Duinker & Bouchertall, 1989; McLachlan & Horstmann, 1998). Metal ions and other water-soluble compounds are deposited on the lichen surface by other mechanisms. Both hydrophobic and hydrophilic compounds can be assimilated by lichens as a result of the volatilization of compounds from soils – or by direct methods such as wind impaction or splashing from the ground during rainfall episodes (Jones & Duarte-Davidson, 1997; Trapp & Matthies, 1997).

Organic air pollutants of relevance include persistent organic pollutants (POPs) that are typically chemicals that partition favorably to organic matter of a non-polar nature (UNEP, 2013). They are either deliberately produced (for example agrochemicals) or form accidentally through processes like combustion (such as polychlorinated dibenzo-*p*-dioxins and polychlorinated dibenzofurans). They are known to have long half-lives and therefore accumulate in organic matter (Jones & de Voogt, 1999). The associated health risks are often related to chronic exposure to POPs, and the effects of exposure include endocrine disruption as well as carcinogenic and mutagenic effects (Kogevinas, 2001).

There are three types of lichens: fruticose, foliose and crustose. The foliose lichens are known to have the highest surface area to dry weight ratio, and are said to accumulate airborne particles more readily than fruticose lichens (Nieboer et al., 1978). The foliose lichens are completely exposed to ambient air as a result of having few points of attachment to the substrate. Fruticose lichens are flatter; having a leaf like structure with defined upper and lower layers, subsequently only the upper layer is in contact with the ambient air. The crustose lichens are tightly attached to their substrates and are thus difficult to remove for analysis and are less exposed to the surrounds (Oksanen, 2006).

Lichens have been utilized to monitor air pollution in three different ways (Garty, 2001):

- (i) to determine the concentration of specific pollutants accumulated in the thallus
- (ii) to use the effect of pollution sources on the life span and presence or absence of lichen species to map out the distribution and effect of pollution in a specific area (such as a recent work (Shukla et al., 2014)) and
- (iii) to take healthy lichens with low background pollutant accumulation and to transplant them into polluted areas to measure the accumulation of pollutants or the consequential degradation of the thallus.

When conducting an experiment that exploits the absorptive nature of compounds by lichens, the choice of lichen species is paramount to the success of the study. The choice must be made with the method of investigation in mind: if a lichen transplant is to be done, a species that is very sensitive to changes in pollution should be chosen, whereas a hardy local species should be used if the concentration of target analytes in the thallus is to be determined at a sampling site. It is important to identify the species of lichen to be studied, as lichen species selectivity for absorption of compounds from the atmosphere has been illustrated by Blasco et al (2011) for example.

A distinction between bioindicators and biomonitors has been made in the literature. Using lichens as bioindicators has, in the past, meant that the apparent health of a specific lichen species is tracked for changes in growth and proliferation (so called 'injury symptoms') to gauge the extent of pollution in the area. On the other hand, biomonitoring is a method to measure the response of lichens to air pollution exposure; meaning that the biomonitor should reflect an integrated exposure over time. For a few years, it was believed that lichens could not be used as biomonitors (Sigal, 1988) because there was little understanding on how to extract the compounds of interest and which analytical methods to use for the analysis. Upon reviewing recent publications, the trend in research leans toward the use of lichens as biomonitors rather than bioindicators and will likewise be the attention of this study, with a specific focus on analytical

chemistry techniques utilized when using lichens as biomonitors, rather than bioindicators, which tends towards the biological sciences.

Utilizing the accumulative nature of lichens is advantageous over direct air sampling because large volumes of air would typically need to be sampled for a long period of time (over 24 hr or more) to obtain detectable concentrations of trace-level organic air pollutants. Direct air sampling also only provides information on the current air quality. Lichens, on the other hand, accumulate the organic air pollutants, thus providing concentrations that are detectable using sensitive instrumentation, as well as an integrated value of the atmospheric organic pollutant levels. This is relevant when studying these pollutants because their associated health risks are often related to chronic exposure (Kogevinas, 2001). Another advantage of utilizing lichens is that it is a simple and cheap sampling method, applicable in developing countries and in locations that are less accessible to sampling with bulky equipment, such as the Pyrenees Mountains (Blasco et al., 2008).

## **2.2 The current trend of using lichens as biomonitors for PAHs**

Investigation into the levels of polychlorinated dibenzofurans (PCDFs) and dioxins (PCDDs), and polycyclic aromatic hydrocarbons (PAHs) in lichens have been performed in countries including Portugal, Spain, Poland, Italy and India (Augusto et al., 2004; Augusto et al., 2007; Augusto et al., 2009; Augusto et al., 2010; Blasco et al., 2006; Guidotti et al., 2003; Migaszewski et al., 2002; Nascimbene et al., 2014; Schrlau, 2011; Upreti & Patel, 2012). A study by Augusto et al (2010) showed that lichens are better accumulators of PAHs than soil and pine needles, whilst their PAH profiles are similar to that of the ambient air. Schrlau et al (2011) also found that lichens were more effective at accumulating POPs than pine needles and mosses. It was shown by Migaszewski et al (2002) that the lichen species studied had a higher PAH concentration than the host bark, and that the same lichen species had consistent PAH concentrations despite growing on the bark of different tree species.

Studies have been performed to identify which lichens are better at absorbing

different organic air pollutants. It has been reported that the fruticose lichens absorb the lower molecular weight POPs better, most likely due to their high surface area to volume ratio, whilst the foliose lichens preferentially absorb the higher molecular weight organic pollutants (Augusto et al., 2009; Blasco et al., 2011; Schrlau, 2011). Despite these differences, it has been found that similar PAH profiles have emerged from studies, where 2- to 4-ringed PAHs are primarily observed in lichens (Augusto et al., 2010; Blasco et al., 2006; Blasco et al., 2008; Domeño et al., 2006; Guidotti et al., 2003; Guidotti et al., 2009; Migaszewski et al., 2002; Nascimbene et al., 2014; Shukla & Upreti, 2009) and the PCDD/F profiles show a tendency towards tetrachlorodibenzodioxin/pentachlorodibenzo-p-dioxin (TeCDD/PeCDD) and pentachlorodibenzofuran (PeCDF) domination (Augusto et al., 2004; 2007; 2009). Blasco et al (2011) investigated the behavior of different lichen species under the same conditions with regard to their abilities to accumulate PAHs. It was generally found that there was a high 3-ring PAH content and a low 6-ring PAH content across all species, as has been found in other studies (Shukla & Upreti, 2009). Phenanthrene, closely followed by naphthalene, fluoranthene and benzo[a]anthracene are often the PAHs occurring at the highest concentrations in lichens (Augusto et al., 2010; Blasco et al., 2011).

Studies have indicated that the substrate from which the lichens are collected may play a role in the accumulation of POPs and other compounds (Sloof & Wolterbeek, 1993). It is unlikely that any POPs are assimilated via bark due to their hydrophobic nature, so the main mechanisms of uptake are due to dry deposition as well as from the soil, as described in Section 2.1. Bauer et al (1992; 1997) showed that the heavier POPs diffuse slower than the lower molecular weight (MW) POPs, and that the higher MW POPs are thus more likely associated with particles that remain on the surface of lichens. It has been shown (Augusto et al., 2013) that the PCDD/F concentrations remain relatively constant after periods of wet conditions (rain or fog, for example) suggesting that some POPs are retained within the lichen thallus and are not rinsed off easily. A parallel study for PAHs has not yet been published, but studies of other organic air

pollutants strongly suggest that a similar trend for PAH integration in lichens is to be expected (Augusto et al., 2013; Kylin & Bouwman, 2012).

## **2.3 Polycyclic Aromatic Hydrocarbons**

### **2.3.1 PAHs: sources and environmental fate**

A process called pyrosynthesis occurs at high temperatures, whereby C-H and C-C bonds in hydrocarbons are broken to form free radicals, which then react to form stable ring structures known as PAHs (Baek et al., 1991). PAHs are made up of two or more fused benzene rings comprised of only carbon and hydrogen. They can also be generated by pyrolysis; from thermal combustion processes in oxygen deficient conditions, such as vehicular emissions, coal burning, household fires including the incineration of waste, biomass burning and various industrial processes (Lima et al., 2005; Simoneit, 1967).

PAHs are released in gaseous and particulate-associated phases as by-products of combustion of organic materials and fuels depending on their low vapour pressure (Ravindra et al., 2008), and enter the food chain, water supply and vegetation by gaseous exchange at the air/water interface, by wet deposition as a result of rainfall, fog or humid weather conditions and by dry deposition of particulate matter (Chen et al., 2004). The phase distribution of different PAHs is shown in Table 2.1. The lighter PAHs such as phenanthrene and anthracene are predominantly found in the gas phase, whereas the heavier PAHs such as the five-ringed benzo[*a*]pyrene (BaP) are found adsorbed to particles, thus the fate of PAHs in the environment depends largely on their physiochemical properties (Baek et al., 1991). Derivatives of PAHs which contain oxygen, nitrogen or sulphur atoms as well as alkyl groups are formed (Lundstedt et al., 2003) and commonly occur in samples alongside PAHs, but are beyond the scope of this study and will not be discussed further. Structures of all PAHs considered in this study are given in Appendix A.



**Table 2.1: The Agency for Toxic Substances and Disease Registry (ATSDR) and US Environmental Protection Agency (US EPA) priority PAHs and their gas/particle phase distribution (Ravindra et al., 2008)**

PAHs	Primary phase distribution
Naphthalene	Gas phase
Acenaphthene	Gas phase
Acenaphthylene	Gas phase
Fluorene	Gas phase
Anthracene	Particle / gas phase
Phenanthrene	Particle / gas phase
Pyrene	Particle / gas phase
Fluoranthene	Particle / gas phase
Benzo[ <i>a</i> ]anthracene	Particle phase
Chrysene	Particle phase
Benzo[ <i>b</i> ]fluoranthene	Particle phase
Benzo[ <i>a</i> ]pyrene	Particle phase
Indeno[1,2,3- <i>cd</i> ]pyrene	Particle phase
Dibenzo[ <i>ah</i> ]anthracene	Particle phase
Benzo[ <i>ghi</i> ]perylene	Particle phase

Due to their ubiquitous nature, it is required in Europe and the USA that particle associated PAHs are monitored and their release into the atmosphere be regulated (ATSDR, 1995, Atlanta, 2011; Commission of the European Communities, 2005), highlighting the importance of understanding the PAH profile and concentrations in the atmosphere around South Africa.

### 2.3.2 Health effects of PAHs

PAHs are analytes of interest in environmental and food analyses as a result of their toxic and carcinogenic nature. Benzo[*a*]pyrene (BaP) was the first PAH to be classified as carcinogenic and since then, benzo[*a*]anthracene, chrysene, benzo[*b*]fluoranthene, benzo[*j*]fluoranthene, benzo[*k*]fluoranthene, indeno[1,2,3-*cd*]pyrene and dibenzo[*ah*]anthracene have also been listed as carcinogenic and teratogenic chemicals (Ravindra et al., 2001).

The mutagenic and suspected carcinogenic activity of numerous PAHs have been studied and pivotal evidence based on the International Agency for Research on Cancer (IARC) criteria suggests PAHs contribute towards the development of lung, laryngeal, bladder and prostate cancers (Clapp et al., 2008). It has been shown in mice that PAHs induce lung cell tumors (Osgood et al., 2013) and PAHs are known to disrupt the reproductive endocrine system (Kim et al., 2011). Toxic Equivalency Factors (TEFs) have been assigned to PAHs (Nisbet & Lagoy, 1992) in order to perform risk assessments of exposure to various PAHs, where the TEF is measured relative to the carcinogenicity of BaP. These are then used to assess the potential health risk per PAH exposure, bearing in mind that the toxic affects are additive, such that an exposure to a range of PAHs with various TEFs will result in an increased risk of harmful health effects. The TEF of a mixture is then calculated and provides the equivalent concentration of BaP, since BaP is considered to be one of the most harmful. As an example: chrysene, with a TEF of 0.01, at a concentration of  $0.1 \text{ mg.l}^{-1}$  would have a BaP toxic equivalent concentration of  $0.001 \text{ mg.l}^{-1}$ . Table 2.2 lists the PAHs of interest and their associated TEFs according to Nisbet and Lagoy (1992) that will be used in the calculations of the toxic equivalence quotient (TEQ), where the TEQ is the sum of the TEF-weighted concentrations of all the PAHs identified and quantified in a mixture.

**Table 2.2: PAHs and their toxic equivalency factors (TEFs) expressed relative to BaP according to Nisbet and Lagoy (1992)**

PAH of interest	TEF
Dibenzo[ <i>ah</i> ]anthracene	5
Benzo[ <i>a</i> ]pyrene	1
Benzo[ <i>a</i> ]anthracene	0.1
Benzo[ <i>b</i> ]fluoranthene	0.1
Phenanthrene	0.001
Indeno[1,2,3- <i>cd</i> ]pyrene	0.1
Anthracene	0.01
Benzo[ <i>ghi</i> ]perylene	0.01
Chrysene	0.01
Acenaphthene	0.001
Acenaphthylene	0.001
Fluoranthene	0.001
Fluorene	0.001
Pyrene	0.001
Naphthalene	0.001

### 2.3.3 Justification of the choice of PAHs to be investigated

Since the US Environmental Protection Agency has listed 16 PAHs as priority pollutants (Table 2.1), these were used as a starting point in the study. These PAHs, as discussed in Section 2.3.2, have cancer and endocrine-disrupting risks associated with them and are therefore of immediate concern to identify and quantify in the atmosphere. Furthermore, in studies focusing on the use of lichens as biomonitors of PAHs in the environment, it was generally found that there was a high 3-ring PAH content and a low 6-ring PAH content across all species (Shukla & Upreti, 2009). Phenanthrene, closely followed by naphthalene, fluoranthene and benzo[*a*]anthracene are often the PAHs occurring at the highest concentrations in lichens (Augusto et al., 2010; Blasco et al., 2011). This served as an indication of which PAHs should be focused on in this study so that

comparisons could be made between studies in different geographic locations as well as studies that had made use of different sample preparation techniques.

Only PAHs of molecular mass lower than  $278 \text{ g}\cdot\text{mol}^{-1}$  were considered since the heavier PAHs are known to have low vapour pressures as well as very low solubility in water (Table 2.3) (Lima et al., 2005; Tobiszewski & Namiesnik, 2012). Considering that gas chromatography-mass spectrometry was the chosen method of separation and identification in this study, heavier PAHs would also be difficult to quantitatively volatilize and analyze.

**Table 2.3: Identity of PAHs of interest in this study, with molecular mass, solubility in water and abbreviations used in this study**

PAH	Abbreviation	Nominal m/z	Water solubility at 25°C ( $\text{mg}\cdot\text{l}^{-1}$ ) <sup>a</sup>
Naphthalene	Nap	128	31
Acenaphthylene	Acy	152	16
Acenaphthene	Ace	154	3.8
Fluorene	Flu	166	1.9
Phenanthrene	Phe	178	1.1
Anthracene	Ant	178	0.04
Fluoranthene	FluAn	202	0.2
Pyrene	Pyr	202	0.13
Benzo[ <i>a</i> ]anthracene	BaA	228	0.011
Chrysene	Chr	228	0.0019
Benzo[ <i>b</i> ]fluoranthene	BbF	252	0.0015
Benzo[ <i>a</i> ]pyrene	BaP	252	0.0015
Indeno[123- <i>cd</i> ]pyrene	IcdP	276	0.00019
Benzo[ <i>ghi</i> ]perylene	BghiP	276	0.00014
Dibenzo[ <i>ah</i> ]anthracene	DahA	278	0.0005

<sup>a</sup> (Tobiszewski & Namiesnik, 2012)

## 2.4 Source diagnostics using PAH ratios

PAHs can be traced back to their origin by using specific PAHs as markers for pollution sources. Furthermore, diagnostic ratios determined from concentrations of different PAHs can be used to identify pollution sources in different regions, as shown below in Table 2.4. An important note to bear in mind when calculating diagnostic ratios is that PAHs with similar physiochemical properties should be used when calculating the ratios, to minimize the bias introduced by differences in volatility, chemical reactivity and

solubility (Ravindra et al., 2008) as well as the fact that they were developed for direct air sampling techniques, not lichens.

**Table 2.4: The diagnostic ratios commonly used to determine PAH pollution sources in direct air sampling studies as well as lichen studies**

Diagnostic ratio	Value	Source	References
IcdP/ (IcdP+ BghiP)	0.18	Vehicle emissions	(Grimmer et al., 1983) (Kavouras et al., 2001)
	0.56	Coal combustion	(Ravindra et al., 2006) <sup>a,b</sup> (Yunker et al., 2002)
	0.62	Wood burning	(Ravindra et al., 2006) <sup>a</sup>
	0.35-0.70	Diesel emissions	(Ravindra et al., 2006) <sup>a</sup> (Yunker et al., 2002) <sup>b</sup>
Flu/ (Flu + Pyr)	>0.5	Diesel, grass, wood combustion	(Rogge et al., 1993) (Mandalakis et al., 2002) (Ravindra et al., 2006) <sup>a</sup>
	<0.5	Petroleum combustion	(Ravindra et al., 2006) <sup>b</sup> (Fang et al., 2004)
BaP/ (BaP + Chr)	0.5	Diesel combustion	(Khalili et al., 1995) (Guo et al., 2003)
	0.73	Petroleum combustion	
Pyr/ BaP	+/- 10	Diesel engine emissions	(Ravindra et al., 2006) <sup>a,b</sup>
	+/- 1	Petrol engine emissions	
FluAn/ Pyr	0.6	Vehicle emissions	(Neilson, 1998)
PAH <sub>comb</sub> / PAH <sub>total</sub>	+/- 1	Combustion	(Guidotti et al., 2003)
BaA/ (BaA + Chr)	0.2-0.35	Coal combustion	(Yunker et al., 2002) (Akyüz & Çabuk, 2010)
	>0.35	Vehicle emission	(Yunker et al., 2002)
	<0.2	Petrogenic	(Yunker et al., 2002)
Ant/ (Ant + Phe)	>0.1	Pyrogenic	(Pies et al., 2008)
	<0.1	Petrogenic	
ΣMW <sub>low</sub> /ΣMW <sub>high</sub>	<1	Pyrogenic	(Zhang et al., 2008)
	>1	Petrogenic	

PAH<sub>comb</sub>: FluAn, Pyr, BaA, Chr, BbF, BkF, BaP, DbA, BghiP

ΣMW<sub>low</sub>: Sum of 2- and 3-ring PAHs

ΣMW<sub>high</sub>: Sum of 4- and 5-ring PAHs

Industrial sources of pollution are typically characterized by a profile rich in 2-, 5-, and 6-ring PAHs (such as naphthalene, benzo[ghi]perylene, indeno[123-cd]pyrene and benzo[k]fluoranthene), whereas urban pollution sources are distinguished by a profile rich in 4-ring PAHs (fluoranthene, pyrene, chrysene and benzo[a]anthracene) which are commonly found in vehicular emissions (Guidotti et al., 2003).

PAH concentration ratios found in lichens may also be used for source apportionment studies. For example: phenanthrene/anthracene (Phe/Ant) and fluorene/pyrene (Flu/Pyr) (Domeño et al., 2006) may be employed where a Phe/Ant ratio higher than 10 and Flu/Pyr ratios higher than 1 indicate a mix of pyrogenic and petrogenic sources (Augusto et al., 2009; Blasco et al., 2011). The number of rings in the PAH structures are also often compared to give an indication of the PAH profiles that the lichens are accumulating: generally increased toxicity is related to an increase in the number of rings.

Domeño et al (2006) found total PAH concentrations of around 340 ng.g<sup>-1</sup> using their dynamic sonication assisted solvent extraction (DSASE) technique, in lichens which were sampled near a river outside of a city in Spain, whereas a study by the same group, using the same technique found total PAH concentrations of between 1.2 – 1.65 µg.g<sup>-1</sup> in lichens in an area with a high density of traffic. Using diagnostic ratios, it was suggested that the traffic was indeed the main contributor towards the high PAH content in the latter lichens (Blasco et al., 2006). These studies have successfully exploited the efficacy of lichens as biomonitors to diagnose pollution sources of specific SVOCs. The large variation in total PAH concentration is heavily influenced not only by the contributing pollution sources at the location, but by many environmental factors such as the season in which sampling was undertaken, the altitude at which samples were collected and the extraction techniques used in the laboratory (Blasco et al., 2006; 2007).

The results from most studies indicate that phenanthrene, fluoranthene, naphthalene and pyrene tend to dominate the PAH profiles in lichens, with higher 2- and 3-ring PAH concentrations than 6-ring compound concentrations (Augusto et al., 2010; Blasco et al., 2006; Blasco et al., 2007; Blasco et al., 2008; Blasco et al., 2011; Shukla & Upreti, 2009). This is possibly due to the fact that the heavier PAHs are less volatile and are generally associated with particulate matter and may therefore be less airborne.

The vast range in total PAH concentrations can be illustrated by the difference between the study (Shukla et al., 2012) where concentrations as high as 187.3  $\mu\text{g}\cdot\text{g}^{-1}$  were detected, compared to 0.058  $\mu\text{g}\cdot\text{g}^{-1}$  total PAH concentration detected in Portugal (Augusto et al., 2013). In India, the samples were taken in an industrial area close to the Himalayas and in Portugal the samples were taken in an industrial area on the coast. Soxhlet extraction was used in sample preparation in a study in India (Shukla et al., 2012), whilst the sample preparation technique was not reported for the study in Portugal, which makes understanding the sources of the differences difficult.

## **2.5 Lichen sampling strategies for monitoring PAHs in the environment**

Lichens typically grow on walls, roof tiles, bark and rocks (Hale, 1974). Studies have indicated that the substrate from which the lichens are collected may play a role in the accumulation of POPs and other compounds (Sloof & Wolterbeek, 1993). This suggests that in order to reduce uncertainties when comparing results between regions, lichens of the same species should be sampled from the same substrate type in order to minimize any influence, however negligible, the substrate may play.

Two distinctly different sampling methods are used when lichens are to be utilized as tools for air pollution monitoring. The technique of transplanting lichens from relatively clean environments to areas of interest has been a popular method when studying heavy metals (Caniglia et al., 1994; Cercasov et al., 2002) but is not readily applied to PAH studies, due to the long term exposure which is required in order to allow for meaningful and detectable results of these low level pollutants.

Direct sampling, on the other hand, is a popular sampling technique that exploits the fact that lichens accumulate pollutants with time directly from the atmosphere. Lichens are mostly sampled at heights of more than 1 m above the ground except for one study (Migaszewski et al., 2002) that sampled from as low as 25 cm above the ground. Such low sampling heights may influence results due to contamination of the lichens by soil, as well as protection of the lichens from

wind by surrounding shrubs and rocks at the base of a tree. In contrast, lichens have been sampled off roof tiles for a study into atmospheric dioxin and furan deposition in Portugal (Augusto et al., 2004).

When atmospheric furans and dioxins have been studied using lichens, the samples have been removed from the substrates, placed in either glass jars (Denys et al., 2012) or stored in plastic bags (Augusto et al., 2004), dried at room temperature and then stored for analysis. For PAH studies, the sampling methodology varies; whereas some groups sample the lichens into amber vials (Augusto et al., 2010; Migaszewski et al., 2002), others sample into paper bags (Nascimbene et al., 2014), wrap samples in aluminum foil (Shukla & Upreti, 2009) or polyester barrier film bags (Schrlau, 2011). Due to the fact that PAHs may photodegrade, the sampling and storage procedures that prevent further chemical changes to the lichen after sampling are most regularly employed. Non-permeable collection containers are also preferable, in order to prevent loss of the more volatile analytes (2- and 3-ring PAHs).

Whereas some studies have meticulously reported sampling conditions, such as avoiding the sampling of fruiting lobes and only sampling the apical lobes of lichen structures (Nascimbene et al., 2014), most studies collect samples of the same species from different tree species (Augusto et al., 2010; Migaszewski et al., 2002), which could increase uncertainty when comparing studies, since different tree species would have different bark and canopy structures, potentially protecting some lichen species better than others from wind and atmospheric deposition.

Large uncertainties are also introduced when the sampling strategy is not representative (Bodnar et al., 2013). This means that samples which are collected, cleaned, stored and dried must be treated in a manner that ensures that the collected sample reflects the average composition of the entire sample (Romanik et al., 2007). Careful consideration therefore needs to be given to the sampling, transport, cleaning and storage strategy in order to ensure that the



concentrations and content of the collected sample remains congruent with the total substrate of interest.

Augusto et al (2012; 2013) showed that seasonal flux influences the concentration of PAHs in lichen samples, with the highest concentrations of PAHs found in lichens sampled during the wet winter months, and the lowest concentrations during the warmer, dry summer months. This may be as a result of increased evaporation of organic air pollutants; an increase in emissions during the cold winter months, or as a result of UV induced photodegradation or other photochemical reactions that may take place (Beyer et al., 2003; Jung et al., 2010; Schauer et al., 2003). It is for this reason that it is important that the season when lichen sampling took place is reported for consistency and to allow for comparison.

Other conditions that need to be considered when sampling are wind speed and direction (laminar boundary will be affected by air flow), air pressure (an increased pressure will mean a higher concentration of compounds in the atmosphere thus higher accumulation rates) as well as humidity (Augusto et al., 2013; Bakker, 2000; Blasco et al., 2011; Oksanen, 2006). For a detailed summary of necessary factors to consider when using lichens as biomonitors for POPs, the reader is referred to a review by Augusto et al (2013).

## **2.6 Sample preparation methods**

Sample preparation is considered the most crucial step of any analysis, and introduces the most uncertainty in the final outcome (Yang et al., 2013). The main objective of optimizing sample preparation is to selectively isolate analytes of interest, reducing the noise and interference from the matrix. Sample preparation steps also often serve as pre-concentration measures for analytes which are present at trace levels, in order to improve the sensitivity of analysis (Romanik et al., 2007).

The European Committee for Standardization (CEN) developed standard extraction methods for the specific application of PAHs as airborne pollutants,

with the stipulation that any recovery above 50% is acceptable (European Committee for Standardization (CEN), 2008). The methods detailed in the EN15549 are shown below in Table 2.5 and were used as a starting point in this study to establish which techniques and solvent schemes would be compared.

**Table 2.5: Standard methods for the measurement of benzo[a]pyrene in ambient air according to EN15549:2008**

Extraction Method	Solvent scheme
Reflux	Toluene
Soxhlet	Toluene, hexane:acetone (1:1), dichloromethane
Microwave extraction	Hexane:acetone (1:1)
Ultrasound extraction	Toluene, dichloromethane

### 2.6.1 Soxhlet extraction

Soxhlet is an old extraction technique, developed in 1879 by von Soxhlet (von Soxhlet, 1879). It has since been used for over a century to leach analytes of interest from their solid matrices into a solvent of choice. The Soxhlet apparatus allows the continual dripping of condensed extracting solvent onto the sample, held in a porous thimble, encouraging extraction of the analytes into the solvent (Luque de Castro & Garcia-Ayuso, 1998; Luque de Castro & Priego-Capote, 2010). A novel siphon aspirates the solvent dripping into the thimble when an over-flow volume level is exceeded and the liquid is poured back into the distillation flask. This process allows the transfer equilibrium to be continually disturbed, improving the extraction efficiency when compared to static leaching techniques (Luque de Castro & Priego-Capote, 2010).

Soxhlet extraction has historically been used to extract organic analytes from lichens (Augusto et al., 2004) and continues to be used for the extraction of PAHs from the lichen matrix (Augusto et al., 2010; 2012; Shukla & Upreti, 2009; 2012). Table 2.6 shows the Soxhlet solvent schemes and various other experimental conditions mentioned in the literature where PAHs were studied in lichens. The method has been applied with great success to the extraction of PAHs from soils, bark and pine needles (Augusto et al., 2010; Dean et al., 1995; Di Lella et al., 2006; Orecchio et al., 2008; Ratola et al., 2006).

A serious disadvantage of using the Soxhlet methodology for sample preparation is the large volumes of wasted solvent that are expensive to purchase and dispose of, many of which are harmful to the environment. Another disadvantage is the long extraction times (2 – 24 hr) – this decreases sample throughput and utilizes more energy and that has environmental as well as financial implications (Augusto et al., 2009; Nascimbene et al., 2014). Boiling solvent over the period of a few hours also introduces the possibility of the degradation of thermo-labile species (Luque de Castro & Priego-Capote, 2010). However, an advantage that Soxhlet extraction has over other techniques is that no further filtration is required which may incur analyte losses, since the thimble keeps the solid material out of the final extract.

**Table 2.6: The different Soxhlet extraction conditions applied in various studies using lichens as biomonitors for PAHs**

Mass of sample (g)	Solvent scheme	Solvent volume (ml)	Extraction time (hr)	References
2	Dichloromethane	100	16	(Bajpai et al., 2013; Shukla & Upreti, 2009; 2012; 2013)
2	Acetonitrile	200	24	(Augusto et al., 2009; 2010; Schauer et al., 2003)
0.2	Dichloromethane	250	6	(Domeño et al., 2006)
0.6 – 0.8	Hexane: acetone (1:1)	150	2	(Nascimbene et al., 2014)

### 2.6.2 Microwave assisted extraction

The earliest use of microwave energy to assist in the extraction of organic analytes dates back to the 1980's, where household equipment was used (Ganzler et al., 1986; Ganzler & Salgo, 1987). Specialized laboratory equipment has since been developed. The principle of microwave-assisted extraction (MAE) is that the analytes of interest adsorbed or absorbed by the substrate are partitioned into the extraction solvent by the application of microwave energy. The microwave energy applied heats and transfers energy as a result of the

dipole rotation of organic solvents (Camel, 2000). Microwaves heat the contents of the vessel simultaneously and nearly instantaneously, without heating the vessel, reducing extraction time significantly.

The extraction efficiency is heavily dependent on the choice of extraction solvent, since the solvent should be able to readily absorb microwave energy (Guo & Lee, 2013). As a result, non-polar solvents produce poor extraction efficiencies, unless they are used in conjunction with solvents that have large dipole moments (Camel, 2000). The dielectric constant is a measure used to define the extent to which the sample can absorb microwave energy, and its loss factor is the degree to which absorbed energy can be dissipated. The ratio of the loss factor to the dielectric constant will then determine to what degree the entire sample is heated (Camel, 2000). The experimental design generally involves the absorption of microwave heat by the solvent and not by the sample within the solvent by selecting a solvent with a high dielectric constant.

MAE has been performed to extract PAHs from numerous environmental matrices such as soils, sediments and even smoked meat samples (Bartolomé et al., 2005; Chee et al., 1996; Purcaro et al., 2009; Srogi, 2006). It has been proposed that the incidence of microwave energy on soils and sediments creates cavities of gas bubbles, which burst and rupture the macrostructure of the substrate that would then increase the extraction of analytes trapped by the matrix (Camel, 2000). A similar mechanism could be expected from lichens that have been finely ground. Guo et al (2013) demonstrated in their study on PAHs in soils that higher temperatures during extraction increases the diffusion rates of the solvent and thus permeates the solid matrix more effectively. The partition coefficient is also affected such that the analytes are released from the solid more readily at higher temperatures (Guo & Lee, 2013). Typically, a clean up and concentration step is necessary after MAE before the extract can be analyzed. The filtration and clean up procedure would also ideally serve to remove interferences and thus reduce any matrix effects (Xu & Lee, 2008).

Studies on sediments and soils have shown that a solvent scheme of hexane: acetone (1:1, v/v) efficiently extracts PAHs with satisfactory recoveries (Chee et al., 1996; Lopez-Avila et al., 1994; Lopez-Avila & Benedicto, 1996). The study by Ratola et al (2009) used the solvent scheme of hexane: dichloromethane (1:1, v/v) successfully. A study on PAHs in spruce needles and pollen (Tomaniova et al., 1998) found MAE to be superior over USAE, with optimized extraction conditions of 140°C for 20 min using a solvent scheme of *n*-hexane: acetone (3:2, v/v).

In order to improve on MAE procedures, stir bar micro extraction (SBME) was developed by Jiang and Lee in 2004 with the purpose of extracting, filtering and pre-concentrating extracts in one process, delivering high extraction efficiencies of pentachlorobenzene and hexachlorobenzene in solvent as model compounds (Jiang & Lee, 2004). This method was then exploited in a study (Guo & Lee, 2013) focusing on PAHs in soils with further extraction potential enhancement by the combined use of microwave assisted extraction. Here the extraction solvent was placed in a short hollow fiber membrane that was lowered into a solution containing the soil sample. It was then exposed to microwave radiation to quantitatively extract PAHs from the sample into the solvent held within the fiber. Direct GC/MS analysis followed the extraction procedure, producing results with recoveries between 81 – 107 % and RSDs below 9.5% (Guo & Lee, 2013). Although the SBME technique has proven successful with soils, the application to lichens might be challenging due to the fact that many pigments could co-extract with the analytes of interest (i.e. PAHs) and thus nullify the apparent advantage that SBME offers regarding minimal required clean up.

A study by Ratola et al (2009) compared the use of MAE and ultrasound assisted extraction (USAE) to determine the PAH content of pine needles in Portugal. The study utilized a modified domestic microwave oven, and 90 ml of dichloromethane:hexane (1:1, v/v) for 30 min for the MAE extraction. The recovery of PAHs using MAE varied widely between 10 – 120 %, with the recoveries of PAHs using USAE reported as being very similar. It was also observed that MAE had better recoveries for the heavier molecular weight PAHs

compared to USAE. Similar findings were seen in the study by Purcaro et al (2009) on smoked meats, where extracts obtained by MAE were found to have higher extraction efficiencies for PAHs than USAE extracts from the same sample. In the study on smoked meats, the extraction times were optimized at 15 min at 115 °C, with recoveries ranging between 70 – 103 % (Purcaro et al., 2009).

In general, when compared to traditional Soxhlet extraction, MAE offers a reduction in extraction time (between 5 – 20 min compared to > 2 – 24 hr for Soxhlet), reduced solvent consumption (10 – 50 ml in MAE compared to 250 ml – 500 ml in Soxhlet) as well as enhanced reproducibility and reduced solvent wastage (Baumard et al., 1998; Camel, 2000). Reduced solvent wastage goes hand-in-hand with a lesser impact on the environment as a result of less harmful solvents being volatilized during the blow down and pre-concentration steps.

### **2.6.3 Quick, Easy, Cheap, Effective, Rugged, Safe (QuEChERS) extraction method**

A relatively new sample extraction technique was developed for the extraction of pesticides in food products that was designed to be quick, easy, cheap, effective, rugged and safe. QuEChERS, as it was cleverly named, was developed by Anastassiades et al in 2003 and quickly found popularity as an effective sample preparation technique for pesticide analysis in foodstuffs (European Committee for Standardization (CEN), 2009). The method serves as an alternative to traditional liquid-liquid extraction and solid phase extraction techniques, reducing the number of steps as well as the total extraction time and solvent consumption (Wilkowska & Biziuk, 2011).

The QuEChERS methodology involves two steps. The first step is the extraction of homogenized samples using an organic solvent of choice with a solution of extraction salts, often made up of magnesium sulphate (absorbs water and increases ionic strength), sodium chloride (improves partitioning to organic phase), trisodium citrate dihydrate (acts as a buffer) and disodium hydrogen citrate sesquihydrate (acts as a buffer), followed by a centrifugation step. The addition of the extraction salts ( $\text{Na}_2\text{SO}_4$  or  $\text{MgSO}_4$ ) is an exothermic process and therefore increases the temperature of the reaction vessel, improving the

extraction efficiency (Anastassiades et al., 2003). The second step then involves the clean up of the supernatant using a dispersive solid phase extraction technique (dSPE) which differs from SPE since it does not use a fixed solid bed. The addition of internal standards, buffers or acids can be done in the first step in order to tweak the extraction efficiencies and track recoveries. The dSPE step involves the use of a drying salt (magnesium sulphate) as well as SPE sorbent packing materials such as primary secondary amine (PSA), often supplemented with graphitized carbon black (GCB) or C<sub>18</sub> material to clean the extracts and remove moisture. The dSPE step also removes sugars, fats, organic acids, proteins, pigments and sterols (Restek Corporation, 2009), thereby simplifying what would be many consecutive clean up steps into one clean up procedure. Instrumental analysis using either gas or liquid chromatography follows, with or without the addition of a pre-concentration step after the clean up.

QuEChERS has gained popularity as a method to analyze foodstuffs, so much so that by 2009, the European Committee for Standardization (CEN) had developed a reference method for pesticides in foods of plant origin (European Committee for Standardization (CEN), 2009) and analyses for pesticides in many matrices has since been undertaken, such as olives, milk, rice, baby food, blood and soils (Cunha et al., 2007; Dagnac et al., 2009; Nguyen et al., 2008; Plössl et al., 2006; Przybylski & Segard, 2009; Zhao et al., 2012).

QuEChERS has been used for extracting PAHs from foodstuffs, with shrimp and fish being the first samples reported (João Ramalhosa et al., 2009; Kalachova et al., 2011). Forsberg et al (2011) followed shortly thereafter, with the analysis of smoked salmon samples for traces of PAHs in which they suggested that increasing the temperature of extraction would enhance the extraction of PAHs from the matrix. Forsberg et al modified an existing QuEChERS method that produced recoveries between 50-200% with relative standard deviations below 10%.

QuEChERS has been applied to PAHs in green, white, black and red teas and a study found that ethyl acetate was a preferred extraction solvent for PAH

analysis (Drabova et al., 2012). Molecularly imprinted polymers (MIPs) designed for PAHs were then used to clean up the tea extracts, reporting highly efficient sample clean up, followed by GC x GC – time-of-flight mass spectrometry (TOFMS) analysis. The studies on teas produced recoveries between 73-103% despite the surprising use of boiling water during the sample extraction procedure that could possibly introduce losses of some of the lighter PAHs (Drabova et al., 2012; Sadowska-Rociek et al., 2014).

Perhaps closer to our samples of interest, the lichen matrix, Sadowska-Rociek and coworkers (2013) evaluated the QuEChERS methodology for the application of PAHs in fresh herbs, cleaned up using 0.15 g PSA, 0.05 g graphitized carbon black (GCB) and 0.9 g MgSO<sub>4</sub>, followed by GC – single ion monitoring - mass spectrometry (SIM-MS) analysis. Acetonitrile, as well as ethyl acetate were used, and it was found that acetonitrile was more efficient at extracting PAHs from the herbs with recoveries between 71.6 – 116.9%. Included in the methodology was a 15 min shaking step, which deviates from the original QuEChERS methodology of extractions between 1 – 5 min (Albinet et al., 2013), but remains, however, a considerably faster extraction technique compared to Soxhlet, USAE and some MAE methods. Although herbs are higher plants than the symbiotic organisms focused on in this study, the work by Sadowska-Rociek et al indicates that PAH analysis on plant matrices has promise.

The importance of solvent choice has been studied for the analysis of PAHs in tea and herbs, where QuEChERS on tea had the greatest extraction efficiency when using acetonitrile, and the same was found to be true for herbs (Sadowska-Rociek et al., 2013; 2014). This emphasizes the need to try a few different solvent schemes on the homogenized lichen samples, since there are no research articles to date that have optimized the extraction of PAHs from lichens using QuEChERS. It has been noted that water should be added to the extract, so that the extraction efficiency is improved as a result of the partitioning of polar compounds between the organic and aqueous phases (Albinet et al., 2013; Zhao et al., 2012).



In a study by Albinet et al (2013), the first research article to be published on QuEChERS as a sample preparation technique for particle-bound PAHs in emission samples (ambient air), PAHs were extracted from particles collected onto filters with dichloromethane, acetonitrile or acetonitrile: water (5:2, v/v). The extraction with acetonitrile was the solvent of choice due to instrumental compatibilities; the different solvent schemes showed negligible differences in extraction efficiencies. In a study on emission samples, simple filtration by the use of a polyethylene terephthalate (PET) syringe filter was considered the 'clean up' step (Albinet et al., 2013). The study on tea by Sadowska-Rociek et al (2014) tested various sorbent types for the clean up procedure including C<sub>18</sub>, PSA, strong anion exchange (SAX), -NH<sub>2</sub> and GCB. PSA is used to remove sugars, fatty acids and sterols, GCB is used to remove pigments (in particular, chlorophyll), C<sub>18</sub> is used to remove non polar compounds such as lipids, NH<sub>2</sub> is a weak anion exchanger and thus extracts acids and charged compounds, and finally SAX is a strong anion exchanger, typically used to extract organic acids (Agilent Technologies, 2013). It was found that SAX was the most appropriate sorbent for the tea samples, with the results indicating that the use of graphitized carbon black resulted in losses of PAHs, in particular the high molecular weight PAHs (Sadowska-Rociek et al., 2014). This could be as a result of intercalation between the graphite structure and the planar structure of PAHs. For this reason, GCB should be avoided in the clean up procedure, despite its success at removing unwanted pigments and sterols.

#### **2.6.4 Ultrasound assisted solvent extraction**

Ultrasound assisted solvent extraction (USAЕ) functions on the basis of cavitation; tiny bubbles formed and popped in liquids and the subsequent erosion of solid materials within the liquids (Szreniawa-Sztajnert et al., 2013). The application of ultrasound energy also creates an interface between solids and liquids by increasing the frictional contact between the extraction solvent and the solid from which analytes are to be extracted, and generally operate at frequencies above 16 kHz and below 500 kHz (de Castro & Capote, 2007). Ultrasound energy can also lead to the rupturing of cells and particles, freeing up analytes of interest trapped within the matrix. Cavitation, friction and the

absorption of ultrasonic energy all generate heat and such an increase in temperature is known to improve extraction efficiency (Babić et al., 1999), as long as the heat is maintained at a temperature below which PAHs are susceptible to degradation (below 35 °C (Blasco et al., 2006)).

The most commonly used ultrasound apparatus is an ultrasonic water bath. However, when using this apparatus it must be remembered that reproducibility and repeatability are negatively influenced by the insufficient uniformity of the distribution of ultrasound energy throughout the bath as well as a loss of power with time, as opposed to the newer techniques utilizing ultrasonic probes (Namieśnik & Szefer, 2008).

Ultrasound assisted solvent extraction techniques are commonly used due to the fact that smaller sample sizes can be accommodated, less solvent is used, and the extractions are relatively fast compared to traditional liquid extraction techniques (Domeño et al., 2006; Guidotti et al., 2003). Whereas Guidotti et al (2003; 2009) used cyclohexane as the solvent for ultrasound assisted extraction of PAHs from lichens (sample mass 2 g), combining only 2 extractions of 30 ml each, Domeño et al (2006) extracted a 0.2 g lichen sample using portions of 15 ml dichloromethane in each of four subsequent extractions. A study, in which a novel dynamic ultrasonic assisted extraction technique for lichens was developed by Domeño et al (2006), found that hexane is the solvent which extracts most PAHs efficiently and the new dynamic sonication-assisted solvent extraction (DSASE) technique has been popular in numerous studies since (Blasco et al., 2006; 2007; 2011). Ultrasonic extraction was also found to be superior over Soxhlet and pressurized liquid extraction (PLE) in a study by Ratola et al (2006) that focused on PAHs in pine needles. The method has grown in popularity because it is a fast method that uses less solvent and less energy, with satisfactory results.

Concern has been raised about the fact that high ultrasound frequencies may increase the degradation of PAHs. A study by Manariotis et al (2011) showed that phenanthrene, naphthalene and pyrene were degraded at frequencies above

582 kHz, particularly when the extractions were performed for long periods of time longer than 30 minutes. This should therefore be carefully considered when planning the experimental procedure, however the ultrasonic bath used in this study operated at frequencies lower than the 582 kHz thresholds.

Other parameters that need to be considered and optimized are the sonication time, amount of sample used, the particle size of the samples as well as the generation of heat and temperature control. In particular, the temperature needs to be monitored in order to ensure that no significant temperature changes occur between extractions, resulting in different extraction efficiencies as well as possible evaporation of volatile extraction solvents. An inbuilt thermometer was therefore valuable to monitor the temperature of the water and indicate when cooling was necessary, when using the ultrasonic bath used for this study.

#### **2.6.5 Thermal desorption of lichens**

The use of thermal desorbers in PAH studies serves as both an extraction method, where PAHs are released from the substrate as a result of thermal energy, and a sample introduction technique – where the desorbed analytes move directly into the GC system. Thermal desorption is a popular sample introduction technique when analyzing PAHs contained in particulate matter on filter papers, soils and dust samples using thermal desorption coupled to gas chromatography - mass spectrometry (TD-GC-MS) (Hays et al., 2003; Robbat Jr et al., 1992; Waterman et al., 2000). It is useful for the study of PAHs because thermal desorption volatilizes both volatile and semi-volatile organic compounds (VOCs and SVOCs) without any sample preparation or clean up. This subsequently reduces the time and the cost of analysis dramatically (van Drooge et al., 2009). A method for the analysis of PAHs in airborne particulate matter was developed by Waterman et al (2000) using TD-GC-MS. The study used a National Institute of Standards and Technology (NIST) Standard Reference Material SRM1649a-Urban Dust to quantify PAHs in dust, with the results showing good linearity as well as exceptional sensitivity (concentration of 2-6 mg.kg<sup>-1</sup>) for 10 of the EPA priority PAHs with standard deviations as low as

0.228 mg.kg<sup>-1</sup>. Indeed, in a study by van Drooge et al (2009), higher recoveries of some of the lighter PAHs were seen compared to traditional liquid extraction.

Thermal desorption requires a thermal desorber to be directly attached to the instrument (eg GC-MS), but the price of the apparatus can be offset with increased throughput due to the absence of sample preparation, the absence of solvents and sample clean up consumables.

The most common use of thermal desorption is in the analysis of filters, polyurethane foam (PUF) or polydimethylsiloxane (PDMS) rubber traps used to sample atmospheric and particle-bound PAHs (Albinet et al., 2007; Forbes & Rohwer, 2009; Hays et al., 2003; Robbat Jr et al., 1992), but it has also been used to determine PAHs directly in a variety of environmental matrices such as clay, water and sand (Potter & Pawliszyn, 1994). These studies used headspace solid-phase microextraction to analyze PAHs deposited in various environmental matrices (Potter & Pawliszyn, 1994; Zhang & Pawliszyn, 1993), followed by thermal desorption of the solid phase. The matrices mentioned above all have high combustion points, rendering thermal desorption a viable candidate for thermal desorption. Finding no literature on the use of thermal desorption techniques on lichens, the investigation into whether PAHs would be released from the symbiotic matrix upon the application of thermal energy could be a valuable step towards faster and greener sample preparation. This technique would, however, only be considered successful if all the PAHs, including the heaviest PAHs are desorbed at temperatures below the combustion point of lichens.

#### **2.6.6 Sample clean up methodology**

With regards to the clean up of sample extracts, either column chromatography or solid phase extraction is commonly used and these are summarized in Table 2.7. Silica column chromatography has been employed extensively to lichen extracts, with the use of different solvent schemes by different groups (Guidotti et al., 2003; Schrlau, 2011; Shukla & Upreti, 2009). A direct comparison between the results of these studies is a challenge as a result of the different affinities solvents have for the different PAHs (Domeño et al., 2006), leading to biased

elutions. The mass of sorbent is not clearly defined in most studies, which also influences the extent to which the analytes are recovered.

More recently, solid phase extraction has been used to clean up the lichen extracts with mainly normal phase  $-NH_2$  columns being used after a study by Blasco et al (2007) found that these columns, combined with an elution solvent of hexane:dichloromethane (65:35) yielded the best recoveries. SPE remains a favourable choice in comparison to traditional column chromatography since it uses less solvents, thus decreasing the cost of analysis, as well as a reduction in harmful solvent volumes that need to be blown off after clean up. The smaller volumes also reduce the likelihood of loss of SVOCs since the blown down and pre-concentration steps are shortened.

The method developed by Blasco et al has not been reviewed since 2007, despite the production of many new sorbents, some of which are PAH specific (Phenomenex Incorporated, 2010). Due to the importance of sample preparation in delivering accurate and reliable analytical results, the extraction methodology as well as sample clean up techniques employed should be continually revised and optimized as new techniques become available, to enhance recoveries of analytes and reproducibility of results. The revision of the currently accepted clean up procedure is, however, beyond the scope of this study.

**Table 2.7: The different clean up procedures for lichen extracts prepared for PAH studies, using various sample extraction techniques found in literature**

Type of clean up	Sorbent Type	Elution solvent	References
Column chromatography	Silica	25 ml methylene chloride:pentane (2:3, v/v)	(Guidotti et al., 2003; Shukla & Upreti, 2009; 2012; 2013)
Column chromatography	Silica	50 ml dichloromethane	(Schrlau, 2011)
Column chromatography	Florisil	30 ml acetonitrile	(Augusto et al., 2009; 2010)
Solid phase extraction	Normal phase – $NH_2$	2 ml hexane:dichloromethane (65:35, v/v)	(Blasco et al., 2007; 2008; 2011; Nascimbene et al., 2014)

## 2.7 Chromatographic techniques for the analysis of lichen extracts

The EPA PAH-related methods all recommend the use of either gas or liquid chromatography (GC and LC) in the analysis of sample extracts including drinking water, sludge, municipal waste, ambient air and foodstuffs (Poster et al., 2006; US Environmental Protection Agency, 1980). Other agencies such as the American Public Health Association (APHA) and the Association of Official Analytical Chemists also incorporate GC and LC techniques into their standard methods for PAH analysis (US Environmental Protection Agency, 1993).

When analyzing lichen extracts for PCDD/Fs and PAHs, the main analytical techniques used are HPLC using reverse phase C18 columns and gas chromatography, using a variety of column configurations with column lengths ranging between 15 – 60 m. GC-MS and fluorescence-based liquid chromatography both have their advantages. If a particular PAH is of concern, such as BaP or Ant, the sensitivity and selectivity of using liquid chromatography with fluorescence detection will be far greater at trace levels, due to the high fluorescence characteristics of these PAHs (Poster et al., 2006). On the other hand, BghiP has poor sensitivity to fluorescence and is therefore better suited to analysis using GC-MS if it is of primary concern in a study (Poster et al., 2006). In general when deciding which method should be applied to a sample, a few judging criteria guide the decision, namely:

- The type of matrix. If the matrix is complex and rich in interferences, GC-MS would be a more appropriate technique due to the enhanced separation achievable using GC techniques, where hundreds of compounds can be separated as opposed to liquid chromatography where the limited peak capacity restricts separation to a few dozen compounds (Fetzer, 1989). In the case of lichens, depending on how efficient the clean up procedure is, it is likely that the resolving power of GC-MS would be advantageous over liquid chromatography techniques.
- The required sensitivity. The expected concentration range should fall within the detection limits of the instrument. Both GC-MS and HPLC coupled to a fluorescence detector are known to be very sensitive,

whereas other LC detectors such as the diode-array detector may not have the desired sensitivity for the analysis.

- Analytes of interest. If a range of PAHs is to be investigated, as opposed to a pair of isobaric compounds, then a more comprehensive technique such as GC-MS should be employed, whereas if individual isomers are of concern, a more selective technique such as HPLC coupled to a fluorescence detector should be considered (Poster et al., 2006).

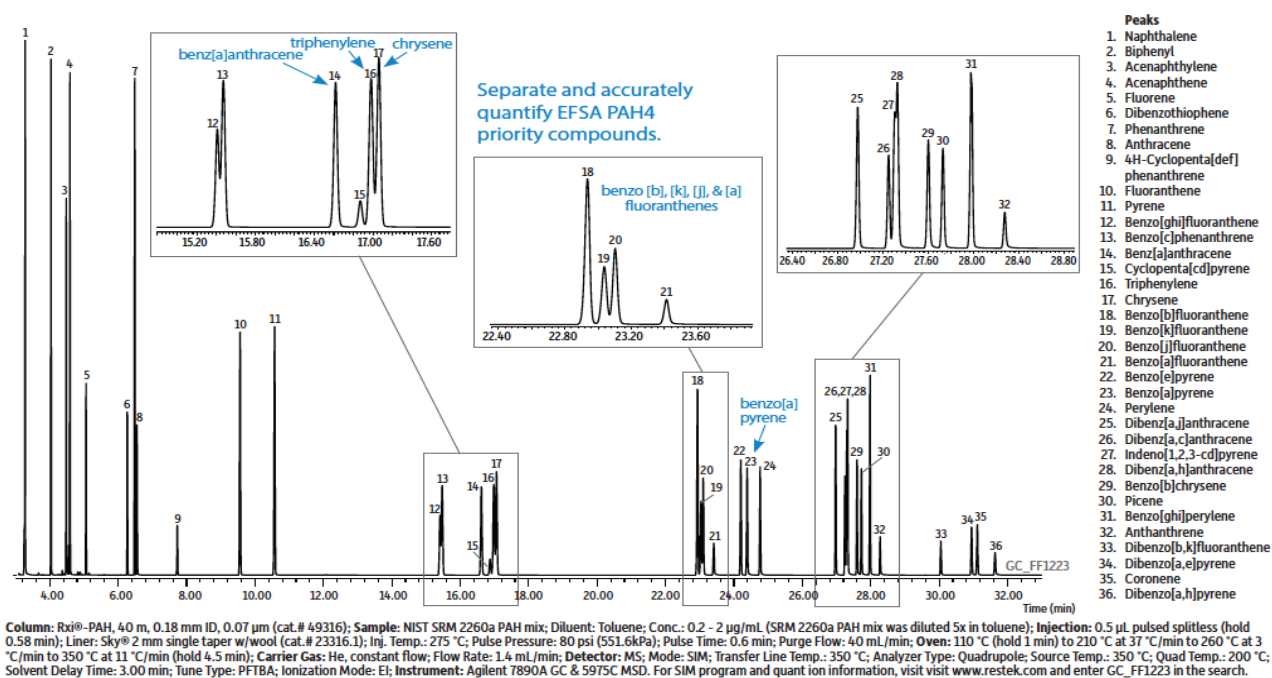
The detection of PAHs is typically accomplished using electron impact (EI) mass spectrometry, yielding few fragments with molecular ion peaks of great intensity (Poster et al., 2006).

Augusto et al (2010) made use of a HPLC coupled to an ultraviolet fluorescence detector (FLD) as well as an visible-ultraviolet detector (DAD/V-UV), which had the advantage of sensitivity as a result of the fluorescence detector with detection limits of 58 ng.g<sup>-1</sup> reported for the sum of all 16 EPA Priority PAHs. Selectivity, as a result of using the DAD/V-UV detector, operating at 254 nm ensured well-resolved peaks, depending on chromatographic optimization. Likewise, Shukla and Upreti (2009; 2012; 2013) also used HPLC coupled to a UV detector with reported detection limits as low as 8 ng.g<sup>-1</sup>. The fluorescence detector yields exceptional sensitivity in PAH analysis due to the fluorescent character of PAHs (Fox & Staley, 1976) with some studies on PAHs in pine needles using HPLC coupled to a fluorescence detector providing detection limits as low as 0.1 ng.g<sup>-1</sup> dried weight (Tremolada et al., 1996).

