DETERMINATION OF POLYCYCLIC AROMATIC HYDROCARBONS ACCUMULATION IN THE LICHEN *Parmotrema tinctorum* BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY COUPLED WITH DIODE ARRAY AND FLUORESCENCE DETECTORS

PRICHUKORN KHONGSATRA

A THESIS PRESENTED TO RAMKHAMHAENG UNIVERSITY
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF MASTER OF SCIENCE
(APPLIED CHEMISTRY)
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การหาปริมาณสารประกอบโพลีไซคลิกอะโรมาติกไฮโดรคาร์บอนที่สะสมในไลเคน *Parmotrema tinctorum* โดยโครมาโทกราฟีของเหลวสมรรถนะสูงควบคู่กับเครื่องตรวจวัดไดโอดอาร์เรย์และฟลูออเรสเซนต์

ไปรชุกรณ์ คงสัตรา

วิทยานิพนธ์เสนอต่อมหาวิทยาลัยรามคำแหง
เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญา
วิทยาศาสตรมหาบัณฑิต (เคมีประยุกต์)
ปีการศึกษา 2561

Thesis Title Determination of Polycyclic Aromatic Hydrocarbons Accumulation in the Lichen *Parmotrema tinctorum* by High Performance Liquid Chromatography Coupled with Diode Array and Fluorescence Detectors

Student's Name Miss Prichukorn Khongsatra

Field of Study Applied Chemistry

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Assoc. Prof. Dr. Kansri Boonpragob

Ramkhamheang University approved this thesis as a partial fulfillment of the requirements for the degree of Master of Science (Applied Chemistry).

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ABSTRACT

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The aim of this research was to develop the analytical method to evaluate eight PAHs included naphthalene (Naph), acenaphthene (Ace), phenanthrene (Phe), anthracene (Anth), fluoranthene (Fluo), pyrene (Pyr), chrysene (Chr) and benzo[a]pyrene (BaPy) accumulated in the lichen *Parmotrema tinctorum*. The analytical method consisted of sample preparation and quantitative analysis. The former included extraction of PAHs from lichens and cleaning up the extracting solution. The latter comprised determination and quantification by using high performance liquid chromatography (HPLC) coupled with Diode array detector (DAD) and fluorescence detector (FLD).

The extraction PAHs from lichen were carried out by ultrasound-assisted extraction (UAE) using hexane solvent. The optimum conditions were 10 minutes extraction for three times at 30 ºC, 100% of power output and mean operating frequency of 37 kHz. The clean up process used solid phase extraction technique (SPE). This was performed by using florisil mini-column, previously rinsed with 30.0 mL of acetonitrile, and then conditioned with 6 mL of dichloromethane and 3 mL of hexane, respectively. The suitable solvent for elution of PAHs compounds comprising of a mixture of hexane and dichloromethane (65 : 35). The eluate was evaporated under nitrogen gas until dry and redissolved by acetonitrile before injection to HPLC. The validation of sample preparation showed that the precision of eight PAHs at a low level gave the relative standard deviation percentage (% RSD) lower than 9.97% and the percentage recoveries of the spiked PAHs into the lichen were within the ranges of 70.51-101.34%.

The optimum HPLC method was validated by using Accliam C18 column (4.6×150 mm i.d., 3 µm particle size), maintained at 25 ºC, with the fluorescence detector in medium sensitivity mode. The excitation (Ex) was performed at 270 nm and emission (Em) was under automatic filter wavelength. Each PAHs had specific spectrum obtained from DAD. A sample solution (15 µL) was injected into the HPLC system by the autosample. It was found that the optimum HPLC condition for the analysis of PAHs consisted of gradient elution using DI water and acetonitrile as mobile phase at flow rate 1.0 mL/min.

The gradient solvent program began with 60% of acetonitrile for 3 minutes, then went to 100% of acetonitrile in 15 minutes with a final hold 5 minutes. The results demonstrated that PAHs compounds had limit of detection of FLD lower than DAD. The LOD by FLD showed that Naph, Ace, Phe, Anth, Fluo, Pyr, Chr and BaPy were 21.22, 9.76, 7.35, 22.83, 1.61, 2.37, 1.27 and 0.54 µg/L, respectively. The linearity (in term of correlation coefficients, r^2) of eight PAHs were higher than 0.9996.

The studied samples were the lichen *Parmotrema tinctorum* collected from Khao Yai National Park (KNP) at Nakhon Ratchasima province. They were collected from three localities: 1) The control site, at Nong Keing in KNP, at about 1,000 m away from the park's road 2) Tourist sites at Khao Yai National Park (TKNP) included the golf course, Nong Keing and Saisorn reservoir. Collections were made in January 2017. 3) Polluted sites in Bangkok at different traffic congestion (PBKT), performed by transplanting lichens from KNP to Lam Sali intersection, Rama 9 road intersection and the garden beside the Science Office Building (SCO) at Ramkhamhaeng University. The transplantations were done from 7 March 2017 to 7 April 2017. The results indicated that PAHs at the golf course had Ace, Phe, Anth, Chr and BaPy higher than the control site. The Nong Keing site had Ace, Phe and BaPy higher than the control site, and The Saisorn site had Chr and BaPy higher than the control site. These could be the products of incomplete combustion form forest fires and fossil fuel combustion from tourist traffic. Interestingly, the lichens from the three different traffic zones in Bangkok showed that those

from Lam Sali intersection had Anth, Pyr, Chr and BaPy higher than the control site at KNP. The lichen from Rama 9 road intersection had Pyr, Chr and BaPy higher than the control site at KNP. The lichen at SCO had Phe, Anth and Pyr higher than those at the control site, whilst Chr and BaPy were under the detection limit, similar to the control site.

บทคัดย่อ

ชื่อเรื่องวิทยานิพนธ์	การหาปริมาณสารประกอบโพลีไซคลิกอะโรมาติกไฮโดรคาร์บอนที่สะสมในไลเคน *Parmotrema tinctorum* โดยโครมาโทกราฟีของเหลวสมรรถนะสูงควบคู่กับเครื่องตรวจวัดไดโอดอาร์เรย์และฟลูออเรสเซนต์
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คณะกรรมการที่ปรึกษาวิทยานิพนธ์

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งานวิจัยนี้มีวัตถุประสงค์เพื่อพัฒนาวิธีการวิเคราะห์สารประกอบโพลีไซคลิกอะโรมาติกไฮโดรคาร์บอน (PAHs) 8 ชนิด ได้แก่ แนฟทาลีน (Naph), อะซีแนปทีน (Ace), ฟีแนนทรีน (Phe), แอนทราซีน (Anth), ฟลูออแรนทีน (Fluo), ไพรีน (Pyr), ไครซีน (Chr) และ เบนโซ(เอ)ไพรีน (BaPy) ที่สะสมในไลเคน *Parmotrema tinctorum* วิธีการวิเคราะห์ประกอบด้วยขั้นตอนการเตรียมตัวอย่าง ได้แก่ การสกัดสาร PAHs ออกจากไลเคนรวมทั้งการทำความสะอาดสารสกัด และขั้นตอนการวิเคราะห์หาปริมาณสาร PAHs โดยวิธีโครมาโทกราฟีของเหลวสมรรถนะสูงควบคู่กับตัวตรวจวัดไดโอดอาร์เรย์และฟลูออเรสเซนต์

การสกัด PAHs ออกจากไลเคนทำโดยวิธีการใช้คลื่นเสียงในตัวทำละลายเฮกเซน เงื่อนไขที่เหมาะสมคือ ใช้พลังงานในการทำงาน 100% และความถี่เฉลี่ยที่ 37 กิโลเฮิรตซ์ ทำการสกัดเป็นเวลา 10 นาที เป็นจำนวน 3 ครั้ง ที่อุณหภูมิ 30 องศาเซลเซียส ขั้นตอนการ

ทำความสะอาดสารสกัดใช้เทคนิคการสกัดด้วยเฟสของแข็ง (SPE) โดยใช้ตัวดูดซับของแข็งเป็นฟลอริซิล บรรจุในคอลัมน์ขนาดเล็ก จากการศึกษาสภาวะที่เหมาะสมพบว่าต้องทำความสะอาดด้วยอะซิโตไนไตรล์จำนวน 30 มิลลิลิตรก่อน แล้วปรับสภาวะให้เหมาะสมด้วยไดคลอโรมีเทน 6 มิลลิลิตรและ เฮกเซน 3 มิลลิลิตร ตามลำดับ ตัวทำละลายที่เหมาะสมสำหรับการชะล้างสาร PAHs คือ ส่วนผสมของเฮกเซนและไดคลอโรมีเทน (65 : 35) สารละลายที่ชะเก็บได้จะถูกนำมาระเหยภายใต้แก๊สไนโตรเจนจนแห้ง จากนั้นละลายกลับด้วยอะซิโตไนไตรล์ ก่อนนำไปฉีดเข้าเครื่องโครมาโทกราฟีของเหลวสมรรถนะสูง วิธีการเตรียมตัวอย่างถูกตรวจสอบการนำไปใช้ได้โดยตรวจหาความแม่นยำ และความถูกต้องของการวิเคราะห์ พบว่า มีค่า % RSD ต่ำกว่า 9.97% และค่าร้อยละของการกลับคืนของการเพิ่มสารประกอบโพลีไซคลิกอะโรมาติกไฮโดรคาร์บอน (PAHs) ลงในไลเคน อยู่ในช่วง 70.51-101.34%

การตรวจสอบวิธีการวิเคราะห์ที่เหมาะสมด้วย HPLC ทำโดยใช้คอลัมน์แอคเคลียม C18 (เส้นผ่านศูนย์กลาง 4.6×150 มิลลิเมตร ขนาดอนุภาค 3 ไมโครเมตร) อุณหภูมิของคอลัมน์ 25 องศาเซลเซียส และใช้เครื่องตรวจวัดฟลูออเรสเซนต์ในโหมดความไวปานกลาง มีความยาวคลื่นกระตุ้น (Ex) ที่ 270 นาโนเมตร และความยาวคลื่นปล่อย (Em) เลือกแบบอัตโนมัติ ซึ่งลักษณะสเปคตรัมเฉพาะของ PAHs แต่ละชนิดสามารถตรวจวัดด้วยเครื่องตรวจวัดไดโอดอาร์เรย์ สารละลายตัวอย่าง (ปริมาตร 15 ไมโครลิตร) ฉีดเข้าสู่ระบบ HPLC โดยเครื่องฉีดสารแบบอัตโนมัติ พบว่าสภาวะที่เหมาะสมสำหรับการวิเคราะห์ PAHs คือ ระบบเกรเดียนต์ โดยใช้น้ำปราศจากไอออนกับอะซีโตไนไตรล์เป็นเฟสเคลื่อนที่ ที่อัตราการไหล 1.0 มิลลิตรต่อนาที โดยเริ่มจากอัตราส่วน 60% ของอะซีโตไนไตรล์เป็นเวลา 3 นาที จากนั้นปรับอัตราส่วนของอะซีโตไนไตรล์เพิ่มขึ้นจนถึง 100% เป็นเวลา 15 นาที และใช้อัตราส่วน 100% ของอะซีโตไนไตรล์ต่อไปอีกจนครบ 5 นาที จากการตรวจสอบค่าขีดจำกัดการตรวจวัด (LOD) ของสาร PAHs พบว่า LOD ที่ได้จากเครื่องตรวจวัดฟลูออเรสเซนต์ มีค่าต่ำกว่าเครื่องตรวจวัดไดโอดอาร์เรย์ โดยพบว่า ค่าขีดจำกัดการตรวจวัด จากเครื่องตรวจวัดฟลูออเรสเซนต์ มีดังนี้ Naph, Ace, Phe, Anth, Fluo, Pyr, Chr และ BaPy มีค่า

21.22, 9.76, 7.35, 22.83, 1.61, 2.37, 1.27 และ 0.54 ไมโครกรัมต่อลิตรตามลำดับ ค่าความสัมพันธ์เชิงเส้น (r^2) ของ PAHs ทั้ง 8 ชนิดมีค่ามากกว่า 0.9996

ตัวอย่างที่ศึกษาคือ ไลเคน *Parmotrema tinctorum* เก็บจากอุทยานแห่งชาติเขาใหญ่ จังหวัดนครราชสีมา โดยมีการเก็บตัวอย่างไลเคนจาก 3 พื้นที่ คือ 1) พื้นที่ของหนองขิง บริเวณที่ห่างไกลจากถนนที่มีการจราจร เป็นระยะทาง 1,000 เมตร ใช้เป็นพื้นที่ควบคุม 2) พื้นที่ใกล้แหล่งท่องเที่ยว ได้แก่ บริเวณสนามกอล์ฟ หนองขิง และอ่างน้ำสายสร เก็บในช่วงเดือนมกราคม 2560 และ 3) ย้ายปลูกไลเคนมายังกรุงเทพมหานคร ที่มีการจราจรหนาแน่นแตกต่างกัน 3 แห่ง ได้แก่ แยกลำสาลี แยกถนนพระราม 9 และสวนหย่อมข้างอาคารคณะวิทยาศาสตร์ (SCO) มหาวิทยาลัยรามคำแหง โดยย้ายปลูกเป็นระยะเวลา 1 เดือน ระหว่างวันที่ 7 มีนาคม ถึงวันที่ 7 เมษายน พ.ศ. 2560 ผลการศึกษาพบว่า ไลเคนที่สนามกอล์ฟ มีปริมาณ Ace, Phe, Anth, Chr และ BaPy สูงกว่าสถานีควบคุม ที่หนองขิงมีปริมาณ Ace, Phe และ BaPy สูงกว่าสถานีควบคุม และที่อ่างน้ำสายสร มีปริมาณ Chr และ BaPy สูงกว่าสถานีควบคุม สาร PAHs เหล่านี้อาจมาจากการเผาไหม้ที่ไม่สมบูรณ์ของไฟป่า และการใช้พลังงานฟอสซิลจากรถยนต์ของนักท่องเที่ยว สำหรับไลเคนที่ย้ายปลูกมายังกรุงเทพฯ พบว่า ไลเคนที่แยกลำสาลี มีปริมาณเฉลี่ยของ Anth, Pyr, Chr และ BaPy สูงกว่าสถานีควบคุม ไลเคนที่แยกถนนพระราม 9 มีปริมาณ Pyr, Chr และ BaPy สูงกว่าสถานีควบคุม ไลเคนที่สวนหย่อมข้างอาคารคณะวิทยาศาสตร์ มหาวิทยาลัยรามคำแหง มี Phe, Anth และ Pyr สูงกว่าสถานีควบคุม ในขณะที่บริเวณนี้ มีปริมาณ Chr และ BaPy น้อยมากจนตรวจไม่พบเช่นเดียวกับที่สถานีควบคุม

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Prichukorn Khongsatra

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LIST OF ABBREVIATIONS

Ace	Acenaphthene
ACN	Acetonitrile
ATSDR	Agency for Toxic Substances and Disease Registry
Anth	Anthracene
BaPy	Benzo[a]pyrene
CC	Column Chromatography
Chr	Chrysene
r^2	Correlation coefficient
DAD	Diode Array Detector
DCM	Dichloromethane
DI water	Deionized water
^oC	Degree Celsius
DSASE	Dynamic Sonic-Assisted Solvent Extraction
Ex	Excitation wavelength
Em	Emission wavelength
Fluo	Fluoranthene
FLD	Fluorescence Detector
G	Golf course
GFF	Glass Fiber Filter
GAC	Green Analytical Chemistry
GR	Gradient System

g	Gram
HPLC	High Performance Liquid Chromatography
IARC	International Agency for Research on Cancer
IS	Isocratic System
KNP	Khao Yai National Park
kHz	Kilohertz
L	Lam Sali Intersection
LOD or DL	Limit of Detection
LOQ	Limit of Quantification
MΩ/cm	Megaohm/centimeter
MeOH	Methanol
MDL	Method Detection Limit
µg	Microgram
µL	Microliter
mL	Milliliter
moL	Mole
Naph	Naphthalene
nm	Nanometer
N	Nong Keing
ppb	Part per billion
Phe	Phenanthrene
PBKT	Polluted sites in Bangkok at different traffic congestion
PAHs	Polycyclic Aromatic Hydrocarbons

PTFE	Polytetrafluoethylene
PUF	Polyurethane foam
Pyr	Pyrene
QFF	Quartz Fiber Filter
RF	Radio frequency
R9	Rama 9 Road Intersection
RSD	Relative Standard Deviation
S	Saisorn Reservoir
SCO	Science Office Building
SPMDs	Semipermeable membrane devices
SPE	Solid Phase Extraction
SD	Standard deviation
TKNP	Tourist sites at Khao Yai National Park
UAE	Ultrasound-Assisted Extraction
UV	Ultraviolet
US-EPA	United States Environmental Protection Agency
VIS	Visible
WL	Wavelength
XAD	Amberlite styrene divinylbenzene copolymer resin

CHAPTER 1

INTRODUCTION

Overview

Polycyclic aromatic hydrocarbons (PAHs) are potentially carcinogenic substances that are persistent in the environment. Increasing concentrations of PAHs were observed due to rapid urbanization, thus; monitoring PAHs concentrations is necessary. However, it is expensive to conduct intensive monitoring activities of the large number of PAHs.[1] The air sampling method for monitoring of airborne PAHs in atmosphere may be classified into two methods[2], they are active air sampling and passive air sampling. In recent years, passive air sampling has popular been used to monitor the concentrations of pollutants in air. The collection of PAHs is based on a free flow of pollutants from the sampled medium (i.e. air) to the collecting medium. Therefore, some plants have also been used to passively sample as bioindicator for air quality monitoring, which is widely used in both urban and rural environments, including lichen[3-5], moss[3,6-7], plant leaves[8-11], pine needles[3,12-13] and tree bark[14-15].

The use of plants as bioindicators has proved very useful for the evaluation of atmospheric levels of PAHs[3] and lichens emerged as particularly suitable for this purpose.[5] A good agreement has been reported for PAHs profile in lichens and atmospheric particulate.[4,13] Epiphytic lichens are the most extensively used material for biomonitors of air quality since 1866. They have superior sensitivity toward specific pollutants and ability to store contaminants in biological tissues. Lichens are often used as bioindicators and/or bioaccumulators of heavy metals, chlorinated hydrocarbons, radionuclides, organic compounds and pesticides, etc.[4]

A wide variety of sample preparation and analytical techniques can be used to determine PAHs accumulation in lichens.[16] The procedure for determination of PAHs consists of three procedures: First, extraction of PAHs from lichens has many techniques, such as soxhlet extraction[13,17-20], Ultrasound-assisted extraction (UAE)[21-22] and Dynamic sonic-assisted solvent extraction (DSASE), etc.[4,17] Each of these methods has been developed to be more effective and have different measurement limitations. Second, clean up sample extract, after extraction of PAHs from lichen, there are many other contaminants that must be purified by passed through solid phase extraction cartridge (SPE) which is one of the most important steps for sample preparation[23]. This technique is increasingly popular, because unlike liquid-liquid extraction it does not require large volumes of (toxic) organic solvents and analysis times can be decreased significantly.[24] The stationary phases most commonly used to clean-up for contaminants of lichens are silica silica[5,18], florisil[25] and normal phases (-NH_2), which addition of florisil to the top of SPE cartridges.[26] Third, the analyzing step, the clean lichen extracts of PAHs can be analyzed by GC-FID, GC-MS, HPLC-UV and HPLC-FLD-DAD/UV.[16,27] Each technical measurement methods have been developed to more effectively continuous. The HPLC (High performance liquid chromatography) is an analytical technique to separate, identify, and quantify components in a mixture. Its suitable for analysis of compounds with higher molecular weights and boiling points and has therefore been widely used for PAH analysis.[28] HPLC with fluorescence detectors (FLD) has the characteristics of high sensitivity and low detection limit for PAHs compounds. The diode array detector (DAD) had to obtain the specific UV spectra of the PAHs for peak identification and peak purity checks.[28] Therefore, HPLC coupled with FLD and DAD is suitable for the analysis of lichen samples, for enabling both data analysis to confirm and comparison the results.

In this work, was to develop the analytical method analysis eight PAHs include naphthalene (Naph), acenaphthene (Ace), phenanthrene (Phe), anthracene (Anth), fluoranthene (Fluo), pyrene (Pyr), chrysene (Chr) and benzo[a]pyrene (BaPy) accumulated in the lichen *Parmotrema tinctorum*. The sample preparation of extraction used ultrasound-assisted extraction due to relatively low-cost method, using small volumes of organic solvent without the need of elaborate instrumentation and faster extraction times. In addition, it can be required many times for more efficient and reproducible extractions. Then using solid phase extraction (SPE) for cleaning-up and concentration of lichen extract. Followed by high performance liquid chromatography (HPLC) coupled with diode array detector (DAD) and fluorescence detectors (FLD) for determination and quantification PAHs compound. The lichen sample was chosen to study as bioindicator/ biomonitor of air quality due to PAHs in the atmosphere as passive air sampling. Lichens are particularly useful to study since they are not dependent on root uptake and receive nutrients directly from the atmosphere. In addition, this work focuses on using lichens as biomonitors of air pollution because of their allows monitoring of several sites at low cost and the fact that monitoring species can integrate pollutants over long period unlike conventionally used instruments.

Air Pollutants

Nowadays, the atmospheres were spreading dust and pollutants, including heavy metals, volatile organic compounds and polycyclic aromatic hydrocarbons (PAHs) have cause to climate change and affecting people's health. Air pollutants can be classified in different ways[29]:

Firstly, by formalism, air pollutants can be divided into two groups, primary pollutants and secondary pollutants.

Primary pollutants are the ones emitted from the pollution sources directly, for instance, carbon monoxide (CO), sulfur dioxide (SO_2), nitric oxide (NO) and etc.

Secondary pollutants are the ones produced by the chemical or photochemical reactions of primary pollutants, for instance, ozone (O_3), H_2SO_4, aerosol and etc.

Secondly, by physical property, air pollutants can also be divided into two groups, gaseous pollutants and particulates. A large proportion of air pollution is caused by gaseous pollutants, for instance, SO_X, NO_X, chlorofluorocarbon (CFCs) and etc., or particulates pollutants, for instance, heavier polycyclic aromatic hydrocarbons (PAHs) or vapour adsorbed into airborne particulate matter, particulate matter ($PM_{2.5}$ and PM_{10}) and etc.

Thirdly, by chemical property, air pollutants can be divided into two groups, organic pollutants, for instance, volatile organic compounds (VOCs) dioxins, furans, polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), organochlorine pesticides such as DDT and etc., for inorganic pollutants, for instance, heavy metals and etc.

Polycyclic Aromatic Hydrocarbons

Polycyclic aromatic hydrocarbons (PAHs) are a large group of organic compounds with two or more fused aromatic rings. There are more than hundreds of individual PAH compounds with different physio-chemical properties. The United States Environmental Protection Agency (US-EPA) has classified 16 of these as priority PAHs (as shown in Figure 1) to be targeted of air monitoring.[24,30-31] The structures of 16 PAHs can be classified by number of rings as shown in Table 1. The general characteristic of PAHs are high melting and boiling points (therefore making them solid), low vapor pressure, and very low aqueous solubility, the latter two tending to decrease with increasing molecular weight. They are highly lipophilic and therefore very soluble in organic solvents.[32]

Each of PAHs possess apecific characteristic UV absorbance spectra. Each ring structure has a unique UV spectrum, and thus each isomer has a different UV absorbance spectrum. This characteristic is especially useful in the identification of PAHs. Most PAHs are also fluorescent, emitting characteristic wavelengths of light when they are excited (when the molecules absorb light).[32-33]

PAHs belong to the group of persistent organic pollutants (POPs), these are organic contaminants that are resistant to degradation[7], can remain in the environment for long periods, and have the potential to cause adverse environmental effects. They are accumulated in the human through both the air, aquatic and terrestrial food chains.[34-36] The mechanism of toxicity was considered to be interference with the function of cellular membranes as well as with enzyme systems which are associated with the membrane. It has been proved that PAHs can cause carcinogenic and mutagenic effects and are potent immune-suppressants.[37]

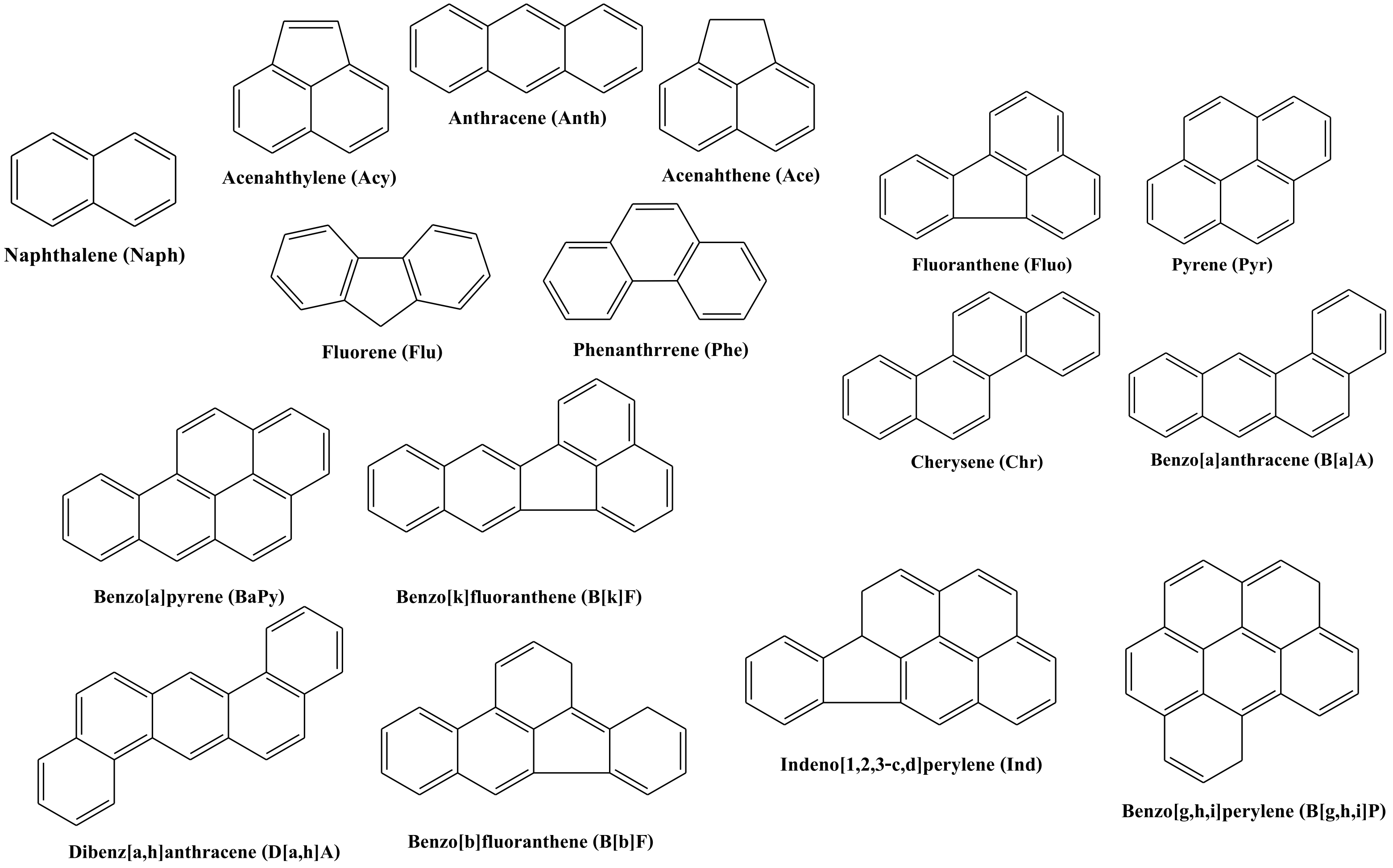


Figure 1 Chemical Structures of the 16 Polycyclic Aromatic Hydrocarbons[31]

Table 1 Classification of 16 PAHs by Molecular Weight and Cyclic Ring Number[30]

Low MW PAHs	MW	Ring No.	Medium MW PAHs	MW	Ring No.	High MW PAHs	MW	Ring No.
$C_{10}H_8$ (Naph)	128	2	$C_{16}H_{10}$ (Fluo)	202	4	$C_{20}H_{12}$ (B[b]F)	252	5
$C_{12}H_8$ (Acy)	152	3	$C_{16}H_{10}$ (Pyr)	202	4	$C_{20}H_{12}$ (B[k]F)	252	5
$C_{12}H_{10}$ (Ace)	154	3	$C_{18}H_{12}$ (B[a]A)	228	4	$C_{20}H_{12}$ (BaPy)	252	5
$C_{13}H_{10}$ (Fl)	166	3	$C_{18}H_{12}$ (Chr)	228	4	$C_{22}H_{14}$ (D[a,h]A)	278	5
$C_{14}H_{10}$ (Phe)	178	3				$C_{22}H_{12}$ (B[g,h,i]P)	276	6
$C_{14}H_{10}$ (Anth)	178	3				$C_{22}H_{12}$ (Ind)	276	6

MW stands for molecular weight (g/mol)

Priority of PAHs

PAHs usually occur naturally in oil, coal and tar deposits, and are produced as by-products of fuel burning (whether fossil fuel or biomass). They are found as pollution in air, water and soil. The toxicity of the PAHs is highly structurally dependent, and isomers may therefore vary from being non-toxic to very toxic. The list of priority PAHs varies in different countries. In the United States, the EPA has listed 16 priority PAHs. Each PAHs has a different risk of causing cancer. The carcinogenic classifications to humans and animals of selected PAHs by specific agencies shows in Table 2.[38-40]

Based on their toxicity, several PAHs are included in “priority” lists and are currently regulated in the number of countries. One of the most famous is the Benzo[a]Pyrene (BaPy) due to its carcinogenic properties. Besides, the European ambient air legislation targets this PAH and recommends an annual maximum guideline value of 1 ng/m^3. Because the PAHs structures are thermally stable, most of these compounds possess high boiling point and low vapor pressure favoring their association to particulate phase in the atmosphere.[30,41-43]

Sources of PAHs

PAHs are produced in all processes of incomplete combustion of organic substances and can diffuse in atmosphere. In general, the following two sources: natural and anthropogenic are the major PAHs sources to the environment.[31,37]

1. Natural sources of PAHs formation include: forest and brush fires, volcanoes, bacterial and algae synthesis, petroleum seeps, erosion of sedimentary rocks containing petroleum hydrocarbons, and decomposition of vegetative.

2. Anthropogenic sources can be divided into two groups: pyrogenic and petrogenic. Pyrogenic PAHs form during fossil fuel and biomass combustion (automobiles, power plants, industrial coal and petroleum burning and waste incinerators), whereas petrogenic PAHs originate from combustion of crude oil and its products, including kerosene, gasoline, diesel fuel, lubricating oil and asphalt.[44-46]

The mode of PAHs formation can be either natural or anthropogenic. Figure 2 illustrate such typical of PAHs contamination sources.

Table 2 The Carcinogenic Classifications of Selected PAHs by Specific Agencies

Agency	PAHs Compound	Carcinogenic Classification	Reference
International Agency for Research on Cancer (IARC)	Benzo(a)fluoranthene Benzo(k)fluoranthene Ideno(1,2,3-c,d)pyrene	Possibly carcinogenic to humans	[38]
	Acenaphthylene Anthracene Benzo[g,h,i]perylene Benzo[e]pyrene Fluoranthene Fluorene Phenanthrene Pyrene	Not classifiable as to their carcinogenicity to humans	

Table 2 (continued)

Agency	PAHs Compound	Carcinogenic Classification	Reference
U.S. Environmental Protection Agency (US-EPA)	Benz[a]anthracene	Probable human carcinogens	[39]
	Benzo[a]pyrene		
	Benzo[b]fluoranthene		
	Chrysene		
	Dibenz[a,h]anthracene		
	Indeno[1,2,3-c,d]pyrene		
	Acenaphthylene	Not classifiable as to human carcinogenicity	
	Anthracene		
	Benzo[g,h,i]perylene		
	Fluoranthene		
	Fluorene		
	Phenanthrene		
	Pyrene		

Table 2 (continued)

Agency	PAHs Compound	Carcinogenic Classification	Reference
Agency for Toxic Substances and Disease Registry (ATSDR)	Benz[a]anthracene Benzo[a]pyrene Benzo[b]fluoranthene Dibenz[a,h]anthracene Indeno[1,2,3-c,d]pyrene	Known animal carcinogens	[40]
	Benz[a]anthracene Benzo[a]pyrene	Probably carcinogenic to human	

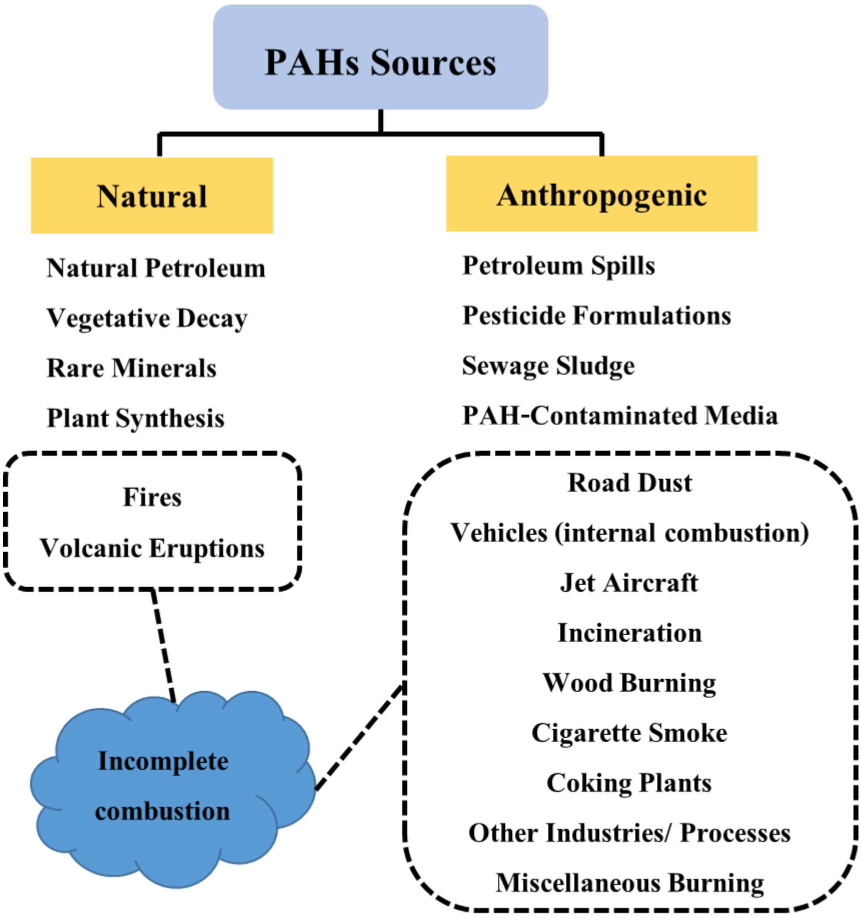


Figure 2 Natural and Anthropogenic Sources of PAHs[37]

PAHs entering the atmosphere derived from the combustion and from volatilization. They are presented in the ambient air as vapours or adsorbed into airborne particulate matter.[31] The gas/particle partitioning of PAHs depends on the molecular weight of the compounds as shown in Table 3, temperature, humidity, and precipitation.[47] In general, lower-molecular weight PAHs with two, three or four benzene rings, are more volatile (with low temperatures of condensation) and exist mainly in the gas phase. PAHs with four or more benzene rings are characterized by relatively with high temperature of condensation. Most of the heavier PAHs occur mainly in the particulate phase in the atmosphere due to their low vapor pressure. A significant correlation was also found between the amounts of dust in the air and PAHs concentrations in the particulate phase.[33] In general measured in the particulate phase, the higher PAHs concentrations in the winter months were related also to the higher emission of these pollutants from domestic heating sources.[31] The adsorption of PAHs onto particulate phases may be affected not only by temperature but by humidity as well, which it was found that the gas-to-particle PAHs ratio decreases with increasing humidity. The range of PAHs adsorption on atmospheric sorbents depends also on the quantity of the suspended particulates and their nature (soot, dust, fly-ash, pyrogenic metal oxides, pollens, etc., of different particle size).[48]

Table 3 Lists the Partition of Phase Distribution of PAHs in the Atmosphere by US-EPA[39]

PAHs[a] (chemical formula)	Mol. Wt. (g.mol^{-1})	Particle/gas phase distribution
Acenaphthylene ($C_{12}H_8$)	152	Gas phase
Acenaphthene ($C_{12}H_{10}$)	154	Gas phase
Fluorene ($C_{13}H_{10}$)	166	Gas phase
Anthracene ($C_{14}H_{10}$)	178	Particle gas phase
Phenanthrene ($C_{14}H_{10}$)	178	Particle gas phase
Fluoranthene ($C_{16}H_{10}$)	202	Particle gas phase
Pyrene ($C_{16}H_{10}$)	202	Particle gas phase
Benz[a]anthracene ($C_{18}H_{12}$)	228	Particle phase
Chrysene ($C_{18}H_{12}$)	228	Particle phase
Benzo[a]pyrene ($C_{20}H_{12}$)	252	Particle phase
Benzo[b]fluoranthene ($C_{20}H_{12}$)	252	Particle phase
Benzo[k]fluoranthene ($C_{20}H_{12}$)	252	Particle phase
Benzo[e]pyrene ($C_{20}H_{12}$)	252	Particle phase
Benzo[g,h,i]perylene ($C_{22}H_{12}$)	276	Particle phase
Indeno[1,2,3-c,d]pyrene ($C_{22}H_{12}$)	276	Particle phase
Dibenz[a,h]anthracene ($C_{22}H_{14}$)	278	Particle phase

[a] Predominant sources are motor vehicles and wood smoke

Transformations and Fate of PAHs in the Environment

PAHs present in the atmosphere are subject to complex physic-chemical reactions and transformations in the atmosphere; dry and wet deposition, photochemical transformations and reaction with other pollutants.[31]

The composition of PAHs in association with airborne particles changes significantly according to their emission sources, e.g. traffic or other processes of combustion. PAH concentration ratios can be used to identify possible emission sources, which are deposited slowly and, depending on atmospheric conditions and chemical reactivity, may be transported over long distances and pollute even remote areas.[8] They are widespread harmful compounds generated by different sources, which have concerns over the carcinogenic and mutagenic properties of PAHs have led to efforts to understand their formation and atmospheric chemistry.[47]

Figure 3 shown some of these processes in broad form and shows how PAHs are added, dispersed, and degraded in the environment.[37] This may affect people exposed to PAHs through inhalation, ingestion, or direct contact dermal contact. In the following sections, main sources of PAHs in ecosystem include, air, water, vegetation, lichens and soils will be described.

