Development of gas chromatographic and mass spectrometric techniques for the analysis of Polycyclic Aromatic Compounds (PACs) in environmental samples

By

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Thesis Abstract

Concerns emanating from the presence of polycyclic aromatic compounds (PACs) in the environment especially as a yardstick for overall environmental health have been a challenge for several decades. Polycyclic aromatic compounds are a complex and structurally diverse class of compounds many of which can exist as isomers. Approaches to measure these compounds in environmental samples have relied on techniques based on gas chromatography coupled to mass spectrometry (GC/MS) at unit mass resolution. For example, the quantitation of the 16 US Environmental Protection Agency priority polycyclic aromatic hydrocarbons (PAHs) uses GC/MS and selected ion monitoring (SIM) of diagnostic *m/z* values of individual PAHs. Although this approach works well for the 16 priority PAHs, for more complex PACs, GC/MS in SIM mode is largely unreliable. Reasons for this include low selectivity of the SIM mode, insufficient chromatographic and mass resolution and the lack of commercially available authentic analytical standards. *The overarching hypothesis of my thesis, therefore, is that more sophisticated chromatographic and MS techniques will provide more accurate measurements of PACs in the environment.* The first advancement I made to the field of PAC research was the development of a one-dimensional GC coupled to MS method in the multiple reaction monitoring (MRM) to measure a suite of PACs. The method I developed was fully validated according to the EURACHEM guide - *The Fitness for Purpose of Analytical methods* and represents a significant improvement in the current SIM approach to quantitation of PACs in environmental samples. The second major advancement I made to the field of PAC research was the validation of a

comprehensive 2D GC (GC×GC) high resolution time-of-flight (HR-TOF) MS method for the separation and quantitation of PACs. In this study I was able to exploit the increased peak capacity of the GC×GC system (relative to 1D) and the specificity of the HR-TOF/MS to quantify individual isomers of PAC compounds that were not attempted before. The methods I developed were paramount to the success of 2 other studies: (*i*) the GC/MS MRM method was used to understand the kinetics of PAH absorption to a highly sorptive medium that is used by industry to remediate the aquatic environment during/after an oil-spill and, (*ii*) the GC×GC-HR-TOF/MS was used in the detection of novel halogenated PACs in biological samples from the Alberta Oil Sands Region. The results of my work will have an impact and influence on future studies on source apportionment and chemical fingerprinting of crude-oil and will support much needed toxicological and monitoring studies of individual PAC isomers in the environment.

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Dedication

This thesis is dedicated to God and to everyone who made it a success.

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Chapter 1

1.0 Introduction

The recent economic advancement in the oil sector has led to an increased industrial and technological development of crude oil deposits to keep the oil flowing to markets. For example, the production from the Oil Sands deposit in northern Alberta, Canada, is projected to reach over 3.3 million barrels per day by 2020.[1] The economic influence of the sector is unarguably positive especially with countries whose economy solely or partly depends on crude oil. But the public concerns about the environmental impact of crude oil exploration and exploitation cannot be overlooked. Globally, combustion of fossil fuels (coal, oil, gasoline, diesel fuel and natural gas) produces the majority of air pollution from electricity production, heating, industry and transportation.[2] In 2017, Environment and Climate Change Canada (ECCC) reported that the oil and gas sector was a major contributor to total national emissions of volatile organic compounds (VOCs) (37%), sulphur oxides (SO_x) (22%), nitrogen oxides (NO_x) (25%) and carbon monoxide (CO) (10%) for the year 2015. These emissions are attributed to upstream activities (exploration, drilling, production and field processing) with downstream activities contributing no more than a fifth of air pollutant emissions.[3]

Crude oil is arguably one of the most complex and multi-component naturally occurring homogeneous mixtures and is made up of paraffins (straight chain and branched chain alkanes), naphthenes, aromatics with contents of non-hydrocarbons (e.g. nitrogen (N), sulfur (S) and oxygen (O)), resin and asphaltene.[4] The aromatic component of crude oil and those containing hetero-atoms (N, S, O) are broadly referred to as polycyclic aromatic compounds (PACs). These PACs

present in crude oil are also produced from other multiple sources including but not limited to incomplete combustion of fossil fuels, natural seeps or leakage, spills from drilling platforms during exploitation and accidental spills during transportation (via rail, road and pipeline). The multiple sources of these compounds account for their ubiquitous nature and their prevalence in the environment has been of health and environmental concern. Research reports have thus far detailed the persistence, bioaccumulation and toxicity (PBT) of some compounds in this group, popularly, the polycyclic aromatic hydrocarbons (PAHs), to both terrestrial and aquatic life. Actually, not all the PAHs have been widely studied except the selected 16 PAHs listed by the US environmental protection agency (EPA) back in the 1970s as priority pollutants.[5]

To aid in understanding PACs transformation, fate and effects in the environment, establishing a method that accurately quantifies individual components is of high importance. This has evidently helped in the analysis of the 16 EPA PAHs. Currently, the major difficulty in quantifying PACs is due, in part, to the inherent complexity of these compounds. The large number of compounds and possible isomers theoretically present in this group makes developing analytical methods challenging.

1.1 Polycyclic aromatic compounds (PACs)

The term PACs define a complex and broad class of organic molecules consisting of two or more fused aromatic rings. These rings contain mainly carbon, hydrogen and sometimes at least one of the heteroatoms: -(N)itrogen, -(S)ulphur and -(O)xygen giving rise to different compound of PACs. These include polycyclic aromatic hydrocarbons (PAHs), alkylated PAHs (alkyl-PAHs), nitrogen-containing polycyclic aromatic compounds (N-PACs), polycyclic aromatic sulphur

heterocycles (PASH) and oxygenated PAHs (oxy-PAHs). PAHs and alkyl-PAHs contain only carbon and hydrogen in their chemical structure. While PAHs are regarded as unsubstituted/native PAHs, alkyl-PAHs have substitution on their PAH ring system with one or more alkyl group. The PACs possessing heteroatoms are sometimes referred to as polar PAHs with varying polarity depending on the attached heteroatom.

A common feature of most PACs is their PAH backbone. This further explains why the PAHs are the most studied of these compound class. Keith and Telliard [5] identified 16 PAH compounds among other known organic chemical compounds for monitoring by the US Environmental Protection Agency (USEPA) and these were tagged 'Priority Pollutants'. The years following have experienced advancement in technology to increase detection and aid the synthesis of more PAH compounds. In 1997, Sander and Wise listed 660 parent PAH compounds ranging from the smallest aromatic molecule, benzene, up to the nine-ringed system.[6]

An expansion to the conventional group of PACs is the report of halogenated and cyano groups in the PAH ring structure. Recently, occurrence of halogenated PAHs (HPAHs) have been reported in food, biota, air, sediment, and fly-ash.[7-13]

1.2 Structure and Physicochemical properties of PACs

The structures of PACs are conventionally classified as cata-annellated (linearly or angularly) or pericondensed[14] with continuous circuits of conjugated π - electron density which accounts for their chemical stability and susceptibility to electrophilic substitution.[15] PACs vary in structure and also in physicochemical properties which determine their environmental transformation, fate and effects. Figure 1.1 shows the 16 EPA parent PAHs commonly analyzed with their carbon atom

numbering. Also included in Figure 1.1 are other PACs. Constitutionally, positions on the parent ring are available for substitution resulting in the potential for a large number of isomers[16]. Although they have numerous theoretical congeners, only a few of these have been successfully synthesized in the laboratory which has aided individual identification in environmental matrices furthering our understanding of their behavior in the environment.

Most PACs are regarded as chemically inert, stable compounds of varying volatility that reduces with increasing aromatic rings. They possess relatively high boiling and melting points with the simplest compound, naphthalene, having a boiling point of 218º C (Table 1). PACs are sparingly soluble in an aqueous medium with their solubility decreasing with increasing number of aromatic rings.[17] PACs are highly lipophilic (hydrophobic) in nature, which makes them partition into the lipid/fatty tissues of living organisms.

The volatility of PACs from a point source, sorption, aqueous solubility and accumulation are largely driven by their hydrophobicity. When in the atmosphere, they are mainly adsorbed on particles. Hydrophobicity can be measured using n-octanol/water (log K_{OW}) and n-octanol/air partition (log K_{OA}) coefficients. Octanol is an ideal surrogate phase for lipids in biological organisms and log K_{ow} and log K_{OA} represent how a chemical compound would thermodynamically partition between the lipids present in biological organisms and water/air.[18]

The main factor that drives their distribution is solubility and hydrophobicity. The K_{ow} of these organic compounds is related to their solubility. K_{ow} determines if the PACs will remain available in the water column or will prefer to partition into lipids, soil and sediment.

These coefficients (log K_{ow} and log $K_{OA)}$ originate from quantitative structure-activity relationships (QSAR) or quantitative structure-property relationships (QSPR) of chemical

compounds. The model obtained from examining these relationships describe the relationship between chemical structures, biological activity and chemical property for a set of chemical compounds. Log K_{ow} coefficient is known to increase with the number of rings while log K_{OA} decreases with the number of rings.[19] The relevance of log K_{OW} and log K_{OA} in predicting the bioaccumulation, bioconcentration, biomagnification and possibility of eventual toxicity of organic pollutants has been widely explored.[20-22]

Some PACs possess characteristic UV absorbance and are photosensitive as they are capable of undergoing structure alteration on exposure to light. Despite their chemical stability some can undergo transformation in the environment under the influence of visible and ultraviolet (UV) radiation; for example, anthracene can be photo-transformed to anthraquinone.

Table 1.1 presents some of the physico-chemical properties of some PACs. All parameters were adapted from Chemspider[23] and toxnet databases[24].

1.3 Occurrence, transport and fate of PACs in environmental samples

Polycyclic aromatic compounds represent a group of compounds that are known for their ubiquity, toxicity and persistence in the environment. They are widely detected in coastal and surface ocean ecosystems, air, soils, sediments, wastes, vegetation and food products. Although these compounds are not listed among Stockholm's Convention on Persistent Organic Pollutants (SC-POPs), they possess many of the same properties as POPs listed in the SC-POPs. These characteristics are as reported in Jones and Voogt.[25]

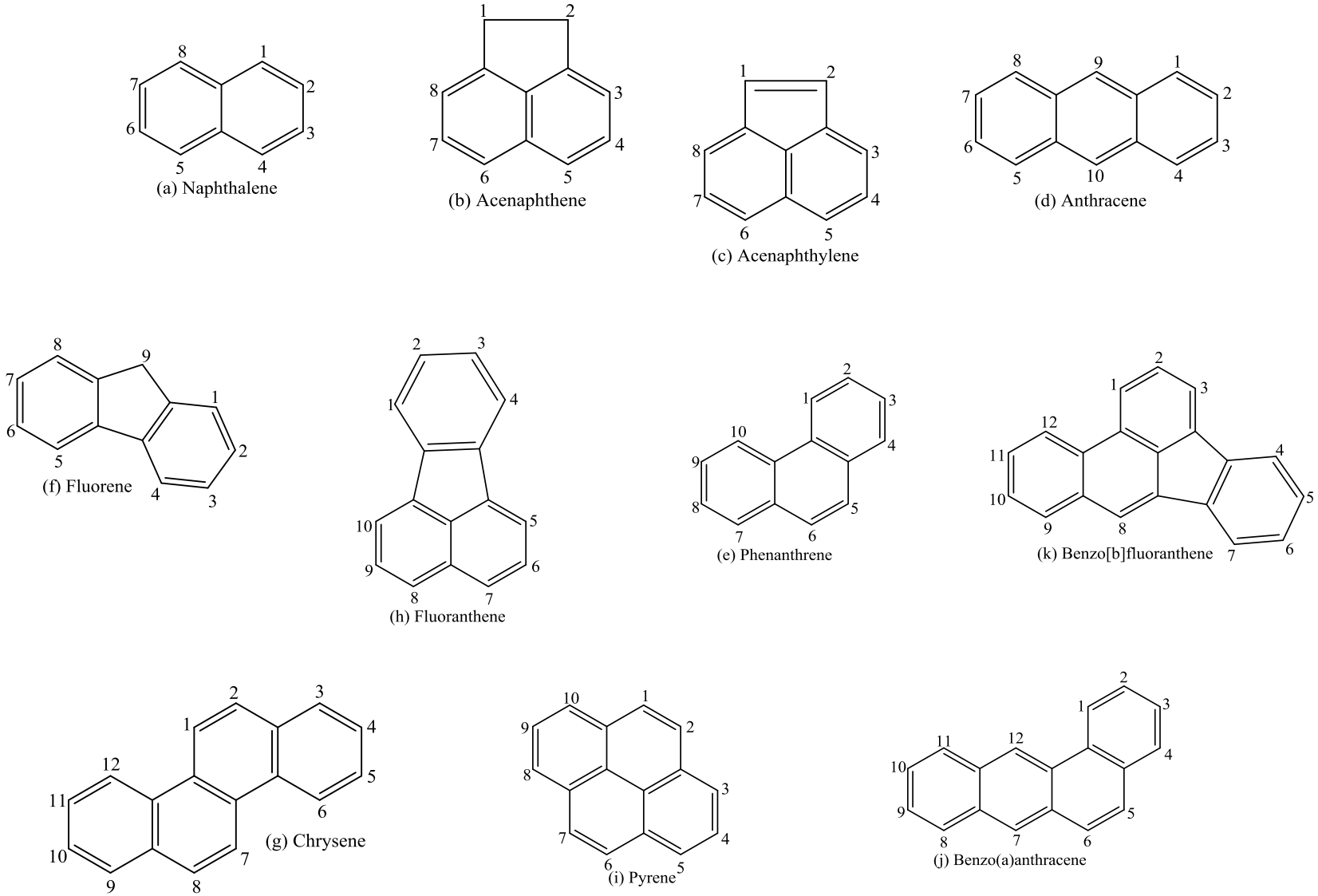

***Figure 1.1**: Structures and names of some representative PACs*

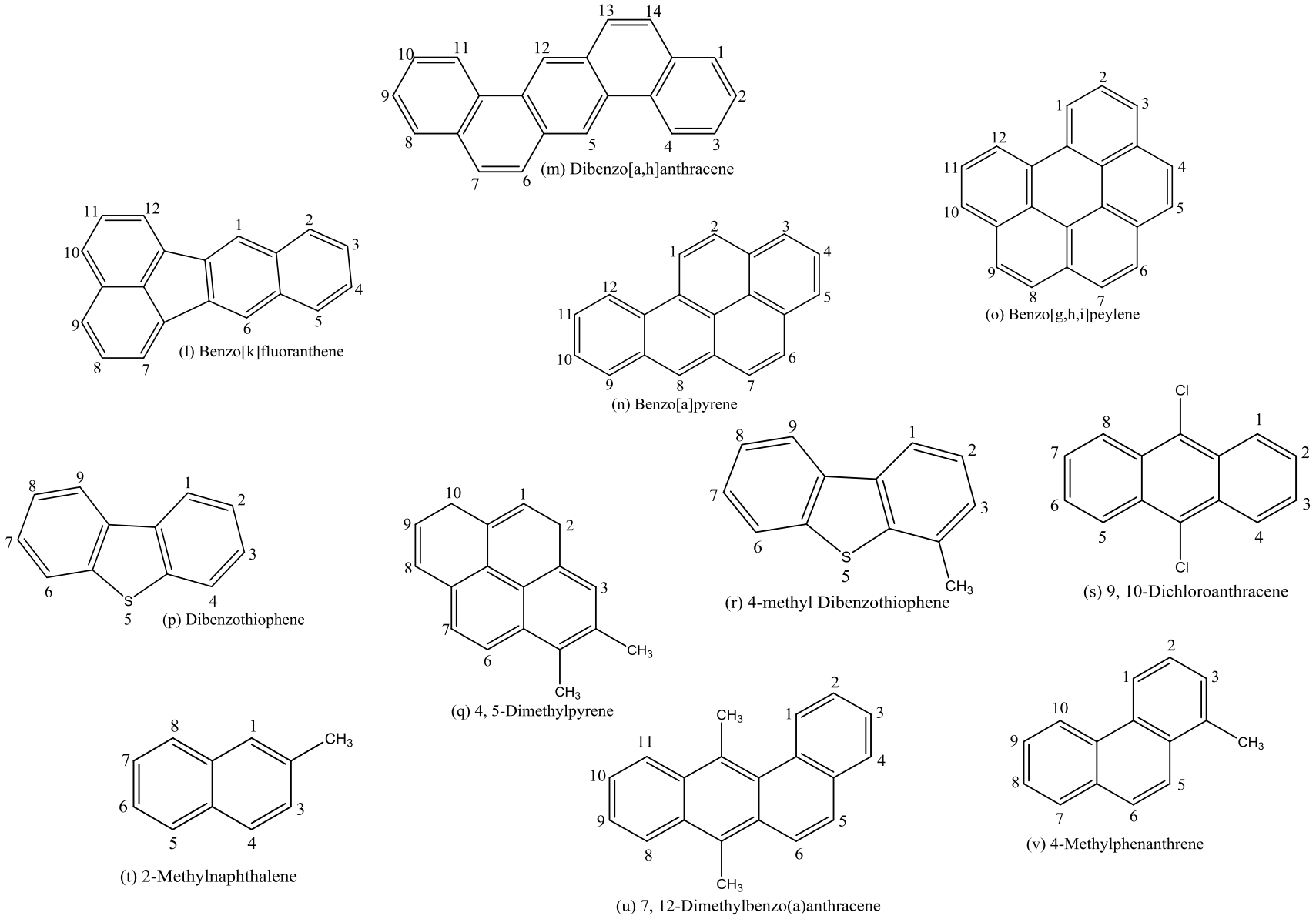

***Figure 1.1**: Structures and names of some representative PACs*

***Table 1.1**: Physicochemical properties of some polycyclic aromatic compounds (PACs). Data taken from references 23 and 24.*

Compound	Molecular weight (MW) Nominal mass	Molecular Formula (MF)	Water solubility (S) mg/L @25 ºC	Boiling point (ºC)	Melting point (ºC)	Vapour pressure (Vp) Pa @ 25 ºC	Log K_{ow}	Log K_{oa}	Henry's constant (H) Pa m^3/mol @ 25 ºC
Naphthalene	128	$C_{10}H_8$	3.10×10^{1}	217.9	80.20	1.13×10^{1}	3.30	5.05	44.58
Acenaphthene	154	$C_{12}H_{10}$	3.93×10^{0}	279.0	93.40	2.87×10^{-1}	3.92	6.31	18.64
Acenaphthylene	152	$C_{12}H_8$	1.61×10^{1}	280.0	92.50	8.91×10^{-1}	3.94	6.23	11.55
Fluorene	166	$C_{13}H_{10}$	1.69×10^{0}	295.0	114.8	7.99×10^{-2}	4.18	6.79	9.747
Phenanthrene	178	$C_{14}H_{10}$	1.15×10^{0}	340.0	99.20	1.61×10^{-2}	4.46	7.57	4.286
Anthracene	178	$C_{14}H_{10}$	6.91×10^{-1}	339.9	215.0	8.71×10^{-4}	4.45	7.55	5.634
Fluoranthene	202	$C_{16}H_{10}$	2.60×10^{-1}	384.0	107.8	1.23×10^{-3}	5.16	8.80	0.898
Pyrene	202	$C_{16}H_{10}$	1.35×10^{-1}	404.0	151.2	5.99×10^{-4}	4.88	8.80	1.206
Benz(a)anthracene	228	$C_{18}H_{12}$	9.40×10^{-3}	437.6	84.00	2.80×10^{-5}	5.76	9.07	1.216
Chrysene	228	$C_{18}H_{12}$	2.00×10^{-3}	448.0	258.2	8.31×10^{-7}	5.81	9.48	0.530
Benzo(b)fluoranthene	252	$C_{20}H_{12}$	1.50×10^{-3}	442.8	168.0	6.67×10^{-5}	5.78	10.4	0.067
Benzo(k)fluoranthene	252	$C_{20}H_{12}$	8.00×10^{-4}	480.0	217.0	1.05×10^{-7}	6.11	-	0.059
Benzo(a)pyrene	252	$C_{20}H_{12}$	1.62×10^{-3}	377.0	176.5	3.25×10^{-4}	6.13	7.82	0.046
Dibenz(a,h)anthracene	278	$C_{22}H_{14}$	2.49×10^{-3}	524.0	269.5	1.27×10^{-7}	6.75	11.2	1.010×10^{-5}
Indeno(1,2,3-c,d)pyrene	276	$C_{22}H_{12}$	2.79×10^{-2}	404.1	161.3	3.52×10^{-5}	6.79	8.79	23.00
Benzo(g,h,i)perylene	276	$C_{22}H_{12}$	2.49×10^{-3}	486.3	199.7	1.17×10^{-7}	6.63	12.0	0.034
1-methylnaphthalene	142	$C_{11}H_{10}$	2.58×10^{1}	244.7	34.00	8.93×10^{0}	3.87	5.55	52.08
2-methylnaphthalene	142	$C_{11}H_{10}$	2.46×10^{1}	241.1	34.40	7.33×10^{0}	3.86	5.53	52.49
2,6-dimethylnapthalene	156	$C_{12}H_{12}$	2.00×10^{0}	262.0	112.0	7.57×10^{-1}	4.31	5.89	64.95
1,6-dimethylnapthalene	156	$C_{12}H_{12}$	9.00×10^{-1}	264.0	-16.90	1.95×10^{0}	4.26	6.02	64.95
1,3-dimethylnaphthalene	156	$C_{12}H_{12}$	8.00×10^{0}	263.0	-6.000	1.12×10^{0}	4.42	6.00	64.95
1,4-dimethylnaphthalene	156	$C_{12}H_{12}$	1.14×10^{1}	268.0	7.600	1.12×10^{0}	4.37	5.95	64.95
1,2-dimethylnaphthalene	156	$C_{12}H_{12}$	1.49×10^{1}	266.5	1.600	1.69×10^{0}	4.31	5.89	64.95
1,5-dimethylnaphthalene	156	$C_{12}H_{12}$	2.74×10^{0}	265.0	82.00	1.12×10^{0}	4.38	6.22	35.46
1,7-dimethylnaphthalene	156	$C_{12}H_{12}$	1.15×10^{1}	263.0	-16.90	1.12×10^{0}	4.44	6.02	64.95
2,3-dimethylaphthalene	156	$C_{12}H_{12}$	1.99×10^{0}	268.0	105.0	1.47×10^{-1}	4.40	5.83	93.22
1-ethylnaphthalene	156	$C_{12}H_{12}$	1.07×10^{1}	258.6	-13.90	3.36×10^{0}	4.40	5.95	69.41

2-ethylnaphthalene	156	$C_{12}H_{12}$	8.01×10^{0}	258.0	-7.400	4.21×10^{0}	4.38	6.04	54.51
1,4,5-trimethylnaphthalene	170	$C_{13}H_{12}$	2.10×10^{0}	282.8	55.60	0.34×10^{0}	4.90	6.44	71.64
1,6,7-trimethylnaphthalene	170	$C_{13}H_{12}$	4.78×10^{0}	282.8	55.60	0.34×10^{0}	4.81	6.35	71.64
2,3,6-trimethylnaphthalene	170	$C_{13}H_{12}$	1.70×10^{0}	282.8	55.60	0.34×10^{0}	4.73	6.27	71.64
1,2,3,4-tetramethynaphthalene	184	$C_{14}H_{14}$	1.39×10^{0}	298.1	72.00	0.10×10^{0}	5.36	6.86	79.03
1-methylfluorene	180	$C_{14}H_{12}$	1.09×10^{0}	307.3	80.00	5.31×10^{-2}	4.97	7.09	18.75
9-methylfluorene	180	$C_{14}H_{12}$	1.22×10^{0}	303.1	46.50	8.27×10^{-2}	4.15	6.19	22.49
4-; 3- & 2-methylfluorene	180	$C_{14}H_{12}$	5.41×10^{-1}	307.3	79.90	5.31×10^{-2}	4.56	6.68	18.75
1,9-dimethylfluorene	194	$C_{15}H_{14}$	3.54×10^{-1}	317.1	86.30	2.69×10^{-2}	4.70	6.70	24.83
2,7-dimethylfluorene	194	$C_{15}H_{14}$	1.57×10^{-1}	321.0	88.50	2.08×10^{-2}	5.11	7.19	20.67
2,3-dimethylfluorene	194	$C_{15}H_{14}$	1.71×10^{1}	297.3	74.40	1.02×10^{-1}	5.20	5.99	396.2
9-ethylfluorene	194	$C_{15}H_{14}$	3.95×10^{-1}	317.1	80.00	3.12×10^{-2}	4.64	6.56	29.89
2,7,9- &2,3,9-trimethylfluorene	208	$C_{16}H_{16}$	1.02×10^{-1}	330.3	94.40	1.10×10^{-2}	5.24	7.20	27.46
3-methylphenanthrene	192	$C_{15}H_{12}$	2.63×10^{-1}	350.0	65.00	8.89×10^{-3}	5.15	7.50	5.745
2-methylphenanthrene	192	$C_{15}H_{12}$	2.80×10^{-1}	339.8	93.61	6.68×10^{-3}	4.86	7.50	5.745
9-methylphenanthrene	192	$C_{15}H_{12}$	2.47×10^{-1}	339.8	93.61	6.68×10^{-3}	4.89	7.53	5.745
4-methylphenanthrene	192	$C_{15}H_{12}$	2.69×10^{-1}	339.8	123.0	1.99×10^{-3}	5.08	7.78	4.995
2-methylanthracene	192	$C_{15}H_{12}$	2.13×10^{-2}	339.8	209.0	7.12×10^{-3}	5.00	7.63	5.745
1-methylphenanthrene	192	$C_{15}H_{12}$	2.69×10^{-1}	339.8	123.0	1.99×10^{-3}	5.08	7.78	4.995
3,6-dimethylphenanthrene	206	$C_{16}H_{14}$	7.10×10^{-2}	351.5	108.9	1.01×10^{-3}	5.44	8.03	6.333
1,8-dimethylphenanthrene	206	$C_{16}H_{14}$	7.10×10^{-2}	351.5	108.9	1.01×10^{-3}	5.44	8.03	6.333
Ethylphenanthrene	206	$C_{16}H_{14}$	7.97×10^{-2}	351.5	102.6	2.82×10^{-3}	5.38	7.89	7.620
Trimethylphenanthrene	220	$C_{17}H_{16}$	2.05×10^{-2}	363.1	115.6	1.09×10^{-3}	5.99	8.54	6.991
1-ethyl-2-methylphenanthene	220	$C_{17}H_{16}$	2.29×10^{-2}	363.1	117.7	1.03×10^{-3}	5.93	8.40	8.410

9-ethyl-10-methylphenanthene	220	$C_{17}H_{16}$	2.29×10^{-2}	363.1	117.7	1.03×10^{-3}	5.93	8.40	8.410
Tetramethylphenanthrene	234	$C_{18}H_{18}$	5.88×10^{-3}	374.7	128.0	4.23×10^{-4}	6.53	9.04	7.721
1-Methylfluoranthene	216	$C_{17}H_{12}$	5.84×10^{-2}	383.5	132.3	2.33×10^{-4}	5.48	8.91	0.928
3-Methylfluoranthene	216	$C_{17}H_{12}$	1.53×10^{0}	333.4	104.9	7.20×10^{-3}	5.12	-	-
7,10-dimethylfluoranthene	230	$C_{18}H_{14}$	1.68×10^{-2}	395.1	137.8	1.07×10^{-4}	6.03	9.41	1.023
2,3-dimethylfluoranthene	230	$C_{18}H_{14}$	4.40×10^{-1}	344.8	115.7	3.04×10^{-3}	5.67	-	-
7.8-dimethylfluoranthene	230	$C_{18}H_{14}$	2.24×10^{-2}	361.4	122.1	1.02×10^{-3}	5.88	8.14	13.68
3-ethylfluoranthene	230	$C_{18}H_{14}$	1.87×10^{-2}	395.1	140.6	9.93×10^{-5}	5.97	9.27	1.236
Methylpyrene	216	$C_{17}H_{12}$	5.84×10^{-1}	383.5	132.3	2.33×10^{-4}	5.48	8.91	0.928
2,7-dimethylpyrene	230	$C_{18}H_{14}$	1.70×10^{-2}	395.1	137.8	1.07×10^{-4}	6.03	9.41	1.023
1-ethylpyrene	230	$C_{18}H_{14}$	1.87×10^{-2}	395.1	140.6	9.93×10^{-5}	5.97	9.27	1.236
1,2,3-trimethylpyrene	244	$C_{19}H_{16}$	4.78×10^{-3}	406.5	148.9	4.20×10^{-5}	6.57	9.91	1.135
1,3,6,8-tetramethylpyrene	258	$C_{20}H_{18}$	1.36×10^{-3}	418.3	159.9	1.63×10^{-5}	7.12	10.4	1.246
Methylbenz(a)anthracene	242	$C_{19}H_{14}$	5.50×10^{-2}	410.8	148.4	3.37×10^{-5}	6.07	9.72	0.560
7,12-dimethylbenz(a)anthracene	256	$C_{20}H_{16}$	6.10×10^{-2}	422.4	122.5	3.37×10^{-5}	5.8	9.40	0.618
8,9-dimethylbenz(a)anthracene	256	$C_{20}H_{16}$	3.79×10^{-3}	422.4	153.9	1.52×10^{-5}	6.62	10.2	0.618
7-ethylbenz(a)anthracene	256	$C_{20}H_{16}$	4.10×10^{-2}	422.4	156.7	1.41×10^{-5}	6.56	10.1	0.743
6,7,8-trimethylbenz(a)anthracene	270	$C_{21}H_{18}$	1.08×10^{-3}	434.0	164.5	5.92×10^{-6}	7.18	10.7	0.683
1,7,12-trimethylbenz(a)anthracene	270	$C_{21}H_{18}$	1.08×10^{-3}	434.0	164.9	5.92×10^{-6}	7.16	10.7	0.683
7-ethyl-12-methylbenz(a)anthracene	270	$C_{21}H_{18}$	1.20×10^{-3}	434.0	161.8	6.43×10^{-6}	7.11	10.6	0.821

7,9,10,12-tetramethylbenz(a)anthracene	280	$C_{22}H_{20}$	3.05×10^{-4}	445.6	170.1	2.68×10^{-6}	7.71	11.2	0.753
5-methylchrysene	242	$C_{19}H_{14}$	6.20×10^{-2}	410.8	118.0	7.27×10^{-5}	6.07	9.72	0.560
3-methylchrysene	242	$C_{19}H_{14}$	1.33×10^{-2}	410.8	148.4	3.37×10^{-5}	6.07	9.72	0.560
1,5-dimethylchrysene	256	$C_{20}H_{16}$	3.79×10^{-3}	422.4	153.9	1.52×10^{-5}	6.62	10.2	0.618
Ethylchrysene	256	$C_{20}H_{16}$	4.23×10^{-3}	422.4	156.7	1.41×10^{-5}	6.56	10.1	0.744
7-methylbenzo(a)pyrene	266	$C_{21}H_{14}$	4.96×10^{-2}	388.6	144.4	1.29×10^{-4}	6.54	8.33	40.12
10-methylbenzo(a)pyrene	266	$C_{21}H_{14}$	3.08×10^{-3}	454.4	174.7	1.44×10^{-6}	6.66	11.1	0.100
7,10-dimethylbenzo(a)pyrene	280	$C_{22}H_{16}$	8.73×10^{-4}	465.9	184.9	5.63×10^{-7}	7.20	11.6	0.100
6-ethylbenzo(a)pyrene	280	$C_{22}H_{16}$	9.74×10^{-4}	465.9	182.4	6.01×10^{-7}	7.15	11.5	0.121

Emissions from anthropogenic activities contribute mainly to the presence of PACs in the environment, although there are some PACs that originate from natural sources. The three sources of PACs are: i) pyrogenic, from pyrolysis of substrates such as fossil fuels, ii) petrogenic, crude oil related sources and iii) biogenic which are naturally occurring in the environment.[26] These different sources determine the composition of each PAC and hence their complexity is largely source-dependent.[26] It is a general opinion that lower molecular weight PACs are formed at low temperatures (approx.100 ºC-150 ºC) while temperatures greater than 400 ºC yield high molecular weight PACs.