When conducting PAH studies, Migaszewski et al (2002), Domeño et al (2006) as well as Blasco et al (2011) have used a GC-MS for analysis, reporting detection limits as low as 21 ng.g<sup>-1</sup>. The use of Rxi®-5Sil MS, HP 5-MS and factor four VF5-MS columns are common due to the non-polar nature of these capillary columns resulting in good separation of PAHs, but run times ranging between 29 min (Blasco et al., 2011) and 81 min (Nascimbene et al., 2014) are reported. The systems are almost always operated in single-ion-monitoring mode (SIM mode) to improve the sensitivity and resolution of the peaks of interest, simplifying the

data processing method (Blasco et al., 2007; Nascimbene et al., 2014; Ratola et al., 2009).

Sacrificing peak separation, particularly of the benzo fluoranthenes, as well as BaA and Chr for the sake of faster run times should be avoided to ensure an accurate representation of the PAH profile, owing to the different toxicities of PAH compounds. Recently, the Restek Corporation (Bellefonte, USA, 2012) has developed the Rxi®-PAH column, specifically for the separation of isobaric PAHs for environmental and food safety applications. The column was developed in response to the notoriously difficult separation and identification of isobaric PAHs. The column was created with a higher phenyl-content stationary phase in order to separate PAHs that cannot be distinguished by mass spectrometry, beyond the capabilities of typical GC columns. The arylene modification in the Rxi®-PAH column improves the surface bonding and thus thermal stability is improved so that the heavier PAHs can move off the column without the interference of column bleed (Restek Corporation, 2012). A chromatogram from the application note using the Rxi®-PAH column on a PAH standard reference material (SRM2260a) showing the separation of some analytes of interest is shown in Figure 2.2.



**Figure 2.2: Chromatogram obtained using the Rxi®-PAH column, for the analysis of a PAH standard mix in toluene, showing separation of isobaric pairs (Restek Corporation, 2012)**



GC x GC-TOFMS has been a popular analytical technique for environmental samples over the past few years as a result of the large resolving power, increased sensitivity and selectivity of the method (Dallüge et al., 2003; Lane et al., 2008). For reviews on the use of comprehensive two dimensional gas chromatography-mass spectrometry for environmental sample analysis, the reader is referred to the papers by Pani & Górecki (2006) and Vreuls et al (1999). The time-of-flight MS detector (TOFMS) provides accurate mass measurements with full scan data at very fast scan rates, resulting in enhanced peak capacity, as well as improved sensitivity as a result of reduced noise levels (Dallüge et al., 2003; Schnelle-Kreis et al., 2005).

GC x GC – TOFMS has been shown to effectively separate hundreds of PAHs and derivatives of PAHs in a study by Hilton et al on a NIST standard reference material for household dust. This illustrates the capabilities of comprehensive two dimensional gas chromatography with regards to the analysis of PAHs and related compounds. Considering the possibilities of interferences and co-eluting compounds in lichen extracts, the separating power of GC x GC-TOFMS might be required to accurately quantify all PAHs of interest (see Section 2.3.3) if the resolving power of the Rxi©-PAH column proves to be insufficient.

## **2.8 Chlorophyll in lichens: potential matrix effects**

In the lichen extract clean up optimization study by Blasco et al (2007), lichens of the type *Parmelia sulcata* were extracted and analyzed. These differ from the *Parmotrema austrosinense* (Zahlbr) Hale species, which is to be used in our study, as it has been successfully used in other South African lichen biomonitoring studies (Forbes et al., 2009; True et al., 2012). This difference is expected to add to the level of uncertainty due to inter-species differences. It was Blasco et al, in 2011, who demonstrated that *P. sulcata* was the best monitor for PAHs of pyrogenic origin, and also described *E. Prunastri* as the most valuable in studying pyrogenic and petrogenic PAHs. This preferential uptake of PAHs highlights the nuances between lichens of different species, and how small biological differences can influence analytical results.

As discussed in Section 2.6.6, the chosen clean up procedure in this study is based upon the work by Blasco et al (2007). The use of hexane as an elution solvent in their study is recorded to ensure 'clear' extracts after the SPE clean up, but makes no mention of the visual colour intensity when the elution solvent scheme was optimized to be hexane: dichloromethane (65:35, v/v), which brings about an uncertainty in terms of whether the extracts should be colourless upon completion of the clean up procedure.

This is a cause for concern; since the presence of interfering substances can greatly influence the quantified results in a phenomenon commonly known as the matrix effect. A major matrix interferent in the analysis of PAHs in plants is chlorophyll; a green pigment held in the chloroplasts of cells, used in the photosynthetic pathways. The species of lichen used in a study could then very well play a significant role in how much chlorophyll is present in the extract, directly influencing the extent to which the clean up procedure would effectively remove any interferences present.

The presence of various acidic organic lichen substances on the surface of different lichen species plays a significant role in chlorophyll degradation in lichens (Brown & Hooker, 1977) and will therefore also impact on the total quantified chlorophyll content across species and thus the removal of chlorophyll from sample extracts will be more, or less, effective. The fact that chlorophyll *a* and chlorophyll *b* initially degrade within the lichen thallus in the presence of pollutants, and then increase in concentration after longer exposure (Canas et al., 1997) means that a direct comparison between species should be made with caution since the degradation will be more or less severe, depending on the age of the lichens, the levels of pollutants in the environment from which the specific samples were taken, as well as the mesoclimate of the sampling area. Chlorophyll content is not affected by both moderate temperatures and extreme cold, as shown by Brown and Hooker (1977), who studied the chlorophyll degradation in lichens naturally frozen in Antarctica, so post-sampling freezing of collected lichen samples should not have an impact on their chlorophyll content.

Another factor that further complicates the direct interpretation of differing chlorophyll content between lichen species if visually examining colour intensity, is the bleaching effect that the lichen thallus undergoes as a result of exposure to pollutants and the subsequent degradation of chlorophyll (Eversman, 1978; Puckett et al., 1973; Showman, 1975). Thus, older lichens which have been exposed to more pollutants over time when compared to younger lichens of the same species, would contain less chlorophyll. There is a common lack of reporting the age of lichens when sampling and this means that directly comparing chlorophyll contents of different lichen species becomes complicated as a result of possible and undefined degradation effects occurring with time, as well as increases in chlorophyll concentration after exposure to pollutants (Canas et al., 1997).

Another factor, which influences whether chlorophyll content between species can be estimated by visual inspection, is the extent of hydration. Having sampled *Parmotrema austrosinense* on a few occasions, it has been noted that they appear bright green in humid and wet weather conditions, whereas they show a duller grey colour in dry, hot conditions. It is therefore misleading to visually compare lichens and make conclusions regarding the chlorophyll content based on images.

Canas et al (1997) also studied the lichen of interest in this study, *Parmotrema austrosinense*, and found that the total chlorophyll content was very similar to *P. conferendum*, following similar patterns of reduction in chlorophyll concentration and a subsequent increase after exposure to pollution. The study did, however, highlight the fact that the total chlorophyll concentration was lower in polluted areas when compared to the non-polluted samples. A study by Carreras et al (1998) where the lichen species *Usnea sp.* was studied suggested that an increase in the total chlorophyll content runs parallel to an increase in pollutant level. This was also suggested by von Arb et al (1990), who saw an increase in chlorophyll content in lichen samples in polluted areas. This is problematic since the study did not comprehensively study the same lichen species using a clean, non-polluted study site as a control as was used in the

study by Canas et al (1997). Studying lichen samples from both non-polluted and heavily polluted areas would therefore be expected to reflect different total chlorophyll concentrations and thus the clean up procedure for sample extracts will be more, or less effective at removing all the unwanted chlorophyll. If we were to observe a matrix effect in this study, it would be affected by the differences in total chlorophyll content and thus the efficiency of the clean up procedure.

A study by Piccotto et al (2011) measured the difference in response of different lichen species to various changes such as mesoclimate and gaseous pollutant concentrations, and measured the chlorophyll fluorescence before and after exposure in these differing environments. The total chlorophyll content prior to exposure was higher in the *Parmotrema perlatum*, when compared to *Xanthoria parietina* (L.) Th. Fr. and *Flavoparmelia caperata* (L.) Hale. The focus of our study centers on the use of *P. austrosinense* (Zahlbr) Hale as the biomonitoring species, which is in the same family as *P. perlatum* which could suggest that the family has higher levels of chlorophyll when growing in unpolluted sites, but this requires verification.

Beltman et al (1980) quantified the chlorophyll content of a large number of different lichen species and found that *Parmelia sulcata* (used by Blasco et al, 2007) had low chlorophyll content when compared to other species. Unfortunately, *P. austrosinense* was not included in the study, but *X. parietina* was, and was found to have large variation in chlorophyll concentration across samples of the same species, leading to large uncertainties which was also a matter of concern in a study by Beekley and Hoffman (1981). This might be a common problem when dealing with the extent of matrix effects in sample extracts from lichens, since it has been observed by Beekley et al (1981) as well as Beltman et al (1980) that there were variations in chlorophyll content even within samples of the same species.

A study by Tretiach and Carpanelli (1992) suggested that when considering lichens and their assimilation of pollutants, it might be more meaningful to

represent the assimilation rates based on the chlorophyll content rather than on the principle of dry weight. They also recommended that the concentration of chlorophyll in samples should always be reported.

It is therefore evident that the chlorophyll content of lichens is not a simple experimental variable that can be easily controlled or accounted for. This should be borne in mind when sample preparation methods are devised for lichen matrices.

## **2.9 Concluding remarks**

This chapter started off with a brief review of the historic use of lichens as biomonitors, particularly for organic air pollutants. The importance of studying PAHs was then highlighted and the specific PAHs to be targeted in this study were identified. The information presented in this chapter was used to inform the choice of extraction techniques in this study with particular reference to the solvents used, extraction times applied and extraction conditions for Soxhlet, USAE, MAE, QuEChERS and thermal desorption. Specialized techniques such as SBME, DSASE and automated Soxhlet techniques were not included as a result of the necessary equipment being unavailable for this study. Different sample extract clean up procedures were discussed which led to the decision to clean up all samples with a SPE-based clean up step using  $-NH_2$  cartridges. Various chromatographic techniques were discussed, including HPLC and GC-MS. Lastly, the importance of performing a chlorophyll study on lichens was mentioned.

Chapter 3 details the sampling procedures, sampling points of interest as well as the storage conditions. The sample preparation techniques will then also be discussed, including the solvents used, number of consecutive extractions and the clean up procedure. The methods of analysis, as well as quality assurance will be detailed, followed by the method used to investigate the chlorophyll content of the different sample extracts.

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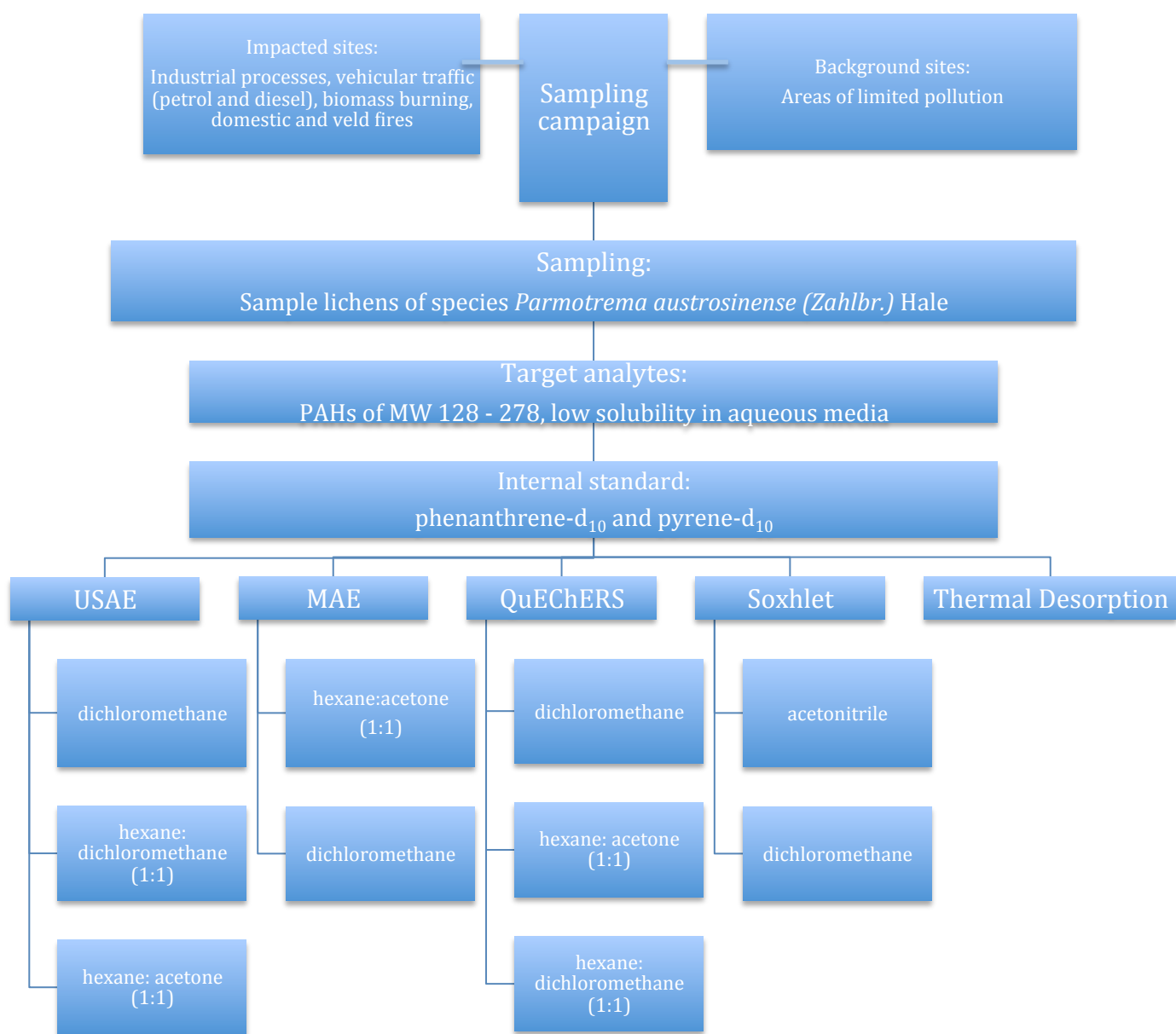
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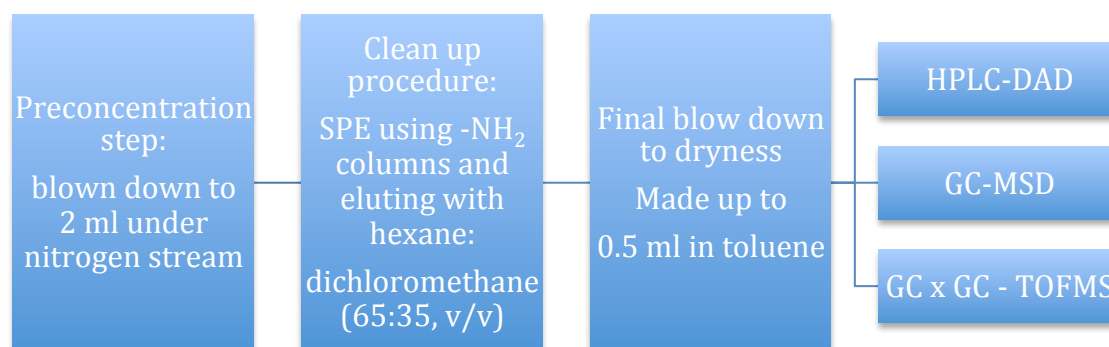
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## Chapter 3: Experimental Methods

The general experimental approach employed in this work using lichens as biomonitors for atmospheric PAHs is shown in Figures 3.1 - 3.2. Figure 3.1 details the sampling process, including the identification of polluted (or non polluted) areas, lichen species choice, then the investigation into 4 sample preparation techniques using various solvent schemes. Figure 3.2 then shows the clean up procedure followed for all extracts prepared according to Figure 3.1, excluding the lichens used in the thermal desorption study.



**Figure 3.1: The sampling and sample extraction experimental design for the use of lichens as biomonitors for atmospheric PAHs employed in this study**



**Figure 3.2: The clean up and analysis experimental design for lichen extracts utilized in this study**

### 3.1 Quality assurance and sample handling

All samples were collected wearing nitrile gloves (Lasec Laboratory Solution Provider, Cape Town) using clean stainless steel tweezers, rinsed with methanol between samples. Amber collection vials were rinsed with dichloromethane and baked overnight in an oven at 150 °C prior to use. The electronic balances used had been calibrated just prior to the study. All solvents used were purchased from Merck (Gauteng, South Africa) and were of analytical grade, except for the acetonitrile, which was from the LiChrosolv range from Merck (Darmstadt, Germany) and was of gradient grade for liquid chromatography applications. All water was purified using a Millipore system (MA, USA) with resistivity ranging between 18.2 and 18.4 Ω. Standards were obtained from Sigma Aldrich (St Louis, MO, USA), and transferred using clean Hamilton syringes and solvents were quantitatively transferred using an Eppendorf Research® Plus pipette and sterilized Eppendorf tips. High purity nitrogen gas for the blown down of extracts was obtained from Afrox (Gauteng, South Africa). The statistical programs StatPlus:mac v5 (AnalystSoft, 2013, StatPlus:mac - statistical analysis program for Mac OS. Version 5. See <http://www.analystsoft.com/en/>) and JMP 10 software were used for statistical analysis.

### 3.2 Sampling strategy

#### 3.2.1 Bulk Sample

##### 3.2.1.1 Sampling site selection

The lichen samples to be used for method development needed to be abundant enough to supply a bulk sample in excess of 100 g. Such a large bulk sample is necessary in order to minimize the variation caused by sampling lichens of different ages, exposed to varying amounts of PAHs and slight differences in chlorophyll content which occurs even amongst lichens of the same subspecies (Beekley & Hoffman, 1981; Beltman et al., 1980). A large sample volume would also improve homogeneity and would ensure that the bulk sample is representative of the PAH content of lichens in the area (Romanik et al., 2007).

Prior to sampling, the lichen species was specifically identified as *Parmotrema austrosinense* (Zahlbr) Hale by comparing the identity of the sample of interest with an image known to be of the *P. austrosinense* species from previous studies in our group (Forbes et al., 2009; True et al., 2012). It is important to sample lichens from only one species in order to minimize variations between samples, since a study by Blasco et al (2011) showed that different lichen species preferentially accumulate different PAHs resulting in an increase in uncertainty as well as a negative impact on the precision of the results if different species were used within the same study.

To further reduce any other variables that could impact on the homogeneity of the sample, it was decided that lichens for the bulk sample would only be sampled from one particular substrate specifically Jacaranda trees (*Jacaranda mimosifolia*), to eliminate any influence the surface may have on the health, growth, nutritional cycle and assimilation of compounds from the air (Augusto et al., 2013), since these mechanisms are still poorly understood but have been shown to play a role in lichen biodiversity (Loppi & Frati, 2004).

It was also decided that lichens for the bulk sample would be sampled during one sampling campaign on one day, so that the humidity, temperature, seasonal and mesoclimate effects described by Piccotto et al (2011) were eliminated. It was determined that lichens for the bulk sample, as well as samples of interest (as far as possible) would be sampled during the winter months according to findings by Augusto et al (2013) that suggest less wash off and revolatilization of

PAHs from the lichen substrate when sampled in the cooler, drier months, coupled to the fact that the concentrations of atmospheric PAHs are expected to peak during the dry, cooler months due to higher evaporation and degradation rates of PAHs in the summer months due to high temperatures and increased sunlight intensity, as well as increased household and veld fires during the winter months.

A location for the collection of the bulk lichen sample needed to be identified. The prerequisites for the bulk sampling site are listed below in Table 3.1, as well as the manner in which our chosen location satisfied these conditions.

**Table 3.1: Requirements of a bulk sampling site and how these conditions were met at the chosen bulk sampling site in Justice Mahomed Street, Pretoria, South Africa**

<b>Prerequisite</b>	<b>Motivation</b>	<b>How it was met at sampling site</b>
<b>Lichens of identical species</b>	Eliminate possibilities of interspecies variations in assimilation of PAHs	All lichens were examined visually and compared to previous studies – all were confirmed to be <i>Parmotrema austrosinense</i>
<b>All trees of same species</b>	Eliminate variability of possible lichen-bark interactions	All trees identified were of the <i>Jacaranda mimosifolia</i> species
<b>High number of trees in sampling area</b>	Smaller sampling area will decrease variability in atmospheric PAH profile and concentrations	Area had high density of Jacaranda trees, on one side of the road for consistency
<b>Area must have significant exposure to PAHs</b>	Sampling in an area that definitely is contaminated is necessary to draw meaningful conclusions from the data regarding extraction efficiencies	Sampling site was on a busy road in a neighbourhood in Pretoria, Gauteng. Continual traffic ensured PAHs were present in the atmosphere
<b>Sizes of lichens on the trees should vary</b>	A broad range of sizes corresponds to different ages of lichens, providing an integrated picture of the atmospheric PAH profile over a number of years	Lichens sampled ranged in size from 1 cm to 10 cm diameter corresponding to young and old lichens being sampled, respectively

After evaluating a number of potential sampling sites, it was decided that a section of Justice Mahomed Street between Stella Street and Brooklyn Road in Pretoria, as indicated by the map in Figure 3.3, was the ideal place to sample

sufficient lichens for the bulk sample. Each tree had multiple lichens growing on its trunk, all located on the southern side of the tree. The choice for location was based on the high density of lichen bearing Jacaranda trees in the area, as well as the fact that Justice Mahomed Street is nearby the University of Pretoria, meaning that subsequent sampling campaigns could be easily undertaken again if necessary. The road has medium traffic density for most hours of the day; thus the lichens are expected to contain PAHs at suitable levels for method development. Despite the suggestion by Zygmunt and Namiesnik (2003) to sample on the windward side, where the pollutants would be incident on the substrate, lichens were exclusively identified, and thus sampled, on the southern side of the tree trunks, that is facing towards the road, despite the wind being incident on the northern side of the trunk on our sampling day, as seen on the sampling sheet (Appendix B).



**Figure 3.3: Location of trees from which the bulk lichen sample was collected on Justice Mahomed Street, Pretoria**

### 3.2.1.2 Lichen sampling and cleaning technique

Lichens were removed from the trunk and low branches of the trees at heights above 1 – 1.7 m to avoid any bias from adsorption of PAHs volatilized from contaminated soil, as suggested in other lichen studies (Augusto et al., 2007; Blasco et al., 2006; Trapp & Matthies, 1997). Lichens were carefully pried from the bark with sterilized stainless steel tweezers (rinsed in methanol) and placed directly into one large amber glass bottle and sealed tightly (Szulejko et al., 2014). Samples were taken midmorning in the dry, winter season (June),

following the studies by Augusto et al (2012; 2013) that reported that PAH concentrations were higher in lichens that had been sampled in the dry, cooler months. The amber glass bottle was then transported to the laboratory immediately and placed inside a resealable plastic bag, and stored in the fridge at 4 °C for 1 day. The detailed sampling sheet can be found in Appendix B.

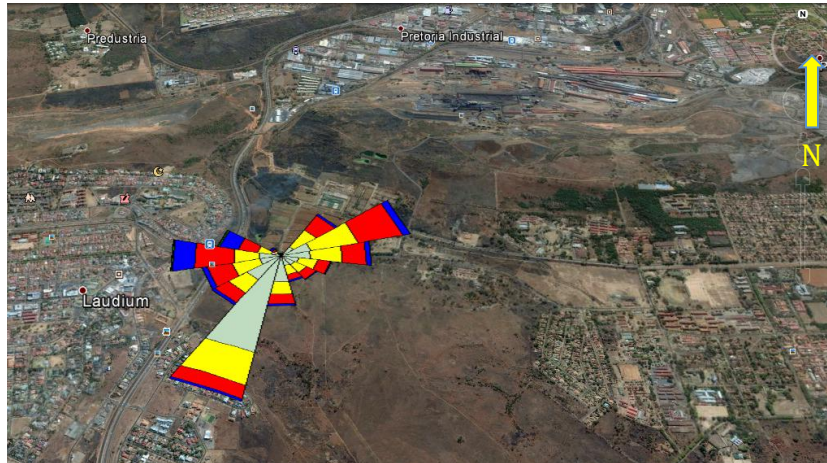
In the laboratory, the bulk sample was inspected for pieces of bark and small stones. The exogenous matter was removed using a sterile tweezers. It has previously been shown that effective drying can be performed at temperatures as low as 40 °C (Market, 1995), therefore the lichen samples were placed in an oven (1.60 kW, Binder, Germany) at 35 °C for 4-5 days, until the mass remained constant and it was certain that all the moisture had been removed.

One small piece of lichen, of dimensions 10 mm x 10 mm, was set aside in a separate amber glass vial and placed in the freezer at -18°C for analysis by Scanning Electron Microscopy (SEM), with full results given in Appendix C. The remaining lichens were ground to a powder using a marble mortar and pestle. The ground lichens were then transferred to a sterile glass bottle, wrapped in aluminium foil, placed in a resealable plastic bag and stored in a freezer at -18°C until required.

### **3.2.2 Pretoria Industrial Area**

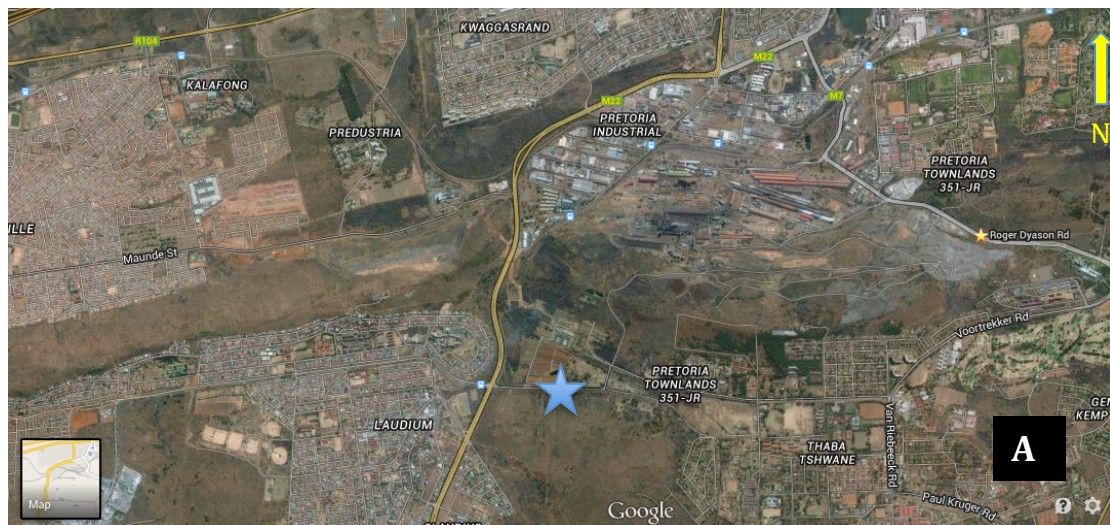
One of the identified sites of interest was the Pretoria Industrial area. The presence of numerous factories such as a nearby zinc refinery, as well as urban areas located near to the industrial areas, suggests that lichens in the area are likely to contain significant amounts of PAHs, since Augusto et al (2009) found that areas with a combination of urban and industrial emissions are the largest contributors towards atmospheric PAHs. Once the region had been scouted, prevailing wind direction information for the area obtained from SAAQIS (South African Air Quality Information System) was used to determine where lichens should be sampled in the area, as shown in Figure 3.4. This was done by taking the hourly wind speed and wind direction readings from the Pretoria West monitoring station collected over a year, and converting the data into a windrose

using WindPlot software. In order for the PAHs released by the industrial works to be reflected in the results, it was decided that lichens would be sampled preferably north east thereof, as shown by indication of blue stars in Figure 3.5 (Google Maps Pretoria Industrial, 2014).



**Figure 3.4: Prevailing wind direction in Pretoria West, superimposed on our sampling site in the Pretoria Industrial area**

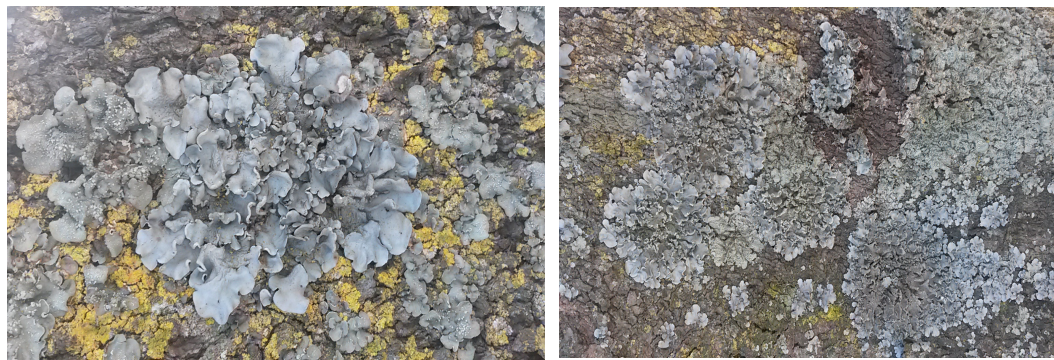
Lichens were only found on one tree on the Pierre van Ryneveld Road in the target areas and are shown in Figure 3.6. The tree was not *Jacaranda mimosifolia* but *Ocotea Bullata* (with the common name Black stinkhout tree), since no trees of the Jacaranda species were growing in the sampling area. Full details from the sampling campaign can be found in Appendix B. The samples were removed at the end of winter (August) before the first rains at a height of 1.5 m, and cleaned using the same protocol as outlined in Section 3.2.1.2.







**Figure 3.5: Sampling point in the Pretoria Industrial area from afar (A) and zoomed in (B)**



**Figure 3.6: Lichens identified prior to removal on trees in Pierre van Ryneveld Road in the Pretoria Industrial area**

### 3.2.3 Cathedral Peak region in the Drakensberg

It was decided that samples that come from areas of low levels of pollution would provide meaningful results that could be used as typical background levels of atmospheric PAHs in South Africa. For this reason, lichens were collected during the winter months (July) in the Cathedral Peak region of the Drakensberg. The samples were collected off *Leucosidea sericea* (Oldwood) on the south and west of the trunk, roughly 1 m from the ground, adjacent to a paved, rural road. Obtaining wind data for the specific sample area is deemed unnecessary since the mountainous nature of the area creates localized variations in the wind direction. Thus, no wind direction considerations were taken into account whilst sampling on the windless day, since the only expected sources of PAHs in the area are from a low density of trucks and light motor vehicle traffic utilizing the road. Samples were stored in amber glass vials and

kept cool until reaching the laboratory. The same sample cleaning protocol was implemented as in Section 3.2.1.2.

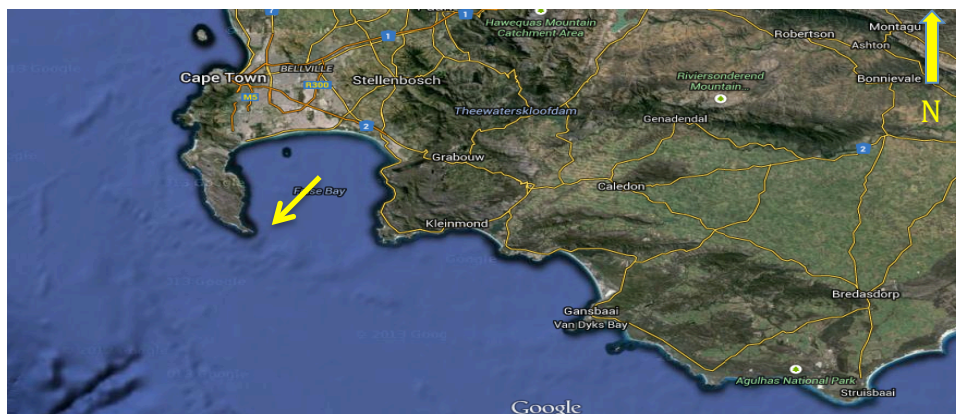
### 3.2.4 Cape Point Nature Reserve

The Cape Point Nature Reserve is situated 60 km southwest of Cape Town and forms part of the Table Mountain National Park. The winds predominantly blow from the southeast during the summer months and northerly winds predominate during the winter months (Scheel et al., 1990). Maritime air masses blown in by the southerly winds represent background air quality, thus we expect the Cape Point Nature Reserve lichen samples to have accumulated low levels of PAHs. However, the *fynbos* northwest of the identified sampling site had been burned in April 2014 (Brunke, 2014, personal communication), which may have impacted on PAH levels in lichens considering that the prevailing winds from April to August (when the lichens were sampled) tend to be northerly (Scheel et al., 1990). Located in the Cape Point Nature reserve is the GAW (Global Atmospheric Watch) station (coordinates: 34°21' S, 18°29' E), built on the sea cliff, below a sampling tower being exposed mostly to maritime air moving over the southern Atlantic Ocean (Brunke et al., 1990). The site is protected from any future development and, to a large extent, industrial pollution since 75 % of the wind blows over the ocean in summer, and 55 % of the winds in winter are recorded as having moved over the ocean, with a general wind direction of south or south easterly winds (Brunke & Halliday, 1983).

Permission was granted by SAN Parks to sample lichens at any point south of the parking lot. Sampling was undertaken on the 16<sup>th</sup> August 2014, which fell at the turn of the wet season (winter) to the dry season (summer). This contradicts the sampling protocol laid out in Section 3.2.1.1 that stipulates the sampling of lichens in the dry season at sampling heights higher than 1 m off the ground. However, the Cape region experiences a wet winter, so a choice had to be made regarding either sampling in warm dry weather, which would likely to show lower PAHs levels than samples taken in winter due to revolatilization and photodegradation (Augusto et al., 2013) or sampling in the wet winter. Since PAHs are hydrophobic, effects of washing off by rain and fog is less likely to

influence the results than revolatilisation (Augusto et al., 2013), so it was decided that sampling would be conducted in August.

The three sampling points were chosen based on varying location, as well as availability of our lichen species of choice. The Cape Point Nature Reserve has rich flora diversity and many different lichen species were seen growing on shrubs and rocks, many of which were growing on top of one another, making the sampling of lichens of only *Parmotrema austrosinense* difficult. The three sampling locations are indicated on the map below in Figure 3.7 to Figure 3.9, courtesy of Google (Google Maps Cape Point, 2014). During the sampling campaign, it was observed that the plant diversity of the Cape Point is limited mainly to shrubs and small bushes, so samples had to be removed very close to the ground contrary to common practice. The samples were removed and stored in glass vials wrapped in foil, carefully placed in a cooler box packed with ice packs until reaching the laboratory, where the same clean up and storage protocol was followed as in Section 3.2.1.2. The sampled lichens from sampling points 1 – 3 are shown in Figures 3.10 to Figure 3.12, and a short table highlighting the sampling conditions is given in Table 3.2.



**Figure 3.7: Location of the Cape Point Nature Reserve in the Western Cape, South Africa, indicated by the yellow arrow**



**Figure 3.8: A closer look at Cape Point with the burn area as well as the sampling area indicated**



**Figure 3.9: The three sampling sites at Cape Point, indicated by their numbers on the map, relative to the parking lot**



**Figure 3.10: The lichen removed at point 1, from the branch of a protea bush, sampled 15 cm above the ground**



**Figure 3.11: The lichen removed at point 2, from the branch of a protea bush, sampled 55 cm above the ground**



**Figure 3.12: The lichen removed at point 3, from an old branch of an unidentified shrub, sampled 45 cm above the ground**

**Table 3.2: Sampling conditions for the three samples taken at the Cape Point Nature Reserve**

Point	Altitude (m)	Height (cm)	Substrate	Details
1	218	15	Protea branch	Next to the GAW station entrance S: 34° 21.204 E: 18° 23.375
2	211	55	Protea branch	Below the GAW station, toward the eastern side S: 34° 21.195 E: 18° 29.401
3	177	45	Unidentified	On the point, lower down on the east face of the cliff S: 34° 21.301 E: 18° 29.598

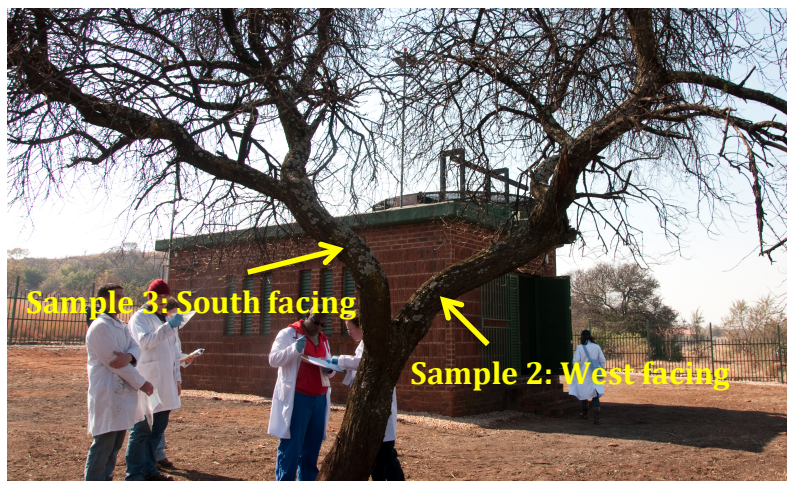
### 3.2.5 Daspoort Tunnel in Pretoria

The Daspoort Tunnel (also known, historically, as the Ollie Denyschen Tunnel) was built in the 1970s in the North Western region of Pretoria. The initial design intent was for the tunnel to service a traffic load of up to 6000 vehicles a day, but a recent study by Forbes et al (2011) showed that up to 2420 vehicles were passing through the tunnel an hour, suggesting that the daily expected limit would be far exceeded every day, which would impact on the efficacy of the original extraction fan which is situated midway in the tunnel. The extraction fan above the tunnel is shown in Figure 3.13.

A few studies have been undertaken in the Daspoort Tunnel to monitor the particulate matter and air quality in the tunnel (Mingay & Stadler, 1988; Wright et al., 2011). Since there are two pedestrian lanes that are used by school children and workers alike, the air quality in the tunnel is of interest because of the health risks associated with exposure to PAHs (Clapp et al., 2008; Osgood et al., 2013; Ravindra et al., 2001).

Similar to a study by Augusto et al (2012), the use of lichens to assess human exposure to PAHs in the surrounds of the Daspoort Tunnel extraction exhaust

may provide an interesting perspective on the effect of vehicle emissions on the pedestrians and other commuters, as well as people living in the vicinity of the extraction exhaust. It was therefore decided that lichens would be sampled above the tunnel, adjacent to the extractor fan within the access-controlled extraction fan area. A dead acacia tree with *Parmotrema austrosinense* lichens on all sides was identified and lichens were sampled from both the south facing side of the trunk as well as the west facing side of the trunk as shown in Table 3.3. Considering the fact that the extractor fan is located close to the tree, the possibility exists that the emission plume of the extracted air would loft over the tree, which would result in lower exposure of the lichens to PAHs. Samples were collected on a dry winter's day (June) and stored in the refrigerator until cleaned, dried and ground for extraction.



**Figure 3.13: The extraction fan above the Daspoort Tunnel in Pretoria West, where lichen samples were removed from a dead acacia tree as indicated by the arrows**

**Table 3.3: Two samples collected from a tree adjacent to the Daspoort Tunnel extraction exhaust**

Identity	Sampling height	Substrate	Details
2	155 cm	Dead acacia tree	West facing on trunk
3	195 cm	Dead acacia tree	South facing on trunk

### 3.3 Standards and calibration

#### 3.3.1 PAH standards

A PAH standard mix of nominal concentration 2000  $\mu\text{g}\cdot\text{ml}^{-1}$  for each PAH in methylene chloride was obtained from Supelco (St Louis, MO) containing 15 PAHs of interest, as shown in Table 2.3. The certificate of analysis is given in Appendix D, showing the analytical concentrations as well as the concentrations by mass of the PAH standard mix. The standards were made up from a stock standard solution in toluene of concentration 200  $\mu\text{g}\cdot\text{ml}^{-1}$ . The standards were not made by sequential dilution but rather from the stock solution in order to prevent the carrying forward of an error. Two different analytical grade solvents were used for the different instrumental analyses employed and the concentrations of the standards are shown in Table 3.4. The standards were made up directly into amber glass vials with PTFE lined screw top lids using a high precision syringe and an autopipette, vortexed for 1 min each and stored in the refrigerator at 4 °C when not in use.

**Table 3.4: Solvent choice and concentrations of PAH mix standards prepared for HPLC and GC analyses**

Instrumental analysis	Solvent	Concentrations
HPLC-DAD	Acetonitrile	0.10, 0.50, 1.00, 2.00, 5.00, 10.00 $\mu\text{g}\cdot\text{ml}^{-1}$
GC-MSD, GC x GC - TOFMS	Toluene	0.01, 0.04, 0.10, 0.21, 0.50, 1.00, 2.00 $\mu\text{g}\cdot\text{ml}^{-1}$

Individual PAH standards were prepared gravimetrically from the pure solids, dissolved in the same solvents as shown in Table 3.4 and stored in amber vials. These were required to confirm retention times on the HPLC, as well as to confirm the identity of PAHs in GC-MSD, since the spectral database software configured to the system could not differentiate between some of the isobaric PAHs. Difficulties were experienced in dissolving the heavier PAHs, namely indeno[123-*cd*]pyrene, dibenzo[*ah*]anthracene and benzo[*ghi*]perylene in acetonitrile, and even after rigorous mixing by means of a vortex, one or two crystals could still be seen at the bottom of the vials. This did not prove to be a problem since these individual standards were not used for quantification, but



merely to confirm spectra and chromatographic retention times for the HPLC analyses. The individual standards prepared for HPLC analysis were in the concentration range of 10 – 200 mg.ml<sup>-1</sup> and the concentration range of the individual standards for GC-MSD analysis was between 1 – 20 µg.ml<sup>-1</sup>.

### 3.3.2 Internal standard choice and preparation

An internal standard is typically used to track recoveries during extraction in order to account for losses either during the sample preparation or even in the column inlet (Bates et al., 2008; Gil-Moltó et al., 2009). Isotopically labeled standards were used in this study as a recovery standard in order to monitor the recoveries of the extraction techniques, as well as identify steps during sample preparation where the loss of analytes may have occurred (Szulejko et al., 2014).

It was decided that both deuterated phenanthrene (phenanthrene-d<sub>10</sub>) and deuterated pyrene (pyrene-d<sub>10</sub>) would be used in this study. Phenanthrene-d<sub>10</sub> has molecular mass of 188 g.mol<sup>-1</sup> and falls within the category of the lighter, more volatile PAHs of molecular mass 128 – 178 g.mol<sup>-1</sup>, thus ensuring that a measure of the volatile PAHs recoveries were accounted for. Pyrene-d<sub>10</sub>, of molecular mass 212 g.mol<sup>-1</sup>, served to represent the heavier PAHs of molecular mass 202 – 278 g.mol<sup>-1</sup>. By using two internal standards instead of one, the varying volatilities and extraction efficiencies of the different PAHs are better represented.

The two standards were obtained from Supelco (St Louis, MO) in their crystalline form and were made up in toluene. 500 µg of each deuterated standard was weighed out and made up to 1000 µl in toluene, resulting in a final standard of concentration 500 µg.ml<sup>-1</sup> (500 ng.µl<sup>-1</sup>). This internal standard mix was stored in the freezer at -18 °C to minimize losses via evaporation. For each extract, 10 µl (5 µg total mass of deuterated standard mix) was spiked onto the lichens, unless otherwise stated.

### 3.3.3 Calibration curves using GC-MSD and the PAH mix standards

The PAH mix standards, made up in toluene as shown in Table 3.4, were used to perform an external calibration on the GC-MSD. Prior to analysis, a solvent as blank was analyzed, and then the same solvent was run in between every standard to ensure no carryover and to use as the  $x = 0$  intercept in the calibration curves, the standards starting at  $0.01 \mu\text{g.ml}^{-1}$  and ending at  $2.00 \mu\text{g.ml}^{-1}$ . The GC conditions used were those that had been determined as optimal in Section 3.10. The calibration curves are given in Chapter 4, as part of the results and discussion. Calibration curves were not drawn up for the HPLC technique since it was decided, after initial studies on individual PAH standards, that the HPLC technique was not suitable for the quantification of PAHs in the sample extracts, as discussed in Chapter 4.

### 3.4 Scanning electron microscope analysis

A cutting from a lichen removed at the bulk sampling site during the bulk sampling campaign was cleaned with sterilized stainless steel tweezers and dried in the oven along with the rest of the bulk sample at  $35 \text{ }^\circ\text{C}$  for 4 - 5 days so that all moisture had been removed. The cutting was then analyzed using the scanning electron microscope (SEM) at the Laboratory of Microscopy and Microanalysis at the University of Pretoria.

The lichen sample was loaded onto an aluminium stub and coated with gold twice using an Emitech K550X sputter coater operated at 10 mA for 5 min (10 min in total). Electrical charge build up on the sample was observed (drifting image) during surface analysis after only one coat, so it became apparent that the lichen needed to be coated twice. The stub was then mounted onto the stage and viewed using a variable pressure SEM (JEOL 5800LV) operated at 5 kV. Figure 3.14 shows the aluminium stub with the lichen loaded and coated with gold, and Figure 3.15 shows the scanning electron microscope instrumentation used at the University of Pretoria.



**Figure 3.14: Segment of the lichen thallus, loaded onto an aluminium stub and coated twice with gold prior to SEM analysis**



**Figure 3.15: JOEL 5800LV SEM used for the analysis of the lichen segments**

In addition, seven lichen samples from the bulk-sampling site were freeze dried and coated in carbon prior to SEM/EDX analysis. The analysis was performed on a Tescan Vega 3 SEM/EDX instrument, operated with an accelerating voltage of 20 keV, monitoring the backscattered signal. The percentage distributions of identified elements were used as an indication of approximate abundance.

### **3.5 Sample Preparation techniques**

#### **3.5.1 Soxhlet Extraction**

##### **3.5.1.1 Dichloromethane extraction**

Dichloromethane (DCM) was investigated as an extraction solvent as suggested by a previous lichen study (Bajpai et al., 2013). 0.200 g dried and ground bulk

lichen sample was measured out into a Whatman glass microfibre thimble (25 mm ID x 90 mm length, tapered, high purity), spiked with 10 µl internal standard mix and extracted with 100 ml dichloromethane using the traditional Soxhlet apparatus for 6 hr, at the boiling point of the solvent (39.6 °C). The extraction was performed in triplicate. Figure 3.16 shows the Soxhlet apparatus configuration in the laboratory.



**Figure 3.16: The Soxhlet apparatus set up in the laboratory for the extraction of PAHs from lichens (0.200 g) using 100 ml dichloromethane**

The advantage of Soxhlet extraction is that the centrifugation step, included in the other extraction procedures, can be omitted since the thimble acts as a sample holder and a filter. Thus, the extracts were simply cooled prior to blow down to 2 ml under N<sub>2</sub>, followed by the clean up step using the procedure as detailed in Section 3.6, after which all extracts were made up to a final volume of 500 µl. A blank extraction using no lichen sample was also performed. All analyses were performed using the GC-MSD method according to the parameters in Table 3.10.

### **3.5.1.2 Acetonitrile extraction**

A study by Augusto et al (2012) used acetonitrile as the extraction solvent for lichens in a traditional Soxhlet sample preparation procedure. It was therefore utilized in this study in order to compare with the dichloromethane extraction (as detailed above in Section 3.5.1.1) and the other sample preparation techniques investigated in this study.

0.200 g dried and ground bulk lichen sample was weighed out into a Whatman glass microfibre thimble (25 mm ID x 90 mm length, tapered, high purity), spiked with 10 µl internal standard mix and extracted with 100 ml acetonitrile using the traditional Soxhlet apparatus for 6 hr, at the boiling point of the solvent (82 °C). The extraction was performed in triplicate.

As with the dichloromethane extracts, the acetonitrile-based lichen extracts were simply cooled prior to blow down to 2 ml under N<sub>2</sub>, followed by the clean up step using the procedure as detailed in Section 3.6, after which all extracts were made up to a final volume of 500 µl. It was observed that the extract obtained using dichloromethane was a darker green colour when compared to the acetonitrile based extract. A blank extraction using no lichen sample was also performed. All analyses were performed using the GC-MSD method given in Table 3.10.

### **3.5.2 Microwave assisted extraction**

#### **3.5.2.1 12 hr equilibrium study of spiked lichen sample**

Microwave assisted extraction was performed using an Anton Paar Synthos 3000 microwave system. Both the power and irradiation time can be varied, and the carousel sample holder can house 8 extraction vessels. Portions of the bulk lichen sample of mass 0.200 g were weighed out into 100 ml quartz tubes with Teflon® lined caps. The lichen portions were spiked with 10 µl of the internal standard mix (as detailed in Section 3.3.2), sealed with the cap, wrapped in foil and stored in a cool, dark place for 12 hr, as this was the equilibration time at which the PAH concentrations were determined to be highest in the USAE integration study detailed in Section 3.5.4.3.

After a 12 hr equilibration period, the lichen samples were extracted with 12 ml *n*-hexane: acetone (1:1) with a 2 min ramp from 0 W to 150 W, held at 150 W for 10 min, then cooled at 0 W for 10 min. The pressure was set to 2.0 bar.s<sup>-1</sup>. The extracts were then removed, decanted into amber vials and concentrated to 2 ml under N<sub>2</sub> before the clean up procedure as detailed in Section 3.6 was followed, after which all extracts were made up to a final volume of 500 µl.

### **3.5.2.2 MAE techniques using acetone, *n*-hexane and dichloromethane**

Microwave assisted extraction was performed using the Anton Paar Synthos 3000 microwave system. Three portions of the bulk lichen sample of 0.200 g mass each were weighed out into 100 ml quartz tubes with Teflon® lined caps. The lichen portions were spiked with 10 µl of the internal standard mix (as detailed in Section 3.3.2) and covered with 12 ml *n*-hexane: acetone (1:1). The vessels were then placed in the carousel, with a 2 min ramp from 0 W to 150 W, held at 150 W for either 5, 10 or 20 min, then cooled at 0 W for 10 min. The pressure was set to 2.0 bar.s<sup>-1</sup>. The extracts were then removed, decanted into amber vials and concentrated to 2 ml under N<sub>2</sub> before the clean up procedure as detailed in Section 3.6 was conducted, after which all extracts were made up to a final volume of 500 µl. Blanks were run alongside every extraction.

The suggestion of using *n*-hexane: acetone (1:1, v/v) for MAE of PAHs was made by Piñeiro-Iglesias et al (2004) who found the best recovery for BaP using this solvent combination on an urban dust certified reference material as well as for an urban particulate matter certified reference material, further supported by work on sewage sludge (Villar et al., 2004).

In order to compare the extraction efficiency of the MAE technique with that of USAE, extractions using 12 ml dichloromethane were also performed according to the method using *n*-hexane: acetone, with a hold time of 10 min. It was observed that the *n*-hexane: acetone based extracts were decidedly brighter green than the dichloromethane extracts. Table 3.5 summarizes the different MAE extractions performed. All extracts were analyzed using the optimized GC-MSD method given in Table 3.10.

**Table 3.5: The different MAE extraction conditions investigated in this study on 0.200 g bulk lichen sample portions, spiked with 10 µl internal standard**

Extract Identity	Solvent	Solvent volume	Hold time at 150 W
MAE 12 hr spike	Hexane: acetone (1:1)	12 ml	10 min
MAE Hex:A 5 min	Hexane: acetone (1:1)	12 ml	5 min
MAE Hex:A 10 min	Hexane: acetone (1:1)	12 ml	10 min
MAE Hex:A 20 min	Hexane: acetone (1:1)	12 ml	20 min
MAE DCM 10 min	Dichloromethane	12 ml	10 min

### 3.5.3 QuEChERS extraction

Plastic centrifuge tubes (volume 15 ml) were obtained from Labcon (Johannesburg, South Africa) for use in QuEChERS sample preparation. The extraction salts were supplied by Restek (Bellefonte, PA), and the details of the Q-Sep™ Q110 packets can be found in Appendix D. The QuEChERS extraction process is shown below in Figure 3.17. The modifications made to the extraction method are specifically related to the use of a different clean up step, increased shaking time as well as the use of ice to cool the tubes down after the addition of the extraction salts.

The dispersive solid phase extraction (dSPE) based clean up methodology recommended by the Restek Corporation (Bellefonte, PA) was not used in this study because it was decided that the clean up methodology would remain constant in order for the extraction efficiencies of different techniques to be compared. Since graphitized carbon black (GCB) is recommended for the removal of pigments, it is commonly used on plant extracts, but it was intuitively decided that the GCB-based dSPE should not be used since the planar structure of graphite would intercalate with the PAH structures and result in adsorption losses (Sadowska-Rociek et al., 2014). The increase in shaking time was adapted from work by Sadowska-Rociek et al (2013; 2014), as well as a suggestion by Jack Cochran (personal communication, 2013), from the Restek Corporation.

Water was added to all the vials prior to the addition of solvent since it has been reported that this improves the partitioning of polar compounds between the organic and aqueous phases (Albinet et al., 2013; Zhao et al., 2012). It was also

decided that the extracts would be cooled down in ice water once the extraction salts had been added and the extracts shaken, in order to minimize losses by evaporation, and to reduce any uncertainties introduced by temperature variations during the exothermic reaction taking place when the salts are added, since an increase in temperature would improve the extraction efficiency (Forsberg et al., 2011).



**Figure 3.17: Schematic representation of the QuEChERS methodology used in this study to extract PAHs from lichens**

### 3.5.3.1 QuEChERS extractions using different solvent schemes

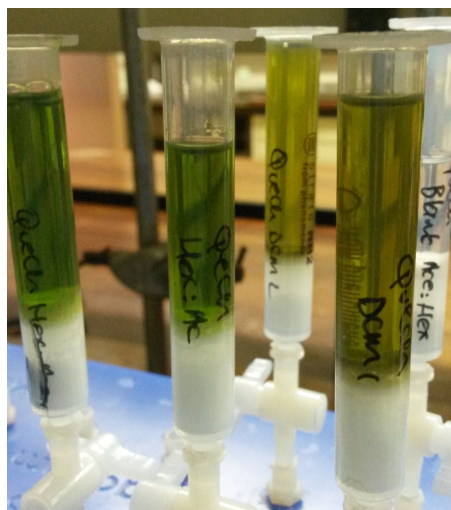
The modified QuEChERS extraction involved a 30 min agitation time, and the use of ice to cool down the extracts in order to consistently control the extraction temperature. A few solvent schemes were investigated, as shown in Table 3.6. The solvent volumes, spike volumes and water volumes were kept constant in all



extractions, and blank extracts were performed alongside each extraction. The clean up methodology is detailed in Section 3.6, and all analyses were performed using the optimized GC-MSD methodology given in Section 3.10. Figure 3.18 clearly shows how the colours of the QuEChERS extracts differ between *n*-hexane: acetone (1:1, v/v) and dichloromethane, loaded onto SPE cartridges prior to elution.

**Table 3.6: The different extraction conditions using QuEChERS for the extraction of PAHs from lichens**

Sample identity	Solvent used	Volume water	Colour intensity
Q Hex:A	Hexane: acetone (1:1, v/v)	2 ml	Bright green
Q Hex:DCM	Hexane: dichloromethane (1:1, v/v)	2 ml	Light green
Q DCM	Dichloromethane	2 ml	Murky light green



**Figure 3.18: The difference in colours of Q Hex: A and Q DCM bulk lichen sample extracts**

### 3.5.3.2 12 hr equilibrium study on spiked lichen

Portions of the bulk lichen sample of mass 0.200 g were weighed out into 15 ml centrifuge tubes. The lichen portions were spiked with 10 µl of the internal standard mix (as detailed in Section 3.3.2), sealed with the cap, wrapped in foil and stored in a cool, dark place for 12 hr. The reason why the spiked samples were left for 12 hr is because it was the equilibration time at which the PAH concentrations were determined to be highest in the USAE integration study detailed in Section 3.5.4.3.