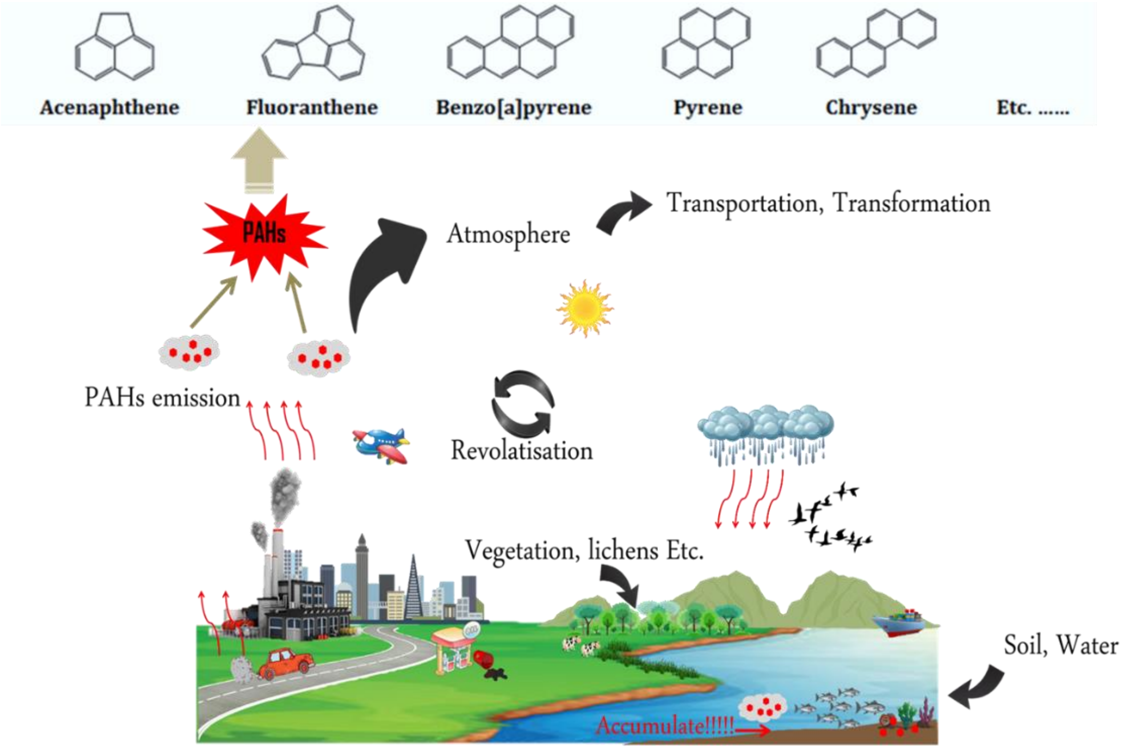


Figure 3 Conceptual Diagram Illustrating Diffusion and Transportation of PAHs in the Environment[49-50]

PAHs in Atmospheric

The atmosphere is the most important means of PAHs dispersal, it receives the bulk of the PAHs environmental load resulting in PAHs being ubiquitous in the environment. PAHs are emitted to the atmosphere primarily from the incomplete combustion of organic matter. The combustion sources can be either natural or anthropogenic. PAHs tend to be found in greater concentrations in urban environments than in rural environments because most PAH sources were located near urban centers. PAHs in the ambient air exist in vapour phase or adsorb into airborne particulate matter depending on the atmospheric conditions (ambient temperature, relative humidity, etc.), the nature and the properties of the individual PAH.[33] It can be concluded that "the atmosphere is not a repository and collector of PAH but more likely to be transporter, dilutor and reactor.[31]

Water

PAHs enter surface waters mainly via atmospheric fallout, urban run-off, municipal effluents, industrial effluents and oil spillage or leakage. Atmospheric fallout includes wet and dry deposition of particles and vapors. PAHs, as semi-volatile organic compounds, exist in both the gaseous and the particulate phase in air, and are subject to both vapor and particle washout from the atmosphere during precipitation. Atmospheric deposition has considered to be an important input of PAHs to surface waters.[8]

Vegetation and Lichens

PAHs from a polluted atmosphere are generally transferred to plants by particle-phase deposition on the waxy leaf cuticle or by uptake in the gas phase through stomata.[50-51] Leaf features (surface, cuticular waxes, hairs, number of stomata) play an important role in PAH uptake and accumulation.[52] Many plants have a relatively large surface area covered with waxes that facilitates the accumulation of hydrophobic chemicals. The use of plants as passive samplers of organic compounds in the atmospheric has been suggested by many authors.[8,12,53-55]

On the other hand, lichens play an important role, because they were able to retain a great amount of pollutants present in the atmosphere, through wet and dry depositions. The particular organisation of lichen thalli, without roots and cuticle, makes them highly sensitive to any change in the environment and provides the ability to accumulate pollutants in their biological tissue.[56-57]

Soils

Atmospheric PAHs are continuously deposited to the earth by dry or wet deposition processes. Some of these PAHs are from nearby sources, such as automotive exhaust from adjacent roadways. Other PAHs are from more distant sources and have been carried various distances through the air.[37] Soil system seems to be the important long-term repository for PAHs and considered to be a steady indicator of the environmental pollution state. Accumulation of PAHs in soils may lead to further potential contamination of vegetables and food chains[8,51,58] and then cause direct or indirect exposure to human. Moreover, leaching, evaporation and migration are possible PAHs sources of atmospheric or groundwater contamination. Sorption and degradation are key processes that affect the fate and transport of PAHs in the environment.[8]

Air Sampling of PAHs

The monitoring methods for airborne PAHs may be classified into those concerned with either direct occupational exposures or with ambient exposures, which air sampling method can be divided in two methods[2] as following:

1. Active Air Sampling

Active sampling methods are the standard methods and the most common method that rely on integration of the sample, require pumping the air stream through a medium consists of filter and solid adsorbent which will retain the target compounds. The filter, often teflon, glass or quartz fiber (GFF or QFF). The solid adsorbent normally consisted of a polyurethane foam (PUF) disks[59-61], XAD-based[5], and SPMDs[59,62], etc. The disadvantages of these samplers are the requirement of deploying for a period of time, less efficient in accumulating particulate compounds and higher cost.

2. Passive Air Sampling

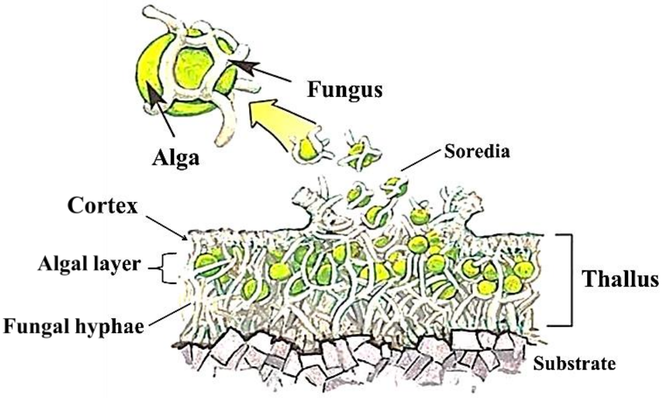
Passive sampling techniques rely on the controlled transport of the analyte material along a concentration gradient towards a surface medium which acts as a sink for the target compound. This technique is not need of a pump and electricity to collect pollutants. Passive air sampling has been applied infrequently to ambient (outdoor) monitoring of PAHs, mainly because of the long sampling times required due to the low PAH levels and limited analytical sensitivity in environments. The low cost and reasonably good sensitivity of this technique for specific compounds, are very useful for sampling surveys requiring the collection of large numbers of samples.[2]

Lichens

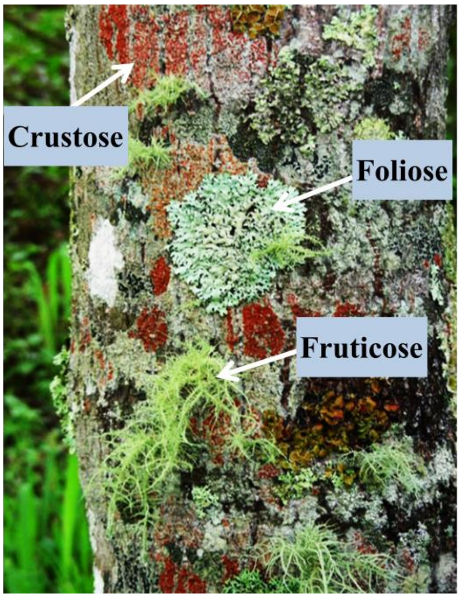
The lichen thallus is a complex symbiotic vegetative lower plant composed of two organisms; a fungus, which are portion as referred to as the mycobiont refers to the thallus or the vegetative body of the lichen, and an alga or cyanobacteriae, which are component also the photobiont and provides the photosynthetic energy.[63-64] The cell wall consists of a multi laminate and a granular layer. Adhering to the outside of the cells is a fibrous polysaccharide layer. 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 (as shown in Figure 4a). It is believed that the porous nature of the epicortex is what enables efficient gas exchange.[16,65]

Lichens can be found from extreme low tide level on the sea shore to the tops of high mountains, and from arctic to tropical regions, which the wide distribution is the more remarkable.[64]

On the basis of their overall habit, lichens are traditionally divided into three main morphological groups: these are the crustose, foliose and fruticose types (as shown in Figure 4b).[63] The crustose lichens are tightly attached to their substrates, are thus difficult to remove for analysis and are less exposed to their surrounds. The foliose lichens are known to have the largest ratio of surface area to dry weight, and are to accumulate airborne particles more readily, which are completely exposed to ambient air as a result of having few points of attachment to the substrate. And the fruticose lichens are hair-like strap-shaped or shrubby and the lobes may be flat or cylindrical[66], with a leaf-like structure with defined upper and lower layers, so only the upper layer is in contact with the ambient air.[67-68] Figure 4 shows the relationships between alga and fungi (a) and three types of lichens (b).



(a) Lichen Thallus



(b) Three Types of Lichens

Figure 4 (a) Lichen Showing Relationships between Alga and Fungi, which Consists of Lichen’s Internal Structure

(b) Three Types of Lichens; are Crustose, Foliose and Fruticose[69]

Lichens as Biomonitors of Air Pollution

Terrestrial lichens have been used as biomonitors for assessing air quality since 1866, which have been widely used to biomonitoring of heavy metals, chlorinated hydrocarbons, radionuclides, organic compounds and pesticides, etc.[56]

Lichens are particularly useful in these studies since they are not dependent on root uptake and receive nutrients directly from the atmosphere; moreover, since they lack a waxy cuticle (lipid surface layer that is present in plant) and stomata, elements are easily incorporated in their tissues, are therefore incapable of controlling gas exchange (as shown in Figure 5).[65,70-71]

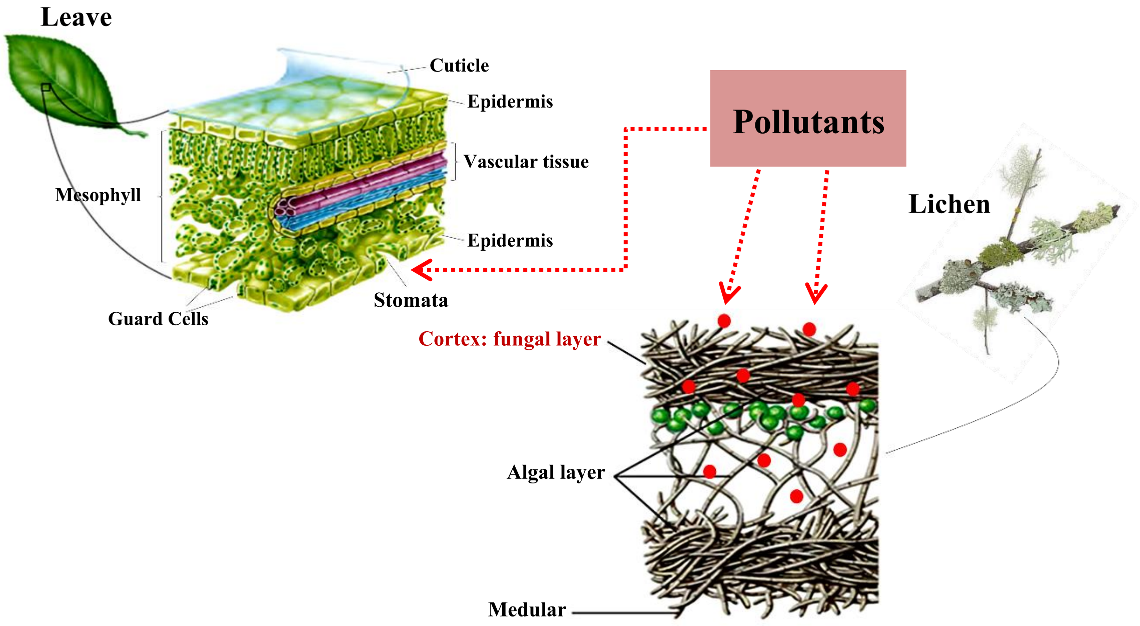


Figure 5 Different of Structures (internal) between Leaves and Lichens in Accumulation of Pollutants[72]

Lichens are long lived organisms and are long term integrators of atmospheric pollution. This characteristic is of crucial importance to evaluate human exposure to pollutants such as PAHs; time integration of these compounds allows relating low levels of pollutants with long-term chronic effects on health.[73-74]

Sampling Methods of Biomonitoring of Air Pollutants using Lichens

Two distinctly different sampling methods are used when lichens are to be utilized as tools for air pollution monitoring.[16]

1. Direct sampling

Direct sampling is a popular sampling technique, which exploits the fact that lichens accumulate pollutants with time directly from the atmosphere. The 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.[26]

2. Lichen Transplants

The technique of transplanting lichens from relatively clean environments to areas of interest has been a popular method when studying heavy metals[75] and sulphur dioxide (SO_2)[76], etc. Interestingly, were applicable to PAHs studied, due to the long-term exposure to the low levels of these pollutants that is required in order to allow meaningful, detectable results.[21-22]

Lichen Samples

Figure 6 The Lichen *Parmotrema tinctorum* (Nyl.) Hale

The foliose lichen *Parmotrema tinctorum* (Nyl.) Hale (as shown in Figure 6) of the family Parmeliaceae was used in this study. It was characterized by green to gray smooth upper surface.[77] This lichen is found throughout tropical and temperate regions including North, Central and South America; Africa, Asia (India, Nepal, China, Thailand, Indonesia, Papua New Guinea, Taiwan, Hong Kong, and Japan), Australia, New Zealand, and the Pacific Island.[78] In Thailand, there are widely distributed in every forest type in the unpolluted site at Khao Yai National Park.[79] It was suitable for monitoring air quality because of these properties: large lobes, large thallus, high growth rates (about 19.32 mm/year), easy to collect from substrates, and etc.[80] This lichen grows on bark, rock and artificial substrates such as bottles, plastic nets, nylon nets, cement blogs, zinc plates, tile roof, and etc.[81] In Thailand, the lichen *Parmotrema tinctorum* has been used for biomonitoring of air pollution (Boonpeng, C.) by transplanted lichens to petrochemical industrial.[82]

Sample Preparation

Direct analysis of pollutants in complex matrices is difficult because in some samples they are present low concentrations and are associated with the sample matrix.[83-84]

Sample preparation was considered to be the most challenging step of the analytical procedure, since it has an effect on the whole analytical methodology, therefore it contributes significantly to the greenness or lack of it of the entire process.[85]

The elimination of the sample treatment steps, pursuing at the same time the reduction of the amount of the sample, strong reductions in consumption of hazardous reagents and energy also maximizing safety for operators and environment, the avoidance of the use of big amount of organic solvents, form the basis for greening sample preparation and analytical methods.[85]

Sample preparation for the determination of trace concentrations of analytes in complex matrices usually includes two steps: extraction of analytes from the matrix, followed by a suitable clean-up, concentration and purification of PAHs from environmental samples mainly.[86-89]

Extraction Techniques

This preparative step aims at extracting PAHs from the sample matrix, which are dissolved by the same solvent as PAHs, as selectively as possible.[86] Extraction techniques reported for PAHs analysis of samples in environment include soxhlet[13,17-20], ultrasound-assisted extraction (UAE)[17,21-22,90-91], mechanical shaking[91-92], soxtec (automated soxhlet)[3], supercritical fluid extraction (SFE)[93], microwave-assisted extraction (MAE)[94-96], pressurised liquid extraction (PLE)[5,93,97] and solid-phase microextraction (SPME)[98-100].

In other literature, the mostly procedures extraction used for lichens have been three techniques. Traditionally, Soxhlet has been employed for extraction of PAHs. Table 4 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.[13,17-20]

Table 4 Reported Experimental Conditions of Sample Preparation for Extraction of PAHs from Lichen using Soxhlet

Mass lichen sample used (g)	Solvent scheme	Solvent volume (mL)	Extraction time (h)	Ref.
0.2	Dichloromethane	250	6	[17]
2	Dichloromethane	100	16	[18, 19]
2	Acetonitrile	200	24	[13]
0.6-0.8	Hexane: acetone (1 : 1)	150	2[a]	[20]

[a] Refers to an automated soxhlet extraction and reflects total extraction time of the extraction in solvent and the reflux in the rinse position

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.[17,21] Guidotti et al. used cyclohexane as the solvent for UAE, combining only two extractions.[21-22] Domeño et al. extracted a 0.2 g lichen sample using portions of 15 mL dichloromethane in each of four subsequent extractions.[17] A study, in which a novel UAE technique for lichens was developed by Domeño and Blasco, found that hexane is the solvent that extracts most PAHs efficiently.[17]

Clean-Up Procedures

Clean up techniques may be divided into pre-separation techniques, which are used to remove the bulk of the co-extracted material, and fractionation techniques, which are used to separate the target analytes in different fractions, and to remove contaminated compounds.[87] A large number of sorbents are used for the isolation and clean-up of organic compounds from the extract solutions. They include alumina[97], florisil[88], $-NH_2$[101], ion-exchange resins[102], silica gel[91-92] and many silica-based sorbents (e.g. octadecyl bonded silica, octyl bonded silica, phenyl bonded silica, cyanopropyl bonded silica, diol bonded silica, etc.).[28,90,102-105]

In other literature, with regards to the clean-up of lichen extracts, column chromatography (CC) or solid-phase extraction (SPE) is commonly used and these are summarized in Table 5.

Table 5 Summarizes Sample Clean-Up Conditions of PAHs in Lichen Extracts

Type of clean-up	Sorbent Type	Elution solvent	Ref.
CC	Silica gel	25 mL methylene chloride:pentane (2 : 3)	[18]
CC	Florisil	30 mL acetonitrile	[25]
SPE	Silica	50 mL dichloromethane	[5]
SPE	Florisil	2 mL hexane : dichloromethane (65 : 35)	[20]
SPE	Normal phase-NH_2	2 mL hexane : dichloromethane (65 : 35)	[57,26,20,106]

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, 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.[16]

Silica column chromatography has been employed extensively, with different solvent schemes as shown in Table 5. Compared to the column chromatography clean-up technique, SPE consumes fewer amounts of toxic solvents which were of a great advantage from the environmental sustainability standpoint as well as saving substantial amount of time.[91]

Ultrasound-Assisted Extraction (UAE)

The application of ultrasound-assisted extraction (UAE), which has gained acceptance as an efficient friendly technique for a greener sample preparation of environmental and food samples has increased in the recent years. UAE sample pretreatments are clearly greener than more classical ones using large amounts of solvents and long operation times. This technique has been used in the development of methods for the analysis of numerous contaminants, including organic compounds (pesticides, pharmaceuticals, polycyclic aromatic hydrocarbons, polyhalogenated flame retardants, etc.) and inorganic compounds (heavy metals).[107-108]

In short, implementation of ultrasound brings about the following advantages from the Green Analytical Chemistry (GAC) perspective[109]

1. Significant shortening of many processes (e.g., digestion, extraction, solubilization) with subsequent saving in energy
2. Use of less solvent and/or at lower concentration
3. Safer procedures, since operation is performed at almost room temperature and atmospheric pressure
4. Fewer opportunities for contamination and/or analyte losses during pretreatment
5. Achievement of ecofriendly and low-cost methods with increased productivity.

Another relevant issue is that recovery may be lower than with other solid-liquid extraction techniques and the need for an efficient clean-up prior to chromatographic separation, mainly when biological samples are involved. The techniques used most for detection and quantification of organic compounds after UAE was GC-MS and HPLC-UV (see Figure 7).[109]

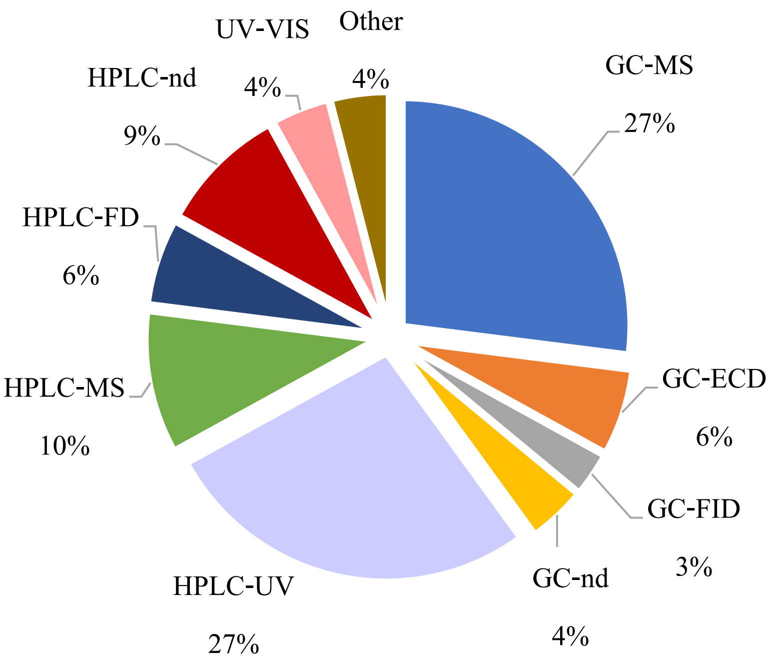


Figure 7 Analytical Techniques most Employed using Ultrasound-Assisted Extraction for Organic Compounds[109] (nd: non-defined detector)

Solid-Phase Extraction (SPE)

Solid-phase extraction (SPE) for liquid samples became a widely used laboratory technique following the introduction in the 1970s of disposable sorbent cartridges containing porous particles sized to allow sample processing by gentle suction.[110] SPE is the most widely used method for the extraction, clean-up, concentration and fractionation of organic compounds from several samples.[111] It has capabilities in a broad range of applications such as environmental analyses, pharmaceutical and biochemical analyses, organic chemistry and food analyses.[112]

The principle of SPE is similar to that of liquid-liquid extraction (LLE), involving a partitioning of solutes between two phases. However, instead of two immiscible liquid phases, as in LLE, SPE involves partitioning between a liquid (sample matrix) and a solid (sorbent) phase.[113] This technique unlike LLE it does not require large volumes of (toxic) organic solvents, analysis times can be decreased significantly and on-line and/or automated procedures are easily designed. Another advantage is the wide variety of extraction conditions which may be used to achieve the desired separation and pre-concentration.[24] In addition, SPE can used after Soxhlet extraction, ultrasonic extraction or accelerated solvent extraction and has also been used in combination with the chromatographic analysis techniques for the determination of analytes in samples.[91]

SPE Phase Types

SPE sorbents are most commonly categorized by the nature of their primary interaction or retention mechanism with the analyte of interest. The three most common extraction mechanisms use in SPE are reversed phase, normal phase and ion exchange (as shown in Figure 8) as followed;

1. Reversed Phase SPE

Reverse-phase SPE the retention mechanisms are the interaction of non-polar groups of the analytes of interest and the non-polar functional groups on the sorbent, via Van der Waals forces. This interaction is facilitated by solvents having very little non-polar character or, in other words, very polar solvents. Thus, the most of applications where reverse-phase SPE has been applied, the extraction was performed in a polar solvent.[114] Common these sorbents contain saturated hydrocarbon chains, for instance, C_{18} and C_8, or aromatoc rings such as phenyl (PH), etc.

2. Normal Phase SPE

Normal-phase SPE the retention mechanisms are commonly employed to extract polar analytes from non-polar organic solvents. The retention mechanisms are based on hydrogen bonding, dipole-dipole and π-π interactions between polar analytes and polar stationary phases such as silica (SiO_2), alumina (Al_2O_3), magnesium silicate ($MgSiO_3$) or florisil.[114]

3. Ion Exchange SPE

Ion exchange mechanisms are used to extract charged analytes from low ionic strength aqueous or organic samples. Charged sorbents are used to retain analytes of the opposite charge.

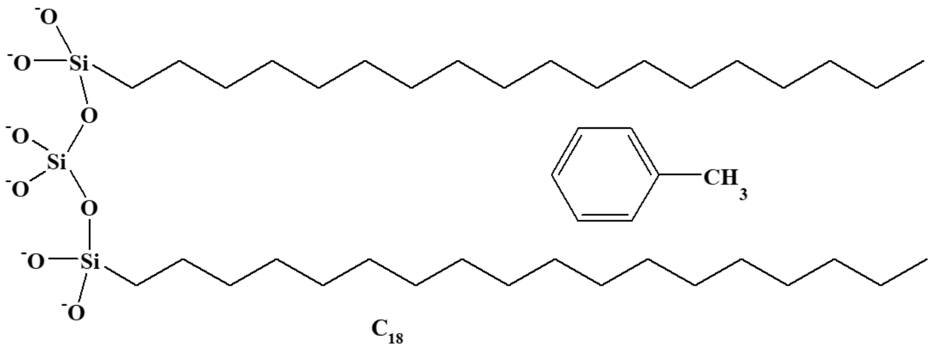
Anion Exchange

Anionic (negatively charged) compounds can be isolated on LC-SAX material is comprised of an aliphatic quaternary amine group that is bonded to the silica surface. A quaternary amine is a strong base and exists as a positively-charged cation that exchanges or attracts anionic species in the contacting solution, thus the term strong anion exchanger (SAX). The LC-NH_2 material is comprised of an aliphatic aminopropyl group bonded to the silica surface and used for normal phase separations is also considered to be a weak anion exchanger (WAX) when used with aqueous solutions.[115]

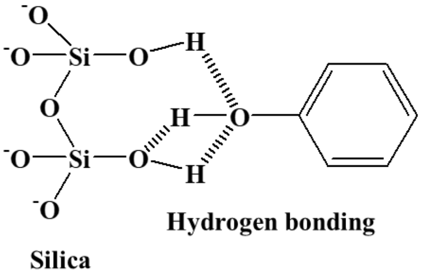
Cation Exchange

Cationic (positively charged) compounds are isolated by using LC-SCX material contains silica with aliphatic sulfonic acid groups that are bonded to the surface. The sulfonic acid group is strongly acidic (pKa <1) and attracts or exchanges cationic species in a contacting solution, thus the term strong cation exchanger (SCX). The LC-WCX material contains an aliphatic carboxylic acid group that is bonded to the silica surface. The carboxylic acid group is a weak anion, and is thus considered a weak cation exchanger (WCX).[115]

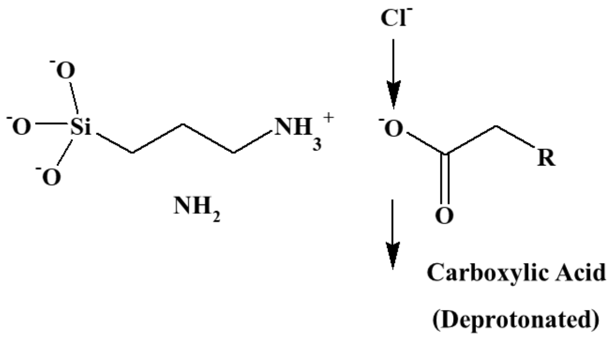
(a) Reversed Phase Retention



(b) Normal Phase Retention



(c) Anion Exchange Retention



(d) Cation Exchange Retention

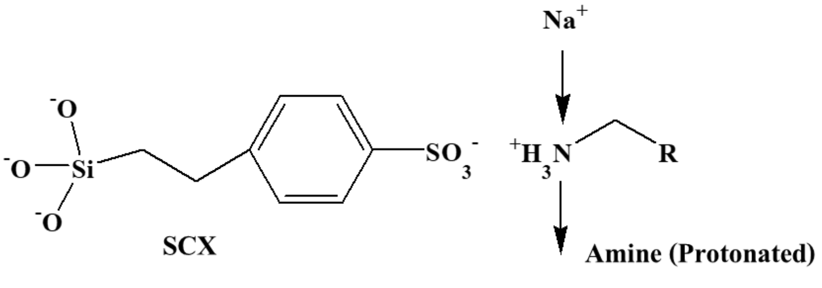


Figure 8 Solid Phase Extraction (SPE) Retention Mechanisms of Reversed Phase (a), Normal Phase (b) and Ion Exchange; Anion Exchange (c), Cation Exchanger (d)[116]

Basic Principles of SPE

Sample preparation with SPE typically consists of four basic steps are conditioning, sample loading, wash, and elution as shown in Table 6.[116]

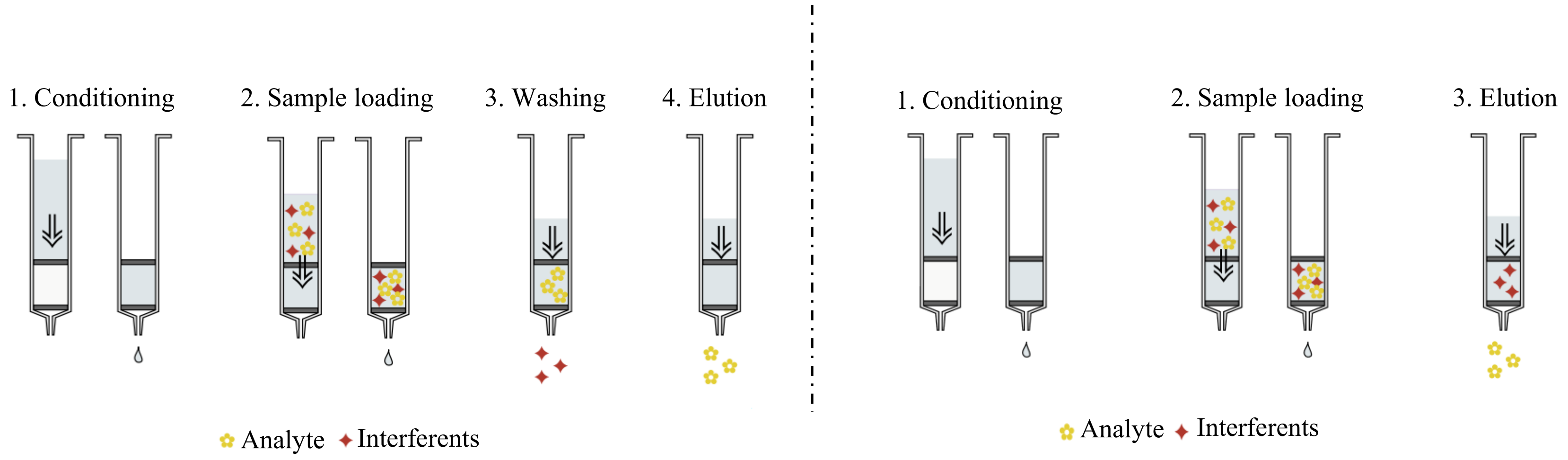
Table 6 Summarizes of Four Basic Steps for Sample Preparation with SPE[116]

Step	Purpose
Conditioning	To prepare the sorbent for effective interactions with the analytes by solvation or activation of the ligands on the chromatographic surface, followed by equilibration in a solvent similar to the sample/matrix.
Sample/Matrix Pretreatment and Loading	To adjust the sample/matrix composition (via dilution, etc.) such that the analytes is quantitatively retained on the sorbent while the amount of bond impurities is minimized.
Wash	To remove impurities that is bound to the sorbent less strongly than the analytes.
Elution	To selectively desorb and recover the analytes by disrupting the analyte-sorbent interactions.

In general, SPE can be used for three important purposes in up-to-date analyses are concentration of the analyte, removal of interfering substances, and changing the matrix of the analyte as needed for subsequent analyses. In most cases these three effects occur together. Since analytes can be either adsorbed on the SPE packing material or directly flow through while the interfering substances are retained, two general separation procedures as shown in Figures 9a and 9b.[112]

The majority of SPE extractions are retentive extraction, typically consist of all 4 steps (as shown in Figures 9a), since a sorbent retains the target analytes, while contaminants simply pass though the column to waste. In contrast, non- retentive extractions may only require 2 or 3 steps (as shown in Figures 9b), since the analytes are effectively eluted during the sample loading and wash steps, which sorbent has no affinity for the analyte, but a high affinity for the sample contaminants.[116]

Specific details on the relative polarity, hydrophobicity and sorbent polarity chart for used each type of extraction mechanism (or sorbent) are provided in Table 7.[112]

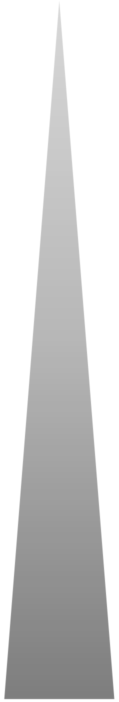


a) Retentive SPE

b) Non-Retentive SPE

Figures 9 Schematic Illustrations of the Retentive and Non-Retentive SPE Mechanisms[112]

Table 7 Detailed on the Relative Polarity, Hydrophobicity and Sorbent Polarity of most Common SPE Sorbents[112,115-116]

Relative Polarity	Sorbent Polarity	Representative Solvents	Miscibility with water
Non-polar		Hexane	No
	C_{18}	Isooctane	No
	C_8/Octyl	Petroleum ether	No
		Cyclohexane	No
	PH/Phenyl	Carbon tetrachloride	No
	CN/Cyano	Chloroform	No
		Methylene chloride	No
	Si/Silica	Tetrahydrofuran	Yes
	NH_2/Amino	Diethyl ether	No
		Ethyl acetate	Poor
	Fl/Florisil	Acetone	Yes
	Al/Alumina	Acetonitrile	Yes
		Isopropanol	Yes
	SCX/Aromatic Sulfonic acid	Methanol	Yes
	SAX/Quaternary amine	Water	Yes
Polar		Acetic acid	Yes

In this work, focuses on the normal phase of solid phase extraction (SPE) for used to clean up a sample before using the chromatographic method to quantify the amount of analytes in the sample. Florisil (Figure 10), in normal phase extractions, target analytes and other compounds with a large proportion of polar functional groups (amines, amides, hydroxyls, carbonyls, heteroatoms) are extracted from non-polar organic solvents (hexane, chloroform) using polar sorbents such as silica (Si), amino (NH_2), cyano (CN), florisil (Fl) and alumina (Al). Retention is facilitated by loading under non-polar solvent conditions. Elution occurs in the presence of more polar solvents than conditioning solvent.