Pyrogenic PACs are formed during incomplete combustion of carbonaceous materials such as herbaceous plant, wood, coal, oil, motor fuels in cars and trucks or fuel oils in heating systems. They are formed from unsaturated hydrocarbons e.g. leaf pigments, lipids or carbohydrates at temperatures higher than 500 ºC under anoxic conditions for a short period of time. During the combustion process, there is an initial formation of fragments at lower temperature which later combine to form PAHs at high temperature. Tsibart and Gennadiev in their review reported that intensive pyrolysis of fossil fuel occurs at 700 ºC, a temperature essential for polyarene formation[27] and this continues to a temperature of 1000 ºC.[28] For example, maximum yield of PAHs from coal combustion was recorded by Liu [29] at 700-800 ºC. Pyrogenic PACs can also be obtained from natural sources. This includes PAC generated during forest fires and volcanic activities. These activities occur at elevated temperatures.

PACs sourced pyrolytically comprise mainly 6 or more fused unsubstituted aromatic hydrocarbon rings ranging from benzo(a)anthracene to coronene although they are known to have 4 and 5 membered ring compounds, for example, pyrene and benzo(a)pyrene.[30] Due to the high number of polycyclic ring (6 or more), they are effectively bound to organic matter (particle/sediments) which

reduces their bioavailability for uptake by organisms. Hence, pyrogenic PACs have not been of much environmental concern as regards to their bioaccumulation and toxicity. While little is known about their toxicity[19] their abundance and persistence have been well documented which can be attributed to stable ring structure which aids their resistance to microbial degradation, photo-oxidation and vaporization.

Petrogenic PACs are associated with petroleum sources and their derivatives. Also included in this category are the groups related to coal sources. They are known to vary as a function of coal rank which is dependent on their carbon content. These include anthracite, bituminous, subbituminous and lignite coal ranks. Petrogenic PACs are formed at low geologic temperatures and pressures following the deep burial of organic matter over millions of years to form petroleum and coal.[31] Petroleum is abundant in substituted PACs compared to their unsubstituted analogs although their concentrations vary with source rock that produces such petroleum.[32] The main PAC components of petroleum consist of the 16 EPA parent PAHs and the alkylated homologues of naphthalene, phenanthrene, dibenzothiophene, fluorene and chrysene, known as the *alkylated five*.[33] Petrogenic PACs are abundant in LMW (2-4 membered ring) compounds which is attributed to their formation. The extensive time required for petroleum maturation, diagenesis, catagenesis and metagenesis, favors the order of the ring formation. Due to their low molecular weight (LMW), they are relatively bioavailable compared to their pyrogenic counterpart. These are also more soluble and have less association with organic matter/sediment after their release.

Point source introduction of PACs in the aquatic environment include accidental spills, natural seepage and leakage, dumping of fresh or used lubricating oils and also urban and rural runoffs. Although, it has been widely reported that anthropogenic activities introduce pyrogenic PACs which are most abundant in the environment, an increased presence of petrogenic PACs over the

pyrogenically derived ones exist which correlates to the rapid development of industries in urban areas and petroleum exploration activities.

Biogenic PAC sources include microbial lipids, leaf wax and insect cuticle.[34] For example, naphthalenes and perylene are produced by the wood of tropical forest and sometimes occur in termite organisms.[35-37] They also possess their characteristic signatures that aids in source apportionment. Tsibart and Gennadiev[27] compiled possible sources of biogenic PACs as reported in various research which include synthesis by algae, some plant organs and stems, higher plants and bacteria.

A less discussed source of PACs in the atmosphere as highlighted in Tsibart and Gennadiev[27] review is the cosmogenic source. They are composed of both LMW and high molecular weight (HMW) PAHs believed to be synthesized in the solar nebula, from pyrolysis of meteorites carbonaceous material found in the earth's atmosphere or by solar wind impact on cosmic bodies' carbonaceous surfaces.

PACs are dispersed in the atmosphere when sorbed onto particulate matter which can be later deposited onto the land or water bodies through dry or wet atmospheric deposition and in the aquatic environment through fluxes in runoff water from urban areas, natural seepage as well as accidental spills on land and sea. Figure 1.2 shows atmospheric emissions, transport, transformation and deposition of gases as adapted from Aneja, et al. [38] for PACs. When present in the atmosphere, they undergo long-range transport resulting in their global distribution such that pristine areas can be affected. The fate, transport, deposition and partitioning of PACs present in the environment are largely influenced by their physico-chemical properties. It is important to note that the high concentrations of PACs are observed near sites of generation; for example, petrogenic PACs dominate and are elevated in samples taken at or close to oil spill locations.

PACs are semi-volatile chemical compounds that are present in both the gas and particulate phase[39]. Their molecular weight and vapor pressures play an important role in which of the phases PAC molecules exist.[39-41] Low molecular weight PACs (< 202 g/mol) possess high vapor pressure and are known to exist more in the free gas phase than high molecular weight compounds (more than 4 aromatic rings) while high molecular weight PACs accumulate on particulate matter (PM) compared to lower molecular weight PACs.[42-44] Their transportation in the atmosphere is largely determined by the partitioning between PM and the gaseous phase. This is understood by examining their gas-partition (Kp) and K_{OA} coefficients. Lohmann and Lammel[45] described the partitioning to be controlled by absorption into organic matter (OM) and /or adsorption onto black carbon (BC). Based on their study, they suggest that K_{OA} is a good approximation for the OM absorption of PAHs although the role of BC adsorption is not totally understood as it was inferred that there might be additional sorption processes occurring for some of the PAHs studied. Because PACs have varying physico-chemical properties due to substitution on the PAHs backbone, it is not surprising that they also have varying affinities to PM.

When PACs partition into the aquatic environment either through atmospheric deposition of particulate matter or fall out from rain, they are distributed between dissolved, colloids, suspended and PM, surface sediment and biota.[46] The main factors that drive their distribution are solubility and hydrophobicity. According to Abdel-Shafy and Mansour[26], a decrease in aqueous solubility leads to an increased K_{ow} and thus a greater tendency for PAC sorption to PM.

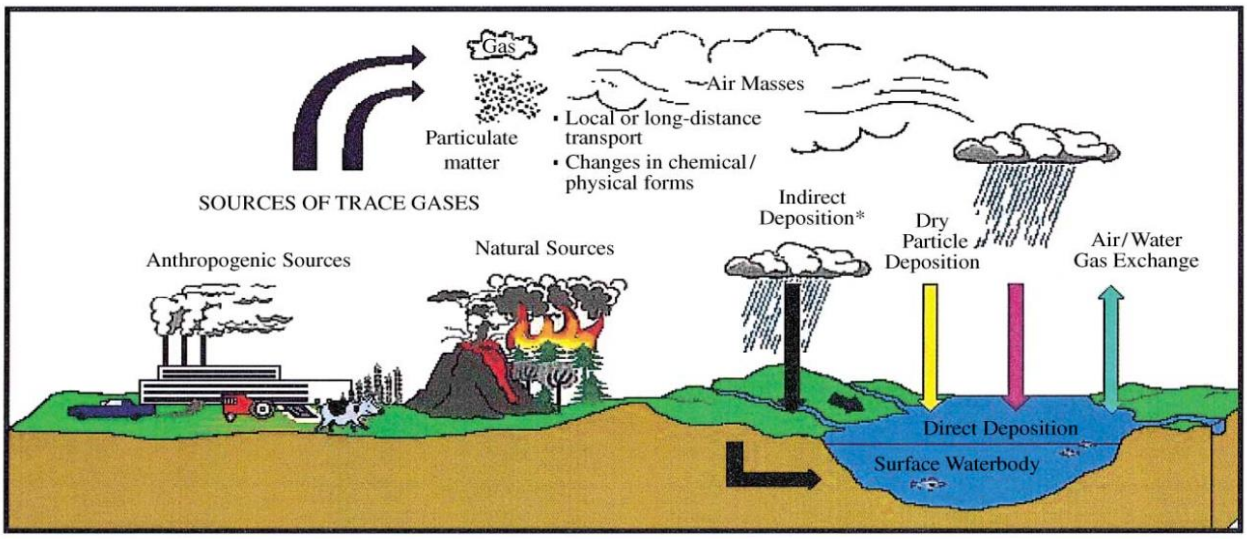

***Figure 1.2**: Atmospheric emissions, transport, transformation and deposition of gases.*[38]

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Idowu, et al. [47] reported that polar PACs (i.e. PAHs containing N, S, O) possess higher solubility and lower lipophilicity than their unsubstituted analogs in aqueous media thus making them more bioavailable. Polar PAHs are also resistant to degradation, hence persist in the environment and their toxicity makes them a concern when present in water bodies.

Understanding PACs in surface soil and sediments is of high importance because they have proven to be an indicator for long-term environmental pollution state. This is further supported by the high number of studies that exist characterizing and quantifying PACs so as to determine the source, transportation and distribution in the environment.[48-51]

Atmospheric deposition of volatile PACs either through wet or dry deposition has been described by Liu, et al. [52] to contribute 30%-40% to the concentration of PAHs in the coastal regions of South China. The same trend was observed for surface soils where Wang, et al. [53] reported that the principal pathway of PAHs to urban surfaces and top soils is via atmospheric deposition. The

volatility of these compounds is explained by Eweis [54] using Henry's Law coefficient. A chemical compound is considered volatile if the coefficient is greater than 3.04×10^{-2} Pa-m^3 mol^{-1} but if less than 3.04×10^{-2} Pa-m^3 mol^{-1}, volatilization is not an important transfer process.[55]

The hydrophobic (lipophilic) nature of PAC leads to strong sorption on OM or suspended sediments in water column which can further undergo sedimentation[56] while the hydrophobic micro pores associated with the sediment particles serve as a reservoir.[57] The ability to retain these contaminants in pores reduces their bioavailability and hence their toxic potential to aquatic organisms.[58] Sediment disturbance events e.g. daily tidal currents, storms in coastal and estuarine systems and wind energies are capable of remobilizing these contaminated sediments and thus release PACs into the water column.[58-59]

When present in sediment, Srogi [60]and Pierzynski [61] describe the fate of PACs to be defined by factors such as sorption, transfer, degradation and sequestration. Others include volatilization, leaching and uptake from plant. Maletic, et al. [62] summarized these factors described by Wick [55] while considering other co-factors e.g. the texture, geochemical properties of pollutants, chemical and biological characteristics of sediment and environmental conditions.

Of these factors, the rate of sorption or desorption of PACs from OM determines their bioavailability for uptake by aquatic life or removal through degradation by either bioremediation or natural attenuation. This rate can be altered by physical, chemical and biological processes which cause changes in the sorbed particles.[55] PACs bound to OM are less bioavailable for mobility (solubility and volatility) and uptake by the microorganisms.

1.4 PACs as environmental biomarkers

PACs are recognized environmental and geological biomarkers.[63-66] Geochemically, biomarkers are useful tools to trace the source, thermal maturity and identify migration pathway of crude oil. [67-71] For the purpose of PAC source correlation, diagnostic ratios are typically evaluated. The technique uses the ratio between two PAC congeners in samples to distinguish PAC origin mostly if pyrogenic or petrogenic, both of which are known to be complex to evaluate. The composition of biogenic PACs is not as complex while it is widely accepted that biogenic PACs do not contribute significantly to the total mass of PAHs in contaminated soils and sediments.[31] Table 1.2 and 1.3 shows the different diagnostic ratios used for source apportionment and oil spill processes.

Other studies have employed the use of multivariate statistics such as principal component analysis (PCA). This has proven to be useful when there are multiple sources contributing to the PAC component in environmental samples.[72-73] Another statistical method adopted in numerous studies is positive matrix factorization (PMF). This is a receptor model different from diagnostic ratios which are source-based. PMF has use for both air and sediment source apportionment. Due to the complexity of PACs, it is advised to combine multiple approaches to identify sources of these chemical compounds in the environment.

Although the selection of the 16 EPA PAHs back in the 1970s was not to primarily be utilized in source identification, they have been the backbone of diagnostic ratio consideration. The common PAHs adopted over the years mainly are naphthalene, phenanthrene, anthracene, pyrene, fluoranthene, benz(a)anthracene, chrysene and benzo(a)pyrene.

These native PAHs cannot, however, be efficient as a stand-alone tool for source apportionment. Stout, et al. [31] suggested combining these diagnostic ratios with petroleum or geological

biomarkers. Also, expanding the list of diagnostic ratios to include other PACs aside from the unsubstituted PAHs may further help with source apportionment.

1.5 Toxicity and Carcinogenicity of PACs

The increased scientific interest in PACs can be attributed to the fact that many of these compounds are carcinogenic, mutagenic, teratogenic, immunotoxic and/ or genotoxic. The knowledge of their carcinogenicity dates back to the late 1700s where Sir Percivall Pott, an English surgeon, described the occurrence of scrotal cancer in chimney sweepers due to exposure to soot.[74] This happens to be the first report of occupational cancer. Although the International Agency for Research on Cancer (IARC) believes there is inadequate evidence of carcinogenicity of crude oil or bitumen (the main source of PAC in oil explored or exploited zone) in humans, they have classified compounds or mixtures e.g. coal, possessing petrogenic PACs as carcinogens on their list. Reports on cancer occurrence as a result of exposure to crude oil have been documented over time.[75-79] Other reports detailing various effects of aquatic organisms exposure to PACs are well recognized.[80-81]

Exposure to PACs includes inhalation, ingestion, and dermal contact. Their health effects to human or aquatic life depend on the duration and route of exposure, concentration and also the toxicity of the PAC. Short term exposure can lead to acute health effects ranging from irritation, diarrhea or nausea in human[82] to narcosis in aquatic organisms.

An example of human exposure route to PACs is through diet derived from marine lives e.g. mussels, clams and lobsters or higher predators like fish and squid.[26] These are organisms are

Table 1.2: *Diagnostic ratios of PACs used for oil spill studies*[83]

Diagnostic ratios	Application
Double ratio plots (C_2D/C_2P vs. C_3D/C_3P)	Distinguishing between sources with similar chemical compositions
	Useful in establishing statistical models for source allocation
	Examples: Exxon Valdez oil spill study; Gulf War oil spill study
Double ratio plots (C_3D/C_3P vs. C_3D/C_3C)	Distinguishing among weathered crude oils
	Distinguishing spilled oil from other sources
	Examples; to describe oil depletion and to identify in subtidal data from the M/C Haven spill in Italy, Exxon Valdez spill, and a North Sea oil spill
4- to 6-ring non-alkylated PAH/ ΣPAH Σ Naphs/Σ PAH Σ Phens/Σ dibenz Phen/Σ phens	Used for multi-source hydrocarbons identification in the study hydrocarbon contamination on the Antarctic Peninsula
Ratios of 3 m-DBT isomers	Used for source identification of unknown spilled oils
	Distinguishing between oils with a similar chemical compositions
	Differentiation between oils due to physical weathering and biodegradation
	Marker of biodegradation
C_0C; C_1C: C_2C: C_3C Σ chrys/ Σ phens Σ chrys/ Σ dibenz	Used for source identification in the Arrow and BIOS spill studies
	Weathering indicator
	Differentiation between composition changes due to physical weathering and biodegradation Relative distribution of PAH in each homologous family

Σ PAH: the sum of total PAH including five target alkylated PAH homologue and the other EPA priority PAH; m-DBT: methyl-dibenzothiophene; 4- to 6-ring non-alkylated PAH include fluoranthene, pyrene, benzofluoranthenes, benzopyrenes, indenopyrene dibenzoanthracene and benzoperyene.

C_0C- C_0 Chrysene; C_1C- C_1 chrysene; C_2C – C_2 Chrysene; C_3C – C_3 Chrysene; C_2D- C_2 Dibenzothiophenes; C_2P – C_2 Phenanthrenes; C_3D- C_3 Dibenzothiophenes; C_3P – C_3 Phenanthrenes; phens – phenanthrenes; dibenz – dibenzothiophenes

Table 1.3: *Diagnostic ratios with reported values used for processes.*[84]

PAC ratio	Value range	Source
Σ LMW PAH/Σ HMW PAH	<1	Pyrogenic
	>1	Petrogenic
Σ COMB/Σ PAHs	~1	Combustion
FL/(FL + PYR)	<0.5	Petrol emissions
	>0.5	Diesel emissions
ANT/(ANT + PHE)	<0.1	Petrogenic
	>0.1	Pyrogenic
FLA/(FLA + PYR)	<0.4	Petrogenic
	0.4-0.5	Fossil fuel combustion
	>0.5	Grass, wood, coal combustion
BaA/(BaA + CHR)	0.2-0.35	Coal combustion
	>0.35	Vehicular emission
	<0.2	Petrogenic
	>0.35	Combustion
BaP/(BaP + BeP)	~0.5	Fresh particles
	<0.5	Photolysis(ageing of particles)
IcdP/(IcdP + BghiP)	<0.2	Petrogenic
	0.2-0.5	Petroleum combustion
	>0.5	Grass, wood and coal combustion
RET/(RET + CHR)	~1	Wood burning
2-methylnaphthalene/PHE	<1	Combustion
	2-6	Fossil fuels
ΣMePHE/PHE	<1	Petrol combustion
	>1	Diesel combustion
BbF/BkF	2.5-2.9	Aluminium smelter emissions
BaP/BghiP	<0.6	Non-traffic emissions
	>0.6	Traffic emissions

Σ COMB e (FLA, PYR, BaA, CHR, BkF, BbF, BaP, IcdP and BghiP); Σ PAHs - sum of total non-alkylated PAHs; Σ LMW - sum of two and three-ring PAHs; Σ HMW - sum of four and five ring PAHs; FLA - Fluoranthene, PYR - Pyrene, BaA – Benz(a)anthracene, CHR - Chrysene, FL - Fluorene BkF – Benzo(k)fluoranthene, BbF – Benzo(b)fluoranthene, BaP – Benzo(a)pyrene, BeP – Benzo(e)pyrene, RET- Retene, PHE – Phenanthrene, ANT – Anthracene, MEPHE – methylphenanthrene, IcdP – Indeno(1,2,3-c,d)pyrene and BghiP - Benzo(g,h,i)perylene

exposed to PACs in sediments and pore water. Other routes of exposures include inhalation of smoke from open fireplaces, smoking cigarettes or eating food containing PAHs. According to Abdel-Shafy and Mansour [26], 17 PAHs have been identified to be of concern with regard to adverse health effect that may be either carcinogenic, mutagenic and/or teratogenic to humans. Some of these PAHs have been classified as known, possibly or probably carcinogens (Group 1, 2A or 2B) to human by the International Agency for Research on Cancer (IARC) e.g. benzo (a) pyrene (Group 1), naphthalene and benz (a) anthracene (Group 2B). Because exposure to these compound class is never to a single compound in the group, an understanding of the dynamics of single metabolism of PAHs is important as it will provide a better insight of metabolism of mixtures.

The high lipophilicity of this class of compounds makes them detectable in almost all internal organs especially those rich in adipose tissue, thus serve as repository from which PACs can be gradually released. Most metabolites of PAHs are excreted in feces and urine where they require multistep metabolic activation by specific enzymes which is responsible for PAHs metabolism.[26]

Acute toxicity of PAHs to fish is generally attributed to narcosis,[85] sometimes death may occur in an oil spill situation. In human, short term health effects is not clear but symptoms such as nausea, eye irritation or diarrhea have been reported.[86] Chronic health effects arise from long term exposure e.g. asthma-like symptoms, lung function abnormalities/ risk of lung cancer, skin inflammation, liver and kidney damage etc.[87] Some toxicity models exist to predict PAH toxicity in aquatic life. Barron, et al. [85] used four mechanism-based models: narcosis, aryl hydrocarbon receptor (AhR) agonism alkyl phenanthrene toxicity and combined toxicity, to determine the toxicity of embryonic exposures to complex petrogenic PACs in two fish species. Acute effects can also sometimes be related to LMW PACs due to their perceived short residence while HMW PACs responsible for chronic health effects.

The most studied PACs for toxicity are Benzo(a)pyrene (BaP), 7, 12-dimethyl benzo(a)anthracene (DMBA) and dibenz[a, l] pyrene (DB[a, l]P). These compounds share a common structural feature, a sterically hindered region (bay) enclosed within four rigidly constrained carbon-carbon bonds, as in the case of BaP and DMBA (Figure 1.3). When all four bonds are part of a six-membered rings, the sterically hindered bay region is regarded as a fjord region, as with DB[a, l]P. PACs which are inert can be metabolized in the liver and other tissue by an enzyme, cytochrome P450, into reactive electrophiles. These reactive electrophiles can either be excreted from cells through the process of detoxification or can form an epoxide capable of binding to cellular macromolecules particularly proteins and nucleic acids such as with DNA forming PAH-DNA adducts.

Presently, there are three major pathways and minor sulfonation pathway that have been established as metabolic activation of PAHs:

i) the widely known bay region theory where a diol-epoxide is formed by successive epoxidations catalyzed by cytochrome P450 (P450) monooxygenases and an intermediate hydrolysis step catalyzed by microsomal epoxide hydrolase (Figure 1.4),

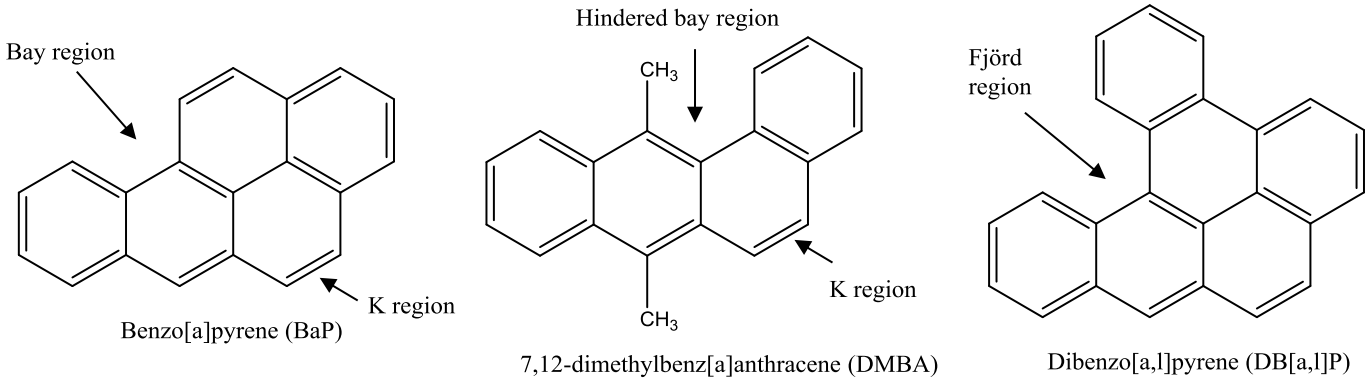

Figure 1.3: *Structural features of PACs that contribute to carcinogenicity.*

ii) enzymatically catalyzed one-electron oxidation by P450 peroxidase leading to reactive radical cation intermediates or adducts.[88-89] (Figure 1.5) and

iii) the more recently proposed pathway which involves an activation through the formation of *o*-quinines catalyzed by dihydrodiol dehydrogenases (DDs) (Figure 1.6)

The region theory involves three enzyme-meditated reactions. First, the oxidation of a double bond catalyzed by P450 enzymes (CYPs) to unstable arene oxides, second, hydrolysis of the arene oxides by microsomal epoxide hydrolase (EH) to *trans* dihydrodiols, and finally, a second CYP-catalyzed oxidation at the double bond adjacent to the diol function to generate a vicinal diol-epoxide.

For BaP, the theory of its carcinogenicity follows an initial oxidation to benzo(a)pyrene-7, 8-oxide(I) via diol-epoxide formation using cytochrome P450 enzymes CYP1A1 or CYP1B1, the most important enzymes in the metabolic activations of PACs. The product formed can then be metabolized to benzo(a)pyrene-7,8-dihydrodiol (II) through epoxide hydrolase. This can form metabolites that are carcinogens capable of damaging genomic DNA. Rybicki, et al. [90] and Rybicki, et al. [91] described that the dihydrodiol can be metabolized by CYP1A1, CYP1A2 or CYP 1B1 to form a diol-epoxide (benzo(a)pyrene -7,8-dihydrodiol-9,10-epoxide, BPDE (III)) which has four possible diastereomers where the (+)-*anti*-BPDE is the most abundant. This can covalently bind to DNA to form BPDE-DNA adduct known to be mutagenic and tumorigenic. Other PAC metabolized through this pathway include 5-methylchrysene,[92] 7, 12-dimethylbenz(a)anthracene,[93] dibenzo(a,l)pyrene,[94] benzo(a)anthracene, phenanthrene and benzo(c)phenanthrene.[95] Some nitrogen containing PACs have been reported to be activated via the bay region pathway. They include benz(a)acridine,[96] 7-methylbenz(c)acridine,[97] benz(c)acridine,[98] dibenz(a,h)acridine[99]. Here, dibenzo (a, j) acridine was metabolized in vitro by

rat liver microsomes to a series of epoxides, phenols and dihydrodiols. Of the diols form, the 3,4-diol-dibenzo(a,j)acridine constituted 35-62% of total metabolic products and is more mutagenic than other isomeric diols towards *Salmonella typhimurium*.[100] The epoxide formed, 3,4-diol-1,2-epoxide, showed the most mutagenic activity in both mammalian and bacterial cells without activation.[101] For BaP-7,8-diol, the diol-epoxide constituted 59-95% of the total metabolites formed by microsomes from both control and induced rats.[102] It is important to be aware of the high stereoselectivity of the formation of PAH metabolites by CYPs and EH.

The second pathway involves the formation of radical cation of PAH by the removal of one electron from the π electron system of the molecule through one electron oxidation. Radical cation intermediates formed are electrophilic in nature and are capable of interacting with nucleophilic centers in cellular macromolecules to yield adducts (IV) or intermediates. Figure 1.4 show one of the pathways for PAH activation as proposed by Cavalieri, *et al*. [103,] Cavalieri and Rogan [104,] Cavalieri and Rogan [105].

The third major metabolic activation pathway is the formation of *o*-quinones by dihydrodiol dehydrogenases (DD)-catalyzed oxidation, proposed in the late 1980s.[106-108] With this pathway, the DD enzyme competes with P450 to oxidize the non-K-region diol, a carcinogenic metabolite of PAH, but not the K-region diol.[109] This mechanism was initially thought to be a detoxification pathway as the *o*-quinones of PAC exhibited strong reactivity towards cellular gluthathione and cysteine leading to water soluble conjugates. However, more recent evidences have shown that PAC-*o*-quinones are highly reactive acceptors that can form stable and depurinating DNA adducts therefore considered to be a metabolic activation pathway.[110-111] Figure 1.6 shows the pathway using BaP as a representative. The redox active PAC can be reduced to reform catechol by a non-

enzymatic two-electron reduction or to reform the semiquinone anion radical via a one-electron enzymatic reduction.

For some methyl PACs, a possible proposed mechanism of metabolic activation is by hydroxylation of the meso-methyl group(s) with subsequent formation of reactive benzylic esters bearing a good leaving group e.g. sulfate.[109] Quite a number of researchers have demonstrated electrophilic sulfuric acid esters of hydroxymethyl PACs, such as 5-methyl chrysene,[112] 9,10-dimethyl-anthracene,[112] 6-methyl BaP,[113] 7, 12-DMBA,[114] 7-methylbenz(a)anthracene[115] and 1-methyl pyrene,[112] catalyzed by rodent hepatic sulfotransferase. Other PACs such as 3-methylchrysene can also be metabolized through sulfuric acid esterification by sulfotransferase by generation of secondary benzylic alcoholic groups.[112]

Although various metabolic pathways have been proposed for these compounds in organisms, there still exist some unclear details about the pathways and their effects especially in human. For example, most PACs are not genotoxic by themselves but need to be metabolized to the diol epoxides which react with DNA, thereby inducing geno-toxic damage. The effects of PAH exposure to human during pregnancy include low birth weight, premature delivery and heart malfunctions.[116] There has also been report of lower IQ at age three, increased behaviour problems at ages six and childhood asthma and also cord blood of exposed babies show DNA damage that has been linked to cancer.[117] These are evidences showing the need to further study and monitor these compounds.