After a 12 hr equilibration period, the lichen samples were extracted using the same extraction conditions detailed in Section 3.5.3.1, using *n*-hexane: acetone (1:1, v/v) as the extraction solvent as it was found to perform best, followed by the clean up procedure detailed in Section 3.6 after which the final volume was made up to 500 µl. All analyses were performed using the GC-MSD method given in Table 3.10.

### **3.5.3.3 Study on increased sample mass to improve homogeneity**

A QuEChERS extraction was performed in which 0.600 g portions of the bulk lichen sample were measured out into three centrifuge tubes, each spiked with 10 µl internal standard mix and extracted with 2 ml water and 12 ml *n*-hexane: acetone (1:1, v/v) as described in Figure 3.17, with an agitation time of 30 min. These samples were denoted 'Q H:A 0.6 g'. This was done in order to determine whether increasing the sample mass would improve the relative standard deviation, which could be impacted more by any sample inhomogeneity in the smaller 0.200 g lichen samples. The clean up procedure is detailed in Section 3.6, after which the final volumes were made up to 500 µl. The analyses of the extracts were performed using the GC-MSD method optimized in Section 3.10. All analyses were performed using the GC-MSD method given in Table 3.10.

### **3.5.4 Ultrasound Assisted Extraction (USAE)**

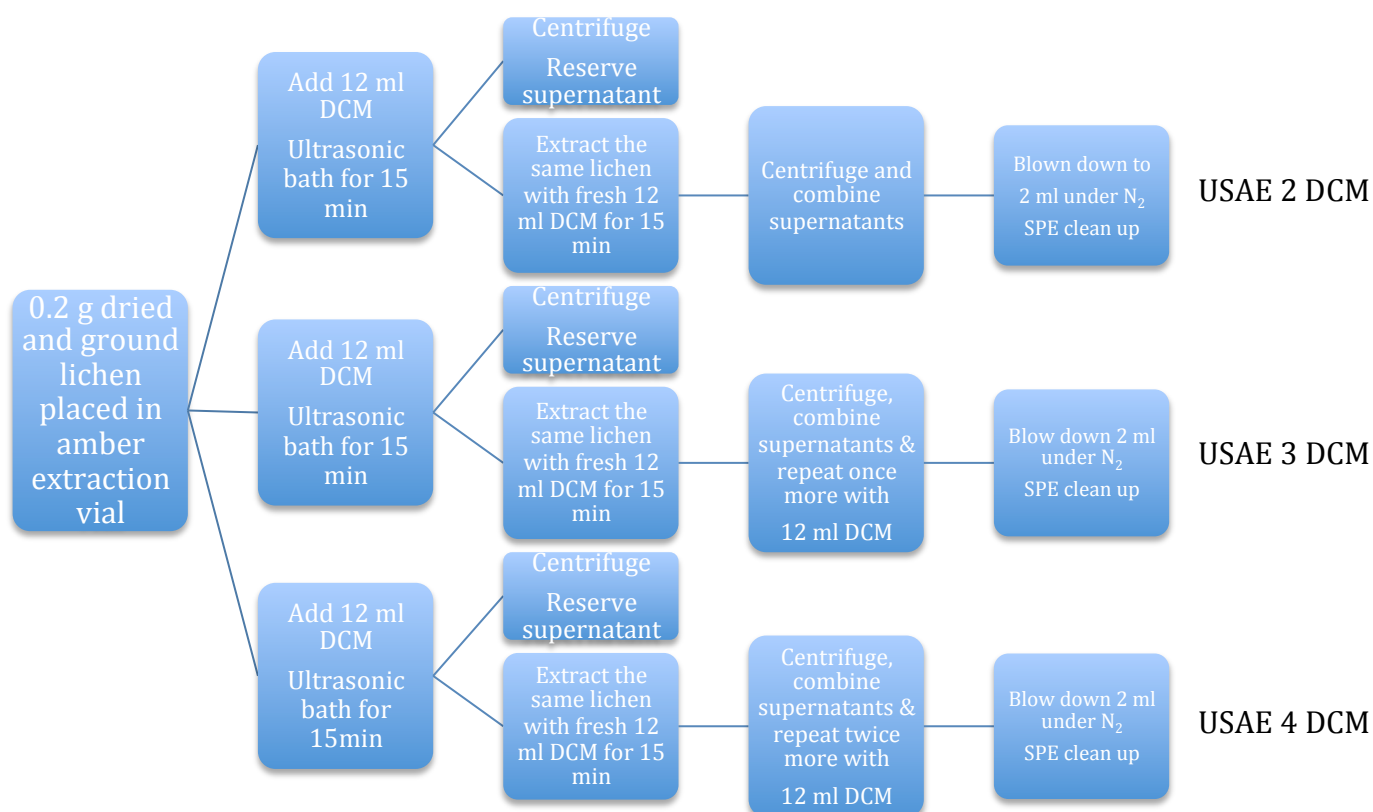
The lichen studies by Blasco et al (2008) (2006) (Domeño et al., 2006) laid the foundation for the USAE study in this project. A 6L ultrasonic bath was obtained from Eumax (UD200 SH-6L, 200W), the centrifuge (model EBA 35 from Hettich, Tuttlingen, Germany), and 15 ml plastic centrifuge tubes, with plastic lids, were bought from Labcon (Johannesburg, South Africa).

#### **3.5.4.1 Native lichen extraction**

To establish how many sequential extractions would be sufficient in quantitatively extracting the PAHs from the lichen matrix, it was decided that two, three and four 12 ml USAE extractions on individual 0.200 g portions of the bulk lichen sample would be performed. A comparison of the PAH profile and

concentrations would then indicate how many sequential extractions are required.

The extraction process is shown below in Figure 3.19. Dried and ground lichen portions of 0.200 g were placed into individual 15 ml amber vials (Supelco, St Louis, MO) and 12 ml DCM was added as extraction solvent. The vial was then placed in an ultrasonic bath and sonicated for 15 min. The contents of the vial were transferred to a clean centrifuge tube and centrifuged at 6000 U.min<sup>-1</sup> for 10 min until the lichen solids and solvent had separated into two clear layers. The solvent was decanted into a new clean vial, and the lichen solids were placed back into the original glass vial to repeat the extraction with fresh extraction solvent, followed by centrifugation. One, two and three subsequent extractions were performed respectively and the extracts of each sample combined. The temperature of the water bath was maintained under 30 °C by adding ice to the bath as necessary. Each unique extraction procedure was prepared in triplicate. The combined extraction solvent for each sample was then blown down under nitrogen gas to 2 ml. The cleanup method is detailed below in Section 3.6, and all extracts were made up to a final volume of 500 µl. No internal standard was used since the results were to be directly compared to one another both qualitatively and quantitatively. Analysis of the extract was performed using the GC-MSD method given in Table 3.10. A blank extraction using no lichen sample was also performed.



**Figure 3.19: Sample preparation procedure to establish how many subsequent extractions are required for lichens using dichloromethane (DCM) and USAE**

### 3.5.4.2 Recovery study on USAE

An inquiry was made into whether PAHs were lost during the USAE process. The internal standard mix, detailed in Section 3.3.2, was used to investigate at which stages the largest losses were occurring. The bulk lichen sample was used for the investigation, in which samples were spiked at different stages in the extraction process, namely:

- At the start of the extraction procedure, directly onto the 0.200 g portion of the dried and ground bulk sample. The spike was made directly onto the lichen sample, without touching the sides of the vial in order to minimize adsorption onto the walls. This would indicate overall recovery.
- After the ultrasound assisted extraction, prior to the SPE clean up procedure (detailed in Section 3.6). This would indicate whether the clean up procedure was a major contributor towards the loss of analytes.

- After the SPE clean up procedure, after the final blow down under N<sub>2</sub>, just prior to analysis. This would serve as a reference point to which the other two types of samples could be compared, since it would reflect any matrix effects identical to the other samples, but not losses as a result of sample preparation.

Dried and ground lichen portions of 0.200g each were placed into twelve 15 ml amber vials (Supelco, Bellefonte, PA). Three vials were labeled as '3A1, 3A2 and 3A3'. These were not spiked. Another three vials were labeled '3B1, 3B2 and 3B3' and were spiked with 10µl of the internal standard of concentration 500 µg.ml<sup>-1</sup> (phenanthrene-d<sub>10</sub> and pyrene-d<sub>10</sub>) as detailed in Section 3.3.2. Three vials were labeled '3C1-3' and the remaining three vials '3D1-3'. 12 ml dichloromethane was then added to each vial. The vials were then placed in an ultrasonic bath and sonicated for 15 min. The contents of the vials were then placed into separate centrifuge tubes and centrifuged at 6000U.min<sup>-1</sup> for 10 min until the lichen solids and solvent had separated into two clear layers. The solvent was then decanted into new clean vials, and the lichen solids were then placed back into the original glass vials to repeat the extractions.

Two additional sequential extractions were performed respectively to provide a total of 3 extractions on each lichen sample. The process is detailed below in Table 3.7. Once the extractions had been performed, the respective supernatants from each individual extraction were combined, and the extracts were then blown down under nitrogen gas to 2 ml. At this point, the 'USAE 3C' series of extracts were spiked with 10 µl internal standard mix. The cleanup method is detailed in Section 3.6. After the clean up and concentration step, just prior to analysis of the 500 µl extract, the 'USAE 3D' series of extracts were spiked with 10 µl internal standard. In this manner triplicate recovery data for each spiking stage was obtained. All analyses were performed using the GC-MSD method given in Table 3.10. A blank extraction using no lichen sample was also performed.

**Table 3.7: Experimental scheme to investigate losses in USAE**

Name	Solvent volume used (ml)	Stage at which extracts were spiked	Spike volume ( $\mu\text{l}$ )	Theoretical concentration
USAE 3A 1-3	36	No spike	0	0
USAE 3B 1-3	36	Before 1 <sup>st</sup> extraction	10	10 ng. $\mu\text{l}^{-1}$
USAE 3C 1-3	36	Before SPE cleanup	10	10 ng. $\mu\text{l}^{-1}$
USAE 3D 1-3	36	Prior to injection	10	10 ng. $\mu\text{l}^{-1}$

#### 3.5.4.3 Study on the integration of PAHs into the lichen matrix over time

The degree to which PAHs might be assimilated into the lichen matrix, and whether that plays a role in how easily the PAHs are then, in turn, extracted out of the matrix is not clearly understood. For this reason, it was decided that an investigation into the integration of PAHs into the lichen matrix with time would be beneficial towards understanding to what extent samples need to be equilibrated prior to extraction.

0.200 g portions of the dried and ground lichens were measured out into twenty-one amber vials. Each sample was then spiked with 10  $\mu\text{l}$  of the internal standard mix (as detailed in Section 3.3.2) and 25  $\mu\text{l}$  of the 200  $\mu\text{g}.\text{ml}^{-1}$  PAH standard mix (as detailed in Section 3.3.1). The vials were sealed, wrapped in foil and placed in a desiccator for different periods of time. The laboratory temperature was controlled at 21 °C for the entire test period. Spiked lichen samples were left for 0 hr, 2 hr, 6 hr, 12 hr, 24 hr, 48 hr and 96 hr. Once the set time had elapsed, extraction was performed using the USAE method detailed above in Section 3.5.4.1, with a total of 3 sequential extractions, all performed in triplicate. Once the extractions had been performed, the extracts were then blown down under nitrogen gas to a volume of 2 ml. The cleanup method is detailed below in Section 3.6, after which all extracts were made up to a final volume of 500  $\mu\text{l}$ . Analysis of the extract was performed using the GC-MSD method given in Table 3.10.

#### 3.5.4.4 Solvent optimization study for USAE

In order to optimize the sample preparation procedure it was necessary for the effect of different solvents on the extraction efficiency to be studied. Therefore,

extractions were performed on 0.200 g portions of dried and ground bulk lichen sample. Three samples were each extracted three times sequentially, with each extraction using 12 ml dichloromethane. Three samples were similarly prepared using 12 ml portions of *n*-hexane: acetone (1:1, v/v). Each sample was spiked with 10 µl internal standard mix prior to extraction, to track recoveries. The recoveries could then be compared in order to aid in the identification of the most efficient extraction solvent scheme.

### 3.5.5 Thermal Desorption of the lichen

The GC-MSD and GC x GC – TOFMS systems were both fitted with Gerstel Thermal Desorption System (TDS) 3 at the inlet to the instruments. PAHs had previously been identified and quantified in urban dust certified reference material (Gil-Moltó et al., 2009) using thermal desorption as a sample introduction technique (which requires no sample preparation) and so it was decided that it should be considered as a possibility in this study.

#### 3.5.5.1 Lichen combustion test

Prior to introducing lichen material directly into any GC instruments, it was decided that a simple combustion test should be performed on the lichen matrix. A temperature sensor was taped to a hot plate using heat resistant glass fiber tape (Scotch 27 glass cloth, 3M, Germany) and a cutting was taken from a large lichen of the species *Parmotrema austrosinense* (Zahlbr.) Hale and placed next to the sensor on the hot plate, as shown in Figure 3.20. The hot plate was switched on and the temperature slowly increased. The lichen was closely watched for any changes in colour or the emission of smoke. This was repeated three times with fresh lichen cuttings of various sizes, and it consistently observed that the lichens turned a light brown colour at 180 °C and are fully blackened by 250 °C.

Since the GC parameters in this studies ranged from 275 °C for the inlet temperature, to 320 °C (final GC oven temperature), it was decided that thermal desorption of the lichen matrix is not suitable for the analytical technique to be employed. This is largely due to the likelihood that new PAHs may be formed as the lichen burns at temperatures as low as 180 °C.



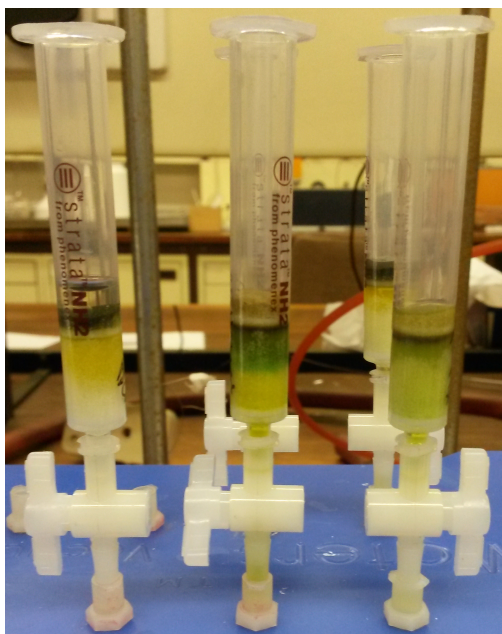
**Figure 3.20: Combustion test on lichens using a hot plate and a thermocouple**

### 3.6 Sample extract clean up procedure

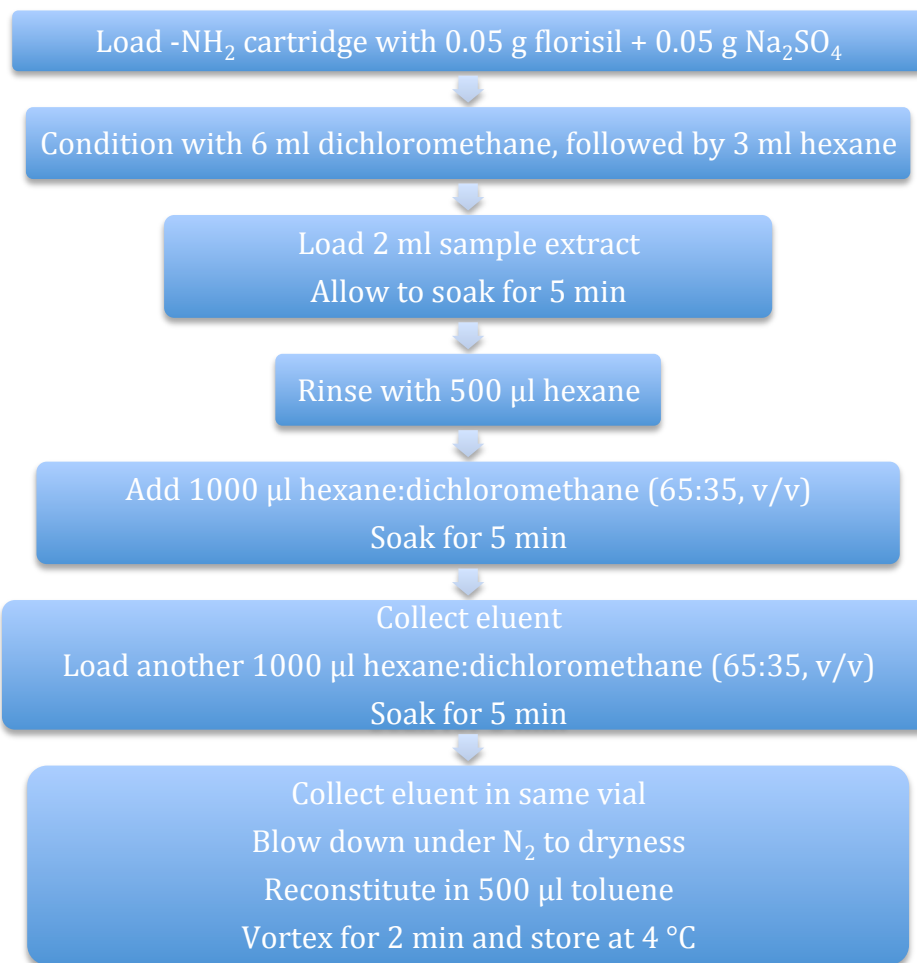
The sample extract clean up procedure was directly taken from a lichen optimization study by Blasco et al (2007). Strata -NH<sub>2</sub> solid phase extraction (SPE) cartridges with sorbent bed mass of 500 mg (bed volume 6 ml) were obtained from Phenomenex (Separations, South Africa). Florisil and anhydrous sodium sulphate were purchased from Merck Chemicals (Darmstadt, Germany). Examples of the appearance of the SPE columns are shown in Figure 3.21, where the bands can be clearly seen in the *n*-hexane: acetone based QuEChERS extracts (seen in the centre) as opposed to the dichloromethane based QuEChERS extract (on the left) which was lighter in colour and only formed one yellow band during the clean up procedure. The colour of the *n*-hexane: dichloromethane (1:1, v/v)



extract fell in between the two extremes, and can be seen on the right hand side of the SPE manifold. The details of the clean up procedure are given in Figure 3.22. The final extracts were collected in amber vials with PTFE lined screw caps, blown down carefully to the exact point of dryness under N<sub>2</sub>, then immediately made up to 500 µl in toluene and stored at 4 °C. The concentration step was deemed necessary to improve the sensitivity of the analysis (Romanik et al., 2007).



**Figure 3.21: Appearance of the SPE columns during the clean up procedure of Q DCM (left), Q Hex:A (centre) and Q Hex:DCM (right)**



**Figure 3.22: SPE clean up methodology used for all lichen extracts, both on the bulk sample and on those for the other sampling sites**

### 3.7 Matrix Matched Standards

After the different sample preparation techniques had been performed and the extracts analyzed using the GC-MSD method optimized in Section 3.10, concern was raised over whether the matrix might be interfering with the individual PAH signals. These concerns mainly centered around the calculated recoveries (above 180 %), suggesting an enhancement effect on the internal ‘recovery’ standards. For this reason, it was decided that matrix matched standards should be prepared and analyzed in order to establish whether any matrix effects were influencing the results. Such a study has not been reported with respect to PAHs in lichens, to date.

The five matrix matched standards were prepared using the modified QuEChERS extraction using 12 ml *n*-hexane: acetone (1:1, v/v) with a 30 min shaking step followed by the clean up procedure as listed in Section 3.6. The five extracts were then spiked, with an increase in concentration of standard PAH mix from 0.01 – 0.5 ng.µl<sup>-1</sup>, as well as a corresponding increase of the internal standard concentration of 0.5 – 10 ng.µl<sup>-1</sup>. A blank or native (with no spike) lichen extract was also prepared. An increase in concentration of the internal standard was studied to see whether any matrix effects were influencing the results of both the internal standard and the PAHs of interest.

The entire matrix matched standard set, as well as the blank extracts were run on the GC-MSD instrument using the optimized method given in Section 3.10. Calibration curves were drawn up plotting the peak area of the SIM chromatogram of each analyte at the given concentration of each individual PAH. The response for each PAH in the blank or native extract was plotted at x = 0. The matrix effect of each PAH was then calculated using Equation 3.1, where a negative % matrix effect implied a suppression of the signal, and a positive % matrix effect implied signal enhancement. The extent to which the matrix was interfering with the response of the analyte on the instrument was then classified as strong, medium or soft (Rajski et al., 2013) (Table 3.8).

$$\% \text{ Matrix Effect} = \left( \frac{\text{Slope of the calibration curve in matrix}}{\text{Slope of the calibration curve in solvent}} - 1 \right) \times 100 \quad \text{Equation 3.1}$$

**Table 3.8: Classification of different % matrix effects for analytes undergoing either signal suppression or enhancement (Rajski et al., 2013)**

Percent matrix effect, x	Type of matrix effect
0 < x < 20	Soft
21 < x < 50	Medium
51 < x	Strong

### 3.8 Extraction of samples from areas of interest

The samples of interest were removed from the refrigerator, and 0.200 g of the sample was weighed into individual plastic centrifuge tubes. The samples were then spiked with 10 µl of the internal standard mix and extracted using the best performing extraction technique, found to be the modified QuEChERS technique (12 ml *n*-hexane: acetone (1:1, v/v) and 30 min shaking), followed by the clean up procedure as given in Section 3.6. The samples were not prepared in triplicate because insufficient sample was collected at some of the sampling sites. The extracts were analyzed on the GC-MSD instrument, using the optimized conditions given in Table 3.10.

### 3.9 Method development for high performance liquid chromatography

High performance liquid chromatography (HPLC) was performed on a Waters 600 Millipore Controller system fitted with a Waters 610 Fluid Unit pump and a Waters 996 photodiode array detector (DAD) (Milford, MA). The Rheodyne® injector fitted to the system had an external loop volume of 20 µl, thus the injection volume was chosen to be 20 µl. The Waters PAH C18 column was employed which has specifically been developed for the analysis of PAHs when using HPLC as the separation technique. The column dimensions are given in Table 3.9.

**Table 3.9: Waters PAH C18 HPLC column dimensions used in the separation of PAHs**

Column Name	Particle size	Inner diameter	Length
Waters PAH C18	5 µm	4.6 mm	250 mm

Individual PAH standards were made up in HPLC grade acetonitrile as detailed in Table 3.4. Individual PAHs were analyzed by HPLC-DAD using Milli-Q water and HPLC grade acetonitrile as mobile phases at room temperature. The equilibrium times between each run were above 20 min in order to make sure retention times were reproducible (Villar et al., 2004). Millennium<sup>32</sup> 4.0 (Waters, USA) software was used for data processing.

The analytes were separated using a set flow rate and programmed mobile phase gradient, starting at 40.0% water and 60.0% acetonitrile at 1.20 ml.min<sup>-1</sup>,

switching to 100.0% acetonitrile at 9.00 min (curve 9 as given in the Millennium<sup>32</sup> software) and maintained there for 14 min, after which the system was returned to starting conditions. The total run time was 30 min. The photodiode array detector acquired data in the wavelength range of 210.0 nm – 400.0 nm, with the resolution set to 1.2.

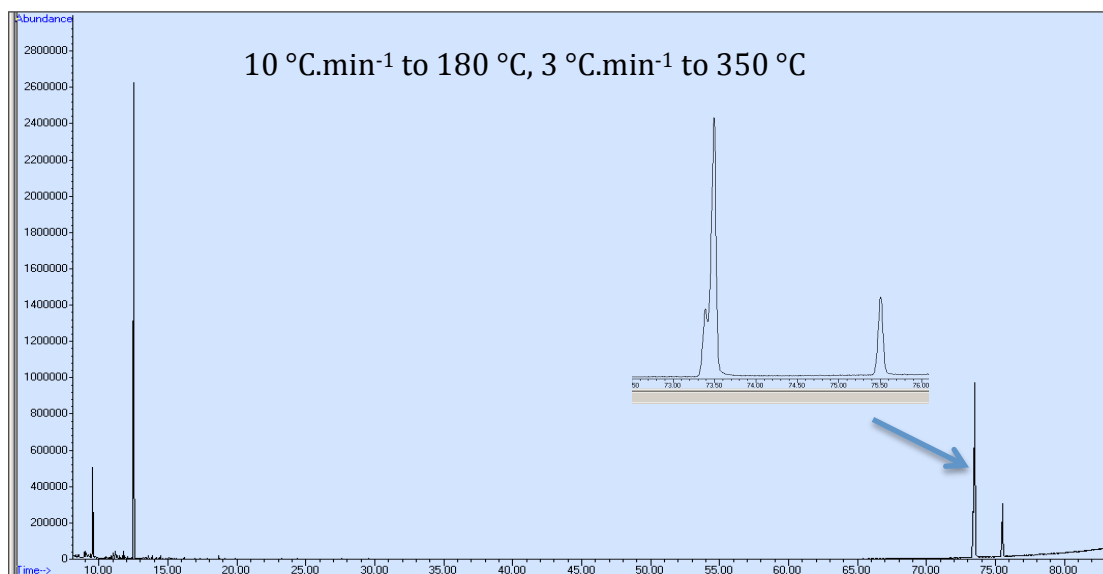
### 3.10 Method development for gas chromatography using PAH standards

All analyses were performed on an Agilent 6890 Series GC coupled to an Agilent 5975C MSD operated in both scan (mass acquisition 45 – 550 m/z) and SIM mode. The ions monitored were m/z 128, 154, 156, 166, 178, 202, 228, 252, 276 and 278, as listed in Table 2.3. The internal standards were also monitored at m/z 188 and 212 (phenanthrene-d<sub>10</sub> and pyrene-d<sub>10</sub>, respectively). Helium gas of high purity (Afrox, Gauteng) was used as the carrier gas at a flow rate of 1.0 ml.min<sup>-1</sup> in constant flow mode. The SIM ion peaks were used for all quantification and identification in all extracts in this study.

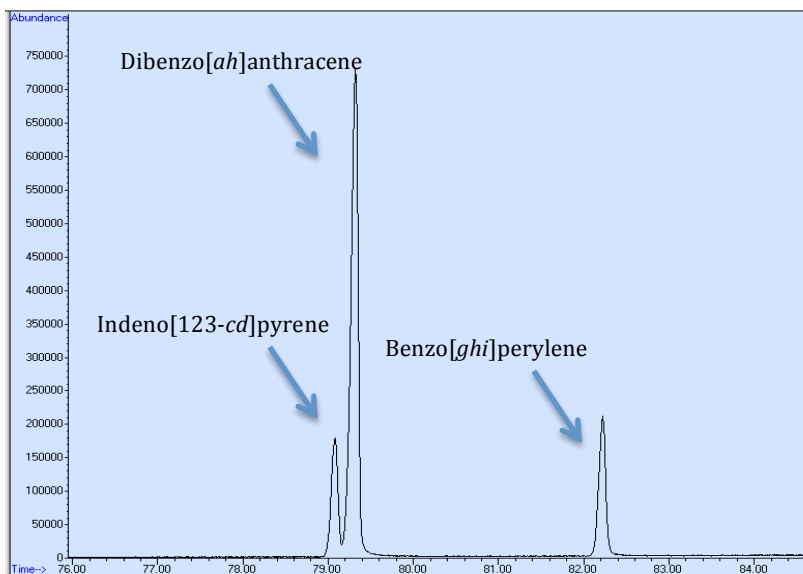
Since multiple repeats of the entire procedure are necessary to estimate the uncertainty in the final measurement, as well as to define the precision of the analysis (Ramsey & Ellison, 2007), extracts prepared in triplicate were analyzed consecutively, with a solvent blank run between each analysis. The full MS spectra as well as the retention times of individual standards were used to confirm the presence of the PAHs of interest, as in an herbal infusion study (Cacho et al., 2014).

The starting point for the GC-MSD method was based on the 1<sup>st</sup> dimension parameters of a developed GC x GC – TOFMS method used in previous work (Geldenhuis et al., 2015). These parameters were tweaked in order to effectively separate the three heaviest PAHs on the Restek Rxi®-PAH column. This was important since IcdP, BghiP and DahA have markedly different TEFs (0.1, 0.01 and 5.0 respectively), implying that the identification and quantification of each heavier PAH would impact the final TEQ value.

In the chromatogram obtained using an oven temperature ramp of  $10\text{ }^{\circ}\text{C}\cdot\text{min}^{-1}$ , only two peaks were observed in the region where the three heaviest PAHs in the standard should be eluting. Thus, the ramp rate was then slowed down from  $180\text{ }^{\circ}\text{C}$ , increasing by  $3.0\text{ }^{\circ}\text{C}\cdot\text{min}^{-1}$  to  $350\text{ }^{\circ}\text{C}$  and the resulting chromatogram is shown in Figure 3.23, where three peaks can be seen in the retention time window of 73 – 76 min, but are poorly resolved. The temperature ramp rate was then slowed down from  $180\text{ }^{\circ}\text{C}$  onwards to  $1.8\text{ }^{\circ}\text{C}\cdot\text{min}^{-1}$ , and efficient separation (resolution of 1.1, calculated according to (Skoog et al., 2004)) was observed as seen in Figure 3.24. A change to  $2.0\text{ }^{\circ}\text{C}\cdot\text{min}^{-1}$  did not change the resolution and was therefore chosen to reduce the final run time to 74 min. The final GC-MSD procedure is given in Table 3.10.



**Figure 3.23: Chromatogram obtained with a slower oven temperature ramp rate from  $180\text{ }^{\circ}\text{C}$  onwards ( $3.0\text{ }^{\circ}\text{C}\cdot\text{min}^{-1}$  to  $350\text{ }^{\circ}\text{C}$ ), showing two peaks in the region where 3 peaks should be observed**

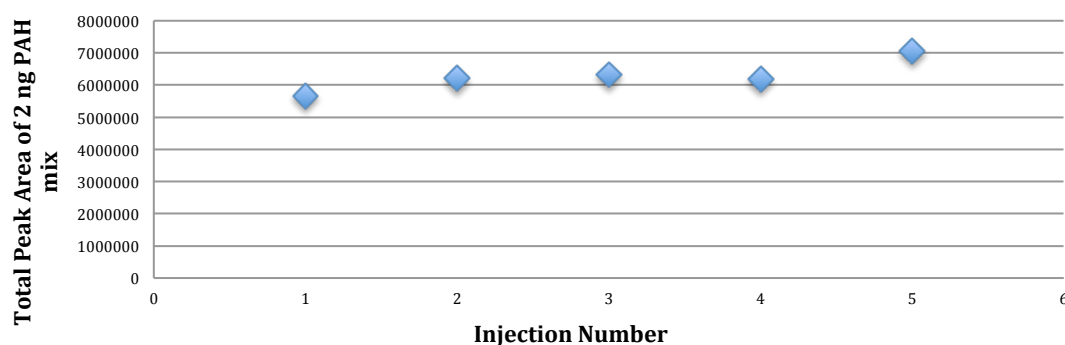


**Figure 3.24: Chromatogram obtained with a 1.8 °C.min<sup>-1</sup> oven temperature ramp rate from 180 °C onwards, showing the separation of the three heaviest PAHs in the standard mix, determined by the retention times of the individual standards**

**Table 3.10: The optimized GC-MSD conditions for the analysis of PAHs in lichen extracts**

Parameter	Conditions
Column	Restek Rxi®-PAH
Column dimensions	60 m, 0.25 mm ID, 0.25 µm d <sub>f</sub>
Oven program	80°C (1 min), 30°C/min to 180°C, 2°C/min to 320°C
Injection	1 µl (manual injection)
Inlet mode	Splitless, purge flow 40 ml.min <sup>-1</sup> (1 min)
Inlet liner	Restek SKY™ Precision splitless liner without wool
Solvent delay	6.5 min
Inlet temperature	275 °C
Carrier gas	Helium, constant flow mode, 1 ml.min <sup>-1</sup>
Transfer line temperature	300 °C
Detector settings	-70 eV, electron impact mode
MS ion source temperature	230 °C (ion source), 150 °C (quad temperature)
Total run time	74 min

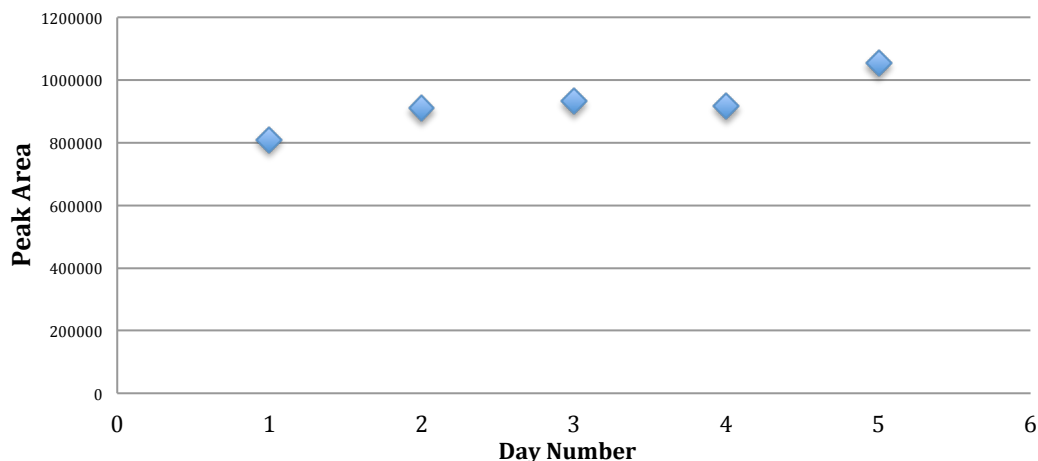
Once the optimized GC-MSD method had been established, a quality control standard ( $2 \text{ ng}\cdot\mu\text{l}^{-1}$ ) was analyzed consecutively, with solvent blanks in between each analysis, five times on one day to establish intraday variations on the instrument. The results are shown below in Figure 3.25, where the total peak area of all the PAHs in the standard was monitored over one day, for consecutive injections. The total peak area remains relatively constant, but the fifth injection appeared to be possibly anomalous, and it was necessary to establish whether it could be discarded from the data. A Q-test was performed on the fifth injection of the day, resulting in a Q value of 0.535. This Q value is significantly lower than  $Q_{\text{crit}}$  for 5 observations at 95% confidence interval ( $Q_{\text{crit}} = 0.710$ ), thus the fifth injection could not be considered as an outlier (Skoog et al., 2004). The relative standard deviation (RSD) of the intraday values was calculated to be 7%.



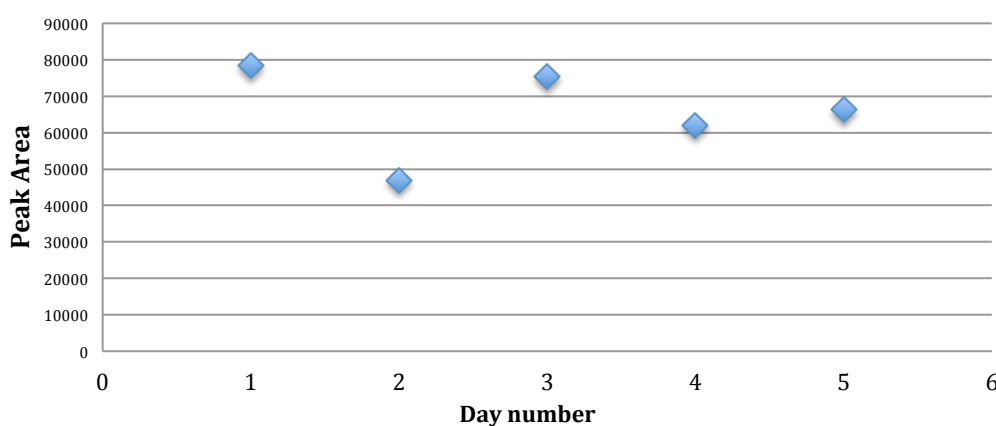
**Figure 3.25: Intraday variations on total peak area of 2 ng PAH standard mix**

The same methodology was followed for an interday study, in which a  $2 \text{ ng}\cdot\mu\text{l}^{-1}$  PAH mix standard was analyzed using the method given in Table 3.10 over five consecutive days in order to establish a day-to-day variation. The results of the interday study are given in Figure 3.26 and 3.27, where the peak area for a light PAH (fluorene, Fig 3.26) has been investigated, as well as a heavy PAH (dibenzo [ah] anthracene, Fig 3.27). It shows that there are variations in the instrument responses, and the response varies more significantly for the heavy PAHs (%RSD of 18% for DahA) than for the lighter PAHs (%RSD of fluorene 9.6%) The sum of the total peak areas for all identified PAHs in the injections made over 5 days had a calculated average RSD value of 23%.





**Figure 3.26: Interday variations of 2 ng fluorene in a PAH mix standard analyzed by GC-MSD**



**Figure 3.27: Interday variations of 2 ng dibenzo[ah]anthracene in a PAH mix standard analyzed by GC-MSD**

The use of a LECO Pegasus 4D TD- GC x GC – TOFMS (LECO Corporation, St Joseph, MI) was considered in this study since the 2<sup>nd</sup> dimension separation would improve the selectivity of the analysis, separating the matrix from the PAHs of interest. A few sample extracts were analyzed using liquid injection GC x GC – TOFMS, but an incongruence between the solvent (toluene) and the 5SilMS column was observed, and thus liquid injection-based GC x GC - TOFMS had to be disregarded in this study. The oven program used was obtained from previous work on PAHs on filters, using a Rxi®-5SilMS column.

The use of thermal desorption sample introduction techniques was considered as a result of the increased purge time, in order to overcome the problems observed with the stationary phase of the column and toluene. Therefore, to compare the results from the liquid-injection GC-MSD to the use of a thermal

desorber as a sample introduction technique, a 1 µl volume of extract was injected into the heated zone of a Gerstel® glass thermal desorption tube, fitted to the TDS system and was run in 1D mode to compare the separation efficiency with the GC-MSD technique. The GC-TOFMS conditions are given below in Table 3.11. This was performed for the DCM Soxhlet, Q Hex:A, Q Hex:DCM, MAE 20 min H:A and USAE 4A extracts prepared from the bulk sample, in order to compare the GC-TOFMS results with the GC-MSD results. Data was processed using ChromaTOF software with PAHs identified based on a similarity greater than 80% compared to the NIST library. Unsuccessful modifications of the oven program were attempted, and it was subsequently decided that further analysis would be performed on the GC-MSD system, since the results were satisfactory using the one-dimensional technique and the Restek® Rxi-PAH column. The advantage of a thermal desorption system being attached to the GC x GC – TOFMS was nullified by the findings in Section 3.5.5.1, where it was decided that lichens are not candidates for thermal desorption. It should also be noted that performing liquid injection into the TD-GC-TOFMS system would incur unnecessary costs of liquid nitrogen, with no analytical benefits, but rather only introduce the possibility of the loss of analytes.

**Table 3.11: The TDS-GC-TOFMS conditions used to analyze lichen extracts**

<b>Parameter</b>	<b>Conditions</b>
Column	Rxi®-5SilMS
Dimensions	29.6 m length, 0.25 mm ID, 0.25 µm d <sub>f</sub>
Inlet mode	TDS splitless, 30 ml.min <sup>-1</sup> flow rate
Inlet liner	Gerstel® glass liners for CIS 4 straight with notch
TDS program	30 °C (3 min), 60 °C.min <sup>-1</sup> to 280 °C (5 min)
CIS program	-50 °C (0.1 min), 12 °C.s <sup>-1</sup> to 280 °C (2.4 min)
Carrier gas	Helium, 1 ml.min <sup>-1</sup>
Oven programme	40 °C (3 min), 10 °C.min <sup>-1</sup> to 315 °C (5 min)
Run time	62 min 30 sec
2ndary oven	20 °C offset
Transfer line temperature	280 °C
MS mode	EI positive
Mass range	50 – 500 amu
Acquisition rate	10 spectra.s <sup>-1</sup>
Detector voltage	1750 eV
Ion source temperature	250 °C

### 3.11 Limit of detection, limit of quantification and toxic equivalency factor determinations

The limit of quantification (LOQ) and limit of detection (LOD) for each individual PAH was calculated using signal-to-noise (S/N) ratio principles (González et al., 2014), where the LOD was based on a S/N value of 3, and the LOQ based on a S/N value of 10.

In order for the toxic equivalency factors (TEFs) weighted concentrations to be calculated, the individual concentration of each PAH identified in the sample first needed to be determined (Nisbet & Lagoy, 1992). The TEF-weighted concentrations were calculated for each PAH identified by Equation 3.2 in order to calculate the TEQ.

$$TEQ = \Sigma(TEF_i \times c_i) \quad \text{Equation 3.2}$$

Where  $TEF_i$  is the TEF for a given PAH and  $c_i$  is the concentration of that PAH in the sample. Where PAHs were identified below the LOQ, the LOQ was used as the concentration in equation 3.2, and if the PAH was not identified, the LOD was used as the concentration value as a worst-case scenario in terms of human health risk.

### 3.12 Chlorophyll studies using fluorescence spectroscopy

As a result of the observed matrix effects, an investigation into the chlorophyll content of lichens was made. It was decided that fluorescence spectroscopy would be used to determine the relative concentration of chlorophyll in the sample extracts of interest. Since chlorophyll exists in two forms: chlorophyll *a* and chlorophyll *b*, both were monitored using a FluoroMax4 Spectrofluorometer (Horiba Scientific, Jobin Yvon Technology, Edison, NJ).

3 ml of toluene was placed inside a clean quartz cell and excited at both 410 nm (chlorophyll *a*) and 452 nm (chlorophyll *b*) separately. This was done in order to establish a 'blank response'. No peaks were observed (420 – 800 nm). The 7 samples of interest from the study sites were analyzed individually at both

excitation wavelengths of 410 nm and 452 nm, by placing 30  $\mu\text{l}$  of the sample extract in a clean quartz cell to which 3000  $\mu\text{l}$  toluene was added with aspiration using the pipette. The quartz cell was rinsed with methanol and toluene between analyses.

Summing the peak heights of both chlorophyll *a* and chlorophyll *b* for each sample of interest gave a comparative indication of the total chlorophyll content of each sample.

### 3.13 Concluding remarks

In this chapter, the sampling procedure for the collection of a bulk lichen sample for method development use was described. The sampling methodology as well as sample storage was clearly defined for both the bulk sample and samples of interest collected in the Pretoria Industrial area, above the Daspoort Tunnel in Pretoria West, at Cathedral Peak in the Drakensberg and at the Cape Point Nature Reserve. The ultrasound assisted extraction, microwave assisted extraction, QuEChERS and Soxhlet extraction techniques were detailed, including extraction times, lichen mass used, solvent schemes and the extraction temperatures. The cleanup procedure using  $-\text{NH}_2$  cartridges and *n*-hexane:dichloromethane (65:35, v/v) followed by a blow down and reconstitution in toluene was given in Section 3.6. The GC-MSD, GC-TOFMS and HPLC methods were discussed, and a final optimized GC-MSD method was given in Section 3.10. It was decided that thermal desorption and GC-TOFMS would not be used further in the study, owing to the low combustion point of lichen and the inefficient separation of all the PAHs of interest respectively on the Rxi®-5SilMS column despite oven program optimization, when compared to the GC-MSD method. The investigation into the chlorophyll content of the different samples of interest was performed using a spectrofluorometer and the details of analysis discussed in Section 3.12. Chapter 4 will discuss the results of the analyses described in Chapter 3, including selected chromatograms, SEM micrographs, calibration curves, LODs, LOQs and the TEQs of the samples of interest.

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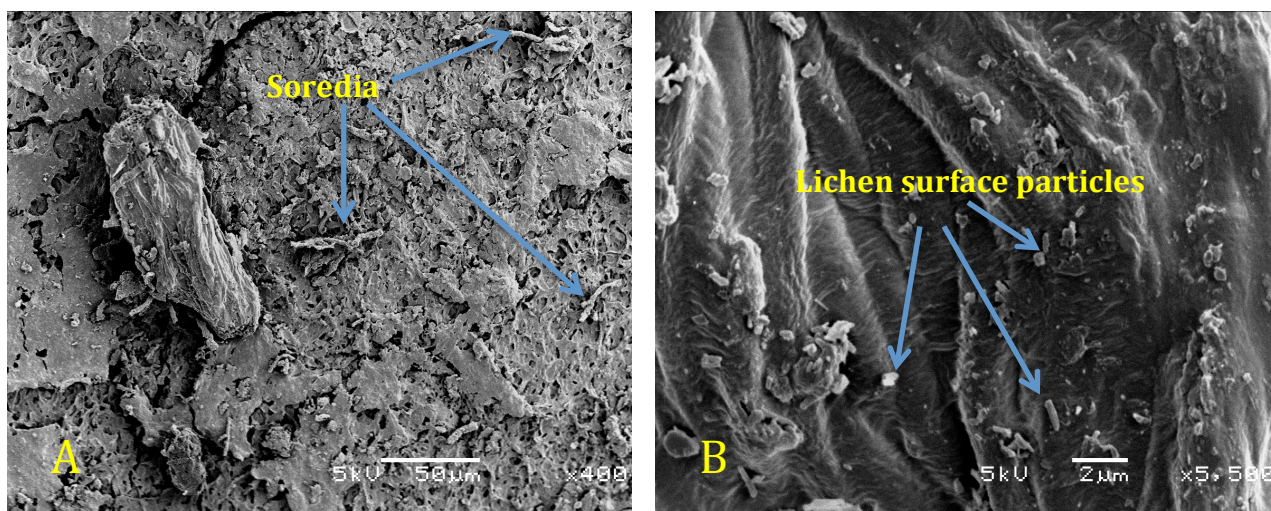
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## Chapter 4: Results and Discussion

### 4.1 Scanning Electron Microscopy

Figure 4.1 shows a SEM micrograph of a section of a lichen thallus cleaned of exogenous dirt and dried at 35 °C at a magnification of 400x (Figure 4.1A) and 5500x (Figure 4.1B), observed at 5 kV. The cortical hyphae are tightly held together and this often causes the surface to appear granular, as seen in Figure 4.1B. Garty et al (1979) studied the localization of metals within the lichen thallus, and the resulting SEM micrographs produced had significant similarities to the SEM micrographs produced in this study, where irregularly shaped particles on the surface were considered likely to be metal-based association. This is complemented by a study by Chaparro et al (2013) that described the presence of magnetite-like minerals on the surface with grain sizes between 0.1  $\mu\text{m}$  and 5  $\mu\text{m}$ , similar in size to particles observed on the lichen upper cortex in Figure 4.1B.



**Figure 4.1: SEM micrographs of a section of a *Parmotrema austrosinense* (Zalhbr) Hale lichen viewed at 5 kV at a magnification: 400x (A) and 5500x (B).**

The SEM micrographs of *Parmotrema austrosinense* (Zalhbr) Hale obtained in this study were less smooth than that of *Pseudevernia furfuracea* (Adamo et al., 2008) and our lichen contained many more cylindrical particles, suggesting that either more spores or more lichen substances (acids) could be present on the surface (Figure 4.1B). These cylindrical particles were not characterized by SEM with energy dispersive X-ray spectroscopy (SEM/EDX). Lichen substances are

generally extracellular acidic compounds, found in the crystalline form and are soluble in organic solvents (Brown & Hooker, 1977).

The surface of the lichens in our studies appeared more inconsistent than the lichens observed by Adamo et al (2008), with ours having many holes and uneven contours. The spherical clusters seen on *P. furfuracea* by Adamo et al were not seen on *P. austrosinense* in our study, suggesting that the lichen under observation had not been exposed to the range of metal-based compounds that theirs had.

The application of SEM/EDX on lichens sampled at the bulk sampling site showed that the irregularly shaped particles had high percentages of Si and Fe on the surface, with moderately high levels of Al, Mg and Cu. This corresponds with studies on lichens by Ward (1989) who found that the source of Cu in lichens is from brake pads (as well as Mn and Zn), lubricating oils and diesel engine emissions and that Al, Ca, Zn and Fe originate from automobile catalysts (Helmers, 1996). Ba was also found on the surface of a few lichen samples, indicative of emissions from the combustion of unleaded gasoline (Monaci & Bargagli, 1997). The likely sources of the high Si levels, as well as the low levels of K are from the resuspension of soil and dust impaction. In a study by Garty et al (2001), high levels of K, Fe, Mg, Zn, Mn, Pb and Cu were found in lichens sampled along busy streets, but were not found on lichens sampled next to highways. The particles studied using SEM/EDX were not produced by the lichens (therefore are not lichen substances) but were the result of pollutant deposition on the lichen thallus. Since our bulk sample was taken from trees next to a busy street away from a highway, it can be concluded that our results were similar to the findings in the study by Garty et al (1996). Some of the SEM/EDX results are given in Appendix C.

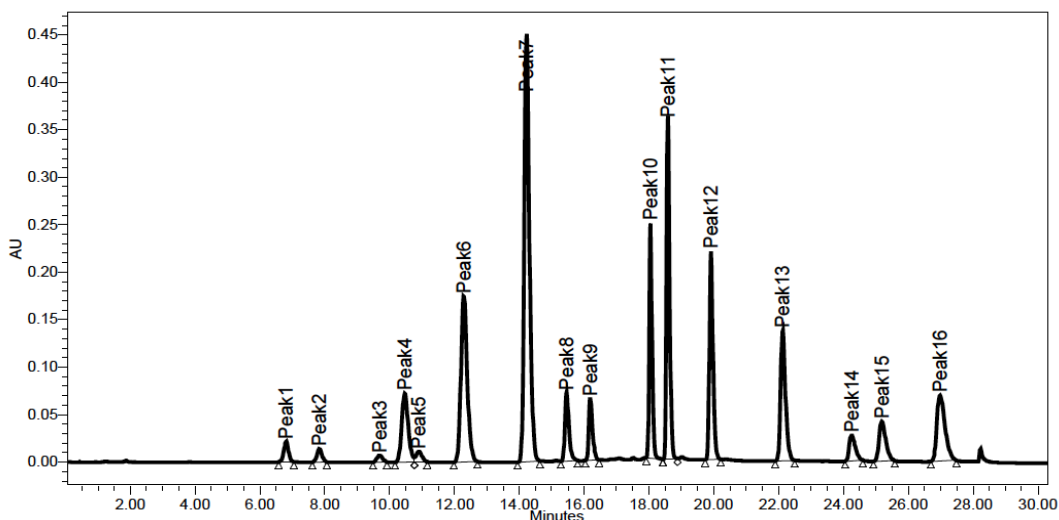
#### **4.2 High performance Liquid Chromatography**

The individual standards were analyzed using the HPLC-DAD and a select few chromatograms are given below. Isobaric pairs such as phenanthrene/anthracene, pyrene/fluoranthene and

chrysene/benzo[*a*]anthracene were efficiently separated in the PAH mix standard, as shown in Figure 4.2. The peak identities and retention times are given in Table 4.1

However, once a PAH standard of low concentration ( $0.1 \mu\text{g}\cdot\text{ml}^{-1}$ ) was analyzed, as shown in Figure 4.3, a few PAHs were no longer identified in the mix. Detection levels were poor at  $0.1 \mu\text{g}\cdot\text{ml}^{-1}$  concentrations where it can be seen that some of the PAH peak responses were at levels comparable with that of the solvent blank (Figure 4.4). The analysis of one of the spiked USAE extracts is shown in Figure 4.5, with peak overlap in the region of 10 – 20 min, where 9 of the PAHs of interest were expected to elute. One can see that the matrix is not separated from the analytes on the column, such that the identification and quantification of PAHs is compromised.

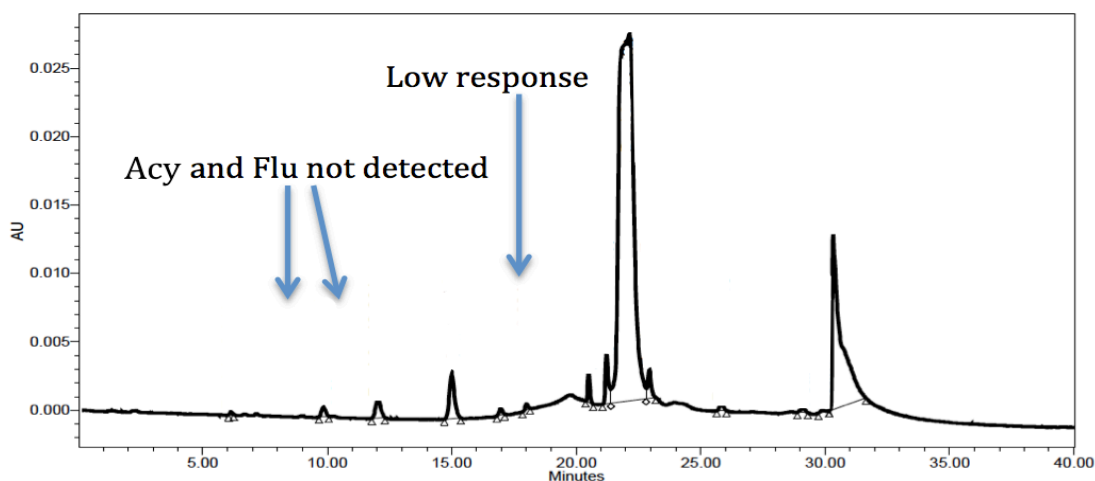
Furthermore, carryover of analytes was observed, despite performing multiple blank runs in between analyses and rinsing the injection port multiple times. Since HPLC was not able to deliver the sensitivity required for the detection of PAHs in our analysis, nor sufficiently separate the analytes from the matrix background, it was decided that HPLC would not be further pursued as an identification or quantification technique for the analysis of PAHs in the prepared lichen extracts.