Florisil

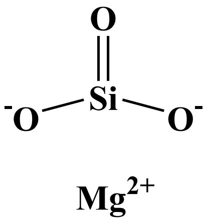


Figure 10 The Structure of Florisil[117]

Florisil has been designated, and are certified, for specific separation. There are highly active, polar sorbent with a slightly basic surface for adsorption of low to moderate polarity species from non-aqueous solution; specifically designed of the adsorption of pesticides using official AOAC and EPA methods; other applications include polychloeinated biphenyls (PCB) in transformer oil[117], organic tin compounds aliphatic carboxylic acids and polyaromatichydrocarbons (PAHs) in lichen.[20]

High Performance Liquid Chromatography

High performance liquid chromatography (or high pressure liquid chromatography, HPLC) is a specific form of column chromatography generally used in chemistry and analysis to separate, identify, quantify, and purify for mixtures, are organic, inorganic, biological, ionic, and polymer materails. It is the most widely used laboratory technique.[118,119]

HPLC is the LC technique with the highest efficiency which has ability to separate different sample compounds in a given time with a given resolution.[120] It is especially suitable for compounds which are not easily volatiles, thermally unstable and have high molecular weights.[119]

Instrumentation of HPLC

Typical high performance liquid chromatography system consists of solvent reservoirs containers for the mobile phase, a pump and degasser to move the mobile phase and auto sample through the system, an injection device to allow sample introduction, a column to provide solute separation, a column oven to controlled-temperature, a detector to visualize the separated components, a waste container for the used solvent, and finally a data collection device to assist in interpretation and storage of results as shown in Figure 11, to obtain separations sufficient for quantitative analysis in liquid chromatography, good equipment is essential.[120]

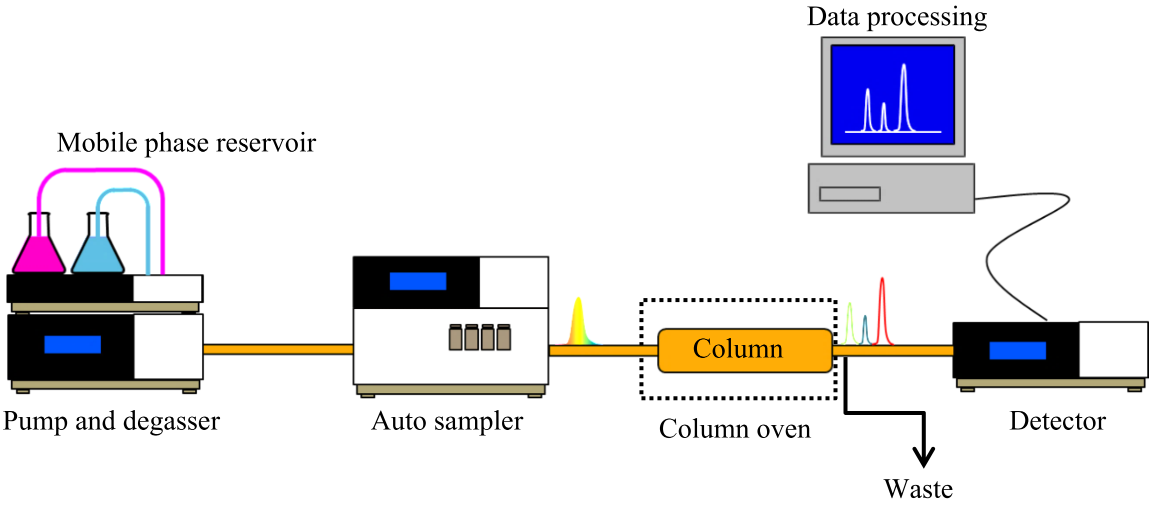


Figure 11 Components of High Performance Liquid Chromatography System

The mobile phase, which may be a single liquid or a variable mixture of two or more liquids, is pumped at high pressures into a controlled-temperature oven, where it passes first through an open coil to bring it to the operating temperature and then through a guard column designed to protect the analytical column from impurities and extend its lifetime. If a differential type of detector is used, the flow may be spilt in this point, with part going directly to the reference side of the detector and part to the analytical column. Ultimately, the column effluent passes through the sample to the detector and on to a collection device or to waste.[121]

In addition, the separation of analytes is performed inside the column, whereas a detector is used to observe the obtained separation. The composition of the eluent is consistent when no analyte is present. While the presence of analyte changes the composition of the eluent. This difference is monitored as a form of electronic signal, which different types of detectors available.[121]

Types of HPLC Detectors

Detector in HPLC is placed at the end of analytical column. Function of detector is to examine the solution which is eluting from the column. Detector is the heart of an instrument and efficiency of system is dependent upon detecting techniques.[122]

An electronic signal (output from detector) is proportional to the concentration of individual components of analyte. Detectors are classified as bulk property detectors and solute property detectors. Bulk property detectors measure the changes in the property of combined eluting mobile phase and eluting solute. Solute property detectors detect the changes in physical and chemical property of eluting component of the mobile phase.

There are many characteristics to consider when choosing a detector, and lists some of them as shown in Table 8. Since no one detector has all of these characteristics, over time a multitude of detectors have been designed, produced, and sold to answer one particular challenge or another. Ease of use, predictability, and reproducibility are all very important characteristics.[123]

Table 8 Desired Detector Characteristics[123]

The desirable features
- High sensitivity and reproducible, predictable response
- Respond to all solutes, or have predictable specificity
- Wide linear dynamic range; Response that increases linearly with the amount of solute Response unaffected by changes in temperature and mobile phase flow
- Respond independently of the mobile phase
- Not contribute to extra-column band broadening
- Reliable and convenient to use
- Nondestructive of the solute
- Provide qualitative and quantitative information on the detected peak
- Fast response

Over the years, HPLC has been combined with numerous detection methods explained below and summarized in Table 9 and has steadily experienced increasing innovations regarding stationary phases which have enhanced its sensitivity and specificity.[121,124]

Table 9 Summary of the most Important HPLC Detectors[124]

Detector	Type*	Maximum sensitivity (µg/ml)	Gradient possible	Advantages	Disadvantages
UV, VIS	S	10^{-9}-10^{-10}	Yes	- Enables detection of a wide variety of compounds containing a chromopheres non-destructively. - Able to monitor co-eluting compounds at variable wavelengths. - Can be used for other measurements or for preparative purposes simple and inexpensive.	- Sensitivity dependent on chromophore in molecule. - Not highly specific for some molecules particularly those absorbing at low wavelengths. - Dependent on solution conditions compound identification based exclusively on retention time and absorbance wavelength.
DAD	S	$< 2\times10^{-5}$	Yes	- As UV-VIS but can acquire all UV-VIS absorbance data in a given run allowing UV-VIS scans for all peaks detected and 3D images (time vs. intensity vs. wavelength). - Characterization of peaks by UV-VIS scans possible. -Simple and more expensive.	- As above but prone to larger noise than UV-VIS detection. - Compound identification based exclusively on retention time and UV-VIS absorbance pattern.

*: S, selective; N, nonselective; SN, selective universal, **: Grams per second

Table 9 (continued)

Detector	Type[*]	Maximum sensitivity (µg/ml)	Gradient possible	Advantages	Disadvantages
FLD	S	10^{-9}	Yes	- Very sensitive. - Very specific (only compounds that fluorescence are detected). - Minimum interference. - Simple and inexpensive	- Few compounds show native fluorescence. - Natively non-fluorescent compounds require pre-column or post-column imitation techniques which are additional steps, often not applicable to certain analytes, and can contribute to quantitation errors.
MS	SN	$< 10^{-9}$[**]	Yes	- Very selective and accurate quantitation, ideal internal standards by using stable isotopes of the analyte. - Ideal for multiple analytes unless isobaric compounds co-elute Sample purity not important.	- High cost of instrument acquisition, operation, and maintenance/repair. - Time-consuming maintenance and troubleshooting.

[*]: S, selective; N, nonselective; SN, selective universal, [**]: Grams per second

Table 9 (continued)

Detector	Type[*]	Maximum sensitivity (µg/ml)	Gradient possible	Advantages	Disadvantages
MS	SN	$< 10^{-9}$[**]	Yes	- Provides structural information when exact masses can be determined (high resolution instruments) and by identifying distinctive fragmentation patterns. - Ideal for multiple analytes unless isobaric compounds co-elute Sample purity not important.	
RI	N	10^{-7}	No	- The response is dependent on changes in refractive index of eluting compounds in the mobile phase.	- Gradient programming is not possible due to resulting changes in refractive index of mobile phase. - Less sensitive than UV-VIS detector. - Typical applications are in Size Exclusion Chromatography.

[*]: S, selective; N, nonselective; SN, selective universal, [**]: Grams per second

The most popular detectors are used to for detection of PAHs accumulation in samples, which are discussed in the following.

Ultraviolet-Visible Detector

The UV-VIS detector is the most frequently used detector in HPLC. It is simple to handle, concentration sensitive, selective, and nondestructive.[120] The most organic compounds of interest absorb light in the UV region (190-400 nm) and visible region (400-950 nm).

There are three different types of UV detectors as follow; fixed wavelength, variable wavelength, and diode array detectors.[123] All the UV-VIS detectors are based on Beer-Lambert law of ability of solute to adsorb light at defined wavelengths based on chemical structure and functional groups present in the solute molecule. Sensitivity of the detector depends on absorbance; A (1%, 1 cm) value of analyte. Source of UV light is a deuterium or high pressure xenon lamp while for visible range tungsten lamp is preferable. A beam of light was allowed to pass through a flow cell mounted at the end of column.[122] Schematic diagram of UV detector is described in Figure 12.

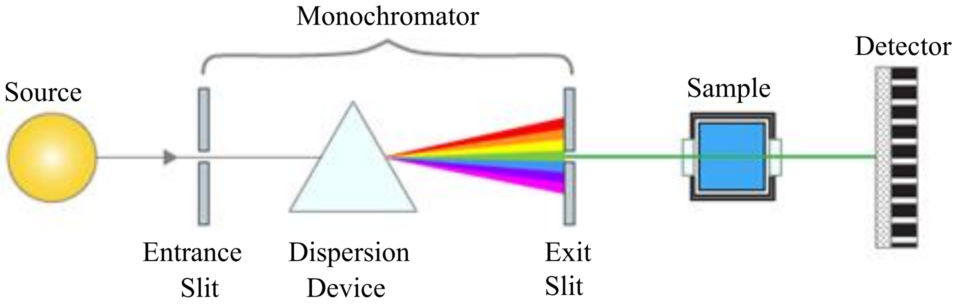


Figure 12 Schematic Diagram of Ultraviolet Detector[125]

1. Fixed Wavelength Detector

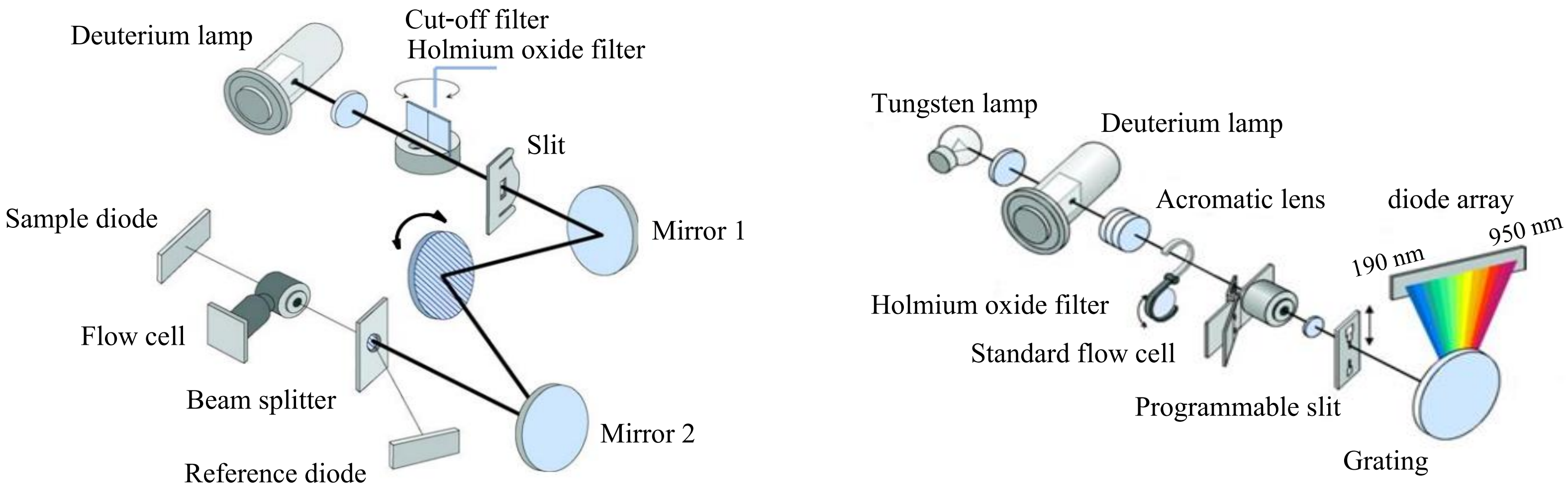
Fixed wavelength detectors operate at single wavelength ether at 254 or 280 nm in the UV region. Most popular lamp is low pressure mercury lamp which generates light at wavelength of 254 nm.[122]

2. Variable Wavelength Detector

These detectors can be adjusted to operate at the absorbance maximum of an analyte or at a wavelength that provides more selectivity. They can also be programmed to change wavelengths during a chromatographic run to compensate for response of different analytes. In a variable wavelength detector, light from a broad spectrum (for UV deuterium is common, tungsten for visible) lamp is directed through a slit to a diffraction grating that spreads the light out into its constituent wavelengths. The grating is then rotated to direct a single wavelength of light through a slit, through the detector cell, to a photodiode. An example schematic for a variable wavelength detector is shown in Figure 13a.[123]

3. Diode Array Detector (DAD)

DAD is an advanced UV-VIS detector commonly used to simultaneously and continuously acquire UV-VIS data over a wide range of wavelengths typically 190-800 nm. This ability enables the measurement of compounds that display absorbance patterns in both the UV and VIS spectrum.[124] There are two advantages of diode array detection: in the first, it allows for the best wavelength(s) to be selected for actual analysis. This is particular important when no information is available on molar absorptivities at different wavelengths. The second major advantage is related to the problem of peak purity. Often, the peak shape in itself does not reveal that it actually corresponds to two (or even more) components. In such a case, absorbance rationing at several wavelengths is particularly helpful in detecting whether the peak represents a single compound or, is in fact, a composite peak.[126] Schematic diagram of diode array detector is described in Figure 13b.



(a) (b)

Figure 13 Schematic Diagrams of Variable Wavelength (a) and Diode Array (b) Detectors[127]

Fluorescence Detection (FLD)

FLD is probably the most sensitive among the existing modern HPLC detectors. It measure the optical emission of light by solute molecules after they have been excited at a higher energy wavelength and can be very sensitive for compounds that have native fluorescence or that can be made to fluorescence through derivatization.[123] Typically, fluorescence sensitivity is 10-1000 times higher than that of the UV detector for strong UV absorbing materials. This ability to reliably achieve low detection limits (e.g. picogram range)[128-129] and is superior to UV or VIS detectors with respect to sensitivity, specificity, and selectivity. This is normally used as an advantage in the measurement of specific fluorescent species in samples.[124] Fluorescence intensity depends on both the excitation and emission wavelength, allowing selectively detect some components while suppressing the emission of others.

Schematically, they described in Figure 14, the grating is replaced by a filter or monochromator at a right angle to the incident light to simplify the optics and reduce background noise. The light source is usually a broad spectrum deuterium or xenon flash lamp. The excitation wavelength (often close to the UV max) is selected by a filter or monochromator between the lamp and the flow cell, always at a higher energy (lower wavelength) than the emission wavelength.[123]

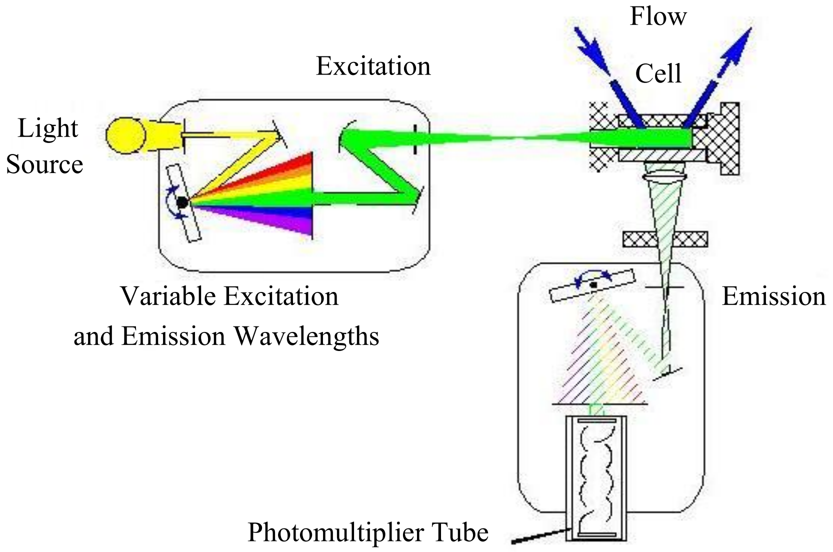


Figure 14 Diagrammatic Illustration of a FLD Detector Optical System[130]

Applications of HPLC for Determination of PAHs in Samples

HPLC has contributed to analytical solutions in diverse fields such as pharmaceuticals, foods, life sciences, environment, forensics, etc. Table 10 showed summarize a review of pre-separation and detection of HPLC to be used for determination of PAHs in samples.[131-132]

Table 10 Application of Pre-Separation and Detection of HPLC to be used for Determination of PAHs in Samples

Sample	Year	Analyte	Sample pre-separation	Detection	Ref.
Smoked meat, smoked fish spreads	1984	PAHs	Saponification with mixture of methanol, water and KOH, extraction with cyclohexane, washing with Na_2WO_4 solution, clean-up on florisil.	UVD 254 nm FLD Ex/Em 250/370 nm	[133]
Smoked meats	1987	PAHs	Saponification with ethanolic KOH, extraction into cyclohexane, washing with saturate NaCl solution, clean-up on silica gel.	FLD Ex: 305, 381 nm Em: 389, 430, 520 nm	[134]
Smoked frankfurters, smoked meats	1996	16 PAHs	Extraction with methanol in Soxhlet app.,+KOH, extraction into *n*-hexane, and clean-up on Pep-Pak florisil.	UVD 230-360 nm FLD Variable Ex (232-302) nm Em (330-484) nm	[135]
Airborne particulates	2000	15 PAHs	Ultrasonic extraction with dichloromethane/hexane, rotary evaporation	FLD Variable Ex (246-300) nm Em (330-500) nm	[136]
	2003	12 PAHs	Ultrasonic extraction with dichloromethane/acetonitrile, evaporate concentration	FLD	[137]

Ex means that excitation wavelength and Em means that emission wavelength

Table 10 (continued)

Sample	Year	Analyte	Sample pre-separation	Detection	Ref.
Edible oils	2003	16 PAHs	Extraction of SPE with reversed-phase.	FLD Variable Ex (250-290) nm Em (330-500) nm	[138]
Airborne particulates	2004	18 PAHs	Ultrasonic extraction with dichloromethane, evaporation under N_2.	FLD	[139]
Lichen, *Parmotrema hypoleucinum (Steiner) Hale*	2009	16 EPA-PAHs	Soxhlet with acetonitrile, rotary evaporation and clean-up on florisil.	FLD-DAD/UVD	[25]
Moss	2010	13 PAHs	Soxtec extraction and clean-up on florisil SPE.	FLD Variable Ex (233-300) nm Em (320-500) nm	[7]
Lichen, *Pyxine subcinerea*	2012	16 PAHs	Soxhlet with dichloromethane, according to the EPA 8310 and clean-up by silica gel column, according to the EPA method 3630.	UVD 254 nm	[140]
Soil	2013	16 PAHs	Soxhlet with n-hexane/acetone, rotary evaporation and clean-up by silica gel column.	DAD 254 nm FLD Variable Ex (252-290) nm Em (323-500) nm	[28]

Ex means that excitation wavelength and Em means that emission wavelength

Aims and Scope of This Work

The aims of this research work were to find the appropriate procedure to analyze eight PAHs, include Naphthalene*,* Acenaphthene, Phenanthrene, Anthracene, Pyrene, Fluoranthene, Chrysene and Benzo[a]pyrene accumulated in the lichen *Parmotrema tinctorum* by HPLC. The analysis consisted of summarized as follows:

1. To study the optimum conditions of HPLC techniques for simultaneous separation of PAHs using diode array and fluorescence detectors.

1.1 To optimize the conditions of HPLC techniques used the two columns, were LiChrospher PAH and Acclaim C18, and solvent systems in isocratic and gradient system.

1.2 To validate the methods by finding the optimum wavelength, limit of detection (LOD), limit of quantification (LOQ), linearity, sensitivity, precision and accuracy. Finally, the suitable column was choosen to analyse the samples.

2. To study the suitable sample preparation method for lichen *Parmotrema tinctorum* sample by using ultrasonic-assisted extraction and solid phase extraction before analysis by HPLC.

2.1 To optimize of ultrasonic-assisted extraction by variable parameter as follows: sample amount, frequency, power, and time.

2.2 To optimize the conditions of florisil absorbent by varying solvent types and volumes for conditioning and elution.

2.3 To compare the efficiency and suitability of the solid phase extraction procedure between two sorbent types, florisil and silica.

2.4 To validate the sample preparation methods; ultrasound-assisted extraction and solid phase extraction technique, for analysis PAHs accumulation in the lichen by finding the method detection limit, the percentage of spiked recoveries, precision, and accuracy of these methods.

3. To apply the appropriate techniques from 1 and 2 for analyzing PAHs accumulation in lichen from three localities including

3.1 The control site, at Nong Keing in Khao Yai National Park (KNP), at about 1,000 m. away from the park's road.

3.2 Tourist sites at Khao Yai National Park (TKNP): by collecting lichen from three areas included the golf course, Nong Keing and Saisorn reservoir.

3.3 Polluted sites in Bangkok at different traffic congestion (PBKT): performed by transplanting lichens from KNP to Lam Sali intersection, Rama 9 road intersection and the garden beside the Science Office Building (SCO) at Ramkhamhaeng University.

CHAPTER 2

EXPERIMENTAL

Instruments

1. HPLC system, Thermo Scientific Dionex Ultimate 3000 series (UHPLC$^+$ focused) consists of LPG-3400SD pumps, a WPS-3000SL auto samplers, a TCC-3000SD column compartments, a DAD-3000 (RS) diode array detector and a FLD-3100 fluorescence detector (Thermo, Waltham, USA).

2. An Accliam C18 column (4.6×150 mm i.d., 3 µm particle size) from Thermo, Waltham, USA and a LiChrospher PAH (4.6×250 mm i.d., 5 µm particle size) from Merck, Darmstadt, Germany were used for separation of PAHs.

3. Branstead Easy Pure RF Compact Ultrapure water system (USA).

4. Fisherbrand® 112101 series ultrasonic bath (Elma-Hans, Germany).

5. Elma ultrasonic bath model Elmasonic P-30-H (Singen, Germany).

6. Chromabond® vacuum manifold (M-N, Germany).

7. Glass vacuum filtering funnel filtration apparatus (Chrom Tech, USA).

8. GAST vacuum pump (USA).

9. Centrifuge (Heracus Christ, Germany).

10. Mettler Toledo analytical balance model MS (Switzerland).

11. Oven (Shandon Southern).

12. Laboratory Refrigerator.

Materials

1. Mortars and pestles.
2. Sieve 500 μm (Retsch, Haan, Germany).
3. 6 mL bond elut empty SPE cartridges (Agilent, USA).
4. 10 mL glass tubes with a Polytetrafluoroethylene (PTFE) screw cap.
5. 2 mL clear glass vials with screw top.
6. 2 mL Amber glass vials with screw top.
7. 0.45 μm Nylon Membrane Filters.
8. 0.45 μm Nylon Syringe Membrane Filters.

Chemicals

1. Deionized Water (DI water)

DI water with specific resistance > 18.0 MΩ/cm was prepared by an Easy Pure RF Compact ultrapure water system. DI water was used for the preparation of all solutions.

2. Liquid Nitrogen

Liquid nitrogen was used for the grinding of lichen samples.

3. Nitrogen gas

99.5% Purity of nitrogen gas was used for evaporating extracted lichens.

4. Reagents

All reagents used in this study were of analytical grade except methanol and acetonitrile which were HPLC grade.

4.1 Acetonitrile from RCI Labscan (Gillman, Australia).

4.2 Dichloromethane from Carlo Erba (Val de Reuil, France).

4.3 Hexane from Merck (Darmstadt, Germany).

4.4 Methanol from Merck (Darmstadt, Germany).

4.5 Sodium sulfate anhydrous from Fluka (Buchs, Switzerland).

4.6 Oxalic acid from Ajax Finechem (Auckland, Newzeland).

4.7 Naphthalene (Naph) from BDH (Poole, England).

4.8 Acenaphthene (Ace) from Merck (Darmstadt, Germany).

4.9 Chrysene (Chr) fromSupelco (Bellefonte, USA).

4.10 Anthracene (Anth) from Sigma-Aldrich (St. Louis, USA).

4.11 Phenanthrene (Phe) from Sigma-Aldrich (St. Louis, USA).

4.12 Pyrene (Pyr) from Sigma-Aldrich (St. Louis, USA).

4.13 Fluoranthene (Fluo) from Sigma-Aldrich (St. Louis, USA).

4.14 Benzo[a]pyrene (BaPy) from Sigma-Aldrich (St. Louis, USA).

5. Adsorbents

5.1 Florisil Adsorbent particle size 30-60 mesh from Fluka (cat.no. 46384-500G-F, St. Louis, USA).

5.2 Silica gel 60 with particle sizes 0.063-0.200 mm from Merck (cat. no. 7631-86-9, Darmstadt, Germany).

Preparation of Standard Solutions and Reagents

1. Stock Standard Solutions

1.1 Stock Standard Solution 1000 µg/mL of Naphthalene, Acenaphthene, Phenanthrene, Anthracene, Pyrene and Fluoranthene

Each stock standard solution was prepared by dissolving 50 mg Naphthalene, Acenaphthene, Phenanthrene, Anthracene, Pyrene, Fluoranthene in acetonitrile and adjusted to 50 mL with acetonitrile in a volumetric flask. These standards solutions were stored in the refrigerator at 4 °C and used throughout this study.

1.2 Stock Standard Solution 100 µg/mL of Chrysene and Benzo[a]pyrene

Each stock standard solution was prepared by dissolving 10 mg Chrysene and Benzo[a]pyrene in acetonitrile and adjusted to 100 mL with acetonitrile in a volumetric flask. This standard solution was stored in the refrigerator at 4 °C and used throughout this study.

1.3 Stock Mixed Standard Solutions 160 mg/L of Naphthalene, Anthracene and 80 mg/L of Acenaphthene, Phenanthrene and 32 mg/L of Fluoranthene, Pyrene and 16 mg/L of Chrysene, Benzo[a]pyrene

This solution was prepared by taking 8 mL of 1000 mg/L Naphthalene, Anthracene and 4 mL of 1000 mg/L Acenaphthene, Phenanthrene and 1.6 mL of 1000 mg/L Pyrene, Fluoranthene and 8 mL of 100 mg/L Chrysene, Benzo[a]pyrene into a 50 mL volumetric flask and then diluting with acetonitrile and mixed thoroughly.

1.4 Stock Mixed Standard Solutions 8 mg/L of Naphthalene, Anthracene and 4 mg/L of Acenaphthene, Phenanthrene and 1.6 mg/L of Fluoranthene, Pyrene and 0.8 mg/L of Chrysene, Benzo[a]pyrene

This mixed solution was prepared by taking 2.5 mL of 160 mg/L of Naphthalene, Anthracene and 80 mg/L of Acenaphthene, Phenanthrene and 32 mg/L of Fluoranthene, Pyrene and 16 mg/L of Chrysene, Benzo[a]pyrene from 1.3 into a 50 mL volumetric flask then diluting with acetonitrile and mixed thoroughly.

1.5 Stock Mixed Standard Solutions 1.6 mg/L of Naphthalene, Anthracene and 0.8 mg/L of Acenaphthene, Phenanthrene and 0.32 mg/L of Fluoranthene, Pyrene and 0.16 mg/L of Chrysene, Benzo[a]pyrene for analysis of PAHs detection wavelength of diode array detector (DAD)

This mixed solution was prepared by taking 0.1 mL of 160 mg/L of Naphthalene, Anthracene and 80 mg/L of Acenaphthene, Phenanthrene and 32 mg/L of Fluoranthene, Pyrene and 16 mg/L of Chrysene, Benzo[a]pyrene from 1.3 into a 10 mL volumetric flask then diluting with acetonitrile and mixed thoroughly.

2. Mixed Standard Solutions for Calibration Curves

2.1 Stock Mixed Standard Solutions 800 μg/L of Naphthalene, Anthracene and 400 μg/L of Acenaphthene, Phenanthrene and 160 μg/L of Fluoranthene, Pyrene and 80 μg/L of Chrysene, Benzo[a]pyrene

This mixed solution was prepared by taking 0.25 mL of 160 mg/L of Naphthalene, Anthracene and 80 mg/L of Acenaphthene, Phenanthrene and 32 mg/L of Fluoranthene, Pyrene and 16 mg/L of Benzo[a]pyrene, Chrysene from 1.3 into a 50 mL volumetric flask then diluting with acetonitrile and mixed thoroughly. This solution was prepared freshly before the construction of standard calibration curve.

2.2 Mixed Standard Solutions for Calibration Curves

Each level of calibration mixed standard solution was prepared by taking the appropriate dilution of stock mixed standard solution from 2.1 into a 10 mL volumetric flask diluted with acetonitrile and mixed thoroughly. The volume of stock solution and concentrations of these PAHs are shown in Table 11.

3. Standard Solutions for Quality Check

This mixed standard quality check was prepared daily by taking 0.5 mL of stock mixed standard solutions from 2.1 into a 10 mL volumetric flask and diluted with acetonitrile. The concentration of PAHs was the same as level 3 that shown in Table 11.

4. Mixed Standard Solutions for Recovery Study on Lichen Samples

4.1 Stock Mixed Standard Solutions of 40 µg/L of Naphthalene, Anthracene and 20 µg/L of Acenaphthene, Phenanthrene and 8 µg/L of Fluoranthene, Pyrene and 4 µg/L of Chrysene, Benzo[a]pyrene

This solution was prepared by taking 50 µL of stock mixed standard solutions from 1.4 into a 10 mL volumetric flask and diluted with acetonitrile and mixed thoroughly.

4.2 Stock Mixed Standard Solutions 160 µg/L of Naphthalene, Anthracene and 80 µg/L of Acenaphthene, Phenanthrene and 32 µg/L of Fluoranthene, Pyrene and 16 µg/L of Chrysene, Benzo[a]pyrene

This solution was prepared by taking 200 µL of stock mixed standard solutions from 1.4 into a 10 mL volumetric flask and diluted with acetonitrile and mixed thoroughly.

4.3 Stock Mixed Standard Solutions 640 µg/L of Naphthalene, Anthracene and 320 µg/L of Acenaphthene, Phenanthrene and 128 µg/L of Fluoranthene, Pyrene and 64 µg/L of Chrysene, Benzo[a]pyrene

This solution was prepared by taking 800 µL of stock mixed standard solutions from 1.4 into a 10 mL volumetric flask and diluted with acetonitrile and mixed thoroughly.

Spike 250 µL of mixed standard solution PAHs from 4.1, 4.2 and 4.3, respectively into the ground control lichen (accurate weight). This sample was later used for validation of SPE sample preparation methods. The concentration of PAHs was the same as level 1, 3 and 5, in Table 11.

5. Mobile Phase

5.1 Mobile phase B: DI water with specific resistance > 18.0 MΩ/cm was filtered through a 0.45 µm membrane filter and degassed about 15 minutes in an ultrasonic bath before use.

5.2 Mobile phase C: Acetonitrile (HPLC grade) was filtered through a 0.45 µm membrane filter and degassed about 15 minutes in an ultrasonic bath before use.

Table 11 The Concentration Levels of Mixed Standard Solutions of Polycyclic Aromatic Hydrocarbons for Calibration Curves

Concentration Level	Volume of Stock Mixed Solution (mL)/10 mL				Concentration of Mixed Standard (μg/L)			
	Naph and Anth 800 μg/L	Ace and Phe 400 μg/L	Fluo and Pyr 160 μg/L	Chr and BaPy 80 μg/L	Naph and Anth	Ace and Phe	Fluo and Pyr	Chr and BaPy
1	0.125				10	5	2	1
2	0.25				20	10	4	2
3	0.5				40	20	8	4
4	1.0				80	40	16	8
5	2.0				160	80	32	16

Procedures

1. Optimization of Chromatographic Conditions for Analysis of PAHs on UHPLC$^+$ Focused

The optimum conditions were evaluated to obtain the highest separation, efficiency, sensitivity and short retention time for analysis of Naphthalene, Acenaphthene, Phenanthrene, Anthracene, Pyrene, Fluoranthene, Chrysene and Benzo[a]pyrene by choosing optimal columns and chromatographic conditions, such as mobile phase composition and detector wavelength.

The following two columns as shown in Figure 15 were used to compare for chromatographic separation of eight PAHs.

- LiChrospher PAH 4.6×250 mm i.d. separation column packed with 5 µm spherica RP-18 silica gel particle size from Merck.
- Acclaim C18 4.6×150 mm i.d. separation column packed with 3 µm particle size from Thermo.

Figure 15 Two Column Types for Chromatographic Separation of Polycyclic Aromatic Hydrocarbons were LiChrospher PAH (a) and Acclaim C18 (b)

The chromatographic conditions for each column were the optimization on the following:

1.1 Optimization of Solvent System

To verify the optimum solvent program of mobile phase between DI water as solvent B and acetonitrile as solvent C for separation of PAHs as following:

1.1.1 Optimization of Isocratic System

The investigation was performed by using mixed standard solution of eight PAHs from 2.1, the concentration was the same as level 3 shown in Table 11. The ratio of solvent B and solvent C in isocratic elution were 25 : 75 and 20 : 80. The mobile phase flow rate was studied as the following: 0.6, 1.0 and 1.5 mL/min. The injection volume was 15 µL, the temperature of the column was maintained at 25 °C, and the fluorescence detector in medium sensitivity mode, excitation (Ex) was performed at 270 nm and emission (Em) was selected filter wavelength as automatic. The PAHs spectra were recorded by DAD in the range 190-400 nm.

1.1.2 Optimization of Gradient System

The investigation was performed by using mixed standard solution of eight PAHs from 2.1, the concentration was the same as level 3 that shown in Table 11. The optimum systems were investigated by varying the composition of solvent B : solvent C and the time of gradient program that shown in Table 12. The injection volume was 15 µL, temperature of the column was maintained at 25 °C, and the fluorescence detector in medium sensitivity mode, excitation (Ex) was performed at 270 nm and emission (Em) was selected filter wavelength as automatic. The PAHs spectra were recorded by DAD in the range 190-400 nm.

Table 12 Gradient Elution System of Solvent and Time for Separation of Polycyclic Aromatic Hydrocarbons

	Time (min)	% B (DI Water)	% C (Acetonitrile)
Gradient system 1 (GR1)	0	40	60
	3	40	60
	15	0	100
	30	0	100
	Flow rate 1.0 mL/min		
Gradient system 2 (GR2)[103]	0	17	83
	5.5	17	83
	9	0	100
	30	0	100
	Flow rate 1.0 mL/min		
Gradient system 3 (GR3)	0	50	50
	3	50	50
	10	0	100
	30	0	100
	Flow rate 1.0 mL/min		

Table 12 (continued)

	Time (min)	% B (MeOH:DI Water = 75:25)	% C (Acetonitrile)
Gradient system 4 (GR4)	0	90	10
	2.5	90	10
	6	0	100
	30	0	100
	Flow rate 1.0 mL/min		

In experiments, PAHs were separated on HPLC using the elution process was gradient system (Method GR1), injection volume was 15 µL, and temperature of the column was maintained at 25 °C, were the optimization wavelength on the following:

1.2 Detection Wavelength for Analysis of PAHs

1.2.1 Optimization Wavelength of Diode Array Detector (DAD)

By using DAD, specific UV spectra was obtained and recorded in the range 190-400 nm for peak identification and purity checks. In this experiment, was performed by using mixed standard solution of eight PAHs from 1.5, run an analysis with DAD, recording a 3D field. These by scan extract the retention times and absorption maximum and thus the optimum excitation wavelengths, of each PAH compounds.

1.2.2 Optimization Wavelength of Fluorescence Detector (FLD)

By using FLD, characteristics of low detection limits and higher sensitivity were obtained for the determination of PAHs exhibiting fluorescent effects. The most important parameters that need to be optimized are the excitation and emission wavelength. In this experiment, was performed by using mixed standard solution of eight PAHs from 1.5, and optimized the wavelength for an analysis as following:

First, selected an excitation wavelength on the absorption maximum of the PAH compounds were investigated by scanning the excitation wavelength in the range 190-400 nm and set the filter wheel setting to a wavelength that is at least 30 nm above the excitation wavelength. Run the PAH compounds in zero order mode to set the emission wavelength property to zero order, will get a peak of most sample compound, provided that the emission wavelength is not smaller than the selected filter wavelength.

Second, perform an emission scan for each PAH compounds by set the excitation wavelength form first step. There were selected an emission wavelength that is at least 20 nm above the excitation wavelength. The optimization was investigated by scanning the emission wavelength for each PAH compounds using the single-channel mode at their retention times.

After optimization of excitation and emission wavelength of each PAHs, the obtained data are combined to setup the time-table for best limit of detection and selectivity, to record data by switching the wavelength in-between the detected peaks of the various PAH compounds. The optimized switching events for the analysis of PAHs are summarized in Table 13.

In practical, running the HPLC chromatogram for the analyte should be in multi-channel mode which switching for suitable wavelengths of each PAHs followed time table in Table 13. Unfortunately, it showed the jumped base line. The optimum chromatogram occurred when selected filter wavelength as automatic or setting on zero order mode.

Table 13 Timetable of Fluorescence Detection Program for the Analysis of Studied Polycyclic Aromatic Hydrocarbons

Time (min)	Excitation WL* (nm)	Emission WL* (nm)	PAH detected
0.0	280	330	Naph
11.0	245	370	Ace
12.0	250	406	Phe
12.3	280	450	Anth
13.5	270	390	Fluo
14.25	270	390	Pyr
15.0	265	380	Chr
16.0	290	430	BaPy

*WL means that wavelength

2. Validation of Chromatographic Methods

The optimum chromatographic conditions of PAHs were validated in the following parameters: limit of detection, limit of quantification, linearity and sensitivity, accuracy and precision.