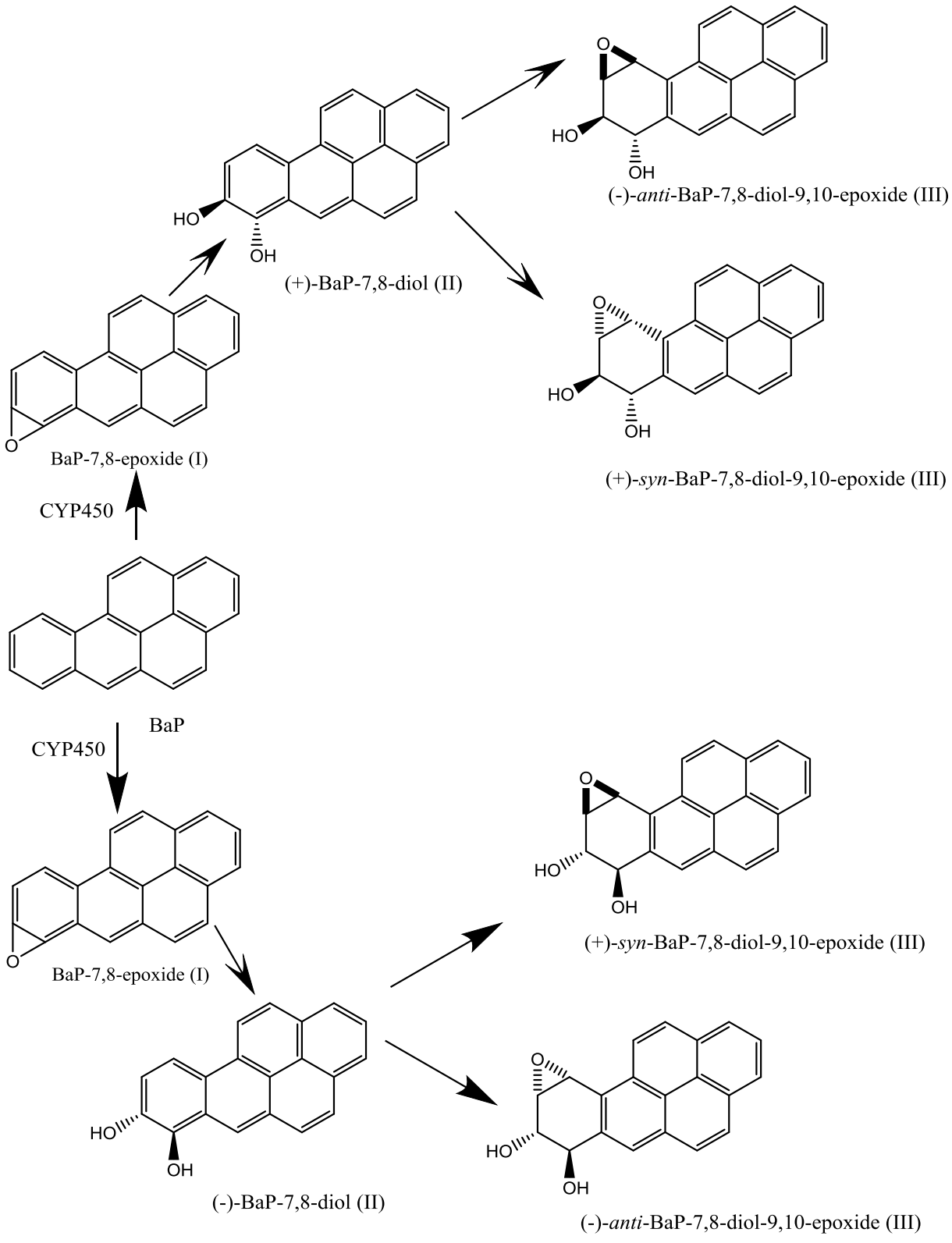

Figure 1.4: *Metabolism of BaP to diol epoxides.*[118]

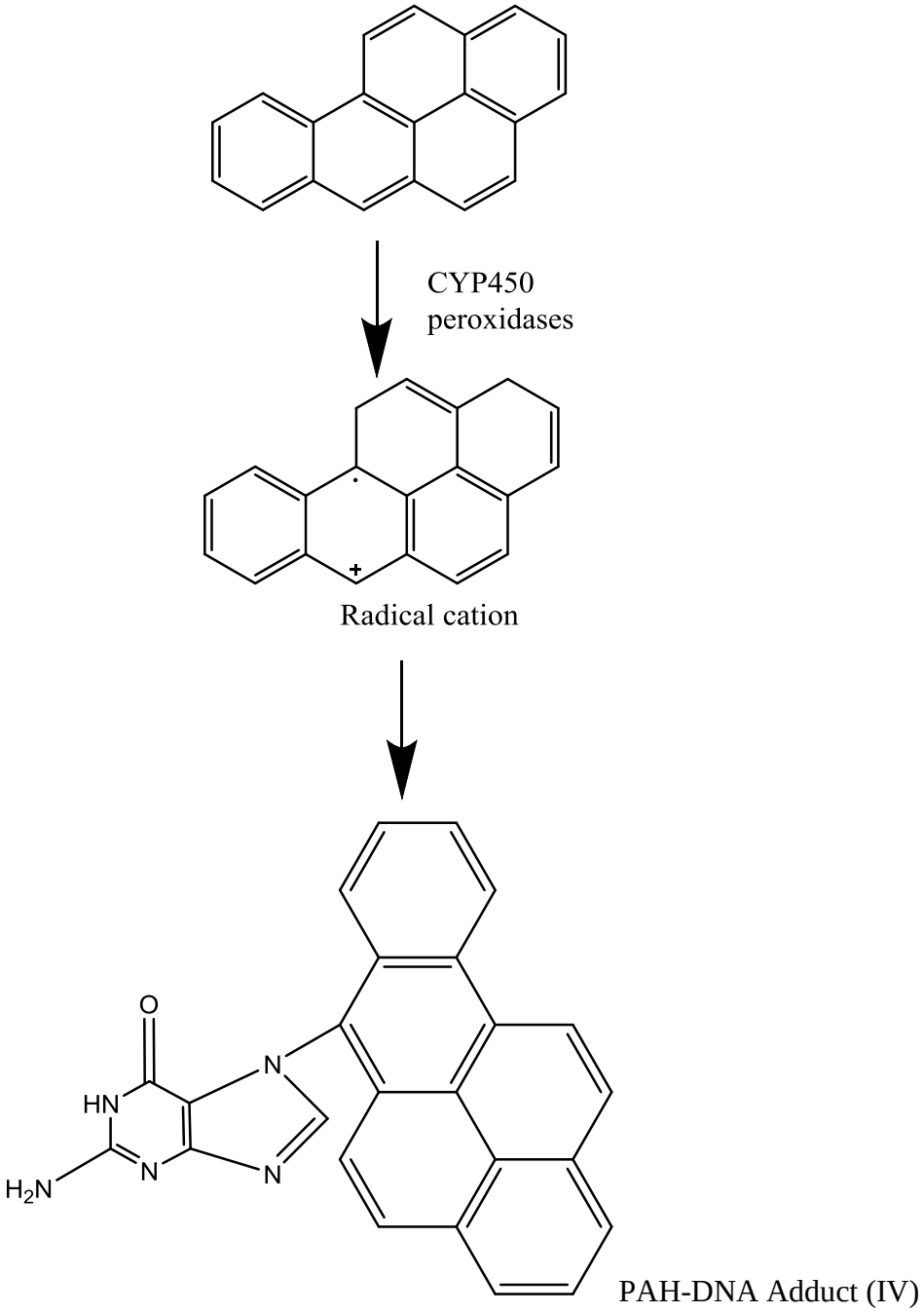

Figure 1.5: *One electron oxidation scheme for the formation of PAH radical cation and DNA intermediate.*[109]

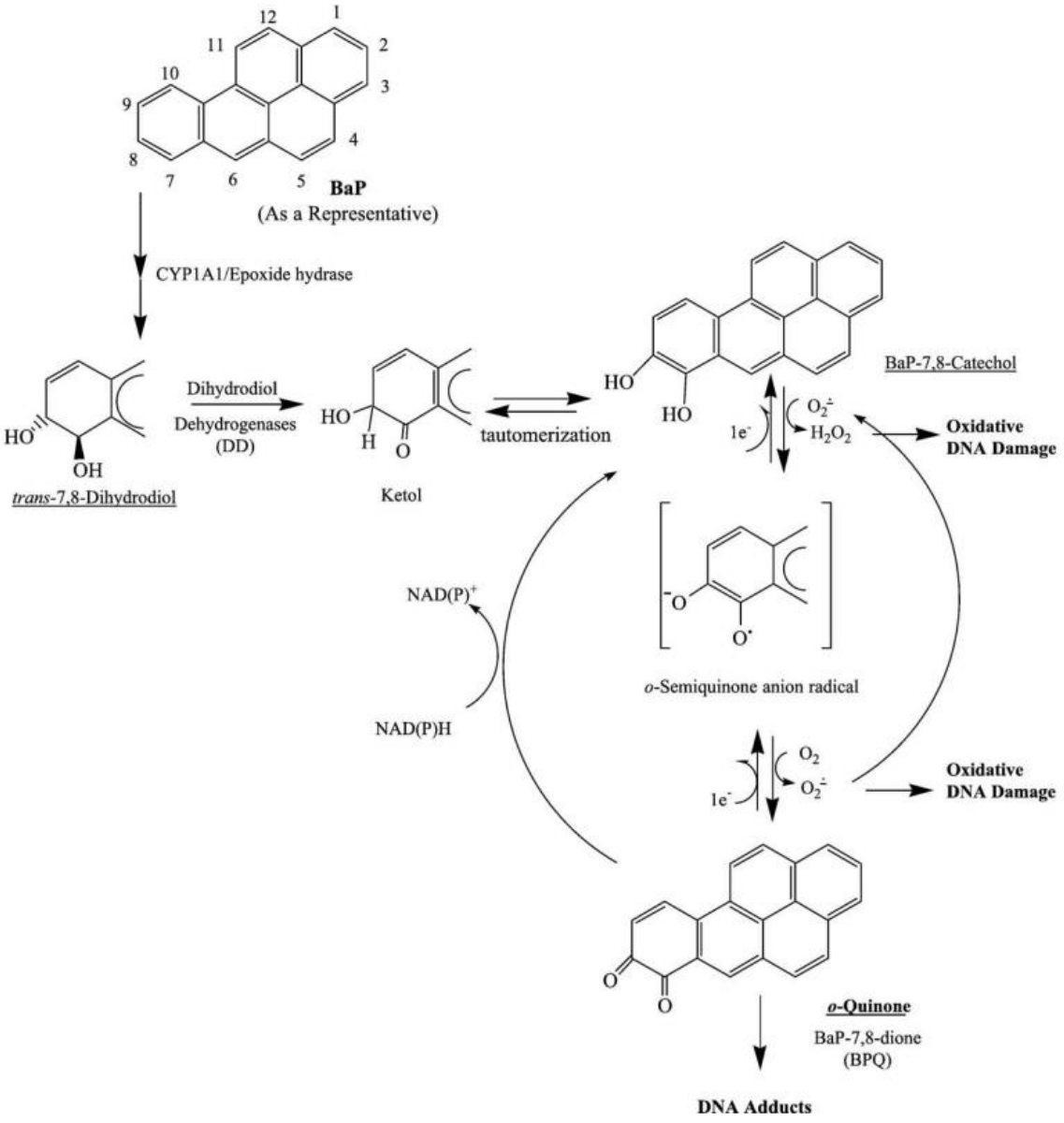

Figure 1.6: *Metabolic activation pathway of PAH via o-quinone.*[109]

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1.6 Challenges of accurately quantifying PACs

Historically, the occurrence of PACs has been monitored by measuring the concentration of the 16 PAHs categorized by the US/EU EPA in various sample matrices.[119-123] These compounds have been analyzed using high-performance liquid chromatography (HPLC) coupled to a diode-array detector (HPLC/DAD),[124-125] ultraviolet (HPLC/UV) detector,[126-127] fluorescence detector (HPLC/FLU)[128-130] and also gas chromatography(GC) coupled with a flame ionization detector (GC/FID) [131-132] or mass spectrometry (GC/MS). [133-135] Variations to GC/MS includes the use of GC tandem mass spectrometry (GC/MS-MS),[136-137] two dimensional GC coupled with mass spectrometry (GCxGC/MS).[138-139] Chromatographic separation of the 16 EPA PAHs is not challenging and Andersson and Achten [140] mentioned the reason for choosing to work with these 16 compounds to include: limited number of analytes and easy evaluation, easy access to analytical standards that are commercially available, good comparability when analyzed by different research laboratories and experience with the list in court cases with easy reference to previous cases.

Evidently, there have been numerous research reports confirming that there are other PACs in addition to the 16 EPA PAHs present in the environment.[140-142] While these additional PACs are not classified as priority pollutants, their abundance, persistence and toxicity have been reasonably well researched. These compounds known as substituted PACs are reported to be present in higher concentration in crude oil and sediments than their parent compounds.[143-145] Andersson and Achten [140] proposed an updated list to include 40 environmentally relevant PAHs (including alkylated PAHs), 23 NSO-heterocyclic compounds and 6 heterocyclic metabolites and 10 oxy-PAHs and 10 nitro-PAHs with future additions to include amino- and cyano- PAHs.

There are a few challenges that have hindered the embrace of expanding the list to include all compounds as suggested by Andersson and Achten [140]. These include limited commercially available standards for quantitation and difficulty with chromatographic separation of individual compounds, unlike the 16 EPA PAHs. These challenges have led to a non-unified approach to quantifying substituted PACs by researchers worldwide.

Perhaps the biggest challenge associated with quantifying PACs is the large number of theoretical congeners possible. For example, Johnson, et al. [16], have calculated that there are 425 constitutional isomers of C_4 phenanthrenes and 376 isomers of C_3 benzo(a)pyrenes. To chromatographically separate these large numbers of isomers if bioavailable in an environmental matrix is hardly imaginable. Efforts to use conventional one-dimensional GC have proven futile for individual separation of these complex clusters.

Apart from the large number of isomers theoretically possible, the limited availability of analytical standards has led to variations in the quantitation methods used i.e., there has either been an over- or underestimation in their concentration in environmental samples.[146-147] These quantitation methods use the parent/unsubstituted PAH to quantify their corresponding alkyl group using the same response factor (RF). Sørensen, et al. [148] highlighted the difference in RF of a parent versus alkyl compounds while Idowu, et al. [149] also showed that even within PAHs with similar alkyl group, there exist differences in RF.

Modifications to the old EPA Method 8270 used to analyze the 16 EPA PAHs to include other PACs are clearly needed. Developing standardized analytical methods that provide accurate measurements of congeners detected in the environment is of high importance as this will drive future work on the potential for these components to adversely affect biological organisms.

1.7 Scope of my research

My research seeks to individually detect, separate and accurately quantify PACs in environmental samples using GC-MS/MS and comprehensive two-dimensional GC high resolution time of flight mass spectrometry (GCxGC-HRToFMS).

Because of the limited availability of PACs my work focused on all 16 EPA PAHs, 16 halogenated-PAHs (HPAHs), C_1 to C_4 alkylated group of naphthalenes, phenanthrenes/anthracenes, fluorenes, pyrenes/fluoranthenes, benzo(a)pyrenes, dibenzothiophenes and chrysenes.

1.8 Aim and Objectives

The overarching aim of my thesis, therefore, is to accurately quantify PACs and their isomers in environmental matrices. Ultimately, my work will aid in the isomer specific identification and separation of these compounds, and to enable ecotoxicology studies and source apportionment on individual compounds.

1.9 Thesis structure

My thesis is divided into eight (8) chapters.

Chapter 1 provides an overview of the thesis highlighting the scope, aim and objectives of the thesis. **Chapter 2** discusses the methodology adopted for sample processing and analysis of analytes of interest. This chapter serves as an introduction to the background study of the extraction

method and gas chromatography mass spectrometric analysis in the following chapters. **Chapter 3** deals with the mathematical concept of constitutional isomer of polycyclic aromatic compounds. This was done by using combinatorics and molecular symmetry and was validated using various classes of chemical compounds. **Chapter 4** presents the validation of a simultaneous quantitative method for analysis of PACs in biota using gas chromatography tandem mass spectrometry. The guideline as published by EURACHEM was meticulously executed to prove the validity of the method developed. **Chapter 5** describes the application of the method developed in the previous chapter to determine the rate of sorption of PAHs by newly developed polymeric beads, Imbiber beads, from the surface of salts and fresh waters at varying temperatures under non-equilibrium conditions. **Chapter 6** focusses on developing a new method based on GCxGC/HRToF-MS to analyze and separate complex PAC in different environmental samples. **Chapter 7** shows the application of 2D GC to biota samples to confirm the presence of a novel class of PACs, the halogenated PAH (HPAH). **Chapter 8** gives an overall conclusion of the study. Suggestions and recommendations for future research in relation to the application of this work are also mentioned.

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Chapter 2

Methodology

2.1 Sample Preparation

Sample preparation is an analytical procedure used to isolate compounds of interest (analytes) from a sample matrix. This may involve sub-sampling, pulverizing, dilution, dissolution, filtration, extraction and chemical digestion to name a few steps. Sample preparation is important because often the least complex of samples can rarely be utilized in their raw state either due to excessive dilution or concentration of target analytes or incompatibility with analytical instruments.[1] Environmental samples can either be solid, semi-solid or liquid and several analytical procedures typically need to be performed prior to instrumental analysis. These procedures can sometimes constitute the principal source of error to acquired data and sample preparation still remains one of the most time-consuming steps.[2] Of the three forms of environmental samples, solid samples are typically the most difficult to process and there is a need to have target analytes in a phase compatible with analytical instruments to be used for analysis. Thus, the first operation in any type of environmental sample preparation is the extraction of compounds of interest into a preferred phase for analysis.

2.2 Extraction

Extraction involves the transfer of target analytes into a liquid phase through partitioning of analytes between the sample matrix and an extracting solvent. The most common extraction types

used in environmental chemistry are liquid-liquid extraction (LLE) and solid-liquid extraction (SLE). LLE is ideal for samples in a liquid state where extraction of target analytes is achieved based on their relative solubilities in two immiscible liquid phases, typically aqueous and organic. Hydrophobic target analytes (the focus of my own research) present in the aqueous phase preferentially partition into the organic layer where they can be removed and analyzed. SLE, on the other hand, is similar to LLE except that the target analytes are present in a solid matrix.

In 1879, von Soxhlet developed an SLE system which was adopted as the conventional and traditional method used for extraction of hydrophobic organic analytes such as PACs, Polychlorinated Biphenyls (PCBs) from biota, soils, and sediments. Although this method is simple and cost-effective, the drawbacks of Soxhlet extraction include the large volume of solvent and long duration (~24 hours) required for efficient extraction. To mitigate these shortcomings modifications to the conventional Soxhlet method were developed and included the use of high pressure (high - pressure Soxhlet extraction),[3-6] automation (Soxtec®),[7] ultrasound effects (ultrasound-assisted Soxhlet extraction)[8-9] and sample compartment-focused microwave irradiation (microwave-assisted Soxhlet) extraction technique. The drive to further shorten extraction times and reduce solvent consumption during extraction led to the latest extraction technique, accelerated solvent extraction (ASE) sometimes referred to as pressurized fluid extraction.[10]

These techniques, particularly ASE, have addressed the limitations of Soxhlet by minimizing the time required for extraction, maintaining extraction efficiency and minimizing the volume of solvent required.

2.3 Accelerated Solvent Extraction (ASE)

For the purpose of my study, ASE was employed for solid and semi-solid environmental matrices analyzed. Ezzell, et al. [11] and Richter, et al. [10] were the first publications on the principles and application of ASE. Since its introduction, documented recovery and precision of the ASE have been shown to be equivalent to those of the United States Environmental Protection Agency (US-EPA) standard extraction methodology for analysis of PACs that was based on Soxhlet extraction.[12]

The ASE technique features low consumption of organic solvent that boils at relatively low temperature at atmospheric pressure. ASE employs high temperatures and pressures which results in increased extraction efficiencies, reduction in the volume of solvent used and overall reduced extraction times. These three salient advantages of extractions based on ASE are due to the fact that it is easier to disrupt the solute-matrix chemical interactions at elevated temperatures and pressures. This is a result of the greater solvating power, decreased solvent viscosity and faster diffusion rates of the extraction solvent. In addition to the decreased solvent viscosity increased temperatures will decrease the surface tension of the solvent. The use of high pressure ensures efficient removal of analytes especially in samples where analytes have been trapped in matrix pores, when normally at atmospheric conditions solvent might not have made contact with these pores.[13]

For example, based on calculations of the temperature dependency of solubility for an ideal solution, the solubility of anthracene increases almost 13-fold as the temperature increases from 50 to 150 ºC.[14] The solubility of hydrocarbons such as *n*-C_{20} can increase several hundred-fold over the same temperature range.[14] Sekine [15] stated that the solubility of water in an organic solvent

increases with increasing temperature and aids the availability of analytes for extraction when present in wet samples. Diffusion rates are estimated to increase by a factor of 2-10 upon increasing the temperature from 25 to 150 ºC.[16] Also, as the temperature rises from 25 to 200 ºC, an organic compound like 2-propanol viscosity decreases by 9-fold.[16] For my study, dichloromethane (DCM) was used as the solvent for extraction. Other solvents such as hexane, acetone or mixture of solvents with varying gradients are sometimes employed.

Caution is needed when working with samples at high temperature because an increase in temperature alone does not always lead to an increase in extraction efficiency. An increase in temperature can sometimes reduce the density of extractant which can lead to decreased extraction efficiencies. Also, thermo-labile species can be denatured or decomposed. Schäfer [17] reported an increase in extraction efficiency for saturated and unsaturated fatty acids from cereals when the temperature was raised from 100 to 120 ºC but noticed a decrease in recoveries when the temperature was increased to 150 ºC. An intermediate temperature is advised to ensure reliable extraction.[18]

The flowchart (Figure 2.1) below shows the steps involved in the ASE process, Figure 2.2 shows the schematic of an ASE system and Figure 2.3 shows an example of the most recent ASE system, Dionex ASE 350.

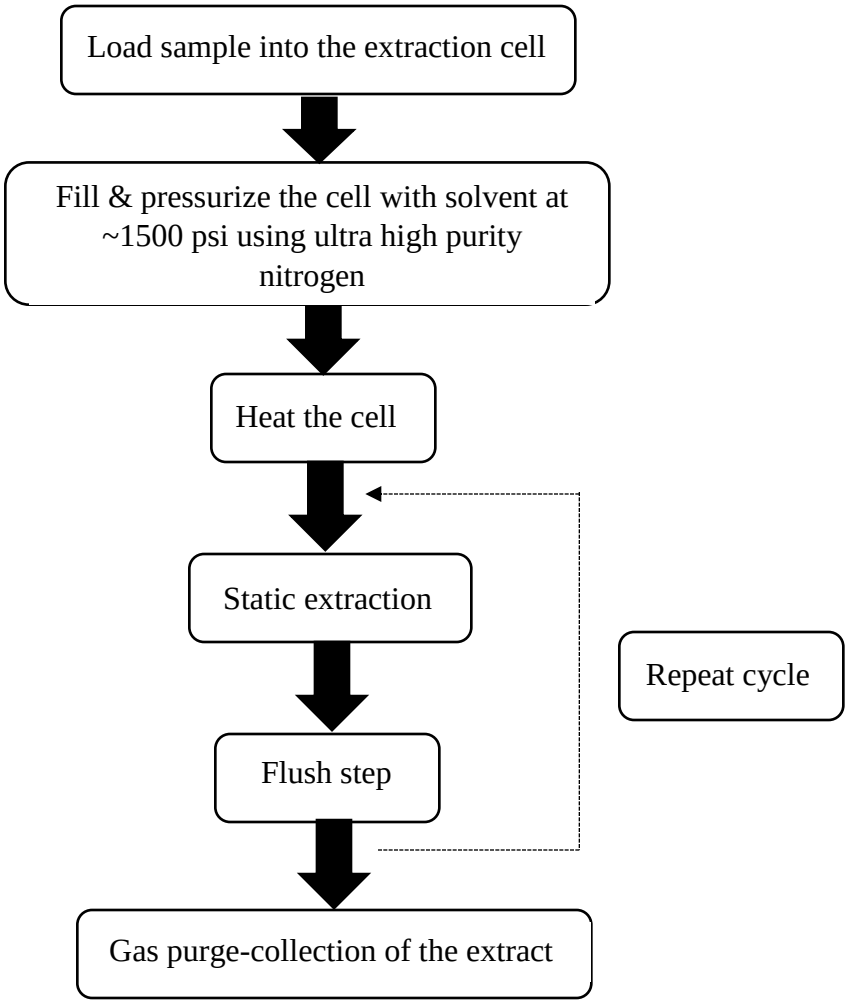

Figure 2.1: *Flowchart showing steps involved in ASE procedure*[13]

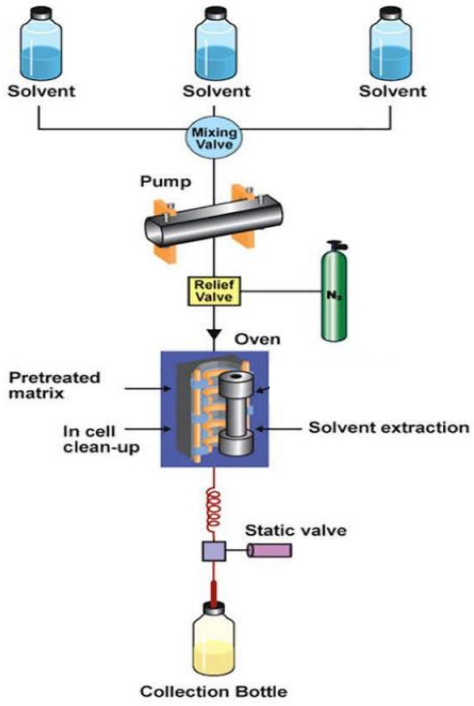

***Figure 2.2**: Operation schematics of an ASE technique*

(www.chromtographyonline.com)

***Figure 2.3:** A Dionex™ ASE 350 system*

(www.thermofisher.com)

For my study, ASE cells were filled with about 0.5 - 1.5 g of freeze-dried biota or sediment sample, weighed into a beaker and mixed with 1.5 g of dispersant (hydromatrix). Method blanks were prepared by using only the dispersant. The mixture was transferred into an extraction cell fitted with a glass fiber filter. It was then spiked with recovery internal standard (RIS) and filled to approximately 0.5 cm below the top of the cell with Ottawa sand purchased from Fisher Chemical (Figure 2.4). Extraction was done with dichloromethane (DCM) at a temperature ≥ 100º C under high pressure (1500-1700 psi). Oven heat-up time was set to 5 min and also the static time while the static cycles were ran twice with a flush volume of 60% using nitrogen gas to purge for 80 s at 150 psi. After the extraction, the extract obtained is further processed using additional cleanup steps. The cleanup steps following the extraction are driven by the type of sample matrix. For example, soils and sediments are subjected to copper treatment to remove elemental sulfur while biotic sample is processed through a gel permeation chromatography (GPC) to eliminate lipids before further cleanup over silica gel/alumina.

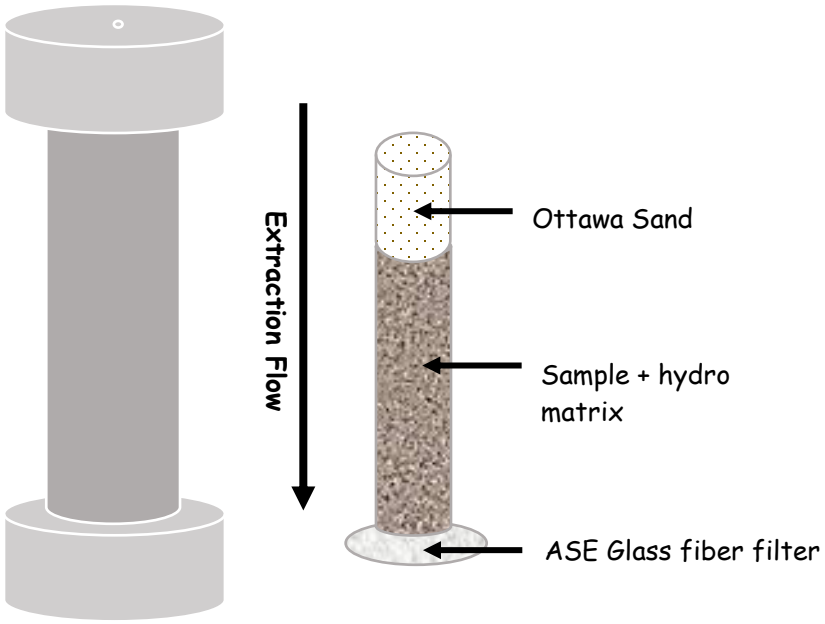

Figure 2.4: *ASE cell filled with sample.*

2.4 Copper treatment

Copper is used to remove elemental sulfur co-extracted with organic compounds. Sulphur is found in environmental media (e.g. soil, sediments, and water) whose organic constituents have been subjected to the action of microorganisms.[19] It can be present in either its native or elemental state and combined with iron, base metals and sulfide minerals[20] with elemental sulfur (Octahedral sulfur- S_8), the form usually found in soil and sediment.