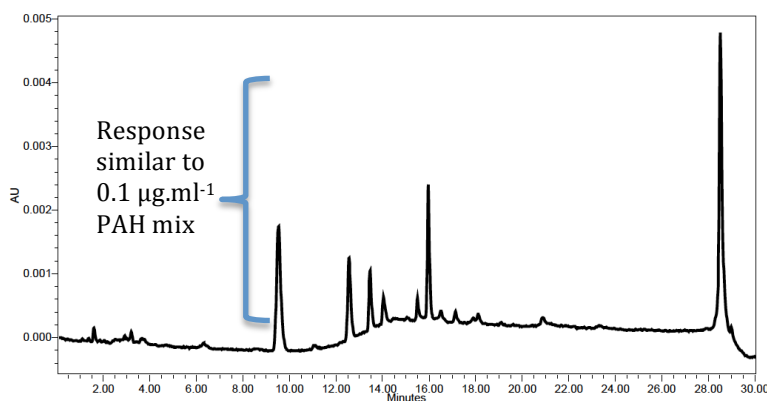
**Figure 4.2: HPLC-DAD chromatogram of a 20  $\mu\text{l}$  injection of the  $10 \mu\text{g}\cdot\text{ml}^{-1}$  PAH mix standard showing efficient separation of all 16 PAHs (refer to Table 4.1 for peak label information)**

**Table 4.1: Identity of the peaks in Figure 4.2 and the corresponding retention times (RT) of the PAHs**

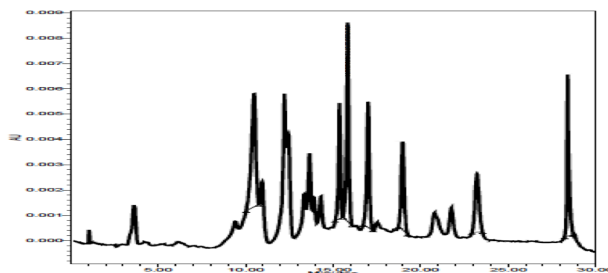
Peak number	PAH identity	RT (min)
1	Nap	6.810
2	Acy	7.834
3	Ace	9.692
4	Flu	10.469
5	2-bromonaphthalene	10.902
6	Phe	12.289
7	Ant	14.227
8	FluAn	15.463
9	Pyr	16.193
10	BaA	18.049
11	Chr	18.586
12	BbF	19.916
13	BaP	22.128
14	DahA	24.255
15	BghiP	25.187
16	IcdP	26.981



**Figure 4.3: HPLC-DAD chromatogram of the 0.1  $\mu\text{g}\cdot\text{ml}^{-1}$  PAH mix standard showing poor separation and low response for a few PAHs**



**Figure 4.4: HPLC-DAD chromatogram of the blank solvent, with absorbance units (AU) of similar magnitude to some PAH peaks in Figure 4.3**

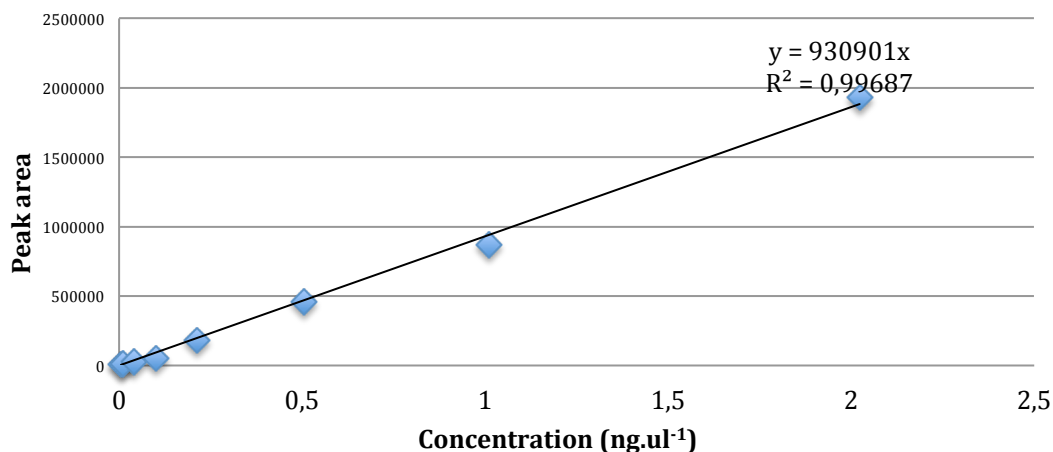


**Figure 4.5: HPLC-DAD chromatogram of a 20 µl injection of the 2 hr equilibrium spiked USAE dichloromethane based lichen extract**

### 4.3 GC-MSD quality control and calibration curves

The precision of the GC-MSD method was studied in terms of both interday and intraday results (Thompson et al., 2002), as discussed in Section 3.10. Figures 3.25 - 3.27 provide an understanding of the precision of the technique, where the results for the intraday variation (n=5) gave a RSD value of 7 %, and a 23 % RSD was observed for the interday (n=5) variation study. These results are similar to a GC/MS study on PAHs in sediments (Rocha et al., 2011).

The individual PAH concentration values used to construct the calibration curves were adjusted according to the accurate mass of each PAH in the standard, as per the certificate of analysis given in Appendix D. The calibration curve for fluorene is given below in Figure 4.6 for example, and the remaining calibration curves for the standards in pure solvent are given in Appendix E. Good linearity was found across the concentration range investigated, with  $R^2$  values between 0.987 – 0.998, using the solvent blank as the y-intercept at x=0.



**Figure 4.6: Calibration curve for fluorene in toluene using GC-MSD**

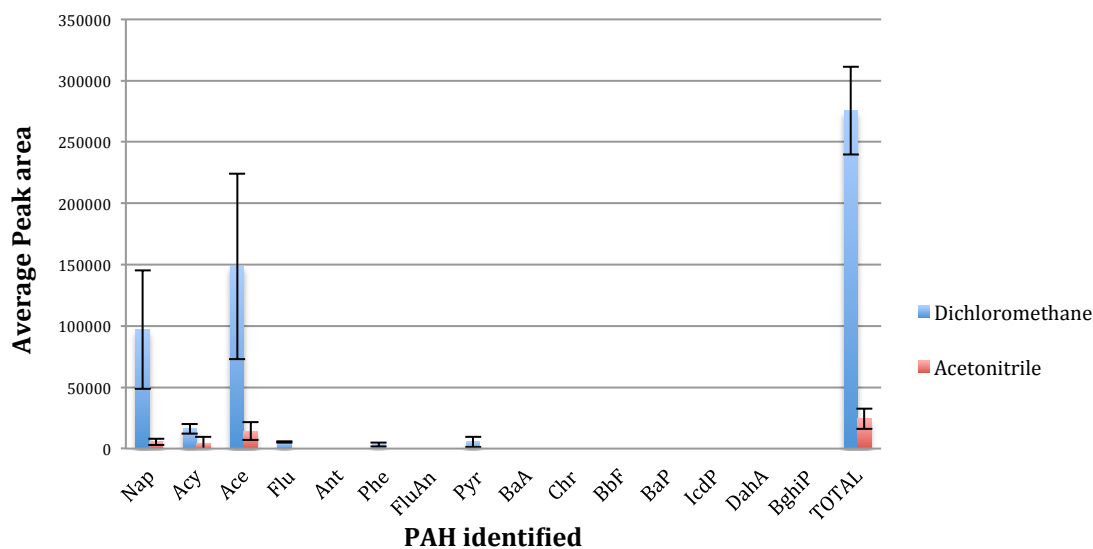
#### 4.4 Soxhlet extraction results

Figure 4.7 shows the results from the analysis of the bulk lichen sample extracts using Soxhlet as the sample preparation technique, with dichloromethane and acetonitrile as extraction solvents. Acetonitrile performed poorly, extracting only 3 PAHs from the lichen matrix, and the recovery of the internal standards was determined to be < 10%. This result is incongruent with other PAH studies using lichens, where acetonitrile was used successfully in studies by Augusto et al (2009; 2010) which found a range of both light and heavy PAHs, with the most abundant PAHs being Phe, FluAn, Pyr and Nap. However, the most common PAHs identified in our lichen samples were similar to the findings by Augusto et al but using QuEChERS, not acetonitrile-based Soxhlet to extract the PAHs of interest (see Section 4.8.3). It is possible that the acetonitrile-based Soxhlet used in this study was not efficiently extracting the heavier PAHs from the lichen matrix to levels above the detection limits.

The results of the dichloromethane-based Soxhlet extraction showed better qualitative and quantitative extraction compared to the acetonitrile-based extracts, extracting 6 PAHs, with a much larger average peak area for the sum of all detected PAHs. The recoveries of the internal standards were also higher for the DCM extracts, at 4.17 % and 19.7 % (phe-d<sub>10</sub> and pyr-d<sub>10</sub>, respectively). However, this recovery is still poor and unacceptable for quantitative work. The results were unexpected since a study using dichloromethane as extraction solvent by Domeño et al (2006) showed recoveries > 65 %. A study by Bajpai et al (2013) used DCM (and a 16 hr extraction time), extracting all 16 EPA priority PAHs from native lichen samples. The %RSD range in peak area for individual SIM ions of the identified PAHs in the DCM based extracts was 11.4 – 78.0 % and for the acetonitrile based extracts, the %RSDs for the identified PAHs were 46.6 – 117%. The observed differences between this study and other successful Soxhlet extractions was possibly due to inter-species differences (Bajpai et al., 2013; Domeño et al., 2006); such that the lichen species used in this study was less susceptible to the extraction of PAHs using the solvents which had been successfully applied in other studies on different lichen species. We conclude

that since the detection limits in both studies were comparable with our detection limits (where Domeño et al used GC-MS and Bajpai et al used HPLC-V-UV) (see Section 4.10.1), the differences are likely to be as a result of inter-species differences and not the analytical conditions.

The extraction efficiency using both solvent schemes is likely to improve with an increase in extraction time, as in studies by Bajpai et al (Bajpai et al., 2013) and Augusto et al (Augusto et al., 2009), but bearing in mind the desired outcomes of this project, if the total extraction time was to be increased to 24 hr, the procedure would no longer meet the requirements of a quick, greener extraction procedure. As a result, Soxhlet was considered to be unacceptable in this study as a result of the poor recoveries, poor extraction efficiencies and required increased extraction times.



**Figure 4.7: Comparison between dichloromethane and acetonitrile as extraction solvents using Soxhlet showing the standard deviation amongst replicate extracts (n=3)**

## 4.5 Microwave Assisted Extraction (MAE) results

### 4.5.1 Solvent optimization for MAE

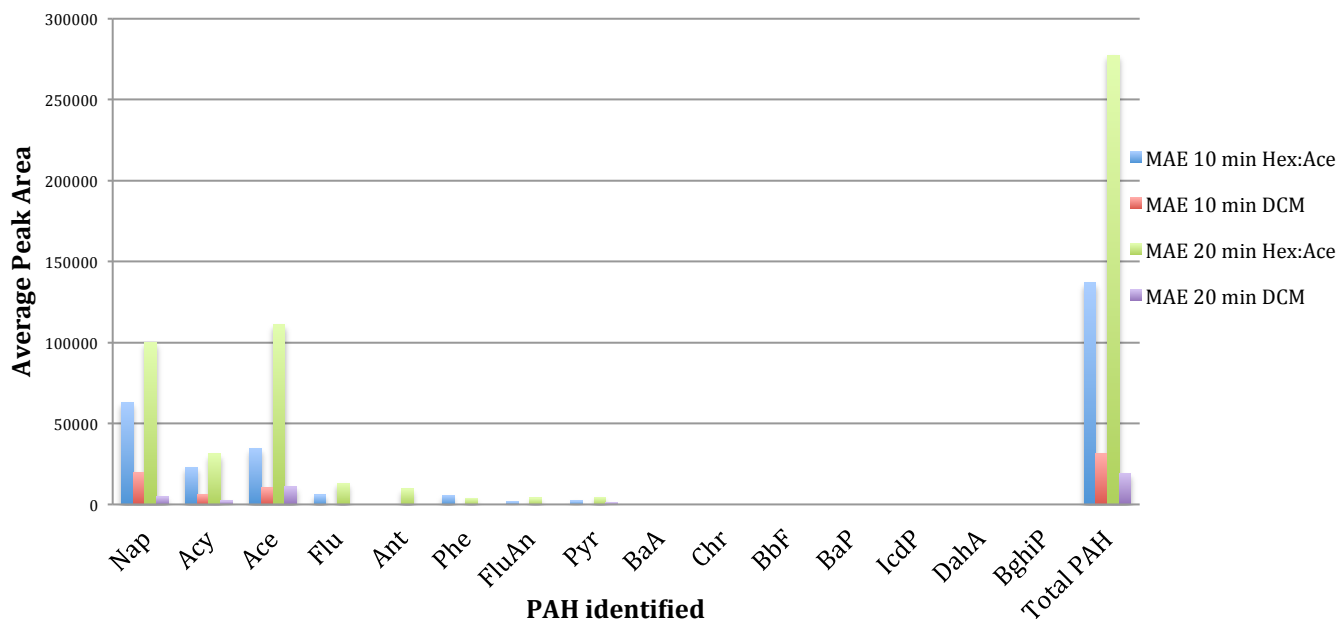
The first investigation using the principles of MAE was to establish which solvent scheme extracts PAHs from the lichen matrix most efficiently. As can be seen in Figure 4.8, the *n*-hexane:acetone (1:1, v/v, abbreviated as MAE Hex: Ace) – based

extraction performed better than when using dichloromethane as solvent (abbreviated as MAE DCM), both qualitatively and quantitatively. The application of *n*-hexane:acetone as extraction solvent extracted more PAHs, extracting heavier PAHs such as Phe, FluAn, Pyr and Ant, as opposed to the dichloromethane extraction which extracted only the lightest PAHs: Nap, Acy and Ace. Comparison of the total PAH peak area confirmed that MAE using *n*-hexane:acetone is preferred, since the sum of the peak areas far exceeded that of the dichloromethane-based extracts.

Comparing the recovery of the internal standards in the dichloromethane and *n*-hexane: acetone extracts in Table 4.2, it can be seen that *n*-hexane: acetone performed better and confirms it as the choice of solvent when using MAE, over dichloromethane. However, the percent relative standard deviation of the identified PAHs is better for the dichloromethane-based extracts. The range of the %RSD suggests that the *n*-hexane:acetone extraction is less precise, but the larger %RSD values in the *n*-hexane:acetone extracts were for the heavier PAHs (FluAn and Pyr) which were present at very low levels, and were unseen in the dichloromethane extracts. At lower levels of analysis, a small deviation has a large impact on the relative deviation.

This suggests addressing the homogeneity of the sample as well as improving the extraction procedure by reducing the introduction of human error is important in using lichens as biomonitors for PAHs. This is a matter which needs to be addressed and will be discussed further in Chapter 5.





**Figure 4.8: Comparison of different solvent schemes for MAE at 10 and 20 min extraction times with error bars omitted to avoid cluttering of the data, %RSD given in Table 4.2**

**Table 4.2: Mean recoveries of the internal standards as well as the %RSD values for identified PAHs for both solvent schemes used during MAE with a total extraction time of 10 min, n=3**

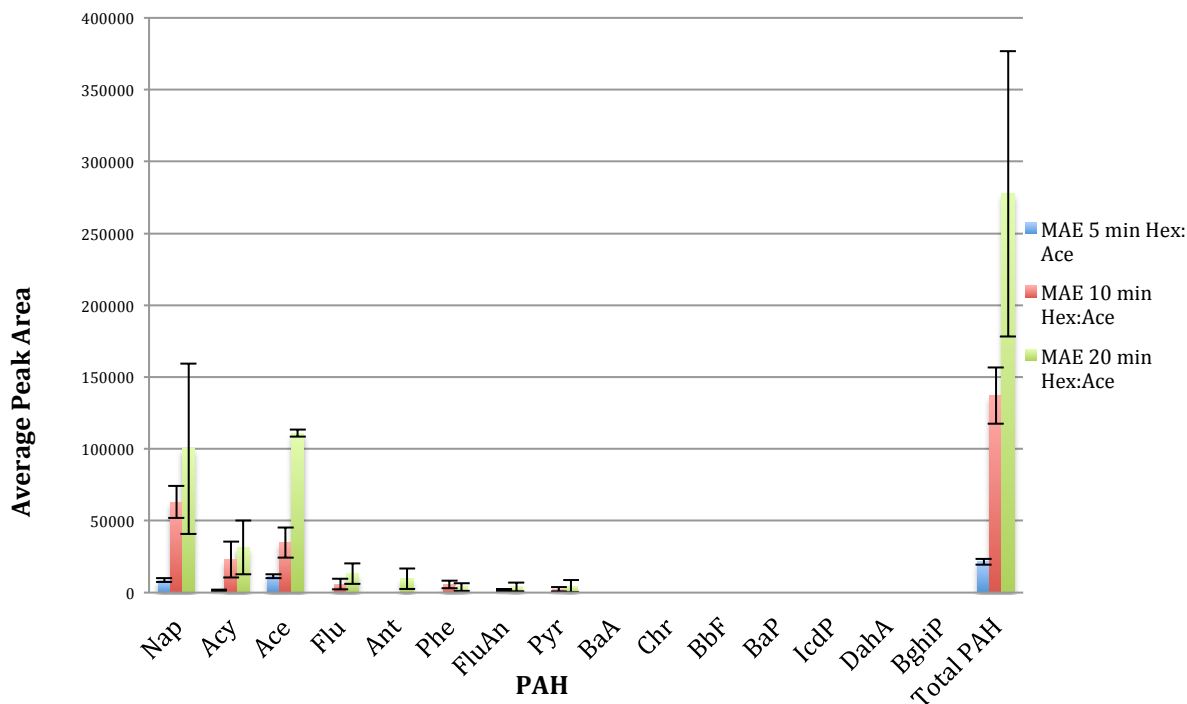
Conditions	% Phe-d <sub>10</sub> recovery	% Pyr-d <sub>10</sub> recovery	%RSD of light weight <sup>a</sup> PAHs	%RSD range of medium weight <sup>b</sup> PAHs
10 min Hex:A	8.46	9.00	12.8 - 51.7	36.2 - 64.5
10 min DCM	4.03	2.19	5.63 - 29.0	Not detected

<sup>a</sup> Light PAHs: Nap, Acy, Ace

<sup>b</sup> Medium weight PAHs: Flu, Ant, Phe, FluAn, Pyr

#### 4.5.2 Optimization of extraction time using *n*-hexane: acetone as solvent

As seen in Figure 4.8, *n*-hexane:acetone performed better than dichloromethane in terms of the amount and total number of PAHs extracted. The extraction time then needed to be optimized, and the results of the analysis can be seen in Figure 4.9. Applying an extraction time of 5 min was seen to be insufficient, compared to the 10 and 20 min extraction times. The total number of PAHs extracted from the bulk sample was only determined to be 3, with the 5 min extraction time, as opposed to 7 (10 min) and 8 (20 min). Comparing the total peak area of all identified PAHs, the 5 min extractions were dwarfed by the other two extraction times. Overall, the 20 min extraction time extracted more PAHs (8 in total) as well as provided the highest total peak area for all identified PAHs.



**Figure 4.9: Comparison between different extraction times for MAE using *n*-hexane:acetone (1:1, v/v) with error bars shown (n=3)**

As in the solvent optimization study, the spike recoveries for the longer extraction times were poor, with recoveries of 8.46 and 9.00 (phe-d<sub>10</sub> and pyr-d<sub>10</sub>, respectively) for the 10-min extraction, and 11.9 and 8.34 for the 20 min extraction. The recovery for the deuterated phenanthrene internal standard in the 5 min extraction was above 127%, suggesting that matrix effects were influencing the analytical response. Lower %RSD values, as well as improved spike recoveries for the 5 min extraction, may be as a result of the non-selective degradation of PAHs at longer extraction times (10 and 20 min extractions) (Camel, 2000). The non-selective degradation increases the variability between extracts, leading to a large %RSD values. Some improvements which could be made to the MAE method in future is the use of a stirrer to ensure mixing during extraction, or even new technologies such as stir-bar sorptive extraction (Alvarez-Avilés et al., 2007) which protects the extracted analytes by the use of a non-microwave absorbing sorbent.

**Table 4.3: Percent recoveries of the internal standards using *n*-hexane:acetone-based MAE and the %RSD range for identified PAHs in the extracts, n=3**

Extraction Time	% Phe-d <sub>10</sub> recovery	% Pyr-d <sub>10</sub> recovery	%RSD range light <sup>a</sup> PAHs	%RSD range medium <sup>b</sup> PAHs
5 min	127	36.6	12.4 - 52.0	Not detected
10 min	8.46	9.00	12.8 - 51.8	36.2 - 64.5
20 min	11.9	8.34	30.0 - 58.7	48.8 - 76.6

<sup>a</sup> Light PAHs: Nap, Acy, Ace

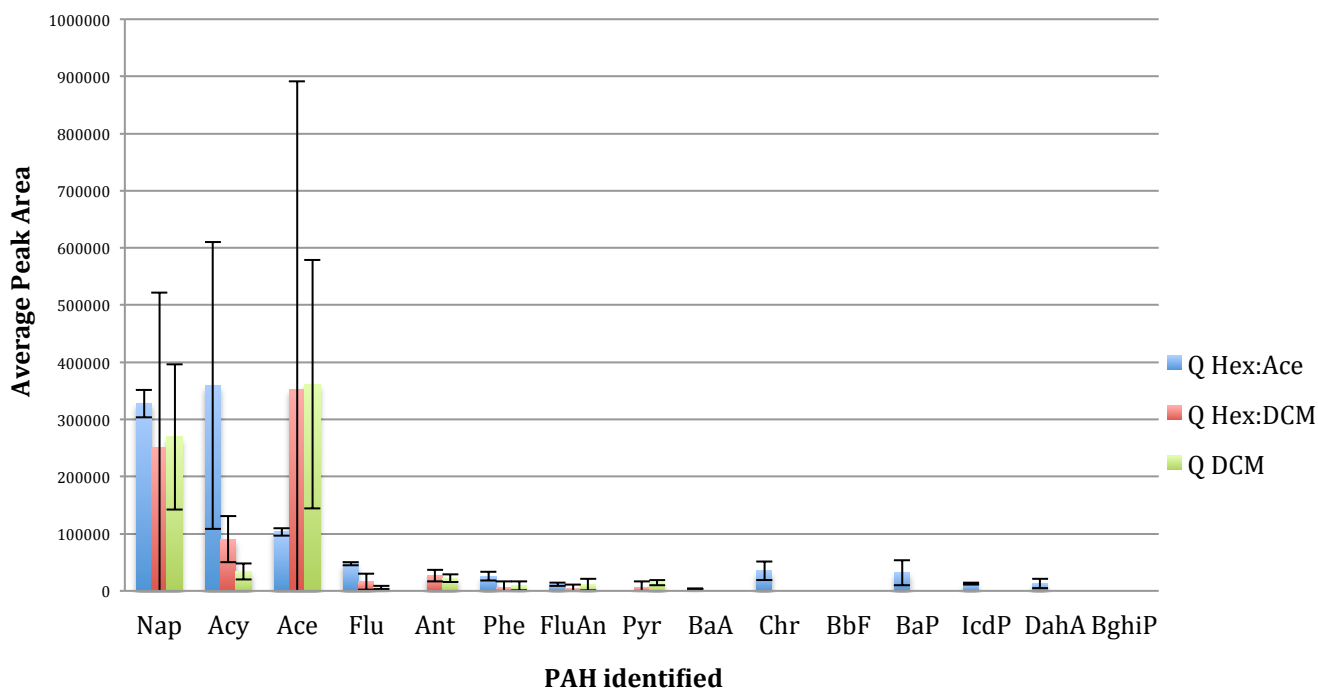
<sup>b</sup> Medium PAHs: Flu, Ant, Phe, FluAn, Pyr

## 4.6 QuEChERS results

### 4.6.1 Solvent optimization for QuEChERS

Three solvent schemes were investigated, and the results can be seen in Figure 4.10. The extraction using dichloromethane only (Q DCM), as well as the extraction with *n*-hexane:dichloromethane (1:1, v/v) (Q Hex:DCM), performed well compared to the previously investigated sample preparation techniques, allowing for the identification of 8 PAHs in the bulk lichen sample. However, the hexane: acetone extraction (Q Hex:Ace) outperformed both of the other solvent schemes using QuEChERS, extracting 11 of the 16 PAHs of interest from the bulk lichen sample. Of particular interest is the extraction of heavier PAHs: BaP, IcdP and DahA, which were identified in none of the other extracts of the native bulk lichen sample.

Quantitatively, the Q Hex:Ace also performed best, as can be seen in Figure 4.10, where the average peak area for most of the identified PAHs is largest in the *n*-hexane:acetone extract. The variability in results indicates that establishing homogeneity of the PAHs across the lichen matrix sample may be an analytical challenge.



**Figure 4.10: Results for the solvent optimization scheme using QuEChERS on the bulk lichen sample using *n*-hexane:acetone, dichloromethane only and *n*-hexane:dichloromethane, with error bars shown, n=3**

Table 4.4 shows the recoveries of the internal standard spiked onto the lichen prior to extraction, for all three solvent schemes. It is clear from the % recovery of both phenanthrene-d<sub>10</sub> and pyrene-d<sub>10</sub> that Q Hex:A extracts had the best extraction efficiency. The recoveries for the internal standards in the DCM and *n*-hexane:DCM extracts were comparable, as are the %RSDs for the internal standard recoveries. The %RSDs for the internal standards were lowest for the Q Hex:A extract (15.2 and 30.6 %), confirming that Q Hex:A should be the solvent scheme of choice.

When considering the %RSD for the PAHs identified in the extracts, the range of RSDs is similar for all three solvent schemes, where the lighter PAHs, present at higher concentrations (refer to Figure 4.10) had lower %RSD values, and the PAHs such as Chr and FluAn which were present at low concentrations had larger %RSDs, since deviations at low levels of the analytes response have a large impact on the %RSD. The varied %RSD across all three solvent schemes again brings the homogeneity of the bulk lichen sample into question, as well as the extent to which the matrix may be interfering with the analyte's response.

**Table 4.4: Percent spike recoveries, with related %RSD shown in brackets, of the different QuEChERS solvent schemes investigated as well as the %RSD range for all identified PAHs in the different QuEChERS extracts, n=3**

	Solvent scheme		
	Q Hex:A	Q Hex:DCM	Q DCM
Phe-d <sub>10</sub> % recovery	96.2 (15.2)	44.1 (23.9)	41.4 (31.8)
Pyr-d <sub>10</sub> % recovery	179 (30.6)	31.3 (46.3)	31.3 (27.0)
%RSD range of light <sup>a</sup> PAHs	6.32 - 69.9	4.12 - 127	41.1 - 111
%RSD range of medium <sup>b</sup> PAHs	5.31 - 29.5	38.3 - 173	29.3 - 94.7
%RSD range of heavy <sup>c</sup> PAHs	13.4 - 70.0	Not detected	Not detected

a Light PAHs: Nap, Acy, Ace

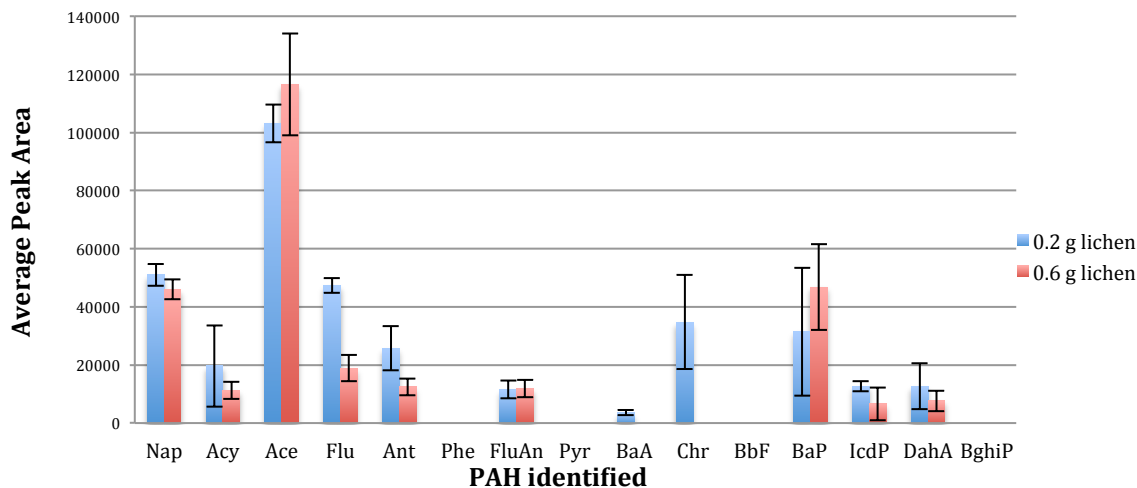
b Medium PAHs: Flu, Ant, Phe, FluAn, Pyr

c Heavy PAHs: BaA, Chr, BbF, BaP, IcdP, DahA, BghiP

Not only did the varying %RSDs necessitate attention, but also observed tailing in the chromatograms indicated that regular changing of the inlet liner was mandatory to ensure that the samples were not contaminating the column and interfering with results. Tailing was observed in both early and later eluting peaks, indicating that matrix effects may have been interfering with the signal. The analysis of PAH standards in clean solvent confirmed that a polarity mismatch did not exist between the column and the solvent, nor was the cutting of the column deemed necessary, since the peaks were symmetrically shaped.

#### 4.6.2 Increasing sample mass to investigate homogeneity

Since the matter of homogeneity was brought into question by the high %RSD values as detailed in Section 4.6.1, a study was performed to investigate whether increasing the sample mass would improve the method detection limits, and reduce the deviation between repeat extractions. The extraction conditions were chosen based on the results from the solvent optimization study as shown in Figure 4.10 and Table 4.4, using *n*-hexane:acetone (1:1, v/v) as the extraction solvent with GC-MSD analysis. The extraction solvent volume was kept constant, and the extracts were blown down to the same final volume (2 ml).



**Figure 4.11: Results from the investigation into whether increasing sample size would improve standard deviation, with error bars shown, n=3**

The results for the extractions using 0.6 g lichen and 0.2 g lichen were similar in PAH profile, where the 0.6 g extractions extracted 9 PAHs and the 0.2 g extract was found to contain 11 of the 15 PAHs of interest, as shown in Figure 4.11, with further data given in Appendix F. Some PAHs reflect higher concentration in the 0.6 g extract, and others reflect higher concentrations in the 0.2 g extract, without any noticeably consistent pattern. Table 4.5 shows the %RSD values for the identified PAHs in both samples. The lowest %RSD out of the two extracts is marked by a green colour, and it can quickly be seen that there is no clear improvement in the %RSD in either sample. The 0.2 g lichen extract has low %RSD for some of the lighter PAHs, and the 0.6 g lichen extracts have lower %RSD values for some of the heavier PAHs, but the results are inconsistent. A reason for the slight improvement in some of the heavier PAHs may be as a result of the improved detection limits resulting from the increased sample mass.

**Table 4.5: The %RSD for each identified PAH in the 0.6 g lichen extract and the 0.2 g lichen extract (n=3) with the lowest %RSD between the two highlighted in green**

PAH	0.6 g	0.2 g
Nap	7.42	7.35
Acy	26.0	69.9
Ace	15.1	6.32
Flu	24.0	5.31
Ant	23.4	29.5
FluAn	25.2	26.2
BaA	ND	25.4
Chr	ND	46.0
BaP	31.5	70.0
IcdP	85.9	13.4
DahA	46.3	62.4

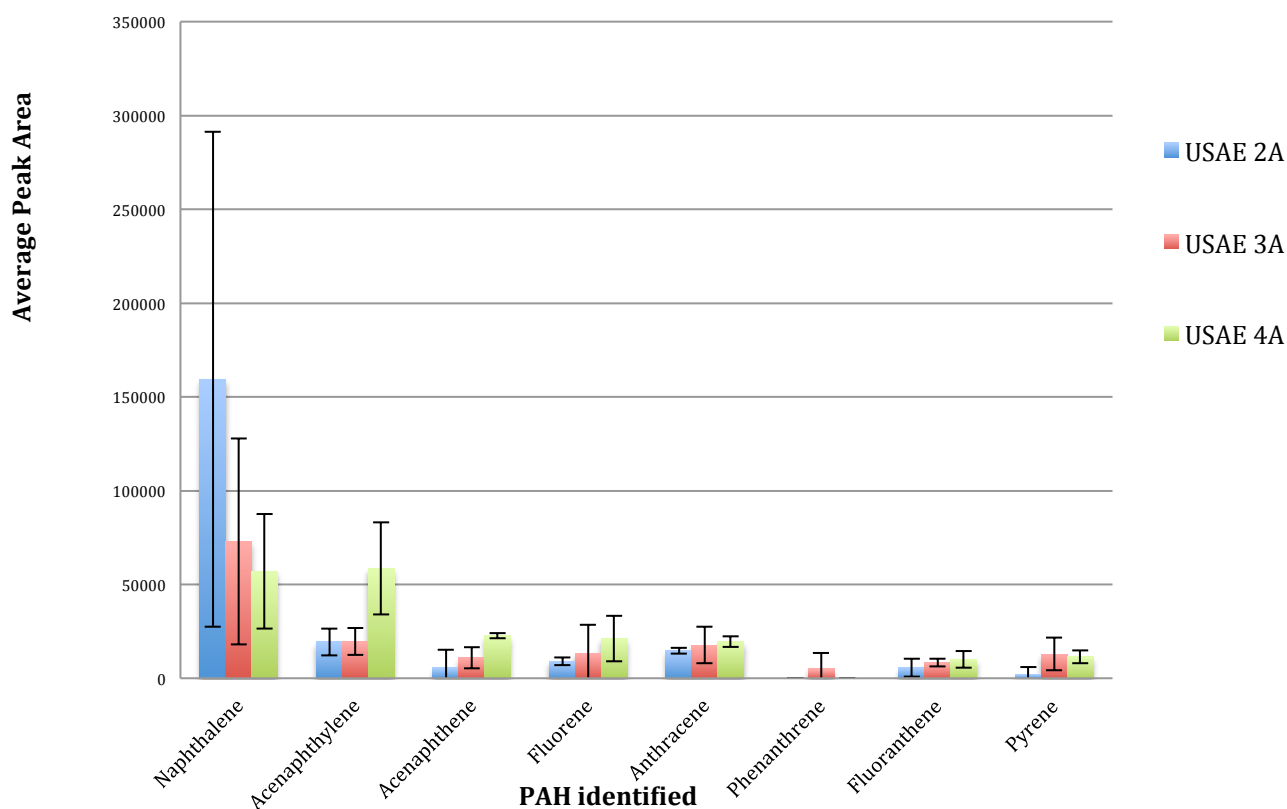
Future improvement of the QuEChERS method should focus on the challenge of homogeneity, and as such, future studies should focus on a thorough study of up scaling the sample mass. This should include the use of a range of solvent volumes, as well as different masses of extraction salts, since these factors all influence the distribution coefficient between PAHs trapped in the lichen matrix and the extraction solvent. Results from a more detailed investigation are necessary to draw meaningful conclusions regarding the homogeneity of the bulk sample.

## 4.7 Ultrasound Assisted Extraction (USAE) results

### 4.7.1 Number of consecutive extractions required

In order to establish how many consecutive repeat extractions were required to quantitatively extract PAHs from the lichen matrix using USAE techniques, the total peak area and standard deviations of PAHs for 2, 3 and 4 extractions (named USAE 2A, USAE 3A and USAE 4A respectively) were given in Figure 4.12. The average peak areas for each identified PAH, as well as the %RSD values are given in Table 4.6. The extracts were all reduced to the same volume prior to the SPE cleanup procedure, followed by the blow down step to produce an extract of 0.5 ml, eliminating any possible dilution effects.

From the results, it can be seen that two extractions are not sufficient to extract the PAHs qualitatively from the lichen matrix, with particular regards to the PAHs from MW 178 g.mol<sup>-1</sup> and higher. The %RSD of the USAE 2A extracts is also very high, suggesting that more than two extractions on the same matrix are required in order to reduce the %RSD, thus improving the precision of the preparation technique, as well as increasing the number of PAHs partitioned into the extract and improving the method limit of detection as a result of the greater number of extractions on the sample. Comparing the results of USAE 3A and USAE 4A, the peak areas are very similar, and the %RSD values do not improve for all PAHs with the extra extraction. It was therefore decided that 3 consecutive extractions on the lichen matrix would be sufficient, similar to other studies on PAHs in environmental matrices (Drabova et al., 2012; Ratola et al., 2006). The peak areas for Nap decrease with the number of subsequent extractions as a result of losses due to evaporation; heat generated as a result of friction caused losses of the most volatile PAH studied.



**Figure 4.12: Comparison between peak areas of identified PAHs in USAE DCM extracts with different number of total extractions, with no heavier PAHs seen n=3**



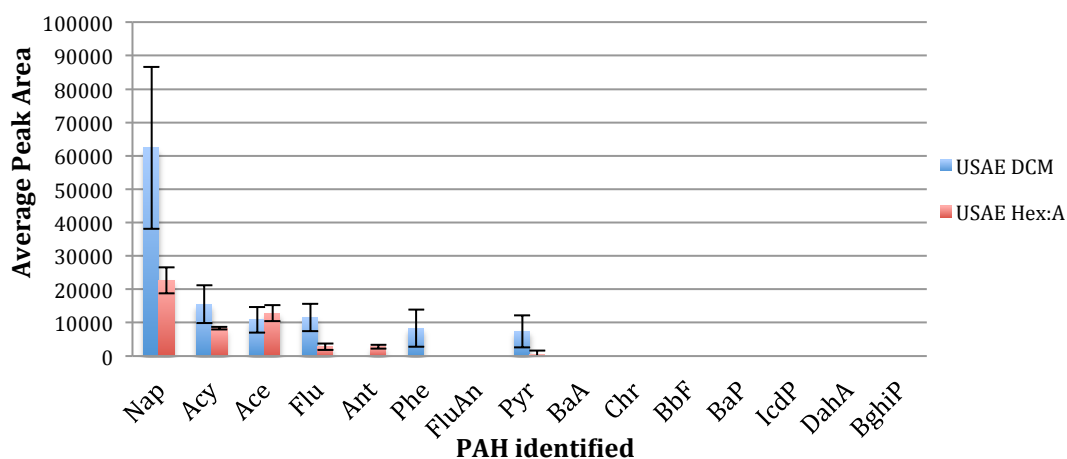
**Table 4.6: The average peak areas and %RSDs for PAHs identified in either 2, 3 or 4 consecutive extractions using dichloromethane on the bulk lichen sample, n=3**

PAH	USAE 2 A		USAE 3 A		USAE 4 A	
	Average	%RSD	Average	%RSD	Average	%RSD
Nap	159420	82.8	72939	75.2	57084	53.6
Acy	19405	37.1	19536	36.6	58580	41.9
Ace	5604	173.2	10879	52.7	22653	6.5
Flu	8973	22.0	13355	113.3	21088	57.8
Ant	14591	10.2	17680	55.1	19561	14.6
Phe	ND	ND	4962	173.2	ND	ND
FluAn	5574	87.1	8253	24.2	10155	44.6
Pyr	2170	173.2	12927	67.8	11460	30.4
BaA	ND	ND	ND	ND	ND	ND
Chr	ND	ND	ND	ND	ND	ND
BbF	ND	ND	ND	ND	ND	ND
BaP	ND	ND	ND	ND	ND	ND
IcdP	ND	ND	ND	ND	ND	ND
DahA	ND	ND	ND	ND	ND	ND
BghiP	ND	ND	ND	ND	ND	ND

#### 4.7.2 Solvent optimization for USAE

Figure 4.13 shows the comparison of the extraction efficiencies of two different solvent schemes using USAE. Hexane:acetone (1:1, v/v) was investigated as an extraction solvent since it had been used in this study for MAE as well as the QuEChERS technique. The investigation then served to establish whether *n*-hexane:acetone (USAE Hex:A) would extract a different PAH profile to DCM, and establish which extraction solvent would be more suitable in terms of recovery. Figure 4.13 clearly shows that dichloromethane extracted PAHs better than *n*-hexane:acetone under ultrasonic energy, even when comparing three consecutive extractions using DCM with four consecutive extractions using *n*-hexane:acetone. DCM extracted a greater quantity of each PAH, as seen when comparing average peak areas. Of the USAE *n*-hexane:acetone extractions, USAE 4 Hex:A provided the largest peak areas and the most number of PAHs extracted, as opposed to the three consecutive extractions which had been optimized for the USAE DCM extracts, as discussed in Section 4.7.1.

The precision of the USAE Hex:A extracts (n=3) was far better, with %RSD values ranging between 35.2 – 67.1 % for USAE DCM, and as low as 4.77 – 34.4 % for the USAE Hex:A extracts. However, the recovery values for the internal standards were far better for DCM (51.7 % and 17.0 % for phenanthrene-d<sub>10</sub> and pyrene-d<sub>10</sub>) when compared to the poor recoveries of Hex:A (8.48 % and 2.63 %). Despite the improved precision that the USAE Hex:A extracts had over DCM, it was decided that DCM would be more beneficial in terms of quantification as well as improved recovery of analytes. The fact that both solvent schemes have poorer recoveries for the heavier deuterated standard suggest that there could be a bias in the extraction method towards the lighter PAHs. This is also supported by the lack of any heavier PAHs (of MW 228 and above) in the extracts.

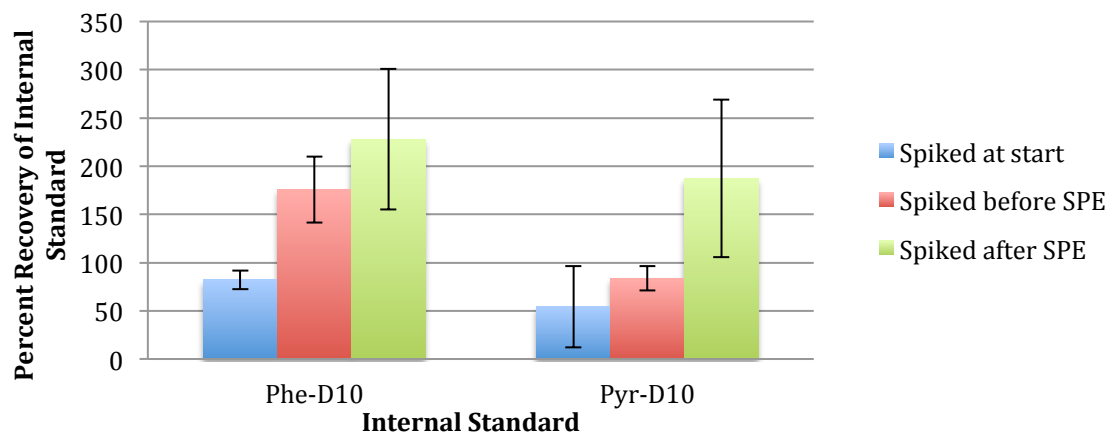


**Figure 4.13: Comparison of extraction efficiencies for USAE with extraction by dichloromethane and *n*-hexane:acetone, respectively, n=3**

#### 4.7.3 Identification of losses in USAE

Errors are introduced in every step of the sample preparation procedure, resulting in a cumulative effect, impacting the final recovery of analytes and extraction efficiency (González et al., 2014) (Bodnar et al., 2013). Therefore, the recovery of the internal standard was tracked in order to establish where PAHs were most likely to be lost during the extraction process for the USAE 3 DCM sample extraction. As shown in Figure 4.14, the recovery of the internal standard phenanthrene-d<sub>10</sub> is most significantly affected by the initial steps in the extraction process: sonication, transfer between vials and centrifuge tubes and the initial blow down to 2 ml. Since phenanthrene is of the lighter, volatile PAHs,

this result is not surprising since blowing down the extract, as well as transferring the solvent from one vial to another are steps that increase the volatilization of lighter PAHs from the solvent.



**Figure 4.14: Percent recovery of two internal standards at different stages of USAE 3 DCM sample preparation, including a spike at the start, a spike after USAE and a spike just after the SPE cleanup (n=3)**

On the other hand, the heavier internal standard, pyrene-d<sub>10</sub>, incurs most losses during the cleanup step, since the recovery of the spike before SPE is half of the recovery of the extract spiked after SPE. Since pyrene is a heavier PAH, it is less likely to be blown off during the concentration steps, or lost during the transfer between vials. This study raised concern regarding the suitability of the established cleanup procedure by Blasco et al (2007) since significant losses were observed for both phenanthrene-d<sub>10</sub> and pyrene-d<sub>10</sub>, implying that the spectrum of PAHs investigated in this study would be similarly affected by the cleanup procedure. The recommendations for addressing this matter are discussed further in Chapter 5.

After it was established that PAHs were being lost to different extents during the sample preparation steps, attention was given to the large % recoveries of the internal standard. Recoveries of over 100 % immediately raised questions about whether matrix effects were enhancing the signal of our analytes of interest. It was then decided that the four different sample preparation techniques applied in this study would be compared, and the respective peak areas of the internal standard (for recovery studies) as well as the peak areas of the PAHs identified, would be directly compared prior to any quantification. This was done in order to establish which sample preparation technique was most suitable before the

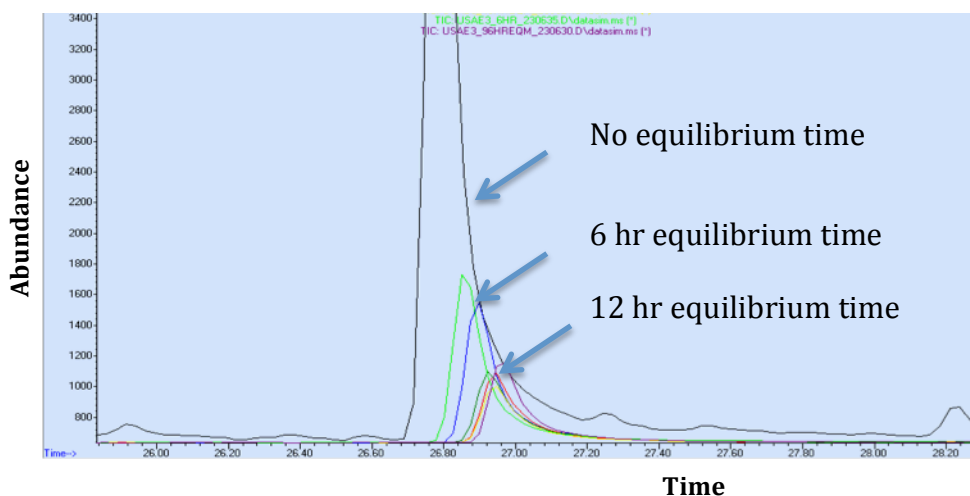
matrix-matched standards were prepared and analyzed, and then the matrix-matched standard curve would be used to quantify the results from the lichen samples of interest from various sampling sites. In order to compare the results from the different sample extraction techniques, it was assumed that the matrix effects would be similar for all the different extraction techniques employed in the analysis of the bulk sample.

## 4.8 Comparison of recoveries of different sample preparation techniques

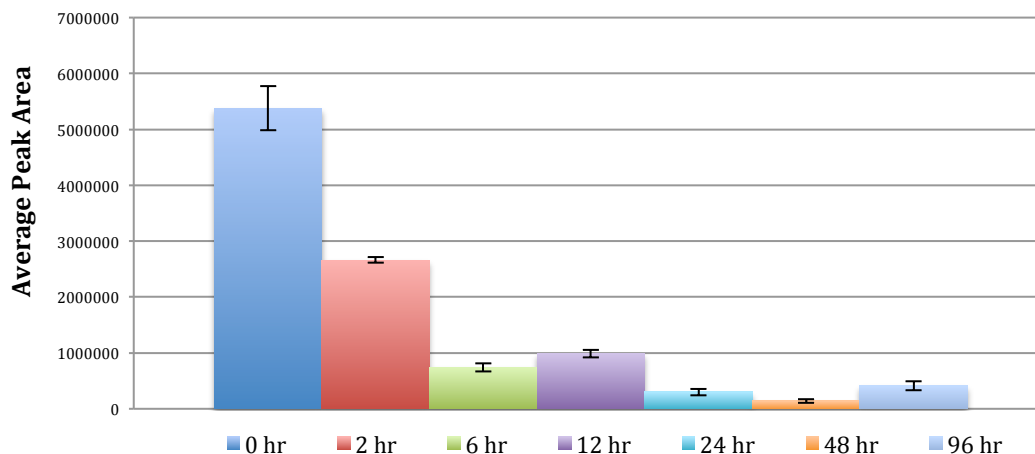
### 4.8.1 Ultrasound assisted extraction 0 – 96 hr equilibration study

Through the process of spiking lichens (with the PAH standard mix and the deuterated standard mix), and leaving the matrix to equilibrate in the dark for different time periods, the incorporation of PAHs into the lichen matrix would be better understood. It would also establish whether it is necessary to spike the internal standard ahead of time for a set period of time, prior to extraction.

Figure 4.15 shows the overlaid chromatograms from the 0 hr equilibrium (extracted immediately after spiking, black trace), the 6 hr equilibrium extract (green trace), and the 12 hr equilibrium extract (blue trace) for FluAn as an example. It can be seen that the analyte response decreases immediately from an equilibrium time of 0 hr, indicating that there appears to be no improvement in terms of recovery of analytes if the lichen sample is spiked and left to reach equilibrium with the spiked analytes prior to ultrasound assisted extraction.



**Figure 4.15: Comparison of response for the fluoranthene peak (MW 202 g.mol<sup>-1</sup>) relating to different equilibrium times (0 – 96 hr) after direct spiking of the lichen matrix with USAE DCM extraction**



**Figure 4.16: Comparison of the total PAH peak area with respect to the incorporation of PAHs into the lichen matrix over 96 hr after spiking with 2 ug PAH mix, n=3**

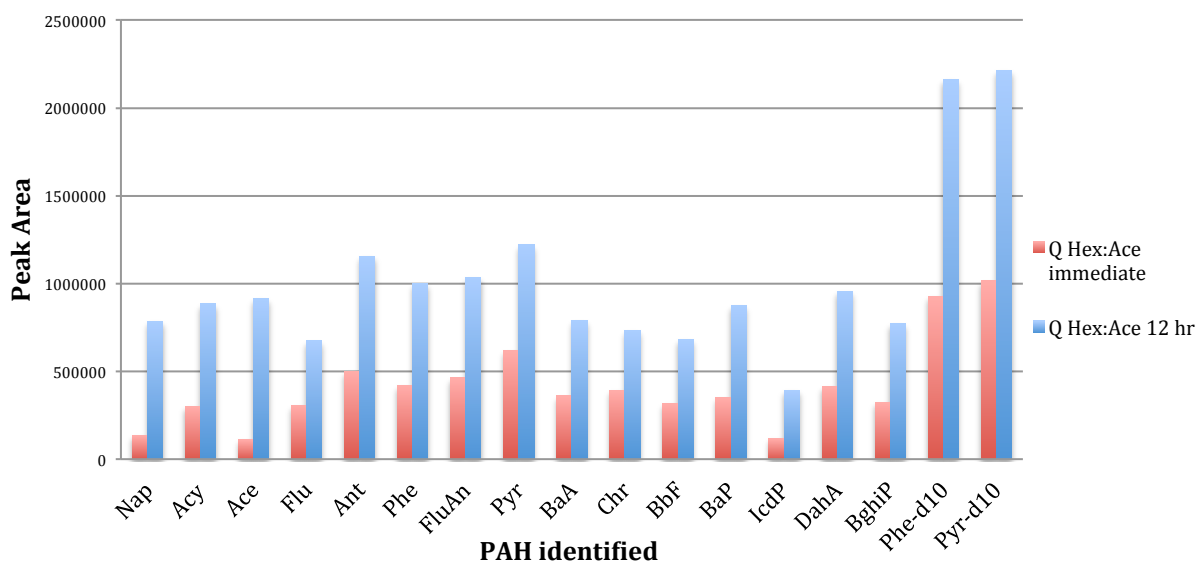
Figure 4.16 summarizes the results from the equilibrium study, showing the decrease in average total PAH peak area from 0 hr to 12 hr, at which there was a slight increase in average total PAH peak area, followed by a decline in peak area again up until 96 hr, where the peak area increased again slightly. This profile suggests a cyclic absorption and revolatilization of the spiked analytes from the lichen matrix. The continual decrease of analytes is likely to be as a result of two factors: losses due to the adsorption of PAHs onto the vial walls and the cap liner, as well as an incorporation of PAHs into the lichen matrix in a manner such that it is beyond the capabilities of the USAE procedure to then extract them from within the matrix.

A table summarizing the standard deviations and average total peak areas of the spiked PAHs is given in Appendix F. From the profile of PAHs identified at different equilibrium times, it is noted that in the 48 hr equilibrium extract (the extract with the lowest recoveries), both Chr and Ant were not identified. This is surprising, as it would be expected that the lighter, more volatile PAHs would suffer from losses to a greater extent than the mid-mass range PAHs. This result could be indicative of the preferential uptake of certain PAHs over others, by our species of interest, *Parmotrema austrosinense* (Zahlbr.) Hale. This type of preferential uptake of certain PAHs by different species has previously been identified (Blasco et al., 2011).

#### **4.8.2 Effect of 12 hr equilibration of spiked lichen samples using different extraction methods**

The results from the investigation on whether the recovery of the internal standard using the optimized QuEChERS extraction improved with a prolonged equilibration time, showed that the recovery of the PAHs spiked onto the matrix improved drastically with time when allowed to equilibrate for 12 hr. In contrast to the results from the USAE equilibrium study, the peak area for each identified PAH as well as the internal standards was nearly double the size of the peak for the extraction performed immediately after spiking onto the matrix, as can be seen in Figure 4.17. These results illustrate the powerful extraction efficiency of the QuEChERS technique, since it was observed that USAE was not as efficient at extracting the PAHs which had been left for periods of time to partition into the matrix, as described in in Section 4.8.1.

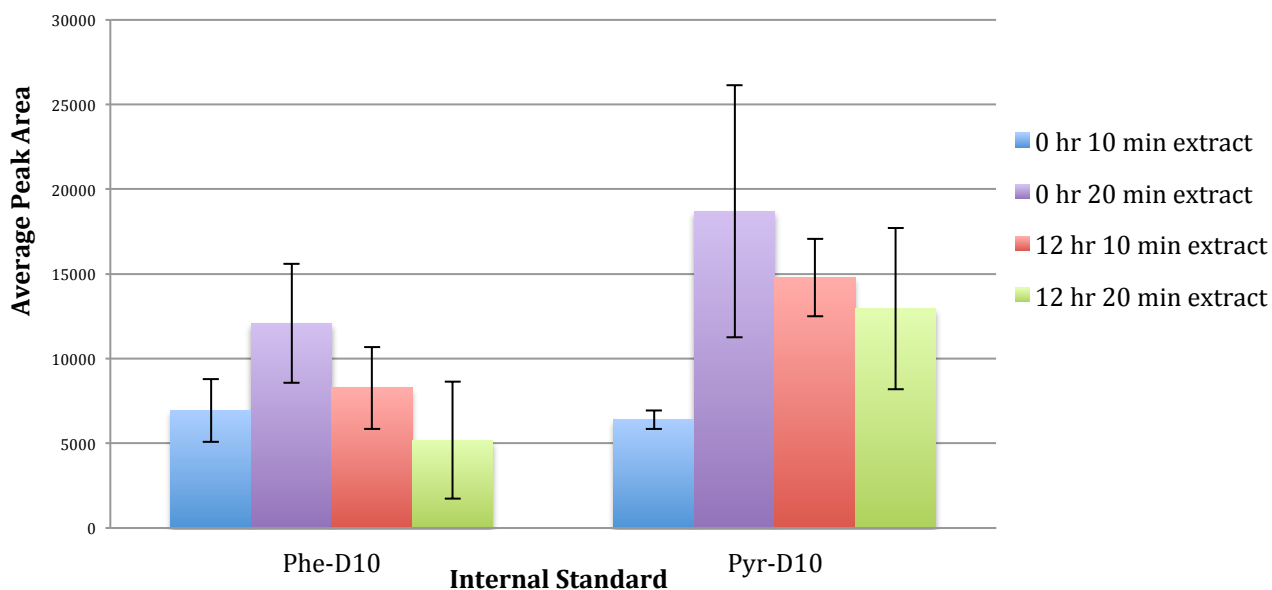
Unfortunately, this investigation was made towards the end of the project, and was thus not applied to the samples of interest (with results given in Section 4.11). Further investigations into what the optimal internal standard spike equilibrium time would be for the QuEChERS extraction is required in order to confidently establish the equilibrium period required prior to extraction, in order to quantitatively extract both the PAHs from the native lichen matrix, as well as the internal standard. Further statistical analysis of the results is also required.