2.1 Limit of Detection (LOD)

Limit of detection (LOD) or detection limit (DL) was determined according to the ICH (Q2B) guidelines[141] by using equation:

$$\text{LOD} = \frac{3.3\sigma}{S} \qquad \ldots\ldots\ldots\ldots\ldots\ldots(2.1)$$

where

σ = the standard deviation of the noise made from several bank injections

S = the slope of the calibration curve

2.2 Limit of Quantification (LOQ)

LOQ was the lowest concentration of analyte that can be determined with an acceptable level of repeatability precision and trueness. The LOQ was determined by using tenfold value the standard deviation of the noise. The approximate LOQ value of analytes were used as the first point of calibration curves.

2.3 Linearity

In this method, the five points of calibration curves were prepared with different concentration levels of each PAH compounds as shown in Table 11. In order to find the linearity, the concentrations of each PAHs were plotted with the peak area versus the concentration of the standard solutions and calculated for their correlation coefficient (r^2).

2.4 Accuracy and Precision

2.4.1 Precision

Precision of the chromatographic method was determined in terms of repeatability by using standard solution of each PAHs containing different concentrations in three level which were analyzed in seven replicates. The concentration of PAHs was the same as level 1, 3 and 5 as shown in Table 11. The precision was expressed as relative standard deviation (% RSD) calculated by the following equation:

$$\% \text{ RSD} = \frac{\text{SD}}{\overline{\text{X}}} \times 100 \qquad \text{................(2.2)}$$

where SD = Standard deviation of seven replicate analyzed

$\overline{\text{X}}$ = Average value

2.4.2 Accuracy

Accuracy of the chromatographic method was determined in terms of observed value to compare with true value of standard solution which were freshly prepared. The concentration of PAHs was the same as level 1, 3 and 5 that shown in Table 11. Accuracy was determined in term of the relative accuracy value. The relative accuracy value was calculated by the following equation:

$$\% \text{ Relative accuracy } = \frac{O}{T} \times 100 \qquad \ldots\ldots\ldots\ldots\ldots(2.3)$$

where O = Observed value or concentration found by HPLC

T = True value or concentration of standard solutions

3. Quality Control Check Standards for HPLC Analysis

The instrument was calibrated when the quality check standard no longer meets the requirements of quality control chart. Quality control check standard was performed at the beginning and end of every analysis run. The accepted result was within ± 10% of the prepared concentration for each PAHs being analyzed. If not, the problem was identified and corrected before proceeding with the analysis. The concentration of quality control check standard of PAHs was the same as level 3 that shown in Table 11.

4. Optimization of Sample Preparation

The lichen samples were extracted PAH compounds by ultrasonic extraction using hexane solvent followed the UAE technique by Domeño and Blasco[17], before clean-up by solid phase extraction. The optimized conditions of sample preparation were applied with lichen sample as follows:

4.1 Optimization of Ultrasound-Assisted Extraction Condition

Grounded lichen samples were brought from refrigerator to keep in desiccators until it has a constant weight. An exactly 0.2 g of lichen sample was spiked with 250 µL of mixed standard solutions PAHs from 4.5, the concentration was the same as level 5 shown in Table 11 for recovery.
The spiked sample was placed with 0.7 mL hexane in a glass vial with a PTFE lined screw cap. Extraction by ultrasonic bath was optimized by variable parameter as shown in Table 14. After extraction it was centrifuged for 10 min and following by decantation. It was continued extraction two times with 0.7 mL hexane, by centrifuged and decanted respectively. Three extracted samples were piled together and loaded into SPE florisil cartridge for clean-up. After elution of PAHs compounds from cartridge, the extracts were evaporated under nitrogen gas until dry and redissolved by 2 mL of acetonitrile. The extract solutions were filtered through 0.45 µm syringe membrane prior analyzed by HPLC coupled with DAD and FLD.

In this work, ultrasound-assisted extraction equipment used for sonication was shown in Figure 16.

Table 14 Experimental Conditions for the Ultrasound-Assisted Extraction

Variable parameter	Studied interval
Sonication frequency (kHz)	37, and 80 kHz
Sonication time (min)	5-10 min
Sonication temperature (°C)	0-30 °C
Sonication power (%)	30, 60, and 100%
Sample amount (g)	0.05-0.25 g

Figure 16 The Fisherbrand® 112101 Series Ultrasonic Bath (Elma-Hans)

4.2 Solid Phase Extraction Sorbent

In this study, to compare the efficiency and suitability of the SPE between two sorbent types, florisil and silica. Sorbent florisil, were studied by varying various parameter whereas sorbent silica gels were succeed studying by Sriviboon[27]. SPE cartridges were prepared by a 5 mL glass syringe as the SPE mini-column. The optimized conditions were applied with lichen sample as follows:

4.2.1 Optimum of SPE Procedure for using Florisil

The optimum procedure of SPE florisil was validated in the following steps: Condition and elution the compounds of interest.

4.2.1.1 Optimization for the Condition Step of Sorbent

SPE cartridges were prepared by packing 1.2 g of florisil, which was dried at 150 ºC for 30 min, into mini columns then added approximately 0.05 g sodium sulfate anhydrous to the top of florisil. In order to clean-up and condition the solid phase cartridges before loading, they were placed on a Chromabond® vacuum manifold (Figure 17). The conditions were optimized by various volume and solvent types as shown in Table 15. The final solvents were collected after condition, the solvent was filtered through 0.45 µm syringe membrane prior analyzed by HPLC coupled with DAD and FLD. In order to test, the cartridge was cleaned and cleared from interference compounds.

Table 15 Conditions for the Clean-Up the Solid Phase Cartridges

Condition	Types of sovent	Volume (mL)
1	Hexane	10
	Methanol	10
2	Hexane	10
	Hexane : Dichloromethane (65 : 35)	10
	Methanol	10
3	Hexane	20
	Hexane : Dichloromethane (65 : 35)	20
	Methanol	30
4	Hexane	20
	Hexane : Dichloromethane (65 : 35)	20
	Acetonitrile	30
5	Acetonitrile	30
	Dichloromethane	6
	Hexane	3

4.2.1.2 Investigation of Solvent Volume for Elution Step

After getting an appropriate conditioning step from 4.2.1.1. The volume of elution solvent was investigated by spiking 250 µL of mixed standard solutions PAHs into the lichen extract solution, then they were loaded into SPE cartridge and the target compounds were eluted by the following solvent: acetonitrile[25] and the mixture of hexane and dichloromethane (65 : 35)[20]. Eluted extract was collected in a glass vial by 0.5 mL fraction. After elution of PAHs compounds, the extracts were evaporated under nitrogen gas until dried and redissolved by 0.5 mL of acetonitrile. Each fraction was filtered through 0.45 µm syringe membrane prior analyzed by HPLC coupled with DAD and FLD to find the elution profile and the optimum collected volume.

4.2.2 Procedure of SPE Condition for using Silica Gel

A 5 mL glass syringe was used as the SPE mini-column, 0.0125 g of oxalic acid was dissolved in 2.5 mL of methanol and impregnated on 1.2 g of silica gel, was dried at 100 °C for 30 min. Impregnated silica was packed in glass syringe and added sodium sulfate anhydrous about 0.05 g on the top of mini column. They were cleaned and conditioned by passing 6 mL of dichloromethane and following by 3 mL of hexane through the cartridge. Once the SPE cartridges were condition, the mixed standard solutions of PAHs were load on the top, and the flow rate was adjusted to 1-2 drop/second. The PAHs compounds were eluted from SPE cartridge by using the mixture of hexane and dichloromethane (65 : 35). The elution was performed by discard the solvent 3.5 mL and continue to collect 2 mL.[27] After elution of PAHs compounds, the extracts were evaporated under nitrogen gas until dried and redissolved by 2 mL of acetonitrile. The extract solutions were filtered through 0.45 µm syringe membrane prior injection to HPLC coupled with DAD and FLD.

Figure 17 The Chromabond® Vacuum Manifold

5. Method Validation of Sample Preparation

5.1 Method Detection Limit (MDL) of Sample Preparation

MDL is the lowest amounts of PAHs prepared by SPE can be analyzed by HPLC with the accepted yield recoveries. The mixed standard solutions of PAHs were also validated by varying concentrations to the lowest concentration that the method can analyze. Two sorbent types, florisil and silica gel, were determined in seven replicates by using the lowest concentration. The MDL of all PAHs were calculated by equation 2.1.

5.2 Precision and Spike Recovery of Sample Preparation Method

5.2.1 Precision of Sample Preparation Method

To use lichen *Parmotrema tinctorum* from the control station to clean, crush and ground into powder with ceramic mortar and pestle by liquid nitrogen. The fine kept in a desiccator until it has a constant weight. The precision was determined by exactly 0.1 g of grounded lichen samples, were accurately weight placed in a PTFE tubes and spiked with 250 μL of mixed standard solution PAHs in three levels. Allow the substances were absorbed into the lichen until dry, added 0.7 mL hexane into a glass vial closed with a PTFE lined screw cap and extracted for 10 min at 30 ºC, using 100% power output and mean operating frequency 37 kHz in an ultrasonic bath. After extraction it was centrifuged for 10 min, followed by decantation. Three extracted samples were piled together and loaded into SPE florisil cartridge for clean-up. The SPE technique was determined for seven times of each level and repeated with non-spiked lichen. After analyzed by HPLC, the precisions were studied in term of relative standard deviation (% RSD) value.

5.2.2 Spike Recovery of Sample Preparation Method

Spiked recovery of sample preparation methods for PAHs were studied in order to calculate the recovery percentage, the procedure was the same as 5.2.1. Recovery percentage value for each spiked sample was calculated by the subtraction of PAHs concentration obtained from the blank sample from PAHs concentration obtained from the spiked sample devided by the amounts of PAHs added into lichen sample before extraction.

$$\text{Spike Recovery (\%)} = \frac{[S - B]}{T} \times 100 \quad \text{................(2.4)}$$

where T = the amount of PAHs added into lichen sample before extraction

S = the amount of PAHs detected from the spiked sample

B = the amount of PAHs extracted from the non-spiked lichen sample

6. Analysis of Lichen Samples

6.1 Sampling and Study Area

The epiphytic foliose lichens, *Parmotrema tinctorum*, were collected from bark of trees at Khao Yai National Park (KNP) in Nakhon Ratchasima province (Figure 18). They were collected into three localities including

6.1.1 The control site, at Nong Keing in KNP, at about 1,000 m. away from the park's road. The lichen collecting sites as shown in Table 16.

6.1.2 Tourist sites at Khao Yai National Park (TKNP)

By collecting lichen from three areas, Golf course (G), Nong Keing (N) and Saisorn reservoir (S), in January 2017. The lichens were collected four different distances from the road, they were 0-20, 100-200, 300-400, > 500 m, and each site was collected 5 samples. Total lichen from collecting sites were 60 samples as shown in Table 16. All samples were collected in brown paper bags brought to laboratory to prepare for PAHs analysis by the suitable sample preparation methods as mention earlier.

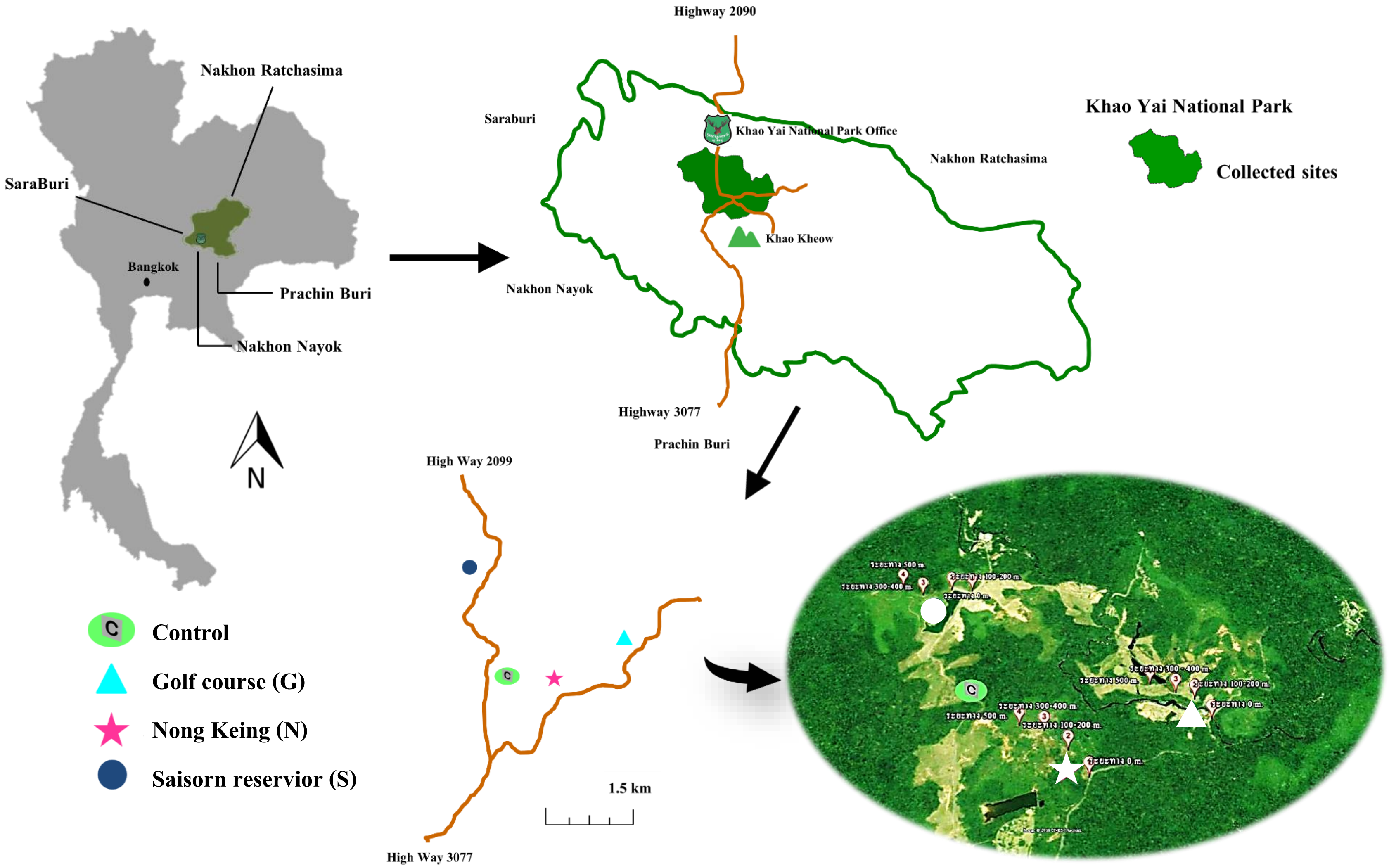


Figure 18 Map of Lichen Collecting Sites at Khao Yai National Park

Table 16 Lichen Samples Collected from Khao Yai National Park

Location & Distance above sea level (m)	Distance (m) from main road	Sample Code	Latitude-Longitude	Location view
Control site 736 m	1,000	Control (C)	N 14°25′ 17.502″ E 101° 22′ 24.336″	Figure 19a
Golf course (G) 709-716 m	0-20	G1-1	N 14°25′ 22.482″ E 101° 23′ 18.420″	Figure 19b
		G1-2	N 14°25′ 22.518″ E 101° 23′ 18.258″	
		G1-3	N 14°25′ 21.204″ E 101° 23′ 18.984″	
		G1-4	N 14°25′ 20.448″ E 101° 23′ 18.834″	
		G1-5	N 14°25′ 21.204″ E 101° 23′ 19.008″	
	100-200	G2-1	N 14°25′ 28.200″ E 101° 23′ 16.434″	
		G2-2	N 14°25′ 28.896″ E 101° 23′ 16.074″	
		G2-3	N 14°25′ 28.896″ E 101° 23′ 16.074″	
		G2-4	N 14°25′ 28.350″ E 101° 23′ 14.832″	
		G2-5	N 14°25′ 28.302″ E 101° 23′ 14.766″	

Table 16 (continued)

Location & Distance above sea level (m)	Distance (m) from main road	Sample Code	Latitude-Longitude	Location view
Golf course (G) 709-716 m	300-400	G3-1	N 14°25′ 29.622″ E 101° 23′ 11.904″	Figure 19b
		G3-2	N 14°25′ 29.646″ E 101° 23′ 11.904″	
		G3-3	N 14°25′ 31.230″ E 101° 23′ 12.576″	
		G3-4	N 14°25′ 30.552″ E 101° 23′ 11.064″	
		G3-5	N 14°25′ 30.552″ E 101° 23′ 11.064″	
	>500	G4-1	N 14°25′ 32.588″ E 101° 23′ 05.172″	
		G4-2	N 14°25′ 32.588″ E 101° 23′ 05.172″	
		G4-3	N 14°25′ 32.588″ E 101° 23′ 05.172″	
		G4-4	N 14°25′ 32.952″ E 101° 23′ 05.232″	
		G4-5	N 14°25′ 32.640″ E 101° 23′ 04.878″	

Table 16 (continued)

Location & Distance above sea level (m)	Distance (m) from main road	Sample Code	Latitude-Longitude	Location view
Nong Keing (N) 719-727 m	0-20	N1-1	N 14°25′ 08.046″ E 101° 22′ 48.468″	Figure 19c
		N1-2	N 14°25′ 07.194″ E 101° 22′ 48.048″	
		N1-3	N 14°25′ 07.176″ E 101° 22′ 47.646″	
		N1-4	N 14°25′ 07.302″ E 101° 22′ 48.588″	
		N1-5	N 14°25′ 07.302″ E 101° 22′ 48.588″	
	100-200	N2-1	N 14°25′ 15.090″ E 101° 22′ 41.334″	
		N2-2	N 14°25′ 10.626″ E 101° 22′ 44.220″	
		N2-3	N 14°25′ 14.442″ E 101° 22′ 41.238″	
		N2-4	N 14°25′ 14.322″ E 101° 22′ 41.874″	
		N2-5	N 14°25′ 14.082″ E 101° 22′ 41.922″	

Table 16 (continued)

Location & Distance above sea level (m)	Distance (m) from main road	Sample Code	Latitude-Longitude	Location view
Nong Keing (N) 719-727 m	300-400	N3-1	N 14°25′ 19.146″ E 101° 22′ 36.480″	Figure 19c
		N3-2	N 14°25′ 19.128″ E 101° 22′ 36.300″	
		N3-3	N 14°25′ 19.386″ E 101° 22′ 36.426″	
		N3-4	N 14°25′ 19.554″ E 101° 22′ 36.600″	
		N3-5	N 14°25′ 19.374″ E 101° 22′ 36.522″	
	>500	N4-1	N 14°25′ 19.662″ E 101° 22′ 30.222″	
		N4-2	N 14°25′ 20.112″ E 101° 22′ 29.760″	
		N4-3	N 14°25′ 20.478″ E 101° 22′ 30.108″	
		N4-4	N 14°25′ 20.412″ E 101° 22′ 30.018″	
		N4-5	N 14°25′ 20.412″ E 101° 22′ 30.018″	

Table 16 (continued)

Location & Distance above sea level (m)	Distance (m) from main road	Sample Code	Latitude-Longitude	Location view
Saisorn reservoir (S) 761-771 m	0-20	S1-1	N 14°26′ 03.816″ E 101° 22′ 19.584″	Figure 19d
		S1-2	N 14°26′ 04.272″ E 101° 22′ 19.242″	
		S1-3	N 14°26′ 04.272″ E 101° 22′ 19.242″	
		S1-4	N 14°26′ 03.864″ E 101° 22′ 18.942″	
		S1-5	N 14°26′ 02.418″ E 101° 22′ 17.705″	
	100-200	S2-1	N 14°26′ 02.550″ E 101° 22′ 12.528″	
		S2-2	N 14°26′ 01.530″ E 101° 22′ 10.836″	
		S2-3	N 14°26′ 00.948″ E 101° 22′ 10.632″	
		S2-4	N 14°26′ 00.948″ E 101° 22′ 10.632″	
		S2-5	N 14°26′ 00.948″ E 101° 22′ 10.632″	

Table 16 (continued)

Location & Distance above sea level (m)	Distance (m) from main road	Sample Code	Latitude-Longitude	Location view
Saisorn reservoir (S) 761-771 m	300-400	S3-1	N 14°25′ 57.876″ E 101° 22′ 05.154″	Figure 19d
		S3-2	N 14°25′ 58.416″ E 101° 22′ 05.826″	
		S3-3	N 14°25′ 58.422″ E 101° 22′ 05.424″	
		S3-4	N 14°25′ 58.422″ E 101° 22′ 05.424″	
		S3-5	N 14°25′ 58.422″ E 101° 22′ 05.424″	
	>500	S4-1	N 14°25′ 59.316″ E 101° 21′ 58.074″	
		S4-2	N 14°25′ 59.208″ E 101° 21′ 57.504″	
		S4-3	N 14°25′ 59.358″ E 101° 21′ 58.368″	
		S4-4	N 14°26′ 00.000″ E 101° 21′ 57.696″	
		S4-5	N 14°25′ 59.310″ E 101° 21′ 57.222″	

Figure 19 Overview Images of the Lichen Collecting Sites at Khao Yai National Park, the Control Site (a), Golf Course (b), Nong Keing (c) and Saisorn Reservoir (d)

Figure 19 (continued)

6.1.2 Polluted Sites in Bangkok at Different Traffic Congestion (PBKT)

The technique of transplanted lichens was prepared by using lichens from the control site, the lichens were fixed on a box of polyethylene net with the thallus of lichen on each side, except the top using nylon string (Figure 20). By transplanting lichen to three traffic areas in Bangkok (Figure 21), include Lam Sali intersection, Rama 9 road intersection and the garden beside the Science Office Building (SCO) at Ramkhamhaeng University. Total lichen from collecting sites were 15 thallus (as shown in Table 17) when exposure periods 1 month in during 7 March 2017 to 7 April 2017 of summer season, all samples were collected in brown paper bags brought to laboratory to prepare for PAHs analysis.

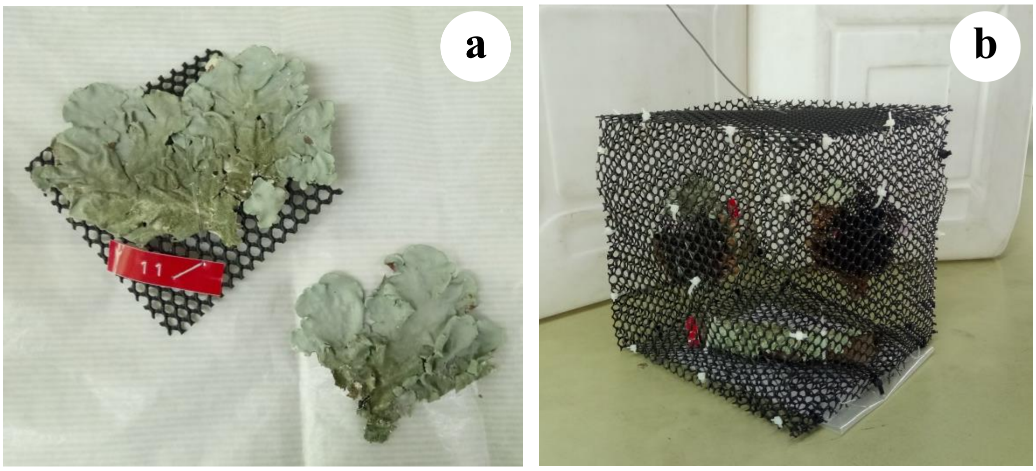


Figure 20 Thallus of the Lichen *Parmotrema tinctorum* (a), Fixing the Lichen Samples on the Box of Polyethylene Net (b)

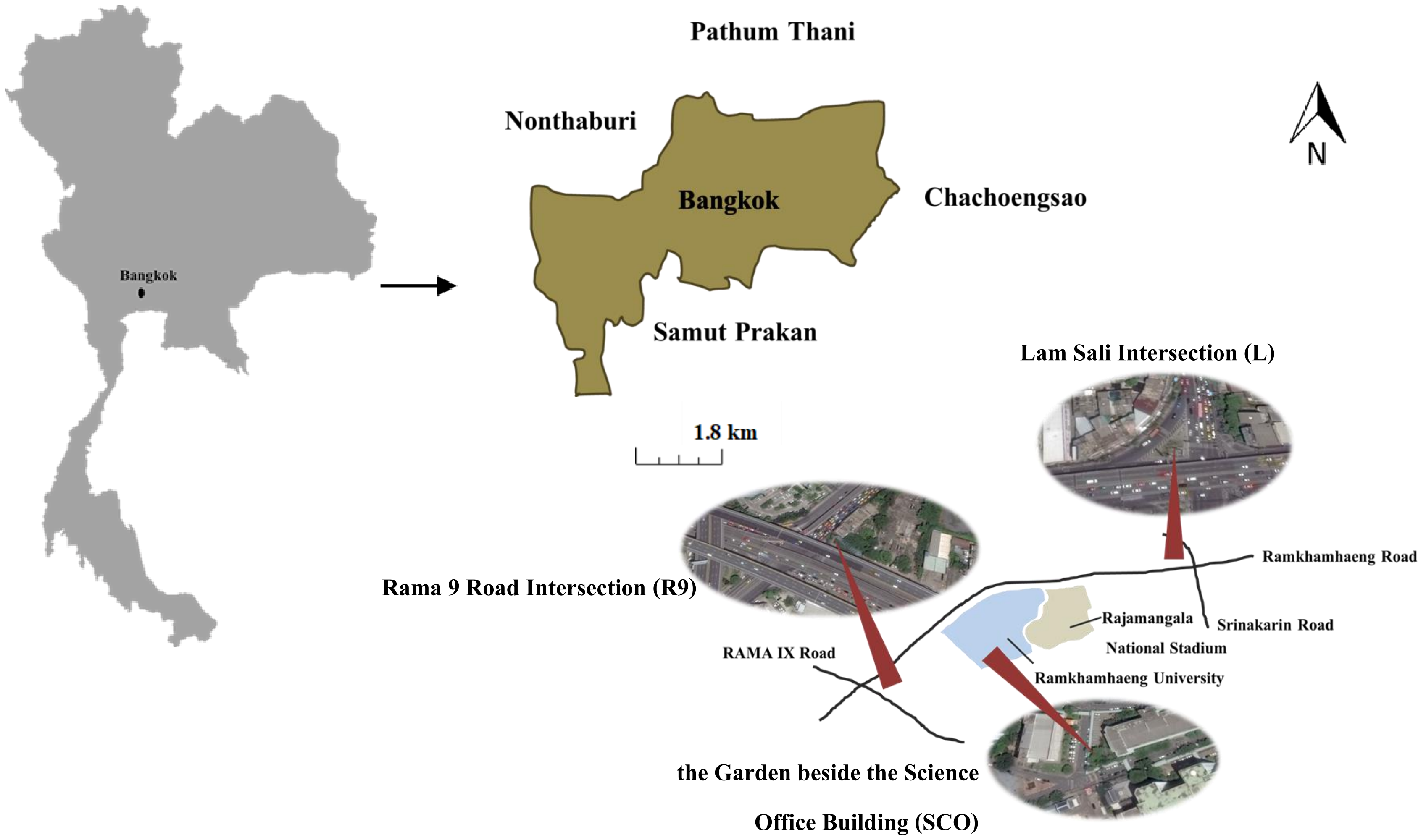


Figure 21 Maps of Three Traffic Areas in Bangkok for Transplanted Lichens

Table 17 Three Locations with Different Traffic Congestion Levels in Bangkok for Transplanted Lichens

Location & Above sea level (m)	Sample Code	Latitude-Longitude	Traffic density	Location view
1. Lam Sali Intersection (L) 9.85 m	L.1-L.5	N 13°45′ 47.560″ E 100° 38′ 43.750″	Traffic congestion	Figure 22a
2. Rama 9 Road Intersection (R9) 3.67 m	R9.1-R9.5	N 13°44′ 51.220″ E 100° 36′ 21.240″	Traffic congestion	Figure 22b
3. the Garden beside the Science Office Building (SCO) 2.68 m	SCO.1-SCO.5	N 13°45′ 15.970″ E 100° 37′ 08.000″	Traffic congestion some time	Figure 22c

Figure 22 Images of the Different Traffic Areas in Bangkok, Lam Sali Intersection (a), Rama 9 Road Intersection (b) and the Garden beside the Science Office Building (SCO) (c)

6.2 Preparation and Storage of Sample

Lichen samples from each collecting should be stored separately in brown paper bags, protected from sunlight and immediately stored at 4 °C. In the laboratory, the lichen samples were kept in air dried condition at room temperature and foreign debris on thalli were manually removed. Lichen samples were cleaned, crushed and grounded into powder with ceramic mortar and pestle by liquid nitrogen. The fine powder samples were kept frozen until analysis. Before analysis the samples were transferred to desiccators and kept until constant weight was achieved.

6.3 HPLC Analysis

All samples were analysis by using a HPLC of Thermo Scientific Dionex Ultimate 3000 series (UHPLC^{+} focused) as shown in Figure 23. The analytical chromatographic condition was performed with a flow rate of 1.0 mL/min using an Acclaim C18 column, the injection volume was 15 μL and the temperature of the column was maintained at 25 °C. The column was equilibrated before running, with the initial mobile phase being 10 min long. The mobile phase was used as a gradient elution prepared from DI water (solvent B) and acetonitrile (solvent C). Details of the chromatographic condition were given in Table 18. The PAHs were determined by using the fluorescence detector in medium sensitivity mode, excitation (Ex) was performed at 270 nm and emission (Em) was selected filter wavelength as automatic. The PAHs spectra were recorded by DAD in the range 190-400 nm.

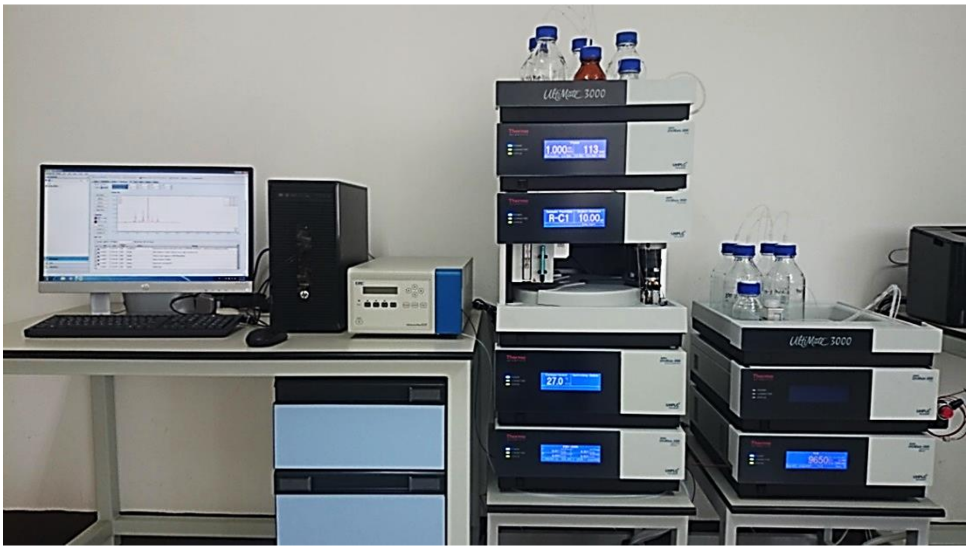


Figure 23 Image of the Ultimate 3000 Series

(UHPLC$^+$ Focused, Thermo Scientific Dionex) was used in This Work

Table 18 The Chromatographic Condition for Analysis of Polycyclic Aromatic Hydrocarbons Accumulation in the Lichen *Parmotrema tinctorum*

Analytical column	Acclaim C-18 (3 µm,4.6×150 mm)		
Column temperature	25 ºC		
Mobile phase	DI water as solvent B and Acetonitrile as solvent C		
Gradient system	Time (min)	% B	% C
	-10	40	60
	0	40	60
	3	40	60
	15	0	100
	20	0	100
Flow rate	1.0 mL/min		
Injection volume	15 µL		
Detectors	DAD in the range 190-400 nm FLD with excitation wavelength 270 nm and emission (Em) was selected filter wavelength as automatic		

CHAPTER 3

RESULTS AND DISCUSSION

Optimization of Chromatographic Conditions for Analysis of PAHs

The optimization of simultaneous chromatographic separation of eight PAHs (Naph, Ace, Phe, Anth, Fluo, Pyr, Chr, and BaPy) were studied by choosing optimal columns and chromatographic conditions, such as mobile phase composition and detector wavelength. This work was to be comparing of chromatographic condition on two columns, LiChrospher PAH and Acclaim C18. The optimum conditions were evaluated to obtain the highest separation, efficiency, sensitivity and short retention time for analysis.

1. Optimization of Solvent System

1.1 Optimization of Isocratic Systems for Separation of PAHs on LiChrospher PAH column and Acclaim C18 Column

Firstly, the method was determined by using the guide line of certificate of analysis of LiChrospher PAH column from literature to compare the efficiency between LiChrospher PAH column and Acclaim C18 column. The chromatographic condition: the injection volume was 15 µL, the temperature of the column was maintained at 25 ºC, and the fluorescence detector in medium sensitivity mode, excitation (Ex) was performed at 270 nm and emission (Em) was selected filter wavelength as automatic. The PAHs spectra were also recorded by DAD in the range 190-400 nm. The isocratic programs were studied by varying flow rate and ratio of DI water : acetonitrile, those gave six different isocratic programs as shown in Table 19. All the obtained chromatograms from FLD detector of two columns were displayed in Figure 24, 25.

Table 19 Six Chromatographic Conditions of Solvent System for Studied in Isocratic System

Method	Condition
IS1	(a) Flow rate 0.6 mL/min and ratio of DI water : Acetonitrile was 25 : 75
IS2	(b) Flow rate 0.6 mL/min and ratio of DI water : Acetonitrile was 20 : 80
IS3	(c) Flow rate 1.0 mL/min and ratio of DI water : Acetonitrile was 25 : 75
IS4	(d) Flow rate 1.0 mL/min and ratio of DI water : Acetonitrile was 20 : 80
IS5	(e) Flow rate 1.5 mL/min and ratio of DI water : Acetonitrile was 25 : 75
IS6	(f) Flow rate 1.5 mL/min and ratio of DI water : Acetonitrile was 20 : 80

The results from chromatograms were compared in term to the efficiency of two columns. From Figure 24 and 25, it was found that Acclaim C18 column taken shorter time and good separation. Among the six methods presented, it showed that method IS4 and IS6 (Figure 25) are suitable for analysis. It can be concluded that the optimum ratio was 20 : 80 (DI water :

Acetonitrile) by using Acclaim C18 column due to it took less run time and provided adequate separation.

1.2 Optimization of Gradient Systems for Separation of PAHs on LiChrospher PAH Column and Acclaim C18 Column

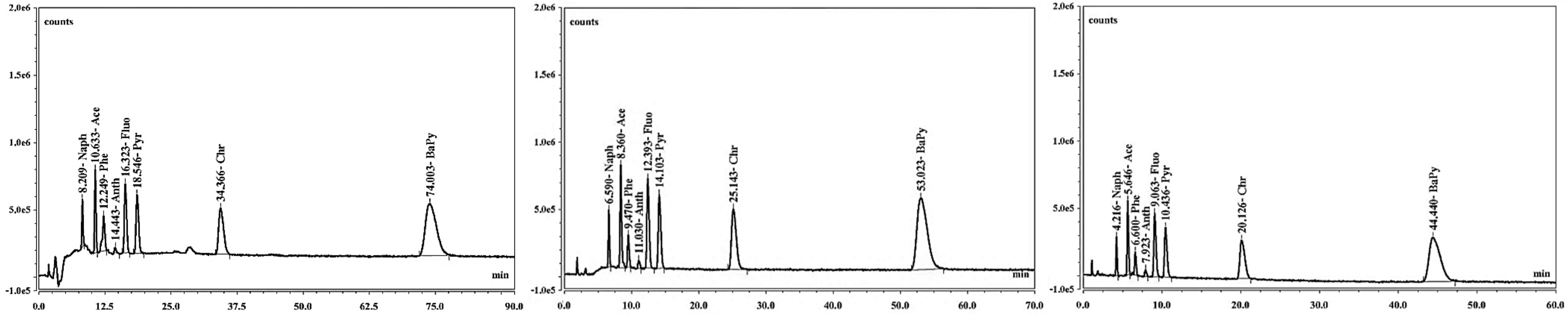
Many gradient programs were studied by varying the composition of solvent B (DI water) : solvent C (acetonitrile), run time, hold time and step in gradient elution upon the injection volume was 15 μL, temperature of the column was maintained at 25 ºC and the fluorescence detector in medium sensitivity mode, excitation (Ex) was performed at 270 nm and emission (Em) was selected filter wavelength as automatic. The PAHs spectra were also recorded by DAD in the range 190-400 nm. In this work, there are four possible gradient programs, they were chosen to reported as shown in Table 20. All the obtained chromatograms of four gradient programs to compare of two columns were shown by FLD in this report. LiChrospher PAH column was shown in Figure 26 and Acclaim C18 column was shown in Figure 27.

From the chromatograms in Figure 26 and 27 was to compare in term to the efficiency of two columns, the results showed method GR1 is the best method for both of LiChrospher PAHs and Acclaim C18 columns, it gave complete resolution, highest the efficiency and separation. This work chosen Acclaim C18 column due to it took less run time and provided adequate separation.