The presence of elemental sulphur in extracts interferes with chromatographic analysis where it either impair or sometimes preclude the proper interpretation of the chromatogram. This is because S_8 (molecular mass of 256 Da) possesses retention characteristics similar to those of polycyclic aromatic compounds (PACs) and other organic compounds (pesticides, polychlorinated biphenyls - PCBs, PBDEs, etc.) thereby masking the peaks of these compounds present in the extract. Certain heavy metals like copper, mercury[21-22] and silver[23] have been explored to eliminate sulphur by precipitation. Although Blumer 1957 [24] acknowledged that mercury is suitable for treating large samples, the hazards involving handling and regenerating of mercury cannot be overlooked. It is also note-worthy to mention that the reaction of S_8 with silver or mercury was reported at elevated temperatures, which can result in a dense precipitate capable of obstructing the flow of analytes through a column.[24] Hence, a variety of copper treatments has been widely done either with copper turnings,[25-26] granules, bars,[27] powder of finely divided copper powder,[28-29] or copper in its reduced form for sulphur removal. This is because copper reacts easily with elemental sulfur even at room temperature using a minimal amount of material.[30-32] (Equation 2.1) Sulfur removal using copper is commonly achieved either by stirring the extract solutions with the copper material or by percolation when the extract is passed through columns packed with copper powder or granules.

$$Cu(s) + S \rightarrow CuS(s) \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (2.1)$$

For my study, reduced copper powder was used to remove sulfur from coal/soil/sediment extracts prior to analysis by gas chromatography mass spectrometry (GC/MS). The metallic copper was prepared by adding 5% nitric acid in excess to approximately 5 - 10 g of copper powder in a flat bottom flask (FBF) to cover the copper powder. A glass stopper was inserted and swirled thoroughly. The copper solution was allowed to settle for approximately 30 minutes with occasional swirling. After 30 min, the nitric acid (5% solution) was decanted and rinsed thoroughly with water, three times while allowing for copper to settle between rinses. It was further rinsed with acetone, 3x, and allowed to dry either by air drying or by rotary evaporation and stored in a vial with screw cap before use in ASE extracts. Rinsing of the acid used in preparation of the copper powder is necessary to avoid degradation of analytes.

2.5 Gel Permeation Chromatography (GPC)

Gel permeation chromatography has been used for sample clean up since the 1960s when it was used for removal of co-extracts, especially fats/lipids for determination of pesticide residues and other organic chemicals such as PACs, PCBs, polybrominated biphenyls (PBB), polychlorinated naphthene (PCN) mixtures in soil and sediments,[33] human adipose tissue samples,[34] fish extracts[35] and river waters and effluents.[36] Natural lipids remain one of the sources of interference in GC/MS analyses of tissues for organic contaminants. Tindle and Stalling [37] were among the first researchers to explore the use of automated GPC to evaluate pesticides and PCB residues in fish extracts.[38] The early development of the technique used cross-linked polydextran gels with varying pore sizes (Sephadex, Pharmacia, Sweden) as stationary phases. Other gel materials include bio-beads, poragel, styragel (all made from polystyrene), bio-gel made from polyacrylamide and a later

marketed product made from polyvinylactate. The choice of gel to be used is dependent on the material that makes up the gel as behaviour differs from one product to another. Thus far, Bio-beads and a type of Sephadex, LH-20, have yielded good results hence their wide adoption for sample clean-up.

Gel permeation chromatography (GPC), also commonly known as size exclusion chromatography (SEC), is a method used to remove high molecular weight compounds which are co-extracted during ASE. These co-extracts can include lipids, organic acids, sugars, pigments and sometimes also elemental sulfur from organic extracts[39-40]. GPC involves the separation of molecules based on their molecular size otherwise referred to as their hydrodynamic volume in solution[41] when the extract is passed through a porous stationary phase. The ability of solvated molecules to travel through the pores of the resin is determined by their molecular size.[42] For my study, there is a need to separate analytes of interest from lipids and pigments coextracted during ASE step. This is because their properties make ease of partitioning into the hydrophilic components. These components can mask, interfere with chromatographic results, lead to fouling of injector liners and the GC column itself.

My study used a J_2 scientific Accuprep™ gel permeation chromatography cleanup system (Figure 2.5) for the cleanup of a wide range of sample matrices which include tissues, foods, grains, plants and hazardous waste for US-EPA analysis.

Figure 2.5: *J_2 Scientific Accuprep™ Gel Permeation Chromatography Cleanup System*

(www.j2scientific.com)

The GPC column was packed with 60g of S-X3 support biobeads purchased from Bio-Rad laboratories, California in a transparent quartz reaction tube, 18 mm outside diameter (O.D) and 2 mm wall. The bio beads were made into a slurry with hexane: dichloromethane (50:50, v/v) and transferred into the column to form a long bed. This was allowed to settle overnight before application of moveable plungers to create the pressure needed to pack the column. This long bed of column is able to handle a load of one gram of lipid per sample on the column, making it useful for the biota extract. Lipids and pigments in biota samples for my study were eluted from the column with hexane: dichloromethane (50:50, v/v) as the eluent under a set pressure (approx. 8 psi) and flow rate (5 ml/min). Prior to the elution, a split check was carried out with PAC standards (all 16 PAHs, 1-methyl naphthalene and 1-methyl coronene) to establish elution window of my compounds of interest. The elution pattern developed for my study utilize volumes collected from 140 mL to 320 mL as the window that contains PACs while the first 140 mL goes to waste as this contains lipids and pigments. The 180 mL collected were then evaporated to 1 mL, ready for

further cleanup. The extract undergo further cleanup using adsorption chromatography prior to GC analysis since GPC removes only interfering matrix based on the size of the molecule.

2.6 Silica gel/ Alumina Chromatography

Silica gel and alumina open column chromatography is widely accepted as a clean-up and fractionation method for environmental samples especially in the early study of the chemical composition of petroleum and petroleum products.[43] The method has also been adopted in previous years to identify pollutants present in environmental matrices[44]. The technique works by separating organic extracts into fractions prior to instrumental analyses. This column chromatography employs the use of polar adsorbents, silica and alumina, loaded either as a powder or slurry in a column of known length and diameter as the stationary phase. Although silica gel and alumina are both polar adsorbents, where the polar components of an organic extract to be fractionated are strongly retained on the stationary phase through Π-electron interaction, they possess different chemical properties. Silica gel is slightly acidic while alumina is slightly basic which makes silica preferentially retain basic compounds and alumina, acidic components.

The common elution solvents for PACs are largely alkane solvents such as hexane, to elute aliphatic fraction while aromatics (PACs) are eluted with solvents such as dichloromethane, toluene or benzene (phased out due to health implications). Solvent mixtures such as dichloromethane and hexane, dichloromethane and toluene can also be used to elute aromatic fraction of an extract from the column. The choice of solvent or solvent mixture is based on the analyte group of interest.

Since its early applications, silica gel/ alumina chromatography has been applied to analyze PACs in different environmental matrices including, soil and sediments,[45] biota,[46] smoke (tobacco, food flavor)[39, 47], dust particles,[48] water and waste water.[49-50]

The choice to use silica gel, alumina or combination of both adsorbents has been explored. The use of alumina chromatography is less popular due to irreproducibility and sometimes decomposition of sensitive compounds.[51] In 1965, Sawicki *et al*.,[52] used alumina column chromatography to isolate fractions rich in PACs containing a nitrogen atom from air particulate matter. Prior to alumina use in any column, it is pretreated by heating in a furnace or oven. The heating is done at low temperature (150 °C) to dehydrate aluminum hydroxide (a mixture of gamma-alumina and alumina monohydrate). When freshly prepared, the former is active, and it then slightly deactivated by addition of water or when exposed to damp atmosphere. The use of only silica/alumina or both adsorbents at differing proportions has been successfully explored by researchers to separate organic compounds in various environmental matrices.[51]

For silica gel/alumina chromatographic cleanup for my study, approximately 11 g of silica gel was made into a slurry with ~ 20 ml dichloromethane. The slurry was then added to a Supelco® chromatographic plugged column containing dichloromethane. The silica gel was allowed to settle down after which approximately 1 g of 5 % deactivated alumina was transferred to the column followed by 0.5 g of sodium sulfate. Dichloromethane was reduced to the head of the column bed and a solvent exchange was done with 25 ml of hexane. The extract (1 ml) off the GPC column was transferred to the head of the column and first eluted with 25 mL of hexane to elute the saturates fraction which for my study was discarded. The next fraction containing PACs was eluted with 25 mL of dichloromethane: hexane (50:50, v/v). This fraction was collected, rotary and

nitrogen evaporated to a final volume of 5 mL. The final extract was then spiked with instrument performance internal standard (IPIS) prior to analysis on the gas chromatograph.

2.7 Gas Chromatography (GC)

The technique of GC was first reported by James and Martin, 1952[53] where volatile fatty acids were separated by partitioning chromatography with silicone-stearic acid supported on diatomaceous earth (DE) as the stationary phase and mobile phase using nitrogen gas. This technique achieves separation by analytes partition which takes place between a constantly moving (mobile) gas phase, usually an inert gas such as helium or an un-reactive gas such as nitrogen, and a microscopic layer of liquid on inert solid support as stationary phase held in a small diameter column. The separation of components in a mixture is influenced by their physico-chemical properties (boiling point, polarity, vapor pressure), the polarity of the stationary phase, column temperature, carrier gas flow rate and column length. These factors affect the retention and elution time of compounds, e.g. a compound possessing similar polarity as the stationary phase will be retained longer due to a stronger interaction with the stationary phase. It is widely believed that the order of separation is driven by differences in boiling point of analytes, especially using dimethyl polysiloxane as a stationary phase, this can be observed for homologous series.[54] The exception includes compounds with a different functional group.[55] GC oven temperature is either maintained isothermally, a constant oven temperature, or ramped during an analysis. Isothermal temperature is adopted for analytes with similar retention time gap but suitable for limited number of analyses because peak width rapidly increases with retention. The use of ramped temperature programming is widely accepted and suitable for various analyses. This temperature program

involves controlled heating which allows faster analysis of analytes with varying retention time gaps, hence, resulting in analyte separation.

The choice of carrier gas varies based on its average linear velocity. The two common carrier gases employed in GC analysis include hydrogen and helium where the latter is commonly used. Hydrogen provides a faster analysis time and better chromatographic resolution compared to helium gas but the safety concerns associated with usage of hydrogen gas makes helium recommended for use.

Since the invention of GC, it has been widely applied to analyze and separate compounds of complex naturally occurring mixtures like petroleum,[56-58] indicator or marker of cell damage in human body[59-60] and food,[61-62] and other natural products like essential oils and fats.[63-64] It is now a standard analytical technique employed in environmental, forensic and fingerprinting, drug residue, and food contaminant analysis.

The application of GC for analysis of PACs in environmental matrices is widely recognized since the early years of the selection of the 16 EPA PAHs.[65-68] Even with its wide applicability, the challenge of separating closely eluting or co-eluting compounds such as those found with constitutional isomers of PACs still exists[69-71] and has led to advent of two dimensional GC.[72]

There are a variety of detectors that can be coupled with GC to monitor the composition of gaseous analytes as they elute off the stationary phase. These detectors include electron capture detector (ECD),[73] flame ionization detector (FID),[74] nitrogen phosphorus detector (NPD),[75] thermal conductivity detector (TCD),[76] flame photometric detector (FPD),[77] photoionization detector (PID),[78] and a mass spectrometer (MS).[79] The choice of detector depends on the target analytes coupled with consideration for detector selectivity, sensitivity and linear range. Some of these

detectors are capable of monitoring PACs in organic extracts e.g. FID and MS where MS is a universal detector capable of detecting any compound that ionizes and produces fragment ions.

For the analysis of PACs in this study, an Agilent 7890B gas chromatograph (Agilent Technologies) was used. Analytes of interests were injected on an Agilent J&W DB-5ms Ultra Inert column of 30 m length, 0.25 mm internal diameter and 0.25 μm film thickness. Helium was used as carrier gas at a constant flow rate of 1.2 mL/min. 1 μL of sample extract is used for injection via a splitless injector kept at a temperature of 250 ºC, temperature sufficient for nearly all samples. The GC oven temperature was ramped from an initial temperature of 60 ºC, held for 1 min, then increased to 120 º at 35 ºC/min then to 220 ºC at 14 ºC/min to 260 ºC and was held at this temperature for 5 ºC/min. The temperature was further ramped to 300 ºC at 10 ºC/min and finally to 310 ºC at 50 ºC/min, held for 5 min. Other set parameters on the gas chromatograph includes temperature of transfer line kept at 320 ºC, gas saver at 20 mL/min at 3 min, split flow at 50 mL/min and septum purge at 5 mL/min.

2.8 Two-dimensional gas chromatography (2D GC /GCxGC)

The history of two dimensional (2D) chromatography dates back to 1944 when Consden *et al*., successfully separated amino acids on a thin sheet of cellulose paper (thin layer chromatography) using two different solvents.[80] Here, the sample mixture was subjected to two displacement processes at a right angle to one another. The fundamental and theoretical concept of two dimensional separation would later be published by Giddings[81] in 1984. Although Giddings acknowledged that the enormous resolving power of 2D separation was an uncharted methodology. His suggestion that the 2D research will become a major element in the field of

advancing analytical separation is evident in modern day chromatography. 2D separation, also referred to as multidimensional separation, enhances the chromatographic resolving power of complex biological and environmental samples. Its potential for increased peak capacity and resolution has been made it a preferred choice for use in gas and liquid chromatography.

The early days of 2D GC employed the heart cutting approach where a portion of unresolved complex eluent from one column was selectively diverted to a second column to improve separation before detection. With this technique, the second column only improves the resolution of those compounds present in the selected region while disregarding other compounds.

In modern day 2D GC, otherwise known as comprehensive two dimensional GC, 2D separation is achieved by utilizing two capillary columns possessing two different stationary phases (orthogonal phases) simultaneously and more often with different column length as well. These columns are connected linearly with a column union (Fig 2.6) through an interface. The connection is made such that all effluent from the first dimension column is injected into the second dimension column by intermittent pulsing with heating and cooling traps and reinjecting the eluent through the modulator interface. Other modes of injection of analytes into the 2nd dimension column exist. This is the primary difference between 'heart-cutting' two dimensional chromatography and comprehensive 2D GC analysis. Begnaud *et al*.[82] referred to a modulator as the 'heart' of a 2D GC system. A thermal cryogenic modulator creates peak segmentation by trapping peaks from the first dimension column by rapid cooling, using liquid nitrogen and cryogenically refocusing before injection on the second column. There are other types of modulator e.g. a heater based modulator. A modulator is necessary for the continuous transfer of effluent from the first column on to the second. The second column usually possesses retention selectivity that differs from that of the first dimension. Three modulation techniques exist: thermal modulation, flow modulation and closed

cycle refrigerated loop modulation. The technique of comprehensive 2D GC was first introduced by Liu and Phillips[83] in 1991 using an on-column thermal modulator interface to analyze hydrocarbon mixture. Since then, the comprehensive 2D technique has evolved and its applications in complex mixtures have increased exponentially.

Resolution in a plane for a 2D (R2D) separation is given as the square root of the sum of the squares of the resolution in the two independent columns according to Giddings [84] (Equation 2 &3).

$$R = \frac{tR(x+1) - tR(x)}{\frac{1}{2}(W(x+1)+Wx)} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (2.2)$$

$$R2D = \sqrt{(R_1^2 + R_2^2)} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (2.3)$$

Analysis of PAC extracts and standard solutions for my study was carried out on a LECO instrument. The front end of the system was fitted with a 7890A gas chromatograph (Agilent Technologies, Wilmington, DE, USA) fitted with a split/splitless injector, GC x GC thermal modulator (operated at -80 ºC) and a secondary oven. The column configurations consist of Rxi -5Sil MS (60 m × 0.25 mm × 0.25 μm) (Restek, Bellefonte, PA, USA) followed by an Rxi-17Sil MS (2 m × 0.25 mm × 0.25 μm) (Restek, Bellefonte, PA, USA). The GC columns were connected using an SGE micro union connector. Standards and samples (2 μL) were injected at 250 ºC in splitless mode. The sample was analyzed in both one dimensional and 2D modes. In one dimensional mode, the oven was programmed from 80 ºC (held for 1 min), heated to 210 ºC at 35 ºC/min, further to 260 ºC at 3◦C/min and finally to 315 ºC at 10 ºC/min (held for 5 min). The temperature ramp in 2D mode was programmed from 55ºC (held for 2 min) heated to 110ºC at 10ºC/min, further to 210ºC at 3 ºC/min and finally to 310 ºC at 8 ºC/min (held for 15 min). The

modulation period was 2 s (hot pulse, 0.7 s; cool time, 0.3 s). Other parameters used in both modes are the MS transfer line temperature set to 300 ºC and helium (carrier gas) was supplied at 1.4 mL/min.

2.9 Mass spectrometry (MS)

Mass spectrometry is a powerful tool for structure determination and analysis of PACs. The beginnings of MS can be traced to FW Aston, who designed the first mass spectrometer in 1919[85] and was able to measure mass defect in 1923,[86] and subsequent applications as a powerful technique for structural identification cannot be overemphasized. MS technology has progressed rapidly during the past years which has led to the advent of new instruments.

MS measurement is carried out in the gas phase of ionized analytes. A mass spectrometer comprises the ion source, mass analyzer, and a detector. The mass analyzer measures the mass-to-charge ratio (*m/z*) of ionized compounds and the detector records the number of ions at each *m/z* value. There have been enormous improvements to the key parameters that make up the mass analyzer which includes the ion source, resolution, mass accuracy and detector.

Presently, GC coupled with MS is widely used for identification and quantification of a broad range of organic compounds occurring in a sample extract. Its applications have been evident in food-related components (e.g. flavors and fragrances),[62, 87] toxicology,[88] pharmaceuticals[89] and clinical (detection of drug abuse and doping control),[90] environmental (oil characterization, fingerprinting and pollution monitoring) and forensic industry[91-92].

In the oil industry, GC-MS has been extensively used in petroleum exploration by analyzing extracts from geological samples such as crude oils, source rocks, coals, sediments and shales for

oil potential assessment and biomarker analysis. The compounds monitored in these geological extracts are mostly saturated hydrocarbons (alkanes) and PACs. In addition to its application in the oil industry, GC/MS can also be used for detection, monitoring and degradation of organic compounds in the environment especially in a situation of environmental pollution.

2.10 Ionization techniques

Prior to detection, neutral analyte molecules eluting off the GC column are ionized in an ion source. Ionization modes can either produce positive or negative ions using various techniques including electron ionization (EI), atmospheric pressure photo-ionization (APPI), field ionization, field desorption, plasma desorption chemical ionization such as atmospheric pressure chemical ionization (APCI) and negative chemical ionization (NCI). The most common ionization technique used in GC-MS is EI and I used this approach for the analysis of PACs in my research.

2.10.1 Electron Ionization

In an EI source, formerly known as electron impact source, gas-phase ions of a chemical compound are produced from the loss of one electron from a neutral, uncharged molecule (M) due to its collision with a fast-moving electron, e^-. The electrons are emitted from a heated filament, an important constituent of an ion source. These electrons are accelerated through a high vacuum electric field of 70 V gaining kinetic energy of 70 eV. The collision results in the generation of excited analyte ions upon interaction referred to as molecular ions designated as $M^{+\cdot}$ (Equation 2.4). For every 1000 molecules entering the ion source, one ion is produced under a usual spectrometer conditions at 70 eV.[93] Out of the 70 eV, about 10 to 20 eV is transferred to molecules during the ionization process where approximately 10 eV is enough to ionize most organic

molecules. The excess energy sometimes leads to extensive fragmentation which can provide structural information for the elucidation of unknown compounds.

$$M + e^- \rightarrow M^{+\cdot} + 2e^- \quad \ldots\ldots\ldots\ldots\ldots\ldots (2.4)$$

The $M^{+\cdot}$ ion (radical cation) is left with extra internal energy after collision with the e^- that is then able to break the ion into many fragments of smaller *m/z* values. EI is regarded as a hard ionization technique. This fragmentation pattern is characteristic of a chemical compound or its compound class.

Mass spectra can be obtained at different electron kinetic energy, either at 70 eV or lower energy. At an e^- energy as high as 70 eV, it referred to as hard ionization while lower than 50 eV can be referred to as soft ionization. Lower ionization energy leads to less fragmentation which can favor some fragments leading to relatively higher intensity of some fragments.

An EI mass spectrum is a graph of the mass to charge ratio of ions (m/z) versus the abundance as measured by the mass analyzer. The ion abundance is the ion current where the peak that corresponds to the highest abundance is called the base peak. The base peak can either correspond to the molecular ion or any fragment ions of an analyte.

EI is the most common ionization technique for GC-MS as it widely capable to detect a large number of compounds. It is the fundamental ionization technique adopted for the determination, structural elucidation and quantification of PACs in different environmental matrices.[94-96] Although, EI technique has quite a vast application for analyzing various samples, it has some drawbacks. These include EI non-applicability to non-volatile and analytes that are not thermally stable.

2.11 Mass analyzers

GC-MS combines the feature and ability of GC to separate compounds based on boiling point and polarity with the efficiency of the mass spectrometer to analyze chemical compounds in sample extracts. Once ions are produced in the source, they are separated based on their *m/z* values in a mass analyzer. A GC with an EI system can be coupled to various mass analyzers as presented in Table 2.1. Another trend in mass analyzer interfaces is the combination of different analyzers in sequence to increase the versatility and allow for multiple experiments to be performed. The performance of a mass analyzer can be measured based on five main characteristics. These characteristics are the mass range limit, the analysis speed (scan speed), the transmission, the mass accuracy and the resolution.

The mass range determines the limit of *m/z* over which the mass analyzer can measure while the scan speed is the rate at which the analyzer measures over a set mass range. The transmission is expressed as the ratio of the number of ions reaching the detector and the number of the ions entering the mass analyzer. The accuracy of the *m/z* value provided by the analyzer indicates the instrument's measurement error. The mass measurement error, otherwise known as the mass accuracy, can be further defined as the observed difference between the theoretical *m/z* ($_{\text{theoretical}}$) and the measured *m/z* ($_{\text{measured}}$) by the spectrometer (Equation 5).

$$\text{Mass accuracy} = \frac{m/z\ (\text{measured}) - m/z\ (\text{theoretical})}{m/z\ (\text{theoretical})} \times 10^6 \text{ in ppm (parts per million)} \quad \ldots\ldots\ldots (2.5)$$

Mass accuracy can be positive or negative where ±5 ppm is often used as a limit for acceptance criterion to retain high level of confidence in compound identification or assigning correct formulae.[97]

The resolving power, R, of a mass analyzer can sometimes be related to the level of its mass accuracy, although caution is needed as a high resolution can lead to measurement errors if the mass analyzer is poorly calibrated. Resolving power is the ability of a mass analyzer to yield distinct signals for two ions with a small *m/z* difference (Equation 2.6). A greater R gives rise to an increased ability to distinguish ions with very small mass difference. A low-resolution instrument will not be able to provide high accuracy as it can only analyze based on the nominal mass of a target analyte compound and not the exact mass. The nominal mass of an ion or molecule is calculated using the mass of the most abundant isotope of each element rounded to the nearest integer value and equivalent to the sum of the mass numbers of all constituent atoms of a compound. The exact (theoretical) mass is the calculated mass of an ion's elemental formula, isotopic composition and charge state.

$$R = \frac{m}{\Delta m} \qquad \text{..... (2.6)}$$

Where Δm is the mass difference between two adjacent peaks of equal intensity that are just resolved, and m is the mass of the first peak or the average of the two peaks.

High resolution MS has become more accessible to researchers and has led to the detection of previously unreported compounds in environmental matrices with varying degrees of certainty.[98-99] Modern mass spectrometers are capable of reporting accurate mass (experimentally determined mass of an ion) measurements to four decimal places (seven figures for masses between 100 and 999 Da). The European Commission Decision 2002/657/EC attributed more identification points to fragment ions detected on a high resolution mass spectrometric analysis compared to the low resolution counterpart.[100]

For my study, a quadrupole and time-of-flight mass analyzers were used. A quadrupole analyzer consists of four set of opposing parallel hyperbolic metal rods. The operation of quadrupole devices is by applying a combination of constantly varying voltages through the axis of the rods. This allows transmission of a narrow mass region (generally <1 amu) of selected *m/z* values or a full scan for a range of *m/z* values. Miller and Denton highlighted that quadrupole mass analyzers are mechanically simple instruments that have relatively short distance between the ion-source and the detector with efficient focusing properties. Another feature that makes quadrupoles more desirable is the capability to resolve ions based on their *m/z*.[101] Although they have high sensitivity and good dynamic range, quadrupoles have slow scan rates compared to TOF.[102]

Table 2.1: *Various mass analyzers coupled to gas chromatograph – EI systems*

Type of mass analyzer	**Symbol**	**Principle of separation**
Electric sector	E or ESA	Kinetic energy
Magnetic sector	B	Momentum
Quadrupole	Q	*m/z* (trajectory stability)
Ion trap	IT	*m/z* (resonance frequency)
Time-of-flight	TOF	*m/z* (flight time)
Fourier transform ion cyclotron resonance	FTICR	*m/z* (resonance frequency)
Fourier transform Orbitrap	FT-OT	*m/z* (resonance frequency)

A quadrupole mass analyzer also popularly known as quadrupole mass spectrometer can be used as a standalone unit, this is commonly referred to as single quadrupole mass spectrometer, containing a single mass analyzer or arranged in linear series to form a triple quadrupole mass spectrometer. A triple quadrupole mass spectrometer consists of three quadrupoles where the first (Q_1) and third (Q_3) quadrupoles serve as mass filters while the second quadrupole (q_2) is used as a collision cell. The collision cell employs the use of Ar, He or N_2 to induce dissociation of parent

ions(s) exiting Q_1. Q_1 is usually set to filter a known mass (parent ions), where the fragments of ions produced after a collision in q_2 are been monitored for in Q_3. The fragment ions are essential for more reliable compound identification from sample extracts. A single and triple quadrupole system are capable of running in multiple detection modes e.g. single ion monitoring (SIM) and multiple reaction monitoring (MRM).

Two of the reasons for the wide acceptance of the single and triple quadrupole analyzers are the ease of scanning and quality quantitative data output. Although both quadrupole types have been successfully used to support numerous applications, a triple quadrupole mass spectrometer will give rise to a more accurate identification, quantitation and reproducible results at low concentrations compared to a single quadrupole.[104] There is a lack of selectivity of single quadrupoles relative to triple quadrupole instruments. Depending on the research goal and available resources, a single quadrupole analyzer has been reported to adequately execute analysis without the need for installation of a triple quadrupole analyzer which comes at an increased cost.[105]

For my research, a triple quadrupole mass analyzer coupled to a gas chromatograph and an EI source was used. Quantitation of analytes was determined using the MS responses of target analytes and these responses depend on the cleanliness of the ion source, ion optics, and the collision cell design, ion suppression, ion source flow rates, collision cell pressure and the ultimate MS vacuum.[106] Another factor contributing to the MS response and recognized limitation of the quadrupole mass analyzers is their low resolution. A single quadrupole offers a unit mass resolution (1 Da) full width at half maximum (FWHM) while the triple quadrupole has both unit mass and an enhanced (<1 Da FWHM) resolution.[107]

The resolving power of an analyzer, as earlier discussed, is the ability of the mass analyzer to distinguish and separate two peaks possessing slightly different mass-to-charge ratios. The mass resolution of compounds possessing similar mass-to-charge, especially isomeric compounds, is difficult to separate on low resolution instruments such as the single quadrupole. Polycyclic aromatic compounds (PACs) found in environmental matrices contain several isomeric compounds such as found in crude oil where they possess more alkylated PAHs than parent PAHs. Even though a triple quadrupole offers an enhanced selectivity, these compounds appear as clusters when analyzed which makes challenging identification and quantitation and the requirement for a higher resolution analyzer.

Table 2.2 list the different operational modes of triple quadrupole mass spectrometers as presented by Pitt, 2009.[106]

Table 2.2: *Different operational modes of triple quadrupole mass spectrometers*[106]

Name	**First Quadrupole**	**Third Quadrupole**	**Applications**
Product scan	Fixed	Scanned	Structural studies, identification of unknowns, confirmation by spectral matching with standard.
Precursor scan	Scanned	Fixed	Detection of structurally related analytes that produce a common fragment ion.
Neutral loss (or gain) scan	Scanned	Scanned with fixed offset relative to first quadrupole	Detection of structurally related analytes that eliminate (or gain) a common neutral molecule on collision.
Multiple reaction monitoring	Fixed	Fixed	High sensitivity detection of a panel of targeted analytes.

The triple quadrupole analyzer can be operated: 1) to fully scan for all ions present, 2) for single ion monitoring (SIM) and multiple reaction monitoring (MRM).

2.11.1 Selected ion monitoring (SIM)

The SIM technique can be carried out on both single and triple quadrupole mass spectrometers. This technique is limited in resolution and also *m/z* ratio range. It is mostly adopted for single ion monitoring in the mass analyzer where the selectivity for individual analytes is enhanced while decreasing the instruments response to background noise. Matrix interferences, co-elution of target analytes with compounds that cannot be mass resolved and accuracy are some of the challenges with this technique of ion monitoring.

2.11.2 Multiple reaction monitoring (MRM)

This is a specific and sensitive technique available on a triple quadrupole system to selectively quantify a complex mixture of compounds that are not easily mass resolved with unit mass resolution.

Multiple reaction monitoring (MRM), otherwise known as selected reaction monitoring makes use of the first and the third quadrupoles to scan for specific precursor/product ion pairs. The first Q1 scans for set precursor ions that undergo collision induced dissociation (CID) in the second quadrupole (q2) giving rise to fragments. Some of the resulting fragments, specific to the *m/z* of the precursor ion, are scanned for in the third quadrupole (Q3). The production of the fragment ions from their precursor ion is referred to as ion transitions. In SIM, a single ion transition is done

which detects only the precursor ion while MRM involves multiple transitions detecting precursor ions and daughter/product ions.

This dual filter approach makes MRM a better choice compared to SIM. Numerous compounds have the same precursor ions, but identical product ions are limited, thereby making the identification of analytes more certain with MRM. These product ions can serve as fingerprints for specific compounds in an analysis.