**Figure 4.17 : Comparison of PAH peak obtained when applying QuEChERS extraction at 0 hr and 12 hr after spiking lichens with 2 ug PAH mix**

The importance of performing a study on the recovery of the internal standard in the optimized MAE sample preparation technique was understood, after obtaining conflicting results from the USAE and the QuEChERS spike equilibrium studies. It can be seen from the results displayed in Figure 4.18, that spiking the lichen matrix with the internal standard, and extracting it immediately for 10 min gave the worst recovery results for both phenanthrene-d<sub>10</sub> and pyrene-d<sub>10</sub>. The best recovery results were obtained for the immediate extraction (no equilibrium time) with a total extraction time of 20 min. These results reiterate the importance of a 20 min extraction time for the MAE technique, showing, as in Section 4.5.2 that 10 min are not sufficient to quantitatively extract the PAHs.

The reason for the lower recoveries of the internal standards left to equilibrate for 12 hr and extracted for 20 min (shown as green in Figure 4.18) could be due to revolatilization from the lichen matrix, or adsorption onto the quartz tube wall and the PTFE cap. It may also be that the PAHs are being incorporated into the lichen matrix in a manner beyond the extraction capabilities of the MAE process. It is therefore recommended that the lichens be extracted for 20 min immediately after being spiked, when using MAE to prepare the extracts for analysis.



**Figure 4.18: Comparison between lichens spiked with internal standard, left for 12 hours as well as spiked lichen extracted immediately, prepared using MAE Hex:Ac with 10 or 20 min extraction times, error bars shown (n=3)**

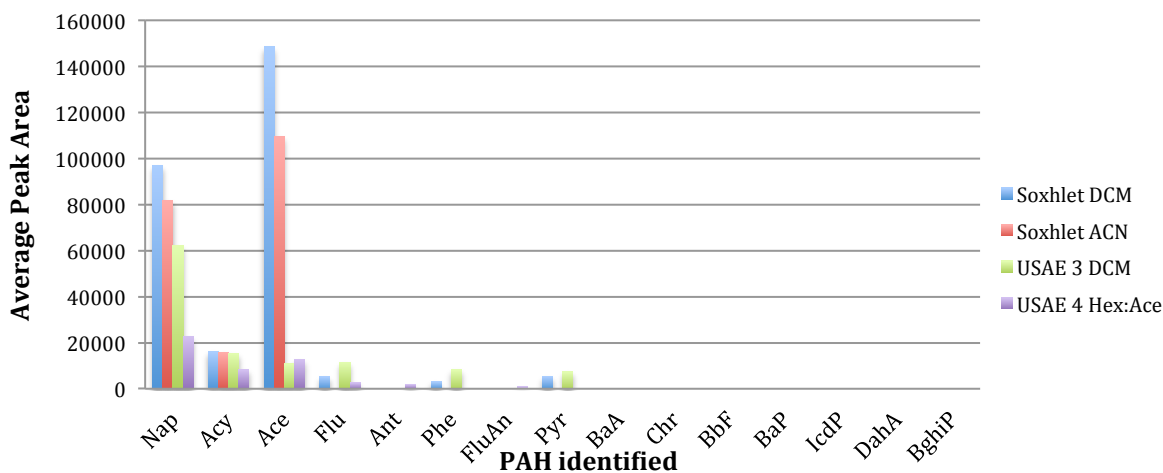
To our knowledge, no studies have previously been performed on the equilibration time required after spiking, prior to extraction when using lichens as biomonitors for PAHs. It has been shown, in this study, that it is imperative to understand the process by which the analytes are being extracted from the lichen matrix, as well as to know how long internal standards need to be allowed to equilibrate with the sample prior to extraction. The extent to which equilibration is required for the representative recovery of analytes varies between each sample preparation technique, which suggests that it is unwise to apply sample preparation techniques blindly without a thorough analytical investigation into the processes occurring during extraction.

#### **4.8.3 Comparison of the recoveries and extraction efficiencies of the different sample extraction techniques**

A comparison between the extraction efficiencies of the different sample extraction techniques was made in order to establish which sample preparation technique was most suited to the extraction of PAHs from our lichen species of choice, *Parmotrema austrosinense* (Zahlbr.) Hale. The profile of PAHs extracted from the native lichen matrix would provide an understanding of the ability of the method to quantitatively and qualitatively extract the analytes of interest. Figure 4.19 shows the results from the traditional sample preparation



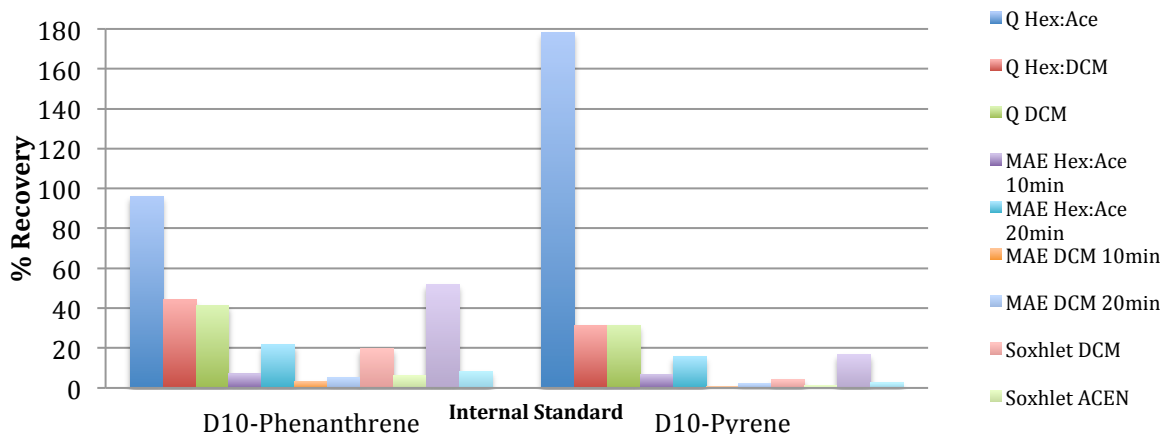
techniques, namely Soxhlet and USAE, previously used by other groups in PAH biomonitoring studies (Augusto et al., 2010; Domeño et al., 2006; Shukla & Upreti, 2013).



**Figure 4.19: Comparison of the extraction efficiencies of the traditional sample preparation techniques: USAE and Soxhlet**

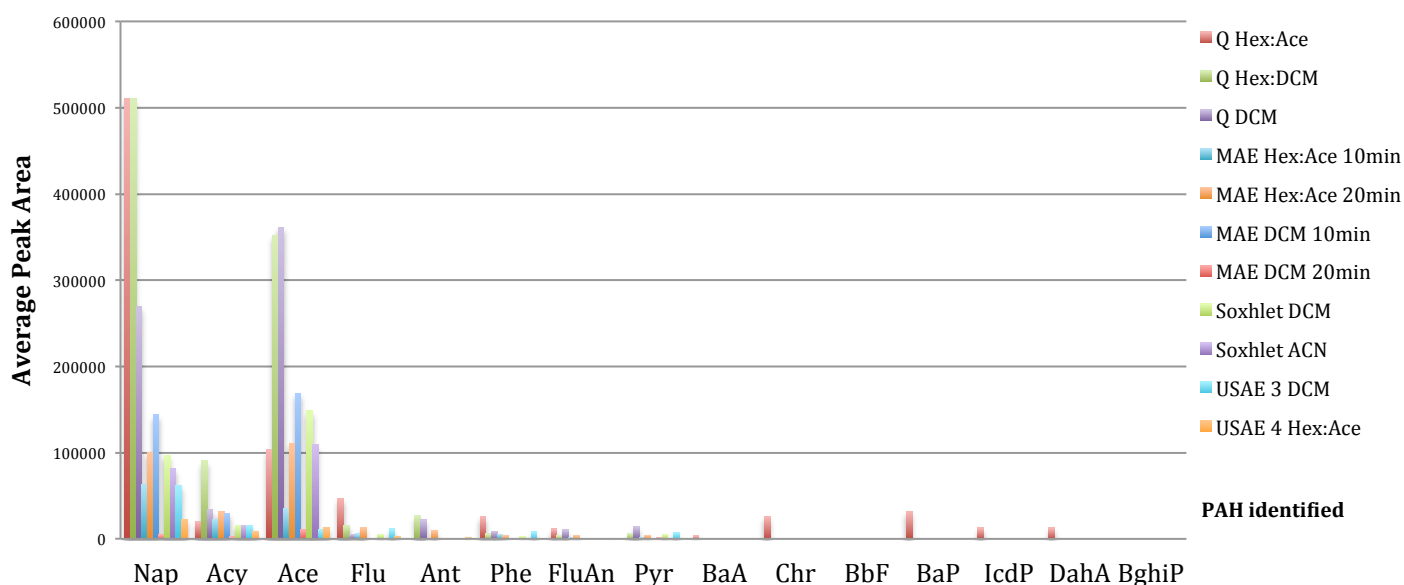
In terms of the number of PAHs extracted, the acetonitrile-based Soxhlet extraction performed worst. Comparing the average PAH peak areas of the USAE extractions as well as the DCM-based Soxhlet extraction, the Soxhlet DCM extract performed the better. However, in terms of recoveries of the internal standard, the USAE technique using DCM as extraction solvent performed the best. For the best performing traditional sample extraction techniques (Soxhlet DCM, Soxhlet acetonitrile, USAE 3 DCM and USAE 4 Hex: Ace), as shown in Figure 4.20, the recoveries were all below 51%.

These results confirm that the traditional sample extraction techniques were not successfully applied in this study on our species of choice, when compared to other studies where the recoveries were found to be above 65% (Domeño et al., 2006). This therefore concluded the investigation into the possibility of the use of USAE or Soxhlet techniques for the extraction of PAHs from the lichens sampled in the areas of interest in this study.



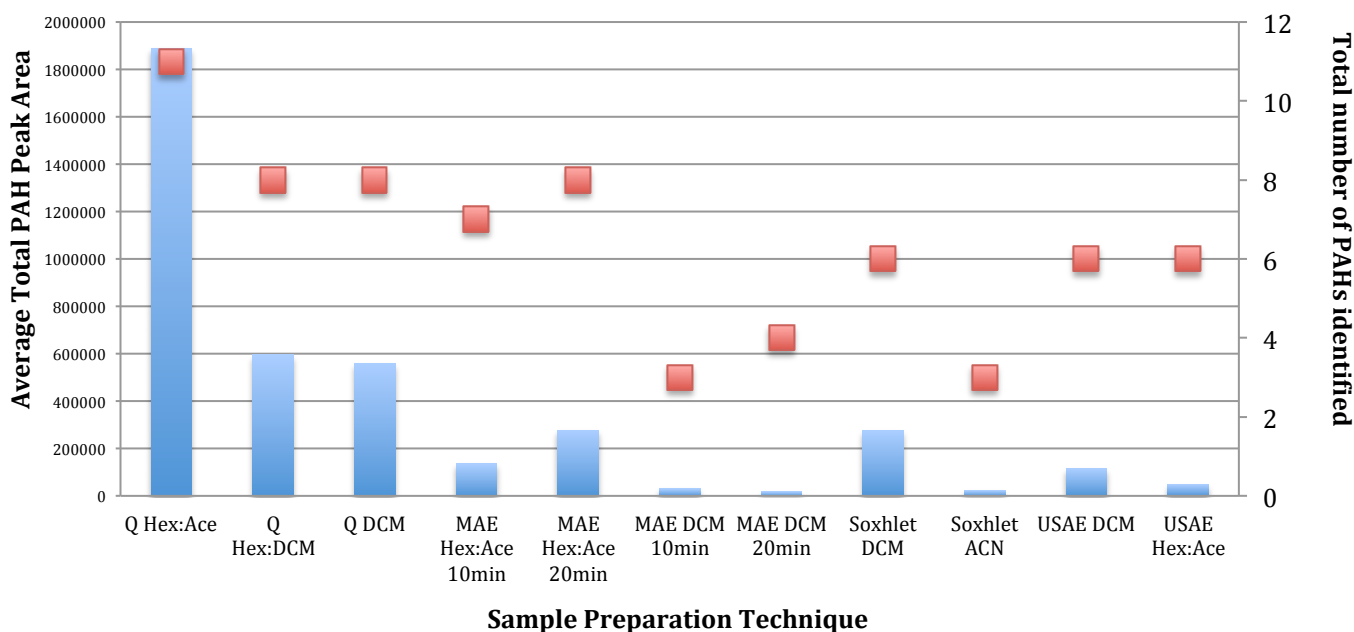
**Figure 4.20: Comparison of the % recovery of all sample extraction techniques using the bulk lichen sample**

The direct comparisons of average peak areas of identified PAHs in the native bulk lichen sample for all the sample extraction techniques investigated are shown in Figure 4.21. QuEChERS Hex:A extract is the only sample extraction method that was able to extract the heaviest PAHs of interest in this study. In terms of average peak areas of each PAH, the QuEChERS Hex:A extract had the highest peak areas, amongst all the extracts, in many cases. The new sample extraction techniques explored in this study (QuEChERS and MAE) performed better in terms of the quantities of PAHs extracted than the traditional techniques (USAE and Soxhlet). Figure 4.22 compares the average total peak areas for all identified PAHs and from this it was concluded that the QuEChERS Hex:A extraction technique outperformed the other sample extraction techniques both qualitatively and quantitatively.



**Figure 4.21: Comparison of the average PAH peak areas for all the sample extraction methods employed with respect to the bulk lichen sample**

In order to conclude which sample extraction technique was best suited for the extraction of PAHs from our lichen species of choice, the internal standard recoveries of each sample extraction technique were compared in Figure 4.20. The % recoveries were calculated according to the matrix-matched calibration curves for the internal standard using a Q Hex:A extract as the matrix-matched solvent. The recovery of pyrene-d<sub>10</sub> is very high (178%) despite being corrected for by the matrix-matched standards. The reason for this could be concentration of the extract by the volatilization of the solvent and lighter PAHs (including phenanthrene-d<sub>10</sub>). If this were true, recoveries for the other sample extraction techniques may have been even poorer than calculated as in Figure 4.20. Another factor which could have influenced the calculated % recoveries was the interday variation (23.3%, given in Section 4.3) since the extracts were analyzed across different days.



**Figure 4.22: Summary of the total PAH peak areas of identified PAHs for all investigated sample preparation techniques on the bulk lichen sample (blue bars) with the total number of identified PAHs found in the extracts (red squares)**

Furthermore, one-way ANOVA was performed on the results shown in Figure 4.22 to identify significant differences between the sample extraction techniques. For the QuEChERS extractions, a significant difference ( $p=0.0064$ ,  $F(2,6)=13.191$ ,  $F_{crit}=10.925$ , 99% confidence level) was observed between the Q Hex: Ace extracts and the Q DCM and Q hexane:DCM extracts. Furthermore, one-way

ANOVA revealed that there were not significant differences between the other sample extraction techniques (MAE, USAE and Soxhlet) where  $p=0.0103$  and  $F(2,6)=10.81$  ( $F_{crit}=10.925$ ) at 99% confidence level. When one-way ANOVA was performed on the QuEChERS Hex: Ace and MAE, USAE and Soxhlet results, the calculated  $p$  value of 0.00004 ( $F(3,8) = 71.66$ ,  $F_{crit} 7.591$ ) showed that it can confidently be claimed that QuEChERS outperformed the other sample extraction techniques.

In order to understand how precise the extraction and analytical methods were, the %RSD of the total peak areas for the different sample extraction techniques were investigated. The calculated %RSDs of the average total peak areas ( $n=3$ ) are given in Table 4.7. The highest %RSD was calculated for the Q Hex:DCM extract, followed by the acetonitrile-based Soxhlet extract. The %RSD of the chosen sample extraction method, QuEChERS with *n*-hexane:acetone, was similar to the other extracts, in the range of 13.0 % -36.3%. It is believed that the %RSD could be reduced in future by establishing criteria for homogeneity as discussed briefly in Section 4.6.2, and further in Chapter 5.

**Table 4.7: Comparison between % relative standard deviation of the total PAHs extracted using different sample extraction techniques**

Extraction Technique	Average Total Area	% RSD
Q Hex:A	9017064	25.0
Q Hex:DCM	1011474	61.4
Q DCM	725813	23.3
MAE Hex:A 10min	135312	19.8
MAE Hex:A 20min	277494	36.3
MAE DCM 10min	31523	9.81
MAE DCM 20min	19299	31.2
Soxhlet DCM	275546	13.0
Soxhlet ACN	24288	42.0
USAE DCM	115982	24.3
USAE Hex:A	49508	16.0

It was therefore concluded that Q Hex:A outperformed the other tested sample extraction techniques in terms of internal standard recovery, number of PAHs extracted as well as the quantity of PAHs extracted. It was therefore selected as the sample extraction technique of choice for the preparation of the lichen

samples from the areas of interest, in Section 4.11. Up until this point, a simple comparison of average total peak areas had been used to estimate extraction efficiencies of the different sample preparation techniques. This was done to identify whether a standout technique would be established. In this manner, *n*-hexane:acetone-based QuEChERS was chosen as the preferred extraction technique and it was further investigated using matrix-matched standards for quantification purposes as detailed in Section 4.10.2.

#### 4.9 Thermal desorption-gas chromatography-time-of-flight mass spectrometry analysis of lichens

Thermal desorption of a 1 µl volume of lichen extract injected into a Gerstel® glass tube using the GC-TOFMS technique detailed in Section 3.10 proved ineffective at desorbing the PAHs in the different sample extraction technique extracts investigated. The presence or absence of PAHs in the sample when analyzing the extracts using TDS-GC-TOFMS is indicated in Table 4.8.

**Table 4.8: The results of TDS-GC-TOFMS analysis on selected sample preparation technique extracts, including all three replicate extractions of Q Hex: Ace (Q H:A 1, Q H:A 2 and Q H:A 3) where red indicates the absence, and green indicates the presence of PAHs, respectively**

PAH	Method						
	DCM Soxhlet	MAE H:A 20min	USAE 4 DCM	Q H:A 1	Q H:A 2	Q H:A 3	Q H:DCM
Nap	Green	Green	Green	Green	Green	Green	Green
Acy	Red	Red	Red	Green	Red	Green	Red
Ace	Red	Green	Green	Green	Red	Red	Red
Flu	Red	Red	Green	Green	Red	Red	Red
Phe	Red	Green	Green	Green	Green	Red	Green
Ant	Red	Red	Red	Red	Red	Green	Red
FluAn	Red	Green	Green	Green	Green	Green	Green
Pyr	Red	Green	Green	Green	Red	Green	Red
BaA	Red	Red	Red	Red	Red	Red	Red
Chr	Red	Red	Red	Red	Red	Red	Red
BaP	Red	Red	Red	Red	Red	Red	Red
BbF	Red	Red	Red	Red	Red	Red	Red
IcdP	Red	Red	Red	Red	Red	Red	Red
BghiP	Red	Red	Red	Red	Red	Red	Red
DahA	Red	Red	Red	Red	Red	Red	Red

The TDS-GC-TOFMS analysis of the bulk lichen sample using dichloromethane-based Soxhlet extraction only identified one PAH: naphthalene. This is much less than the number of PAHs identified when using the liquid-injection GC-MSD method for analysis of the same extract where Nap, Acy, Ace, Flu, Phe and Pyr were identified. The same can be said when comparing the TDS results with the liquid injection results for the other samples prepared using other techniques.

A direct comparison between the number of PAHs identified in the respective extracts using TDS-GC-TOFMS or GC-MSD is made in Table 4.9. The same extracts were run on both instruments on the same day, in order to minimize any variations, concentration effects or losses. Theoretically, the PAHs identified in the extracts should be identical when using either TDS or liquid injection as the sample introduction technique, but this was not observed. The reason for this is likely ineffective desorption, as well as loss of analytes as a result of the nature of the thermal desorption process.

For this reason, thermal desorption of the extracts was not further pursued as it was a less sensitive technique, and the GC-MSD technique was decided to be the most suited for the analysis of the extracts prepared in this study, since more PAHs were seen in any given extract using this analysis technique. Furthermore, the expensive liquid nitrogen needed for thermal desorption incurs costs which cannot be justified by the poor performance compared to the GC-MSD results.

**Table 4.9: Comparison between TDS and liquid injection sample introduction methods and the number of identified PAHs thus obtained from identical extracts**

Extract identity	Number of PAHs detected	
	Thermal Desorption	Liquid Injection
Soxhlet DCM	1	6
Q Hex:Ace 1	7	11
Q Hex:Ace 2	3	11
Q Hex:Ace 3	5	11
Q Hex:DCM 1	3	8
MAE Hex:Ace 20 min	5	8
USAE 4 Hex:Ace	6	6

#### 4.10 Quantification of PAHs in lichen extracts

##### 4.10.1 LOQ and LOD values for solvent based standards using GC-MSD

The LOD and LOQ values for all 15 PAHs of interest are shown in Table 4.10 obtained from the analysis of solvent based PAH standards (8 standards of varying concentration range 0.0051 – 2.0 ng.µl<sup>-1</sup> in toluene) using the GC-MSD method detailed in Section 3.10 and the S/N = 3 (for LOD) and S/N = 10 (LOQ) criteria (Skoog et al., 2004). The calculated instrumental LOD and LOQ values are a reflection of the sensitivity of the method of analysis, and are more sensitive for the lower molecular weight PAHs. The analytical limits of detection and quantification in this study, using clean solvent based standards, were lower than those determined by Domeño et al (2006) (8.4 pg.µl<sup>-1</sup> – 12.8 pg.µl<sup>-1</sup>, using GC-MS) and similar to the range of 0.01 pg.µl<sup>-1</sup> – 0.03 pg.µl<sup>-1</sup> reported by Shukla and Upreti (2013), using HPLC-UV-V.

**Table 4.10: Calculated instrumental LOD and LOQ values of all 15 PAHs of interest in this study for the GC-MSD method using solvent based standards based on SIM ions**

PAH	MW (g.mol <sup>-1</sup> )	LOD (pg.µl <sup>-1</sup> )	LOQ (pg.µl <sup>-1</sup> )	R <sup>2</sup>
Nap	128	0.010	0.022	0.998
Acy	152	0.14	0.45	0.995
Ace	154	0.018	0.062	0.992
Flu	166	0.12	0.40	0.997
Phe	178	0.26	0.87	0.991
Ant	178	0.13	0.44	0.992
FluAn	202	0.21	0.70	0.987
Pyr	202	0.17	0.57	0.989
Chr	228	0.73	2.4	0.994
BaA	228	0.52	1.7	0.987
BbF	252	0.90	3.0	0.988
BaP	252	1.4	4.7	0.989
IcdP	276	1.3	4.2	0.989
BghiP	276	1.4	4.8	0.990
DahA	278	1.5	5.1	0.998

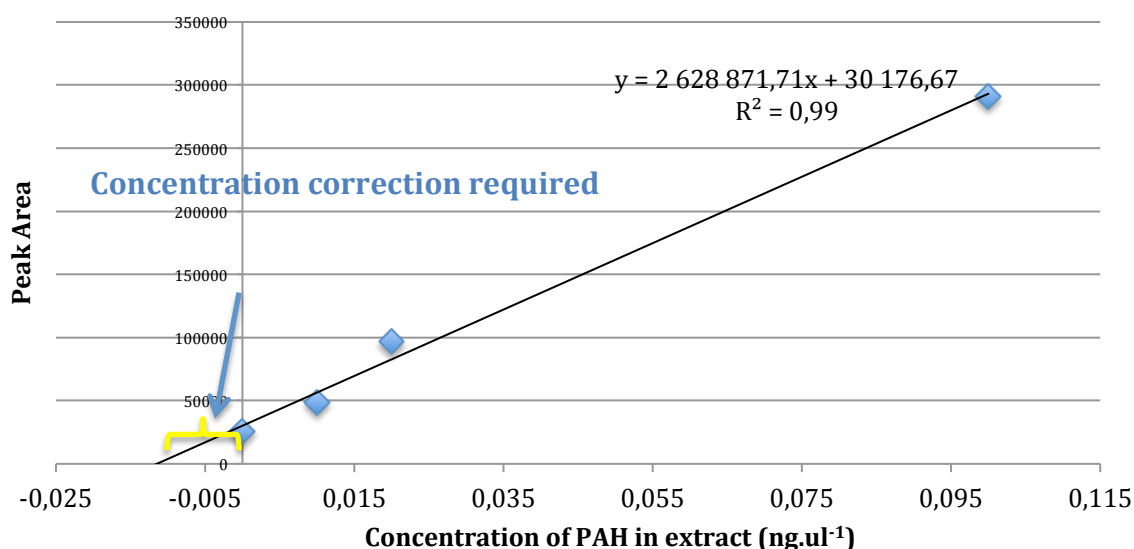
#### 4.10.2 Matrix effects and matrix-matched standards

As a result of the range of internal standard recovery percentages obtained as discussed previously, the tailing observed in the chromatograms, as well as the visual difference between extracts, with some extracts appearing a clear, bright green (Q Hex: Ace based extracts), and other extracts appearing a murky, olive green colour (Q DCM extracts), it was concluded that matrix matched standards should be investigated with respect to the quantification of PAHs in lichen extracts.

Acetone is often used by analysts when studying the chlorophyll content of lichens (Brown & Hooker, 1977; Vernon, 1960), thus it can be expected that acetone-based extracts would have a markedly different chlorophyll content compared to acetonitrile, or *n*-hexane based extractions. For this reason, the use of the matrix-matched standard calibration curves for quantitation can strictly only be applied to extracts that have been prepared using an identical sample preparation method.

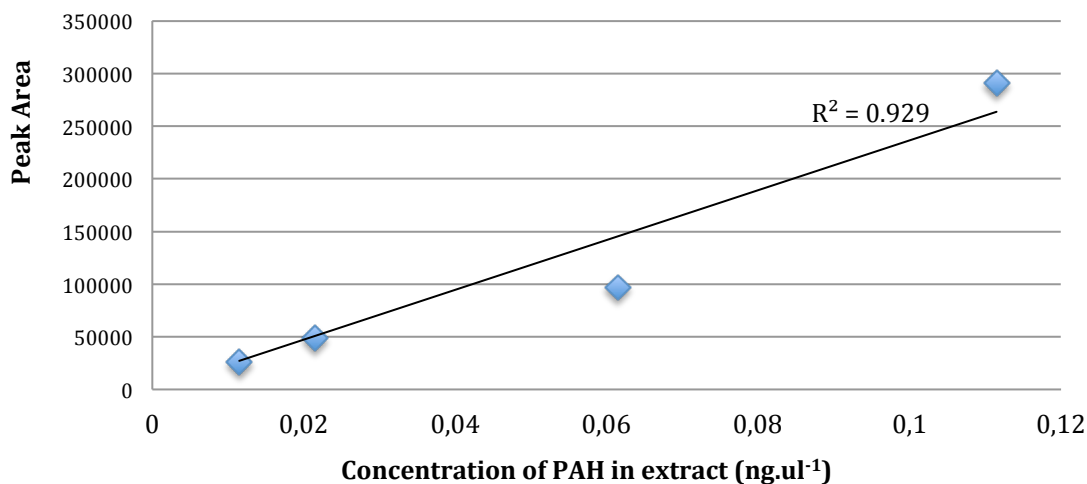


Since Q Hex: Ace produced significantly better PAH extractions than the other sample preparation techniques, it was also chosen as the matrix-matched 'background' for the matrix-matched standards in the investigation of the matrix effect. The bulk lichen sample collected on Justice Mahomed Street was used in the preparation of the matrix-matched standards, since no clean (PAH free) lichen reference material is available commercially. The calibration results obtained for Phe using matrix matched standards are shown in Figure 4.23. The remaining calibration curves are given in Appendix E.



**Figure 4.23: Calibration curve for phenanthrene using matrix matched standards, with trend line equation and R<sup>2</sup> value shown**

The concentrations represented at the x-intercepts (as indicated in Figure 4.23) for each individual PAH of interest in this study, was used to recalculate the calibration curves for all the PAHs that had been identified in the native sample. The resulting calibration curves were then used to formulate the line of best fit using the trend line functionality from which the concentrations of all identified PAHs in sample extracts would be calculated, as shown in Figure 4.24.



**Figure 4.24: Recalculated calibration curve for phenanthrene using matrix matched standards**

The LOD and LOQ values for the matrix-matched standards were calculated using the signal-to-noise method (Skoog et al., 2004). The calculated LOD and LOQ values for the matrix-matched standards are similar in terms of orders of magnitude to the LOD and LOQ values calculated using the clean solvent-based standards (Table 4.10). The two types of LODs however, describe different analytical features: the matrix-matched LOD values describe the ability of the sample preparation method to extract the analytes of interest in addition to the instrumental detection limits, thus being a ‘method LOD’, whereas the clean solvent-based LOD values are a feature of the analytical sensitivity only, thus giving an indication of the capabilities of the instrumental technique. Since the ‘method LOD’ values are mostly comparable with the instrumental LOD, it can be concluded that the sample preparation technique is appropriate for the analytical technique used in this study.

**Table 4.11: The LOD and LOQs of the matrix-matched standards, using the optimized GC-MSD method based on SIM ions**

PAH	LOD (pg.µl <sup>-1</sup> )	LOQ (pg.µl <sup>-1</sup> )
Naphthalene	0.41	1.36
Acenaphthylene	0.12	0.39
Acenaphthene	0.17	0.58
Fluorene	0.22	0.75
Anthracene	0.15	0.49
Phenanthrene	0.43	1.42
Fluoranthene	0.12	0.39
Pyrene	0.04	0.14
Benzo[ <i>a</i> ]anthracene	0.60	1.99
Chrysene	0.54	1.79
Benzo[ <i>b</i> ]fluoranthene	0.23	0.78
Benzo[ <i>a</i> ]pyrene	2.95	9.84
Indeno[123 <i>cd</i> ]pyrene	1.04	3.46
Dibenzo[ <i>ah</i> ]anthracene	2.14	7.13
Benzo[ <i>ghi</i> ]perylene	0.86	2.85

A comparison of the calculated y-intercept from the best fit line equation, with the response of each PAH in the native extract, showed that for some of the calibration curves, notably Nap, Acy and Phe, the y-intercepts were similar, with % differences between 9 and 23 %, as shown in Table 4.12. Some % differences were as high as 100% when the PAH was not identified in the native lichen sample, but since the PAHs were being detected in SIM mode, the matrix effect may have been elevating the signal, not the actual presence of the particular PAH in question. Working with environmental samples, particularly living organisms such as lichens, introduces variations that result in complications when identifying and quantifying analytes at trace levels, as can be seen by the straying of data from R<sup>2</sup> values of 1. The differences between each extract contribute towards the R<sup>2</sup> values ranging between 0.925 and 0.993. It should be noted that although matrix-matched standards were analyzed at higher concentrations, only the results that fell into the linear range in the calibration curve were used (0.01 – 0.1 ng.µl<sup>-1</sup> for PAHs of MW 128 – 228 g.mol<sup>-1</sup> and BbF, and 0.01 – 0.5 ng.µl<sup>-1</sup> for PAHs of MW 252 – 278 g.mol<sup>-1</sup>).

Table 4.12 shows the results from the calculation of the % matrix effect for each of the PAHs of interest, as detailed in Section 3.7, as proposed by Rajski et al (2013). The matrix effects range from strong suppression (IcdP) to strong enhancement (Nap, Acy, Flu, Ant, Phe, FluAn, Pyr, BaA, Chr and BbF).

The PAHs experiencing the most severe matrix effects were Ant and Pyr, with % matrix effects above 300%, indicating a very strong enhancement. These results heavily impact on the interpretation of the analysis, since any diagnostic ratio or toxic equivalence quotient would be severely changed if the matrix effects were not accounted for. The observed suppression for Ace, IcdP, BghiP and DahA would also be problematic since the suppression of analytes, already present at low levels would mean that they may not be identified in a sample, despite their presence in the atmosphere and indeed the sample. This type of matrix effect is most difficult to overcome, since a corrected calibration is not able to correct for the interference if the analyte is being suppressed to the extent that it is not identified in a sample extract. For this reason, in Section 4.11, if an analyte was not detected, the LOD value from Table 4.11 was used.

**Table 4.12: Analysis of matrix matched standards, including the calculated % matrix effect, the type of matrix effect observed and the % difference between the native PAH y-intercept as well as the y-intercept calculated from the trend line equation**

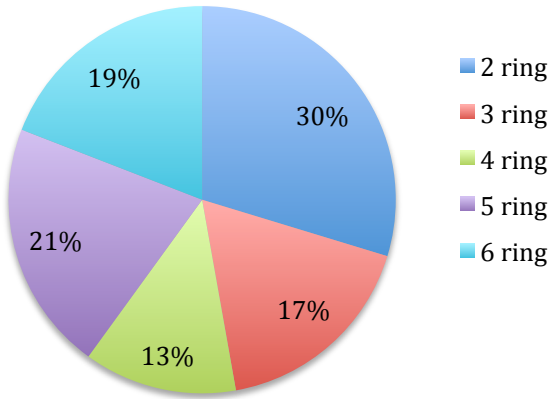
PAH	% Matrix Effect	Type of effect	% Difference between calculated and native y-intercepts
Nap	82	Strong enhancement	23
Acy	104	Strong enhancement	9
Ace	-40	Medium suppression	-39
Flu	74	Strong enhancement	-11
Ant	316	Strong enhancement	<b>100</b>
Phe	113	Strong enhancement	15
FluAn	96	Strong enhancement	50
Pyr	301	Strong enhancement	<b>100</b>
BaA	93	Strong enhancement	64
Chr	57	Strong enhancement	-25
BbF	71	Strong enhancement	<b>100</b>
BaP	6	Soft enhancement	44
IcdP	-67	Strong suppression	-67
DahA	-38	Medium suppression	88
BghiP	-33	Medium Suppression	67

## 4.11 Results of the analysis of the lichen samples from areas of interest

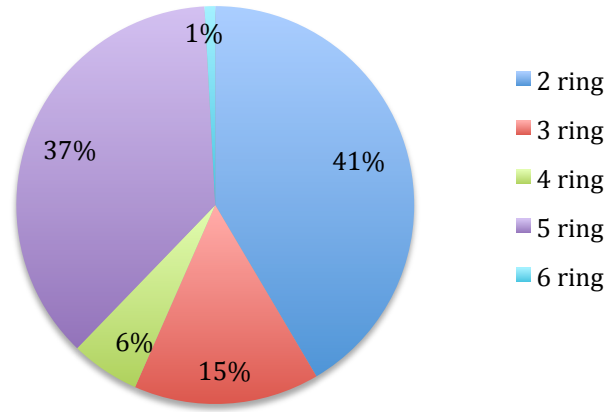
### 4.11.1 Quantitative and qualitative results

The corrected trend line for the matrix-matched standards of each individual PAH was used to calculate the concentration of each PAH identified in the samples from areas of interest, using the LOD values and LOQ values when the PAH was not detected, or detected below the level of quantification respectively. The concentrations were then expressed as  $\text{ng.g}^{-1}$  lichen at dried weight (hereafter referred to as  $\text{ng.g}^{-1}$  dw).

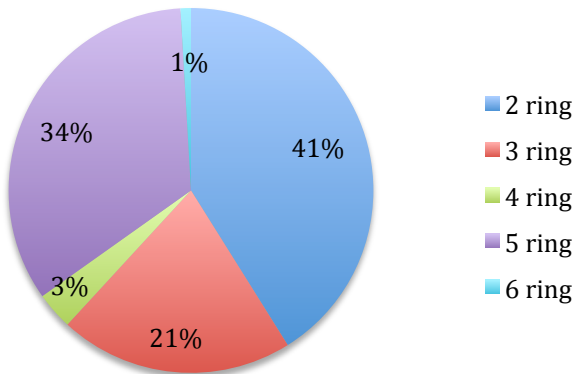
A summary of the PAHs identified in the different samples of interest is given in Figures 4.25 – 4.33 and Tables 4.13-4.14. The results show that many PAHs were identified in the samples of interest, ranging from the light PAHs, to the heavy PAHs. Of the PAHs detected in each sample, the highest concentrations were calculated for Ace and BaP. Higher concentrations of the heavier PAHs were seen in the samples from Pretoria Industrial, as well as the Daspoort Tunnel samples. As a result of high traffic flow and industrial processes occurring in the areas, these sampling regions are expected to be more polluted than the other samples. As the Pretoria Industrial area's chosen sampling area was devoid of lichens with the exception of the one tree; it supported other studies which found a decrease in lichen species in areas with high air pollution levels (LeBlanc & Sloover, 1970; Pinho et al., 2004). The highest total PAH content was also found for these three samples, with total concentrations of 1001, 737 and 693  $\text{ng.g}^{-1}$  dw for Pretoria Industrial, Daspoort Tunnel Site 2 and Daspoort Tunnel Site 3, respectively. The results from these impacted areas are higher than those reported in studies sampling lichens in industrial areas, such as Augusto et al (Augusto et al., 2013), who reported a highest total PAH concentration of 556  $\text{ng.g}^{-1}$  dw in a highly industrialized region in Portugal, but similar to the values found by Nascimbene et al (785  $\text{ng.g}^{-1}$ ) who studied lichens collected next to a road in the Eastern Italian Alps (Nascimbene et al., 2014), as well as the results from Blasco et al who found concentrations of 352 – 1654  $\text{ng.g}^{-1}$  dw next to a national highway in Spain (Blasco et al., 2007).



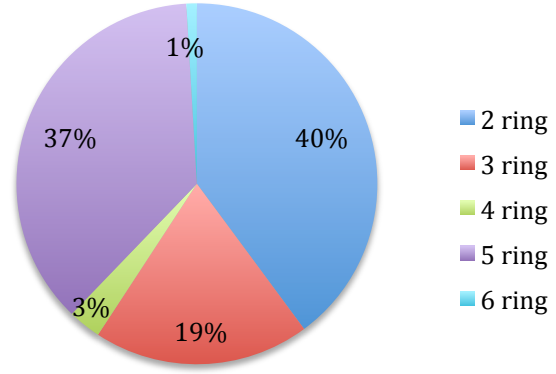
**Figure 4.25: Pie chart showing contribution of ring sizes to total PAH concentration in the bulk sample**



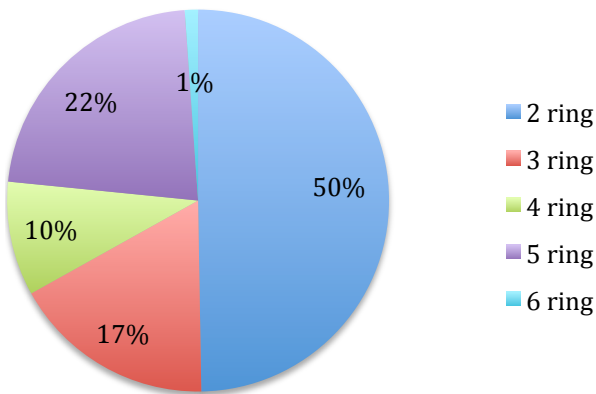
**Figure 4.26: Pie chart showing contribution of ring sizes to total PAH concentration in the Cathedral Peak region sample**



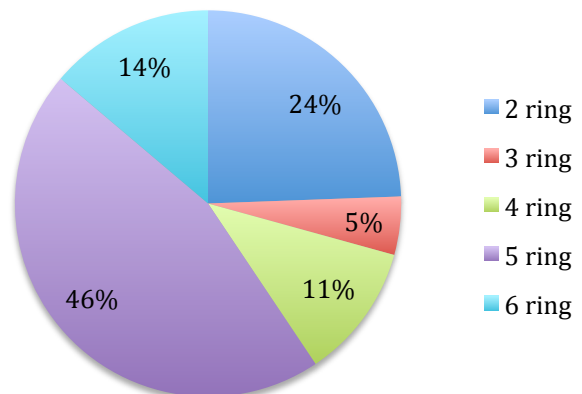
**Figure 4.27: Pie chart showing contribution of ring sizes to total PAH concentration in the Cape Point 1 sample**



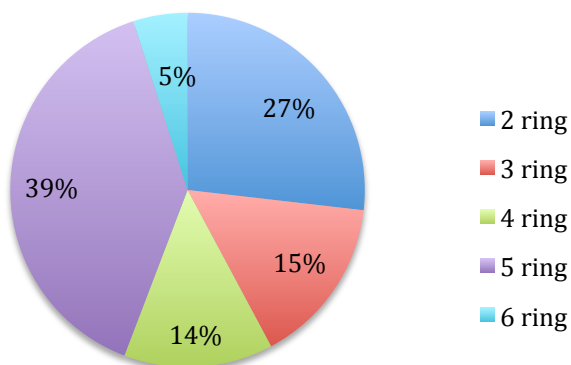
**Figure 4.28: Pie chart showing contribution of ring sizes to total PAH concentration in the Cape Point 2 sample**



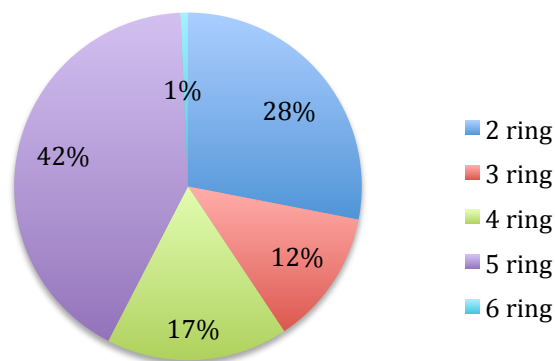
**Figure 4.29: Pie chart showing contribution of ring sizes to total PAH concentration in the Cape Point 3 sample**



**Figure 4.30: Pie chart showing contribution of ring sizes to total PAH concentration in the Pretoria Industrial sample**

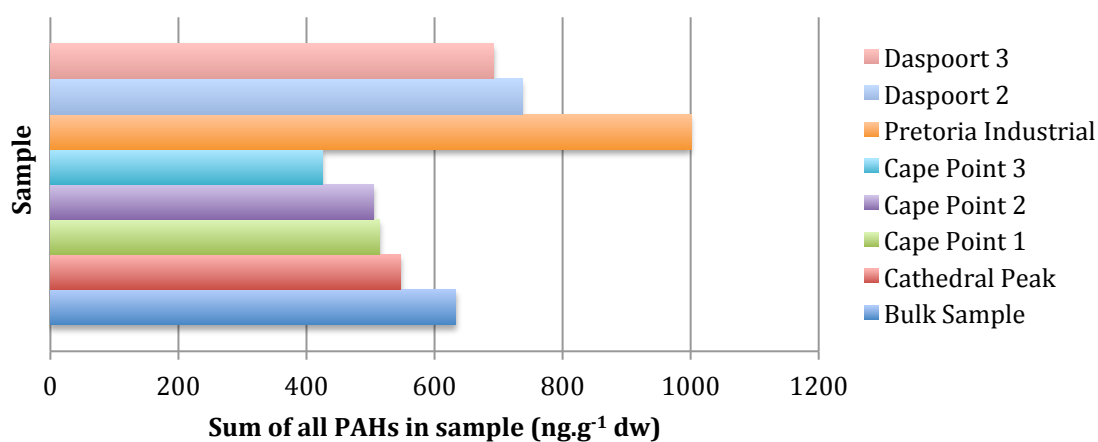


**Figure 4.31: Pie chart showing contribution of ring sizes to total PAH concentration in the Daspoort Tunnel Site 2 sample**



**Figure 4.32: Pie chart showing contribution of ring sizes to total PAH concentration in the Daspoort Tunnel Site 3 sample**

Figures 4.25 – 4.32 show the contribution of the PAHs of different ring sizes to the total PAH concentration. This visual representation provides a quick insight into similarities in the PAH profiles between the samples of interest. Similarities between the ring-size profiles of the lichen samples can be seen between samples from Cathedral Peak, Cape Point site 1 and Cape Point site 2, and then similarities between the Daspoort Tunnel samples. The bulk sample (from an urban area in Pretoria) as well as the Pretoria Industrial sample appear different from all the other samples, where the Pretoria Industrial sample had the largest % contribution of 5-ring PAHs, as well as high 6-ring PAH % contribution, with a low 2-ring PAH contribution, compared to the other samples. The bulk sample had the most even distribution profile in terms of PAH ring size out of all the samples analyzed.



**Figure 4.33: Comparison of the total PAH concentration in each lichen sample from areas of interest**

**Table 4.13: The concentration of individual PAHs in the samples of interest, as well as the total PAH concentration in each sample, expressed in ng.g<sup>-1</sup> of lichen at dried weight**

Concentration of PAHs in lichen samples in ng.g <sup>-1</sup> dw								
PAH	Bulk Sample	Cathedral Peak Region	Cape Point 1	Cape Point 2	Cape Point 3	Pretoria Industrial	Daspoort 2	Daspoort 3
Nap	48,0	60,2	64,2	69,2	52,8	69,9	48,1	55,4
Acy	37,0	41,0	19,3	15,4	36,5	27,2	39,3	22,3
Ace	103	126	128	117	122	147	110	117
Flu	83,3	56,9	38,2	50,4	40,3	18,4	59,1	53,8
Ant	0,375*	19,5	13,3	17,5	6,37	19,6	8,08	0,375*
Phe	27,2	6,16	55,1	29,8	26,3	10,8	46,0	33,1
FluAn	14,8	6,11	5,72	0,300*	0,300*	37,9	18,0	12,1
Pyr	0,100*	21,7	8,43	11,7	37,9	15,0	41,5	44,6
BaA	5,89	1,50*	1,50*	1,50*	1,50*	4,98**	7,86	4,98**
Chr	60,2	1,35*	1,35*	1,35*	1,35*	56,3	32,9	57,3
BbF	0,575*	1,95**	0,575*	1,95**	4,18	10,4	3,21	2,77
BaP	99,0	195	145	166	85,1	440	281	269
IcdP	119	2,60*	2,60*	2,60*	2,60*	80,7	29,3	2,60*
DahA	32,9	5,35*	28,2	17,8**	5,35*	5,35*	5,35*	17,8**
BghiP	2,15*	2,15*	2,15*	2,15*	2,15*	57,5	7,13**	2,15*
<b>Total PAHs</b>	<b>633</b>	<b>547</b>	<b>514</b>	<b>505</b>	<b>425</b>	<b>1001</b>	<b>737</b>	<b>693</b>

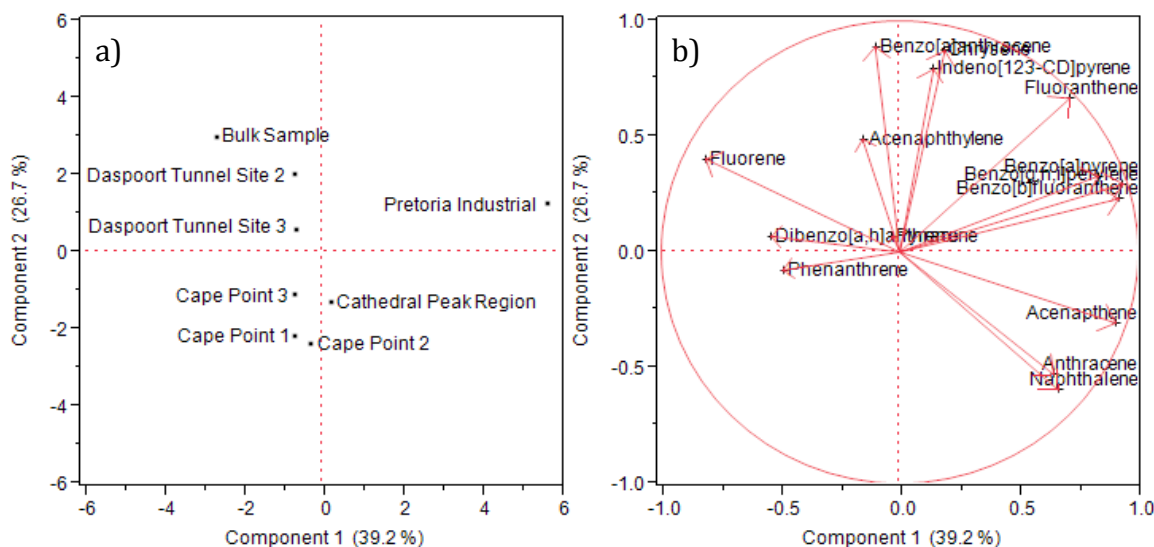
\* Indicates a value below the LOD

\*\* Indicates a value above the LOD but below the LOQ (refer to Table 4.11)

Principal component analysis (PCA) was performed using JMP 10 software on the results given in Table 4.13, using mean-centered data and correlation-based variance. Although PCA is often used for modeling purposes, it was used in this study strictly to identify differences between our samples. The 1<sup>st</sup> principal component accounted for 39.2 % of the variance between samples, and the 2<sup>nd</sup> principal component accounted for 26.7 % of the variance, as shown in Figure 4.34. The results show that the three Cape Point Reserve samples were very similar, as well as the Cathedral Peak region sample. It was also observed that the Pretoria Industrial sample was very different from all the other samples. The two Daspoort Tunnel sites were similar, and had similarities with the bulk



sample, suggesting that the emissions from vehicles are significantly contributing towards the PAH profile at both sites.



**Figure 4.34: Graphs showing a) results from the Principal Component Analysis (PCA) of all the samples of interest and b) the loading plot for all the PAHs identified in the samples of interest**

The loading plot shown in Figure 4.34b indicates the PAHs contributing most to the calculated factors, and thus provides an indication of which PAHs contribute most to the inter-sample variations. The loading plot shows that the increased concentration of IcdP, BbF, FluAn, BaP, Chr and BghiP accounts for the variation of the Pretoria Industrial sample from the other sites. These PAHs are commonly associated with both petroleum and diesel emissions, and both FluAn and IcdP are reported to be produced by the burning of lubricating oil, suggesting nearby industrial processes (Ravindra et al., 2008). High levels of BaP, BghiP and Chr are also reported to be indicative of nearby steel industry emissions (Ravindra et al., 2006). The loading plot shows that Phe, Ant, Nap and Ace are the PAHs that distinguish the cleaner samples (Cape Point and Cathedral Peak region samples) from the more contaminated samples. This indicates that low molecular weight PAHs dominate in cleaner areas, where less PAHs are being emitted.

These results vary from a study by Augusto et al (2013) who sampled lichens in an industrial region in Portugal, which found that 4-ring PAHs dominated the

PAH profile, followed by 3- and 2-ring PAHs. This study was performed alongside a total suspended particulate (TSP) air sampling campaign, and showed that the TSP results showed a dominance of 4-, 5- and 6-ring PAHs in the total PAH profile. This highlights the preferential uptake of PAHs, and suggests that the lichen species chosen in our study better accumulates heavier PAHs, as seen in Figures 4.30-4.32, compared to other studies. This makes the comparison between different studies in different countries difficult, since the lichen uptake mechanisms are still poorly understood; it is difficult to quantify any existing species bias.

It was suggested by Augusto et al (2009) that a PAH profile dominated by 4-ring PAHs is indicative of urban pollution, and that a profile dominated by 5- and 6-ring PAHs is indicative of industrial pollution. It can thus be seen from Figure 4.30 that the Pretoria Industrial site is most likely contaminated by industrial PAH emissions, whereas the Daspoort Tunnel sites (Figure 4.31 and 4.32) are polluted by both urban and industrial sources. It was also suggested (Augusto et al., 2009) that the most polluted sites were the regions affected by both urban and industrial pollution sources, which is supported in this study, since the Pretoria Industrial site is located close to factories, but also to urban areas.

The results suggest that the least polluted sites include the sample from the Cathedral Peak Region and all three Cape Point samples, with corresponding total PAH concentrations of 547, 514, 505 and 425 ng.g<sup>-1</sup> dw respectively. The Cathedral Peak region sample had a higher total concentration of PAHs, compared to the Cape Point samples, likely due to the fact that sample collection was performed next to a road used by automobiles and the occasional heavy-duty motor vehicle. These results are supported by a study by Blasco et al (2011) that found that lichens sampled close to a road had higher PAH levels than those sampled further away. Sampling lichens farther from the road would provide a meaningful understanding of the general ambient atmospheric PAH levels for tourists who visit the region for hiking and outdoor activities, yet the large contribution by BaP to the total PAH concentration (35.6% of total PAH concentration) is surprising and needs to be investigated further, due to the toxic

nature of this compound. The result indicates that *Parmotrema austrosinense* (Zahlbr.) Hale may bioaccumulate BaP, making it a good biomonitor for PAHs.

The total PAH concentration from Cape Point site 3 was the lowest out of the three Cape Point samples, which is similar to the findings by Blasco et al (2011) which stated that total PAH concentration, as well as total PAH<sub>comb</sub> (PAH<sub>comb</sub>: FluAn, Pyr, BaA, Chr, BbF, BkF, BaP, DbA, BghiP, refer to Section 2.4), decreased as the distance from roads increased. Cape Point site 3 was furthest away from any roads as shown in Figure 3.9. The total PAH<sub>comb</sub> decreased with the distance from the car parking area and the funicular, where the values were calculated to be 193 and 203 ng.g<sup>-1</sup> dw at site 1 and site 2, compared to 138 ng.g<sup>-1</sup> dw at site 3, concluding that site 3 was the least impacted sampling site in this study. The relatively high concentration of BaP in the lichen samples from Cape Point sites 1 and 2 (145 and 166 ng.g<sup>-1</sup> dw, respectively) is likely due to the *fynbos* burning event which had taken place earlier in the year, from which sampling point 3 was largely shielded as a result of the presence of a large cliff, sheltering the lichens from the northerly winds expected to have blown during the time of the *fynbos* burning event (Scheel et al., 1990). The PAHs present at the highest concentrations were BaP, Ace, Nap and Flu in all three Cape Point samples. This suggested that the wood and grass burning, as well as petroleum combustion were the main contributors towards atmospheric PAHs in the area since the N, NE and NW winds blow impacted air masses in from the Cape urban areas during the winter months (Brunke & Halliday, 1983; Fang et al., 2004; Ravindra et al., 2006; Rogge et al., 1993; Rogge et al., 1993).

Figure 4.33 shows the total PAH concentration of each of the samples of interest. The bulk sample had a total concentration value of 633 ng.g<sup>-1</sup> dw, having a higher value than the cleaner samples from the Drakensberg and the Cape Point Nature Reserve, and a lower value than the samples from the industrial area in Pretoria, as well as the Daspoort Tunnel samples. These results were expected, considering that the bulk lichen samples was taken next to a road, carrying mainly light motor vehicles and a few small trucks. Emissions from a few domestic fires and household emissions may also be expected therefore this

sampling site is expected to be more contaminated than the Cape Point Nature Reserve and Drakensberg samples, but less contaminated than the samples from the industrial area. These results validate our choice of sampling site for our bulk sample on which the sample preparation methods were developed, since the bulk sample was neither highly polluted, nor significantly free of PAHs, limiting any possible bias. The range in total PAH concentration (425 – 1001 ng.g<sup>-1</sup> dw) between samples demonstrates the ability of the analysis method developed in this study to distinguish between lichens of varying PAH content.