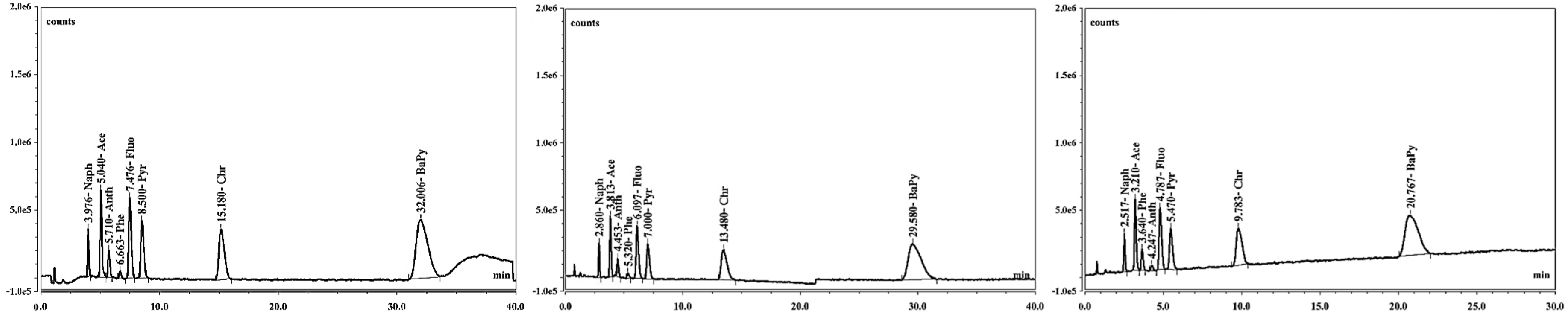
Table 20 Condition of Possible Four Gradient Programs

Method	Condition
GR1	(a) The run start at flow rate 1.0 mL/min with 60% of acetonitrile for 3min, then went to 100% of acetonitrile in 15 min with a final hold 15 min
GR2	(b) The run start at flow rate 1.0 mL/min with 83% of acetonitrile for 5.5 min, then went to 100% of acetonitrile in 9 min with a final hold 21 min
GR3	(c) The run start at flow rate 1.0 mL/min with 50% of acetonitrile for 3 min, then went to 100% of acetonitrile in 10 min with a final hold 20 min
GR4	(d) The run start at flow rate 1.0 mL/min with 90% of mixed solvent of methanol : DI water (75 : 25) for 2.5 min, then went to 100% of acetonitrile in 6 min with a final hold 24 min

Before each run, it started with equilibrate time for 10 min.

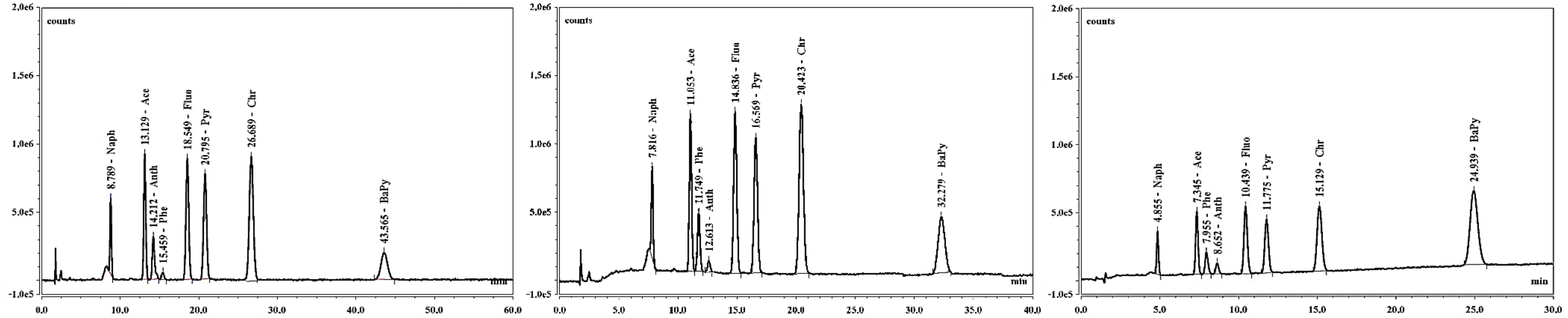


(a) Method IS1 (b) Method IS2 (c) Method IS3

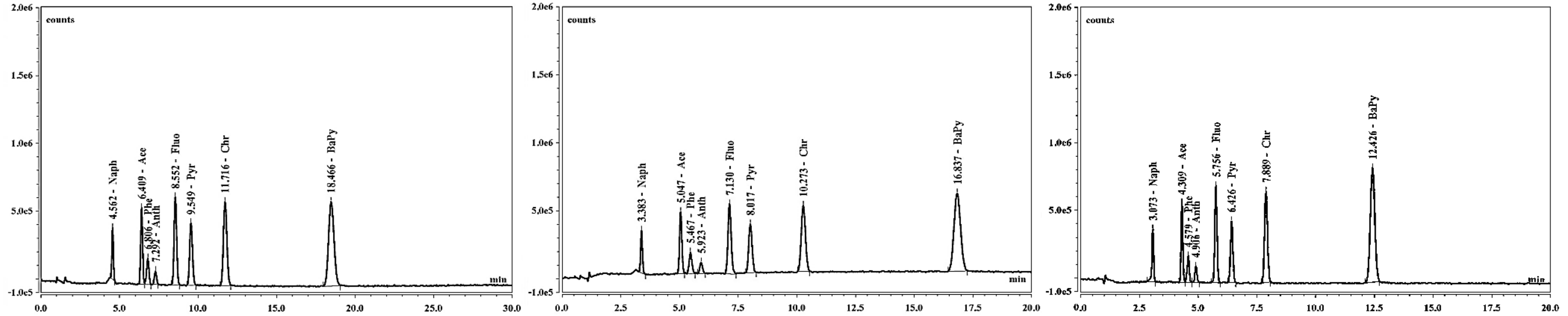


(d) Method IS4 (e) Method IS5 (f) Method IS6

Figures 24 The Chromatograms of PAHs by using Isocratic Systems of LiChrospher PAH Column, the injection volume was 15 µL, the temperature of the column was maintained at 25 ºC, and the fluorescence detector in medium sensitivity mode, Ex was performed at 270 nm and Em was selected filter wavelength as automatic. The PAHs spectra were also recorded by DAD in the range 190-400 nm

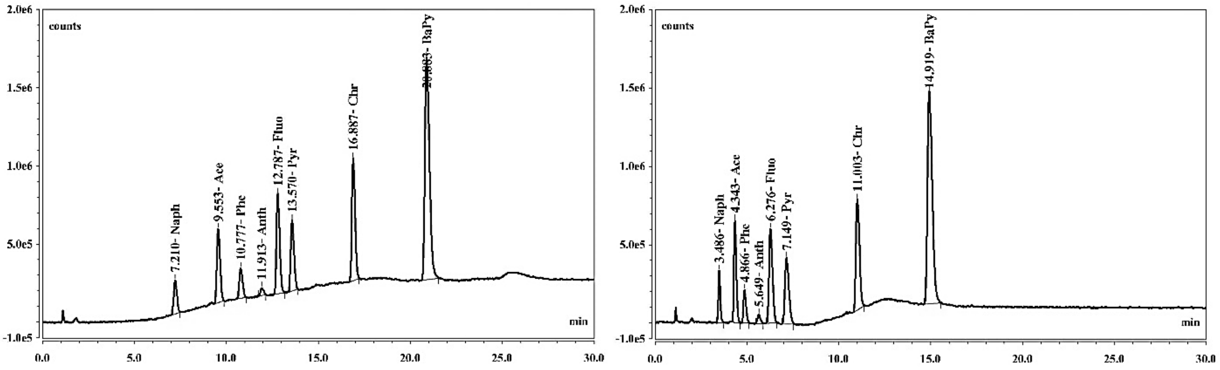


(a) Method IS1 (b) Method IS2 (c) Method IS3



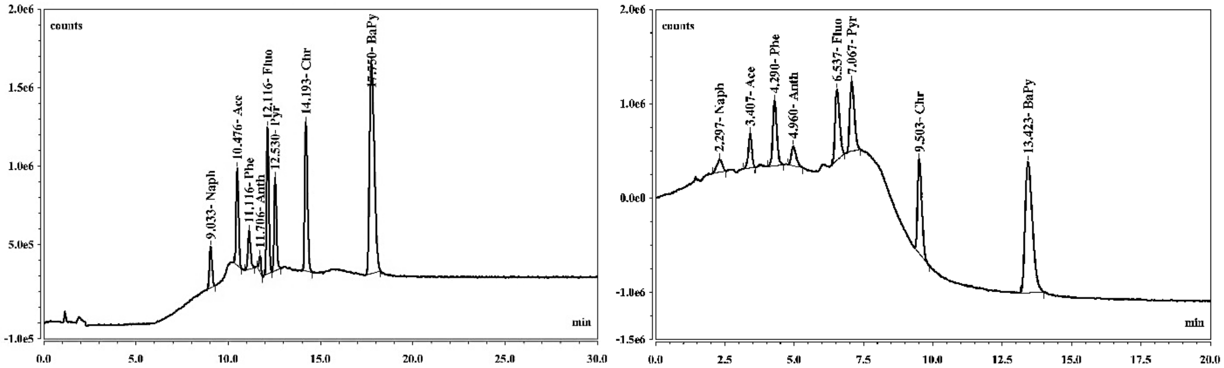
(d) Method IS4 (e) Method IS5 (f) Method IS6

Figures 25 The Chromatograms of PAHs by using Isocratic Systems of Acclaim C18 Column, the injection volume was 15 µL, the temperature of the column was maintained at 25 ºC, and the fluorescence detector in medium sensitivity mode, Ex was performed at 270 nm and Em was selected filter wavelength as automatic. The PAHs spectra were also recorded by DAD in the range 190-400 nm



(a) Method GR1

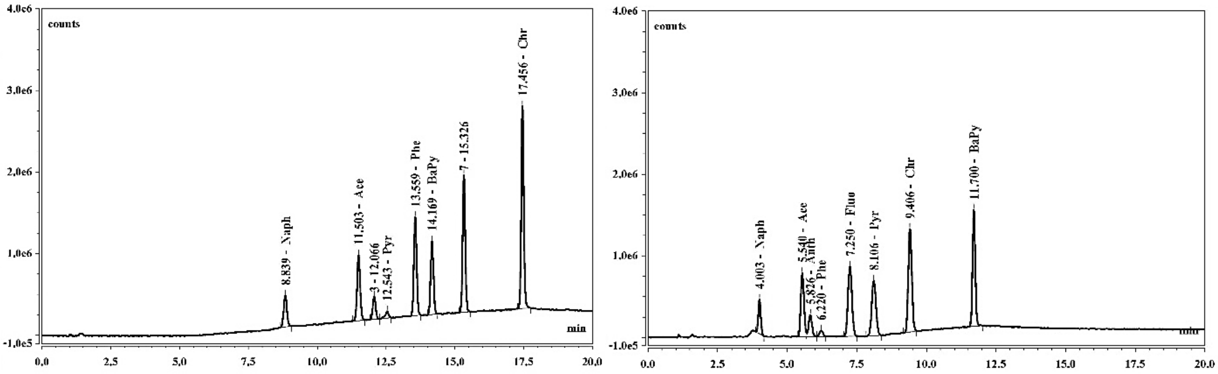
(b) Method GR2



(c) Method GR3

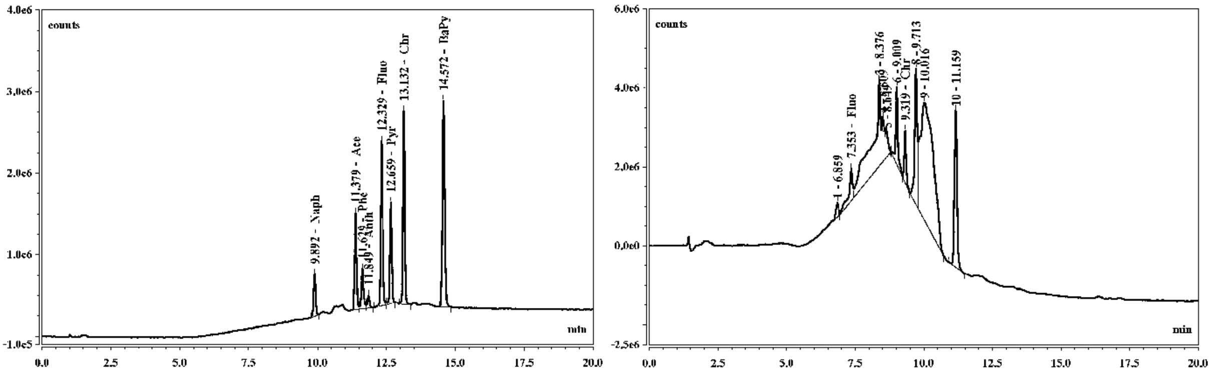
(d) Method GR4

Figures 26 The Chromatograms of PAHs by using Gradient Systems of LiChrospher PAH Column, the injection volume was 15 µL, the temperature of the column was maintained at 25 ºC, and the fluorescence detector in medium sensitivity mode, Ex was performed at 270 nm and Em was selected filter wavelength as automatic. The PAHs spectra were also recorded by DAD in the range 190-400 nm



(a) Method GR1

(b) Method GR2

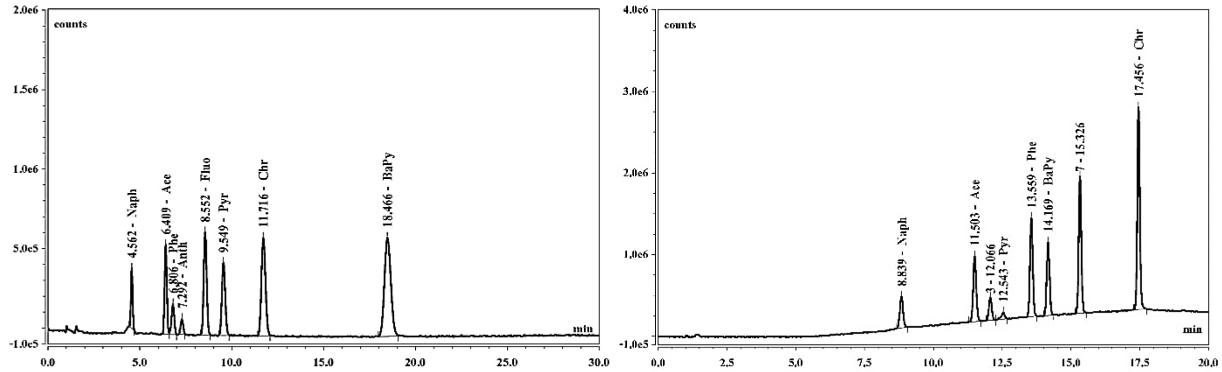


(c) Method GR3

(d) Method GR4

Figures 27 The Chromatograms of PAHs by using Gradient Systems of Acclaim C18 Column, the injection volume was 15 µL, the temperature of the column was maintained at 25 ºC, and the fluorescence detector in medium sensitivity mode, Ex was performed at 270 nm and Em was selected filter wavelength as automatic. The PAHs spectra were also recorded by DAD in the range 190-400 nm

When comparing between isocratic system of method IS4 and gradient system of method GR1 as shown the chromatogram in Figure 28 (a) and Figure 28 (b). It can see that the method GR1 gave better resolution, peak shape although the retention times for eight PAHs were almost the same (below 20 minutes).



(a) (b)

Figure 28 The Comparison of Chromatographic Condition of Two Systems on Acclaim C18 Column

(a) Isocratic system of Method IS4: Flow rate 1.0 mL/min and ratio of DI water : Acetonitrile was 20 : 80

(b) Gradient system of Method GR1: The run start at flow rate 1.0 mL/min with 60% of acetonitrile for 3 min, then went to 100% of acetonitrile in 15 min with a final hold 15 min

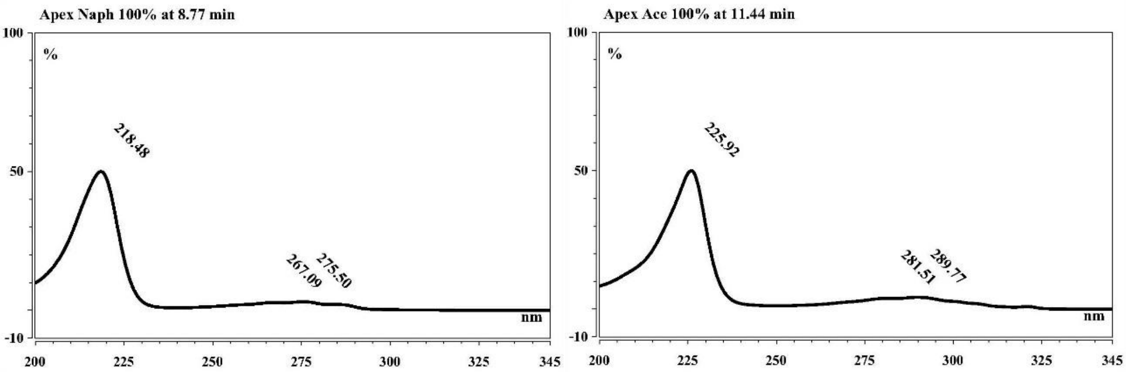
It can be concluded that the optimum solvent B was 100% DI water and solvent C was 100% acetonitrile. The gradient solvent program began at flow rate 1.0 mL/min with 60% of acetonitrile for 3 minutes, then went to 100% of acetonitrile for 15 minutes with a final hold 15 minutes. Before each run, it started with equilibrate time for 10 minutes.

2. Detection Wavelength for Analysis of PAHs on Acclaim C18 Column

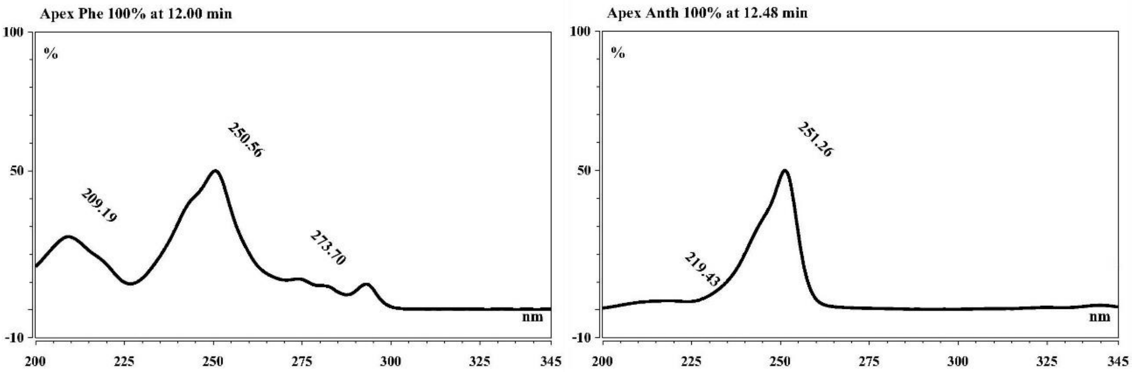
2.1 Selection of Quantitative and Sensitivity Wavelength Coupled with Diode Array Detector (DAD) and Fluorescence Detector (FLD)

2.1.1 Detection by Diode Array Detector (DAD)

The result obtained from the injection of 15 µL of mixed standard solution of 1.6 mg/L of Naph, Anth and 0.8 mg/L of Ace, Phe and 0.32 mg/L of Fluo, Pyr and 0.16 mg/L Chr, BaPy detected by DAD gave the chromatogram at various wavelength. Each PAH compounds has different maximum absorption wavelength (λ_{max}), the suitable wavelength to detect each PAHs should be selected. The seven wavelengths, 245, 250, 254, 265, 270, 280 and 290 nm, were selected for analysis to yield highest peak area. At 270 nm of the DAD spectrogram, almost eight PAHs had high absorption as shown in Table 21. Although almost of PAHs have absorption of characteristics UV wavelength at 270 nm but can be used to as the suitable wavelength in analysis PAHs by HPLC. In analysis of PAHs using HPLC with DAD, measurement at characteristics UV wavelength can use for peak identification and peak purity checks, as well as for quantitative analysis of PAHs at each characteristics UV wavelength. In the present study DAD provided the match with UV spectra for the eight PAHs by scanning from 190 to 400 nm (Figure 29). These show the absorption spectrum of PAHs from elution peak that can imply to purity check. For example, at 218 nm Naph has a sharp absorption peak and the highest sensitivity but Chr shows little or no absorption at this wavelength and has a sharp absorption peak at 267 nm.

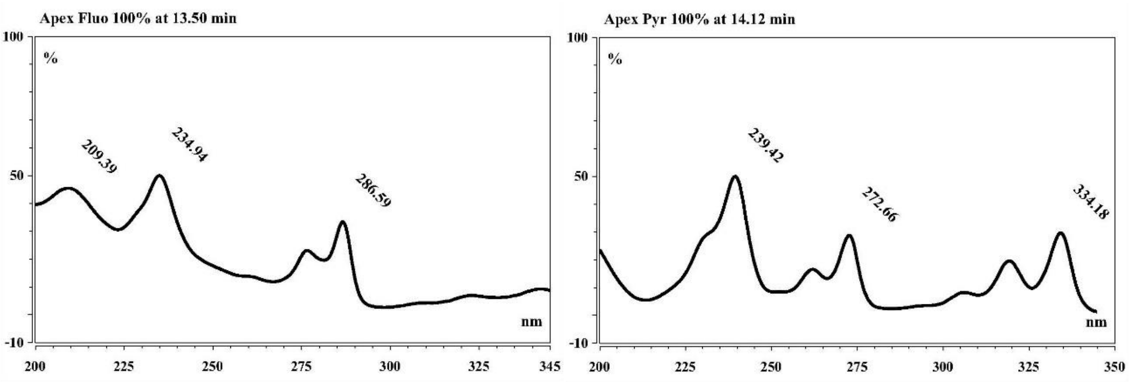


Naphthalene (Naph) Acenaphthene (Ace)



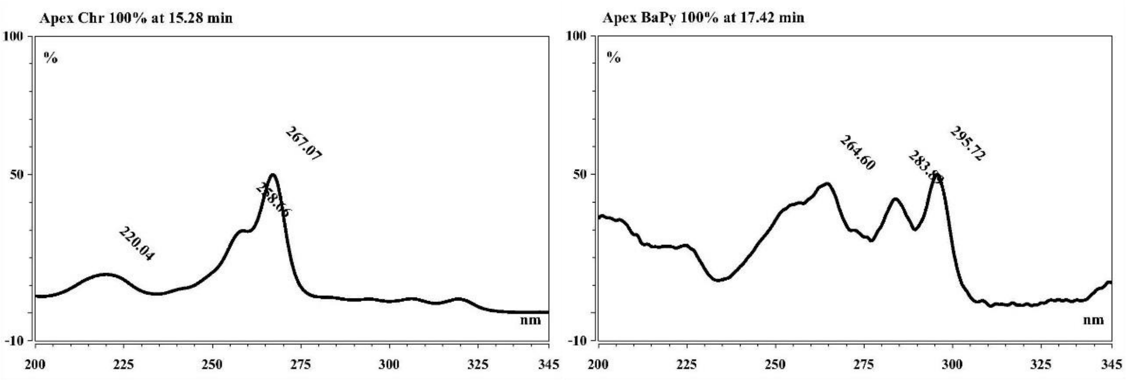
Phenanthrene (Phe) Anthracene (Anth)

Figure 29 The Characteristics UV Spectra of Wavelength for PAH Compounds



Fluoranthene (Fluo)

Pyrene (Pyr)



Chrysene (Chr)

Benzo[a]pyrene (BaPy)

Figure 29 (continued)

Table 21 Wavelengths Detection and Peak Area of Diode Array Detector on Acclaim C18 Column (N = 7)

Wavelength (nm)	Peak area of PAHs (mAU)							
	Naph 1.6 µg/L	Ace 0.8 µg/L	Phe 0.8 µg/L	Anth 1.6 µg/L	Fluo 0.32 µg/L	Pyr 0.32 µg/L	Chr 0.16 µg/L	BaPy 0.16 µg/L
245	0.31 ± 0.03	0.25 ± 0.04	3.28 ± 0.02	12.89 ± 0.06	0.44 ± 0.01	0.57 ± 0.02	0.32 ± 0.05	0.22 ± 0.00
250	0.39 ± 0.00	0.21 ± 0.01	3.97 ± 0.01	18.70 ± 0.14	0.36 ± 0.01	0.25 ± 0.01	0.42 ± 0.04	0.29 ± 0.01
254	0.50 ± 0.01	0.19 ± 0.01	3.21 ± 0.01	13.75 ± 0.03	0.31 ± 0.00	0.25 ± 0.00	0.58 ± 0.03	0.34 ± 0.00
265	0.82 ± 0.02	0.23 ± 0.02	1.07 ± 0.00	0.46 ± 0.00	0.26 ± 0.01	0.43 ± 0.01	1.10 ± 0.02	0.40 ± 0.01
270	0.85 ± 0.01	0.28 ± 0.01	0.88 ± 0.00	0.29 ± 0.00	0.27 ± 0.00	0.64 ± 0.01	0.89 ± 0.01	0.29 ± 0.01
280	0.75 ± 0.01	0.42 ± 0.00	0.70 ± 0.00	0.17 ± 0.01	0.42 ± 0.01	0.11 ± 0.00	0.15 ± 0.01	0.28 ± 0.00
290	0.38 ± 0.00	0.46 ± 0.01	0.54 ± 0.00	0.08 ± 0.00	0.27 ± 0.01	0.08 ± 0.00	0.12 ± 0.00	0.27 ± 0.00

2.1.2 Detection by Fluorescence Detector (FLD)

In this experiment 15 µL of mixed standard solution of 1.6 mg/L of Naph, Anth and 0.8 mg/L of Ace, Phe and 0.32 mg/L of Fluo, Pyr and 0.16 mg/L Chr, BaPy was used. From the results of experiment can be discussed in two ways as following:

The result from scanning the excitation wavelengths of eight PAHs were performed at 245, 250, 265, 270, 280 and 290 nm, were selected for analysis to yield highest peak area. A wavelength at 270 nm gave the highest peak area for almost PAHs which are suitable for use as a wavelength of excitation, as shown in Table 22 and a wavelength of emission selected filter wavelength as automatic or zero order mode gave independently for each PAHs.

The result of switching events for the analysis of PAHs are summarized in Table 13 (Chapter 2). Figure 30 showed the chromatograms of switching wavelength programs for fluorescence detector, it showed the jumped base line and cannot be separated completely.

Table 22 Excitation Wavelengths and Peak Area of Fluorescence Detector on Acclaim C18 Column (N = 7)

Excitation Wavelength (nm)	Peak area of PAHs* (counts)							
	Naph 1.6 μg/L	Ace 0.8 μg/L	Phe 0.8 μg/L	Anth 1.6 μg/L	Fluo 0.32 μg/L	Pyr 0.32 μg/L	Chr 0.16 μg/L	BaPy 0.16 μg/L
245	59.69 ± 2.53	55.63 ± 1.35	947.86 ± 1.95	11,616.00 ± 9.54	570.58 ± 2.11	264.14 ± 7.86	124.80 ± 1.66	527.33 ± 6.68
250	101.28 ± 4.65	66.26 ± 0.82	1050.79 ± 7.50	12,697.45 ± 5.04	519.05 ± 2.75	189.87 ± 0.92	196.73 ± 1.22	730.16 ± 4.83
265	211.41 ± 1.01	179.65 ± 0.38	397.86 ± 0.76	727.67 ± 0.89	466.02 ± 0.09	355.71 ± 0.61	487.03 ± 0.63	1018.07 ± 3.96
270	208.23 ± 1.85	220.41 ± 0.44	301.38 ± 3.20	193.71 ± 3.30	536.38 ± 0.25	364.52 ± 2.68	349.04 ± 3.00	806.89 ± 11.82
280	178.10 ± 0.95	302.33 ± 0.49	218.03 ± 0.17	75.14 ± 0.06	746.68 ± 1.05	134.20 ± 0.73	85.02 ± 0.45	805.11 ± 1.35
290	70.42 ± 0.43	260.85 ± 1.21	139.42 ± 0.31	56.61 ± 0.27	407.87 ± 1.03	43.47 ± 3.23	45.11 ± 3.19	748.61 ± 2.50

*Peak area of FLD reported was $\overline{X}$/1000

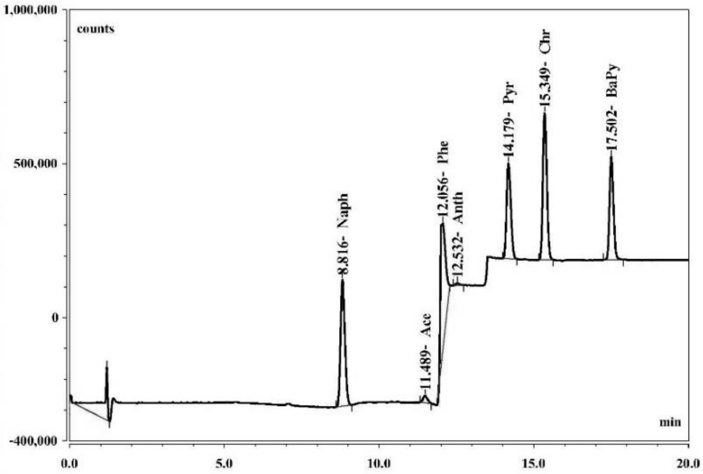


Figure 30 The Chromatograms of Switching Wavelength Programs for Fluorescence Detector on Acclaim C18 Column, using Gradient Systems of Method GR1, the injection volume was 15 µL, the temperature of the column was maintained at 25 ºC

It can be concluded that the optimum of FLD was a wavelength excitation at 270 nm and a wavelength of emission was selected filter wavelength as automatic in order to gave independently wavelength of each PAHs for optimum chromatogram.

In this study, was concluded that perform of the chromatograms of mixed standard of PAHs analyzed by the proposed conditions as shown in Figure 31. In analysis, thalli of the lichen *Parmotrema tictorum* were collected from bark of trees at Khao Yai National Park in Nakhon Ratchasima province and brought to laboratory to prepare for PAHs by HPLC used solvent B was 100% DI water and solvent C was 100% acetonitrile. The gradient solvent program began at flow rate 1.0 mL/min with 60% of acetonitrile for 3 minutes, then went to 100% of acetonitrile for 15 minutes with a final hold 15 minutes. The fluorescence detector in medium sensitivity mode, excitation (Ex) was performed at 270 nm and emission (Em) was selected filter wavelength as automatic.

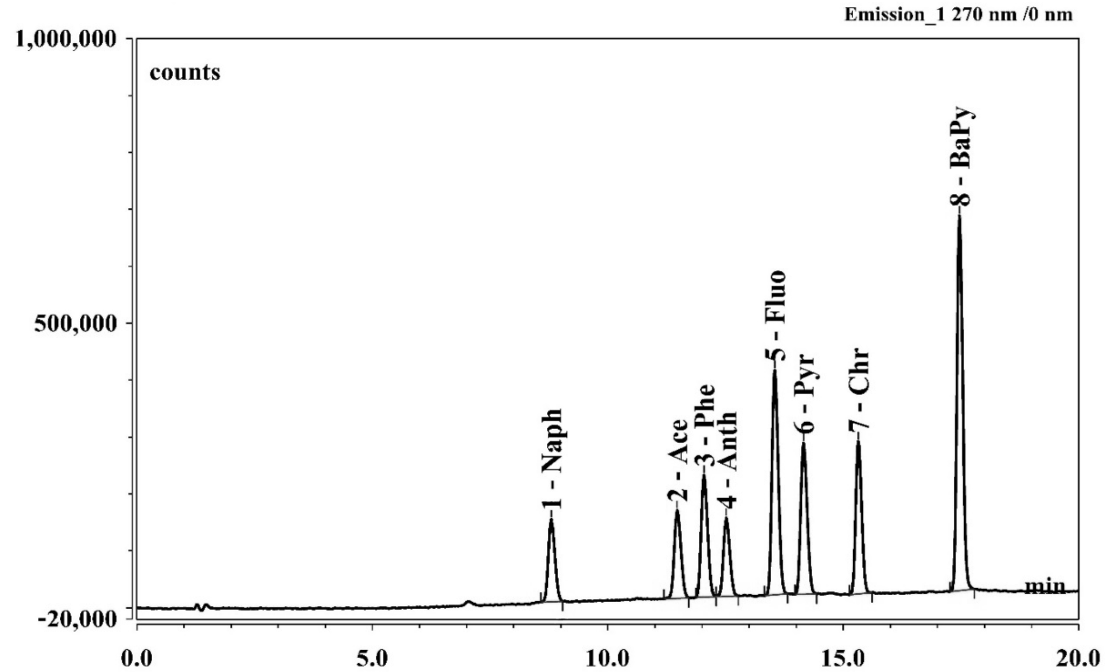


Figure 31 The Chromatogram of a Standard Mixture of Eight PAHs. Conditions: Accliam C18 column (4.6×150 mm i.d., 3 μm); temperature, 25 °C; using Gradient Systems of Method GR1, flow rate, 1.0 mL/min; injection volume 15 μL; detected by FLD. Peaks: (1) Naphthalene, (2) Acenaphthene, (3) Phenanthrene, (4) Anthracene, (5) Fluoranthene, (6) Pyrene, (7) Chrysene, (8) Benzo[a]pyrene

2.1.3 Limit of Detection of PAHs Analyzed by HPLC Coupled with Diode Array Detector (DAD) and Fluorescence Detector (FLD)

From the result of detection wavelength for Analysis of PAHs on Acclaim C18 column to achieve the highest sensitivity for PAHs analysis, the results demonstrated that most compounds had limit of detection from FLD lower than DAD, considering at wavelength 270 nm as shown in Table 23. For the most effective analysis, all PAHs are analyzed with FLD at excitation wavelength 270 nm and a wavelength of emission was under automatic filter wavelength or zero order. For DAD at wavelengths 254 nm was suitable to check impurity from lichen substance.

Table 23 Limit of Detection of Eight PAHs Detected by FLD and DAD at Wavelength 270 nm and 254 nm

PAHs	LOD by FLD* (μg/L)	LOD by DAD (μg/L)	
		270 nm	254 nm
Naph	21.22	135.10	497.70
Ace	9.76	191.10	39.48
Phe	7.35	64.26	10.86
Anth	22.83	378.8	18.01
Fluo	1.61	80.02	ND**
Pyr	2.37	35.83	283.80
Chr	1.27	12.13	34.20
BaPy	0.54	34.59	68.41

*Detected by FLD at excitation wavelength 270 nm and a wavelength of emission selected filter wavelength as automatic

**ND means that non-defined detected

Validation of Chromatographic Methods

The optimum chromatographic conditions of HPLC analysis were validated for Naphthalene, Acenaphthene, Phenanthrene, Anthracene, Pyrene, Fluoranthene, Chrysene and Benzo[a]pyrene in the following parameters: limit of detection, limit of quantification, linearity and sensitivity, accuracy and precision. The results of the analytical characteristics of validation method for eight PAHs obtained from mixed standard solution using fluorescence detector were explained as follows.

1. Limit of Detection (LOD) and Limit of Quantification (LOQ)

The LOD of each PAHs was investigated by calculating from standard deviation of the noise and slope of the calibration curve of seven injections were calculated from equation 2.1. The range of LOD were showed the value of limit of detection for eight PAHs within range 0.54 to 22.83 μg/L.

The LOQ of each PAHs was investigated by calculating from tenfold value the standard deviation of the noise of seven injections. The range of LOQ were showed the value of limit of quantification for eight PAHs within range 1.60 to 69.18 μg/L. The results of LOD and LOQ are shown in Table 24.

2. Linearity

The linearity of each PAH compounds was determined by using mixed standard solution in the following range: Naph and Anth were in 10-160 μg/L, Phe and Ace were in 5-80 μg/L, Pyr and Fluo were in 2-32 μg/L, Chr and BaPy were in 1-16 μg/L. The calibration curves were obtained by plotting the peak area versus the concentration of the standard solutions. The linearity of calibration curve is defined in term correlation coefficient (r^2) as shown in Table 24 and Figure 32. The results showed a good linearity of the eight PAHs with r^2 higher than 0.9996.

3. Accuracy and Precision

3.1 Precision

Precision of the chromatographic method was determined in terms of repeatability by using standard solution of each PAHs containing different concentrations which were analyzed in seven replicates. The results of precision were expressed in percentage of relative standard deviation (% RSD) calculated from equation 2.2. The obtained results are shown in Table 25. The precision of this method gave % RSD less than 3.59%.

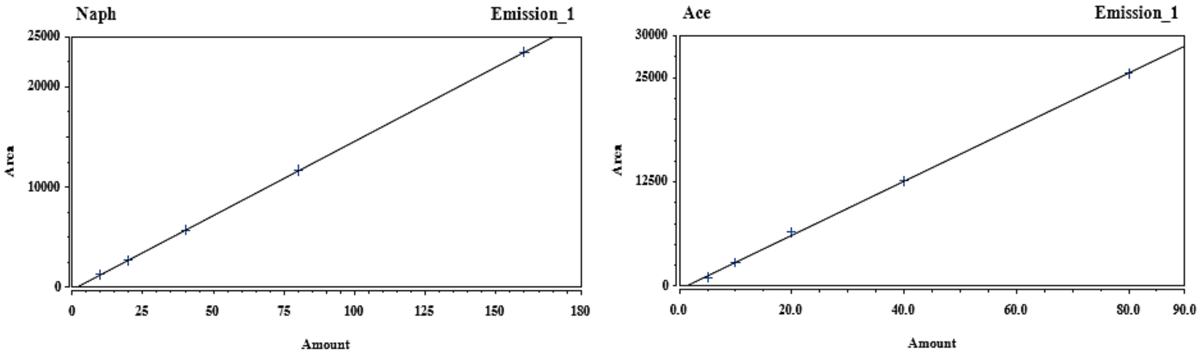
3.2 Accuracy

Accuracy of the chromatographic method was determined in terms of observed value to compare with true value. The results of relative accuracy value were calculated from equation 2.3. In this study, accuracy was determined in term of the relative accuracy value. The obtained results are shown in Table 26 and showed a good accuracy with % relative accuracy (% $RE_{accuracy}$) higher than 99.61%.