2.12 Time of flight mass analyzer

The time of flight (TOF) mass analyzers were designed to improve resolving power. Initially developed by Wiley and McLaren in 1955, renewed interest since its earliest design has led to progress in its applications, not just only for biomolecules but also for synthetic polymers and polymer conjugates, electronics components and handling the high data flow. TOF analyzers separate ions produced in the source according to their *m/z* when they drift in a flight tube. The flight tube is a field-free region. These ions are expelled from the source by application of a potential difference on the source focusing lenses or by an intermittent process such as found in plasma or laser desorption techniques. The application of a potential between an electrode and the extraction grid accelerates ions towards the flight tube where they acquire the same kinetic energy. Ions then enter the field-free region where they are separated according to time of flight before reaching the detector. Ion *m/z* values are obtained by measuring the time it takes to move an ion through the flight tube between the source and the detector.

Initially, an ion with mass, m, and charge, q (where $q=ze$), before it leaves the source, is accelerated by a potential, Vs. It electric potential energy, E_p, is then converted to kinetic energy, E_k.

$$Ep = qVs \qquad \text{……..……..} \quad (2.7)$$

$$Ek = ½\ mv^2 \quad \text{…..…….……} \quad (2.8)$$

If $E_p = E_k$, then:

$$½\ mv^2 = qVs \quad \text{……...……..……} \quad (2.9)$$

Hence, the velocity of the ion leaving the source is obtained from equation 2.9.

$$v = (2zeVs / m)^{½} \quad \text{……} \quad (2.10)$$

After the initial acceleration, the velocity of the charged particle is constant since its movement is in a field-free tube, hence, the velocity can be determined from the length of the path (L) of the flight of the ion and the time of flight of the ion *(t).*

$$v = L/t \text{ where } t = L/v \quad \text{…….……} \quad (2.11)$$

Substituting for v from equation 2.10

$$t^2 = m/z\ (L^2/2eVs) \quad \text{……..….} \quad (2.12)$$

The terms in parentheses are constant, (k), hence,

$$t^2 = m/z\ (k) \quad \text{…….} \quad (2.13)$$

where *t* becomes:

$$t = k\sqrt{(m/z)} \quad \text{…….} \quad (2.14)$$

Equation 2.14 shows that, all other factors being equal, the lower the mass of an ion, the faster it is for the ion to reach the detector.

TOF instruments have a broad mass range which makes them also suitable for soft ionization techniques where masses above 300kDa have been detected using a MALDI-TOF and can be obtained in micro-seconds. Another characteristic of TOF analyzers that makes them highly sensitive is their high transmission efficiency. Here, temporal separation of the ions produced allows them all to be directed to the detector contrary to what is observed on scanning analyzers that transmit ions successively along a time scale.

The ease of mass calibration on TOF analyzer can be attributed to the fact that it uses only two reference points. The calibration relates and converts flight time of the ions, a physical property, to a mass value. With the conversion, two known molecules (standards) can be calibrated by using their *m/z* ratios and their measured flight times. Mass calibration can be either internal or external, although the highest degree of mass accuracy is achieved through internal calibration. An internal calibration method uses the flight times of a standard and unknown ions from the same samples while external calibration method generates its calibration from two standards in an experiment that does not include an unknown molecule.

The mass resolution of TOF analyzers is proportional to the flight path and time.[93] Thus, an increased resolution can be obtained by increasing the length of flight tube. The downside to increased flight tube length is that it increases loss of ions by scattering after collision with gas molecules hence decreasing the performance of the analyzer. Ways to circumvent the poor resolution has led to the development of delayed pulsed extraction and ion reflectors. With delayed pulsed extraction, a time lag between ion formation and extraction is introduced after which a voltage pulse is applied to extract the ions outside the source. In continuous extraction mode, voltage is applied for immediately extraction. Reflectors on the other hand use an electrostatic reflecting lenses and were first proposed by Mamyrin.[108] The reflector is located in the field-free region opposed to the ion source. The detector in this set up is situated on the source side of the ion mirror to capture the arrival of the ions after are reflected. The reflectron has the ability to correct the kinetic energy of the dispersed ions leaving the ion source with same *m/z* ratio. Although, the increase in flight path has a positive effect on the mass resolution of the analyzer, it does this at the expense of the sensitivity and introduces a mass range limitation.

For the purpose of my study, the TOF instruments used has a novel flight path called the folded flight path (FFP^{TM}) technology (Figure 2.7). As it has been established that increased flight tube length favors increased resolution, the FFP^{TM} technology is designed not to extend the size of the system but the ion optics permits the creation of a hyperextended flight path. Ions generated in the source pass through a parallel set of electrostatic mirrors that refocus the ions, thereby minimizing dispersion based on energy. The ions are focused in two dimensions (perpendicular to travel) as they transverse between the mirrors. This leads to higher transmission efficiency of the ions, improves sensitivity, throughput and quality of the results.

The FFP^{TM} technology in combination with the KADAS, a patented ion statistics-based, acquisition system on LECO instrument permits mass accuracy better than 1ppm, with acquisition rates of 200 spectra/second in the two-dimensional mode.

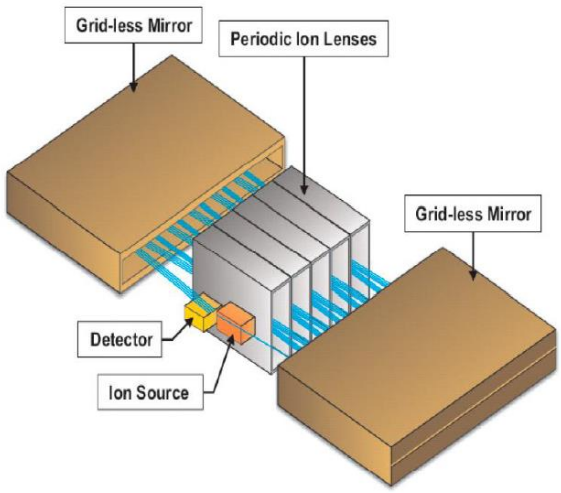

Figure 2.6: *The FFPTM Time of flight mass analyzer*

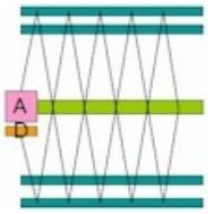

Figure 2.7: *Flight paths for TOF mass analyzer up to R=50,000 and mass accuracy <1 ppm*

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Chapter 3

Enumeration of the Constitutional Isomers of Environmentally Relevant Substituted Polycyclic Aromatic Compounds

A version of this chapter has already been published as Johnson, W., Idowu, I.; Francisco, O., Marvin, C., Thomas, P.J., Stetefeld, J. and Tomy. G.T. Enumeration of the constitutional isomers of environmental relevant substituted polycyclic aromatic compounds. Chemosphere, 202: 9-16, 2018.

My role in this manuscript was to review and interpret all the peer-reviewed literature and I wrote parts of the manuscript. The lead author, Wesley Johnson, was responsible for the mathematical and molecular symmetry approach to evaluating theoretical isomers. This manuscript was added to show the complexity of the compounds discussed in my thesis.

Abstract

Polycyclic aromatic compounds (PACs) are a diverse group of environmentally relevant compounds which can be persistent, bioaccumulative and toxic. The cyclic backbone of PACs can be substituted with halogens or hydrocarbon chains. The amount and positions of these substituents influence their toxicity. For many classes of PACs, substitution creates mixtures containing large numbers of isomers. For example, 209 theoretical isomers of chlorinated biphenyls are possible. Many other classes of environmentally relevant PACs exist where the number of theoretical isomers are unknown. Here, a mathematical approach using molecular symmetry and the binomial coefficient is presented that determines the number of theoretical isomers of PACs. The approach was validated on PACs with known isomer numbers and then applied to PACs with unknown isomer numbers. When the approach was applied to alkylated polycyclic aromatic hydrocarbons, the possible theoretical isomers ranged from 2 for C_1 naphthalene up to 19 502 for C_6 dibenzo (a,h)anthracene. Heterocyclic PACs had similar numbers ranging from 4 isomers for C_1 dibenzothiophene to 13 938 for C_6 dibenzo (a,i)carbazole. The work presented will aid analytical chemists and ecotoxicologists in their efforts to develop methods to measure these compounds, and in attempting to assess the toxicity and environmental fate of individual isomers.

3.1 Introduction

Concerns regarding the possible adverse effects of toxic environmental contaminants have received considerable global attention during the past half-century. A commonly studied group of organic contaminants include polycyclic aromatic compounds (PACs).[1-3] Many of the compounds in this diverse group are known to be persistent, bioaccumulative and toxic (PB&T) and have been detected in remote regions of the world.[4-11] In fact, since 2000, polycyclic aromatic hydrocarbons (PAHs) have dominated the summed contaminant burdens in lower trophic biota in the Arctic.[12]

The PACs that have received the most attention include PAHs, polychlorinated biphenyls (PCBs), polychlorinated dibenzo-p-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs) and polybrominated diphenyl ethers (PBDEs). With the exception of PAHs, all the aforementioned compounds have been recognized by the Stockholm Convention as persistent organic pollutants (POPs). Many chemicals listed in the Stockholm Convention list of persistent organic pollutants are classified as industrial chemicals and by-products that are sourced from either anthropogenic or pyrogenic origins.[1, 3, 13]

It is now widely acknowledged that the number of substituents (defined here as the moieties attached to the aromatic backbone) and their position on the aromatic molecule are major drivers of the environmental fate and toxicities of these compounds. For example, while 209 congeners are possible for PCBs, less than half of these congeners are environmentally significant with resultant adverse health effects.[14-16] Isomer-based toxicity studies on PCBs suggest the coplanar PCBs, 3,3',4,4'-tetrachlorobiphenyl (tetraCB), 3,3',4,4',5-pentaCB, 3,3',4,4',5,5'-hexaCB, and their monoortho analogs are aryl hydrocarbon(Ah)-receptor agonists and contribute significantly to the toxicity of PCB mixtures.[17] Another example that highlights how the amount and positioning of substituents impacts toxicity is for PCDDs. The 2,3,7,8- TCDD (2,3,7,8-tetrachlorodibenzo-p-

dioxin) isomer has repeatedly been shown as the most toxic compound of the PCDDs.[17-18] In fact, the toxicities of all other PCDDs are reported relative to the toxicity of 2,3,7,8-TCDD.

There is also compelling evidence showing that the position and extent of alkylation on PAHs can affect overall chemical toxicities. For example, benz(a)anthracene is a potent genotoxic compound which has been classified as a human carcinogen.[19] However, the 7,12-dimethyl benz(a)anthracene derivative has been reported to be the most potent mutagenic and carcinogenic PAHs.[20] It has also been shown that the position of a single methyl group on the benz(a)anthracene backbone can strongly affect metabolic activation, mutagenicity, tumor-initiating ability and carcinogenicity.[21-25] Similarly, 5-methylchrysene has also been shown to be more carcinogenic than its parent and even more toxic than any other methyl substituted isomer.[26-27]

Clearly, knowledge of the substitution pattern and degree of substitution of PACs is paramount to assessing their PB&T potential. In fact, substitution patterns and degree of substitution are the basis of toxicity and physico-chemical quantitative structure activity relationship (QSAR) predictive models. Perhaps equally important to knowing the position of substituents and degree of substitution on a cyclo-aromatic molecule is knowledge of the number of theoretical isomers (i.e. isomers) of any PAC class.

Efforts to enumerate the isomers for a particular PAC have largely relied on manually drawing and counting. For PCDDs and PCDFs this task is not overly daunting considering that the number of constitutional isomers are 75 and 135 respectively. For PCBs (and PBDEs) deciphering the number of isomeric structures becomes more tedious. There are some PACs whereby manually enumerating the possible constitutional isomers becomes impossible. This is especially true for alkyl-substituted PAHs. Alkylated PAHs (aPAHs) are substituted PAHs possessing vast structural diversity. They consist of two or more aromatic rings with an alkyl-substituent(s) attached at

different positions. The alkyl-substituted polycyclic aromatic compounds which include the C1 to C4 homologues of the 16 USEPA priority parent PAHs (including dibenzothiophene; DBT) have significantly greater effects on the toxicity of mixtures than their parent PAHs, especially in crude oils where they are present at greater concentrations relative to their parent group.[28-30] Alkyl PAHs comprise up to 98% of the total PAH fraction in crude oil, constituting as much as 70% of the total PAH fraction of the sediment in aquatic environments in the event of a spill.[31-33] There are other groups of scarcely studied substituted heterocyclic aromatic PAH-like compounds i.e., where a carbon atom is replaced by nitrogen (aza-arenes) and sulphur atoms (thia-arenes) that can also contain alkyl substituents. More reports exist on the fate and effect of the unsubstituted parent compounds[34-36] with few focusing on aPAHs despite their known abundance.

This paper uses combinatorics and molecular symmetry to enumerate the theoretical constitutional isomers of PACs. The equations were validated with PCBs, PCDDs, PCDFs and PBDEs where the number of constitutional isomers have already been determined. With a clearer understanding of the number of possible isomers for PACs, especially aPAHs, more isomer-specific analytical separations and detections could be undertaken, resulting in increased environmental relevance for various ecotoxicological studies.

3.2 Mathematical approach

In order to develop mathematical relationships to determine the number of unique constitutional isomers for PAC an understanding of the binomial coefficient and some basic symmetry of these molecules is first necessary.

Consider a set of the form:

$$A = \{a_1; a_2; a_3 \ldots a_n\} \quad \text{..... (3.1)}$$

The number of unique subsets of size k that can be made from A is given by the binomial coefficient formula:

$$\binom{n}{k} = \frac{n!}{(n-k)!k!} \quad \text{..... (3.2)}$$

This relation is valid providing that all elements of the set A are unique. The binomial coefficient was used to count the arrangements of substituents on a parent molecule backbone. The binomial coefficient is not specific to chemistry and when applied to molecular systems, molecular symmetry needs to be considered separately.

Since most PACs exist in planar form, they should have a mirror plane cutting through the plane of the molecule. This is the least amount of symmetry and if no other elements of symmetry are present the point group is C_s. Different point groups can be made by adding symmetry elements, though the majority of the molecules discussed in this paper belong to the C_{2h}, C_{2v} or D_{2h} point groups. These point groups each contain a principal axis. When rotation around the principal axis is 180º, it produces the identical chemical structure. A molecule belonging to the C_{2h} point group also possesses an inversion center, while a molecule belonging to the C_{2v} point group contains an additional mirror plane perpendicular to the original mirror plane. The D_{2h} point group contains three orthogonal mirror planes and three C_2 axes where the mirror planes intersect. Four alkylated versions of naphthalene are shown in Fig. 3.1 as representations of the different point groups.

3.3 Proof of concept

Before applying our mathematical approach to enumerate the number of isomers for substituted PACs, we will first ground truth our method for compounds in which the number of constitutional isomers has already been confirmed.

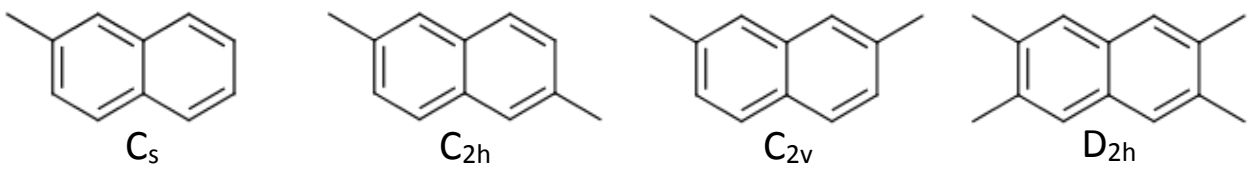

Figure 3.1. *Symmetries of alkylated naphthalenes. From left to right are 2-methylnaphthalene, 2,6-dimethylnaphthalene, 2,7-dimethylnaphthalene and 2,3,6,7-tetramethylnaphthalene with their corresponding symmetries.*

3.3.1 Polychlorinated Dibenzofurans (PCDFs)

The simplest case for determining the number of constitutional isomers is when all substituents attached to the parent molecule are identical. One example of this is when multiple chlorines are attached to dibenzofuran, giving rise to the various PCDFs. The structure of dibenzofuran is shown in the bottom right of Fig. 3.2 and the symmetry of the molecule is C_{2v}.

There are four unique placements of a single chlorine on dibenzofuran: positions 1, 2, 3, or 4. Positions 6, 7, 8, and 9 are equivalent to the first four when considering molecular symmetry. This can also be viewed as a combination problem where there are eight possible positions for the substituent, with one chosen for its actual location. This can represented by the binomial coefficient $\binom{8}{1}$ which equals eight. Since the C_{2v} symmetry of the parent molecule gives it two

equal segments (four if you include the plane of symmetry that cuts through the atoms, although this plane is ignored throughout the following discussion), the eight substitution positions are divided by two to yield four possible isomers. The next step in complexity is to place two identical substituents instead of one.

Using the same approach as above, placing two chlorines would result in 14 different isomers ($\binom{8}{2}$ is 28, 28 divided by 2 is 14). However, there are 16 known isomers of dichlorinated

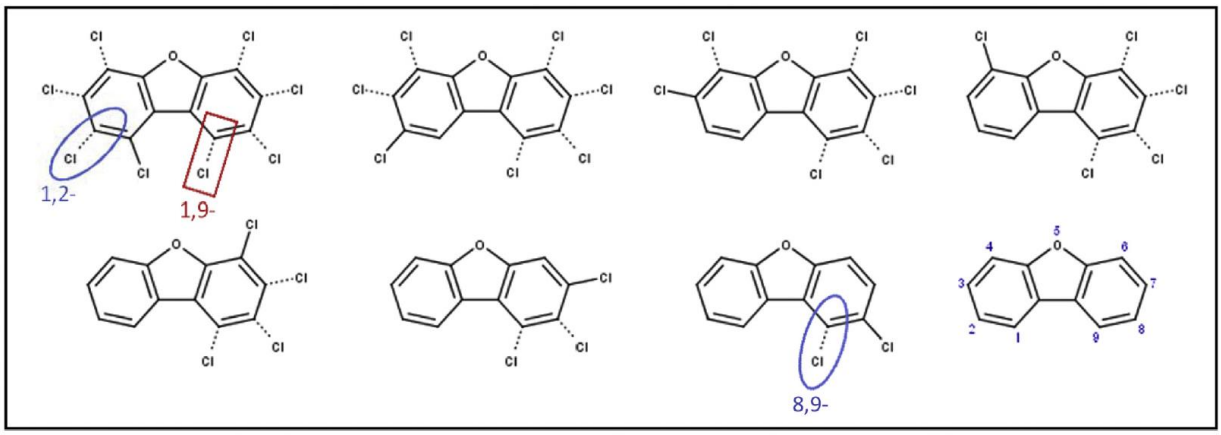

Figure 3.2 *Combinations for dichlorodibenzofuran. The solid chlorines are the fixed chlorines and the dotted chlorines are the options for the placement of the second chlorine. There are seven options with the first fixed chlorine, six for the second, five for the third, four for the fourth, three for the fifth, two for the sixth and one for the seventh for a total of 28.*
The highlighted positions of the second chlorine show groupings of equivalent isomers.

dibenzofuran. Why there are 16 isomers instead of 14 can be explained by more carefully examining all placements the binomial coefficient returns. The placement of two chlorines on dibenzofuran by the binomial coefficient can be visualized by fixing one chlorine on position one and looking at the available options for the second. When the second chlorine has no more available placements, the first fixed chlorine is shifted to position two and the process repeated. A schematic of this is shown in Fig. 3.2.

Identical isomers present in Fig. 3.2 demonstrate the binomial coefficient not accounting for equivalent positions on the molecule. Highlighted in Fig. 3.2 are two isomers along with any

equivalents: 1,2-PCDF (equivalent to 8,9-PCDF) and 1,9-PCDF. Each pair of equivalent isomers represents only one unique isomer. Therefore, pairs of identical isomers should be divided by two while singularly occurring isomers should be left as one to determine the number of unique isomers. However, when looking at the 28 isomers given by the binomial coefficient, the pairing of the isomers is unclear. When single isomers are divided by two, it results in one-half, which is less than the correct value of one. The original division of the 28 isomers by 2 assumes that all isomers are occurring in identical pairs. Since some isomers do not have equivalents, the division of all possibilities by two results in an underestimation of the possible dichlorodibenzofuran isomers. A simple correction can be made by adding one half for every singularly occurring isomer. Determining how many isomers that have no equivalents requires examining the molecular symmetry of the isomers.

The molecular symmetry dictates whether or not isomers occur in pairs. Isomers in pairs have Cs symmetry, while lone isomers have C_{2v} symmetry. The C_2 rotational axis of the parent molecule creates equivalent positions on either side of the molecule. When two chlorines are placed on non-equivalent carbons, the symmetry is reduced to C_s, such is the case with 1,2-PCDF. When 1,2-PCDF is rotated by 180º, it gives 8,9-PCDF. They are both the same molecule but together count as two of the arrangements given by the binomial coefficient. When two chlorines are placed on equivalent carbons as they are in 1,9-PCDF, the original symmetry of C_{2v} is maintained. When 1,9-PCDF is rotated by 180º, the same molecule is produced (a property of C_{2v} symmetry). Therefore, isomers with C_{2v} symmetry occur without any equivalents.

Determining the number of unique symmetric isomers needs to be done by hand, but is much simpler than the total number of isomers due to the limited combinations that give rise to molecular symmetry. In the case of dichlorodibenzofuran, there are 16 unique isomers, while only 4 of them

are symmetric. Symmetric isomers cannot be created when placing an odd amount of chlorines on dibenzofuran since an even number of substituents is needed to have two equivalent halves. When symmetrical isomers cannot be created, the total number of isomers is simply the result of the binomial coefficient divided by two. Therefore, the number of isomers for a trichlorinated dibenzofuran would be $\binom{8}{3}$ divided by two which is 28.

This process can also be applied to the tetrachlorinated dibenzofuran isomers. Any count for greater chlorination degree will equal one of the values for the lower chlorination amounts given the symmetric nature of the binomial coefficient. Instead of viewing the binomial coefficient as a method to pick a set number of hydrogens to be replaced with a substituent, it can be viewed as a method to make two groups out of the available hydrogens; one group for replacement and the other to remain as hydrogens. The number of ways to create the two groups remains the same regardless of which group is replaced and which group remains hydrogens. Putting all the values generated by this method in Table 1 shows that the correct number of isomers for each chlorination level has been obtained. This process also holds true for molecules with C2h symmetry.

Table 3.1 *Calculated number of isomers for PCDFs, PCDDs, PCBs and PBDEs.*

Halogenation #	1	2	3	4	5	6	7	8	9	10	Total
PCDF	4	16	28	38	28	16	4	1	-	-	135
PCDD	2	10	14	22	14	10	2	1	-	-	75
PCB or PBDE	3	12	24	42	46	42	24	12	3	1	209

3.3.2 Polychlorinated Dibenzo-p-dioxins (PCDDs)

Polychlorinated dibenzo-p-dioxins share features with PCDFs: chlorines are the only substituents, and they have eight available positions for placement. The main distinction between the two classes is that the parent molecule dibenzofuran has C_{2v} symmetry while dibenzodioxin has D_{2h} symmetry.

The process of enumerating the isomers for PCDFs is similar to PCDDs with two additional considerations. Since the parent molecule now starts with D_{2h} symmetry, there are four equal quarters of the molecule. This implies the count from the binomial coefficient should be divided by four instead of two as it was with C_{2v} molecules. It is also possible that some arrangements of four chlorines would have D_{2h} symmetry. Rotation about any of the three axes of symmetry by 180° would produce the original molecule so there are no equivalent isomers when this symmetry occurs. In other words, D_{2h} symmetric isomers have no equivalents when counted by the binomial coefficient. Division by four gives one quarter, implying that three quarters needs to be added for every way a D_{2h} symmetric isomer can be made. Putting the above considerations together with the previous method will give an equation for the unique arrangements of identical substituents:

$$\begin{matrix}Number\ of\ Arrangements \\ of\ similar\ substituents\end{matrix} = \frac{\binom{H}{S}}{P} + (0.5)\begin{pmatrix}\#\ of\ C_2\ symmetric \\ arrangements\end{pmatrix} + (0.75)\begin{pmatrix}\#\ of\ D_{2h}\ symmetric \\ arrangements\end{pmatrix} \quad \ldots. (3.3)$$

where H is the number of replaceable hydrogens, S is the number of substituents being placed on the molecule, P is a factor based on the parent molecule symmetry which is four when the parent molecule symmetry is D_{2h} and two when the symmetry is C_{2v} or C_{2h}.

Substituted isomers will never be able to gain more symmetry than the original parent molecule. For example, a parent molecule with C_{2v} symmetry cannot become C_{2h} after substitution. Similarly, a parent molecule with C_{2h} symmetry cannot become C_{2v} after substitution. Parent molecules with D_{2h} symmetry, however, can become any of C_{2v}, C_{2h} or D_{2h} as the required

elements of symmetry for each are contained in the original molecule. The change to other symmetries occurs when certain substitution patterns regain some, but not all, of the elements of symmetry. From this, the terms of the equation that apply to symmetries that are unobtainable can be ignored.

This equation is limited to molecular systems with one of the four symmetries previously discussed. The equation can be expanded to work with other symmetries, however, P could take on different values and additional terms would need to be added. Additional terms pertaining to D_{6h} symmetry are presented in the supplemental information.

The values for the number of isomers for PCDDs obtained with Equation 3.3 are stated in Table 4.1 and match the known values.

3.3.3. PCBs and PBDEs

Both PCBs and PBDEs contain two phenyl rings. The rings of PCBs are linked directly, whereas PBDEs are linked by an ether. In both cases, the bond between the two rings can rotate despite steric hindrance. The rotation makes PCBs and PBDEs unlike the other molecular systems that have been examined so far. They are similar to one another in that they both have a known amount of 209 isomers, and the following explanation applies to both.

While the molecule has a rotatable bond in the middle, the two phenyl rings attached are planar and rigid. The proposed counting method can be applied to each half with subsequent consideration to how the two halves can be combined. The planar ring that makes up half the molecule is given in Fig. 3.3. The ring has C_{2v} symmetry with five replaceable hydrogens. Equation 3.3 can be applied to the system to find the arrangements of up to five halogens on the ring given in Table 3.2.

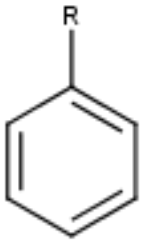

Figure 3.3 *Phenyl ring that is half of either PCB or PBDE. The R group is the other phenyl ring for PCB, and is an ether linked phenyl group for PBDE.*

Table 3.2 *Number of arrangements of up to five halogens on the phenyl functional group.*

Halogenation Number	1	2	3	4	5
Number of Arrangements	3	6	6	3	1

Overall, ten positions are available for halogens to be placed. Only the arrangements for up to five need to be calculated since the binomial coefficient is symmetrical as previously mentioned. When considering the first five halogens, two different situations could occur. The first is when all halogens occur on a single ring. When this happens, the number of possible arrangements can be found in Table 2. The other situation is when the halogens are split between the two rings. When different amounts are on each ring, the available options for each from Table 2 are simply multiplied together. When the same number of halogens are on each ring, multiplying the available arrangements together is no longer appropriate. When the available options are multiplied together, it is approaching the problem from a permutation standpoint where order matters. Since the overall molecule has two equal halves, it makes no difference if the first ring has one arrangement and the second another, or vice versa. The problem is more accurately represented as a combination with

replacement. This is still calculated using the binomial coefficient, but $\binom{n-1+k}{k}$ is used instead of the usual $\binom{n}{k}$.

Based on the above, when one halogen is present, it can only be on one ring and there are three arrangements as shown in Table 2. When two halogens are present, one can be on each of the rings. This is a combination with replacement problem where n is 3 and k is 2 equating to six arrangements. Those six arrangements are added to the six possibilities when they are on the same ring for a total of twelve arrangements for two halogens. The same is done for increasing amounts of halogens to generate the known number of isomers for PBDEs or PCBs shown in Table 3.1.

3.4. Application of the methodology to other cyclic compound classes

3.4.1. Alkylated PAHs

Alkylated PAHs present a new challenge of placing non-identical substituents since carbon based substituents can form chains. The first step in solving this problem is to determine the ways the carbon atoms involved in the alkylation can be arranged into unique sets of substituents. The second step is to place those substituents onto the PAH backbone, building on the method already established. As the extent of the alkylation increases, the number of unique substituent combinations as well as the difficulty in determining them grows exponentially. With the difficulty and relevance in mind, the following discussion has been limited to aPAHs with an alkylation number (carbon number) up to 6 (i.e., C_6). The simplest case for alkyl substituents is if all the carbons are grouped together as one single substituent.

The total number of single substituents for each carbon number are listed in the first column in Table 3. Counting the unique attachment points for all structural isomers of each carbon number

will yield the values found in the table. A visual example for the unique attachment points of C_5 is given in Fig. 3.4.

Table 3.3 *Number of single and multiple substituent possibilities for each carbon number*

	1 Substituent	2 Substituents	3 Substituents	4 Substituents	5 Substituents	6 Substituents
C_1	1	0	0	0	0	0
C_2	1	1	0	0	0	0
C_3	2	1	1	0	0	0
C_4	4	3	1	1	0	0
C_5	8	6	3	1	1	0
C_6	17	15	7	3	1	1

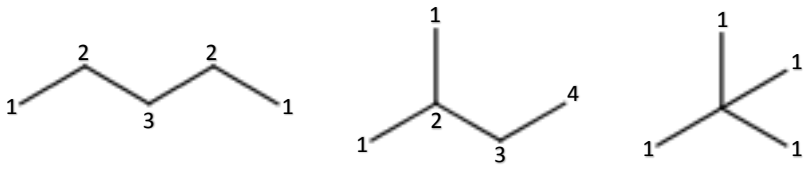

Figure 3.4 *Unique attachment points for C5. n-Heptane has three unique attachment points, 2-methylbutane has four, and 2,2-dimethylpropane has one for a total of eight.*

While the first column of Table 3.3 describes how many single substituents can be made with the corresponding number of carbons, it does not account for multiple substituents. To find the number of possibilities for multiple substituents, it is first necessary to determine all the sets of numbers that add up to a given carbon number. Using C_4 as an example, there are four sets of numbers that add up to four: 3 + 1, 2 + 2, 2 + 1 + 1, and 1 + 1 + 1 + 1. The number of single substituent possibilities for each of the numbers in a set are multiplied together to get the number of ways for that set to happen. Continuing with the first set from the same example, C_3 can be made a single

substituent in two ways, and there is one way C_1 can be made a single substituent. When these values are multiplied together, the result of two indicates there are two ways to group four carbons into a three carbon substituent and a one carbon substituent. There is only a single way to make all other sets of numbers as C_1 or C_2 can be made a single substituent in only one way. The total of all sets is the total number of ways that four carbons can be arranged into multiple substituents. When the same process is repeated for each of the carbon numbers, the rest of Table 3 is generated which contains the number of ways that multiple substituents can be created for each of the carbon numbers.