It should be highlighted that this study focused on a targeted analysis method, and thus the total PAH concentration in each of the samples should be interpreted with caution, since many other PAHs present in the atmosphere, and thus potentially in the lichens, were not identified and quantified. The reason for this is that PAHs show differences in their susceptibility towards photodegradation as a result of the differing wavelengths at which PAHs absorb UV radiation, affecting the profile of PAHs transported in the atmosphere (Lima et al., 2005). Photochemical reactions can lead to the formation of oxygenated PAH species or photodimerisation (Pozzoli et al., 2004), ultimately influencing the PAH profile detected and quantified. Another consideration when interpreting the results should be that the lichen matrix may preferentially assimilate different PAHs, since the mechanisms for the uptake of PAHs into the lichen matrix is not yet clearly understood. This results in a possible bias being introduced and necessitates inter-species comparisons from the same sampling region using the same extraction technique.

#### **4.11.2 Toxic equivalence quotients (TEQs) and source diagnostic ratios for samples of interest**

The TEQ values for all the samples from areas of interest in this study are given in Table 4.14. The TEQ values provide an understanding of the toxic equivalency of a mixture of PAHs, by weighting the calculated concentration of a PAH according to its toxicity relative to BaP, as discussed in Chapter 2 (Section 2.3.2) (Nisbet & Lagoy, 1992). The calculations suggest that the most toxic PAH environment is in the Pretoria Industrial area, with a TEQ value of 478 ng BaP equivalent.g<sup>-1</sup> dw, much higher than any of the other regions. These results, in

combination with the high total PAH concentration value shown in Figure 4.33; suggest that the Pretoria Industrial region was the worst impacted site studied during this project.

Using the calculated TEQ values as an indication of the toxicity of the atmosphere is useful to distinguish between the sampling sites at the Cape Point Nature Reserve, where site 3 had half the calculated TEQ of sites 1 and 2. This further supports the theory that site 3 had been protected by the cliff from the combustion PAHs produced during the  *fynbos*  burning event and impacted inland air masses, since the toxic equivalency of the site as well as the total PAH<sub>comb</sub> (as defined in Section 4.11.1 above) was lower than the two other Cape Point sites.

The calculated TEQ values for all the samples in this study were higher than the values found by Augusto et al (2012) who studied lichens in an industrial area in Portugal that ranged from 6.90 and 46.0 ng BaP equivalent.g<sup>-1</sup> dw concentration. The study used a different lichen species, as well as the Soxhlet sample preparation technique, shown in this study to be less efficient at extracting PAHs from lichens than QuEChERS. No mention was made of accounting for any possible matrix effects either, and these factors all contribute towards the calculated TEQ, which was much lower than any calculated TEQ from any of the sample sites in this study.

**Table 4.14: The calculated TEQ values for all the samples from areas of interest in this study**

Sample Identity	TEQ of sample (ng BaP equivalent/g dw)
Bulk Sample	277
Cathedral Peak Region	222
Cape Point 1	288
Cape Point 2	257
Cape Point 3	113
Pretoria Industrial	478
Daspoort Tunnel Site 2	312
Daspoort Tunnel Site 3	359

In this study, diagnostic ratios were applied to the results in order to assess the applicability of many reported source diagnostic indicators, originally developed for direct air sampling techniques, which have been applied to lichens (Guidotti et al., 2003; Shukla & Upreti, 2009; Shukla et al., 2012). The pollution sources contributing towards the atmospheric PAH levels are known for the Pretoria Industrial, bulk sample and Daspoort Tunnel sites, and can then be compared to the pollution sources indicated by the diagnostic ratios as detailed in Table 2.4.

Sources of PAHs can be identified using the diagnostic ratio of  $\Sigma\text{PAH}_{\text{comb}}/\Sigma\text{PAH}_{\text{total}}$ , which indicates whether PAH levels in the lichens are predominantly from a combustion source or not (Ravindra et al., 2008). The results of the diagnostic ratio, shown in Table 4.15, suggest that only the Pretoria Industrial and Daspoort Tunnel sites are impacted by combustion sources. The ratios also suggest that combustion does not contribute to the bulk sampling site or Cape Point site 3. It should be noted that for the sum of all the PAHs identified used in this diagnostic ratio, we only used the 15 PAHs under investigation in this study and should thus only be used as an indication of the contribution of combustion towards the PAH profile and not a definitive source indicator. Although the diagnostic ratio correctly identifies that combustion processes did not significantly affect the Cape Point site 3, the other sites, including Cape Point 1 and 2 and the bulk-sampling site should reflect combustion processes. This diagnostic ratio should thus be used with caution if applied to lichen studies. The contribution of other sources to the overall PAH profile makes the interpretation of the results from applying diagnostic ratios difficult.

The diagnostic ratio of  $\text{Flu}/(\text{Flu} + \text{Pyr})$  is used to distinguish between different combustions sources. The results from the application of this ratio to the different samples from areas of interest indicate that the source is the same for all samples, namely diesel, grass and wood combustion. Although these results are not particularly misleading, the ratio is not useful in distinguishing between sources and petroleum based vehicles are not accounted for at sites such as the bulk sampling site, Pretoria Industrial and the Daspoort Tunnel sites which are known to be contaminated by a combination of diesel and petroleum engine

sources. The same could be said for the diagnostic ratio of Pyr/BaP which indicated that all the samples were contaminated by PAHs of a petrogenic source, which proved to be contradictory to the Flu/(Flu + Pyr) as well as the Ant/(Ant + Phe) diagnostic ratio results, which had suggested pyrogenic and diesel engine based sources for the samples.

The ratio of IcdP/(IcdP + BghiP) has been applied to distinguish between emission sources in a detailed manner, and the use of the ratio identified the main sources for PAHs as coal and diesel (Pretoria Industrial), diesel (Daspoort Tunnel site 2) and diesel and coal (Daspoort Tunnel site 3), as shown in Table 4.15. These results are congruent with which processes are known to contribute to the atmospheric PAH levels in the sites which were more polluted in the study, but were not useful in the source apportionment of the results from the cleaner areas (Cathedral peak region and the Cape Point Nature Reserve samples). It has been shown that vehicle exhaust fumes are the largest contributors of PAHs in urban areas (Ravindra et al., 2006), thus vehicular emissions are expected to dominate in the samples from the Daspoort tunnel, which experiences congested traffic daily.

**Table 4.15: Results for diagnostic ratio calculations and the most abundant PAHs in each sample of interest**

Site Identity	PAH <sub>comb</sub> /PAH <sub>total</sub> *	IcdP/(IcdP+ BghiP)**	Most abundant PAH
Bulk Sample	0.340	0.982	IcdP > Ace > BaP > Flu
Cathedral Peak Region	0.429	At LOD level	BaP > Ace > Nap > Flu
Cape Point 1	0.376	At LOD level	BaP > Ace > Nap > Phe
Cape Point 2	0.403	At LOD level	BaP > Ace > Nap > Flu
Cape Point 3	0.325	At LOD level	Ace > BaP > Nap > Flu
Pretoria Industrial	0.626	0.584	BaP > Ace > IcdP > Nap
Daspoort Tunnel 2	0.538	0.805	BaP > Ace > Flu > Nap
Daspoort Tunnel 3	0.590	0.547	BaP > Ace > Chr > Nap

\* PAH<sub>comb</sub>: FluAn, Pyr, BaA, Chr, BbF, BkF, BaP, DbA, BghiP

If PAH<sub>comb</sub>/PAH<sub>total</sub> ≈ 1 then combustion is a major source of PAHs

\*\* IcdP/(IcdP+ BghiP):  
 ≈ 0.18 indicates vehicle emissions  
 ≈ 0.37 indicates diesel combustion  
 ≈ 0.56 indicates coal combustion  
 ≈ 0.62 indicates wood burning  
 ≈ 0.35-0.70 indicates diesel emissions

Reference: (Ravindra et al., 2008)

Comparing the diagnostic ratios results reported in other studies, such as by Augusto et al (2010) relating to a petro-industrial area, the IcdP/(IcdP+BghiP) diagnostic ratio gave similar source apportionment results as our Pretoria Industrial site but vastly different results for Flu/(Flu+Pyr) and Pyr/BaP, which further suggests that existing diagnostic ratios may need to be applied cautiously, or developed independently for lichens in general as well as for specific species thereof. Our source diagnostic results are similar to the results reported in a study by Shukla et al (2012) which found that lichens sampled in Haridwar city were contaminated by PAHs of both diesel and gasoline emission sources. Another similarity was that the industrial and city center samples had high PAH<sub>comb</sub>/PAH<sub>total</sub> ratios (Shukla et al., 2012), suggesting that this ratio may indeed be useful since the ratios for a petro-industrial area (Augusto et al., 2010), industrial areas (Shukla et al., 2012) and the urban-industrial areas presented in our study were all similar, pointing towards a large contribution of combustion-source PAHs in the atmospheres around the sampling sites.

The application and interpretation of diagnostic ratios with respect to lichens has been shown to require caution, since distinguishing between sources can be difficult, due in part to the fact that various PAHs display different reactivity with oxidizing atmospheric compounds and in addition PAHs may be degraded or volatilized during the lichen sampling period (Ravindra et al., 2008; Tsapakis & Stephanou, 2003).

As suggested by Galarneau (2008), the conservation of the PAH profile emitted at the pollution source at the point of sampling cannot be guaranteed. This was not verified in this study since no direct air sampling campaign was undertaken at the suspected sources of PAH pollution, nor downwind at the lichen sampling sites. Another assumption in the common application of source diagnostics is that the relative PAH profiles are unique to only one source, yet it has been shown that there exists variability between PAH emissions from the same sources, such as coal (Galarneau, 2008; Yunker et al., 2002). It can therefore not be stated with certainty that a particular diagnostic ratio result could indicate only one source, as has been commonly incorrectly described in literature (Ravindra et al., 2008; Shukla et al., 2012). This could also account for the



unclear source apportionment results from the application of diagnostic ratios to the lichen PAH concentrations found in this study.

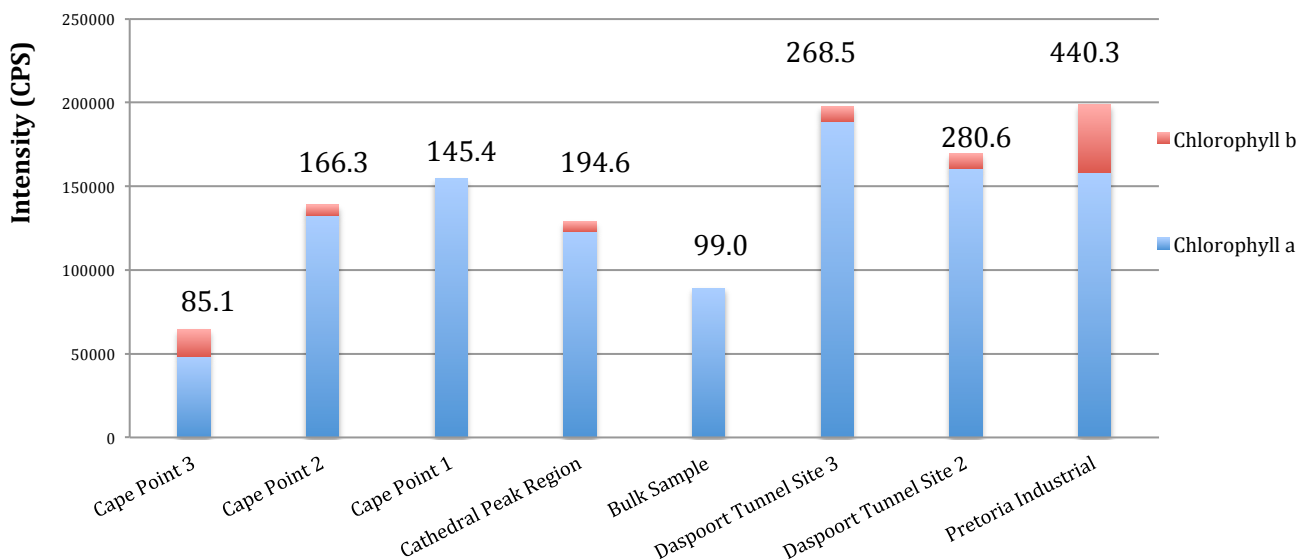
It has been established that different lichen species absorb metals (Bergamaschi, 2004), as well as PAHs (Blasco et al., 2011) to different degrees, some favoring pyrogenic PAHs, and others the absorption of lighter PAHs. This is likely what had been observed with our selected lichen species. A preferential absorption of a given profile of PAHs by the species is common to all samples collected at vastly different sites: from a coastal cliff (Cape Point Nature Reserve), mountains found inland of South Africa (Drakensburg sample) as well as heavily polluted areas such as the Daspoort Tunnel. The PAHs common to all these areas were found to be BaP, Ace, Nap, Acy, Flu and Phe. Understanding the uptake mechanisms of various PAHs by the lichen thallus, and comprehensively investigating the affinity of a particular species of lichen to the full range of PAHs (*P. austrosinense* (Zahlbr.) Hale in this study), would improve the confidence with which the results could be interpreted. The initial results indicate that our lichen species of choice is possibly a good biomonitor for PAHs, since the most toxic PAH, BaP, appears to be bioaccumulating within the lichen thallus and to be well preserved by our chosen biomonitor.

#### **4.11.3 Chlorophyll study results**

The efficiency of the clean up procedure as outlined by Blasco et al (2007) which claimed to produce extracts which were 'enriched and free from interferences', had been brought into question as a result of an apparent incomplete removal of chlorophyll in the prepared extracts. All the extracts prepared in this study ranged in colour from murky, olive green to a clear, bright emerald colour, suggesting that chlorophyll was still present in the samples, to different degrees. This was a cause for concern, since it is important to minimize this background interference in order to reduce the matrix effect observed in the samples. The dispersive-SPE cleanup step in the conventional QuEChERS technique was not effective at cleaning up the lichen extracts either since the graphitized carbon

black (GCB) formulated to remove pigments, has been found to result on losses of PAHs, as discussed in Section 2.6.4 (Sadowska-Rociek et al., 2013).

The species used in the clean up optimization study by Blasco et al (2007) was lichens of the type *Parmelia sulcata*, which differ from the *Parmotrema austrosinense* (Zahlbr) Hale used in this study, which added a level of uncertainty due to inter-species differences. In order to establish whether the different sample extracts had similar chlorophyll content after the clean up procedure, a spectrofluorometer was used to semi-quantify the chlorophyll in each extract. The results for the relative total chlorophyll content (the sum of chlorophyll *a* and chlorophyll *b*, fluorescence intensity expressed in counts per second) are shown in Figure 4.35 below. The results are ordered from the sample with the lowest total PAH concentration to the highest, as given in Table 4.13, with the concentration of BaP in each sample given.



**Figure 4.35: The results from the spectrofluorometer comparing chlorophyll content of each extract of samples of interest with each corresponding BaP concentration shown ( $\text{ng.g}^{-1} \text{dw}$ ), arranged by increasing total PAH concentration, from left to right**

The contributions of chlorophyll *a* and chlorophyll *b* were not consistent between sampling points, as seen in Figure 4.35, where some sites, namely Cape Point 1 and the bulk sampling site, had no chlorophyll *b* contribution, whereas all the other sampling sites had varying contributions towards the total chlorophyll content. This shows that the amount of different chlorophyll forms is not consistent within our lichen species. A similar result was observed by Beekley

and Hoffman (1981) and Beltman et al (1980) who found that chlorophyll content varies within the same species of lichen. Even within the Cape Point samples, a large variation existed between the total chlorophyll contents, illustrating the variation between samples. There is a common lack of reporting of the age (and by implication, the size) of lichens when sampling and this means that variations in chlorophyll content introduced by aging affects cannot be accounted for. The lack of reporting the age of lichens means that comparing chlorophyll contents of different lichen species becomes complicated as a result of possible and undefined degradation effects occurring with time, as well as possible increases in chlorophyll content after exposure to pollutants (Canas et al., 1997).

Although there appears to be no link between the chlorophyll content in each sample and the total PAH concentration, it is clear that the more polluted samples (Daspoort Tunnel sites and Pretoria Industrial) have higher total chlorophyll content than the cleaner samples. This supports work by Canas et al (1997) that found that in the presence of pollution, chlorophylls *a* and *b* degrade within the lichen thallus, after which the concentrations increase after heavier and extended exposure as well as the studies by von Arb et al (1990) and Carreras et al (1998). The additional degradation of chlorophyll, known as the bleaching effect, which occurs with time and cannot be reversed, would also impact older lichens, causing a reduction in total chlorophyll and thus introducing more variability in the chlorophyll content of lichens. It can be concluded that direct comparison of the chlorophyll content of our samples should be undertaken with caution, since without an understanding of the age of the lichen, the levels of atmospheric pollutants and the mesoclimate, a large variation may exist between samples (Eversman, 1978; Puckett, 1988; Showman, 1975).

In order to understand why the chlorophyll content was not proportional to the increase in total PAH concentration, particularly for Cathedral Peak and the bulk lichen sample, the concentration of individual PAHs were studied. It was observed that the samples that had lower concentrations of BaP also had lower chlorophyll content, as illustrated by the Cathedral Peak sample, which had a

PAH<sub>total</sub> of 547 ng.g<sup>-1</sup>, but a BaP of 99.0 ng.g<sup>-1</sup>, much lower than the Cape Point samples, with a corresponding low total chlorophyll result. It appears as though the levels of BaP and chlorophyll may be related, and additional studies are required in order to firmly establish a connection between these two variables.

In a study by Piccoto et al (2012), it was shown that different lichen species have different amounts of chlorophyll, and that the levels of chlorophyll are affected to different degrees by exposure to pollutants. As a result, the comparison of PAH results in lichens of different species need to be made with consideration towards the chlorophyll content, since the varying chlorophyll content would influence the extent to which the sample clean up procedure was effective. Considering that the study on which our sample clean up procedure was based (Blasco et al., 2007) used *Parmelia sulcata*, which was found to have a lower chlorophyll content than many other species (Beltman et al., 1980), the clean up procedure used in this study with specific application to *P. austrosinense* (Zahlbr) Hale requires further optimization.

A study by Tretiach and Carpanelli in 1992 suggested that when considering lichens and their assimilation of pollutants, it might be more meaningful to represent the assimilation rates based on the chlorophyll content rather than on the principle of dry weight. It was furthermore recommended that the concentration of chlorophyll in samples should always be reported (Tretiach & Carpanelli, 1992), and in light of the observed matrix effects and consequent varying chlorophyll content in the different extracts from regions of interest, and as well as the fact that photochemical activity is highest in the marginal lobes of the lichen, it appears that listing the chlorophyll content of lichens alongside any pollutant concentrations is paramount to interpreting the collected data (Baruffo et al., 2008).

Not only does the presence of chlorophyll influence the matrix effect, but also other pigments present in lichens such as pulvinic acid derivatives and terphenylquinones, are also likely to contribute towards the colour and the observed matrix effects in the extracts (Rundel, 1978). These results show the

importance of the standard addition method when quantifying PAHs in extracts from lichens. The standard addition method, suggested as a result of this study, would account for the varying matrix effect which cannot be predicted in lichens and therefore cannot be accounted for when using matrix matched standards, since each extract would have a unique chlorophyll content. The use of standard reference materials would also be obsolete due to the variation in chlorophyll content of lichens, even amongst samples of the same species and from the same region.

#### 4.12 Concluding remarks

The SEM analysis showed mineral-like substances as well as sporeidia on the surface of the bulk sample lichens collected in this study. The HPLC technique was not able to provide the necessary sensitivity or selectivity required for the study, and was thus not used further.

The GC-MSD instrument performance was studied and the intraday %RSD was found to be 7% and the interday %RSD was 23%. The LOD and LOQ values for the PAHs of interest in neat solvent were as low as 0.01  $\text{pg}\cdot\mu\text{l}^{-1}$  and 0.02  $\text{pg}\cdot\mu\text{l}^{-1}$ , respectively. Calibration curves were calculated and found to show good linearity within the required range of quantification.

Using Soxhlet as a sample preparation technique, it was found that dichloromethane had better extraction capabilities compared to acetonitrile as extracting solvent. Extractions using Soxhlet were, however, found to be poor in comparison to the other investigated methods. Microwave assisted extraction performed best when using *n*-hexane:acetone as the extraction solvent for a 20 min extraction, as shown in Figure 4.8. Using QuEChERS proved to be successful, outperforming other sample preparation techniques, as shown in Figure 4.22, with the *n*-hexane:acetone (1:1, v/v) ratio providing the best extraction efficiency and recovery of internal standard, confirmed by one-way ANOVA analysis of the data.

It was found that the homogeneity of the samples was inconsistent, however this was not improved by increasing the mass of sample three-fold. Furthermore, the importance of the use of matrix-matched standards and the standard addition method when quantifying was found to be paramount to the quality of the results. The type of matrix effects on the PAHs of interest ranged from strong enhancement to strong suppression, as given in Table 4.12.

In future, a 12 hr equilibration after spiking the lichen with the internal standard should be performed, as a result of the improved recoveries seen in Figure 4.17 for Q Hex: Ace extractions. Another improvement would be to quantify the amount of chlorophyll in each extract and express the PAH concentration relative to the chlorophyll content, and not only the dried weight, as discussed in Section 4.11, since the chlorophyll content was found to vary from sample to sample.

The QuEChERS extraction method developed and optimized in this study was successfully applied to lichens of interest. The concentrations of PAHs in the lichen samples from areas of interest were calculated using matrix matched standards, and the order of total PAH concentration increased from Cape Point Site 3 < Cape Point Site 2 < Cape Point Site 1 < Cathedral Peak region < Daspoort Tunnel Site 3 < Daspoort Tunnel Site 2 < Pretoria Industrial. The obtained results were meaningful with respect to both the impacted and non-impacted (background) sites.

In Chapter 5, overall conclusions as well as suggestions towards further work are given.

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## Chapter 5: Conclusions

This study covered the use of lichens as biomonitors for atmospheric PAHs in selected regions of South Africa. The particular species chosen was *Parmotrema austrosinense* (Zahlbr.) Hale, since it had been successfully used in a previous project in South Africa (True et al., 2012). This study compared different sample preparation techniques, by means of a bulk lichen sample, collected along a busy road in an urban area of Pretoria. Once the best performing sample preparation technique had been identified, it was used on samples collected from the Cape Point Nature Reserve, the Daspoort Tunnel, the Pretoria Industrial area and the Cathedral Peak region in the Drakensberg. The chlorophyll content of each of the samples was also investigated, to understand whether the matrix effect influenced the analysis of the samples of interest to varying degrees.

### 5.1. Conclusions regarding the sampling procedure

The entire lichen organism was removed from the bark of the tree and incorporated into the sample batch. As a result, the older lobes as well as the younger portions were mixed together. It was expected that collecting a range of lichens of varying size (and thus, varying age) would produce a sample that is representative of the atmospheric PAH profile over several years. This sampling protocol, however, increased the inhomogeneity within our bulk sample, since it has been shown that the older parts of the lichen thallus are degraded first in polluted conditions (Langmann et al., 2014).

Since the sampling methodologies in lichen studies do not always specify whether samples were normalized by age and size, it follows that the comparison of results from different studies is difficult since the apical lobes have been suggested to produce the most homogenous results (Adamo et al., 2008; Nascimbene et al., 2014; Tretiach & Carpanelli, 1992). This should therefore be considered in further studies in that the 5 – 6 mm apical portions of the lichens should be sampled. Furthermore, attention should be given to the meteorological conditions throughout the sampling campaign since it is likely to influence the uptake rates of PAHs (Augusto et al., 2013; Terzaghi et al., 2015). In

terms of sample handling after the sampling campaign, the removal of bark, old leaves and stones was undertaken as per other studies, as well as the drying process and grinding of the lichens (Augusto et al., 2010; Blasco et al., 2008).

The homogeneity of the sample was brought into question by the high %RSD values seen in all the extracts (up to 96% for MAE extractions, up to 173% for QuEChERS and up to 173% for USAE). It can be concluded that the high %RSDs came about as a result of inhomogeneity, and not as a result of one of the sample preparation techniques. No other studies have investigated the challenges regarding homogeneity of their lichen samples, despite noticeable standard deviations amongst replicates, except for a recent paper which sampled specific lobes of the lichen (Nascimbene et al., 2014). Increasing the sample mass and reporting the lichen age (this is a laborious task of measuring growth rates of lichens or using isotope dating), as well as sampling specific portions of the lichen structure consistently, should substantially address the issue of sample inhomogeneity.

The portion of the thallus reserved for scanning electron microscopy (SEM) analysis was found to have more surface cylindrical particles as well as a coarser surface, compared to a study on *Pseudevernia furfuracea* (Adamo et al., 2008), suggesting the presence of more spores or lichen substances on the surface of *Parmotrema austrosinense* (Zahlbr.) Hale. SEM/EDX spectroscopy showed that Si, Fe and Al were present on the surface of lichens from the bulk sampling site, as well as lower levels of Mg, Cu and Ti, all associated with diesel, lubricating oils and automobile catalysts (Garty, 2001).

## **5.2 Conclusions regarding the sample preparation and analytical techniques employed in this study**

HPLC with a diode-array detector was investigated as an analytical tool. However, the sensitivity and resolving power was found to be insufficient for this application. The use of an ultraviolet-visible detector, as in the study by Shukla and Upreti (2013), would dramatically improve detection limits as well as resolution as a result of the fluorescent character of PAHs.

The direct thermal desorption of spiked lichens directly into a GC-TOFMS instrument was disproved by a thermal combustion test, which found that lichens start to combust at temperatures, below the elution temperature of the PAHs of interest.

The GC-MSD method developed for this study, using the Restek Rxi®-PAH column had an interday variation of 23% (n=5) and an intraday variation of 7.0% (n=5). Calibration curves showed good linearity in the desired concentration range for solvent standards ( $R^2$  values ranging between 0.987-0.998), and the PAH standard mix used was efficiently separated, where the resolution of peaks of greatest concern for overlap was calculated to be 1.1. The LOD values ranged between 0.01 – 1.53  $\text{pg}\cdot\mu\text{l}^{-1}$  and the LOQ values were found to range between 0.02 – 5.10  $\text{pg}\cdot\mu\text{l}^{-1}$  in matrix matched standards, comparable to values in studies by Shukla et al (2013) and Upreti and Patel (2012) using HPLC UV-V, and lower than other lichen studies using GC-MS and solvent based standards (Domeño et al., 2006).

As a result of an equilibration study, involving spiking of the lichen matrix with either immediate extraction or 12 hr thereafter, it was concluded that spiking 12 hr prior to extraction is required if using the QuEChERS extraction technique. This would ensure that the internal standard is quantitatively recovered. However, for MAE or USAE extraction of lichen samples, immediate extraction yielded better recoveries. It is therefore recommended that an equilibrium spike study be performed for each unique sample preparation technique over a range of equilibrium periods in order to establish whether an equilibration time is required for the internal standard, prior to commencing with routine sample preparation. An interesting phenomenon was observed for the spike on the lichens left for 0 – 96 hr, where the peaks for Chr and Ant had disappeared. This suggests a preferential uptake of certain PAHs more than others, as had been identified for other lichen species (Blasco et al., 2011). A cyclic increase and decrease of total PAH peak area was also observed, which suggested that a cyclic adsorption/revolatilization process was at play in our lichen species.

To our knowledge, no studies have been performed on the equilibration time required after spiking the lichen matrix, prior to the addition of solvent and the extraction of PAHs from the lichen thallus. Our spike studies for MAE, USAE and QuEChERS illustrate the importance of understanding the optimal spiking conditions and equilibration time required to ensure that the recoveries of the internal standards and standard additions are representative.

Soxhlet extractions were performed on the bulk lichen sample using both dichloromethane and acetonitrile. Both the poor recoveries (<10% for acetonitrile and <20% for dichloromethane) and the total number of PAHs extracted (only 3 for acetonitrile extraction and 6 for dichloromethane extraction) lead to the conclusion that Soxhlet was not efficiently extracting the PAHs under investigation, despite its application in other lichen studies with moderate recoveries reported (Augusto et al., 2010; Bajpai et al., 2013; Shukla & Upreti, 2013). The principles of Soxhlet extraction also did not conform to the desired outcomes of this study, since it uses large solvent quantities and has long extraction times, rendering it unsuitable as a fast, environmentally friendly extraction procedure.

Ultrasound assisted extraction (USAE) was undertaken on the bulk lichen sample and 3 consecutive extractions were found to be optimal, and the optimized solvent was found to be dichloromethane, although no heavy PAHs (MW 228 g.mol<sup>-1</sup> and higher) were detected in any of the extracts. Dichloromethane also provided better recoveries of the internal standard and greater peak intensities for the identified PAHs, compared to the *n*-hexane:acetone extracts. Upon investigation it was found that the initial sample extraction steps prior to SPE were the sample preparation steps introducing the most losses of the internal standard. It can thus be expected that the PAHs associated with the lichen thallus may be affected in a similar manner.

Upon optimization of the solvent for microwave assisted extraction (MAE), it was found that *n*-hexane:acetone (1:1, v/v) produced better extraction of the PAHs of interest from the bulk lichen sample than dichloromethane, extracting

both light and heavier PAHs (Nap, Acy, Ace, Flu, Ant, Phe, FluAn and Pyr) as opposed to dichloromethane which only extracted light PAHs (Nap, Acy and Ace) and a total extraction time of 20 min was required. It was observed, however, that the recoveries of the internal standards as well as the %RSDs were best in the samples that were only extracted for 5 min. This suggested a non-selective degradation of PAHs with longer extraction times (Camel, 2000). Despite this, 5 min was not suitable because it was insufficient in extracting PAHs from the matrix, extracting only 3 PAHs from the matrix, compared to 7 and 8 (for the 10- and 20-min extraction), respectively. High %RSD values as a result of inhomogeneity were also observed, and again highlights the need for further investigation. MAE performed well compared to Soxhlet and USAE, but was outperformed by QuEChERS in terms of the number of PAHs extracted as well as the total peak area of the PAHs extracted.

It was found that *n*-hexane:acetone (1:1, v/v) extracted the most PAHs from the bulk lichen sample when using the QuEChERS technique and produced the largest total peak area amongst all the different sample preparation technique extracts. Out of the three QuEChERS solvent schemes, the *n*-hexane:acetone (Q Hex: Ace) extracts also produced the best recoveries of the internal standards (96% and 178%), as well as the lowest %RSD values of 15.2% and 30.6% for phenanthrene-*d*<sub>10</sub> and pyrene-*d*<sub>10</sub> respectively. However, the inhomogeneity of the bulk sample was found to also be problematic in the QuEChERS extractions, since some of the replicate analyses did not produce the same PAH profile, despite the sample being drawn from the same batch and being prepared in an identical manner.

In making these comparisons between the different sample extraction techniques and conditions, it was assumed that the matrix effects were similar for all the extracts. The application of the QuEChERS approach was the most successful sample preparation technique used in this study. From the results of all the sample extraction techniques, it was clear that a challenge of inhomogeneity regarding the bulk sample was impacting on the precision in all

the different extracts. Increasing the sample mass from 0.2 g to 0.6 g using the optimized QuEChERS extraction did not improve the %RSDs however.

The comparison of the results from the extraction of the bulk lichen sample using a range of techniques showed that Q Hex: Ace was the only extract to contain the heavier PAHs of interest in this study (Chr, BaP, IcdP and DahA). Q Hex: Ace performed better than the conventional sample extraction techniques in terms of internal standard recoveries, total targeted PAHs extracted (total PAH peak area), as well as the largest total number of individual PAHs extracted (11 PAHs in the native bulk sample, compared to 6 using either Soxhlet DCM or USAE). ANOVA analysis at a 99% confidence level showed that there was a significant difference between the Q Hex: Ace extract and all the other sample extraction techniques. It was thus concluded that Q Hex: Ace was the most successful sample extraction procedure in this study, specifically for the analysis of PAHs from the lichen *Parmotrema austrosinense* (Zahlbr.) Hale. The extraction procedure met the requirements for an ideal method, since it is fast, uses little solvent compared to conventional techniques, is efficient and it was found to selectively and quantitatively extract the PAHs of interest from the lichen thallus.

Since heavier PAHs were extracted using the Q Hex: Ace technique, it was concluded that the lichen species chosen in this study was a good choice for the biomonitoring of PAHs, since it accumulates both lighter and heavier PAHs, reflecting the presence of both gas phase and particulate phase PAHs in the atmosphere.

### **5.3 Conclusions regarding the observed matrix effects**

The observed tailing in the chromatograms for all peaks, as well as the differing visual appearance of the extracts and the elevated % recovery for some of the extracts, when using solvent-based standard calibration curves, necessitated the investigation into whether matrix effects were influencing the analytes' responses with particular respect to the PAHs of interest extracted from the lichen thallus.



Matrix-matched standards derived from the bulk lichen sample were prepared using the Q Hex: Ace extraction method. The extracts were spiked with different concentrations of a PAH mix standard and the resulting method LODs and LOQs were of a similar order of magnitude as the solvent-based (instrumental) LODs and LOQs. Since the instrumental and the method LODs are of similar magnitude, it can be concluded that the sample extraction procedure is not significantly reducing the method sensitivity. Calibration curves for the matrix-matched standards had  $R^2$  values ranging between 0.925 and 0.993. The linearity of the matrix-matched standard curves was poorer than the linearity of the calibration curves obtained in neat solvent. This highlights the complexity of the matrix effect.

Calculations were performed in order to describe the extent of the matrix effect for each PAH of interest. The type of matrix effects ranged from strong enhancement to strong suppression. Benzo[*a*]pyrene (BaP), known to be carcinogenic (Clapp et al., 2008; Nisbet & Lagoy, 1992), experienced only a soft enhancement (% matrix effect of 6%) which suggested that the BaP concentration calculations would be least affected by any matrix effects, thus providing an accurate representation of the presence of this harmful PAH in the environment in which the lichen was sampled. Its ubiquitous presence is of concern towards human and ecological health (Ravindra et al., 2001) and the correct quantitation of BaP is paramount to understanding and interpreting the impact it may have on the environment.

The PAHs experiencing the most severe matrix effects were Ant and Pyr, with % matrix effects above 300%, indicating a very strong enhancement. These results heavily impact the interpretation of the analysis, since any diagnostic ratio or toxic equivalence quotient would be severely changed if the matrix effects were not accounted for. This would lead to misleading conclusions about the main sources of PAH contamination in the atmosphere.

The observed matrix suppression for Ace, IcdP, BghiP and DahA is also problematic since the suppression of analytes, already present at low levels

means that they might not be detected in a sample, despite their presence in the atmosphere. This type of matrix effect is most difficult to overcome since a corrected calibration is not able to correct for the interference, if the analyte is being suppressed to the extent that it is not detected in a sample extract. The matrix matched calibration curves were used to quantify the identified PAHs in the samples from areas of interest in this study. It should be noted that no non-impacted lichen standard reference material is available which can be used as a PAH-free matrix, where even lichens sampled in remote valleys in the Alps have been found to contain PAHs (Nascimbene et al., 2014). The practical implications of the severity of the matrix effects include regular liner replacements and the cutting of the column as a result of column damage, impacting both on the cost of analysis and time usage.

The relative amount of chlorophyll in each of the extracts from the lichens sampled in areas of interest was investigated using fluorescence spectrophotometry. As in studies by Beltman et al and Beekley and Hoffman (Beekley & Hoffman, 1981; Beltman et al., 1980), it was found that the chlorophyll content varied amongst the sample extracts, even though the samples were of the same lichen species. Although it is expected that an increase in pollution will result in an increase in chlorophyll content (Carreras et al., 1998), it was observed that this reported trend was not followed in the samples of interest, where results from the bulk sampling site as well as the Cathedral Peak region had lower total chlorophyll content than the Cape Point sites which had lower total PAH concentrations. Since it was observed that the chlorophyll content was higher in the samples with higher BaP concentrations, it is suggested that the chlorophyll content might be correlated to the amount of BaP incorporated into the lichen thallus, and this should be investigated further.

In order to account for the variations induced by the mesoclimate and pollution levels of the sampling area, the age of the lichens sampled as well as the differences in chlorophyll content amongst extracts (Canas et al., 1997), the use of standard additions is considered paramount to the accurate determination of the concentration of PAHs incorporated into the lichen thallus. A thorough

investigation of matrix interferences is vital and should be covered in all studies using lichens as biomonitors for atmospheric PAHs.

#### **5.4 Conclusions regarding the samples of interest**

The matrix-matched calibration curves were used to quantify the identified PAHs in the lichen samples from the areas of interest, and the calculated concentrations were expressed in terms of the dried weight of the lichens used in the extraction. A range of PAHs was identified in the samples of interest, ranging from the lightest PAH of interest, Nap, to the heaviest PAH of interest, BghiP. All the extracts from the samples of interest contained Nap, Acy, Ace, Flu, Phe and BaP, supporting our suggestion that there existed a preferential uptake of particular PAHs, specific to the lichen species used in this study, as in previous studies (Blasco et al., 2011).

High BaP bioaccumulation in all the samples was found, since BaP was amongst the PAHs at highest concentration in all the samples of interest at concentrations as high as 440 ng.g<sup>-1</sup> dw. This suggested that our biomonitor of choice is well suited towards atmospheric PAH studies as a result of the apparent bioaccumulation of BaP, since it is a highly toxic PAH and its presence is a cause for major concern in human health studies. Further studies relating to human health risk assessments should be developed from these results, as seen in a study by Augusto et al (2012).

The most highly impacted sites in this study were the Pretoria Industrial (total PAH content of 1001 ng.g<sup>-1</sup> dw), Daspoort Tunnel Site 2 (737 ng.g<sup>-1</sup> dw) and Daspoort Tunnel Site 3 (693 ng.g<sup>-1</sup> dw), which had the highest total PAH concentrations, as well as the highest concentrations of BaP. The least impacted sites were found to be the Cape Point 3 (425 ng.g<sup>-1</sup> dw), Cape Point 2 (505 ng.g<sup>-1</sup> dw) and Cape Point 1 (514 ng.g<sup>-1</sup> dw) sites, as expected due to the dominant south easterly wind that blows cleaner maritime air masses over the Cape Point Nature Reserve (Brunke & Halliday, 1983). Wood and grass burning, as well as petroleum combustion were suggested to be the main sources of atmospheric PAHs in this region, as a result of the high concentrations of BaP, Flu, Ace and

Nap in the Cape Point Nature Reserve samples (Ravindra et al., 2006; Rogge et al., 1993). The large range in total PAH concentration of all the samples (425 – 1001 ng.g<sup>-1</sup> dw) demonstrated the applicability of the sample preparation technique developed in this study to quantitatively and qualitatively distinguish between lichens sampled from regions with different PAH profiles.

The lichen sample from the Cathedral Peak region (547 ng.g<sup>-1</sup> dw) was more impacted than the Cape Point Nature Reserve samples, likely due to the passing traffic, as the lichens had been sampled on a roadside. Sampling lichens farther from the road in a future study would provide a meaningful understanding of the ambient atmospheric PAH levels for tourists who visit the region for hiking and outdoor activities. The PAH ring-size profiles of the Cape Point Nature Reserve sites 1 and 2 and the Cathedral Peak region samples were found to be similar, with a large % contribution by 2-, 5- and 3-ring PAHs, in order of prevalence respectively. The Daspoort Tunnel samples had similar PAH ring-size profiles with a dominance of 5- and 2-ring PAHs. The bulk sample as well as the Pretoria Industrial sample had unique PAH ring-size contribution profiles, where the sample from the industrial region had a high % contribution of 5- and 6-ring PAHs and a low 2-ring PAH contribution. The bulk sample had an evenly distributed PAH ring-size profile.

Principal Component Analysis (PCA) results showed that the three Cape Point Nature Reserve samples were very similar, with high similarities between the Cape Point and Cathedral Peak region samples. The Pretoria Industrial sample was identified as being different from all the other samples, and the Daspoort Tunnel samples were grouped close together. The fact that the bulk sample was categorized close to the Daspoort Tunnel sites suggested that vehicular emissions were significantly contributing towards the atmospheric PAH profile in the region of the Daspoort Tunnel, since the main sources of PAHs at the bulk-sampling site are known to be vehicular emissions from both automobiles and small trucks.

The loading plots obtained from the PCA indicated that the increased concentration of IcdP, BbF, FluAn, BaP, Chr and BghiP accounted for the variation of the Pretoria Industrial Sample compared to the other samples. It was therefore suggested that these PAHs might be indicative of industrial works as well as heavy-duty trucks. The loading plot also indicated that Nap, Phe, Ant and Ace were the distinguishing PAHs that provided an indication of the difference between the impacted (Daspoort Tunnel sites, bulk sample site and Pretoria Industrial site) and less-impacted sites (Cathedral peak region and the Cape Point Nature Reserve sites). These results differed from the results of a study that sampled lichens from an industrial area in Portugal that found that 4-ring PAHs dominated the PAH profile, followed by 3- and 2-ring PAHs (Augusto et al., 2013). This again suggested a variation in the preferential uptake of PAHs by different lichen species, making the comparison of results using different species problematic, since some lichens tend towards 2-, 3- and 4-ring PAH accumulation, whereas our lichen of choice appeared to accumulate 5- and 2-ring PAHs preferentially. Since it has been shown that lower MW PAHs are photodegrade more easily than the higher MW PAHs when exposed to UV radiation (Nadal et al., 2006), we can say that photodegradation was not largely affecting the results, since 2- and 5-ring PAHs were preferentially accumulated by our lichen species.

The lichen samples were taken during the winter months, where dry weather prevailed, excepting for the Cape Point Nature Reserve samples that had been sampled at the end of the wet winter experienced in this region. The presence of 5- and 6-ring PAHs at some of the more polluted sites was indicative of dry deposition, which is commonly associated with particulate matter (Blasco et al., 2011). The open structure of *Parmotrema austrosinense* (Zahlbr.) Hale results in the possibility of trapping a full volatility range of PAHs, since the entire upper surface is exposed to the atmosphere and can thus trap gas phase as well as particle phase PAHs, similar to another study (Blasco et al., 2011).

The toxic equivalency quotients (TEQs) for the samples from areas of interest gave an indication of the toxicity of the atmosphere. Cape Point site 3 had the

lowest TEQ, with a BaP equivalent concentration of 113 ng.g<sup>-1</sup> dw, and the site with the highest TEQ was found to be the Pretoria Industrial site, with a BaP equivalent concentration of 478 ng.g<sup>-1</sup> dw. This leads to the conclusion that not only was the Pretoria Industrial site the most polluted in terms of highest total PAH concentration, but also the most toxic site covered in this study. The TEQs were higher in our study compared to the values presented by Augusto et al (2102), possibly due to the fact that the matrix effect had not been investigated in the Augusto study, as well as due to differences between the two different lichen species used. Soxhlet extraction was utilized in the Augusto study, which was proven to be ineffective in this study for our specific lichen species.

Since the main sources of PAH contamination were known for the regions of interest in this study, diagnostic ratios were investigated to assess their potential use in terms of accurately apportioning PAH sources in lichen based biomonitoring studies, as they have been successfully applied to direct air sampling data (Geldenhuys et al., 2015). The diagnostic ratio used to describe the contribution of combustion processes to the PAH profile,  $\Sigma\text{PAH}_{\text{comb}}/\Sigma\text{PAH}_{\text{total}}$ , correctly indicated that combustion was not the main source of PAHs for the Cape Point site 3 sample. However, it did not reflect combustion processes for the bulk sampling site impacted by household fires, as well as Cape Point sites 1 and 2, which were known to have been impacted by a *fynbos* burning event. The diagnostic ratio of Flu/(Flu + Pyr) indicated that all the samples from different regions had the same source: diesel, grass and wood combustion. This diagnostic ratio was concluded to therefore not be useful for our results, as was the ratios Ant/(Ant + Phe) and Pyr/BaP that were unable to distinguish between our samples of interest, despite the results from ANOVA indicating clear differences.

The diagnostic ratio of IcdP/(IcdP + BghiP) was useful for the impacted sites, indicating that coal and diesel combustion were likely sources of PAHs at the Pretoria Industrial region, diesel combustion at Daspoort Tunnel site 2 and diesel and coal combustion at Daspoort Tunnel site 3. This source apportionment aligned with the processes known to have occurred at the sampling sites.

Differing source diagnostic results between this study and a study by Augusto et al (2010) highlight that caution should be applied when using diagnostic ratios to identify PAH pollution sources from biomonitoring studies. Overall, the use of diagnostic ratios such as  $IcdP/(IcdP + BghiP)$  as well as the general ratio  $\Sigma PAH_{comb}/\Sigma PAH_{total}$  were concluded to be useful for highly impacted sites, such as the Pretoria Industrial site, but none of the diagnostic ratios were successfully applied to the less impacted sites to identify the likely sources of pollution.

In general, the results of such lichen biomonitoring studies may be affected by artefacts such as the volatilization of lighter PAHs between sampling and final extract analysis, possible over-estimation of the heavier particle-associated PAHs due to sorption onto the lichens, or by chemical degradations by nitrogen oxides, halogens, ozone, or the hydroxyl radical, as well as UV degradation (Ravindra et al., 2008). It should also be taken into consideration when interpreting the results that the targeted analysis of only 15 PAHs in this study results in data that omits other PAHs that were included in other studies. In general, the comparison of results between studies should be made with caution unless the chlorophyll content is given alongside the concentration expressed in relation to the dried weight of the lichen, as well as details regarding the age of the lichen, the section of the lichen sampled, the meteorological data when sampling as well as the details of any preferential uptake by the specific species.

### **5.5 Additional recommendations towards future studies**

A future consideration regarding the use of lichens as biomonitors for PAHs, is the full validation of the sample preparation technique (QuEChERS, using *n*-hexane:acetone (1:1, v/v)) by means of interlaboratory proficiency tests to provide repeatability data (van Zoonen et al., 1999).

Dynamic sonication assisted solvent extraction (DSASE) as described by Domeño et al (2006) has been reported to extraction PAHs from lichens efficiently, and could be useful in reducing the number of steps involved in the USAE procedure, which was found to introduce most losses during the initial extraction steps (transferring between vials and the blown down step), since only one vessel is

used and the total extraction volume would be reduced significantly. In terms of improving the microwave assisted extraction technique, the use of stir-bar sorptive extraction is likely to improve the extraction of PAHs if a PAH selective sorbent material is used, as described by Alvarez-Aviles et al (2007) for aerosols, which would protect the PAHs from possible degradation occurring in the solvent extracts (Camel, 2000).

The clean up procedure used in this study was based on an optimized clean up described by Blasco et al (2007). However, in order to improve on the recoveries of the internal standards as well as reduce losses of PAHs, the clean up procedure needs to be further optimized for the QuEChERS extraction procedure to produce colourless extracts and reduce the problematic matrix effects observed. The use of conventional column chromatography as a clean up step should also be investigated, as applied in other studies (Shukla & Upreti, 2013; Upreti & Patel, 2012).

The lack of availability of a standard reference material for organic pollutants in lichens is problematic and suggests that standard addition should always be used in any lichen study, to account for the combined effect of many uncertainties (the presence of pigments and other interfering peaks). Furthermore, the complex contributions of gaseous pollutants and their interplay with the mesoclimate should be studied further in order to start a trend of consistently reporting contributing factors alongside biomonitors results (Terzaghi et al., 2015; Tretiach et al., 2007).

The use of HPLC with ultraviolet-visible detection, as in the study by Shukla and Upreti (2013), would improve the sensitivity as well as resolution of the HPLC method, as a result of the fluorescent properties of PAHs. Even though the GC-MSD method developed in this study was shown to sufficiently separate and identify PAHs in the extracts, improved sensitivity would further lower the detection levels and provide meaningful data on the PAHs known to undergo strong suppression by the matrix. The Restek Rxi®-PAH column used for the GC



analysis provided efficient separation of all the PAHs of interest in this study, including the problematic isobaric pairs often reported together in literature.

Further investigations into what the optimal spike equilibrium time would be for the QuEChERS extraction is required in order to confidently establish the equilibrium period required prior to extraction and to quantitatively extract both the PAHs from the native lichen matrix, as well as the internal standard.

One of the biggest challenges in this study was the matter of inhomogeneity in the bulk sample, which was used to develop the sample extraction methods. As discussed, the main contribution towards this inhomogeneity is likely to be as a result of the varying chlorophyll content of the different portions of the lichen structure, as well as the combination of both old and young portions of the lichen thallus. Calculating back trajectories at all sampling points could also be meaningful towards understanding the sources.

Further studies should be conducted to optimize the upscaling of the QuEChERS extraction method across a range of sample masses, solvent volumes and extraction salt masses, in order to improve repeatability. This is necessary since the distribution coefficient would dictate that more PAHs would be extracted when the sample mass-to-solvent volume ratio is lower.

A study into the photosynthetic activity of different lichen species by Baruffo et al (2008) showed that there exists a variability in this regard between lichens of the same species of different ages, and the suggestion was thus made to introduce a protocol by which a standard section of the marginal lobe be sampled for lichen studies. This protocol should be standardized across all studies using lichens as biomonitors for atmospheric PAHs in order to allow for the comparison of results from different regions across the world.

Furthermore, the suggestion by Tretiach and Carpanelli (1992) to represent the assimilation rates of pollutants based on chlorophyll content should be adopted in order to establish a standardized approach for reporting PAH concentrations in lichens, in light of the matrix effects observed in this study. It is therefore

recommended that the chlorophyll content of each extract should be reported alongside the concentration of each PAH, with quantification based on calculated standard addition principles (Baruffo et al., 2008). Further studies should also include a full scan study, with the inclusion of oxygenated and alkylated PAH species and PAHs of higher molecular mass, since photochemical reactions can lead to the formation of PAH-related species that were not targeted in this study (Lima et al., 2005; Pozzoli et al., 2004). These broader studies would provide a clearer, holistic understanding of the atmospheric PAH profile in each of the regions of interest.

This study has shown the potential of lichens as biomonitors for atmospheric PAHs in South Africa for the first time. No work has been done in this field across the continent of Africa, and serves as an illustration of the use of simple sample preparation techniques to extract a full spectrum of PAHs from these perennial organisms. The results from this study add value to the global database on PAH levels in lichens, including some PAH concentration levels from impacted and non-impacted sites across South Africa. The novel use of QuEChERS to extract PAHs from the lichen matrix has been shown here for the first time, as well as the use of MAE for the extraction of PAHs from the lichen matrix. Other new developments applicable to the field of lichens as biomonitors for PAHs, forged in this study, show that an equilibration period, prior to extraction, is necessary when performing QuEChERS extraction, as well as a possible cyclic process of volatilization/adsorption of PAHs occurring over a period of 96 hr, as seen in an equilibration of a PAH spike study. Matrix effects were found to influence quantification, and in this study we suggest for the first time the use of matrix-matched standards for all calibrations or the use of standard additions in order to ensure consistent quantification results across different studies. The investigation into the chlorophyll content of extracts in this work also led to a new conclusion in the field of lichens as biomonitors for PAHs, in that the chlorophyll content varies across samples regardless of PAH content, and therefore should be given alongside PAH concentration data in the future, in order to further improve the comparison of global results of PAH levels in lichens.