Table 24 Limit of Detection (LOD), Limit of Quantification (LOQ) and Linearity (r^2) of Standard Solution PAHs using FLD Detector (N = 7)

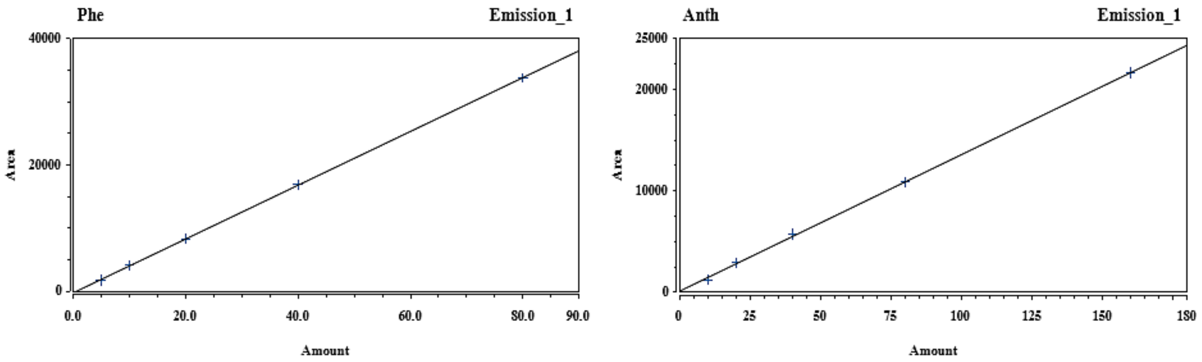
PAHs	LOD (µg/L)	LOQ (µg/L)	Concentration range (µg/L)	Linearity (r^2)
Naph	21.22	64.30	10-160	1.0000
Ace	9.76	29.58	5-80	0.9997
Phe	7.35	22.26	5-80	0.9999
Anth	22.83	69.18	10-160	0.9996
Fluo	1.61	4.88	2-32	0.9999
Pyr	2.37	7.18	2-32	0.9999
Chr	1.27	3.84	1-16	1.0000
BaPy	0.54	1.60	1-16	0.9999

*Detected by FLD at wavelength 270 nm



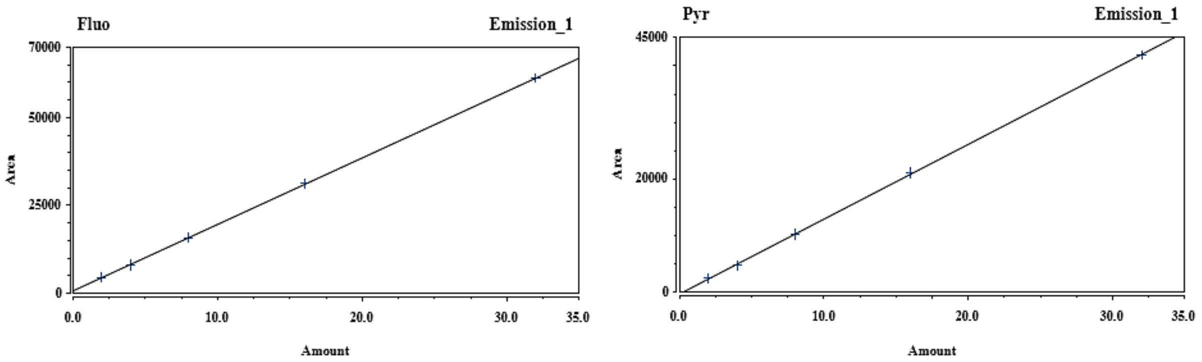
Naphthalene (Naph) (1)

Acenaphthene (Ace) (2)



Phenanthrene (Phe) (3)

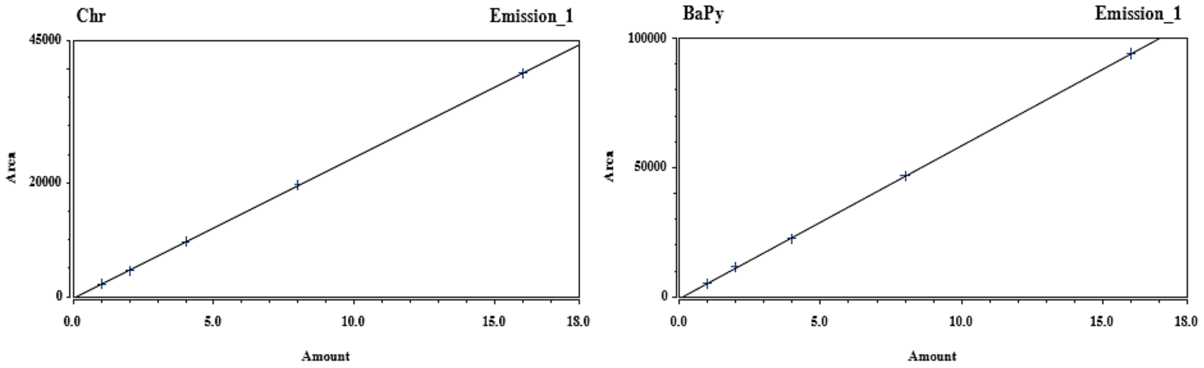
Anthracene (Anth) (4)



Fluoranthene (Fluo) (5)

Pyrene (Pyr) (6)

Figure 32 Calibration Curve of Naph (1), Ace (2), Phe (3), Anth (4), Pyr (5), Fluo (6), Chr (7) and BaPy (8)



Chrysene (Chr) (7) Benzo[a]pyrene (BaPy) (8)

Figure 32 (continued)

Table 25 Precision Data of Concentration and Retention Time of Polycyclic Aromatic Hydrocarbons of the Chromatographic Method (N = 7)

PAHs	Retention Time (min)		Concentration of PAHs (µg/L)								
			Level 1			Level 3			Level 5		
	$\overline{X}$ ± SD	% RSD	Conc.	$\overline{X}$ ± SD	% RSD	Conc.	$\overline{X}$ ± SD	% RSD	Conc.	$\overline{X}$ ± SD	% RSD
Naph	8.83 ± 0.33	0.33	10	10.04 ± 0.14	1.37	40	40.81 ± 0.72	1.76	160	159.79 ± 0.69	0.43
Ace	11.50 ± 0.03	0.28	5	5.10 ± 0.03	0.54	20	20.62 ± 0.27	1.30	80	79.90 ± 0.38	0.47
Phe	12.07 ± 0.03	0.26	5	5.03 ± 0.09	1.81	20	20.00 ± 0.28	1.42	80	80.23 ± 0.44	0.55
Anth	12.55 ± 0.03	0.24	10	9.97 ± 0.20	2.01	40	39.84 ± 1.31	3.30	160	159.83 ± 0.45	0.28
Fluo	13.57 ± 0.03	0.24	2	2.05 ± 0.03	1.56	8	8.24 ± 0.16	1.94	32	32.07 ± 0.19	0.58
Pyr	14.19 ± 0.03	0.22	2	2.08 ± 0.05	2.44	8	8.33 ± 0.21	2.56	32	32.03 ± 0.07	0.23
Chr	15.35 ± 0.03	0.20	1	1.06 ± 0.04	3.36	4	4.22 ± 0.15	3.59	16	16.08 ± 0.27	0.40
BaPy	17.50 ± 0.03	0.18	1	1.08 ± 0.02	1.58	4	4.23 ± 0.05	1.28	16	16.10 ± 0.07	0.42

Table 26 Relative Accuracy Value of Polycyclic Aromatic Hydrocarbons of the Chromatographic Method (N = 7)

PAHs	Level 1			Level 3			Level 5		
	Conc. of Std. PAHs (µg/L)	Conc. Found ($\bar{X}$ ± SD)	% $\mathrm{RE}_{accuracy}$	Conc. of Std. PAHs (µg/L)	Conc. Found ($\bar{X}$ ± SD)	% $\mathrm{RE}_{accuracy}$	Conc. of Std. PAHs (µg/L)	Conc. Found ($\bar{X}$ ± SD)	% $\mathrm{RE}_{accuracy}$
Naph	10	10.04 ± 0.14	100.36	40	40.81 ± 0.72	102.04	160	159.79 ± 0.69	99.87
Ace	5	5.10 ± 0.03	101.90	20	20.62 ± 0.27	103.10	80	79.90 ± 0.38	99.87
Phe	5	5.03 ± 0.09	100.67	20	20.00 ± 0.28	100.02	80	80.23 ± 0.44	100.29
Anth	10	9.97 ± 0.20	99.71	40	39.84 ± 1.31	99.61	160	159.83 ± 0.45	99.89
Fluo	2	2.05 ± 0.03	102.32	8	8.24 ± 0.16	102.98	32	32.07 ± 0.19	100.21
Pyr	2	2.08 ± 0.05	104.05	8	8.33 ± 0.21	104.09	32	32.03 ± 0.07	100.09
Chr	1	1.06 ± 0.04	105.94	4	4.22 ± 0.15	105.45	16	16.08 ± 0.27	100.48
BaPy	1	1.08 ± 0.02	107.50	4	4.23 ± 0.05	105.71	16	16.10 ± 0.07	100.65

Quality Control Check Standards for HPLC Analysis

Prior to analyzes the samples; quality controls were performed to ensure the stability of the HPLC system by injection standard solution of mixed standard PAHs, the concentration was the same as level 3. For monitoring stability throughout the experiment, control chart of the quality standard for PAHs were plotted as shown in Figure 33.

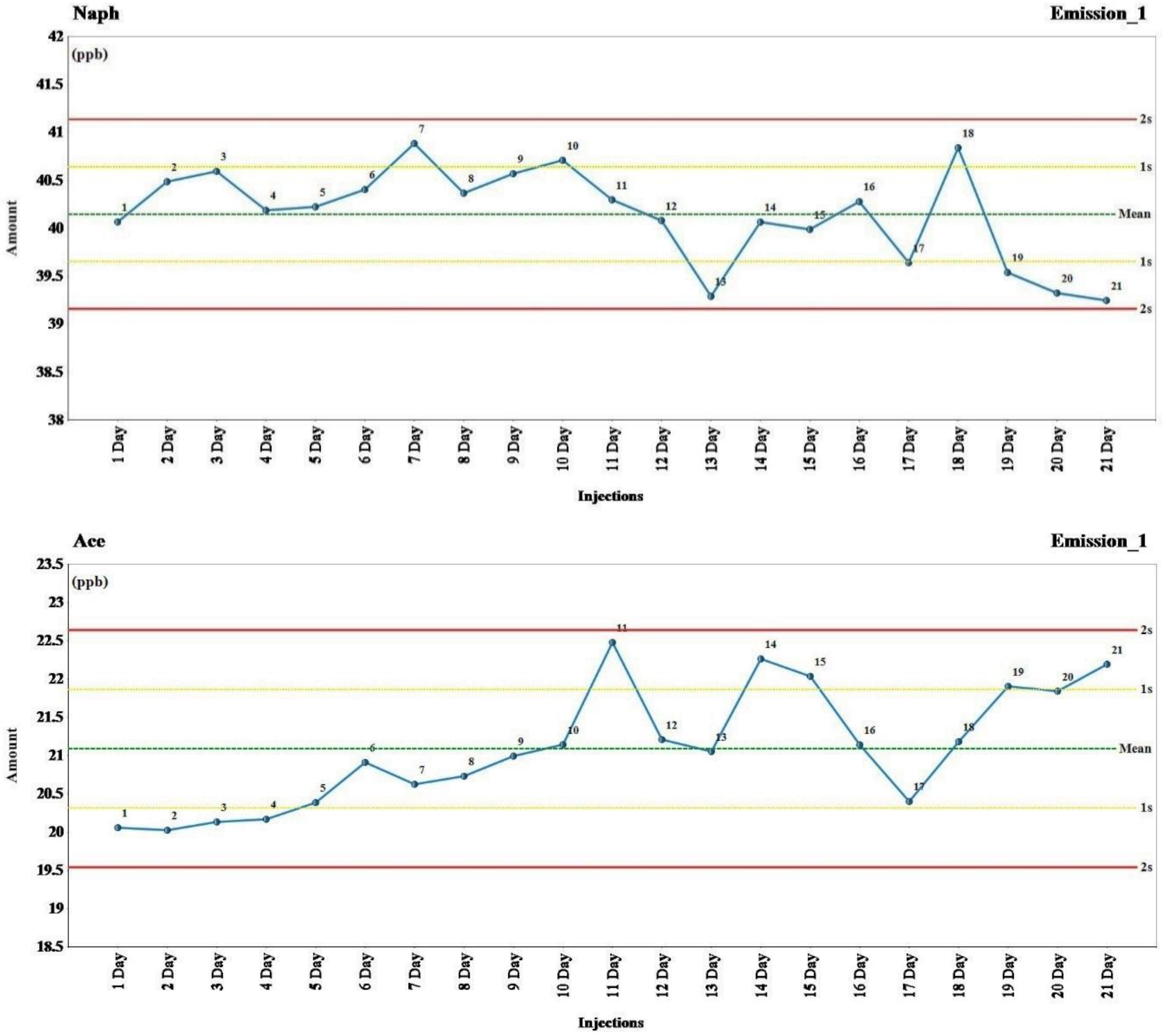
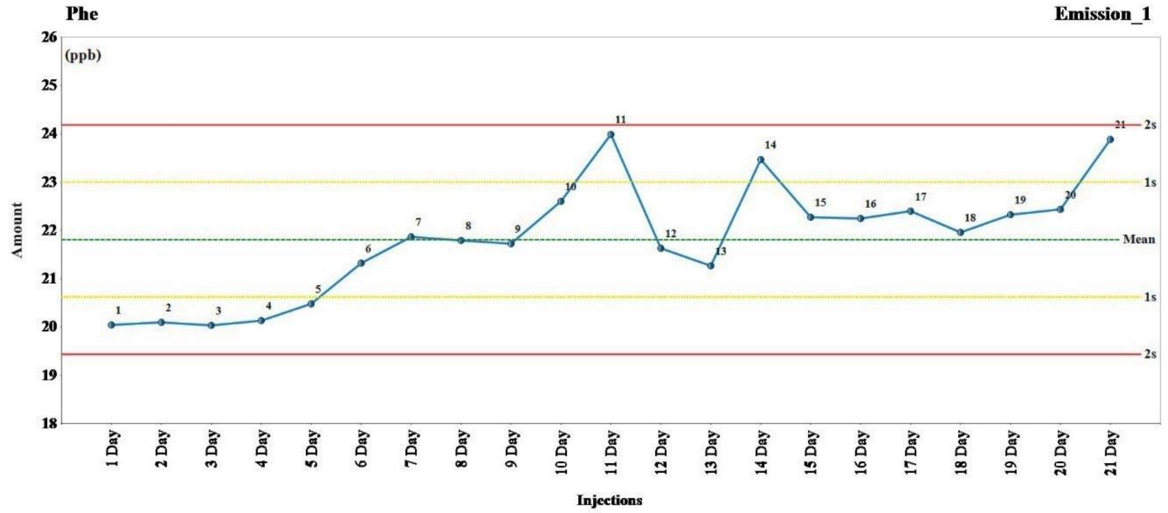
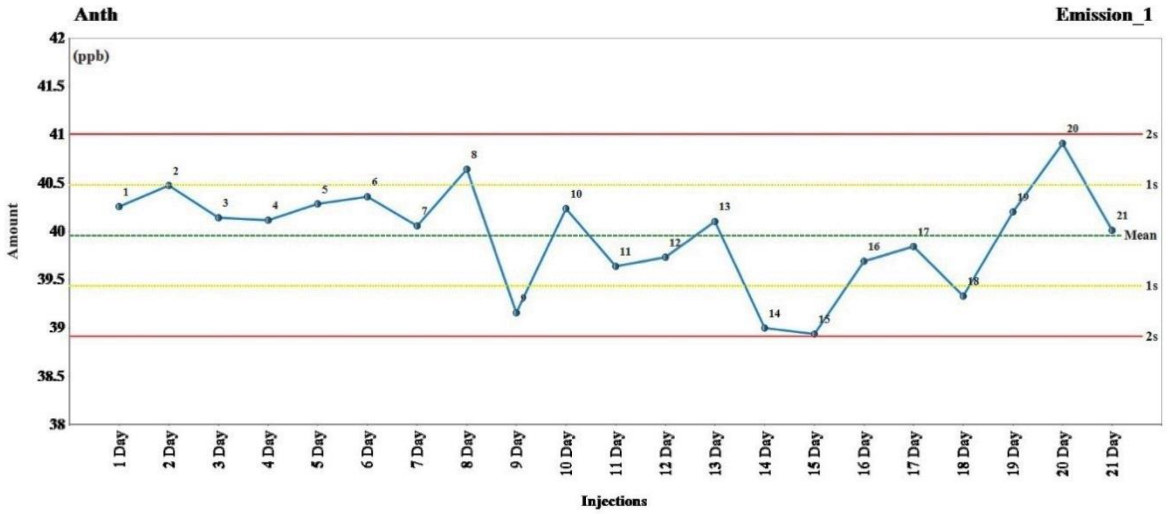


Figure 33 Control Chart for Check Standard of PAHs





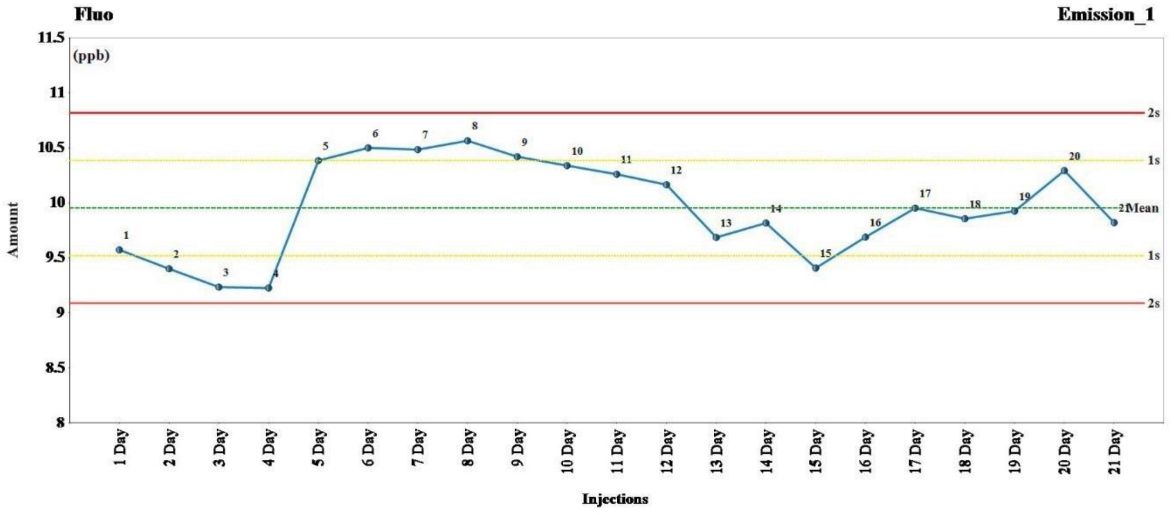
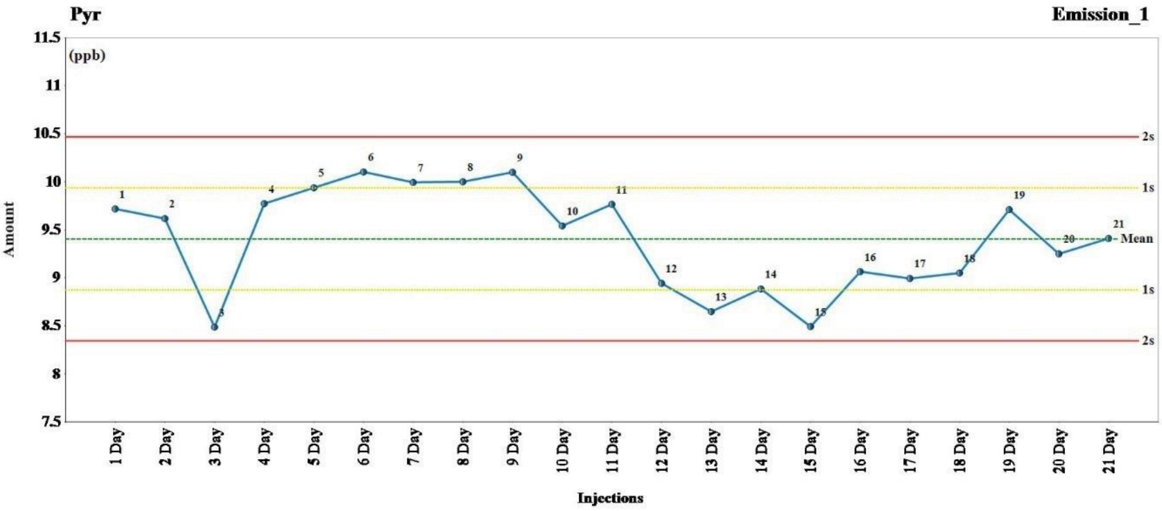
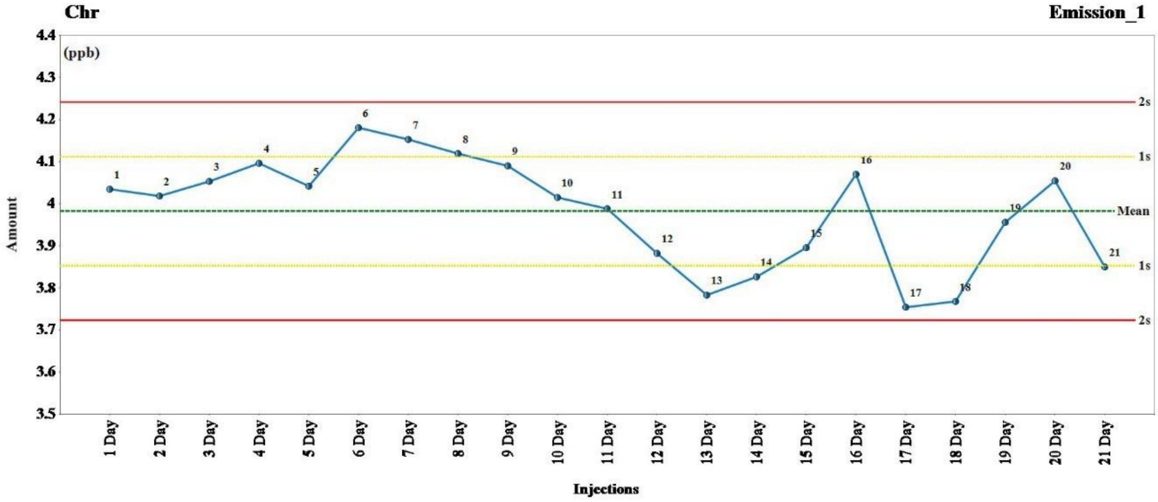


Figure 33 (continued)





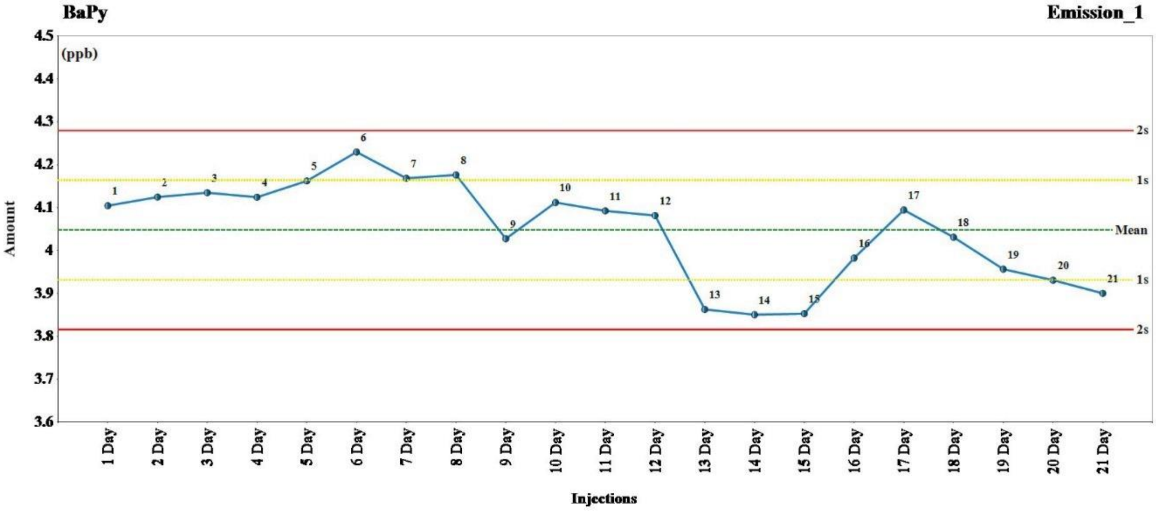


Figure 33 (continued)

Optimization of Sample Preparation

The sample preparation methods were investigated to find the optimum condition. The methods consisted of two steps. First step is ultrasound-assisted for extraction PAHs from lichen, using hexane solvent followed the UAE technique for lichen of Domeño and Blasco[17] and second step is clean-up crude extract by SPE method.

1. Ultrasound-Assisted Extraction Method

The lichen *Parmotrema tinctorum* collected from Khao Yai National Park was used for optimization experiments as shown in Table 15 (Chapter 2). The accuracy of the proposed samples preparation was tested by using spiked recovery experiments with concentrations of mixed standard solutions PAHs from 4.3 (Chapter 2), the concentration was the same as level 5. The validation results of conditions for ultrasound-assisted extraction method were the following:

1.1 Influence of Sonication Frequency

To find out the optimum sonication frequency which were extracted in three times, power was 60% at 30 °C in 10 min. The studied frequency was 37 kHz and 80 kHz. The result was shown in Figure 34, it was found that 37 kHz was a suitable sonication frequency for extraction of PAHs accumulation in lichen samples. The suitable frequency was decided from chrysene and benzo[a]pyrene due to these compounds has the most toxicity to human.

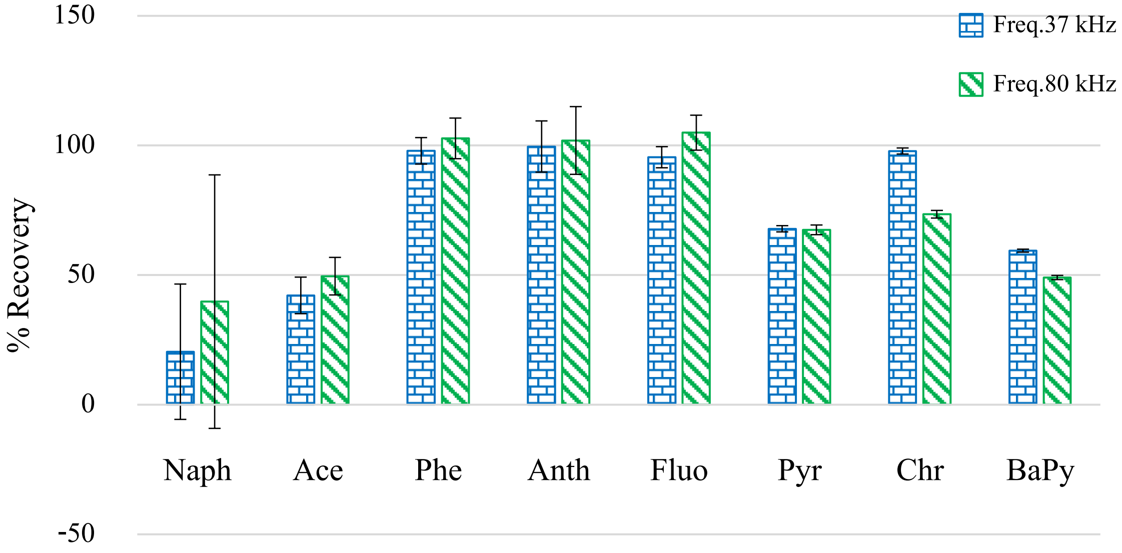


Figure 34 Recovery of PAHs from Ultrasound-Assisted Extraction Method at 37 and 80 kHz Sonication Frequency

1.2 Influence of Sonication Temperature

To find out the optimum sonication temperature which were extract in three times, extraction time was 10 min, frequency was 37 kHz and power was 60%. The temperature studied were 0 °C and 30 °C, respectively. The results were shown in Figure 35, it was found that 30 °C was suitable sonication temperature for lichen samples. The further reason is difficulty to control the temperature to maintain at zero.

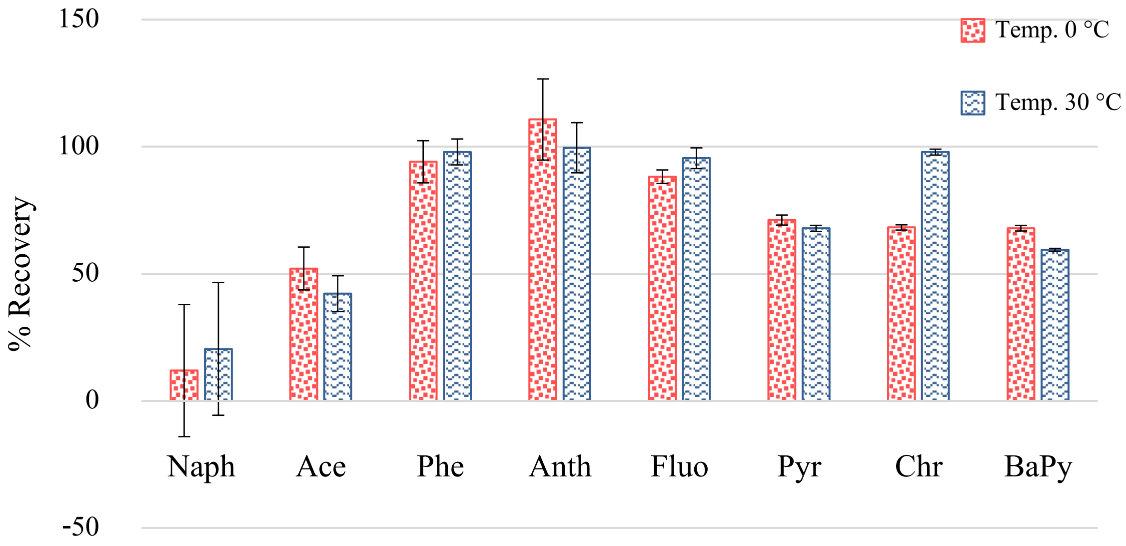


Figure 35 Recovery of PAHs from Ultrasound-Assisted Extraction Method at Various Temperature

1.3 Influence of Sonication Time

To find out the optimum extraction time of all PAHs accumulation in lichen sample which were extracted in three times, frequency was 37 kHz, power was 60% and temperature was 30 °C. The sonication extraction times were 5 min, 8 min and 10 min, respectively. The results were shown in Figure 36, it was found that 10 min gave highest yield of chrysene and then selected as the suitable sonication time for extract of PAHs.

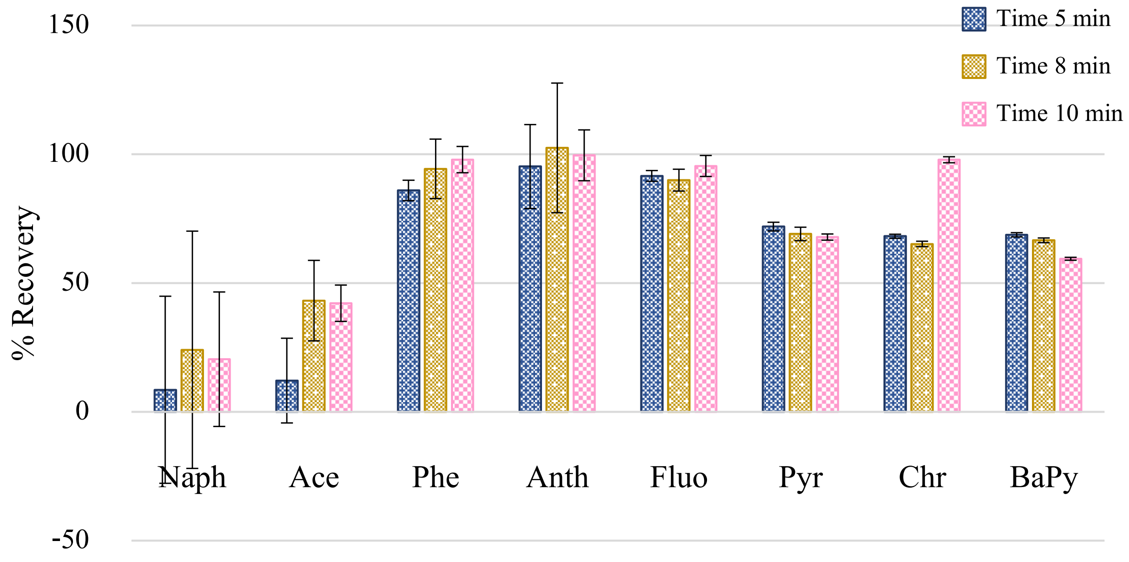


Figure 36 Recovery of PAHs from Ultrasound-Assisted Extraction Method at Various Time

1.4 Influence of Sonication Power

To find out the optimum sonication power which were extracted in three times, extraction time was 10 min frequency was 37 kHz and temperature was 30 °C. The studied sonication powers were 30, 60 and 100%, respectively. The results were shown in Figure 37, it was found that 100% gave highest yield almost of all the PAHs, except Chr. Then selected power 100% as the suitable sonication power for extract of PAHs accumulation in lichen samples owing to higher precision.

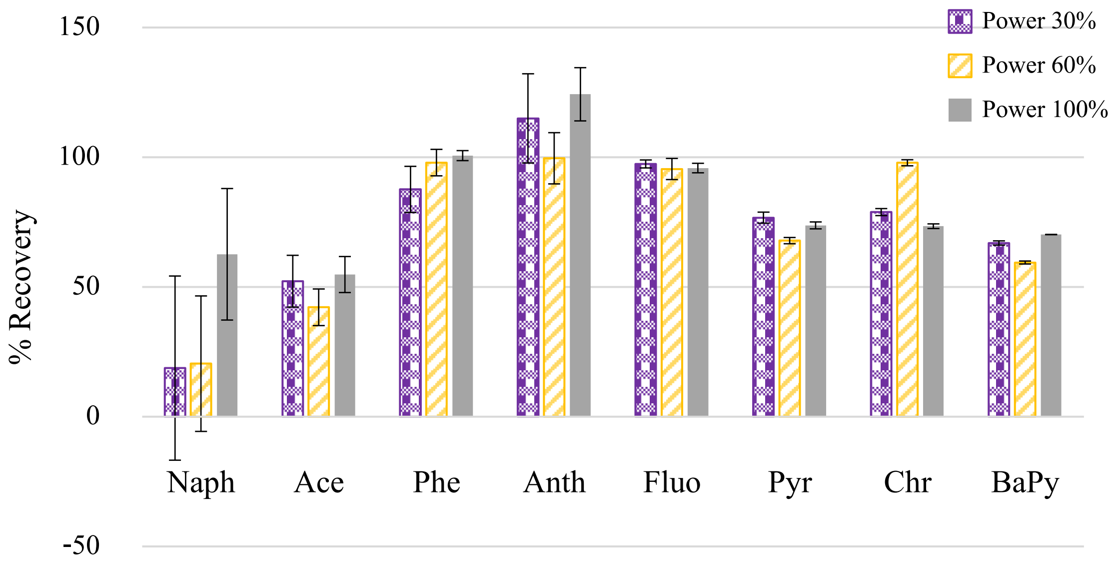


Figure 37 Recovery of PAHs from Ultrasound-Assisted Extraction Method at Various Sonication Power

1.5 Influence of Sample Amount

In this study, indicate that the quantitative recovery with good precision (lower % RSD) for all of PAHs in lichen was achieved when the sample weight is 0.1 g of grounded lichen. The precision of PAHs of ultrasound-assisted extraction method were shown in Figure 38. It was found that the weight of sample as 0.1 g gave highest precision for all PAHs and then selected as the suitable weight for extract of PAHs accumulation in lichen samples.