Since the goal of determining how many arrangements substituent carbons can take is to place them onto the backbone of a PAH, the distinction between two substituents that are the same and two that are different needs to be made as it influences the possible combinations. As stated in Table 3.3, there are three ways to make two substituents with four carbons. One of those ways involves two ethyl groups, the other two involve a methyl with either a propyl or an isopropyl group. Of the three options, one contains a pair of identical substituents, and the other two contain pairs of substituents that differ. When the distinction is made between like or unlike substituents, Table 3.3 is expanded to create Table 3.4.

Table 4 contains all the information necessary to proceed with the addition of the alkyl substituents onto the PAH backbone. However, before placing substituents, Equation 3 needs to be applied to each of the PAHs serving as a backbone. The number of substituents used in the equation should only go up to six, as it is impossible to make more than six substituents with six carbons. As a note, there are replaceable hydrogens that lie on an axis of rotation in anthracene and pyrene. If working with an odd number of substituents, one of these hydrogens can be replaced leaving an even number for the remaining positions. This makes it possible to create symmetric isomers using

an odd number of substituents for these compounds. The results of the calculations for each of the PAHs are presented in Table 3.5.

So far only identical substituents have been considered, but it is also possible that unlike substituents will be attached to the parent molecule, as noted in Table 3.4. When different substituents are placed on the same molecule, the symmetry of their arrangement plays a role in how many possibilities are created. Each type of substituent from Table 3.4 (three of one and one of another; two groups of two; etc.) needs to be considered individually. The way substituent arrangement affects the number of possibilities is shown in Fig. 3.5 using three methyl groups and an ethyl group being placed onto naphthalene as an example.

The first row in Fig. 3.5 shows a C_s symmetry placement of the substituents. When three like and one unlike substituent are placed in this arrangement, four different outcomes are possible. This is because all positions being substituted with a substituent are unique, giving the unlike substituent four different positions that would result in unique isomers. The second row shows a C_{2v} symmetry placement of the substituents which is also representative of C_{2h} arrangements. In either of these arrangements, there are only two unique substituent positions, leaving two isomers that could be created. The last row shows a D_{2h} symmetry placement of the substituents. In this case, all substitution positions are identical leaving only one possible isomer. To end with the total number of ways that the three methyl groups and one ethyl group could be placed onto naphthalene, the number of ways to place substituents in C_s, C_{2v} or C_{2h}, and D_{2h} arrangements should be multiplied by four, two and one respectively. When this is done, the result is 70 different ways to arrange the said substituents onto naphthalene.

Table 3.4 *Number of possible substituent combinations.*

# of substituents	1	2		3			4			5		6
Type of Substituents	-	All Same	All Unique	All Same	Two Same	All Unique	All Same	Three Same	Two Pairs of Two	All Same	Four Same	All Same
C_1	1	0	0	0	0	0	0	0	0	0	0	0
C_2	1	1	0	0	0	0	0	0	0	0	0	0
C_3	2	0	1	1	0	0	0	0	0	0	0	0
C_4	4	1	2	0	1	0	1	0	0	0	0	0
C_5	8	0	6	0	3	0	0	1	0	1	0	0
C_6	17	2	13	1	4	2	0	2	1	0	1	1

This process can be repeated for all non-identical groupings of substituents found in Table 3.4. The number of C_{2v}, C_{2h} or D_{2h} arrangements for each of the PAHs will be the same as those used in Equation (3) to create Table 3.5. Table 6 shows the results of those calculations which is the number of isomers possible for non-identical substituents.

Table 3.6 complements Table 3.5 and provides the last information needed to determine the total number of isomers for the alkylated versions of the 16 priority PAHs. Table 4 contains the number of ways that the different number of carbons can be grouped into different arrangements of substituents. Tables 5 and 6 contain the number of ways those substituents can be placed onto the PAHs. By multiplying each of the values in Table 4 by the corresponding values in Tables 3.5 and 3.6, the result is the total number of possible isomers. The calculation for the number of isomers of C3 naphthalene will be discussed as an example.

Table 3.4 shows there are two ways to make one substituent, one way to make two unique substituents, and one way to make three identical substituents for C3. Table 3.5 shows there are two ways a single substituent can be placed on naphthalene. The two ways to create a single substituent multiplied by the two ways to place them gives four isomers. Table 5 also shows there are 14 ways to place three identical substituents. The one way to make three identical substituents multiplied by the 14 ways to place them on naphthalene gives an additional 14 isomers. Lastly, Table 6 shows there are also 14 ways to place two unique substituents on naphthalene. The one way to create two unique substituents multiplied by the 14 ways to place them adds another 14 isomers for a total count of 32. This is repeated for all other alkylation levels of the 16 priority PAHs.

Table 3.5 *Isomer possibilities using identical substituents.*

Number of Substituents	**1**	**2**	**3**	**4**	**5**	**6**
Naphthalene	2	10	14	22	14	10
Acenaphthylene	4	16	28	38	28	16
Acenaphthene	5	25	60	110	126	110
Fluorene	5	25	60	110	126	110
Phenanthrene	5	25	60	110	126	110
Anthracene	3	15	32	60	66	60
Fluoranthene	5	25	60	110	126	110
Pyrene	3	15	32	60	66	60
Benz(a)anthracene	12	66	220	495	792	924
Chrysene	6	36	110	255	396	472
Benzo(b)fluoranthene	12	66	220	495	792	924
Benzo(k)fluoranthene	6	36	110	255	396	471
Benzo(a)pyrene	12	66	220	495	792	924
Dibenzo(a,h)anthracene	7	49	182	511	1001	1519
Indeno(1,2,3-c,d)pyrene	12	66	220	495	792	924
Benzo(g,h,i)perylene	6	36	110	255	396	472

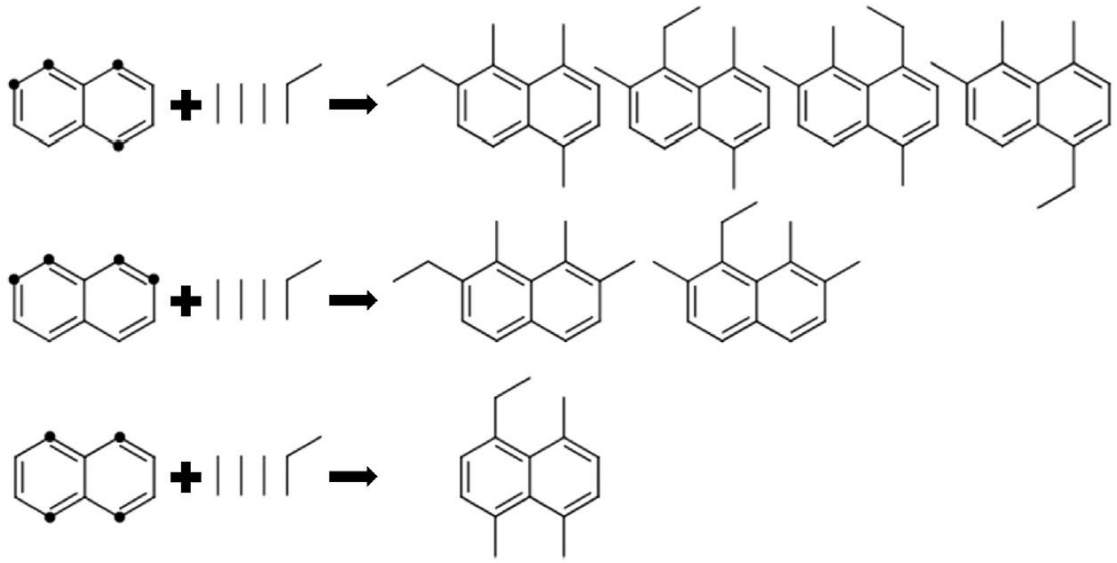

Figure 3.5. *Unlike methyl and ethyl substituents being placed on naphthalene.*

Table 3.4 shows there are two ways to make one substituent, one way to make two unique substituents, and one way to make three identical substituents for C3. Table 3.5 shows there are two ways a single substituent can be placed on naphthalene. The two ways to create a single substituent multiplied by the two ways to place them gives four isomers. Table 5 also shows there are 14 ways to place three identical substituents. The one way to make three identical substituents multiplied by the 14 ways to place them on naphthalene gives an additional 14 isomers. Lastly, Table 6 shows there are also 14 ways to place two unique substituents on naphthalene. The one way to create two unique substituents multiplied by the 14 ways to place them adds another 14 isomers for a total count of 32. This is repeated for all other alkylation levels of the 16 priority PAHs.

Table 3.6. *Isomer possibilities using non-identical substituents.*

	2 Substituents	**3 Substituents**		**4 Substituents**		**5 Substituents**
	(1:1)	**(1:1:1)**	**(1:2)**	**(1:3)**	**(2:2)**	**(1:4)**
Naphthalene	14	84	42	70	114	70
Acenaphthylene	28	168	84	140	216	140
Acenaphthene	45	360	180	420	640	630
Fluorene	45	360	180	420	640	630
Phenanthrene	45	360	180	420	640	630
Anthracene	23	180	92	212	330	318
Fluoranthene	45	360	180	420	640	630
Pyrene	23	180	92	212	330	318
Benz(a)anthracene	132	1320	660	1980	2970	3960
Chrysene	66	660	330	990	1500	1980
Benzo(b)fluoranthene	132	1320	660	1980	2970	3960
Benzo(k)fluoranthene	66	660	330	990	1500	1980
Benzo(a)pyrene	132	1320	660	1980	2970	3960
Dibenzo(a,h)anthracene	91	1092	546	2002	3024	5005
Indeno(1,2,3-c,d)pyrene	132	1320	660	1980	2970	3960
Benzo(g,h,i)perylene	66	660	330	990	1500	1980

Table 3.7 *Theoretical number of isomer for aPAHs and alkyl heterocyclics.*

	C1	**C2**	**C3**	**C4**	**C5**	**C6**
Naphthalene	2	12	32	110	310	920
Acenaphthylene	4	20	64	210	620	1816
Acenaphthene	5	30	115	425	1396	4440
Fluorene	5	30	115	425	1396	4440
Phenanthrene	5	30	115	425	1396	4440
Anthracene	3	18	61	225	716	2272
Fluoranthene	5	30	115	425	1396	4440
Pyrene	3	18	61	225	716	2272
Benz(a)anthracene	12	78	376	1533	5640	19366
Chrysene	6	42	188	777	2820	9714
Benzo(b)fluoranthene	12	78	376	1533	5640	19366
Benzo(k)fluoranthene	6	42	188	777	2820	9714
Benzo(a)pyrene	12	78	376	1533	5640	19366
Benzo(e)pyrene	6	42	188	777	2820	9714
Dibenzo(a,h)anthracene	7	56	287	1316	5243	19502
Indeno(1,2,3-c,d)pyrene	12	78	376	1533	5640	19366
Benzo(g,h,i)perylene	6	42	188	777	2820	9714
Benzothiophene	6	21	62	144	474	1263
Dibenzothiophene	4	20	64	210	620	1816
Benzofuran	6	21	62	144	474	1263
Benzo(b)naphthofuran	10	55	230	835	2792	8840
Benz(a)acridine	11	66	297	1034	4015	13244
Dibenzo(a,i)carbazole	7	49	238	1023	3901	13938
Benzo(b)naphthothiophene	10	55	230	835	2792	8840

Table 3.7 shows the number of theoretical aPAH isomers (C1-C6) possible for the 16 priority PAHs. It is important to note that multiple operations were used to produce these numbers and a single formula, while desirable is too ambitious. Although no single formula exists, the step-by-step process described here can still be easily applied to other molecular systems.

3.4.2. Heterocyclics

There are other important alkylated heterocyclic compounds that can be present in oil for which little is known about the number of constitutional isomers.[37] They include molecules containing sulfur, nitrogen, and oxygen atoms. Two representative compounds containing each of the listed atoms are shown in Fig. 3.6.

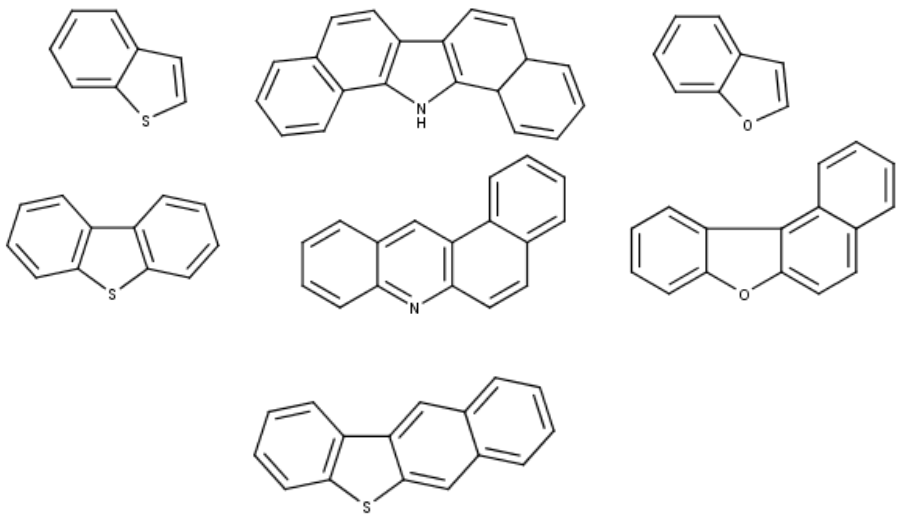

Figure 3.6. *Structures of (a) benzothiophene, (b) dibenzo[a,i]carbazole (c) benzofuran (d) dibenzothiophene, (e) benz[a]acridine, (f) benzo[b]naphthofuran, and (g) benzo[b]naphthothiophene.*

The step-by-step approach described in the previous section can be applied to these molecules. Benzothiophene and Benzofuran share the same structure differing by a sulfur or oxygen atom and will therefore have the same number of theoretical isomers. Dibenzothiophene has C_{2v} symmetry

and eight possible substitution positions meaning it has the same properties as acenaphthylene implying it will have the same number of theoretical isomers. Benzo[b]naphthofuran, benz[a]acridine and dibenzo[a,i]carbazole are unique when compared to previously examined molecules and must be calculated independently. Using the approach above, the number of theoretical isomers for each of the heterocyclic compounds are included in Table 3.7.

3.5. Conclusion

The method described here to determine the number of constitutional isomers of PACs combines combinatorics and molecular symmetry. The approach works best when the aromatic portion of the molecule is fully conjugated, but can still be applied when there are multiple aromatic segments. The number of isomers for some PACs can reach into the thousands, demonstrating the ability of the method to work for systems where manual drawing and counting of isomers is clearly not possible. Our method also highlights the inherent complexity of the substituted PAC systems and the challenge that exists to analytical chemists and ecotoxicologists in understanding the environmental fate and behaviour of individual isomers.

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Chapter 4

Validation of a Simultaneous Method for Determining Polycyclic Aromatic Compounds and Alkylated Isomers in Biota.

A version of this chapter has already been published as Idowu, I., Francisco, O., Thomas, P. J., Johnson, W., Marvin, C., Stetefeld, J. and Tomy. G.T. Validation of a simultaneous method for determining polycyclic aromatic compounds and alkylated isomers in biota. *Rapid Communications in Mass Spectrometry* **2018,** *32* (3), 277-287.

My role in this manuscript was to research, review and interpret all the peer-reviewed literature used for this manuscript as well as writing of the manuscript. The validation study was designed by myself with contributions from my supervisor, Gregg Tomy, Olga Francisco and Wesley Johnson.

Abstract

There is a need for a validated method to improve detection limits and simultaneously quantify polycyclic aromatic compounds (PACs, both parent and alkylated homologues) in biota by gas chromatography-tandem mass spectrometry because of their environmental significance. The validation of the method was performed in accordance to the Eurachem Guide to Quality in Analytical Chemistry. Gas chromatography coupled with a triple quadrupole mass spectrometer used in multiple reaction monitoring (MRM) mode was used for detection and quantification. Retention time windows and selective MRM ion transitions were optimized for a suite of PACs. The developed method was validated by comparing our measurements made on a reference material of freeze-dried mussel tissue (*Mytilus edulis*) to the certified values. Linearity was observed between 10-1000 pg/µL (PAHs) and 2-500 pg/µL (alkyl-PACs including S-based PACs). The overall mean (± SD) for the limits of detection of 43 PACs studied were 0.305 ± 0.276 and 2.69 ± 1.10 ng/g, respectively. For the 14 certified target analytes, the percent relative error ranged from 1.3 to 33%. With the exception of benzo(*a*)pyrene, the between day and within-day repeatability for all target analytes was smaller than 15% RSD. This is the first report of a fully validated method to simultaneously quantify PACs in biota performed in an ISO accredited laboratory.

4.1 Introduction

Polycyclic aromatic compounds (PACs) are environmental contaminants originating from either petrogenic, biogenic or pyrogenic origin.[1] They are mostly sourced from the incomplete combustion of organic matter, including coal, wood or petroleum products but can also be released into the environment from accidental spills of crude oil.[1-2] Numerous studies have established their relative persistence in the aquatic environment, their tendency to bioaccumulate in lipid-rich tissues and their inherent toxicity to aquatic organisms (as carcinogenic, teratogenic and mutagenic compounds) over the past decades.[2-7] Although the European Union and the United States Environmental Protection Agency (USEPA) included 16 parent polycyclic aromatic hydrocarbons (PAHs) as priority pollutants, the need to expand the list to include particularly the alkylated PAHs (APAHs) and heterocyclic PACs has been identified.[8]

Alkylated PAHs are substituted PAHs possessing vast structural diversity and congeners with different molecular weights. Alkylated PAHs are more abundant than their parent compounds in petroleum [9-10] where the two (C_2) to five (C_5) alkyl carbons are most abundant.[11] There have been few studies that have established their distribution, resistance to weathering, diffusion across biological membranes and enhanced toxicity compared to their parent compound.[12-13] Preferential bioaccumulation of APAHs has been reported in bivalve tissues when compared to their concentration in sediments.[14] In this study, total (Σ) APAH in bivalves were *ca*. 2 to 10 times greater than ΣPAHs concentration although ΣAPAHs were 1.2 – 4.5 times smaller in sediments at each sampling site. A similar trend was reported for vertebrates and invertebrates from the Northern Gulf of Mexico after the *Deep Water Horizon* (DWH) oil spill and *Dubai Star* Bunker fuel oil spill in San Francisco Bay.[15-17] An increase in APAHs relative to parent PAHs was also observed in benthic invertebrates from the North Chukchi Sea.[18]

Although the unsubstituted PAHs have received more attention, evidence is mounting that the alkylated congeners are more inherently toxic than their parent analogs.[19-23] Because APAHs seem to be more persistent, bioaccumulative and inherently toxic, recent effort in analytical chemistry have focused on improving methods to accurately quantify these compounds in environmental samples. It is widely acknowledged that the biggest challenge to quantifying APAHs is the large number of constitutional isomers that are theoretically possible. For example, the number of constitutional isomers theoretically possible for C4-phenanthrene is 425.[24] The large number of constitutional isomers within any given alkyl-substituted PACs (APACs, this includes heterocyclic PACs) makes separation of individual isomers impossible with modern day one-dimensional chromatographic techniques. Another challenge with quantifying APACs is the lack of authentic standards for most isomers. PACs and their alkyl congeners have been previously quantified by comparing appropriate peaks in an ion chromatogram to either the area of an internal or external standard peaks injected under similar condition.[25-30] Taken together, these challenges can lead to significant systematic biases in the quantification of APACs.[30-32]

The most common approach to quantify APACs is based on GC/MS with electron ionization typically operated in selective ion monitoring (SIM).[30, 33-35] For SIM, the M^+ ion is used for quantitation and loss of either a H-atom or alkyl group ($[M\text{-}CH_3]^+$, $[M\text{-}C_2H_5]^+$ and so on) is used for confirmation[30]. Because APACs elute over a large retention time, the response obtained for a particular APAC cluster is summed over the entire elution time and then compared to the response of a commercially available APAC isomer injected separately. The criteria for selecting appropriate APACs as external standards have been described by Yang *et al*.[30] Even with the SIM approach to quantifying APACs and despite judicious selection of standards, biases are still known to exist.[33-34].

Recently, GC/MS/MS technique using multiple reaction monitoring (MRM) has been reported for the quantitation of APACs.[36-37] Under product-ion scanning conditions, John *et al*. [36]first reported on the mass spectral fragmentation behavior of seven commercially available alkylated chrysenes. By doing so, they were able to establish selective MRM ion transitions for C_1-C_4 chrysenes that were then used to quantify these species in crude oil samples from the 2010 DWH accident. In a more recent study, Sørensen *et al*. [37] optimized MRM conditions for 24 APACs which were then used to measure alkylated phenanthrenes, dibenzothiophenes, fluorenes, pyrenes, chrysenes and fluoranthenes in biological samples.

The Centre for Oil and Gas Research and Development (COGRAD) is a newly ISO 17025 accredited laboratory created to advance analytical methods on chemicals related to oil and gas (O&G) activities. In this study, we present an analytical method validated at COGRAD based on GC/MS/MS using MRM to simultaneously determine PACs (i.e., PAHs, APAHs and S-based APACs) in environmental biological samples. Method validation was performed in strict accordance with the Eurachem Guide to method validation[38]. The data quality objectives of the method reported here include measurement uncertainty, working range, trueness, detection limits, robustness and precision.

4.2 Experimental Section

4.2.1 Materials

All organic solvents used were of high-purity (Optima grade) and purchased from Fisher Chemicals (Ottawa, Ontario, Canada). Twenty-five (25) individual APAHs including C_1-naphthalene (1-methylnaphthalene; 2-methylnaphthalene), C_2-naphthalene (2,6-

dimethylnaphthalene), C_3-naphthalene (2,3,5-trimethylnaphthalene), C_4-naphthalene (1,4,6,7-tetramethylnaphthalene), C_1-fluorene (1-methylfluorene), C_1-chrysene (5-methylchrysene), C_3-chrysene (1,3,6-trimethylchrysene), C_1-pyrene (1-methylpyrene; 4-methylpyrene), C_2-pyrene (4,5-dimethylpyrene) C_1-fluoranthene (1-methylfluoranthene), C_1-phenanthrene (1-methylphenanthrene; 2-methylphenanthrene; 3-methylphenanthrene; 9-methylphenanthrene), C_2-phenanthrene (1,3-dimethylphenanthrene, 2,6-dimethylphenanthrene, 1,7- dimethylphenanthrene, 1,8- dimethylphenanthrene, 3,6- dimethylphenanthrene), C_3-phenanthrene (1,2,6-trimethylphenanthrene), C_4-phenanthrene (1,2,6,9-tetramethylphenanthrene), retene, C_1-benzo[a]pyrene (7-methylbenzo[a]pyrene); 4 heterocyclic S-based PACs including dibenzothiophene, C_1-dibenzothiophene (4-methyl dibenzothiophene), C_2-dibenzothiophene (2,8-dimethyldibenzothiophene), C_3-dibenzothiophene (2,4,7-trimethyldibenzothiphene) and 1-methyl coronene standards were purchased from Accustandard Inc. (New Haven, Cincinnati, USA) and Caledon Laboratory Chemicals (Georgetown, Ontario, Canada). Sixteen (16) unsubstituted PAHs as a native mix and deuterium mass labeled d_{10}-anthracene were purchased from Accustandard Inc. while their surrogates from Cambridge Isotope Laboratories Inc. (Tewsbury, Massachusetts, USA). All standards were >98% purity. In-house PAH and APAH standards mix in hexane was prepared with varying concentrations as required. The standard reference material, SRM-2974a, organics in freeze dried mussel tissue (*Mytilus edulis*) was purchased from the National Institute of Science and Technology (NIST: Gaithersburg, Maryland, USA). The suite of labeled internal standard used for isotope dilution were d_8-naphthalene, d_8-acenaphthylene, d_{10}-acenaphthene, d_{10}-fluorene, d_{10}-phenanthrene, d_{10}-pyrene, d_{12}-benz(*a*)anthracene, d_{12}-chrysene, d_{12}-benzo(*b*)fluoranthene, d_{12}-benzo(*k*)fluoranthene, d_{12}-benzo(*a*)pyrene, d_{12}-indeno(*1,2,3-c,d*)pyrene, d_{14}-dibenz(*a,h*)anthracene, d_{14}-benzo(*g,h,i*)perylene. Labeled anthracene was used as

the instrument performance internal standard (IPIS). The C_{18} powder (HPLC sorbent) was purchased from Sigma Aldrich (St Louis, MO, USA) and the size-exclusion S-X3 Biobeads were purchased from Bio-Rad Laboratories (Mississauga, Ontario, Canada). An Ultra Grade 19 oil sample (Edwards, Wilmington, USA) was used to establish retention time (r_t) windows for the APAHs. Silica gel (923 grade, 100-200 mesh), alumina (60-325 mesh), Ottawa sand and anhydrous sodium sulphate were all purchased from Fisher Chemical. Diatomaceous earth (DE) dispersant was purchased from Fisher Scientific (Ottawa, ON, Canada).

4.2.2 Sample preparation

4.2.2.1 Accelerated Solvent Extraction (ASE)

SRM 2974a (~1.5 g; *n=9*) was accurately weighed on a Sartorius Cubis MSE225P-100-DI (Fisher Scientific) and mixed with DE dispersant (baked at 600°C for 6 hours), Sartorius Cubis MSE225P-100-DI (Fisher Scientific) and mixed with DE dispersant (baked at 600°C for 6 hours). The dead volume was filled with Ottawa sand (baked at 600°C for 6 hours) to approximately 0.5 cm below the top of the cell. Procedural blanks (*n=10*) were the C_{18}-matrix (surrogate for biota) spiked with the labeled internal standards and processed identically to the SRM material. Conditions for the ASE system were as follows: system pressure: 1500 psi; oven temperature: 100 ^{0}C; oven heat-up time: 5 min; two static cycles of 5 min each; followed by 60% volume flush of extraction cell and nitrogen purge of 150 psi for 60 sec. The extraction solvent used was dichloromethane (DCM). The total extraction time was *ca.* 30 min. Following extraction, approximately 5g of sodium sulfate was added to each collection vial to remove excess water and transferred to round bottom flask. The extract volume was then reduced by rotary evaporation to approximately 1 mL. Reduced extracts were then transferred to a glass test tube then reduced again in volume to approximately

2.6 mL under gentle stream of ultra-high purity (UHP) nitrogen and made up to a final extract volume of ~5.2 mL with hexane. 200 µL of the extract was transferred to a pre-weighed aluminum boat for lipid determination. The solvent was allowed to evaporate in the fume hood and the boat was weighed afterwards. Lipid content was determined gravimetrically (based on total weight of the dried DCM extract) using Equation 4.1.

$$\%\ lipid = \frac{\frac{(weight\ of\ lipid\ in\ 0.2\ mL)}{0.2mL} \times 5.2\ mL}{weight\ of\ whole\ sample} \times 100 \qquad \text{.....(4.1)}$$

4.2.2.2 Automated Gel Permeation Chromatography

Removal of lipids from the extracts was achieved using an automated GPC (J_2-scientific AccuPrepMPS^{TM} Columbia, Missouri, USA). Collection volumes from the GPC for PACs were established using naphthalene and 1-methyl coronene. Five mL of the extract was loaded onto the head of the GPC column packed with 60 g of S-X3 Biobeads, at a pressure of 8 psi and flow rate of 5 mL/ min. The mobile phase was a mixture of hexane and DCM (1:1, v/v). For the purpose of this study, the first 140 mL was discarded while the following 120 mL (lipid-free) was collected in a pre-rinsed (hexane) 250 mL round bottom flask for further sample processing. The lipid-free extracts were reduced to about 1 mL by rotary evaporation and transferred to a glass tube by rinsing three times with hexane (~1 mL). The final extracts were reduced to 1 mL under gentle stream of UHP nitrogen for further clean-up using silica/alumina column chromatography.

4.2.2.3 Silica/Alumina Column Chromatography

Adsorption chromatography was used as a final clean-up step for the lipid–free extract. The stationary phase was made up of silica gel (baked at 150°C for 16 hours), alumina (baked at 250°C for 3 hours) and anhydrous sodium sulphate (baked at 600°C for 6 hours). Approximately 11g of silica gel prepared as a slurry in DCM followed by ~1 g of 5% deactivated alumina and about 1 g of anhydrous sodium sulphate were added to a pre-rinsed glass column (30 × 1 cm) plugged with glass wool. The anhydrous sodium sulphate was added to remove any residual moisture/water. The packed column was preconditioned with approximately 25 mL of hexane before transferring the extract to the head of the column. The PAC containing fraction was eluted with 25 mL DCM: Hexane (1:1, v/v). Eluted extracts were reduced in volume by rotary- and nitrogen evaporation to 1 mL and fortified with IPIS (10 µL of 10 ng/µL). The final extract was stored under refrigerated conditions at 4 ^{0}C in an amber GC vial prior to instrumental analyses.