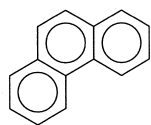
## 5.6 References

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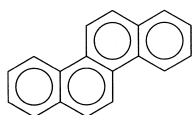
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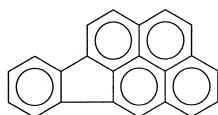
## Appendix A: PAH structures of PAHs of interest in this study



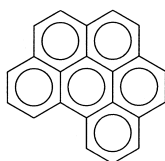
Phenanthrene (Phe)



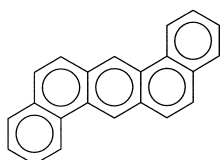
Chrysene (Chr)



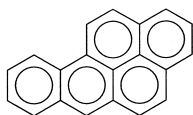
Indeno[123-*cd*]perylene (IcdP)



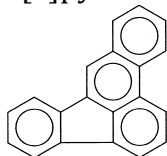
Benzo[*ghi*]perylene (BghiP)



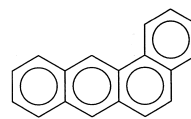
Dibenzo[*ah*]anthracene (DahA)



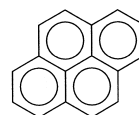
Benzo[*a*]pyrene (BaP)



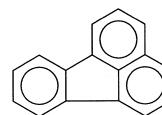
Benzo[*b*]fluoranthene (BbF)



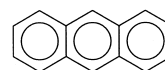
Benzo[*a*]anthracene (BaA)



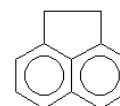
Pyrene (Pyr)



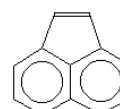
Fluoranthene (FluAn)



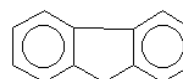
Anthracene (Ant)



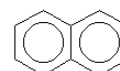
Acenaphene (Ace)



Acenaphthylene (Acy)



Fluorene (Flu)



Naphthalene (Nap)

## Appendix B: Examples of lichen sampling campaign sheets

**LICHEN SAMPLING SHEET - PAH STUDIES**

Name of sampler: LEANDRI VAN DER WAT  
 Date: 26 August 2014  
 Time: 11:42  
 LICHEN IDENTITY/NUMBER: PRETORIA INDUSTRIAL 1  
 Humidity: 0.2%  
 Altitude: 1914 m  
 Wind speed and direction: 1.7 m/s  
 Temperature: 23.1 °C  
 Season: Winter Dry  
 Sampling height (m): 1.5 m  
 Tree detail (lichen NESW): SE of TREE  
 Photo number (if photos are taken) \_\_\_\_\_

Location details - (give GPS co-ordinates of every tree):

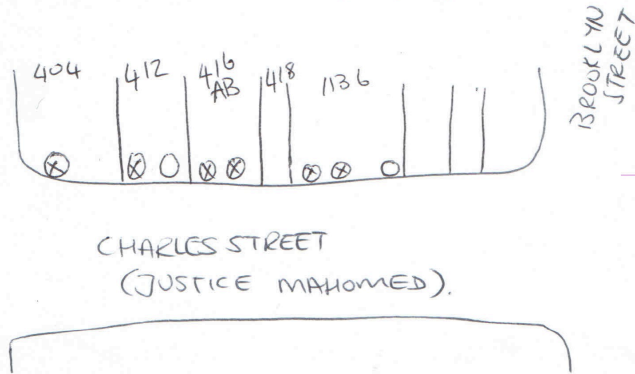
Pieter v Ryneveld Rd.  
 Next to entrance  
 on RHS

Further Comments:

LICHEN SAMPLING SHEET - PAH STUDIES

Name of sampler: Leandri  
 Date: 03/06/2008  
 Time: 09h30 - 10h30  
 LICHEN IDENTITY/NUMBER: BULK SAMPLE  
 Humidity: 10.0%  
 Altitude: 1312m  
 Wind speed and direction: 1.0 m/s NE  
 Temperature: 17.8°C  
 Season: WINTER (DRY)  
 Sampling height (m): 1.5m upwards  
 Tree detail (lichen NESW): JACARANDA

Lichen sampled 1.5m and up; on S facing side of trunk.  
 Location details - (give GPS co-ordinates of every tree):



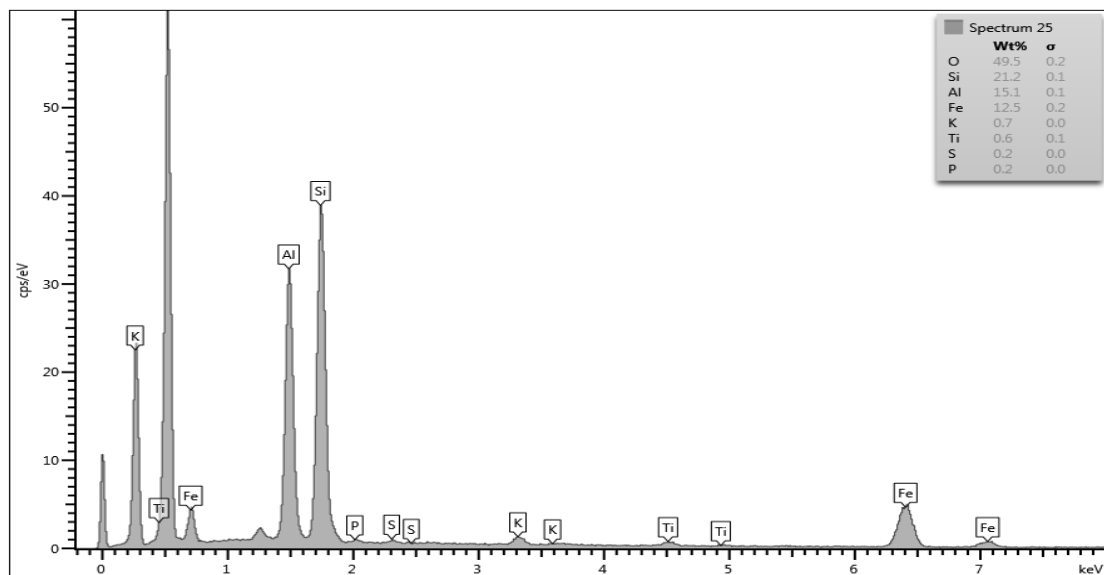
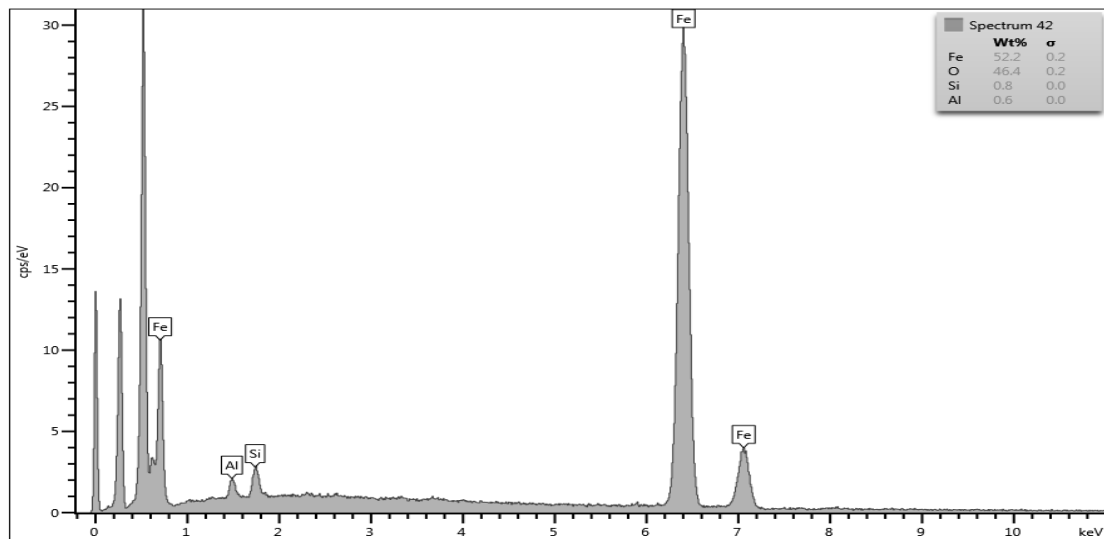
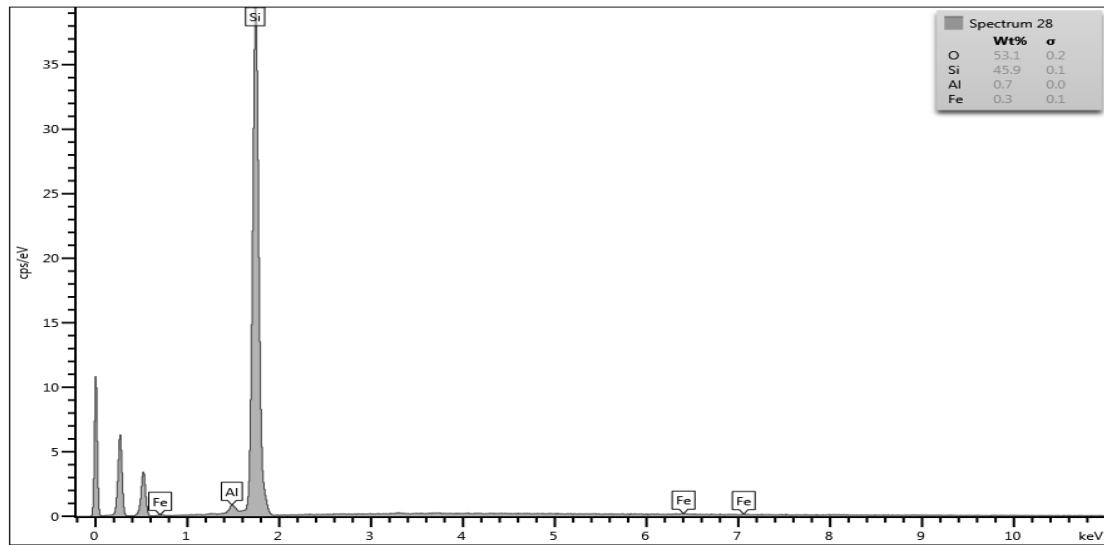
no nearby bus stops or taxi ranks / train stations.  
 no nearby land fill or factories.  
 2 Fuel stations in 2km vicinity

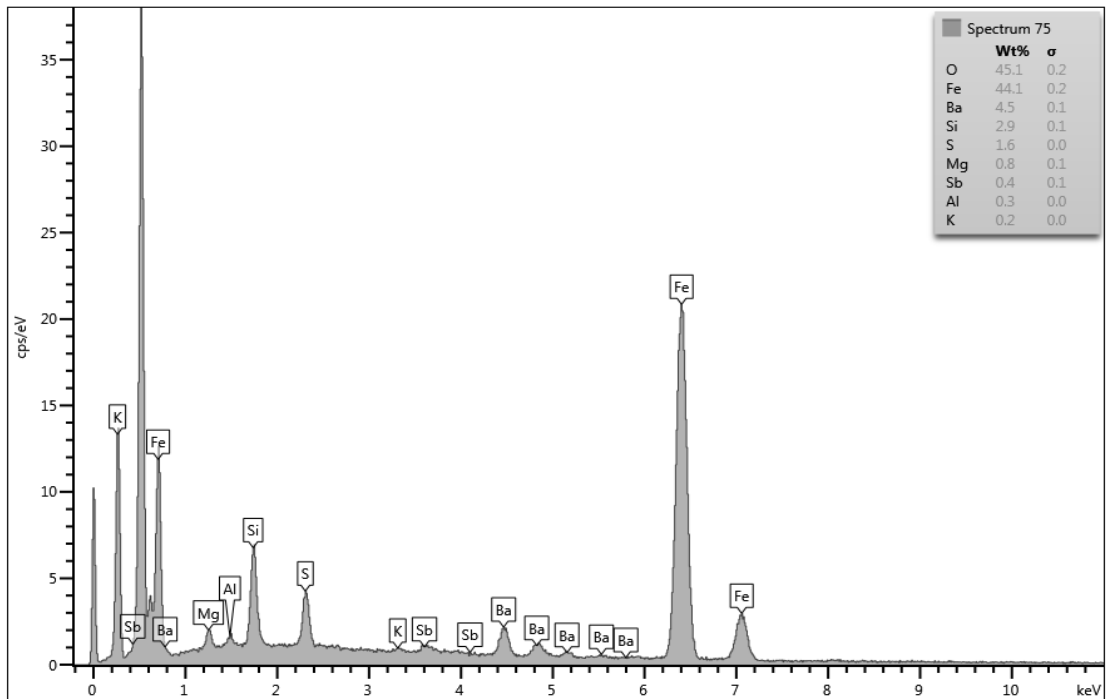
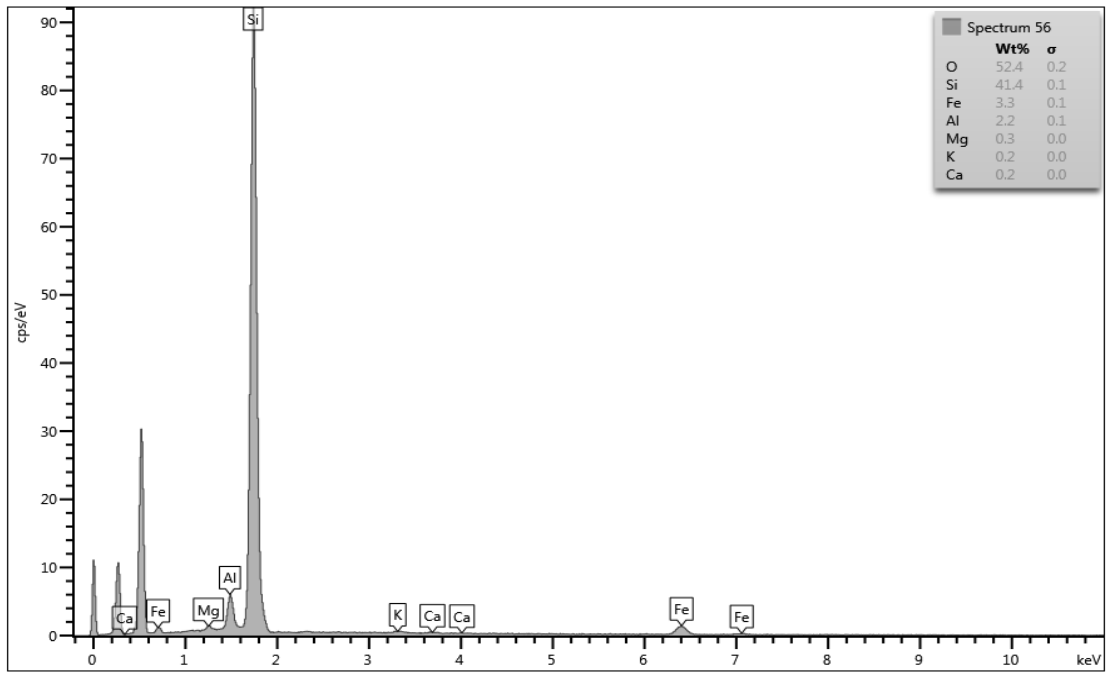
Further Comments:

One tree (only 1 sample taken from it)  
 had a termite colony on the lower trunk.



## Appendix C: SEM/EDX results from lichens sampled at bulk-sampling site









strata®

Certificate of Analysis



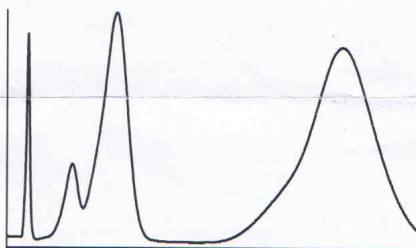
Strata products for solid phase extraction are manufactured with the highest purity reagents and raw materials. Every lot must conform to our strict specification ranges and is subjected to a wide range of rigorous quality control tests and physicochemical characterization methods. These tests include (but are not limited to) the following:

Strata® NH2

Sorbent Lot Number: S209-094

Particle Characterization		Sorbent Characterization	
Parameter	Results	Parameter	Results
Surface Area (m <sup>2</sup> /g)	479	Carbon Loading (% C):	5.0
Pore Size (Å)	73	Nitrogen Loading (% N)	1.8
Pore Volume (ml/g)	0.87	Hydrogen Loading (% H)	1.6
Average Particle Size (µm)	54	Sulfur Loading (% S)	N/A
Particle Size Distribution		Surface Coverage (µmol/m <sup>2</sup> )	3.0
Particles < 20 µm (%)	9	Residue (mg/g):	0.5
Particles < 31 µm (%)	14	Turbidity (NTU)	0.8
Particles > 80 µm (%)	13	Ion Exchange Capacity (meq/g)	1.3
		Physical Appearance	White to off white powder

HPLC Sorbent Test



Conditions:

Column: 100 x 4.6 mm  
Mobile Phase: 40mM sodium phosphate, pH 7  
Flow Rate: 0.5 mL/min  
Detector: UV @ 220 nm  
System: HP 1100  
Injection Volume: 25 µL

Analytes:

1. Acetone
2. L-Tartaric Acid
3. Isophthalic Acid
4. Citric Acid

Sorbent Purity Tests

Sorbent Purity (HPLC): Pass  
Sorbent Purity (GC): Pass  
Dye Tests: Pass

Approved By: K. Keith  
Quality Assurance

Date Approved: May 17, 2013

Note:

In addition to the media tests performed above, the final product is also tested for Mass, Flow, and Media Identity. These parameters are tested and verified in compliance with Phenomenex's ISO 9001:2008 certified Quality Management System prior to packaging to ensure that finished goods also meet finished product specifications.



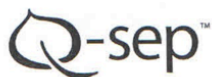
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support@phenomenex.com

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QUALITY MANAGEMENT SYSTEM  
CERTIFIED BY DNV  
ISO 9001:2008



**Certificate of Composition**

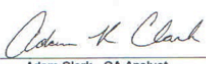
Description: Q-sep™ Q110 Packets plus Empty 50mL Centrifuge Tube  
 Catalog No.: 26235  
 Batch Lot No.: 22455  
 Weight: Compliant with EN15662  
 Contents List:

Component	Method Specification (g)	Cleanliness (P/F)	Packing Lot
Magnesium Sulfate	4.0	P	C05W027
Sodium Chloride	1.0	P	K13U034
Trisodium Citrate Dihydrate	1.0	P	10148754
Disodium Hydrogen Citrate Sesquihydrate	0.5	P	10151505
		P	10149554
Finished Product	-	P	22455

Notice: Material Safety Data Sheets are available by following the link below:

<http://www.restek.com/armdocs.asp>

This product has passed Restek quality control specifications for cleanliness.

Signature:   
 Adam Clark - QA Analyst

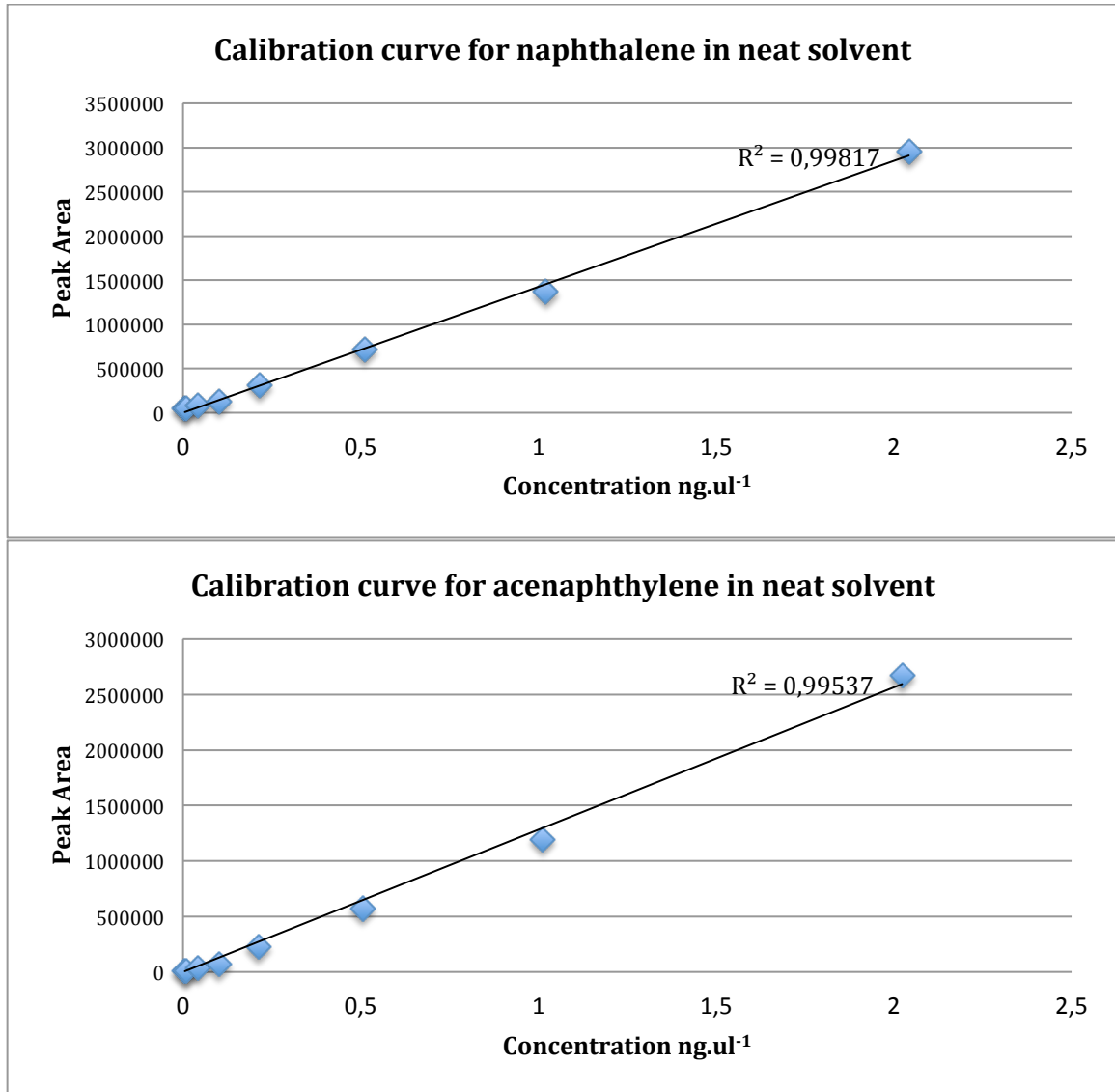
Date: 07-July-2010

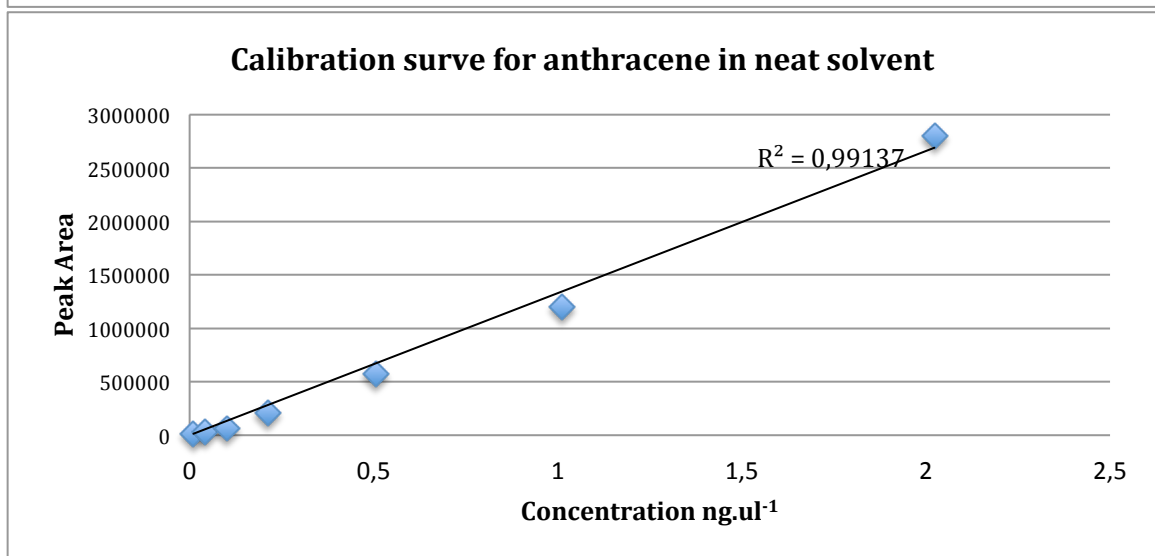
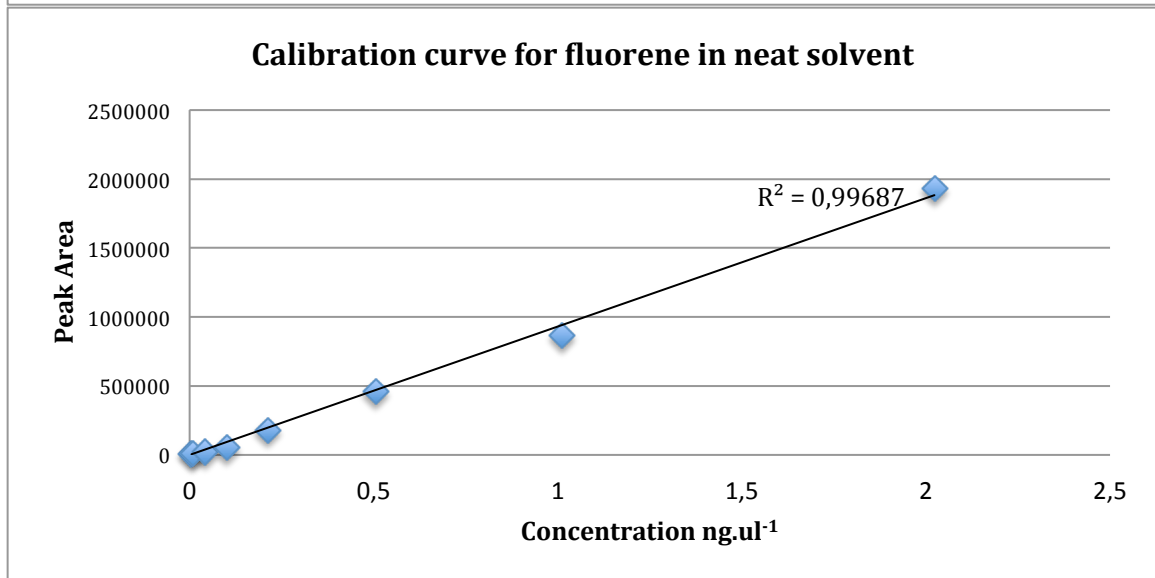
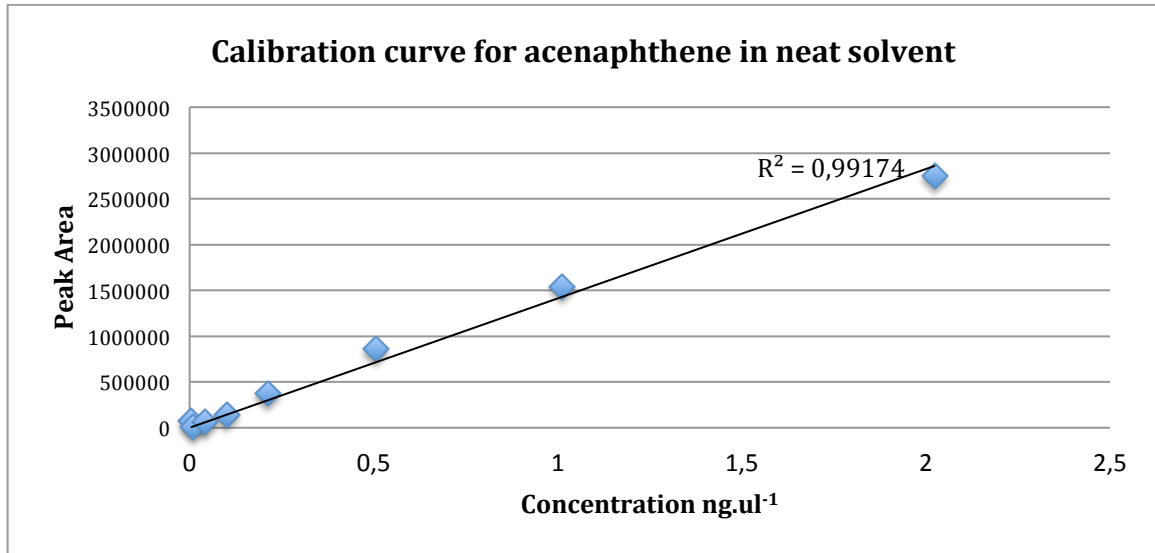
The Q-sep™ sample preparation product above was produced or procured in accordance with the most current edition of the Restek Corporation Quality Management Manual. Restek Corporation's Quality System is registered to ISO 9001:2008 (BSI certificate # FM 80397)

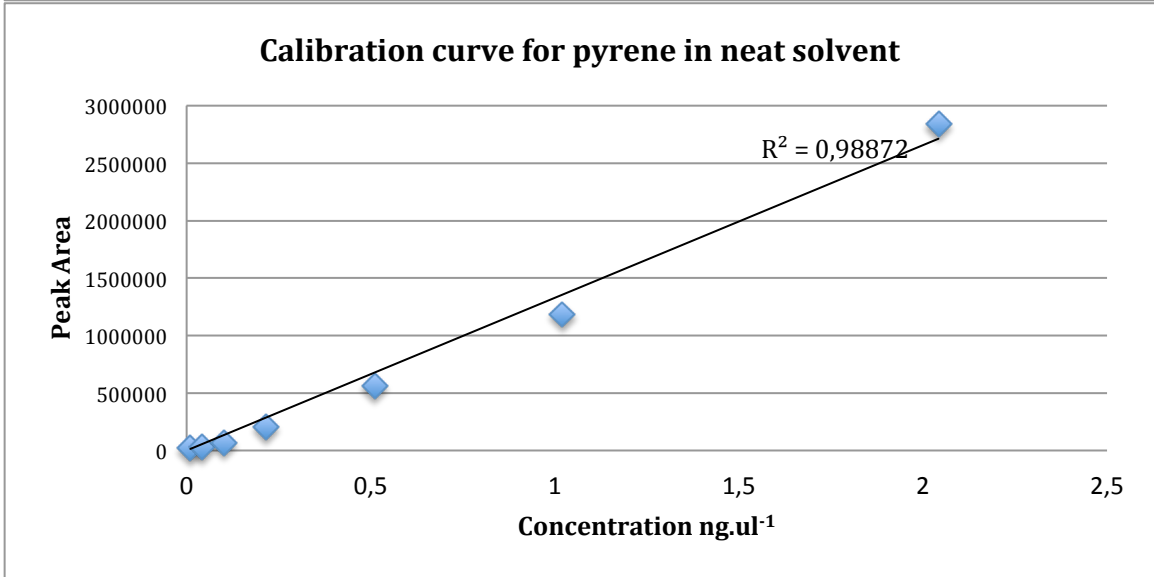
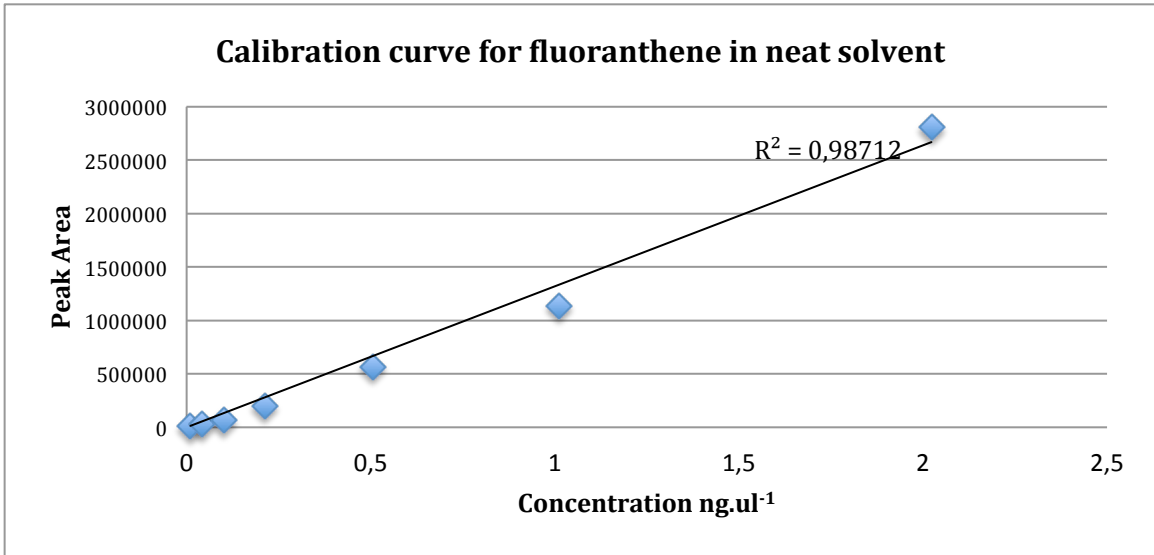
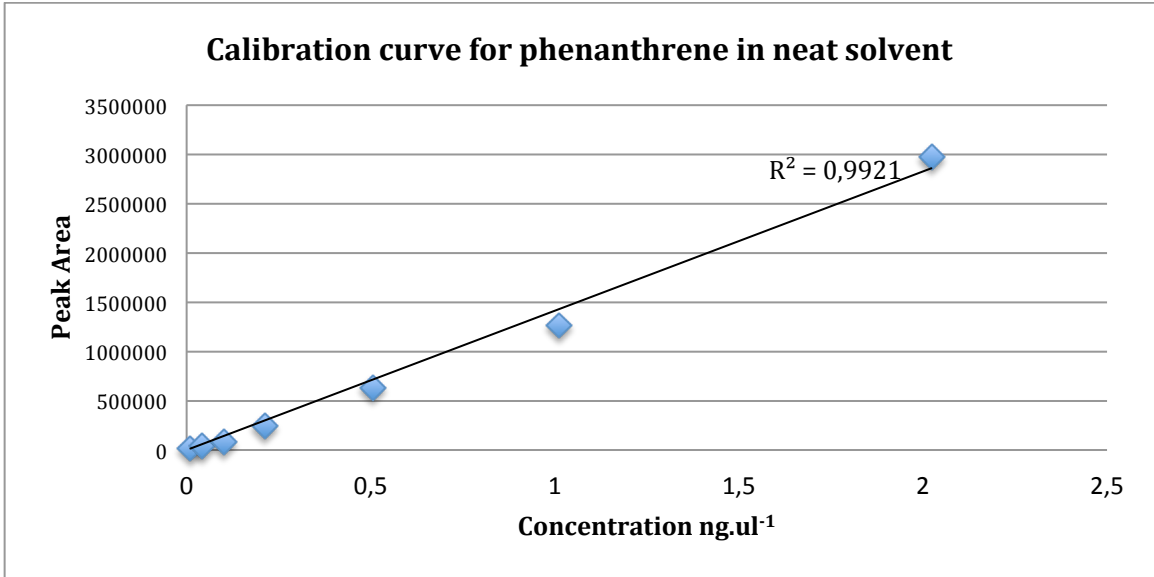
110 Benner Circle ♦ Bellefonte PA 16823 ♦ 814-353-1300 ♦ 800-356-1688 ♦ fax: 814-353-1309 ♦

## Appendix E: Calibration curves for all 15 PAHs of interest using GC-MSD

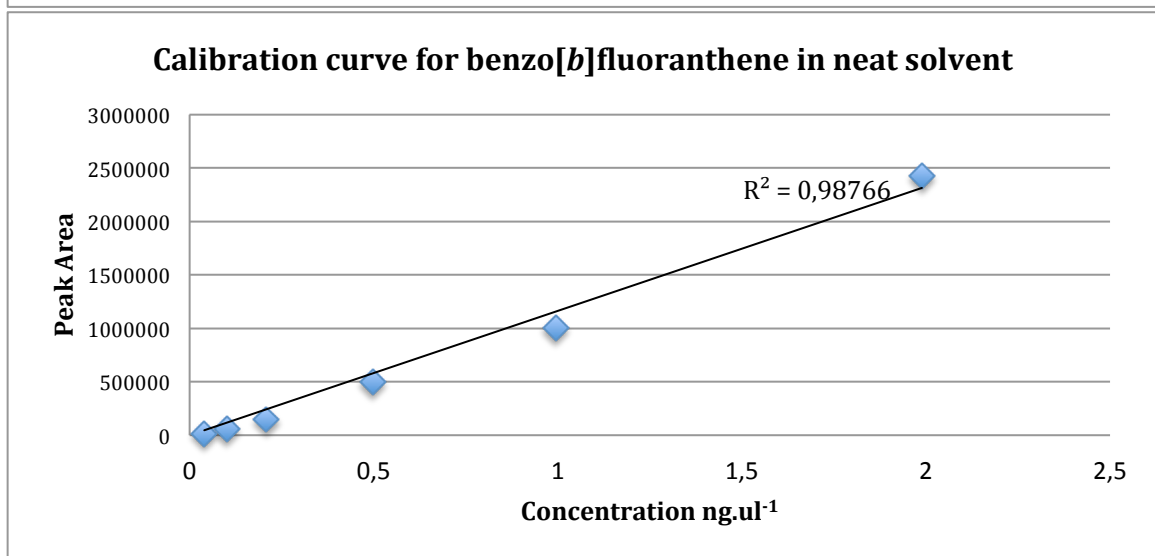
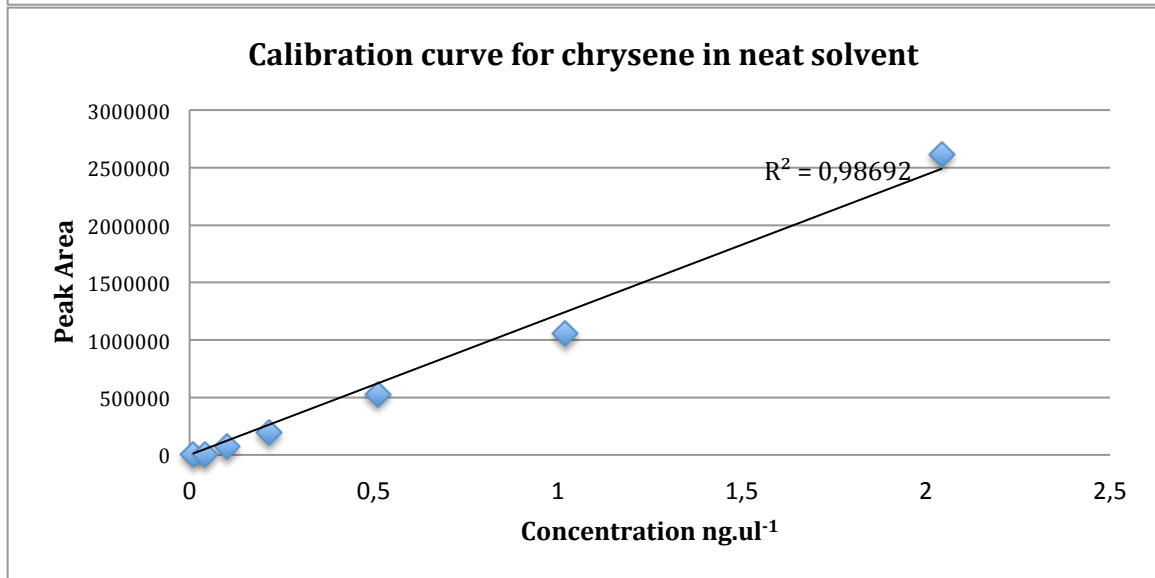
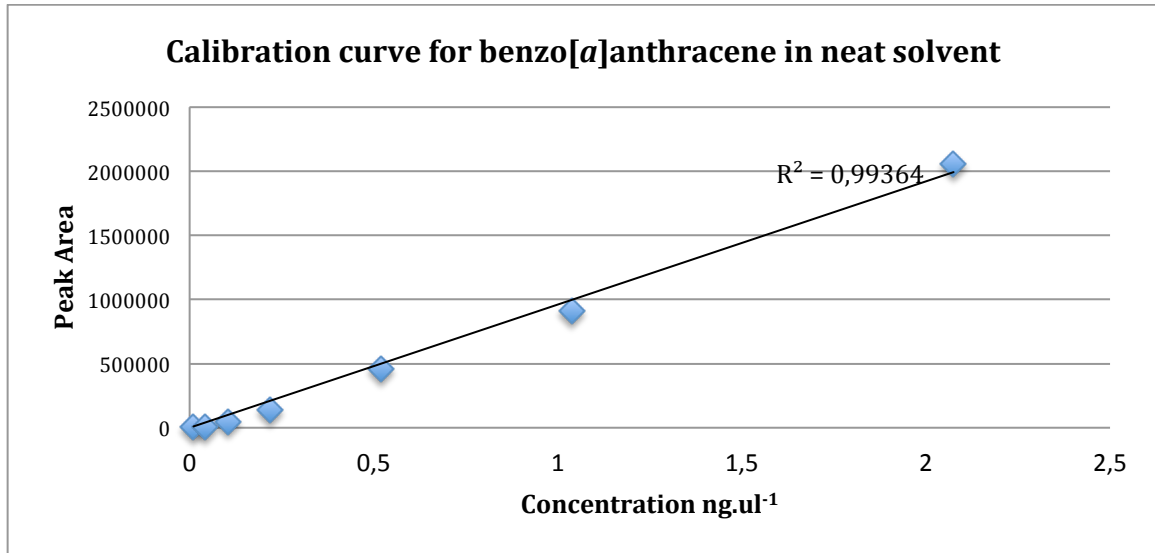
Calibration curve for individual PAHs in PAH mix made up in neat toluene

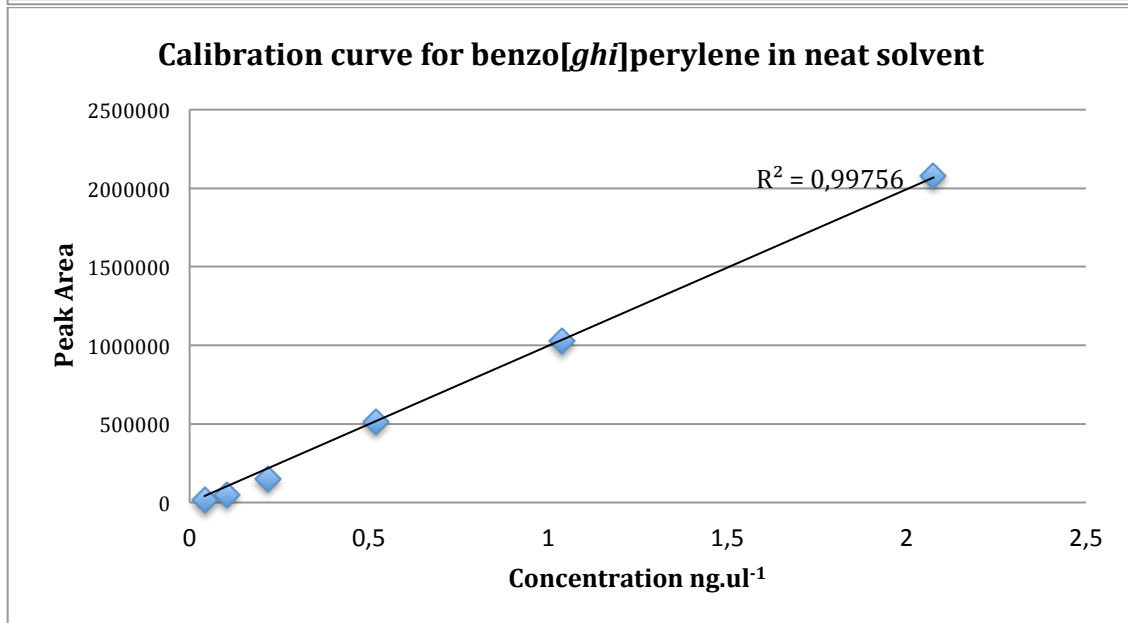
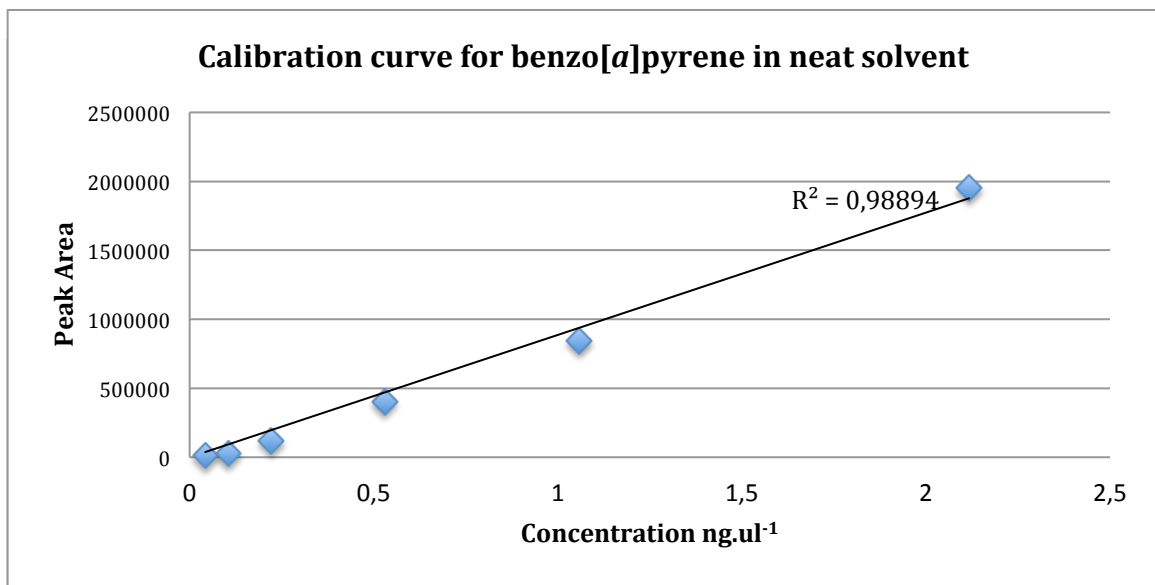
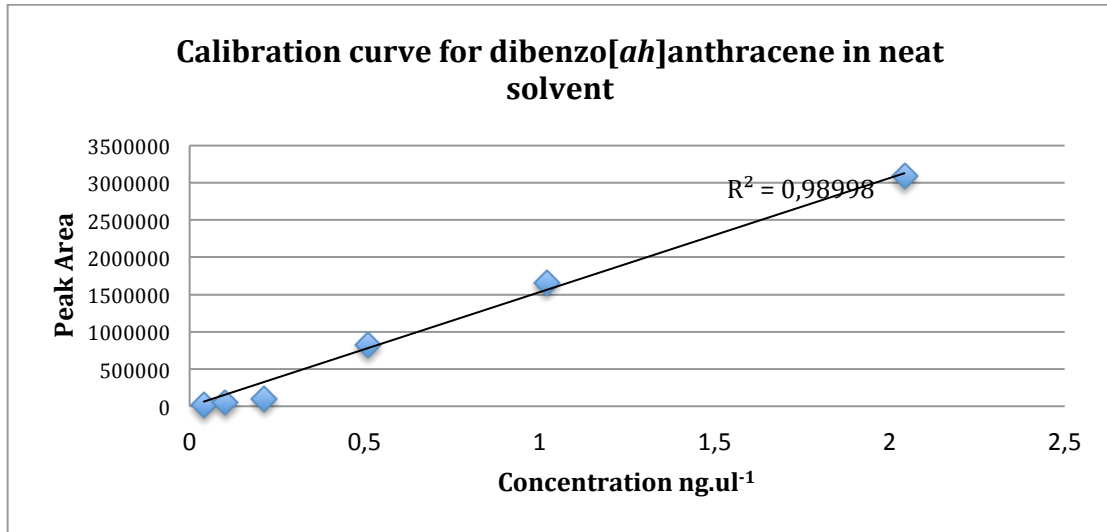








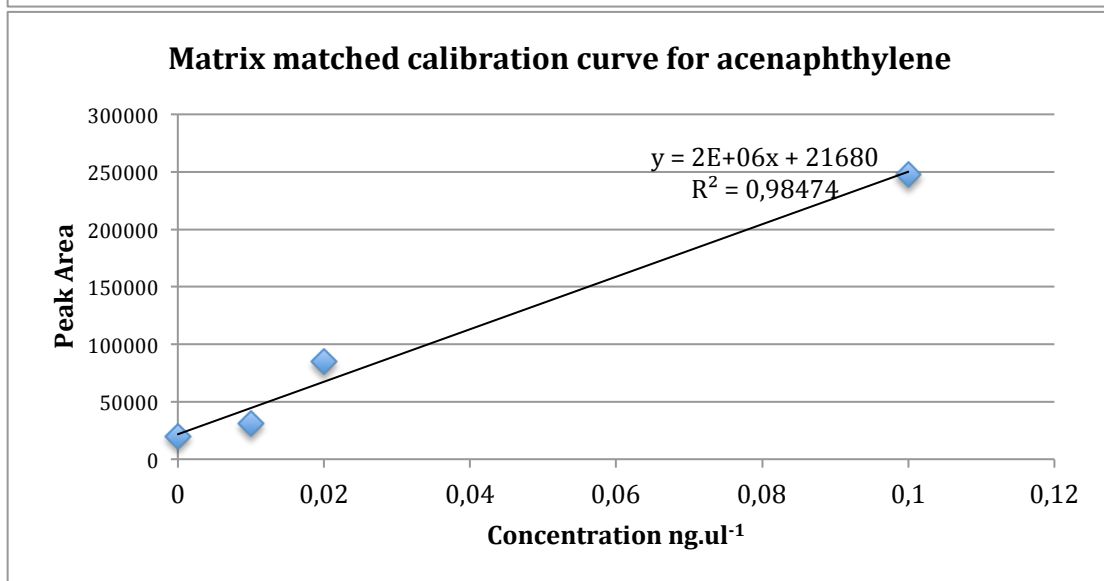
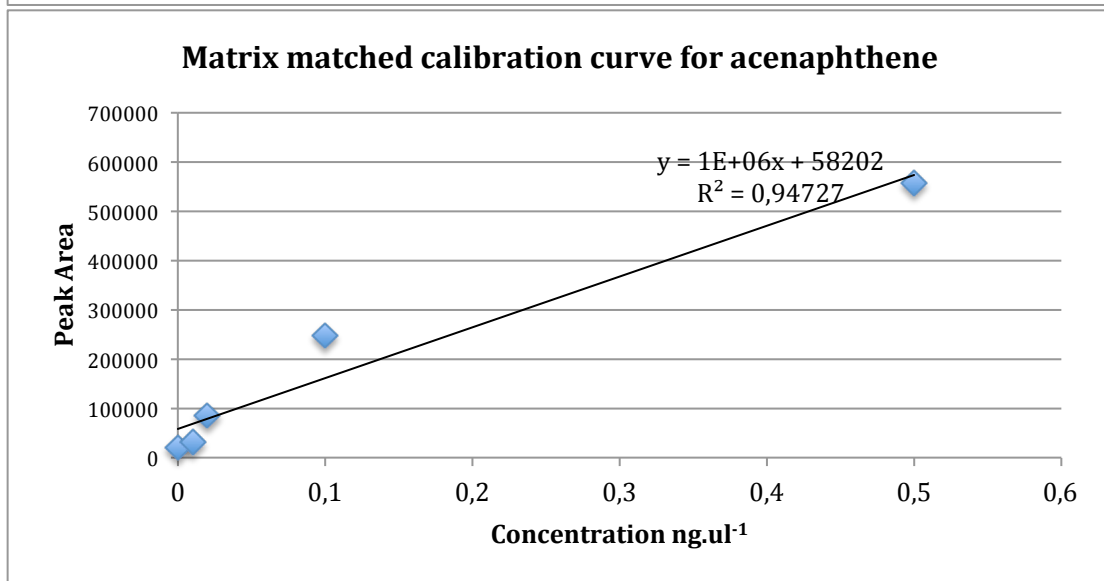
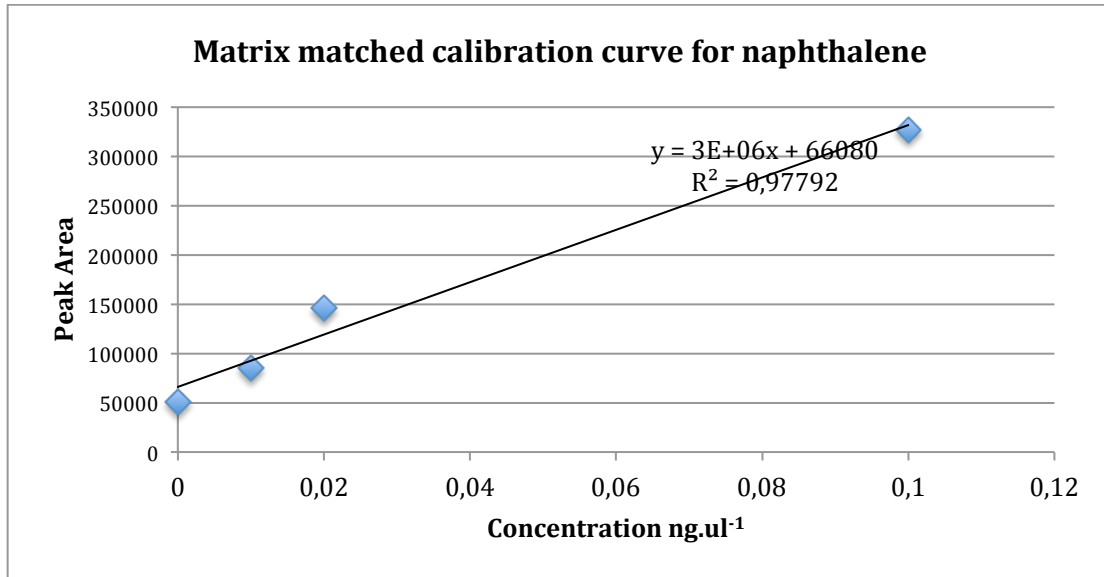


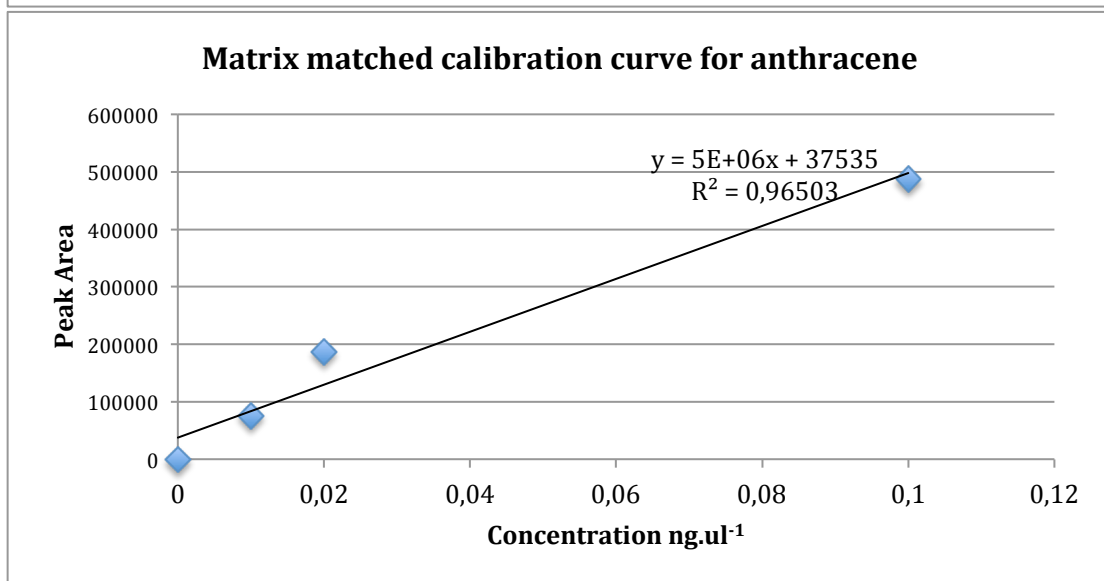
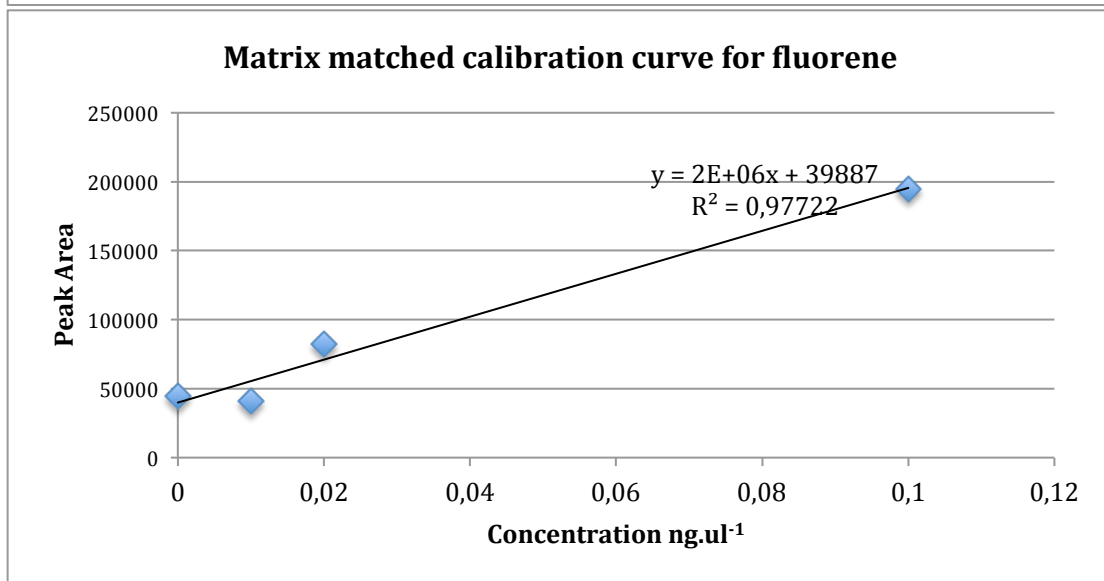
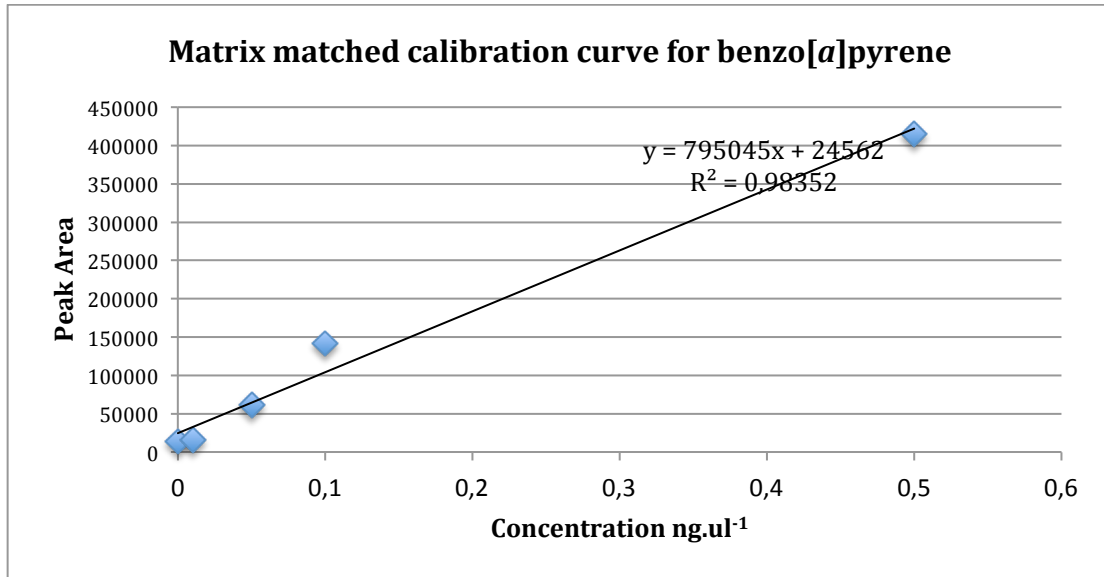