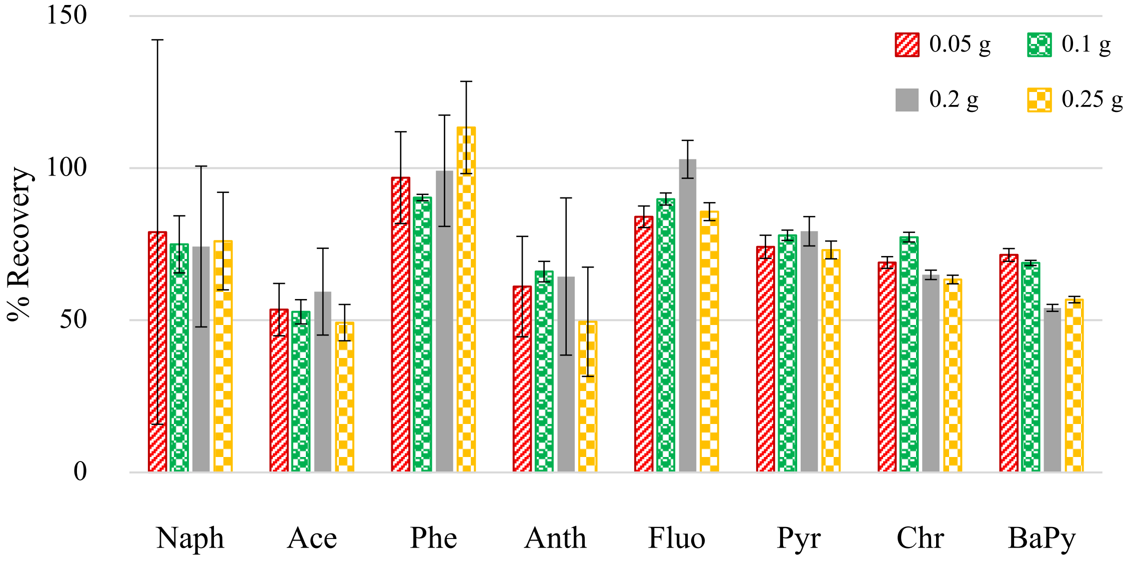


Figure 38 Recovery of PAHs from Ultrasound-Assisted Extraction Method at Various Sample Amount

From the optimization data 1.1-1.5, it can be concluded the optimum condition for sample preparation method as shown in Table 27. The accuracy and the precision of optimum condition was studied by spiking an appropriate amount of analyte PAHs in lichen samples. The recovery and precision data of this sample preparation method were shown in Table 28.

Table 27 The Variable Parameter, Studied Interval and Optimized Parameter of Ultrasound-Assisted Extraction Method

Variable parameter	Studied interval	Optimized parameter
Sonication frequency (kHz)	37, and 80 kHz	37 kHz
Sonication temperature (°C)	0-30 °C	30 °C
Sonication time (min)	5, 8, and 10 min	10 min
Sonication power (%)	30, 60, and 100%	100%
Sample amount (g)	0.05-0.25 g	0.1 g

Table 28 The Validation Results of Ultrasound-Assisted Extraction Method (N = 7)

PAHs	Conc. of Std. PAHs (μg/L)	Conc. Found $\overline{X}$ ± SD (μg/L)	% RSD	% REV
Naph	160	119.93 ± 29.39	8.50	74.96
Ace	80	42.23 ± 3.98	9.43	52.78
Phe	80	72.28 ± 11.04	9.99	90.35
Anth	160	105.59 ± 13.37	9.66	66.00
Fluo	32	28.76 ± 1.99	6.93	89.86
Pyr	32	24.94 ± 1.70	6.83	77.94
Chr	16	12.37 ± 1.59	9.83	77.34
BaPy	16	11.02 ± 0.85	7.72	68.85

2. Solid Phase Extraction (SPE)

In this study was to compare the efficiency and suitability of two sorbent types, florisil and silica. Sorbent florisil, were studied by varying various parameter, using the method of Blasco, M., C. Domeño, and C. Nerín.[106], used weight is 1.2 g of florisil for mini column of SPE. Whereas sorbent silica gels were succeeded studied by Sriviboon[27], used as the SPE mini-column, 0.0125 g of oxalic acid was dissolved in 2.5 mL of methanol and impregnated on 1.2 g of silica gel.

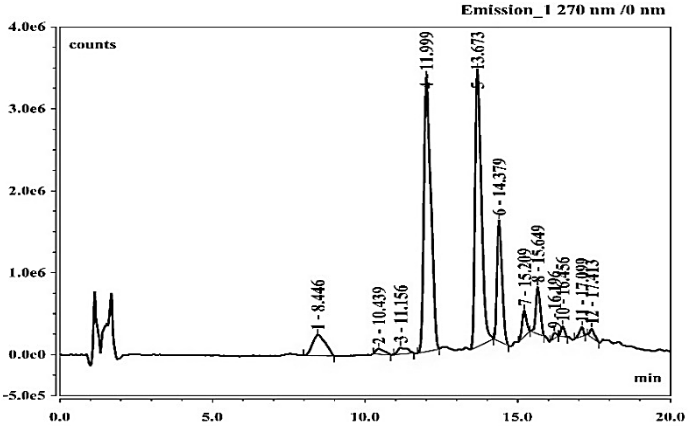
2.1 SPE Procedure for using Florisil

The samples were investigated to find the optimum procedure of SPE florisil which was validated in the following steps: condition and elution the compounds of interest.

2.1.1 Suitable Solvent for Condition Step

Condition step are used to remove the bulk of the co-extracted material and to remove contaminated compounds. This step make it ready activate to absorb samples.

The optimum of five conditions (Table 15 in Chapter 2) of solvent volume types were investigated to condition the sorbent. The last portion of the elution solvent were brought to analysis by HPLC. Figure 39 showed the chromatograms of five conditions from various elution solvents. It was found that Condition 5 as show in Figure 39 (e) is the most suitable condition due to there should not be a substance attached or not showing the peaks. Therefore, it can be concluded that the cartridge was rinse with 30 mL of acetonitrile to remove contaminated compounds and then conditioned with 6 mL of dichloromethane followed 3 mL of hexane.



(a)

Figure 39 Effect of the Condition on Clean-Up of Florisil Sorbent from the Following Condition

(a) Condition 1; Hexane 10 mL, Methanol 10 mL

(b) Condition 2; Hexane 10 mL, Hexane (65) : Dichloromethane (35) 10 mL, and Methanol 10 mL

(c) Condition 3; Hexane 20 mL, Hexane (65) : Dichloromethane (35) 20 mL, and Methanol 30 mL

(d) Condition 4; Hexane 20 mL, Hexane (65) : Dichloromethane (35) 20 mL, and Acetonitrile 30 mL

(e) Condition 5; Acetonitrile 30 mL, Dichloromethane 6 mL, and Hexane 3 mL

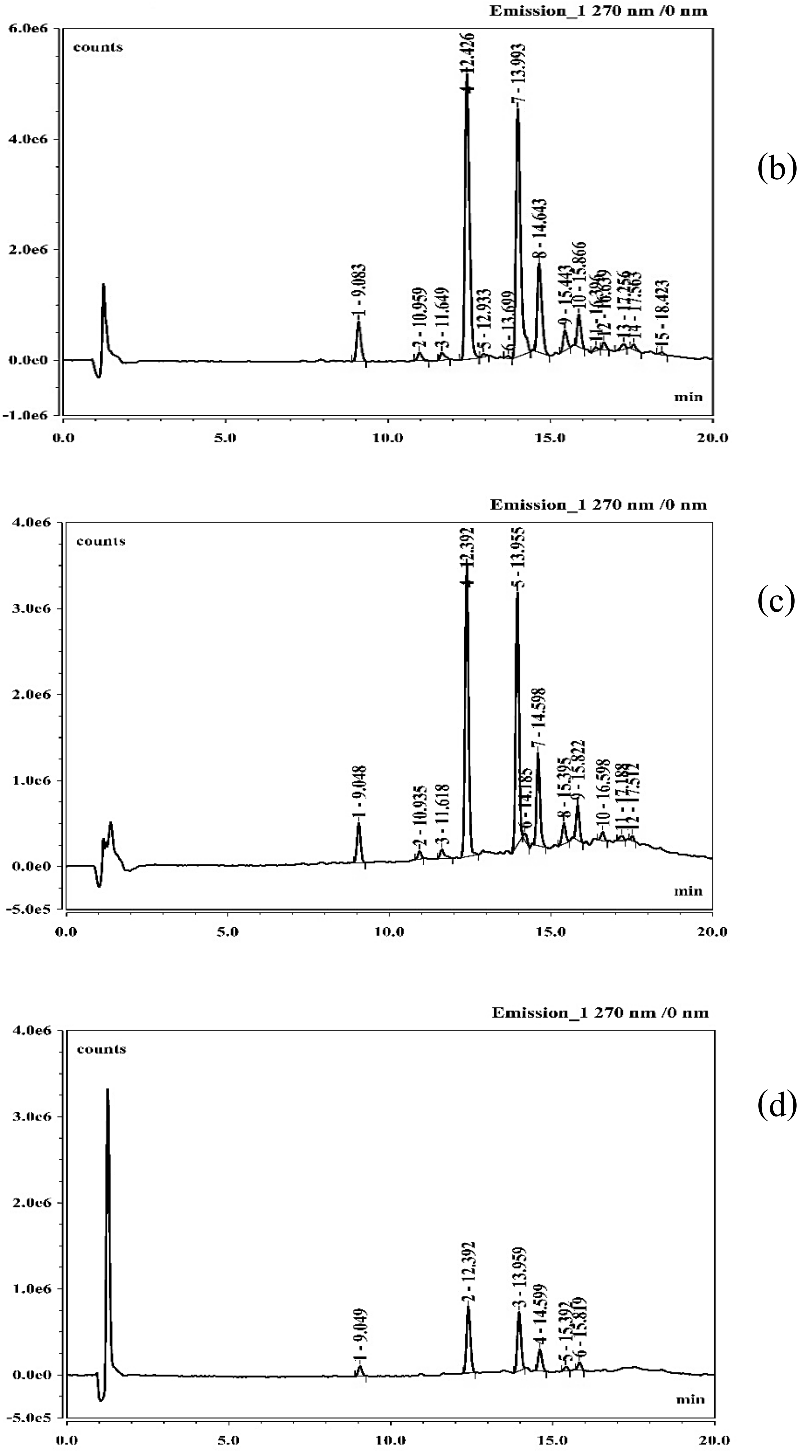
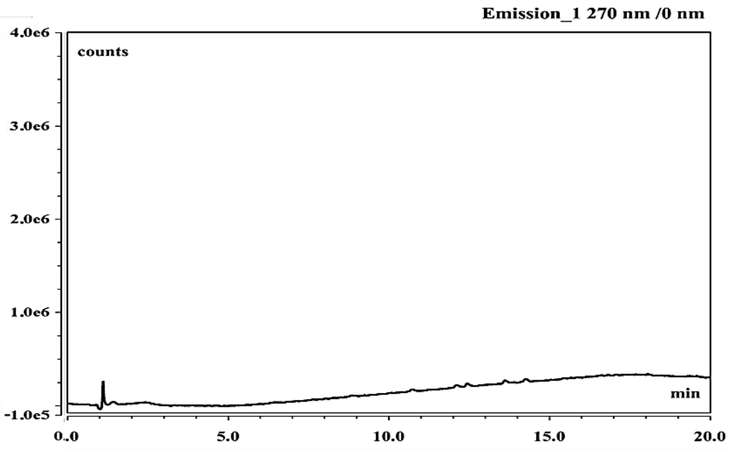


Figure 39 (continued)



(e)

Figure 39 (continued)

2.1.2 Suitable Solvent Volume for Elution Step

To compare elution solvents between acetonitrile and the mixture of hexane and dichloromethane (65 : 35) for elution recovery of mixed standard solution PAHs were investigated. Each elution was determined amount of PAHs by optimum HPLC conditions. Table 29 showed the % recovery of all fraction for elution of mixed standard solution. It was found that % recovery of PAHs eluted simultaneously by acetonitrile better than the mixture of hexane and dichloromethane (65 : 35).

Table 29 Recovery of All Fraction for Elution of Mixed Standard Solution PAHs by Acetonitrile and the Mixture of Hexane and Dichloromethane (65 : 35)

PAHs	Conc. of Std. PAHs (µg/L)	Std. PAHs were loaded into SPE (µg/2 mL of hexane)	% REV	
			ACN[a]	Hexane : DCM[b] (65 : 35)
Naph	100	8.0	88.79	95.41
Ace	100	8.0	94.29	108.89
Phe	50	4.0	102.14	88.56
Anth	100	8.0	86.91	92.03
Fluo	10	0.8	98.05	86.18
Pyr	50	4.0	99.24	103.22
Chr	20	1.6	98.19	103.20
BaPy	5	0.4	89.78	83.85

[a] ACN is the abbreviation for acetonitrile

[b] DCM is the abbreviation for dichloromethane

However, when the SPE procedure was evaluated by spiking with 250 µL of mixed standard PAHs into raw lichen (0.2 g) extract. It was found that substances in lichen, atranorin, was co-elute with PAHs in SPE procedure when eluted with acetonitrile as showed in Figure 40 (a). From this reason, the mixture of hexane and dichloromethane (65 : 35) was chosen as elution solvent.

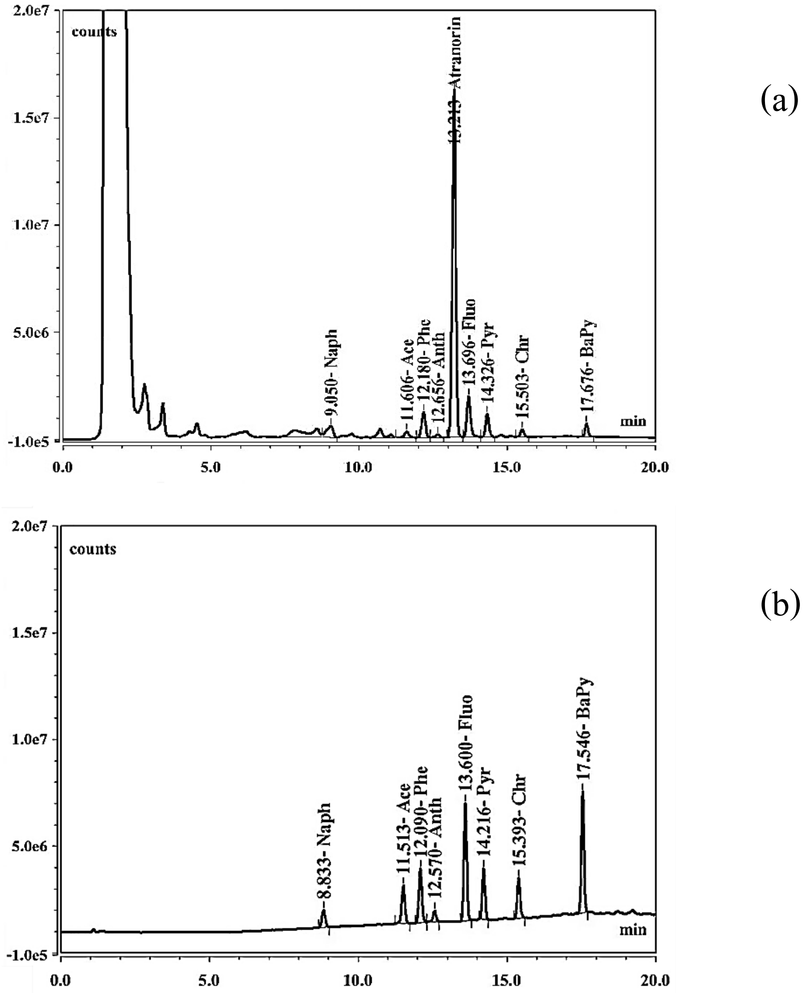


Figure 40 The Chromatogram of SPE Clean-Up Lichen Extract Eluted by Acetonitrile (a) and the Mixture of Hexane and Dichloromethane (65 : 35) (b)

The 0.5 mL fraction of eluate was collected simultaneously by the mixture of hexane and dichloromethane (65 : 35). Each fraction was determined amount of PAHs by optimum HPLC conditions. Figure 41 showed the elution profile by plotting the number of the fraction versus weight. Table 30 as showed the % recovery all fraction for elution of PAHs compound. The result showed the eluate was to discard 2.5 mL and continue to collect 4 mL, were fraction no. F2-F9 to collected.

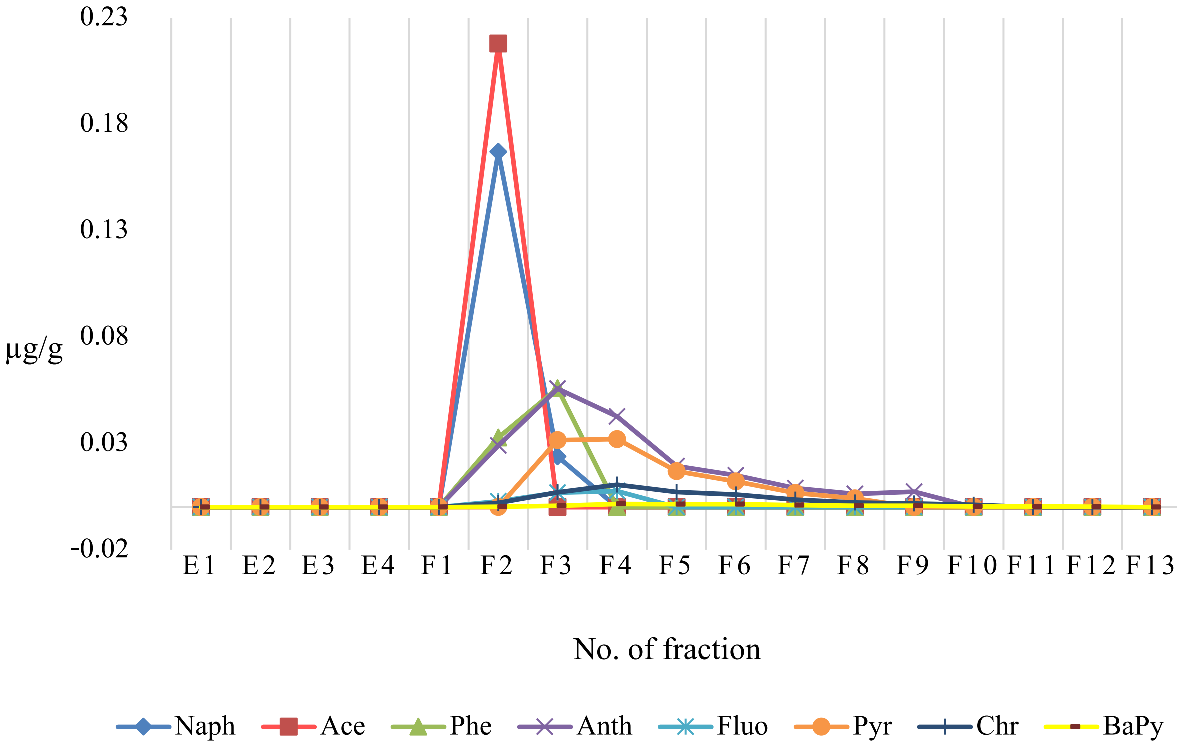


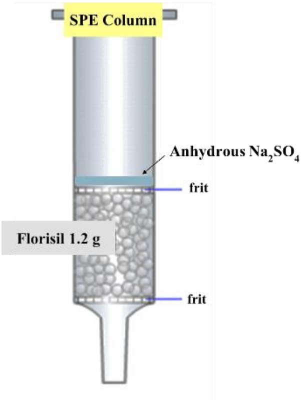
Figure 41 Elution Profile by Plotting the No. of Fraction Versus Weight of PAHs

Table 30 Recovery of Elution All Fraction of PAHs from SPE Procedure by the Mixture of Hexane and Dichloromethane (65 : 35)

PAHs	Conc. of Std. PAHs (µg/L)	Std. PAHs were loaded into SPE (µg/2 mL of hexane)	Fraction of Found (µg/g)	% REV
Naph	100	0.2	0.1670	83.49
Ace	100	0.2	0.2178	108.89
Phe	50	0.1	0.0886	88.56
Anth	100	0.2	0.1466	73.31
Fluo	10	0.02	0.0187	93.69
Pyr	50	0.1	0.0925	92.54
Chr	20	0.04	0.0343	85.69
BaPy	5	0.01	0.0073	73.48

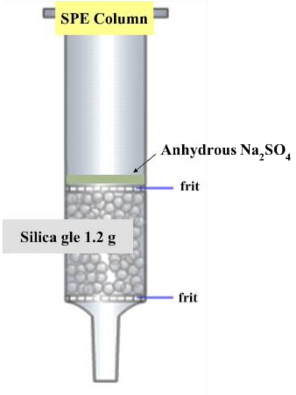
From the optimization data 2.1.1-2.1.2, it can be concluded the optimum condition of sample preparation method, SPE procedure for using florisil as shown in Figure 42 (a) and Figure 42 (b) as shown SPE procedure for using silica gel was succeeded studied by Sriviboon.[27]

(a) Florisil SPE Procedure



- Conditions: 30 mL of ACN, 6 mL of DCM and 3 mL of Hexane
- Load: 2 mL of raw lichen extract containing PAHs plus interferences
- Wash: 0.5 mL of Hexane : DCM (65 : 35) and then discarded the solvent 2.5 mL
- Elution: 4 mL of Hexane: DCM (65 : 35)

(b) Silica Gel SPE Procedureh



- Conditions: 6 mL of DCM and 3 mL of Hexane
- Load: 2 mL of raw lichen extract containing PAHs plus interferences
- Wash: 1.5 mL of Hexane : DCM (65 : 35) and then discarded the solvent 3.5 mL
- Elution: 2 mL of Hexane: DCM (65 : 35)

Figure 42 Schematic of Sample Preparation by Solid Phase Extraction (SPE) Procedure;

(a) Florisil SPE (b) Silica Gel SPE

Method Validation of Sample Preparation

1. Method Detection Limit (MDL) of Sample Preparation

In the experiment, two sorbent types, florisil and silica gel were evaluated. The MDL of all PAHs were determined in seven replicates by using of the lowest concentration and calculated by equation 2.1. The results of MDL of PAHs were shown in Table 31, it can conclude that florisil SPE was the suitable method and gave low concentration below than SPE from silica gel for preparation the lichen samples.

Table 31 The Method Detection Limit (MDL) of PAHs by using Florisil and Silica Gel for SPE (N = 7)

PAHs	MDL (μg/L)	
	Florisil SPE	Silica gel SPE
Naph	22.23	33.35
Ace	10.18	15.26
Phe	7.75	15.50
Anth	24.51	30.64
Fluo	1.74	2.62
Pyr	2.49	3.74
Chr	1.34	2.00
BaPy	0.56	0.84

2. Precision and Spiked Recovery of Sample Preparation

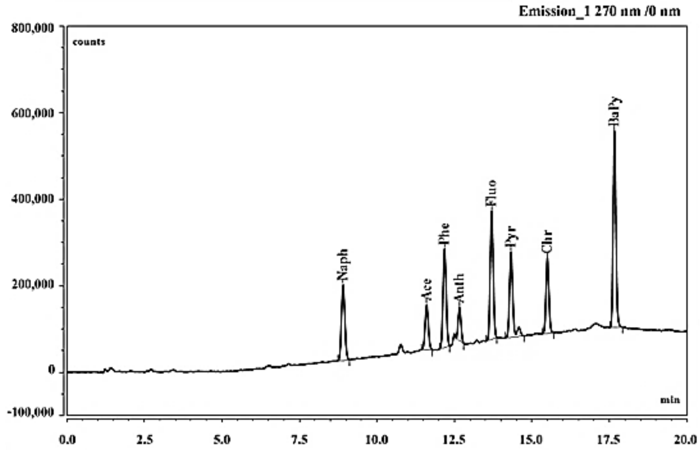
Florisil and silica gel, were evaluated by spiking with 250 µL of mixed standard PAHs into grounded lichen (0.1 g) samples. The precision and recovery data of this sample preparation method of SPE, florisil and silica gel, are shown in Table 32 and Table 33. Figure 43 compared the chromatogram of PAHs compounds in lichen after using two sorbents of sample preparation methods. From the result, it was found that florisil SPE gave a highest % recovery and precision. It can be concluded that cleanup procedure for solid phase extraction (SPE) of florisil is the suitable method for preparation the lichen samples.

Table 32 Precision and Recoveries of Spiked Lichen PAHs by using Florisil SPE (N = 7)

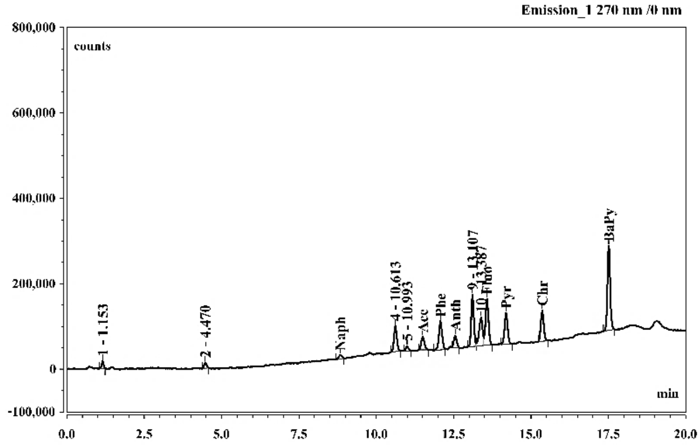
PAHs	Level 1			Level 3			Level 5		
	Conc. (μg/L)	% RSD	% REV	Conc. (μg/L)	% RSD	% REV	Conc. (μg/L)	% RSD	% REV
Naph	10	6.43	74.20	40	8.00	76.93	160	1.46	95.84
Ace	5	5.31	70.51	20	5.36	93.72	80	6.00	85.88
Phe	5	8.67	81.54	20	3.90	91.92	80	5.67	95.21
Anth	10	9.97	89.52	40	8.68	88.93	160	3.14	93.94
Fluo	2	8.13	94.00	8	6.79	89.98	32	9.82	84.91
Pyr	2	2.36	101.34	8	3.64	85.41	32	6.76	86.17
Chr	1	8.96	92.16	4	6.10	90.61	16	6.38	83.27
BaPy	1	9.87	73.36	4	3.74	91.52	16	5.40	81.08

Table 33 Precision and Recoveries of Spiked Lichen PAHs by using Silica Gel SPE (N = 7)

PAHs	Level 1			Level 3			Level 5		
	Conc. (µg/L)	% RSD	% REV	Conc. (µg/L)	% RSD	% REV	Conc. (µg/L)	% RSD	% REV
Naph	10	4.86	55.90	40	4.83	46.92	160	3.08	53.46
Ace	5	13.04	50.01	20	7.43	46.15	80	4.94	60.08
Phe	5	6.05	64.45	20	9.71	47.67	80	6.73	64.90
Anth	10	8.60	61.96	40	6.24	43.78	160	3.09	51.16
Fluo	2	23.60	66.97	8	10.66	65.87	32	4.84	56.67
Pyr	2	11.95	66.58	8	19.08	40.47	32	18.78	27.86
Chr	1	4.90	50.74	4	8.68	34.46	16	9.14	52.44
BaPy	1	7.10	45.11	4	9.03	40.65	16	8.86	55.33



(a) Florisil SPE



(b) Silica Gel SPE

Figure 43 Comparison the Chromatograms of PAH Compounds in the Lichen after using Two Sorbents, (a) Florisil SPE and (b) Silica Gel SPE

Summary procedures of the optimum condition for sample preparation were extraction by ultrasound-assisted and cleanup crude extract by florisil SPE method for analysis PAHs accumulated in lichen sample by HPLC shown in Figure 44 and Table 18 showed the chromatographic condition for analysis of PAHs accumulation in the lichen *Parmotrema tinctorum*.

0.1 g of homogenized lichens samples

↓

Extraction

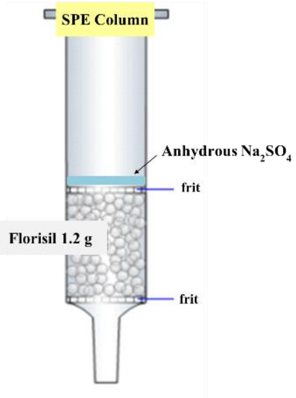
UAE

- With 0.7 mL of hexane/ 3 times
- Extraction time: 10 minutes, T 30 °C, Power 100%, Frequency 37 kHz.
- Centrifuge time: 10 minutes at room temperature

↓ 2 mL of raw lichen extract containing PAHs plus interferences

Clean up

SPE Procedure



- Conditions: 30 mL of ACN, 6 mL of DCM and 3 mL of Hexane
- Load: 2 mL of raw lichen extract
- Wash: 0.5 mL of Hexane : DCM (65 : 35) and then discarded the solvent 2.5 mL
- Elution: 4 mL of Hexane: DCM (65 : 35)

↓ Purified extract containing PAHs

Concentration

- Evaporation under Nitrogen gas
- Add 2 mL of Acetonitrile

↓

Quantitative analysis by HPLC (Chromatographic Condition in Table 18)

Figure 44 Schematic of Determination of PAHs Accumulation in Lichen Samples

Analysis of Lichen Samples

The lichen, *Parmotrema tinctorum*, were collected from bark of trees. They were collected from three localities:

1) The control site, at Nong Keing in Khao Yai National Park (KNP), at about 1,000 m. away from the park's road.

2) Tourist sites at Khao Yai National Park (TKNP) included the golf course, Nong Keing and Saisorn reservoir. They were collected from four different distances from the road, 0-20, 100-200, 300-400, > 500 m in January 2017. Each site was collected 5 samples.

3) Polluted sites in Bangkok at different traffic congestion (PBKT), performed by transplanting lichens from KNP to Lam Sali intersection, Rama 9 road intersection and the garden beside the Science Office Building (SCO) at Ramkhamhaeng University. The transplantations were during 7 March 2017 to 7 April 2017.

In this study was to compare the average amount of PAHs accumulated in lichen between control site with Tourist sites at Khao Yai National Park (TKNP) and control site with Polluted sites in Bangkok at different traffic congestion (PBKT).

From TKNP, The results were shown in the Table 34, 35, 36 and Figure 45, it can be concluded that at the golf course accumulated PAHs in the range as follow: naphthalene 666.32-768.42 ng/g, acenaphthene 688.41-1134.83 ng/g, phenanthrene 340.06-386.74 ng/g, anthracene 185.10-234.51 ng/g, fluoranthene

191.20-300.10 ng/g, pyrene 158.97-192.61 ng/g, chrysene ND-187.58 ng/g and benzo[a]pyrene 167.59-255.44 ng/g. Among these, acenaphthene, phenanthrene, anthracene, chrysene and benzo[a]pyrene were higher than the control site. Lichen at Nong Keing accumulated PAHs in the range as follow: naphthalene 602.53-937.10 ng/g, acenaphthene ND-973.07 ng/g, phenanthrene 367.86-1161.34 ng/g, anthracene ND-241.20 ng/g, fluoranthene 212.01-289.89 ng/g, pyrene 165.90-180.95 ng/g and benzo[a]pyrene 170.86-212.25 ng/g. These sites had acenaphthene, phenanthrene and benzo[a]pyrene higher than the control site. At Saisorn reservoir found PAHs in the range as follow: naphthalene 497.75-623.12 ng/g, phenanthrene 270.98-326.45 ng/g, fluoranthene 198.54-239.01 ng/g, pyrene 159.80-182.04 ng/g, chrysene ND-172.76 ng/g and benzo[a]pyrene ND-213.71 ng/g. However, chrysene and benzo[a]pyrene were higher than the control site. Concentration difference of each PAH within or among sites could cause by the processes of incomplete combustion of forest burning and fossil fuel combustion from tourist traffic, which were the effects of human activities.

Table 34 The Average Amount of PAHs (ng/g) in the Lichen, *Parmotrema tinctorum*, at Four Different Distances from the Road at Golf Course (G) from Khao Yai National Park (N = 5)

PAHs	Control ($\bar{X}$ ± SD) (ng/g)	G1 $\bar{X}$ ± SD (ng/g)	G2 $\bar{X}$ ± SD (ng/g)	G3 $\bar{X}$ ± SD (ng/g)	G4 $\bar{X}$ ± SD (ng/g)	Concentration Range of PAHs Detected (ng/g)	Average
Naph	863.41 ± 274.94	666.32 ± 96.48	701.46 ± 87.35	768.42 ± 169.21	754.54 ± 135.10	666.32-768.42	722.68
Ace	ND	ND	1134.83 ± 343.47	688.41 ± 22.78	ND	ND-1134.83	455.81
Phe	326.32 ± 30.84	342.63 ± 102.73	352.64 ± 99.93	386.74 ± 93.53	340.06 ± 31.78	340.06-386.74	355.52
Anth	169.15 ± 79.26	185.10 ± 58.26	234.51 ± 90.77	209.21 ± 125.02	ND	185.10-234.51	157.20
Fluo	331.91 ± 42.93	191.20 ± 12.66	239.26 ± 13.31	230.51 ± 31.17	300.10 ± 24.65	191.20-300.10	240.27
Pyr	283.98 ± 48.34	158.97 ± 4.93	184.36 ± 9.00	179.76 ± 23.20	192.61 ± 20.36	158.97-192.61	178.93
Chr	ND	ND	187.58 ± 9.59	ND	ND	ND-187.58	46.90
BaPy	ND	ND	255.44 ± 19.92	167.59 ± 10.33	ND	167.59-255.44	105.76

ND means that non-defined detected

Distance from road; G1 = 0-20 m, G2 = 100-200 m, G3 = 300-400 m, G4 = >500 m and Control = 1,000 m

Table 35 The Average Amount of PAHs (ng/g) in the Lichen, *Parmotrema tinctorum*, at Four Different Distances from the Road at Nong Keing (N) from Khao Yai National Park (N = 5)

PAHs	Control ($\overline{X}$ ± SD) (ng/g)	N1 $\overline{X}$ ± SD (ng/g)	N2 $\overline{X}$ ± SD (ng/g)	N3 $\overline{X}$ ± SD (ng/g)	N4 $\overline{X}$ ± SD (ng/g)	Concentration Range of PAHs Detected (ng/g)	Average
Naph	863.41 ± 274.94	920.04 ± 1114.94	937.10 ± 333.02	739.49 ± 152.02	602.53 ± 7.15	602.53-937.10	799.79
Ace	ND	ND	ND	973.07 ± 490.10	ND	ND-973.07	243.27
Phe	326.32 ± 30.84	1161.34 ± 133.73	410.21 ± 163.22	393.11 ± 187.21	367.86 ± 90.31	367.86-1161.34	583.13
Anth	169.15 ± 79.26	ND	ND	241.20 ± 80.36	ND	ND-241.20	60.30
Fluo	331.91 ± 42.93	289.89 ± 11.00	212.01 ± 10.27	237.24 ± 12.86	216.71 ± 4.04	212.01-289.89	238.96
Pyr	283.98 ± 48.34	180.95 ± 4.08	165.90 ± 7.18	172.55 ± 9.62	174.97 ± 16.65	165.90-180.95	173.60
Chr	ND	ND	ND	ND	ND	ND	ND
BaPy	ND	ND	ND	212.25 ± 26.70	170.86 ± 2.88	170.86-212.25	127.70

ND means that non-defined detected

Distance from road; N1 = 0-20 m, N2 = 100-200 m, N3 = 300-400 m, N4 = >500 m and Control = 1,000 m

Table 36 The Average Amount of PAHs (ng/g) in the Lichen, *Parmotrema tinctorum*, at Four Different Distances from the Road at Saisorn Reservoir (S) from Khao Yai National Park (N = 5)

PAHs	Control ($\bar{X}$ ± SD) (ng/g)	S1 $\bar{X}$ ± SD (ng/g)	S2 $\bar{X}$ ± SD (ng/g)	S3 $\bar{X}$ ± SD (ng/g)	S4 $\bar{X}$ ± SD (ng/g)	Concentration Range of PAHs Detected (ng/g)	Average
Naph	863.41 ± 274.94	623.12 ± 14.22	606.27 ± 20.96	606.94 ± 23.32	497.75 ± 282.28	497.75-623.12	583.52
Ace	ND	ND	ND	ND	ND	ND	ND
Phe	326.32 ± 30.84	270.98 ± 27.84	285.51 ± 18.05	271.82 ± 19.03	326.45 ± 29.03	270.98-326.45	288.69
Anth	169.15 ± 79.26	ND	ND	ND	ND	ND	ND
Fluo	331.91 ± 42.93	234.35 ± 8.91	226.14 ± 14.72	198.54 ± 4.99	239.01 ± 9.76	198.54-239.01	224.51
Pyr	283.98 ± 48.34	170.63 ± 5.46	171.72 ± 7.22	159.80 ± 4.61	182.04 ± 7.88	159.80-182.04	171.05
Chr	ND	ND	ND	ND	172.76 ± 5.89	ND-172.76	43.19
BaPy	ND	165.06 ± 6.23	179.70 ± 5.04	ND	213.71 ± 13.80	165.06-213.71	139.62

ND means that non-defined detected

Distance from road; S1 = 0-20 m, S2 = 100-200 m, S3 = 300-400 m, S4 = >500 m and Control = 1,000 m

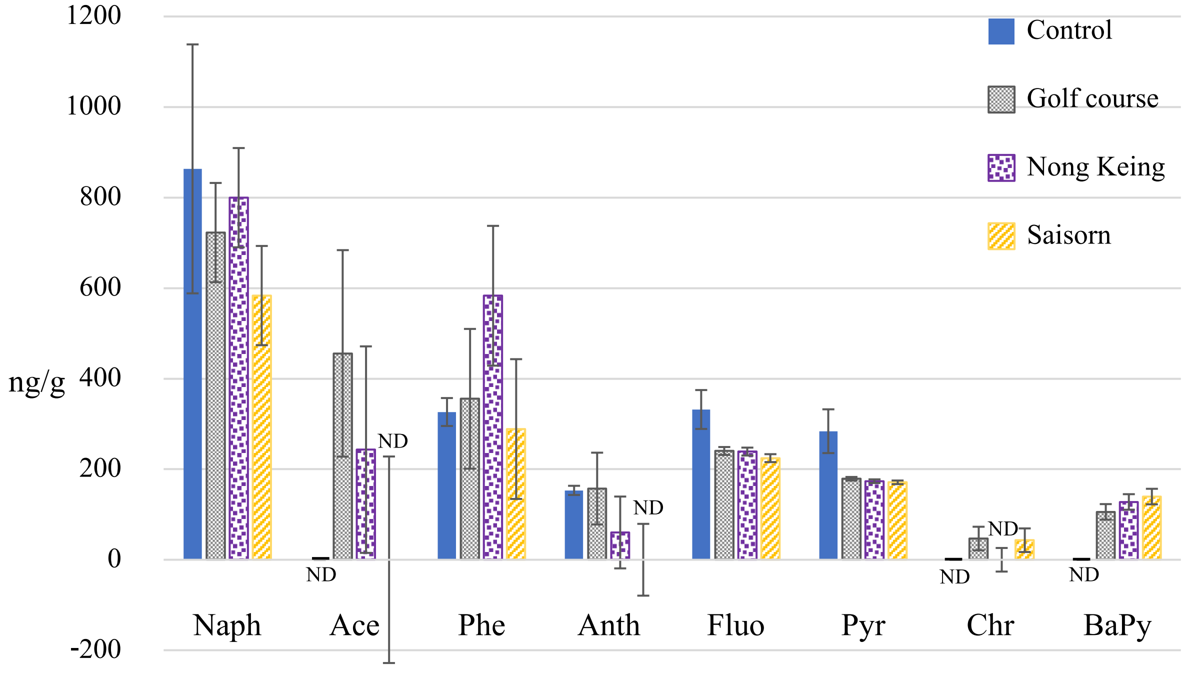


Figure 45 Comparison of the Total Average Amount of PAHs (ng/g) in the Lichen *Parmotrema tinctorum* at Three Sites, Golf Course (G), Nong Keing (N) and Saisorn Reservoir (S), from Khao Yai National Park

From PBKT, The results were shown in the Table 37, 38, 39 and figure 46, it can be conclude that the lichen at Lam Sali intersection had PAH~~s~~ concentrations in the range as follow: naphthalene ND-617.91 ng/g, phenanthrene 240.66-420.78 ng/g, anthracene 241.67-547.51 ng/g, fluoranthene 244.82-313.36 ng/g, pyrene 259.92-334.82 ng/g, chrysene 177.96-208.68 ng/g and benzo[a]pyrene 159.64-169.21 ng/g. Interestingly, anthracene , pyrene , chrysene and benzo[a]pyrene were higher than the control site. The transplanted lichen Rama 9 road intersection had PAH concentrations in the range as follow: naphthalene 576.41-614.05 ng/g, phenanthrene 211.40-265.34 ng/g, fluoranthene 262.69-364.91 ng/g, pyrene 265.42-380.07 ng/g, chrysene 189.82-219.35 ng/g and benzo[a]pyrene 179.63-204.98 ng/g. Among these pyrene, chrysene, and benzo[a]pyrene were higher than the control site. Lichens at the garden beside the Science Office Building at RU accumulated PAHs in the range as follow: naphthalene 587.84-611.33 ng/g, phenanthrene 347.12-479.14 ng/g, anthracene 352.65-541.75 ng/g, fluoranthene 252.80-424.33 ng/g and pyrene 277.21-352.02 ng/g. Notably, phenanthrene, anthracene and pyrene were higher than the control site.