4.2.3 GC-MS/MS conditions

An Agilent 7890 GC coupled with a 7000C triple quadrupole mass spectrometer fitted with electron ionization (EI) source was used for the MS/MS acquisition. An Agilent J&W HP-5ms ultra inert column (30 m × 0.25 mm × 0.25 µm), was used with helium as the carrier gas at a constant flow rate of 1.2 mL/min. The sample (1 µL) was injected with a PAL RSI 85 auto-sampler at 250°C in splitless mode. The oven temperature was held at 60°C for 1 min then raised to 120°C at 35°C/min, further ramped up to 220°C at 14°C/min, 260°C at 5°C/min and held for 5min, to 300°C at 10°C/min and finally to 310°C at 50°C/min. Both transfer line and source temperature were set at 320°C and UHP nitrogen was used as the collision gas at 60 psi.

4.2.3.1 MS analysis

Before MRM conditions could be established it was first necessary to record full-scan EI mass spectra (*m/z* 50-250) for each analyte (5ng/µL, 1 µL injection). Once the most abundant precursor ion was determined it was then purposely selected and fragmented in our collision cell (Q2) to generate product ions. Defined low and high collision energies ranging between 15 eV to 45 eV were used to acquire the product-ion spectra of selected precursor ions (Table 4.1). Ultra-high purity nitrogen was introduced into the Q2 to aid collision-induced dissociation (CID) of the precursor ion. The collision energy (CE) required to obtain the most abundant product ions was selected. In this manner, multiple reaction monitoring (MRM) precursor → product ion transitions along with their associated CEs were established. To improve detection limits, pseudo-MRM transitions were used for indeno(*1,2,3-c,d*)pyrene, dibenz(*a,h*)anthracene and benzo(*ghi*)perylene as described by Shang *et al*.[39]. Table 4.1 shows the MRM ion transitions and CEs used in our study.

Table 4.1. *MS/MS ion transitions, RT windows and CE for native PACs and d-PAH analytes grouped into 3 separate MRM injections.*

Target Analyte	**Quantitation Transitions**	**RT window (min)**	**CE (eV)**
APAH MRM Injection 1:			
C_0 Naphthalene	127.9/102.1	4.0 - 5.2	45
C_1 Naphthalene	141.8/141.1	5.2 - 6.0	15
C_2 Naphthalene	155.8/141.1	6.0 - 7.0	15
C_3 Naphthalene	169.8/155.1; 169.8/141.1	7.0 – 8.18	15
C_4 Naphthalene	183.8/169.1; 183.8/155.1; 183.8/141.1	8.18 – 11.0	15
C_0 Phenanthrene	177.8/152.1	8.18 – 11.0	40
C_1 Phenanthrene	191.8/191.1	8.18 – 11.0	15
C_2 Phenanthrene	205.8/191.1	11.0 – 16.5	15
C_3 Phenanthrene	221.8/205.1; 221.8/191.1	11.0 – 16.5	15
C_4 Phenanthrene	233.8/219.1; 233.8/205.1; 233.8/191.1	11.0 – 16.5	15
C_0 Chrysene	227.8/226.1	16.5 – 17.1	40
C_1 Chrysene	241.8/239.1	17.1 – 27.0	15
C_2 Chrysene	255.8/241.1	17.1 – 27.0	15
C_3 Chrysene	269.8/255.1; 269.8/241.1	17.1 – 27.0	15
C_4 Chrysene	283.8/269.1; 283.8/255.1; 283.8/241.1	27.0 – 32.0	15
APAH and S-based PACs MRM Injection 2:			
C_0 Dibenzothiophene	183.8/139.1	7.9 – 9.4	45
C_0 Fluorene	165.8/163.1	7.9 – 9.4	45
C_1 Fluorene	179.8/165.1	7.9 – 9.4	25
Anthracene-d_{10}	188.0/160.1	9.4 - 11.2	40
C_2 Fluorene	193.8/179.1	9.4 – 11.2	25
C_3 Fluorene	207.8/193.1; 207.8/179.1	9.4 – 11.2; 11.2 – 11.87; 11.87 – 17.0	25
C_1 Dibenzothiophene	197.8/197.1	9.4 – 10.6	15
C_2 Dibenzothiophene	211.8/197.1	9.4 – 11.2; 11.2 – 11.87	15
C_3 Dibenzothiophene	225.8/211.1; 225.8/197.1	11.2 – 11.87; 11.87 – 17.0	15
C_4 Dibenzothiophene	239.8/225.1; 239.8/211.1; 239.8/197.1	11.87 – 17.0	15
C_3 Pyrene	243.8/229.1; 243.8/215.1	17.0 – 23.6	15
C_4 Pyrene	257.8/243.1; 257.8/229.2; 257.8/215.1	17.0 – 23.6	15

C_0 Benzopyrene	251.8/250.1	17.0 – 23.6	40
C_1 Benzopyrene	265.8/263.1	23.6 – 32.0	35
C_2 Benzopyrene	279.8/265.1	23.6 – 32.0	35
APAH and PAH MRM Injection 3:			
Naphthalene	127.9/102.0; 127.8/77.0[1]	4.0-6.8	45
Naphthalene-d_8	136.0/108.0; 136.0/134.0[1]	4.0-6.8	35
Acenaphthylene	151.9/150.0; 151.9/151.0[1]	6.8-7.0	45
Acenaphthylene-d_8	160.0/158.1; 160.0/156.1[1]	6.8-7.0	40
Acenaphthene	152.9/152, 152.9/151.0[1]	7.0-7.88	35
Acenaphthene-d_{10}	162.0/160.2; 162.0/158.1[1]	7.0-7.88	35
Fluorene	164.9/163, 164.9/115.0[1]	7.88-9.4	40
Fluorene-d_{10}	176.0/174.2; 176.0/172.1[1]	7.88-9.4	40
Anthracene	177.9/176.1; 177.9/151.0[1]	9.4-11.72	40
Anthracene-d_{10}	188.0/160.1; 188.0/184.1[1]	9.4-11.72	40
Fluoranthene	201.9/200.1; 201.9/201.1[1]	11.72-12.2	40
Fluoranthene-d_{10}	212.0/208.2; 212.0/210.1[1]	11.72-12.2	40
Pyrene	201.9/200.1; 201.9/201.1[1]	12.2-12.58	45
Pyrene-d_{10}	212.0/208.1; 212.0/210.2[1]	12.2-12.58	45
C1 Pyrene	215.8/215.1	12.58 – 16.3	15
C2 Pyrene	229.8/215.1	12.58 – 16.3	15
Chrysene	227.9/226.1; 227.9/202.1[1]	16.3-21.3	40
Chrysene-d_{12}	240.0/236.2; 240.0/212.2[1]	16.3-21.3	40
Benzo(*b*)fluoranthene	251.9/250.1; 251.9/226.1[1]	21.3-23.0	35
Benzo(*b*)fluoranthene -d_{12}	264.0/260.2; 264.0/236.1[1]	21.3-23.0	45
Benzo(*a*)pyrene	251.9/250.1; 251.9/226.1[1]	23.0-29.5	40
Benzo(*a*)pyrene-d_{12}	264.0/260.2; 264.0/236.2[1]	23.0-29.5	45
Indeno(1,2,3-c,d)pyrene	275.9/275.9; 274.1/274.1[3]	29.5-31.0	15
Indeno(1,2,3-c,d)pyrene-d_{12}	286.2/286.2[2]; 288.0/288.0[3]	29.5-31.0	15
Dibenz(*a*,*h*)anthracene	277.9/277.9[2]; 276.1/276.1[3]	29.5-31.0	15
Dibenz(*a*,*h*)anthracene-d_{14}	292.0/292.0[2]; 288.2/288.2[3]	29.5-31.0	15
Benzo(*ghi*)perylene	275.9/275.9[2]; 275.1/275.1[3]	31.0-32.0	15
Benzo(*ghi*)perylene-d_{12}	288.0/288.0[2]; 286.2/286.2[3]	31.0-32.0	15

[1]Conformation transitions established for PAHs; [2]Pseudo-MRM quantitation ion transitions; [3]Pseudo-MRM confirmation ion transitions[39]

4.2.4 Method Performance Characteristics

4.2.4.1 Detection Limits

The method limit of detection (LOD) and limit of quantification (LOQ) were estimated for individual PACs following Eurachem guideline[38]. Ten (10) replicate samples of C_{18} matrix were spiked with 50 ng of PACs and 500 ng of the suite of mass labeled PAH internal standards. Procedural blanks (*n=8*) consisting of C_{18}-matrix were fortified with the mass labeled PAH internal standard solution and taken through the whole analytical procedure.

Standard deviation s_0' was calculated from the replicate measurements (*n=10*) based on Equation 4.2:

$$s_0' = s_0 \sqrt{\frac{1}{n} + \frac{1}{n_b}} \quad \text{.....(4.2)}$$

Where s_0 is the estimated standard deviation of 10 single results, s_0' is the standard deviation used to calculate LOD and LOQ, *n* is the number of replicate observations averaged when reporting results where each replicate is obtained following the entire measurement procedure, n_b is the number of blanks averaged when calculating the blank correction according to the measurement procedure[38]. Method detection and quantification limits (LODs and LOQs) were calculated as $3 \times s_0'$ and $10 \times s_0'$, respectively.

4.2.4.2 Working Range

Eleven (11) calibration standards were prepared to cover the proposed range of interest of 2-1000 pg/µL for the PACs (25 APAHs and 4 S-based PACs) studied. Calibration standard concentrations run included 2, 5, 10, 20, 50, 100, 200, 400, 600, 800 and 1000 pg/µL for PACs and 10, 100, 200,

300, 400, 500, 600, 700, 800, 900 and 1000 pg/µL for PAHs. Concentration of the IPIS was constant at 100 pg/µL for each calibration level. The purpose of the IPIS was to correct for any small fluctuations in the response of the instrument between injections. Calibration standards were run randomly and in triplicate along with a solvent blank injection. The electronically integrated response of each analyte was normalized to that of d_{10}-anthracene and plotted as a function of concentration and visually examined to identify approximate linear range and upper and lower boundaries.

Subsequently, 6 calibration standards for PACs (2, 100, 200, 300, 400 and 500 pg/µL) and PAHs (10, 200, 400, 600, 800 and 1000 pg/µL) were randomly run in triplicate across the linear range based on the results of the previous injections. Linearity was evaluated by the magnitude of the R^2 (correlation coefficients) and the level of significance (i.e., *p*-value) for each of the analytes of PACs. Residual plots were also examined to ensure that data was randomly distributed about zero.

4.2.4.3 Trueness

The trueness of the method was evaluated using the NIST SRM 2974a which has certified values for 6 APAHs and 8 PAHs. Replicate measurements ($n=9$) of this matrix were analyzed and our measured values were compared to the stated certified values.

4.2.4.4 Precision

Intermediate (i.e., between day) and within-day repeatability was determined by analyzing the SRM 2974a sample on separate days ($n=4$) and on the same day ($n=5$).

4.2.4.5 Measurement Uncertainty (MU)

The combined method uncertainty (U_C) was determined with the uncertainty of the bias (U_{bias}) combined with the uncertainty from the intermediate precision (U_{IP}) based on the following equation:

$$U_C = \sqrt{U_{bias}^2 + U_{IP}^2} \tag{4.3}$$

The uncertainty from intermediate precision (U_{IP}) was estimated through the use of an ANOVA Table[40]. The intermediate precision was calculated based on the combination of the uncertainty from repeatability (U_r) and the between day uncertainty, referred to as the between groups uncertainty (U_{BG}), according to the following equation:

$$U_{IP} = \sqrt{U_r^2 + U_{BG}^2} \tag{4.4}$$

The estimate of the uncertainty from repeatability was taken as the square root of the mean square error (MSE).

$$U_r = \sqrt{MSE} \tag{4.5}$$

The between group uncertainty was determined by use of the following formula:

$$U_{BG} = \sqrt{(MSG - MSE)/n} \tag{4.6}$$

where MSG is the mean square of groups and n is the number of replicates used on a single day. The uncertainty of the bias (U_{bias}) is a combination of the uncertainty from the laboratory estimate of the bias ($U_r/\sqrt{n}$) and the uncertainty from the certified reference value (U_{CRM}) and was estimated based on the following equation[38]:

$$U_{bias} = \sqrt{U_{CRM}^2 + \frac{U_r^2}{n}} \quad (4.7)$$

where n is the number of replicates used on a single day.

4.2.4.6 Ruggedness

The ruggedness of our analytical method was tested by purposely making small deliberate changes to the method and assessing whether the performance characteristics remained unaffected. The following changes to the test method were examined:

1. *ASE conditions*. Extraction temperatures of 120°C, 125°C, and 130°C (*n=3* for each) and ASE cell sizes of 10 mL and 34 mL (*n=3* for each) were employed.
2. *GPC conditions*. Fractions of 100-320 mL, 140-320 mL and 160-320 mL (*n=3* for each) were collected.
3. *Adsorption chromatography conditions*. Different mass of silica gel (10g, 11g and 12g, n=3 each) volumes of hexane: DCM 22mL, 25mL and 28mL (*n=3* for each) were explored.

4.3 Results and Discussion

4.3.1 Detection Limits (DL)

The LODs and LOQs (ng/g, lipid weight) for the PACs examined in this study are shown in Table 4.2. In general, LODs (and LOQs) were an order of magnitude smaller for APACs than PAHs. LODs for individual APACs ranged from 0.048 to 1.39 ng/g for 1,3,6-trimethylchrysene to dibenzothiophene, respectively. The overall mean (± SD) LODs for the 23 APAHs and 4 S-based PACs studied was 0.305 ± 0.276 ng/g. For the PAHs, the overall mean LODs for the 16 PAHs was 2.69 ± 1.10 ng/g and ranged from 1.39 to 5.67 ng/g for naphthalene and indeno(*1,2,3-c,d*)pyrene, respectively. Sørensen *et al.* reported LODs in biota that were only slightly greater than those reported here[37].

4.3.2 Working Range

Calibration standards for PACs were injected randomly and in triplicate. The correlation coefficients were higher than 0.99 in all cases with p-values <0.0001. Residual plots also showed the data to be randomly distributed about zero. Taken together these results verify the use of our linear regression model.

4.3.3 Method of Quantitation

4.3.3.1 PAHs.

The MRM ion transitions to detect and quantify PAHs are shown in Table 4.1. In general, the MRM ion transitions monitored correspond to the $[M]^+$ to $[M - H]^+$ ions. Three notable exceptions are indeno(*1,2,3-c,d*)pyrene, dibenz(*a,h*)anthracene and benzo(*ghi*)perylene in which case we performed a pseudo-MRM monitoring parent ion → parent ion transitions as described in Shang *et al.*[39]

Table 4.2 *Detection Limits for PACs studied.*

Analyte	**LOD (ng/g)**	**LOQ (ng/g)**	**Linear Range Calibration Standards (pg/µL)**
Acenaphthene	3.369	11.230	10-1000
Acenaphthylene	2.820	9.400	10-1000
Anthracene	2.170	7.234	10-1000
Benz[a]anthracene	2.951	9.836	10-1000
Benzo[a]pyrene	2.745	9.149	10-1000
Benzo[b]fluoranthene	1.478	4.926	10-1000
Benzo[g,h,i]perylene	1.634	5.447	10-1000
Benzo[k]fluoranthene	2.194	7.314	10-1000
Chrysene	3.952	13.175	10-1000
Dibenzo[a,h]anthracene	2.292	7.639	10-1000
Fluoranthene	2.625	8.750	10-1000
Fluorene	1.739	5.797	10-1000
Indeno[1,2,3-c,d]pyrene	5.666	18.887	10-1000
Naphthalene	1.387	4.624	10-1000
Phenanthrene	3.802	12.674	10-1000
Pyrene	2.269	7.562	10-1000
1-Methylnaphthalene	0.285	0.950	2-500
2-Methynaphthalene	0.284	0.946	2-500
2,6-Dimethylnaphthalene	0.247	0.823	2-500
2,3,5-Trimethylnaphthalene	0.222	0.740	2-500
1,4,6,7-Tetramethylnaphthalene	0.194	0.648	2-500
1-Methylfluorene	0.240	0.801	2-500
1-Methylphenanthrene	0.161	0.537	2-500
2-Methylphenanthrene	0.165	0.549	2-500
3-Methylphenanthrene	0.167	0.556	2-500
9-Methylphenanthrene	0.148	0.493	2-500
1,3-Dimethylphenanthrene	0.324	1.080	2-500
2,6-Dimethylphenanthrene	0.282	0.938	2-500

1,7- Dimethylphenanthrene	0264	0.878	2-500
1,8- Dimethylphenanthrene	0.339	1.130	2-500
3,6- Dimethylphenanthrene	0.368	1.226	2-500
1,2,6-Trimethylphenanthrene	0.506	1.686	2-500
1,2,6,9-Tetramethylphenanthrene	0.695	2.314	2-500
Dibenzothiophene	1.394	4.652	2-500
4-Methyldibenzothiophene	0.068	0.227	2-500
2,8-Dimethyldibenzothiophene	0.261	0.870	2-500
2,4,7-Trimethyldibenzothiophene	0.136	0.453	2-500
1-Methylpyrene	0.159	0.530	2-500
4-Methylpyrene	0.184	0.613	2-500
4,5-Dimethylpyrene	0.872	2.907	2-500
1-Methylfluoranthene	0.145	0.484	2-500
5-Methylchrysene	0.194	0.647	2-500
1,3,6-Trimethylchrysene	0.048	0.160	2-500
7-Methylbenzo[a]pyrene	0.178	0.594	2-500

Quantitation of PAHs was done using isotope-dilution and was based on the average relative response factors (ARRFs) of the native PAHs relative to authentic d-PAH surrogates.

Prior to calculating ARRFs, the responses of the native and d-PAHs (*i.e.*, electronically integrated peak areas) were normalized to the response of the IPIS, d_{10}-anthracene. The IPIS was added to the calibration solutions and to sample extracts prior to GC-injection at a concentration of 100 pg/µL.

ARRFs were determined by preparing solutions of native PAHs standards at nominal concentrations of 10, 200, 400, 600, 800, and 1000 pg/µL and each concentration level was spiked with a constant amount of d-PAHs (100 pg/µL) and injected onto the GC/MS/MS system using the optimized parameters shown in Table 4.1. After normalizing for the d_{10}-anthracene response, the relative response factors of native PAH at each concentration level were then calculated using equation (8) and the data shown in Table 4.3.

$$RRF_{Native\ PAH} = \frac{\left(\frac{Area_{Native\ PAH}}{Area_{d-PAH}}\right)}{\left(\frac{Conc_{Native\ PAH}}{Conc_{d-PAH}}\right)} \tag{4.8}$$

ARRFs for the 16 PAHs monitored ranged from 0.380 ± 0.02 for fluorene to 2.33 ± 0.23 for benzo(b)fluoranthene and there were no statistically significant differences for the RRFs at any of the calibration levels tested for any of the PAHs. These results imply that as long as the concentration of native PAHs in samples falls within the range of 1-1000 pg/uL that quantitation based on isotope dilution using a constant amount of d-PAH is acceptable.

Table 4.3 *Relative response factors (RRF) at each calibration level (10-1000 pg/µL), average relative response factors (ARRF) and SD for native PAHs.*

Analyte	**RRF**						**ARRF**	**SD**
	10	**200**	**400**	**600**	**800**	**1000**		
Acenaphthene	1.05	1.11	1.14	1.15	1.16	1.17	1.13	0.04
Acenaphthylene	0.80	0.87	0.90	0.90	0.90	0.90	0.88	0.04
Anthracene	1.29	1.43	1.48	1.51	1.51	1.53	1.46	0.09
Benz(*a*)anthracene	1.24	1.43	1.48	1.73	1.60	1.60	1.51	0.17
Benzo(*a*)pyrene	1.87	1.89	2.07	2.27	2.28	2.33	2.12	0.21
Benzo(*b*)fluoranthene	1.96	2.17	2.32	2.40	2.51	2.60	2.33	0.23
Benzo(*ghi*)perylene	1.31	1.48	1.55	1.55	1.62	1.68	1.53	0.13
Benzo(*k*)fluoranthene	1.93	1.86	1.91	1.97	1.95	2.03	1.94	0.06
Chrysene	1.15	1.26	1.29	1.37	1.32	1.34	1.29	0.08
Dibenz(*a,h*)anthracene	1.18	1.15	1.22	1.30	1.33	1.38	1.26	0.09
Fluoranthene	1.42	1.57	1.61	1.63	1.66	1.67	1.59	0.09
Fluorene	0.35	0.37	0.39	0.39	0.39	0.40	0.38	0.02
Indeno(*1,2,3-c,d*)pyrene	1.27	1.17	1.22	1.33	1.33	1.41	1.29	0.09
Naphthalene	0.41	0.45	0.46	0.46	0.46	0.46	0.45	0.02
Phenanthrene	1.10	1.19	1.20	1.24	1.25	1.26	1.21	0.06
Pyrene	1.66	1.80	1.87	1.90	1.94	1.96	1.86	0.11

Two criteria were used to confirm the presence of PAHs. First, the ratio of the quantitation and confirmation MRM ion transitions in the samples were to be within ±15% of their respective theoretical values. Second, the retention times of PAHs in samples must be within ±1 sec to those in standard solutions.

Because of the large number of MRM ion transitions monitored and to increase the detection limits of our method, it was necessary to: (*i*) create retention time (r_t) windows in which only select MRM ion transitions were monitored and, (*ii*) make 3 injections per sample. Elution profiles for PAHs are shown in Figure 4.1. PAHs were monitored in the third injection between r_t: 4-32 min. Concentrations (mass per volume) of native PAHs in sample extracts and finally the concentrations on a mass per mass were determined using equation 9 and 10 respectively.

$$Extract\ Conc_{Native\ PAH} = \left[\frac{\left(\frac{Area_{Native\ PAH}}{Area_{d-PAH}}\right)}{ARRF_{Native\ PAH}}\right] x\ Conc_{d-PAH} \quad (4.9)$$

$$Native\ PAH\ \ (ng/g) = \frac{\left(\frac{Extract\ Conc_{Native\ PAH}\ x\ Extract\ volume\ (\mu L)}{Sample\ mass\ (g)}\right)}{(1000\ pg/ng)} \quad (4.10)$$

4.3.3.2 APAHs and S-based PACs.

The MRM ion transitions used to detect and quantify these APACs are shown in Table 4.1. Given that the EI mass spectra for constitutional isomers within the same C_x-PAC cluster were noticeably dissimilar it is not surprising that multiple MRM transitions were required for each cluster [36-37]. This must be considered when quantifying these compounds in environmental samples.

In general, there are two approaches to quantifying APAH and S-based PACs in environmental samples. One approach is to report on concentrations on an individual APAH and S-based PAC. This is practical if authentic standards for the target analyte were available or alternatively, if the

response of the target analyte is compared to that of a surrogate compound. However, caution needs to be exercised when using the latter approach as EI fragmentation of APAHs and S-based PACs constitutional isomers are different and, as such, differences in response factors will lead to method bias[30].

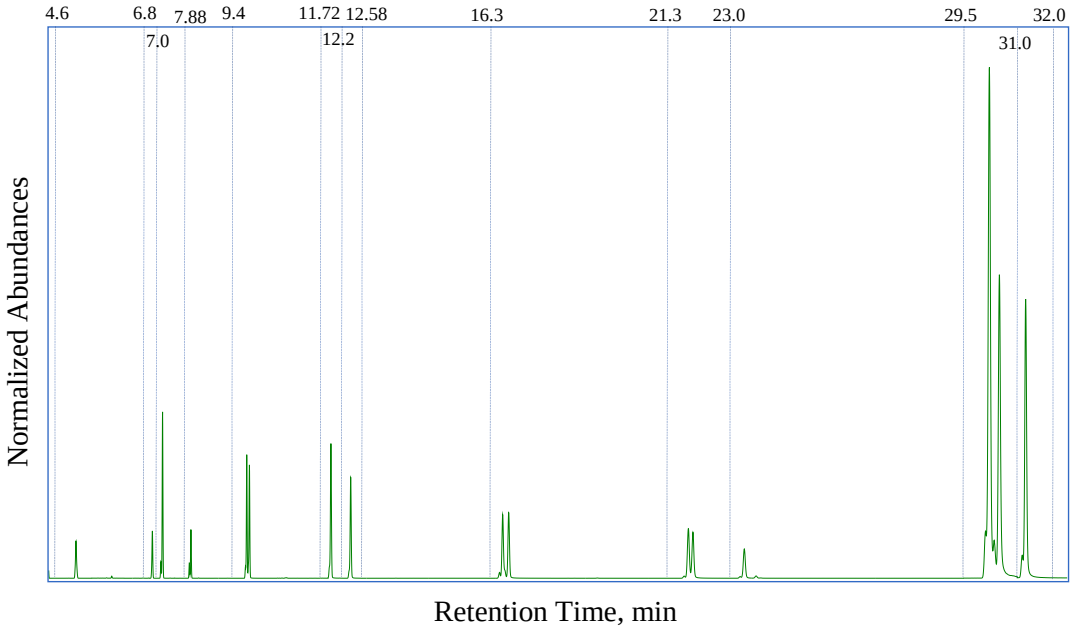

Figure 4.1 *MRM Injection 3 - PAHs with retention time windows.*

The second and perhaps more common approach, referred to as cluster analysis, involves reporting concentrations on a particular C_x-PAC cluster. Because of the large number of possible theoretical isomer for APAHs, for example, any C_x-PAC were expected to elute over a broad retention time range (see Figures 4.2 and 4.3). As an example, Figure 4.2 shows the elution profile of the C_x-Phenanthrenes. As mentioned earlier, different constitutional APAH and S-based PAC isomers fragment differently, and a single MRM transition for one C_x-PAC will not adequately represent the total response of any single C_x-PAC cluster. Instead, John *et al*. and Sørensen *et al*.

recommends summing the ion signals for all characteristic MRM transitions used for a particular C_x-PAC cluster[36-37].

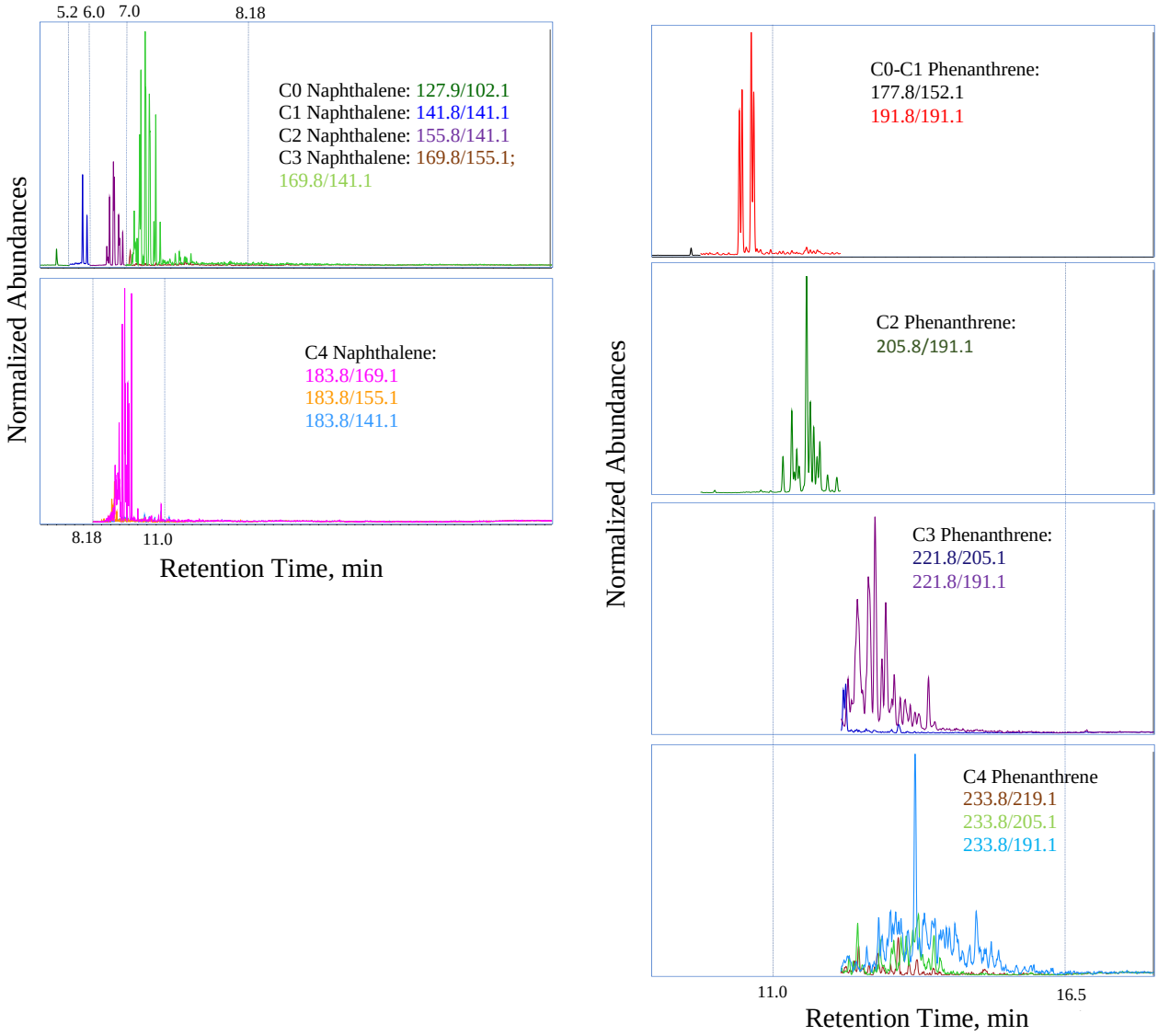

Figure 4.2 *MRM Injection 1- PACs with retention time windows: C_0-C_4 Naphthalene (left panels), C_0-C_4 Phenanthrene (right panels)*

For example, 3 MRM ion transitions are used to quantify the C_4-phenanthrene cluster: $[M]^+ \rightarrow [M - CH_3]^+$; $[M]^+ \rightarrow [M - C_2H_5]^+$; $[M]^+ \rightarrow [M - C_3H_7]^+$ (*see* Table 4.1 and Figure 4.2). These

transitions are summed and the electronically integrated areas are taken from the start to the end of the GC-elution to get a total response.