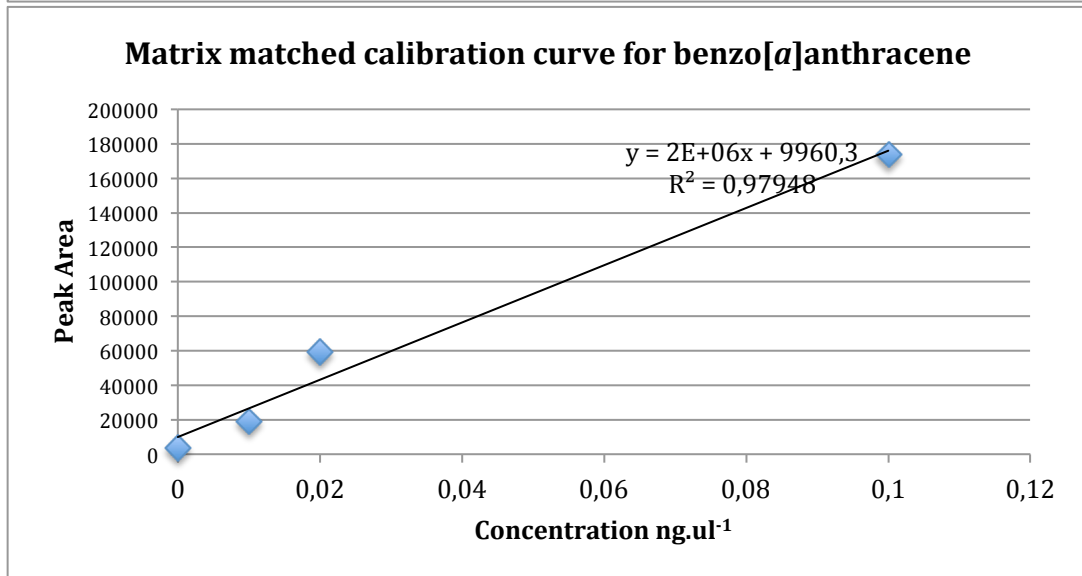
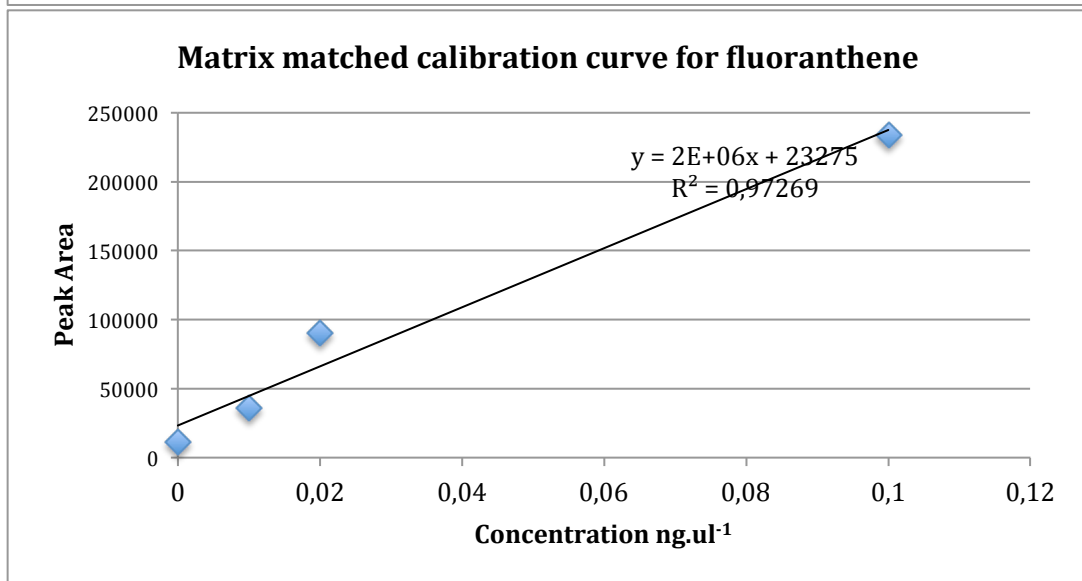
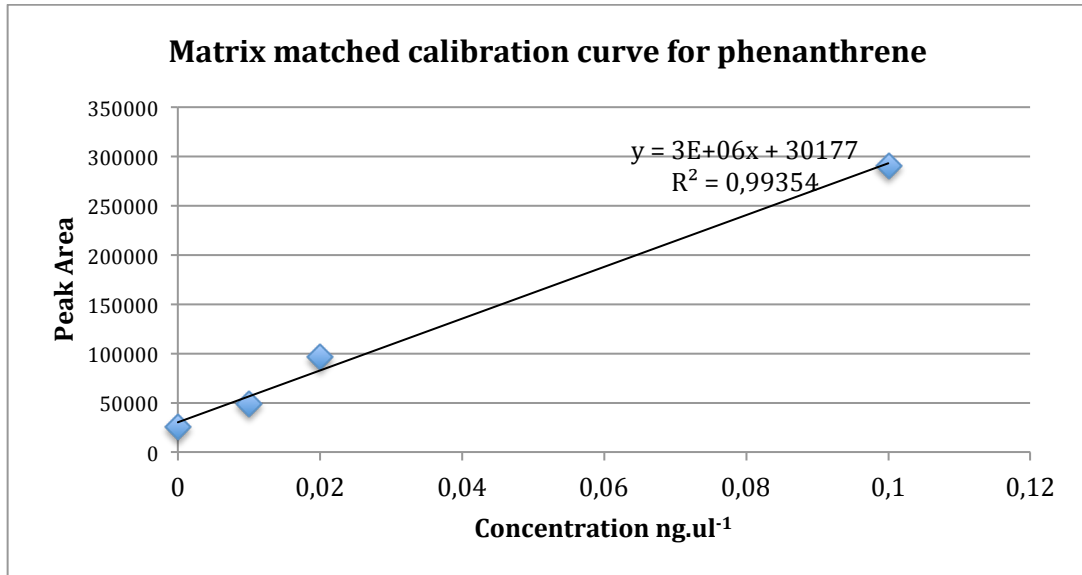
## Calibration curves for individual PAHs in PAH mix in matrix matched extracts

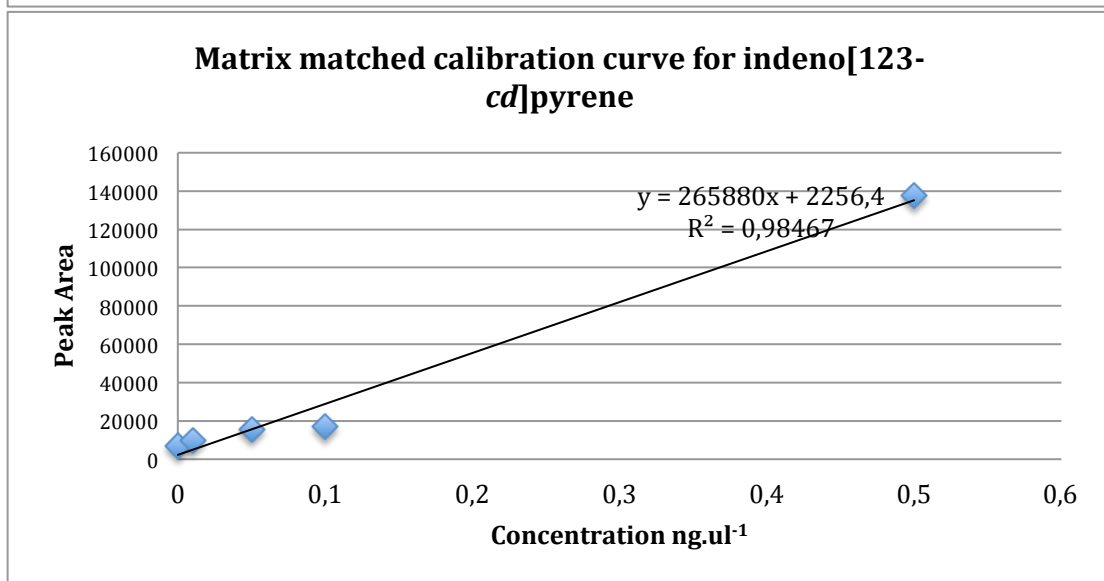
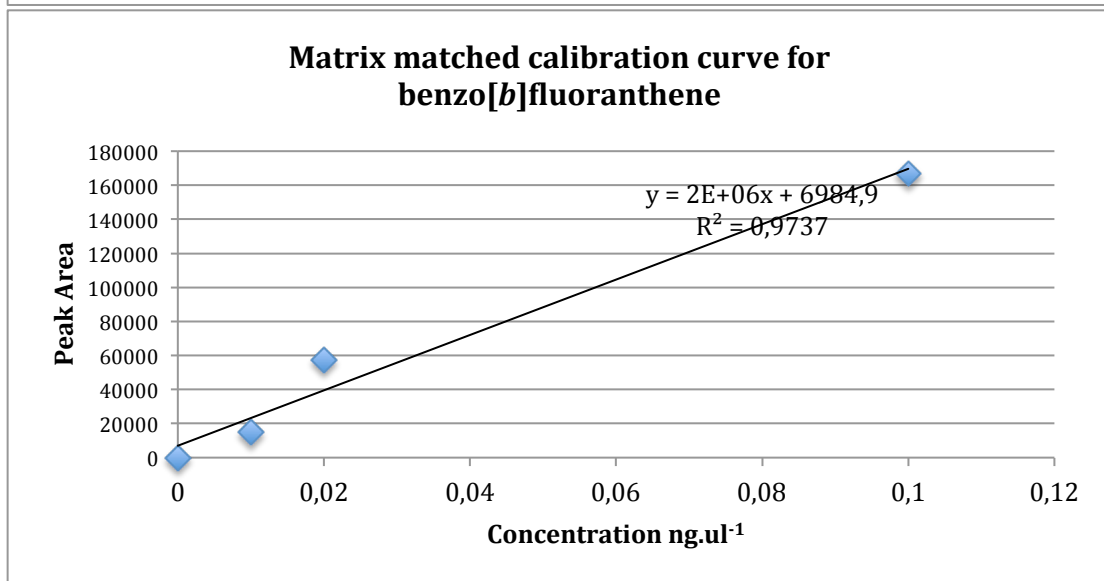
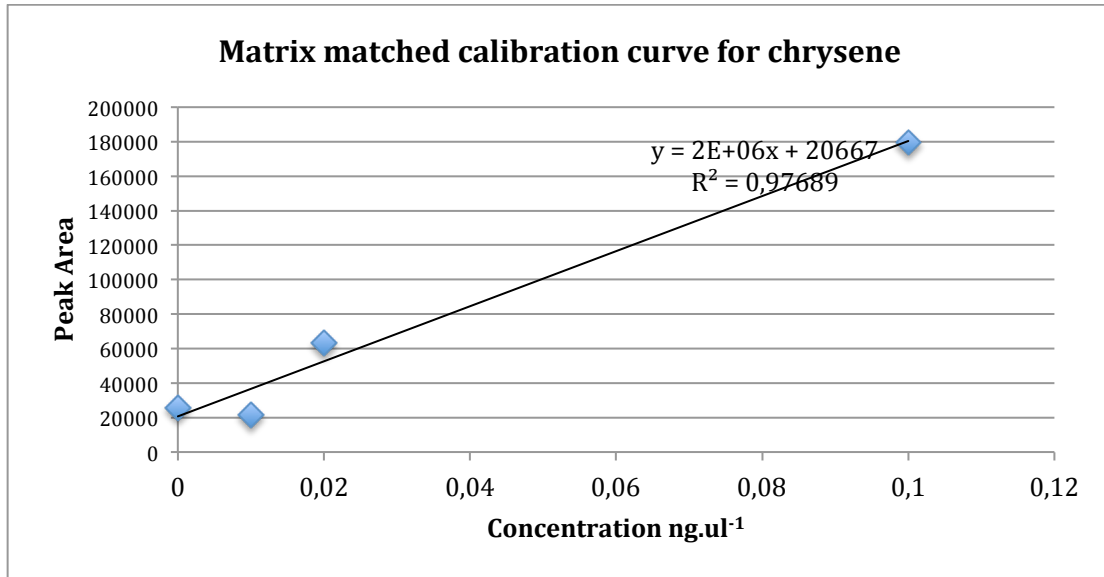
Table of the results from the calibration curves, given below, with the concentration that needed to be corrected for, for each individual PAH in the matrix matched standard-based concentration calculations

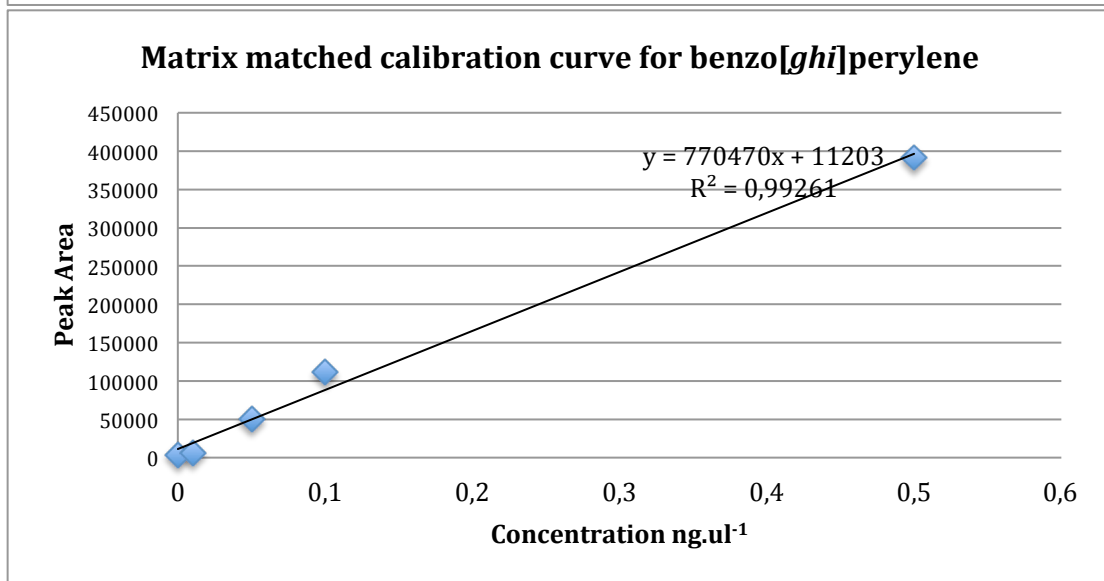
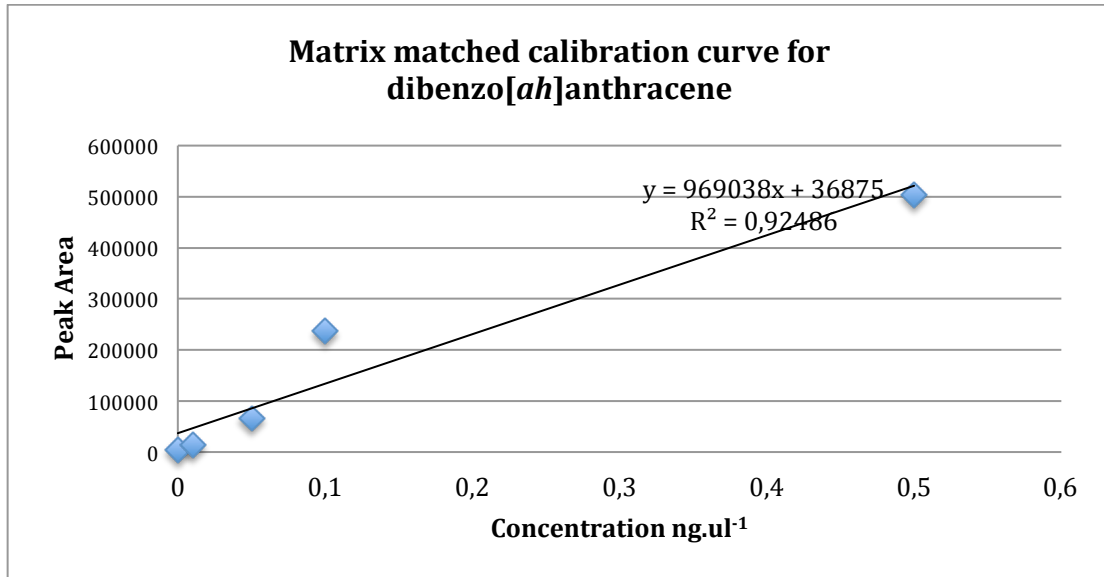
PAH name	R <sup>2</sup>	y-intercept	Gradient	x intercept (conc at y = 0)
Nap	0.977	66080	2657398	-0,0249
Acy	0.984	21680	2284086	-0,0095
Ace	0.947	58202	1030948	-0,0565
Flu	0.977	39887	1556413	-0,0256
Ant	0.965	37535	4603634	-0,0082
Phe	0.993	30177	2628872	-0,0115
FluAn	0.973	23275	2142360	-0,0109
Pyr	0.973	12992	4365985	-0,0030
BaA	0.979	9960	1660592	-0,0060
Chr	0.977	20667	1597221	-0,0129
BbF	0.974	6984	1626511	-0,0043
BaP	0.983	24562	795045	-0,0309
IcdP	0.985	2256	265880	-0,0085
DahA	0.925	36875	969038	-0,0381
BghiP	0.993	11203	770470	-0,0145













## Appendix F: Supplementary sample preparation results

### USAE 0 – 96 hr spike equilibrium study

Time	Total Peak Area	Std Dev
0 hr	5376556	392257
2 hr	2662425	47279
6 hr	739867	70078
12 hr	987274	66390
24 hr	302128	59545
48 hr	141958	33827
96 hr	414238	80672

QuEChERS study using 0.2 g and 0.6 g ground lichens to establish whether increasing the sample mass would reduce the standard deviation: Average peak area of SIM ions, n=3

	0.6 g			0.2 g		
	Average	Std dev	%RSD	Average	Std dev	%RSD
<b>Nap</b>	45976	3412	7.4	50977	3745	7.3
<b>Acy</b>	11229	2914	26	19657	14010	71
<b>Ace</b>	116476	17539	15	103144	6519	6.3
<b>Flu</b>	18872	4518	24	47276	2511	5.3
<b>Ant</b>	12412	2907	23	25740	7582	30
<b>Phe</b>	ND	-	-	ND	-	-
<b>FluAn</b>	11827	2982	25	11550	3027	26
<b>Pyr</b>	ND	-	-	ND	-	-
<b>BaA</b>	ND	-	-	3541	898	25
<b>Chr</b>	ND	-	-	34748	16240	47
<b>BbF</b>	ND	-	-	ND	-	-
<b>BaP</b>	46815	14760	32	31472	22040	70
<b>IcdP</b>	6583	5652	86	12645	1698	13
<b>DahA</b>	7635	3538	46	12738	7944	62
<b>BghiP</b>	ND	-	-	ND	-	-

## Appendix G: Research Outputs

### Conference outputs

*Oral presentation:* Lichens as biomonitors of atmospheric PAHs: A comparison between traditional and new sample preparation techniques

Analitika conference, 7 – 11 September 2014, Khaya iBhubesi, Parys

### Journal articles from this work in print

Lichens as biomonitors for organic air pollutants

*L van der Wat, P. B. C. Forbes*

TrAC Trends in Analytical Chemistry (2015), volume 65, pages 165 – 172 (attached)

### Journal articles from this work in progress

The use of *Parmotrema austrosinense* (Zalhbr.) Hale lichens as biomonitors for atmospheric PAHs in urban, industrial and nature reserves in South Africa

*L van der Wat, P. B. C. Forbes*

In preparation for: Atmospheric Environment

### AND

Development of a QuEChERS approach for the use of lichens as biomonitors for atmospheric PAHs

*L van der Wat, P. B. C. Forbes*

In preparation for: Analytical and Bioanalytical Chemistry



Contents lists available at ScienceDirect

# Trends in Analytical Chemistry

journal homepage: [www.elsevier.com/locate/trac](http://www.elsevier.com/locate/trac)



## Lichens as biomonitors for organic air pollutants

L. Van der Wat, P.B.C. Forbes\*



Laboratory for Separation Science, Department of Chemistry, University of Pretoria, Private Bag X20, Hatfield 0028, South Africa

### ARTICLE INFO

#### Keywords:

Air pollution  
Biomonitor  
Lichen  
Organic air pollutant  
Polyaromatic hydrocarbon  
Polychlorinated dibenzofuran  
Polychlorinated dibenzo-*p*-dioxin  
Semi-volatile organic air pollutant  
Soxhlet extraction  
Ultrasound-assisted extraction

### ABSTRACT

Lichens are useful biomonitors for semi-volatile organic air pollutants, particularly polyaromatic hydrocarbons (PAHs), as a result of their ability to respond to air pollutants at different levels, their slow growth rate, their longevity and their ability to indicate the presence and the concentrations of these pollutants. Consequently, there has been a recent global trend in environmental analytical research to utilize lichens in this way, with Soxhlet and ultrasound-assisted extractions being the most common analyte extraction techniques. A wide range of total PAH concentrations has been determined in lichens from different environments, although phenanthrene, fluoranthene, naphthalene and pyrene tend to dominate the PAH profiles, with higher 2-ring and 3-ring PAH concentrations than 6-ring. In order to facilitate inter-study comparison, there is a need to develop a reproducible, sensitive analytical method for organic pollutants in lichens.

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### 1. Introduction

Lichens are symbiotic organisms found on trees, rocks, in soils and even on weevils and giant Galapagos turtles [1]. They are

perennial, resilient and are able to live for many years in extreme conditions – being found in locations from the icy Himalayas to deserts [2,3]. The use of lichens as biomonitors comes as a result of the ability of lichens to respond to air pollutants at different levels, their slow growth rate, their longevity and their ability to indicate the presence and the concentrations of these pollutants [4,5].

Lichens have the unusual capability of taking up ions and substrates at concentrations beyond their needs. Metal ions are typically absorbed in a passive, extracellular manner and are bound reversibly by an ion-exchange mechanism. It has been found that lichens are able to bind cadmium, lead, tin and zinc at higher concentrations than higher plants, even mosses [6]. It has been suggested that these trace elements are absorbed and stored by particulate entrapment as well as passive and active intracellular uptake in addition to ion exchange [7,8]. There are many factors that determine the absorption and release processes in lichens, namely the chemical nature of the compound, the presence and the influence of other

*Abbreviations:* Ace, Acenaphthylene; Ant, Anthracene; BaA, Benzo[*a*]anthracene; Chry, Chrysene; DbA, Dibenzo[*ah*]anthracene; DAD/V-UV, Diode-array detector/ultraviolet-visible detector; DSASE, Dynamic sonication-assisted solvent extraction; FluAn, Fluoranthene; Flu, Fluorene; FLD, Fluorescence detector; GC-MS, Gas chromatography-mass spectrometry; HpCDD, Heptachlorodibenzo-*p*-dioxin; HPLC, High-performance liquid chromatography; LOD, Limit of detection; MW, Molecular weight; Naph, Naphthalene; OCDD, Octachlorodibenzodioxin; OCDF, Octachlorodibenzofuran; PeCDD, Pentachlorodibenzo-*p*-dioxin; PeCDF, Pentachlorodibenzofuran; Phe, Phenanthrene; POP, Persistent organic pollutant; PAH, Polyaromatic hydrocarbon; PCDD/F, Polychlorinated dibenzo-*p*-dioxin and polychlorinated dibenzofuran; Pyr, Pyrene; SPE, Solid-phase extraction; SVOC, Semi-volatile organic compounds; TeCDF, Tetrachlorodibenzofuran.

\* Corresponding author. Tel.: +27 12 420 5426; Fax: +27 12 420 4687.

E-mail address: [patricia.forbes@up.ac.za](mailto:patricia.forbes@up.ac.za) (P.B.C. Forbes).

<http://dx.doi.org/10.1016/j.trac.2014.09.006>

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compounds, the size of the particles to be absorbed, and the chemical composition of the particles [9].

One of the earliest successful uses of lichens as biomonitors was by Sloof and Wolterbeek [10], who studied the concentrations of pollutants in lichens and compared them qualitatively with the atmospheric concentrations of suspended and deposited particulate matter. Sloof and Wolterbeek [10] performed some elemental analyses (including cobalt, scandium and zinc) on lichens and successfully related the determined concentrations with atmospheric concentrations. In addition, a  $^{137}\text{Cs}$  study after the Chernobyl accident on both lichens and wet and dry depositions showed good correlations with dispersion model data sets [11]. It was consequently shown by Sloof [9] that elemental concentrations within lichens appear to equilibrate with the concentration levels of the surrounding atmosphere, and proved that lichens are suitable candidates for biomonitoring air pollution.

The absorption of atmospheric pollutants by lichens has consequently been a field of interest for many years, including investigation into the uptake of sulfur dioxide by Hawksworth and Rose [12] and Rogers [13]. It is understood that lichens absorb pollutants (metals and organic air pollutants) by wet or dry deposition. The absence of a cuticular wax layer on lichens means that they are able to absorb pollutants much more easily than other higher plants.

The absorption of lead by lichens has been extensively studied due to the toxic nature of the heavy metal. An early application in this regard was a study by Garty, who used lichens as biomonitors to track the lead emissions from automobiles along highways [14]. Over the years, lichen biomonitoring research has been conducted into a range of inorganic analytes, including mercury, most transition metals, radionuclides, fluoride, sulfur, nitrogen and acid rain, which all accumulate in the lichen thallus [15–20].

More recently, the use of lichens as biomonitors for organic air pollutants has been investigated. Although lichens do not have a waxy cuticle or stomata, they produce and release onto their surfaces lipid metabolites, which are suspected of behaving in a manner similar to the cuticle in plants [21]. The lipophilic nature of the surface of lichens then readily attracts lipophilic compounds, such as atmospheric semi-volatile organic compounds (SVOCs), and facilitates their uptake, where their incorporation into cells would be metabolically controlled [22,23].

Organic air pollutants are typically hydrophobic and this means that uptake tends toward dry deposition [24,25]. Metal ions and other water-soluble compounds are deposited on the lichen surface by other mechanisms. Both hydrophobic and hydrophilic compounds can be assimilated by lichens as a result of the volatilization of compounds from soils – or by direct methods, such as wind impaction or splashing from the ground during rainfall episodes [26,27].

Here, we review the current trend of using lichens as biomonitors of SVOC air pollutants, focusing on polyaromatic hydrocarbons (PAHs) and persistent organic pollutants (POPs). POPs are typically chemicals that partition favorably to organic matter of a non-polar nature. They are deliberately produced (e.g., agrochemicals) or form accidentally through processes, such as combustion [e.g., polychlorinated dibenzo-*p*-dioxins and polychlorinated dibenzofurans (PCDD/Fs)]. They are known to have long half-lives and therefore accumulate in organic matter [28]. The associated health risks often relate to chronic exposure to POPs, and the effects of exposure include endocrine disruption as well as carcinogenic and mutagenic effects [29].

In this article, we discuss the use to date of lichens as biomonitors for SVOC air pollutants (primarily PAHs), and we review the sample preparation and analytical techniques employed for such biomonitoring. We also present a comparison of the levels of organic pollutants found in lichens from different studies and geographical areas. Reviews on the progress made in biomonitoring of metal pollution using lichens are available [30,31].

## 2. Lichens as bioindicators and biomonitors of air pollution

The lichen thallus is a complex symbiotic vegetative lower plant composed of two organisms: a fungus and an algae or cyanobacteria. The cell wall consists of a multilaminar and a granular layer. Adhering to the outside of the cells is a fibrous polysaccharide layer. The lichen hyphae can orientate randomly or regularly in a parallel manner – these two types form the basic structure of the layers. The cortex (outer layer) of the lichen serves as a regulator for gas exchange and protective support of the lichen and it is in this layer that small gaps are found, allowing the soredia (the reproductive structures of lichens) to pass into the atmosphere. This layer is covered by an epicortex, which is a porous, non-cellular polysaccharide. It is believed that the porous nature of the epicortex is what enables efficient gas exchange [32]. Fig. 1 shows the cross section of a foliose lichen.

There are three types of lichens: fruticose, foliose and crustose. The foliose lichens are known to have the largest ratio of surface area to dry weight, and are said to accumulate airborne particles more readily than fruticose lichens [33]. The foliose lichens are completely exposed to ambient air as a result of having few points of attachment to the substrate. Fruticose lichens are flatter, with a leaf-like structure with defined upper and lower layers, so only the upper layer is in contact with the ambient air. The crustose lichens are tightly attached to their substrates and are thus difficult to remove for analysis and are less exposed to their surroundings [21].

Lichens have been utilized to monitor air pollution in three different ways [30]:

- to determine the concentration of specific pollutants accumulated in the thallus;
- to use the effect of pollution sources on the lifespan and the presence or the absence of lichen species to map out the distribution and the effect of pollution in a specific area; and,
- to take healthy lichens with little background pollutant accumulation and to transplant them into polluted areas to measure the accumulation of pollutants or the consequential degradation of the thallus.

When conducting an experiment that exploits the absorptive nature of compounds by lichens, the choice of lichen species is paramount to the success of the study. The choice must be made with the method of investigation in mind: if a lichen transplant is to be done, a species that is very sensitive to changes in pollution should be chosen, whereas a hardy local species should be used if the concentration of target analytes in the thallus is to be determined at a sampling site. It is important to identify the species of lichen to be studied (e.g., the selectivity of lichen species for absorption of

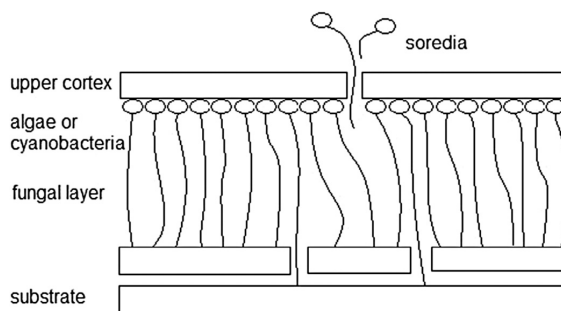


Fig. 1. Simplified cross section through a foliose lichen.

compounds from the atmosphere has been illustrated by Blasco et al. [34]).

A distinction between bioindicators and biomonitors has been made in the literature. Using lichens as bioindicators has, in the past, meant that the apparent health of a specific lichen species is tracked for changes in growth and proliferation (so called “injury symptoms”) to gauge the extent of pollution in the area. However, biomonitoring is a method to measure the response of lichens to air pollution exposure, meaning that the biomonitor should reflect integrated exposure over time. For a few years, it was believed that lichens could not be used as biomonitors [35], because there was little understanding on how to extract the compounds of interest and which analytical methods to use for the analysis. Upon reviewing recent publications, the trend in research leans towards the use of lichens as biomonitors rather than bioindicators, and that is therefore the focus in this article.

### 3. Biomonitoring of organic air pollutants using lichens

Utilizing the accumulative nature of lichens is advantageous over direct air sampling because large volumes of air would need to be sampled for a long period of time (over 24 h or more) to obtain detectable concentrations of trace-level organic air pollutants. Direct air sampling also provides information on the current air quality only. Lichens, on the other hand, accumulate the organic air pollutants, thus providing concentrations that are detectable using sensitive instrumentation, as well as an integrated value of atmospheric organic pollutant levels. This is relevant when studying these pollutants because their associated health risks are often related to chronic exposure [29]. Another advantage of utilizing lichens is that it is a simple, cheap sampling method, applicable in locations that are less accessible to bulky equipment, such as the Pyrenees Mountains [36].

Investigation into the levels of PCDD/Fs and PAHs in lichens have been performed in countries including Portugal, Spain, Poland, Italy and India [37–48]. A study by Augusto et al. [48] showed that lichens are better accumulators of PAHs than soil and pine needles, whilst their PAH profiles are similar to that of the ambient air. Schrlau et al. [45] also found that lichens were more effective at accumulating POPs than pine needles and mosses. It was shown in 2002 by Migaszewski et al. that the lichen species studied had a higher PAH concentration than the host bark, and that the same lichen species had consistent PAH concentrations, despite growing on different tree bark species [43].

Studies have been performed to identify which lichens are better at absorbing different organic air pollutants. It has been reported that the fruticose lichens absorb the low molecular weight (LMW) POPs better, most probably due to their large surface area-to-volume ratio, whilst the foliose lichens preferentially absorb the high molecular weight (HMW) organic pollutants [34,45–47]. Despite these differences, it has been found that similar POP profiles have emerged from studies where 2- to 4-ringed PAHs are primarily observed in PAH studies with lichens [36–38,42–44,48–50] and the PCDD/F profiles show a tendency towards TeCDD/PeCDD and PeCDF domination [39,41,46,51]. Blasco et al. [34] investigated the behavior of different lichen species under the same conditions with regard to their abilities to accumulate PAHs. It was generally found that there was a high three-ring PAH content and a low six-ring PAH content across all species, as found in other studies [38]. Phenanthrene, closely followed by naphthalene, fluoranthene and benzo[a]anthracene are often the PAHs occurring at the highest concentrations in lichens [34,48] (Table 1).

Studies have also indicated that the substrate from which the lichens are collected may play a role in the accumulation of POPs and other compounds [56]. It is unlikely that any POPs are assimilated via bark due to their hydrophobic nature, so the main

**Table 1**  
Summary of results from reported polycyclic aromatic hydrocarbon (PAH) studies using lichens as biomonitors

Most Common PAHs <sup>a</sup>	Location	Year	Lichen species	Sample mass used (g)	Extraction method	Analysis method	LOD	Total PAH concentration detected	Ref.
Phe, Ant, Pyr, FluAn	Dolomites, Italy	2014	<i>Pseudevernia furfuracea (L.) Zopf</i>	0.6–0.8	Automated Soxhlet	GC-MS	–	758.0 ng.g <sup>-1</sup>	[37]
Phe, Pyr, FluAn	Sines, Portugal	2010	<i>P. hypoleucinum</i>	2	Soxhlet	HPLC-FID-DAD/UV	–	95.5–873.8 ng.g <sup>-1</sup>	[48]
3- and 4-ring	Sines, Portugal	2009	<i>Parmotrema hypoleucinum (Steiner) Hale</i>	2	Soxhlet	HPLC-FID-DAD/UV	–	93.56–599.24 ng.g <sup>-1</sup>	[47]
2-, 3- and 4-ring	Sines, Portugal	2013	<i>Parmotrema hypoleucinum (Steiner) Hale</i>	2	Soxhlet	HPLC-FID-DAD/UV	–	58–536 ng.g <sup>-1</sup>	[52]
4 ring	South Central Poland	2002	<i>Hygyginia physodes</i>	–	Soxtec liquid-solid extraction	GC-MS	–	3 – 1887 ppb	[43]
Naph, Ace, Phe, Pyr, FluAn, Naph, Phe, FluAn	Aragon Valley, Spain	2007	<i>Parmelia sulcata</i>	0.2	DSASE	GC-MS	–	352–1654 ng.g <sup>-1</sup>	[53]
Naph, Phe, FluAn	Pyrenees, Spain	2008	<i>Evernia prunastri</i>	0.2	DSASE	GC-MS	–	692–6420 ng.g <sup>-1</sup>	[36]
Naph, Phe, FluAn, BaA	Somport Tunnel, Spain	2006	<i>Parmelia sulcata</i>	0.2	DSASE	GC-MS	–	0.91–1.92 µg.g <sup>-1</sup>	[42]
	Pyrenees, Spain	2011	<i>P. sulcata</i> , <i>E. prunastri</i> , <i>R. farinacea</i> , <i>P. furfuracea</i> , <i>Usnea sp.</i> and <i>L. pulmonaria</i>	0.2	DSASE	GC-MS	–	2.38–6240 ng.g <sup>-1</sup>	[34]
Flu, BaA, DbA, Ace, Phe	Zaragoza City, Spain	2006	<i>Xanthoria parietina</i>	0.2	DSASE	GC-MS	0.021–0.032 µg.g <sup>-1</sup>	+/-330 ng.g <sup>-1</sup>	[50]
	Kanpur City, India	2012	<i>R. sordidus (Ach.) Massal</i>	2	Soxhlet	HPLC-V-LUV	0.008–0.050 µg.g <sup>-1</sup>	0.189–0.494 µg.g <sup>-1</sup>	[40]
	Dehra Dun, Himalayas	2009	<i>Phaeophyscia hispidula</i>	2	Soxhlet	HPLC-V-LUV	10–30ppt	3.38–25.0 µg.g <sup>-1</sup>	[38]
	Hardwar, India	2012	<i>Pyxine subcinerea</i>	2	Soxhlet	HPLC-V-LUV	10–30 ng.L <sup>-1</sup>	1.25–187.3 µg.g <sup>-1</sup>	[54]
Mixed	Himalayas, India	2013	<i>Demmatocarpon vellereum Zschacke</i>	2	Soxhlet	HPLC-V-LUV	10–30 ng.L <sup>-1</sup>	0.136–4.96 µg.g <sup>-1</sup>	[55]
Phe, Ant, FluAn	Rieti, Italy	2003	<i>Pseudevernia furfuracea</i>	2	UAASE	GC-MS	0.5–3.6 µg.kg <sup>-1</sup>	56–159 µg.kg <sup>-1</sup>	[44] <sup>b</sup>
Pyr, FluAn	Viterbo, Italy	2009	<i>Pseudevernia furfuracea</i>	2	UAASE	GC-MS	0.01–0.03 µg.g <sup>-1</sup>	168.0–395.0 ng.g <sup>-1</sup>	[49] <sup>b</sup>

<sup>a</sup> PAHs are abbreviated as follows: Fluoranthene (FluAn); Pyrene (Pyr); Phenanthrene (Phe); Dibenzo [ah]anthracene (BaA); Benzo[a]anthracene (BaA); Anthracene (Ant); Naphthalene (Naph); Acenaphthylene (Ace); Fluorene (Flu).

<sup>b</sup> These studies were lichen transplant studies.

mechanisms of uptake are due to dry deposition and the soil, as described in Section 1. Baur et al. [57] as well as Bauer et al. [58] showed that the heavier POPs diffuse slower than the LMW POPs, and that the HMW POPs are thus more probably associated with particles that remain on the surface of lichens. It has been shown by Augusto et al. [52] that the PCDD/F concentrations remain relatively constant after periods of wet conditions (e.g., rain or fog), suggesting that some POPs are retained within the lichen thallus and are not rinsed off easily. A parallel study for PAHs has not yet been published, but studies of other organic air pollutants strongly suggest that a similar trend for PAH integration in lichens is to be expected [59,60].

#### 4. Experimental procedures employed in the analysis of semi-volatile organic pollutants in lichens

##### 4.1. Sampling

Two distinctly different sampling methods are used when lichens are to be utilized as tools for air pollution monitoring. The technique of transplanting lichens from relatively clean environments to areas of interest has been a popular method when studying heavy metals [61,62] but is not readily applied to PAH studies, due to the long-term exposure to the low levels of these pollutants that is required in order to allow meaningful, detectable results.

Direct sampling, on the other hand, is a popular sampling technique, which exploits that, over time, lichens accumulate pollutants directly from the atmosphere. Lichens are mostly sampled at heights of more than 1 m above the ground, except for Migasewski et al. [43], who sampled from as low as 25 cm above the ground. Such low sampling heights may influence results due to contamination of the lichens by soil, and protection of the lichens from wind by surrounding shrubs and rocks at the base of a tree. In contrast, Augusto et al. [41] sampled lichens off roof tiles for a study into atmospheric dioxin and furan deposition in Portugal.

When atmospheric furans and dioxins have been studied using lichens, the samples have been removed from the substrates, placed in glass jars [63] or stored in plastics bags [41], dried at room temperature and then stored for analysis. For PAH studies, the sampling methodology varies; whereas some groups sample the lichens into amber vials [43,48,60,64], others sample into paper bags [37], or wrap samples in aluminum foil [38] or polyester barrier-film bags [45]. Because PAHs may photodegrade, the sampling and storage procedures that prevent further chemical changes to the lichen after sampling are most regularly employed. Non-permeable collection containers are also preferable, in order to prevent loss of the more volatile analytes (2-ring and 3-ring PAHs).

Whereas some studies have meticulously reported sampling conditions, such as avoiding the sampling of fruiting lobes and only sampling the apical lobes of lichen structures [37], most studies collect samples of the same species from different tree species [43,48], which could increase uncertainty when comparing studies, since different tree species would have different bark and canopy

structures, potentially protecting some lichen species better than others from wind and atmospheric deposition.

Augusto et al. [60,64] showed that seasonal flux influences the concentration of PAHs in lichen samples, with the highest concentrations of PAHs found in lichens sampled during the wet winter months, and the lowest concentrations during the warmer, dry summer months. This may be as a result of increased evaporation of organic air pollutants; an increase in emissions during the cold winter months, or as a result of UV-induced photodegradation or other photochemical reactions that may take place [65–67]. It is for this reason that it is important that the season when lichen sampling took place is reported for consistency and to allow comparison.

Other conditions that need to be considered when sampling are wind speed and direction (laminar boundary will be affected by air flow), air pressure (increased pressure will mean a higher concentration of compounds in the atmosphere, so higher accumulation rates), and humidity [21,34,60,68]. For a summary of factors necessary to consider when using lichens as biomonitors for POPs, the reader is referred to a review by Augusto et al. [52].

##### 4.2. Sample preparation techniques

Once the lichens have been sampled, an extraction process is selected to remove and to concentrate the organic analytes from the sample matrix. Traditionally, Soxhlet extraction has been employed for the extraction of both PAHs and PCDD/Fs [38–40,46]. Table 2 shows the common experimental conditions when using Soxhlet, which is currently less popular due to its high solvent and energy consumption requirements, and the time required for efficient extraction. Migasewski et al. [43] used a modified Soxhlet technique, Soxtec, for PAH extractions, using dichloromethane. Although it performed successfully as one of the first PAH studies using lichens as biomonitors, this method has not been applied in any other similar studies reported to date, probably due to the move towards greener chemistry practice utilizing methods that are faster and use less energy and lower solvent volumes.

Pressurized liquid extraction (PLE) has been employed by Schrlau et al. [45] on lichens followed by a silica-based SPE clean-up in the analysis of certain pesticides and PAHs in the USA. A mass of 2.0 g lichen was extracted using dichloromethane as the solvent at 100 °C for 5 min. A comparative study between PLE and other extraction methods for PAHs in lichens does not appear in literature; however, Ratola et al. [70] performed PLE on pine needles in a PAH study, but found that ultrasonic extraction was the most successful technique (over PLE and Soxhlet).

Ultrasound-assisted extraction (UAE) techniques are commonly used because smaller sample sizes can be accommodated, less solvents are used, and the extractions are relatively fast [44,50]. Whereas Guidotti et al. [44,49] used cyclohexane as the solvent for UAE, combining only two extractions, Domeno et al. [50] extracted a 0.2 g lichen sample using portions of 15 mL dichloromethane in each of four subsequent extractions. A study, in which a novel UAE technique for lichens was developed by

**Table 2**  
Reported experimental conditions for lichen sample preparation using Soxhlet extraction

Analyte studied	Mass lichen sample used (g)	Solvent scheme	Solvent volume	Extraction time	Ref.
PCDD/Fs <sup>a</sup>	10	Methylene chloride:hexane (1:1)	Not reported	18–24 h	[39,41]
PAHs	2	Dichloromethane	100 mL	16 h	[38,54,55,69]
PAHs	2	Acetonitrile	200 mL	24 h	[47,48,65]
PAHs	0.2	Dichloromethane	250 mL	6 h	[50]
PAHs	0.6–0.8	Hexane: acetone (1:1)	150 mL	2 h <sup>b</sup>	[37]

<sup>a</sup> Refers to EPA 1613B method.

<sup>b</sup> Refers to an automated Soxhlet extraction and reflects total extraction time of the extraction in solvent and the reflux in the rinse position.

**Table 3**  
Reported sample clean-up conditions for lichen extracts

Analyte studied	Type of clean-up	Sorbent Type	Elution solvent	Ref.
PCDD/Fs	Column and gel chromatography	Column: Mixed silica and aluminum oxide Gel: Bio Beads S-X3A	Not reported	[41,46]
PAHs	Column chromatography	Silica	25 ml methylene chloride:pentane (2:3)	[38,44,54,55]
PAHs	Column chromatography	Silica	50 ml dichloromethane	[45]
PAHs	Column chromatography	Florisil	30 ml acetonitrile	[47,48]
PAHs	Solid-phase extraction	Normal phase – NH <sub>2</sub>	2 ml hexane:dichloromethane (65:35)	[34,36,37,53]

Domeno and Blasco [50], found that hexane is the solvent that extracts most PAHs efficiently and the new dynamic sonic-assisted solvent extraction (DSASE) technique has been popular in numerous studies since [34,42,53]. The method has grown in popularity because it is a fast method that uses less solvent and energy, with satisfactory results.

With regards to the clean up of sample extracts, column chromatography or solid-phase extraction (SPE) is commonly used and these are summarized in Table 3.

Silicon column chromatography has been employed extensively, with different solvent schemes. A direct comparison between the results of these studies is a challenge as a result of the different affinities that solvents have for different PAHs [50], leading to biased elutions. The volume of sorbent beds is not clearly defined in most studies, and that also influences the extent to which the analytes are recovered.

More recently, SPE was used to clean up the lichen extracts with mainly normal-phase – NH<sub>2</sub> columns being used after a study by Blasco et al. [53] found that these columns, combined with an elution solvent of hexane dichloromethane (65:35), yielded the best recoveries. This method has not been reviewed since 2007, despite the production of many new sorbents, of which a few are PAH specific.

Due to the importance of sample preparation in delivering accurate, reliable analytical results, the extraction methodology and the sample clean-up techniques employed should be continually reviewed and optimized, as new techniques become available, to enhance analyte recoveries and reproducibility of results.

#### 4.3. Analytical techniques

When analyzing lichen extracts for PCDD/Fs and PAHs, the main analytical techniques used are HPLC with reversed-phase C18 columns and gas chromatography (GC), using a variety of column configurations. Augusto et al. [48,60] made use of HPLC coupled to an ultraviolet fluorescence detector (FLD) as well as a diode-array ultraviolet/visible detector (DAD/V-UV), which had the advantage of sensitivity as a result of the fluorescence detector with limits of detection (LODs) of 58 ng.g<sup>-1</sup> being reported for the sum of all 16 EPA Priority PAHs. Selectivity, as a result of using the DAD/V-UV detector, operating at 254 nm, ensured well-resolved peaks, depending on chromatographic optimization. Likewise, Shukla and Upreti also used HPLC coupled to a UV detector [38,40,54,55] with reported LODs as low as 8 ng.g<sup>-1</sup>.

In studies with the explicit focus on PCDD/F content in lichens, high-resolution GC coupled to high-resolution mass spectrometry (MS) is used so that dioxins and furans can be efficiently separated and accurately identified [41,46,63]. Use of a DB dioxin column [41,46] has been found to improve resolution of PCDD/Fs further and should be used where possible.

When conducting PAH studies, Migaszewski et al. [43], Domeno et al. and Blasco et al. [34] have used a GC-MS for analysis, reporting LODs as low as 21 ng.g<sup>-1</sup> [50]. The use of Rxi-5Sil MS, HP 5-MS and factor four VF5-MS columns is common, due to the non-polar nature of these capillary columns resulting in good separation of

PAHs, but run times range between 29 min [34] and 81 min [37]. Sacrificing peak separation, particularly of the benzo[fluoranthenes], as well as BaA and Chry, for the sake of faster run times should be avoided to ensure accurate representation of the PAH profile, due to the different toxicities of PAH compounds.

#### 5. Levels of PAHs and PCDD/Fs found in lichens

PAHs, which are by-products of combustion processes, are receiving significant attention in environmental science research, due to their inclusion in the Convention on Long-Range Transboundary Air Pollution Protocol [71], and the carcinogenic potential of some PAHs, such as benzo[*a*]pyrene, dibenzo[*ah*]anthracene and the benzo[fluoranthenes] [72–74]. A number of studies have therefore been conducted to determine the PAH content of lichens in different environments (Table 1).

PAH concentration ratios found in lichens may also be used for source apportionment studies. For example, phenanthrene/anthracene (Phe/Ant) and fluorene/pyrene (Flu/Pyr) [50] may be employed where a Phe/Ant ratio higher than 10 and Flu/Pyr ratios higher than 1 indicate a mix of pyrogenic and petrogenic sources [34,47]. Another popular application of the concentration data is to look at the toxicity equivalent factors (TEFs), as detailed by Nisbet and Lagoy [75], where the product of the concentration of a specific PAH and its toxicity compared to benzo[*a*]pyrene gives an indication of the extent to which the specific PAH contributes to the carcinogenic potency [60]. The number of rings in the PAH structures are also often compared to give an indication of the PAH profiles that the lichens are accumulating: generally increased toxicity is related to an increase in number of rings.

Domeno et al. [50] found total PAH concentrations of around 340 ng.g<sup>-1</sup> using their DSASE technique in lichens, which were sampled near a river outside a city in Spain, whereas a study by the same group, using the same technique found total PAH concentrations of 1.2–1.65 µg.g<sup>-1</sup> in lichens in an area with a high density of traffic. Using the diagnostic ratios, it was suggested that the traffic was indeed the main contributor towards the high PAH content in the lichens [42]. These studies have successfully exploited the efficacy of lichens as biomonitors to diagnose pollution sources of specific SVOCs. The large variation in total PAH concentration is heavily influenced by not only the contributing pollution sources at the location, but also many environmental factors, such as the seasons in which sampling was undertaken, the altitude at which samples were collected and the extraction techniques used in the laboratory.

When comparing the results of the studies presented in Table 1, it is evident that phenanthrene, fluoranthene, naphthalene and pyrene tend to dominate the PAH profiles, with higher 2-ring and 3-ring PAH concentrations than 6-ring compound concentrations [34,36,38,42,48,53]. This is possibly because the heavier PAHs are less volatile, are generally associated with particulate matter and are less airborne by nature.

The vast range in total PAH concentrations can be illustrated by the difference between the study by Shukla et al. [54] where concentrations as high as 187.3 µg.g<sup>-1</sup> were detected, compared to

**Table 4**  
 Summary of results from reported studies of polychlorinated dibenzo-*p*-dioxins and polychlorinated dibenzofurans using lichens as biomonitors

Most common PCDD/Fs	Location	Year	Lichen species	Extraction method	Analysis method	Total PCDD/Fs concentration detected	Ref.
TeCDF, PeCDF	Setubal Peninsula Portugal	2004	<i>Xanthoria parietina</i>	Solvent Extraction	GC-HRMS	427.74 ng.kg <sup>-1</sup>	[41]
TeCDF, PeCDF, PeCDD	Setubal Peninsula Portugal	2007	<i>Xanthoria parietina</i>	Soxhlet	HRGC-HRMS	197.5–1218.7 ng.kg <sup>-1</sup> d.w. <sup>a</sup>	[39]
OCDD, OCDF, HpCDD in <i>Xanthoria parietina</i> and less chlorinated in <i>Ramalina canariensis</i>	Setubal Peninsula Portugal	2009	<i>Xanthoria parietina</i> and <i>Ramalina canariensis</i> Steiner	Soxhlet	HRGC-HRMS	170.8–1058.6 ng.kg <sup>-1</sup>	[46]

<sup>a</sup> d.w. is dry weight.

0.058 µg.g<sup>-1</sup> total PAH concentration detected by Augusto et al. [52] in Portugal. In India, the samples were taken in an industrial area close to the Himalayas, and, in Portugal, the samples were taken in an industrial area on the coast. Shukla et al. [54] used Soxhlet in their sample preparation, whilst the sample preparation technique was not reported for the study in Portugal.

Few studies using lichens as biomonitors for PCDD/Fs have been undertaken, as summarized in Table 4 [39,41,46]. The specificity of the lichen species towards furan and dioxin uptake is illustrated clearly in the study by Augusto et al. [46] and highlights the importance of consistency in sampling strategies. The profiles of PCDD/Fs contained in lichens vary between different species, so that *X. parietina* contained a higher number of chlorinated pollutants (e.g., OCDD, OCDF and HpCDD) when compared to *R. canariensis*, which accumulated the lesser chlorinated furans and dioxins [46]. The concentrations detected varied between industrial, urban, agricultural and forested areas, but it was suggested that lichens accumulate PCDD/Fs at higher concentrations than other biomonitors, such as pine needles and mosses [41]. It was further shown that the PCDD/F content in lichens was similar to that of soil, without any soil-particle contamination [41]. More studies in different regions need to be undertaken to understand better the potential application of lichens as biomonitors for PCDD/Fs in reaching global understanding of atmospheric levels of PCDD/Fs.

There is generally a lack of LODs and analyte recoveries in the reported studies, which makes comparison of techniques difficult, since laboratories cannot directly compare their findings to others if analytical parameters are not evident. Little mention is made of replicate analyses and variation within bulk samples. Large differences in analyte concentrations are seen within studies and in addition to differences in sampling environments; these could be due in part to lack of homogeneity within samples. Lichens are perennial and grow continually, resulting in a difference in organic pollutant concentration across the thallus as the lichen grows and integrates airborne pollutants, which would in turn affect the homogeneity of the lichen samples. This needs to be remembered when interpreting the results.

## 6. Conclusions and future trends

Lichens are useful biomonitors for SVOC air pollutants, particularly PAHs, as a result of their ability to respond to air pollutants at different levels, their slow growth rate, their longevity and their ability to indicate the presence and the concentrations of these pollutants. Consequently, there has been a recent global trend in environmental analytical research to utilize lichens in this manner.

An area that requires further research regarding lichens as biomonitors for organic air pollutants is determination of relationships between the concentration of pollutants found in the lichens and atmospheric levels thereof, which has only recently begun to be addressed [60]. Understanding that environmental factors, such as humidity, altitude and wind direction, affect the amount of organic air pollutants retained by lichens is also starting to influence how results are interpreted [52], and that should improve comparisons

between different samples across continents. The importance of sample homogeneity must also be emphasized.

Despite many PAH-extraction techniques being established, there is scope for development of quicker techniques, which are greener with higher recoveries. In terms of the analysis of sample extracts, several new products designed specifically for PAH analysis, such as SPE cartridges and GC columns, have been recently developed, and should assist in improving selectivity and sensitivity. Establishing a method that is reproducible and sensitive would improve understanding of the organic pollutant levels in air globally, and this would facilitate comparison of results between studies.

Full understanding of the organic pollutant levels in our global atmosphere can be reached only once gaps in the global use of lichens as biomonitors are filled in. This understanding would facilitate the development of a global distribution map of these atmospheric pollutants, which would allow identification of potential hotspots of pollution – an important step in the process of developing environmental protection and management policies.

## Acknowledgments

The financial assistance of the National Research Foundation (NRF) towards this research is hereby acknowledged. Opinions expressed and conclusions arrived at are those of the authors and are not necessarily to be attributed to the NRF.

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