Table 37 The Average Amount of PAHs (ng/g) in the Lichen, *Parmotrema tinctorum*, Transplanted from Khao Yai National Park to Lam Sali Intersection (L) in Bangkok during 7 March 2017 to 7 April 2017 (N = 5)

PAHs	Control ($\overline{X}$ ± SD) (ng/g)	L.1 $\overline{X}$ ± SD (ng/g)	L.2 $\overline{X}$ ± SD (ng/g)	L.3 $\overline{X}$ ± SD (ng/g)	L.4 $\overline{X}$ ± SD (ng/g)	L.5 $\overline{X}$ ± SD (ng/g)	Concentration Range of PAHs Detected (ng/g)	Average
Naph	863.41 ± 274.94	ND	617.91 ± 0.01	ND	ND	ND	617.91	123.58
Ace	ND	ND	ND	ND	ND	ND	ND	ND
Phe	326.32 ± 30.84	244.30 ± 3.43	240.66 ± 0.89	241.28 ± 1.32	257.49 ± 3.04	420.78 ± 11.56	240.66-420.78	282.88
Anth	169.15 ± 79.26	241.67 ± 7.20	303.62 ± 6.06	255.47 ± 7.81	279.58 ± 0.81	547.51 ± 13.04	241.67-547.51	323.58
Fluo	331.91 ± 42.93	248.82 ± 0.09	249.10 ± 0.77	271.02 ± 1.15	313.36 ± 2.78	244.82 ± 0.61	244.82-313.36	265.42
Pyr	283.98 ± 48.34	259.92 ± 1.81	267.89 ± 3.97	295.45 ± 0.24	334.82 ± 0.66	274.18 ± 3.42	259.92-334.82	288.45
Chr	ND	177.96 ± 7.14	190.45 ± 5.53	188.90 ± 3.00	208.68 ± 2.43	181.62 ± 5.79	177.96-208.68	189.52
BaPy	ND	160.57 ± 2.79	157.92 ± 1.19	159.64 ± 5.89	163.66 ± 1.62	169.21 ± 6.50	159.64-169.21	162.20

ND means that non-defined detected

Table 38 The Average Amount of PAHs (ng/g) in the Lichen, *Parmotrema tinctorum*, Transplanted from Khao Yai National Park to Rama 9 Road Intersection (R9) in Bangkok during 7 March 2017 to 7 April 2017 (N = 5)

PAHs	Control ($\bar{X}$ ± SD) (ng/g)	R9.1 $\bar{X}$ ± SD (ng/g)	R9.2 $\bar{X}$ ± SD (ng/g)	R9.3 $\bar{X}$ ± SD (ng/g)	R9.4 $\bar{X}$ ± SD (ng/g)	R9.5 $\bar{X}$ ± SD (ng/g)	Concentration Range of PAHs Detected (ng/g)	Average
Naph	863.41 ± 274.94	ND	590.00 ± 7.64	576.41 ± 1.22	614.05 ± 7.79	ND	576.41-614.05	356.09
Ace	ND	ND	ND	ND	ND	ND	ND	ND
Phe	326.32 ± 30.84	255.14 ± 0.37	211.40 ± 2.25	265.10 ± 5.84	265.34 ± 2.57	263.38 ± 3.25	211.40-265.34	252.07
Anth	169.15 ± 79.26	ND	ND	ND	ND	ND	ND	ND
Fluo	331.91 ± 42.93	262.69 ± 1.88	281.57 ± 3.12	364.91 ± 2.36	280.56 ± 0.81	272.42 ± 0.99	262.69-364.91	292.43
Pyr	283.98 ± 48.34	265.42 ± 0.99	292.68 ± 0.35	380.07 ± 2.16	279.08 ± 3.19	266.71 ± 2.65	265.42-380.07	296.79
Chr	ND	203.53 ± 3.24	198.45 ± 2.37	215.54 ± 8.50	219.35 ± 1.57	189.82 ± 8.27	189.82-219.35	205.34
BaPy	ND	202.41 ± 1.73	186.60 ± 0.49	194.41 ± 8.97	204.98 ± 1.67	179.63 ± 0.06	179.63-204.98	193.60

ND means that non-defined detected

Table 39 The Average Amount of PAHs (ng/g) in the Lichen, *Parmotrema tinctorum*, Transplanted from Khao Yai National Park to the Garden beside the Science Office Building (SCO) in Bangkok during 7 March 2017 to 7 April 2017 (N = 5)

PAHs	Control ($\overline{X}$ ± SD) (ng/g)	SCO.1 $\overline{X}$ ± SD (ng/g)	SCO .2 $\overline{X}$ ± SD (ng/g)	SCO .3 $\overline{X}$ ± SD (ng/g)	SCO .4 $\overline{X}$ ± SD (ng/g)	SCO .5 $\overline{X}$ ± SD (ng/g)	Concentration Range of PAHs Detected (ng/g)	Average
Naph	863.41 ± 274.94	611.33 ± 3.79	ND	ND	587.84 ± 8.61	597.08 ± 4.85	587.84-611.33	359.25
Ace	ND	ND	ND	ND	ND	ND	ND	ND
Phe	326.32 ± 30.84	352.12 ± 2.75	352.70 ± 2.25	347.12 ± 0.75	399.56 ± 1.04	479.14 ± 3.13	347.12-479.14	386.13
Anth	169.15 ± 79.26	422.97 ± 5.86	352.65 ± 0.73	361.95 ± 5.57	440.81 ± 2.41	541.75 ± 11.30	352.65-541.75	424.03
Fluo	331.91 ± 42.93	252.80 ± 3.62	294.82 ± 1.60	280.72 ± 3.98	424.33 ± 2.45	347.63 ± 1.88	252.80-424.33	320.06
Pyr	283.98 ± 48.34	277.21 ± 1.77	326.81 ± 0.39	287.40 ± 9.92	291.78 ± 5.97	352.02 ± 1.50	277.21-352.02	307.04
Chr	ND	ND	ND	ND	ND	ND	ND	ND
BaPy	ND	ND	ND	ND	ND	ND	ND	ND

ND means that non-defined detected

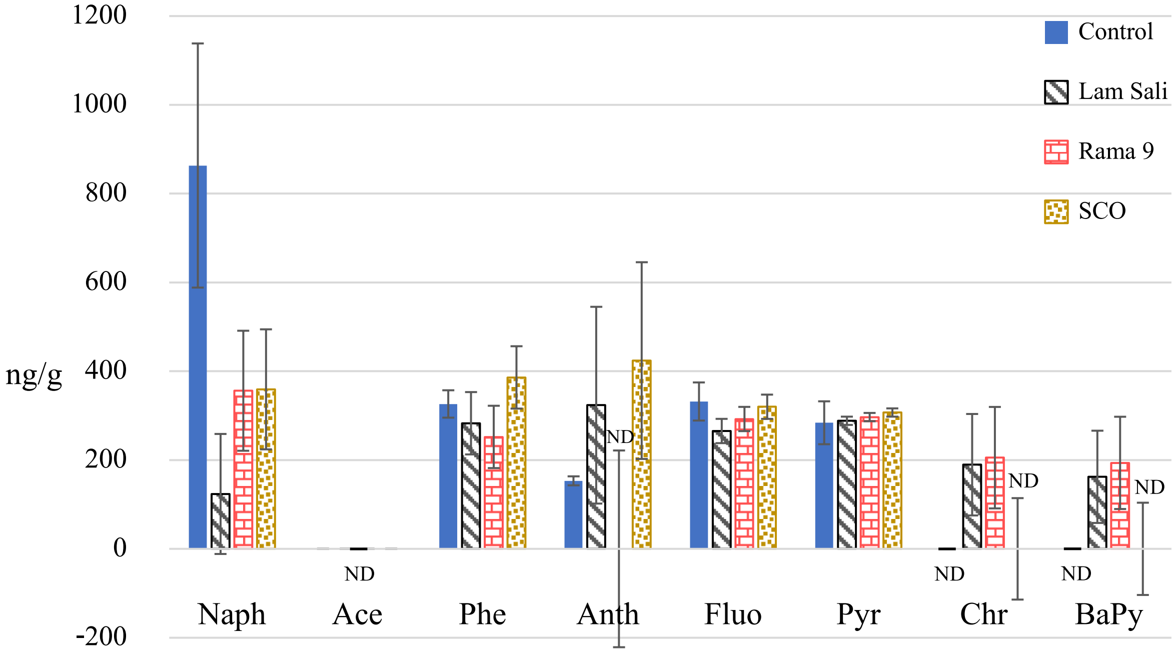


Figure 46 Comparison of the Total Average Amount of PAHs (ng/g) in the Lichen *Parmotrema tinctorum* Transplanted from Khao Yai National Park to Three Traffic Congestion Areas, Lam Sali Intersection (L), Rama 9 Road Intersection (R9) and the Garden beside the Science Office Building (SCO) in Bangkok

Although this study could be quantified the amounts of PAHs from different sites and localities, however concentration of each PAH among sites could also be the effect of local condition and seasonal variation such as wind direction and others.

CHAPTER 4

CONCLUSION

In this study, the analytical techniques were developed to determine PAHs accumulation in the lichen *Parmotrema tinctorum*. The studied PAHs were naphthalene (Naph), acenaphthene (Ace), phenanthrene (Phe), anthracene (Anth), fluoranthene (Fluo), pyrene (Pyr), chrysene (Chr) and benzo[a]pyrene (BaPy). The analytical method consisted of sample preparation and quantitative analysis. The procedure of sample preparation has two steps: Firstly, extraction of PAHs from lichens by ultrasound-assisted extraction (UAE) using hexane solvent. The optimum condition was 10 minutes extraction for three times at 30 ºC, 100% power output and mean operating frequency of 37 kHz. The suitable amount of lichen sample was 0.1 g. Secondly, clean up the extract solution using florisil mini-column as solid phase extraction cartridge (SPE). Before using the SPE cartridge, rinsing with 30.0 mL of acetonitrile and then conditioned with 6 mL of dichloromethane and 3 mL of hexane were done. After loading 2 mL of the sample, the optimum condition for elution of PAH compounds was the mixture of hexane and dichloromethane (65 : 35). The eluate was discarded 2.5 mL, and continue to collect 4 mL, which was then evaporated under nitrogen gas until dry. Subsequently, it was redissolved by 2 mL of acetonitrile before injection to HPLC. The validation results of sample preparation method comprised of detection limit (MDL) ranged 0.56 to 24.51 µg/L, the precision at a low level concentration showed % RSD lower than 9.97% and the percentage of spiked recovery analyte fall between 70.51% and 101.34%.

The analysis and quantification of lichen samples included high performance liquid chromatography (HPLC) coupled with diode array detector (DAD) and fluorescence detector (FLD). The optimum HPLC condition was mobile phase at flow rate 1.0 mL/min, the gradient solvent program began with 60% of acetonitrile for 3 minutes, then went to 100% acetonitrile in 15 minutes with a final hold of 5 minutes. The results of HPLC validation in terms limit of detection and limit of quantification were within the ranges of 0.54-22.83 μg/L and 1.60-69.18 μg/L. A linearity (r^2) was higher than 0.9996, an accuracy was greater than 99.61%, and the precision in term of % RSD was less than 3.59%. The HPLC with FLD has the characteristics of high sensitivity and low detection limit for PAHs compounds. The DAD had to obtain the specific UV spectra of the PAHs for peak identification and peak purity checks. It can be concluded that the developed method was suitable for analysis PAHs accumulated in the lichen *Parmotrema tinctorum*.

The lichen samples, *Parmotrema tinctorum*, collected from Khao Yai National Park (KNP) at Nakhon Ratchasima province were used to compare the amounts of PAHs among localities. There were three localities:
1) The control site, at Nong Keing, KNP, it is about 1,000 m. away from the park’s road. 2) The Tourist sites at Khao Yai National Park (TKNP), at the golf course, Nong Keing and Saisorn reservoir. The lichens were collected from four different distances from the road, 0-20, 100-200, 300-400, > 500 m in January 2017. It was found that lichens at the golf course accumulated PAHs as follow: naphthalene 666.32-768.42 ng/g, acenaphthene 688.41-1134.83 ng/g,

phenanthrene 340.06-386.74 ng/g, anthracene 185.10-234.51 ng/g, fluoranthene 191.20-300.10 ng/g, pyrene 158.97-192.61 ng/g, chrysene ND-187.58 ng/g and benzo[a]pyrene 167.59-255.44 ng/g. Among these, acenaphthene, phenanthrene, anthracene, chrysene and benzo[a]pyrene were higher than the control site. Lichen at Nong Keing accumulated PAHs as follow: naphthalene 602.53-937.10 ng/g, acenaphthene ND-973.07 ng/g, phenanthrene 367.86-1161.34 ng/g, anthracene ND-241.20 ng/g, fluoranthene 212.01-289.89 ng/g, pyrene 165.90-180.95 ng/g and benzo[a]pyrene 170.86-212.25 ng/g. These sites had acenaphthene, phenanthrene and benzo[a]pyrene higher than the control site. At Saisorn reservoir found PAHs as follow: naphthalene 497.75-623.12 ng/g, phenanthrene 270.98-326.45 ng/g, fluoranthene 198.54-239.01 ng/g, pyrene 159.80-182.04 ng/g, chrysene ND-172.76 ng/g and benzo[a]pyrene ND-213.71 ng/g. However, chrysene and benzo[a]pyrene were higher than the control site. Concentration difference of each PAH within or among sites could cause by the processes of incomplete combustion of forest burning and fossil fuel combustion from tourist traffic, which were the effects of human activities. 3) The Polluted sites in Bangkok at different traffic congestion (PBKT), included Lam Sali intersection, Rama 9 road intersection and the garden beside the Science Office Building (SCO) at Ramkhamhaeng University (RU). The lichens were transplanted to these sites during 7 March 2017 to 7 April 2017. The analysis showed that the lichen at Lam Sali intersection had PAH~~s~~ concentrations as follow: naphthalene ND-617.91 ng/g, phenanthrene 240.66-420.78 ng/g, anthracene 241.67-547.51 ng/g, fluoranthene 244.82-313.36 ng/g, pyrene 259.92-334.82 ng/g, chrysene 177.96-208.68 ng/g and benzo[a]pyrene 159.64-169.21 ng/g. Interestingly, anthracene, pyrene, chrysene and benzo[a]pyrene were higher than the control site. The transplanted lichen Rama 9 road intersection had PAH concentrations as follow: naphthalene 576.41-614.05 ng/g, phenanthrene 211.40-265.34 ng/g, fluoranthene 262.69-364.91 ng/g, pyrene 265.42-380.07 ng/g, chrysene 189.82-219.35 ng/g and benzo[a]pyrene 179.63-204.98 ng/g. Among these pyrene, chrysene and benzo[a]pyrene had higher than the control site. Lichens at the garden beside the Science Office Building at RU accumulated PAHs as follow: naphthalene 587.84-611.33 ng/g, phenanthrene 347.12-479.14 ng/g, anthracene 352.65-541.75 ng/g, fluoranthene 252.80-424.33 ng/g and pyrene 277.21-352.02 ng/g. Notably, phenanthrene, anthracene and pyrene were higher than the control site. Whereas chrysene and benzo[a]pyrene were under the detection limit, similar to the control site.

The analytical methods developed from this study for detecting PAHs accumulated in lichen revealed that it was efficient to distinguish concentration differences among sites, which had diverse traffic problem. Therefore, lichen can be used to assess air pollution caused by PAHs with more confidence.

APPENDIX A

The PAH Compounds (ng/g) in the Lichen, *Parmotrema tinctorum*, at Four Distances from the Road at Three Sites from Khao Yai National Park

The PAH Compounds (ng/g) in the Lichen, *Parmotrema tinctorum*, at Four Distances from the Road at Golf Course (G) from Khao Yai National Park

Site	Naph	Ace	Phe	Anth
G1-1	831.93 ± 0.18	ND	323.23 ± 0.01	289.04 ± 0.01
G1-2	639.98 ± 0.24	ND	270.26 ± 0.002	166.37 ± 0.11
G1-3	659.80 ± 0.20	ND	277.34 ± 0.31	156.04 ± 0.14
G1-4	611.37 ± 0.01	ND	321.16 ± 0.82	156.95 ± 0.22
G1-5	588.53 ± 0.77	ND	521.17 ± 0.64	157.09 ± 0.32
G2-1	639.69 ± 0.08	770.46 ± 0.01	282.60 ± 0.55	170.33 ± 0.43
G2-2	720.20 ± 0.06	1007.56 ± 0.05	323.92 ± 0.21	205.46 ± 0.66
G2-3	634.10 ± 0.71	1430.45 ± 0.11	310.45 ± 0.99	298.69 ± 0.41
G2-4	763.23 ± 0.61	1561.72 ± 0.33	529.19 ± 0.99	199.01 ± 0.01
G2-5	750.09 ± 0.91	903.94 ± 0.54	317.06 ± 0.98	299.04 ± 0.32
G3-1	687.89 ± 0.31	712.35 ± 0.01	551.18 ± 0.01	290.22 ± 0.31
G3-2	904.33 ± 0.56	666.99 ± 0.06	329.39 ± 0.04	177.44 ± 0.01
G3-3	990.89 ± 0.55	685.89 ± 0.09	374.51 ± 0.02	159.96 ± 0.08
G3-4	602.33 ± 0.67	ND	341.83 ± 0.67	ND
G3-5	656.64 ± 0.08	ND	336.78 ± 0.77	ND
G4-1	916.56 ± 0.01	ND	318.12 ± 0.75	ND
G4-2	615.89 ± 0.03	ND	386.29 ± 0.08	ND
G4-3	575.44 ± 0.02	ND	327.57 ± 0.03	ND
G4-4	672.03 ± 0.22	ND	309.72 ± 0.04	ND
G4-5	754.54 ± 0.11	ND	358.59 ± 0.01	ND

The PAH Compounds (ng/g) in the Lichen, *Parmotrema tinctorum*, at Four Distances from the Road at Golf Course (G) from Khao Yai National Park

Site	Fluo	Pyr	Chr	BaPy
G1-1	183.05 ± 0.22	155.37 ± 0.09	ND	ND
G1-2	175.85 ± 0.11	154.94 ± 0.03	ND	ND
G1-3	201.26 ± 0.03	165.48 ± 0.07	ND	ND
G1-4	206.52 ± 0.01	160.08 ± 0.01	ND	ND
G1-5	189.32 ± 0.04	ND	ND	ND
G2-1	248.68 ± 0.01	191.10 ± 0.01	192.32 ± 0.02	258.89 ± 0.09
G2-2	237.75 ± 0.08	186.73 ± 0.01	189.21 ± 0.44	285.28 ± 0.04
G2-3	256.54 ± 0.032	194.03 ± 0.003	184.13 ± 0.67	243.95 ± 0.01
G2-4	225.73 ± 0.31	173.66 ± 0.04	198.90 ± 0.31	257.12 ± 0.33
G2-5	227.58 ± 0.34	176.29 ± 0.01	173.35 ± 0.72	231.99 ± 0.01
G3-1	267.99 ± 0.82	213.78 ± 0.02	ND	176.67 ± 0.08
G3-2	197.03 ± 0.52	160.58 ± 0.04	ND	156.42 ± 0.55
G3-3	210.60 ± 0.63	161.91 ± 0.05	ND	160.87 ± 0.56
G3-4	258.92 ± 0.90	193.61 ± 0.02	ND	163.76 ± 0.61
G3-5	218.00 ± 0.07	168.95 ± 0.12	ND	180.24 ± 0.91
G4-1	296.38 ± 0.02	179.99 ± 0.78	ND	ND
G4-2	272.09 ± 0.03	178.80 ± 0.77	ND	ND
G4-3	320.90 ± 0.01	223.77 ± 0.05	ND	ND
G4-4	329.43 ± 0.05	203.00 ± 0.01	ND	ND
G4-5	281.70 ± 0.01	177.50 ± 0.08	ND	ND

The PAH Compounds (ng/g) in the Lichen, *Parmotrema tinctorum*, at Four Distances from the Road at Nong Keing (N) from Khao Yai National Park

Site	Naph	Ace	Phe	Anth
N1-1	286.72 ± 0.03	ND	1013.04 ± 0.21	ND
N1-2	511.90 ± 0.05	ND	1044.23 ± 0.81	ND
N1-3	210.84 ± 0.99	ND	1159.15 ± 0.71	ND
N1-4	706.94 ± 0.01	ND	1291.63 ± 0.34	ND
N1-5	2883.79 ±0.89	ND	1298.63 ± 0.56	ND
N2-1	1047.71 ± 0.08	ND	314.87 ±0 .14	ND
N2-2	1377.10 ± 0.67	ND	686.82 ± 0.27	ND
N2-3	580.37 ± 0.01	ND	339.29 ± 0.12	ND
N2-4	1054.31 ± 0.05	ND	284.87 ± 0.24	ND
N2-5	626.02 ± 0.31	ND	425.22 ± 0.06	ND
N3-1	870.57 ± 0.41	1034.43 ± 0.11	368.22 ± 0.45	ND
N3-2	598.19 ± 0.54	893.50 ± 0.51	325.95 ± 0.43	ND
N3-3	617.85 ± 0.61	ND	240.54 ± 0.33	298.02 ± 0.01
N3-4	ND	ND	717.73 ± 0.56	ND
N3-5	871.33 ± 0.81	991.29 ± 0.31	313.09 ± 0.78	184.38 ± 0.09
N4-1	601.95 ± 0.90	ND	362.67 ± 0.44	ND
N4-2	595.68 ± 0.00	ND	276.46 ± 0.87	ND
N4-3	ND	ND	319.28 ± 0.61	ND
N4-4	ND	ND	515.77 ± 0.21	ND
N4-5	609.95 ± 0.01	ND	365.14 ± 0.11	ND

The PAH Compounds (ng/g) in the Lichen, *Parmotrema tinctorum*, at Four Distances from the Road at Nong Keing (N) from Khao Yai National Park

Site	Fluo	Pyr	Chr	BaPy
N1-1	282.24 ± 0.01	178.94 ± 0.01	ND	ND
N1-2	279.60 ± 0.04	176.26 ± 0.90	ND	ND
N1-3	286.24 ± 0.05	179.62 ± 0.78	ND	ND
N1-4	306.73 ± 0.56	186.74 ± 0.07	ND	ND
N1-5	294.64 ± 0.06	183.22 ± 0.01	ND	ND
N2-1	206.11 ± 0.02	157.56 ± 0.06	ND	ND
N2-2	216.91 ± 0.01	163.24 ± 0.03	ND	ND
N2-3	226.97 ± 0.08	176.15 ± 0.02	ND	ND
N2-4	209.59 ± 0.01	169.77 ± 0.05	ND	ND
N2-5	200.47 ± 0.06	162.79 ± 0.07	ND	ND
N3-1	234.96 ± 0.03	ND	ND	257.40 ± 0.81
N3-2	258.59 ± 0.08	185.81 ± 0.01	ND	215.67 ± 0.42
N3-3	225.24 ± 0.07	166.21 ± 0.33	ND	195.21 ± 0.52
N3-4	237.67 ± 0.01	173.43 ± 0.32	ND	199.08 ± 0.45
N3-5	229.74 ± 0.03	164.75 ± 0.09	ND	193.86 ± 0.07
N4-1	216.75 ± 0.41	169.64 ± 0.06	ND	172.53 ± 0.01
N4-2	213.90 ± 0.89	166.93 ± 0.34	ND	166.73 ± 0.81
N4-3	217.23 ± 0.51	204.39 ± 0.56	ND	174.27 ± 0.01
N4-4	212.60 ± 0.01	163.68 ± 0.67	ND	169.57 ± 0.61
N4-5	223.06 ± 0.23	170.22 ± 0.78	ND	171.23 ± 0.01

The PAH Compounds (ng/g) in the Lichen, *Parmotrema tinctorum*, at Four Distances from the Road at Saisorn Reservoir (S) from Khao Yai National Park

Site	Naph	Ace	Phe	Anth
S1-1	614.96 ± 0.01	ND	312.96 ± 0.91	ND
S1-2	613.06 ± 0.05	ND	282.14 ± 0.51	ND
S1-3	615.47 ± 0.04	ND	241.10 ± 0.45	ND
S1-4	638.92 ± 0.02	ND	264.32 ± 0.78	ND
S1-5	633.17 ± 0.11	ND	254.38 ± 0.01	ND
S2-1	628.18 ± 0.21	ND	308.70 ± 0.08	ND
S2-2	492.19 ± 0.31	ND	265.85 ± 0.03	ND
S2-3	698.47 ± 0.89	ND	268.89 ± 0.02	ND
S2-4	621.11 ± 0.43	ND	295.01 ± 0.21	ND
S2-5	591.42 ± 0.61	ND	289.09 ± 0.01	ND
S3-1	623.43 ± 0.41	ND	270.28 ± 0.11	ND
S3-2	ND	ND	299.05 ± 0.09	ND
S3-3	ND	ND	251.18 ± 0.90	ND
S3-4	590.45 ± 0.03	ND	280.84 ± 0.78	ND
S3-5	ND	ND	257.74 ± 0.51	ND
S4-1	569.86 ± 0.05	ND	313.47 ± 0.71	ND
S4-2	600.97 ± 0.01	ND	353.22 ± 0.21	ND
S4-3	ND	ND	337.35 ± 0.41	ND
S4-4	619.33 ± 0.09	ND	281.98 ± 0.21	ND
S4-5	698.61 ± 0.01	ND	346.22 ± 0.21	ND

The PAH Compounds (ng/g) in the Lichen, *Parmotrema tinctorum*, at Four Distances from the Road at Saisorn Reservoir (S) from Khao Yai National Park

Site	Fluo	Pyr	Chr	BaPy
S1-1	244.00 ± 0.01	178.50 ± 0.04	ND	172.67 ± 0.01
S1-2	228.80 ± 0.81	163.06 ± 0.07	ND	157.78 ± 0.05
S1-3	224.06 ± 0.76	170.57 ± 0.01	ND	163.01 ± 0.51
S1-4	243.30 ± 0.54	170.60 ± 0.02	ND	161.53 ± 0.06
S1-5	231.57 ± 0.43	170.44 ± 0.21	ND	170.29 ± 0.01
S2-1	247.47 ± 0.05	179.60 ± 0.41	ND	178.77 ± 0.43
S2-2	216.29 ± 0.78	168.97 ± 0.56	ND	173.85 ± 0.23
S2-3	208.98 ± 0.98	160.75 ± 0.07	ND	176.40 ± 0.01
S2-4	227.58 ± 0.90	175.38 ± 0.09	ND	183.24 ± 0.22
S2-5	230.37 ± 0.01	173.92 ± 0.01	ND	186.26 ± 0.01
S3-1	192.95 ± 0.02	154.26 ± 0.54	ND	ND
S3-2	200.06 ± 0.08	156.99 ± 0.34	ND	ND
S3-3	193.54 ± 0.34	158.83 ± 0.88	ND	ND
S3-4	203.32 ± 0.31	163.54 ± 0.66	ND	ND
S3-5	202.83 ± 0.41	165.40 ± 0.63	ND	ND
S4-1	241.68 ± 0.23	193.79 ± 0.31	174.73 ± 0.71	222.42 ± 0.01
S4-2	247.95 ± 0.08	185.47 ± 0.11	178.68 ± 0.61	232.14 ± 0.41
S4-3	239.86 ± 0.64	180.39 ± 0.21	171.50 ± 0.034	212.22 ± 0.56
S4-4	222.40 ± 0.01	173.73 ± 0.81	163.27 ± 0.01	198.13 ± 0.66
S4-5	243.16 ± 0.08	176.83 ± 0.07	175.64 ± 0.02	203.66 ± 0.01

APPENDIX B

The PAH Compounds (ng/g) in the Lichen, *Parmotrema tinctorum*, Transplanted from Khao Yai National Park to Three Different Traffic Congestion Areas in Bangkok during 7 March 2017 to 7 April 2017

The PAH Compounds (ng/g) in the Lichen, *Parmotrema tinctorum*, Transplanted from Khao Yai National Park to Lam Sali Intersection (L) in Bangkok

Site	Naph	Ace	Phe	Anth	Fluo	Pyr	Chr	BaPy
L1-1	ND	ND	246.73 ± 0.07	246.76 ± 0.17	248.47 ± 0.31	258.64 ± 0.05	183.01 ± 0.01	158.60 ± 0.08
L1-2	ND	ND	241.87 ± 0.03	236.58 ± 0.37	249.17 ± 0.24	261.20 ± 0.04	172.91 ± 0.08	162.54 ± 0.34
L2-1	617.91 ± 0.01	ND	240.04 ± 0.11	307.91 ± 0.30	248.56 ± 0.34	270.70 ± 0.01	194.36 ± 0.003	158.75 ± 0.24
L2-2	617.90 ± 0.01	ND	241.29 ± 0.07	299.34 ± 0.05	249.64 ± 0.11	265.09 ± 0.09	186.54 ± 0.06	157.08 ± 0.56
L3-1	ND	ND	240.35 ± 0.08	260.99 ± 0.23	270.21 ± 0.01	295.61 ± 0.19	191.02 ± 0.05	155.48 ± 0.67
L3-2	ND	ND	242.21 ± 0.31	249.95 ± 0.11	271.84 ± 0.01	295.28 ± 0.23	186.79 ± 0.01	163.81 ± 0.04
L4-1	ND	ND	259.64 ± 0.21	279.01 ± 0.06	311.39 ± 0.08	345.29 ± 0.32	210.40 ± 0.00	162.51 ± 0.02
L4-2	ND	ND	255.34 ± 0.01	280.16 ± 0.01	315.32 ± 0.07	344.35 ± 0.67	206.96 ± 0.04	164.80 ± 0.01
L5-1	ND	ND	412.61 ± 0.05	538.29 ± 0.02	244.39 ± 0.21	276.60 ± 0.09	177.52 ± 0.14	173.81 ± 0.05
L5-2	ND	ND	428.95 ± 0.21	556.73 ± 0.03	245.25 ± 0.24	271.76 ± 0.01	185.71 ± 0.21	164.62 ± 0.89

The PAH Compounds (ng/g) in the Lichen, *Parmotrema tinctorum*, Transplanted from Khao Yai National Park to Rama 9 Road Intersection (R9) in Bangkok

Site	Naph	Ace	Phe	Anth	Fluo	Pyr	Chr	BaPy
R9.1-1	ND	ND	254.87 ± 0.15	261.36 ± 0.05	261.36 ± 0.38	266.12 ± 0.30	205.83 ± 0.03	201.19 ± 0.01
R9.1-2	ND	ND	255.40 ± 0.45	264.02 ± 0.19	264.02 ± 0.18	264.72 ± 0.34	201.24 ± 0.04	203.63 ± 0.01
R9.2-1	595.41 ± 0.21	ND	209.81 ± 0.05	279.36 ± 0.17	279.36 ± 0.001	292.92 ± 0.23	196.77 ± 0.06	186.94 ± 0.02
R9.2-2	584.60 ± 0.01	ND	212.99 ± 0.06	283.77 ± 0.56	283.77 ± 0.02	292.43 ± 0.11	200.12 ± 0.13	186.25 ± 0.03
R9.3-1	575.55 ± 0.05	ND	269.23 ± 0.08	366.58 ± 0.01	366.58 ± 0.01	378.54 ± 0.18	209.53 ± 0.02	200.75 ± 0.06
R9.3-2	577.27 ± 0.07	ND	260.97 ± 0.01	363.25 ± 0.45	363.25 ± 0.38	381.60 ± 0.09	221.55 ± 0.01	188.06 ± 0.07
R9.4-1	608.54 ± 0.07	ND	267.15 ± 0.01	279.98 ± 0.78	279.98 ± 0.08	276.82 ± 0.35	220.47 ± 0.07	206.16 ± 0.04
R9.4-2	619.55 ± 0.08	ND	263.52 ± 0.75	281.13 ± 0.17	281.13 ± 0.06	281.33 ± 0.12	218.24 ± 0.03	203.79 ± 0.02
R9.5-1	ND	ND	265.68 ± 0.05	273.12 ± 0.18	273.12 ± 0.09	268.59 ± 0.13	183.96 ± 0.04	179.58 ± 0.11
R9.5-2	ND	ND	261.08 ± 0.03	271.71 ± 0.10	271.71 ± 0.01	264.84 ± 0.45	195.67 ± 0.01	179.67 ± 0.00

The PAH Compounds (ng/g) in the Lichen, *Parmotrema tinctorum*, Transplanted from Khao Yai National Park to the Garden beside the Science Office Building (SCO) in Bangkok

Site	Naph	Ace	Phe	Anth	Fluo	Pyr	Chr	BaPy
SCO.1-1	608.65 ± 0.91	ND	350.18 ± 0.01	427.12 ± 0.21	255.36 ± 0.01	275.96 ± 0.06	ND	ND
SCO.1-2	614.01 ± 0.01	ND	354.07 ± 0.21	418.83 ± 0.09	250.24 ± 0.81	278.46 ± 0.07	ND	ND
SCO.2-1	ND	ND	354.29 ± 0.11	352.14 ± 0.41	293.68 ± 0.08	327.09 ± 0.04	ND	ND
SCO.2-2	ND	ND	351.11 ± 0.01	353.17 ± 0.06	295.95 ± 0.07	326.53 ± 0.01	ND	ND
SCO.3-1	ND	ND	346.58 ± 0.41	358.02 ± 0.01	277.90 ± 0.01	280.39 ± 0.03	ND	ND
SCO.3-2	ND	ND	347.65 ± 0.05	365.89 ± 0.08	283.53 ± 0.05	294.41 ± 0.04	ND	ND
SCO.4-1	581.75 ± 0.31	ND	398.82 ± 0.07	439.11 ± 0.51	426.07 ± 0.04	287.55 ± 0.06	ND	ND
SCO.4-2	593.93 ± 0.11	ND	400.30 ± 0.31	442.52 ± 0.67	422.60 ± 0.02	296.00 ± 0.07	ND	ND
SCO.5-1	593.65 ± 0.01	ND	476.92 ± 0.51	549.74 ± 0.81	346.31 ± 0.22	353.08 ± 0.01	ND	ND
SCO.5-2	600.51 ± 0.01	ND	481.35 ± 0.01	533.76 ± 0.01	348.96 ± 0.33	350.95 ± 0.89	ND	ND

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