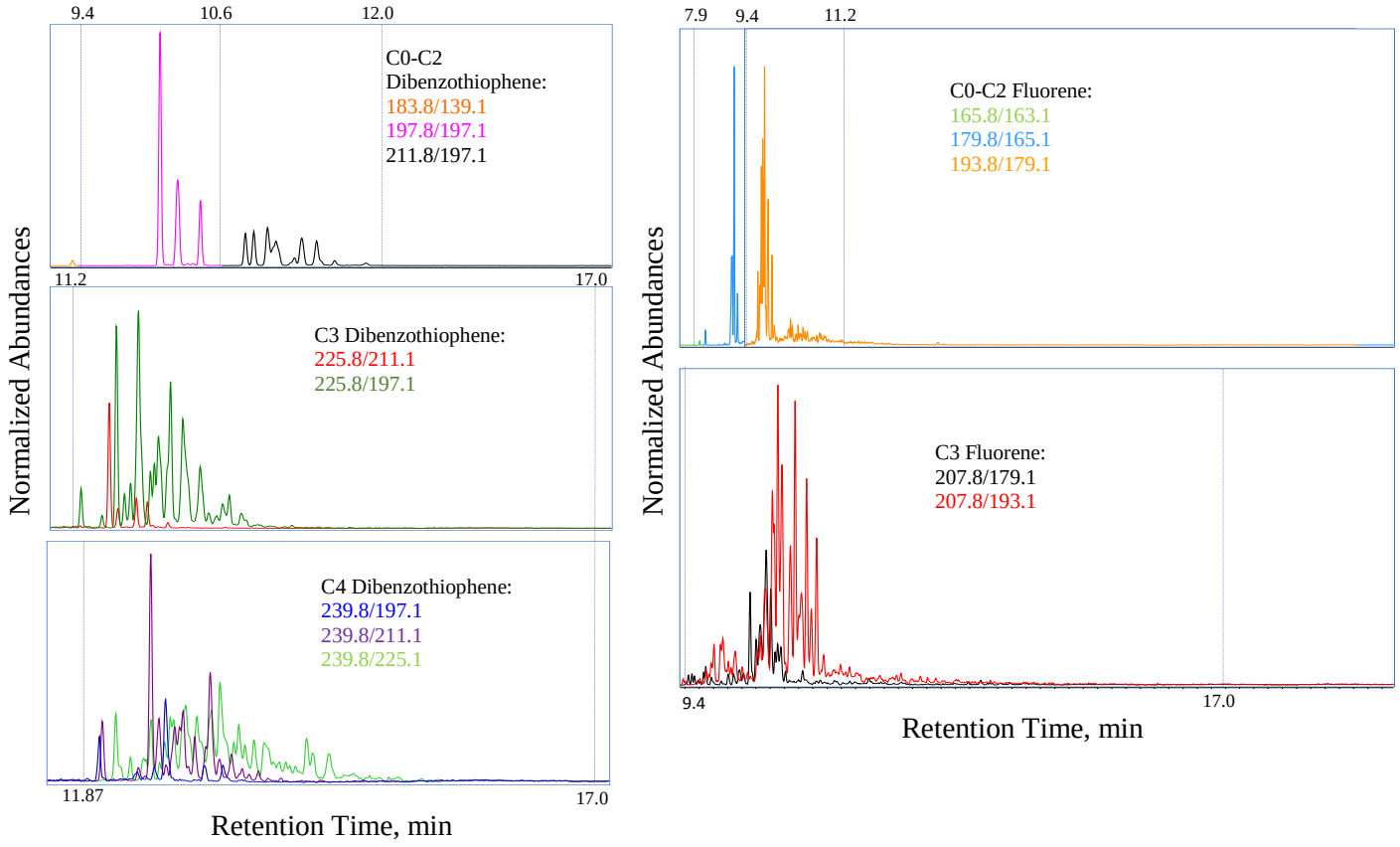

Figure 4.3 *MRM Injection 2 - PAC clusters with retention time windows: C_0-C_4 Dibenzothiophene (left panels), C_0-C_3 Fluorene (right panels)*

Because of their inherent complexity, it was necessary to monitor C_x-PACs in all 3 sample injections (see Figure 4.1-4.3 and Table 4.1). The r_t for cluster analysis were established by diluting an oil sample and injecting into the GC. Multiple injections were made and individual MRM ion transitions characteristic of a particular C_x-PAC cluster were then monitored. The key

criterion when building our final method was to minimize the number of MRM ion transitions in any one r_t window.

To increase the detection limits of my analytes, and to accommodate PAHs in our method, we decided it was necessary to make 3 injections per sample. The final method showing the MRM ion transitions, r_t windows and method number is shown in Table 4.1. It should be noted that even though $C_{1\text{-}4}$-Chrysene, $C_{1\text{-}2}$-Benzo[a]pyrene and $C_{1\text{-}4}$-Pyrene were not detected in our oil sample, based on the study by Yang *et al.*, we were still able to build these compounds into our final method[30].

Our approach to quantifying C_x-PACs (as both individual isomers and through cluster analysis) is by the external standard approach. Calibration solutions were first prepared at nominal concentrations of 2, 100, 200, 300, 400 and 500 pg/µL for all 27 APACs. To each solution, d_{10}-anthracene was added at a concentration of 100 pg/µL. At each calibration level, the response of each APAH and S-based PAC was normalized to the response of d_{10}-anthracene. The corrected response was then used to generate the calibration curves slope and intercept.

To correct for analyte losses during the extraction and work-up of samples, the manually integrated peak areas of each C_x-PAC cluster was corrected by the recovery of the labeled d-PAH. We chose to use the d-PAHs that eluted closest to the C_x-PAC as the internal standard to correct for these losses. These responses are referred to as the corrected peak areas.

Unlike PAHs it is difficult to set suitable criteria that aid in the positive identification of APAHs and S-based PACs in samples. This is true especially if the total concentration of a particular C_x-PAC cluster is desired. The large number of theoretical isomers results in differences in EI mass spectra and using the comparison of quantitation to confirmation MRM ion transitions measured in a sample to a 'theoretical value' is not meaningful. However, in cases where concentrations of

individual APAC isomers were desired and standards were available then the criteria for PAHs was acceptable and can be used.

The concentration (mass per volume) of APACs in sample extracts was then be determined using Equation 4.11.

$$Conc\ in\ Extract\ \ (pg/\mu L) = \frac{(Corrected\ Peak\ Area - Intercept)}{Slope} \qquad (4.11)$$

Finally, the concentration (mass per mass) of APACs in samples was determined using Equation 4.12.

$$Conc\ in\ Sample\ \ (ng/g) = \frac{\left(\frac{Extract\ Conc\quad x\ Extract\ volume\ (\mu L)}{Sample\ mass\ (g)}\right)}{(1000\ pg/ng)} \qquad (4.12)$$

4.3.4 Trueness

The results of our trueness tests are shown in Table 4.4 and presented quantitatively as bias. A 95% confidence interval was constructed using the calculated uncertainty of the bias to determine whether or not the existing bias is statistically significant. A confidence interval containing zero results in a statistically insignificant bias. For certified APAH values, the bias was not significant for all analytes except for 4-Methylpyrene, while there was a statistically significant bias present for all certified PAHs. As part of our laboratory ISO 17025 accreditation, we have successfully completed 3 rounds of international proficiency testing using the described method. Although there was a bias, the method still performed within the acceptance criteria of the proficiency testing coordinator (i.e., Canadian Association for Laboratory Accreditation).

However, for method validation, our laboratory sets our own acceptance criteria such that every analyte must lie within 100 ± 50% of the certified value with 95% confidence. The confidence

interval is constructed with the equation below where, $\bar{x}$ is the mean, U_c is the combined measurement uncertainty, *n* is the number of replicates and t is the critical value:

$$CI = \bar{x} \pm \frac{t^* U_c}{\sqrt{n}} \qquad (4.13)$$

Based on this, the measured target analyte concentrations reported in Table 4.3 all meet our acceptance criteria.

4.3.5 Precision

The between day and within-day repeatability is listed for each target analyte in Table 4.5. With the exception of benzo(a)pyrene, the relative standard deviation (%RSD) for all our target analytes was smaller than 15% for both between and within-day repeatability studies. The day-to-day repeatability ranged from 4.9 % (pyrene and benz(*a*)anthracene) to 9.5 % (benzo(*b*)fluoranthene). Within-day repeatability ranged from 2.6 % (benzo(*ghi*)perylene) to 12.5% (benz(*a*)anthracene).

4.3.6 Measurement Uncertainty (MU)

Combined MU for each of the target analytes is reported in Table 4.3. The listed combined measurement uncertainties are expanded uncertainties about the mean with the coverage factor of 2. It includes uncertainty of the bias and uncertainty from the intermediate precision. Our combined MU for all target analytes was larger than the certified MU which was expected as it incorporates MU from the certified compounds as well as the variability from our own analysis. As this developed method will be routinely used in our laboratory, we will continuously monitor intermediate precision from repeated extractions of SRM2974a. This will be accomplished through

the use of control charts to calculate method uncertainty that takes into account the long term precision.

4.3.7 Ruggedness

The three variables that suggested that could affect the performance of our method include ASE extraction temperatures and cell size, mass of silica gel used in our adsorption chromatography step and the volume of GPC extracts collected. The Eurachem Guide on method validation states explicitly that ruggedness test for in-house developed methods must be performed[38]. There were no significant differences in the performance of our method as a result of any of the deliberate changes undertaken implying that our developed method is robust.

4.4 Conclusion

To our knowledge, the method validated in this study represents the first simultaneous determination of PACs in biota. The method performance characteristics were rigorously tested and the validation parameters were established in accordance with *Eurachem Guide to Method Validation and Quality in Analytical Chemistry*. Efforts to determine certified values on additional C_x-PAC clusters are further warranted. Additionally, future work on quantifying individual APAH and S-based PACs (and N-based PACs) isomers using two dimensional GC will enable isomer specific APACs measurements that will drive much needed ecotoxicity studies and field monitoring programs.

Table 4.4 *Certified and Measured APAH and PAH concentrations reported to include Measurement Uncertainty for SRM 2974a*

Analyte	Measured SRM 2974a Mass Fraction (ng/g)	U_{IP}[1]	Bias[2]	U_{bias}[3]	95% Confidence interval for Bias[4]		Combined MU (U_c)[5]	Certified SRM 2974a Mass Fraction (ng/g)	Certified MU for SRM 2974a[6]	% Relative Error
					lower limit	upper limit				
1-Methylphenanthrene	19.9	2.46	2.31	1.4	-041	5.03	± 5.6	17.6	± 1.6	6.4
2-Methylphenanthrene	28.7	2.56	0.53	1.7	-2.94	3.99	± 6.2	28.2	± 2.6	1.8
3-Methylphenanthrene	24.8	2.52	0.73	1.3	-1.92	3.39	± 5.7	24.1	± 1.4	2.9
9-Methylphenanthrene	19.7	1.85	3.66	1.1	1.56	5.76	± 4.3	15.9	± 1.3	23
1-Methylpyrene	10.55	0.92	-0.14	0.6	-1.30	1.03	± 2.2	10.69	± 0.83	1.3
4-Methylpyrene	21.19	1.72	1.42	0.9	-0.36	3.20	± 5.5	19.77	± 0.89	7.2
Fluoranthene	215	17.80	-71.73	18.77	-109.27	-34.18	± 52	287	± 34	25
Phenanthrene	64.9	4.56	-9.44	3.11	-15.66	-3.21	± 11	74.4	± 4.7	13
Pyrene	127	10.62	-39.17	11.52	-62.22	-16.13	± 31	166	± 21	23
Benz(*a*)anthracene	20.7	2.77	-10.37	2.31	-14.99	-5.75	± 7.2	31.1	± 3.9	33
Benzo(*b*)fluoranthene	34.3	3.38	-7.16	2.00	-11.15	-3.17	± 7.9	41.5	± 2.6	17
Benzo(*k*)fluoranthene	14.98	1.37	-3.97	0.67	-5.30	-2.63	± 3.0	18.95	± 0.54	21
Benzo(*a*)pyrene	7.05	1.96	-2.68	0.90	-4.48	-0.87	± 4.3	9.73	± 0.43	27
Benzo(*ghi*)perylene	16.1	1.14	-7.61	1.21	-10.04	-5.19	± 3.3	23.7	± 2.2	32

[1] The uncertainty from intermediate precision was estimated through the use of the one-way ANOVA table (refer to Equation 10)

[2] Bias was calculated as the difference between laboratory obtained value for the SRM2974a and the certified SRM2974a value

[3] The uncertainty of the bias was determined by combining the uncertainty from the laboratory estimate of the bias and the uncertainty from the certified reference number (refer to Equation 13)

[4] 95% confidence interval for the bias includes lower and upper limits which were estimated as Bias $\pm 2 \times U_{bias}$

[5] The uncertainty listed with each compound for SRM 2974a is the expanded uncertainty about the mean with the coverage factor of 2. The combined uncertainty includes uncertainty of the bias and intermediate precision.

[6] The uncertainty listed with each value is an expanded uncertainty about the mean, with coverage factor 2 (95% confidence), calculated by combining a between-method variance incorporating inter-method bias with a pooled within method variance following ISO/NIST Guide to Expression of Uncertainty in Measurement (SRM 2974a CoA).

Table 4.5. *Between day and within day repeatability for target analytes.*

Analyte	**Repeatability (% RSD)**	
	Between day	**Within day**
1-Methylphenanthrene	8.4	9.0
2-Methylphenanthrene	7.1	5.4
3-Methylphenanthrene	7.5	6.8
9-Methylphenanthrene	6.2	7.1
1-Methylpyrene	7.7	4.0
4-Methylpyrene	7.4	3.3
Fluoranthene	4.6	6.9
Phenanthrene	5.3	4.6
Pyrene	4.9	6.8
Benz(*a*)anthracene	4.9	12.5
Benzo(*b*)fluoranthene	9.5	2.5
Benzo(*k*)fluoranthene	8.0	4.4
Benzo(*a*)pyrene	24.0	13.7
Benzo(*ghi*)perylene	6.6	2.6

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CHAPTER 5

Absorption of Polycyclic Aromatic Hydrocarbons by a Highly Absorptive Polymeric Medium

A version of this chapter has already been published as Olga Francisco, Ifeoluwa Idowu, Kelsey L. Friesen, Matthew McDougall, Sara Seoin Choi, Patrique Bulloch, Oluwadamilola Daramola, Wesley Johnson; Vince Palace, Stetefeld, J. and Tomy. G.T. Absorption of Polycyclic Aromatic Hydrocarbons by a Highly Absorptive Polymeric Medium. Chemosphere, 201: 441-447, 2018.

My role in this manuscript was to review and interpret all the peer-reviewed literature, prepare and review parts of the laboratory data with graphical illustrations and writing parts of the manuscript. The lead author, Olga Francisco, was primarily responsible for interpreting data obtained at varying temperatures that the experiments were carried out. This manuscript was included in my thesis to represent a scenario where the method I developed could be used.

Abstract

The efficacy of a lightly cross-linked polymeric bead to absorb polycyclic aromatic hydrocarbons (PAHs) from the surface of fresh- and salt-water in a simulated oil-spill scenario was assessed in this study. A layer of PAHs at the water surface was created by first preparing the PAHs in hexane and then carefully spiking this mixture onto the surface of water. Beads were then applied to the surface of the organic phase and the amount of hydrocarbons absorbed by the beads was examined at prescribed time intervals and at different temperatures. Absorption of PAHs into the beads was exhaustive with ~86 ± 4% being selectively removed from the organic phase by 120 s. First order reaction rates best described the uptake kinetics and absorption rates ranged from 0.0085 (naphthalene) to 0.0325 s^{-1} (dibenzo[a,h]anthracene). Absorption of PAHs into the beads was driven by molecular volume (A^3). Uptake rates increased markedly for PAHs with molecular volumes between 130 A^3 and 190 A^3. Beyond this molecular volume there was no apparent change in the rate of uptake. This study shows that these polymeric beads have a high affinity for PAHs and can be used under various environmental conditions with negligible difference in absorptive efficacy.

5.1 Introduction

Canada's Oil & Gas industry is a major driver of Canada's economy. Annually, this sector generates more than $52 B or 4.2% of Canada's Gross Domestic Product (GDP).[1-2] Canada's largest export product is crude oil which accounts for one quarter of all Canadian exports. Canada currently exports ~3M barrels per day of oil and that volume is projected to increase by 2-fold by 2030.[3] Transportation of crude oil within Canada, to coastal regions for off-shore shipment, or to the United States is done by rail, tanker trucks, and pipelines with the latter being regarded as the safest mode of transportation. The proper functioning of pipelines is also aided by highly sophisticated computer-based monitoring systems that run through the inner walls of the pipeline itself.[4] This allows for automated shut down of flows in the event of ruptures or leaks.

Despite these best practices, ruptures and leakage of oil from pipelines do still occur. A number of spills have been recorded resulting from pipeline transport, one of the most recent pipeline breach spilled about 2×10^5 L.[5] The records from these spills into both the terrestrial and aquatic environments in Canada have led to government actions which have resulted in improvements to clean-up and remediation procedures.

Physical response tools or clean-up methods used in the initial phase of superficial oil spill response include, although are not limited to: booms, skimmers, and adsorbents.[6-8] These tools are widely used, however, their effectiveness for oil remediation is limited by their mechanism of action: they adsorb oil or fill interstitial spaces within their framework restricting any allowance for expansion beyond their constructed size.[8-9] Additionally, booms used to contain spilled oil to a constrained area are known to be ineffective at restricting some oil components especially those that are of high volatility and solubility. There is also a risk of secondary contamination from the currently used adsorption media due to leaching.[9]

Dispersants are also widely used to remediate oil spilled into the aquatic marine environment. In fact, Corexit 9500® was applied to the Gulf of Mexico in an attempt to remediate oil from the Deep Water Horizon oil spill.[10-13] However, there is also evidence that some dispersant formulations can, in the short term, increase the toxicity of spilled oil by creating small oil droplets in the water column thereby increasing exposure to resident organisms.[14-15] Additionally, the dispersant product itself can exert significant toxicity to aquatic animals.[14, 16-17] Based on multiple species testing, Corexit 9500® was classified as "moderately toxic" to aquatic species especially when free in solution.[18] A settlement failure and complete larval mortality was observed after exposure of *M. faveolata* to 50 and 100 ppm Corexit 9500® and *P. astreoides* to 100 ppm concentrations.[17] Other studies have supported that exposure of aquatic lives to dispersants such as Corexit 9500® has the potential to negatively impact their settlement and survival.[19-22]

Clearly, there is a need for a more effective and safer means for cleanup and remediation of crude oil components in the aqueous environment. Imbiber Beads® are made from a synthetic superabsorbent, lightly cross-linked polymer that has a strong affinity for hydrophobic compounds in aqueous media. The beads themselves are non-porous and inherently non-toxic and non-reactive.[9] During the absorption process, the beads expand to 27 times their original volume and float on the surface of water. Because the beads work by absorption and not adsorption, they do not release entrapped compounds after the sorption process and therefore reduce the risk of secondary contamination.[9]

Here, we examine the rates of absorption of 16 polycyclic aromatic hydrocarbons (PAHs) using Imbiber beads® from the surface of salt- and fresh-waters and at varying water temperatures under non-equilibrium conditions. Our project was designed to simulate a real-world spill scenario as closely as possible. As such, a two phase system consisting of water and an organic layer of hexane

fortified with PAHs was used to study the uptake kinetics. This biphasic system in saltwater was tested at a range of environmentally relevant temperatures to assess whether absorption by the beads is impacted by temperature. Because absorption is a diffusion phenomenon, we hypothesized that this would be driven by compound hydrophobicity. In this study we present results of a controlled laboratory study performed under competitive substrate and static water conditions to test our hypothesis.

5.2. Materials and methods

5.2.1. Chemicals

All organic solvents used were of high-purity (Optima grade) and purchased from Fisher Chemicals (Ottawa, Ontario, Canada). Sixteen (16) unsubstituted PAHs as a native mix and deuterium mass labeled d_{10}-anthracene were purchased from Accustandard Inc. while their surrogates were obtained from Cambridge Isotope Laboratories Inc. (Tewksbury, Massachusetts, USA). All standards were >98% purity. The suites of labeled internal standards used for isotope dilution were d_8-naphthalene, d_8-acenaphthylene, d_{10}-acenaphthene, d_{10}-fluorene, d_{10}-phenanthrene, d_{10}-pyrene, d_{12}-benz[a]anthracene, d_{12}-chrysene, d_{12}-benzo[b]fluoranthene, d_{12}-benzo[k]fluoranthene, d_{12}-benzo[a]pyrene, d_{12}-indeno[1,2,3- c,d]pyrene, d_{14}-dibenz[a,h]anthracene and d_{14}-benzo[g,h,i]perylene. Labeled anthracene was used as the instrument performance internal standard (IPIS). Fluval® Sea Marine Salt Formula was purchased from a local pet store (Winnipeg, Manitoba, Canada) and 1.44 kg of it was dissolved in 4 L of milliQ water to prepare salt water used for the experiments. One (1) kg of Imbiber Beads® was kindly donated by Imbibitive Technologies (Welland, Ontario, Canada). The beads range in size from 150 - 400 μms.

5.2.2. Solid-phase extraction (SPE)

Prior to the extraction, fresh and saltwater, spiking solutions and 20 mL SPE glass cartridge vials were pre-equilibrated overnight at determined temperatures (saltwater: 5, 10, 15, 20 and 25 ºC; freshwater: 25 ºC) in a controlled temperature room. Frit-less SPE glass cartridges were packed with glass wool (heated to 300 ºC for 4 h) at the bottom. Each SPE cartridge was then fortified with native PAH mixture in hexane (200 µL of 4 ng/µL each). After that, 25.0 ± 2.5 mg of Imbiber® Beads was added to the top of each SPE cartridge. At time 10, 20, 30, 40, 50, 60, 70, 80, 90,100,110, and 120 s, 18 mL of water was passed through the cartridge and collected (*n=5* for each time point) using a 24-port manifold connected to a vacuum pump. Beads remained trapped in the glass wool. Control water samples (*n=5*) were collected at each temperature with no PAH added.

5.2.3. Liquid-liquid extraction (LLE)

Target analytes were extracted from water using liquid-liquid extraction (LLE) with 5 mL of hexane (x 2) as the extracting solvent. Prior to extraction, the suite of mass labeled PAH internal standards (10 µL of 5 ng/µL) were spiked into each sample. One (1) mL of toluene was added to the extract as a keeper solvent prior to solvent reduction with gentle stream of UHP nitrogen. Final extracts (100 µL) were then spiked with an IPIS (d_{10}-anthrancene, 10 µL of 5 ng/µL) prior to the instrumental analysis.

5.2.4. GC-MS/MS conditions

An Agilent 7890 GC coupled with a 7000C triple quadrupole mass spectrometer fitted with electron ionization (EI) source was used for the MS/MS acquisition. An Agilent J&W HP-5ms ultra inert column (30m x 0.25mm x 0.25 mm), with helium as the carrier gas at a constant flow rate of 1.2 mL/min 1 μL of sample was injected with a PAL RSI 85 auto sampler at 250 ºC in splitless mode. The oven temperature was held at 60 ºC for 1 min then raised to 210 ºC at 35 ºC/min, further ramped up to 260 ºC at 3 ºC/min and finally to 300 ºC at 10 ºC/min and held for 20 min. Both transfer line and source temperature were set at 320 ºC and UHP nitrogen was used as the collision gas at 60 psi.

5.2.5. MS analysis

Before MRM conditions could be established it was first necessary to record full-scan EI mass spectra (m/z 50 - 250) for each analyte (5 ng/μL, 1 μL injection). Once the most abundant precursor ion was determined it was then purposely selected and fragmented in our collision cell (Q2) to generate product ions. Defined low and high collision energies were used to acquire the product-ion spectra of selected precursor ions. Ultra-high purity nitrogen was introduced into the Q2 to aid collision-induced dissociation (CID) of the precursor ion. The collision energy (CE) required to obtain the most abundant product ions was selected. In this manner, multiple reaction monitoring (MRM) precursor/product ion transitions along with their associated CEs were established. To improve detection limits, pseudo-MRM monitoring parent ion / parent ion transitions were used for indeno[1,2,3-c,d]pyrene, dibenz[a,h]anthracene and benzo[g,h,i]perylene as described by Shang, *et al*. [23]

5.2.6. Method of quantitation

The approach to quantifying native PAHs used was described previously by Idowu, et al. [24]. In brief, the MRM ion transitions used to detect and quantify PAHs correspond to the $[M]^+$ to $[M - H]^+$ ions. Quantitation of PAHs was done using isotope-dilution and was based on the average relative response factors (ARRFs) of the native PAHs relative to authentic d-PAH surrogates. Prior to calculating ARRFs, the responses of the native and d-PAHs (i.e., electronically integrated peak areas) were normalized to the response of the IPIS, d_{10}-anthracene. The IPIS was added to the calibration solutions and to sample extracts prior to GC-injection at a concentration of 500 pg/µL.

5.2.7. Statistical analysis

Calculations were performed using the statistical package in SigmaPlot Version 13 (Systat Software Inc. San Jose, CA, USA). Tests for significance of individual slopes used an alpha of 0.05. A Z-test for the difference in slopes was used to compare temperature and salinity effects and significance was based on an alpha of 0.01.

5.3. Results and discussion

Preliminary batch studies were performed to determine the optimum mass of the polymer beads needed to facilitate meaningful kinetic studies. We found that absorption rates were too fast when bead masses of greater than 25 mg were used. As such, absorption studies were based on 25 mg of beads which translates to a ratio of approximately 1:32 000 mass PAH to mass polymer beads. App Table 1 shows the amount of PAHs (nmoles) measured in our test system after exposure to beads at prescribed exposure times and water temperatures in both freshwater and saltwater. As an example, the uptake profile of benz[a]anthracene by the beads at 25 ºC is shown in Fig. 5.1. Fig.

5.2 shows the relationship between the natural log of concentration of all 16 PAHs (seawater and fresh water data combined) measured vs time (sec) and Table 5.1 shows the first-order uptake rates (s^{-1}) of all 16 PAHs. Fig. 5.3 shows the change in first-order uptake rates (s^{-1}) with increasing molecular volume (A^3) in saltwater at 25 ºC.

The polymeric beads used in our study are less dense than water and as such will primarily absorb chemicals near to or at the surface of water. The relative densities of 12 PAHs have been reported in the literature; no data is available for benzofluoranthene, benzoperylene, benzo[k]fluoranthene, and indeno[1,2,3-c,d]pyrene.[25] With the exception of acenaphthylene all the other PAHs studied have relative densities greater than 1. This was an important consideration in the design of our experiment. To prevent sinking and to simulate a real-world spill in which typically a layer of oil is noticeable on the surface of water, a PAH mixture prepared in hexane was carefully spiked onto surface of water (200 µL of 4 ng/µL), this equated to 800 ng of each PAH that was spiked onto the surface of water and enabled us to create an organic layer immiscible with water. The equivalent mass of hexane based on 200 µL spike and a density of 0.659 g/mL is 2.6×10^6 ng. Although there was a significant discrepancy in the mass of hexane available for uptake compared to PAHs, diffusion into the polymeric beads is thought to be driven by molecular volume of hydrocarbon (see below) and as such the amount of hexane is unlikely to affect the mass transfer of PAHs into the polymeric beads.

It could also be argued that PAHs are migrating into the beads bound to hexane. If this were the case we would expect to see no differences in the absorption rates of individual PAHs into the beads. However, we did not observe this in our study. For example, the rate of absorption of naphthalene and acenaphthene were significantly smaller (z-test, 99% CI level) than any of the other PAHs. This implies that PAHs are diffusing into the beads unbound to hexane.

While the concentration of PAHs in the aqueous phase was not measured after spiking, the low solubility [0.001 (benzofluoranthene) to 32 mg/L (naphthalene)] and disequilibrium between the two phases made it difficult to predict the extent of partitioning between the phases. Admittedly a separate experiment could have been performed to estimate PAH concentration in the aqueous phase. However the extent of PAH absorption by the polymeric beads was exhaustive (see below) and the combination of low PAH solubility and high affinity for hexane it was reasonable to assume negligible partitioning of PAHs from hexane to the aqueous phase.

Because of their hydrophobicity, the fugacity of PAHs from the hexane phase into water is energetically not favorable. Further, the partitioning of PAHs into the beads is likely to be independent of water temperature or salinity. However, we carried out the experiments at varying water temperatures and in saline (5, 10, 15, 20 and 25 ºC) and freshwater (25 ºC) to simulate different environmental scenarios that can arise in a spill-event.

If partitioning of PAHs out of the organic phase into the bulk water was significant then differences in the amount of hydrocarbon in the organic phase at different temperatures at the start of the experiment would be evident. However, no differences in the molar amounts of PAHs were observed (z-test, 99% CI level) at any of the tested temperatures which supports our hypothesis that partitioning out of the organic layer to water is energetically not favorable. Similarly, there were no differences in molar amounts of PAHs in saltwater and freshwater implying that salinity did not have a significant effect on partitioning of PAHs into water.

While partitioning of PAHs between the layers is negligible, it was thought that diffusion of PAHs from the organic layer into the beads would be temperature dependent. As such, the PAH mixture in hexane was also equilibrated at each study temperature prior to spiking into water.

For all the compounds tested, the amount remaining in the organic phase decreased with longer exposure times to the beads implying that the diffusion process is not limited by the mass of beads used at the ratio tested (see Appendix). App Table 1 also shows that there is appreciable uptake of all PAHs for the first 90 s followed by a levelling off until 120 s. As an example, the uptake profile of benz[a] anthracene into the beads at 25 ºC in saltwater is shown in Fig. 5.1. The overall mean amount of PAHs removed from the organic layer after 120 s at all temperatures tested and for both freshwater and saltwater was 86 ± 4% suggesting that the uptake is effectively exhaustive.

The data in App Table 1 was then fitted to both a zero- and first order rate reaction. There was a greater level of significance when the data was fitted to a first-order reaction rate and so we used this approach to describe our kinetics.

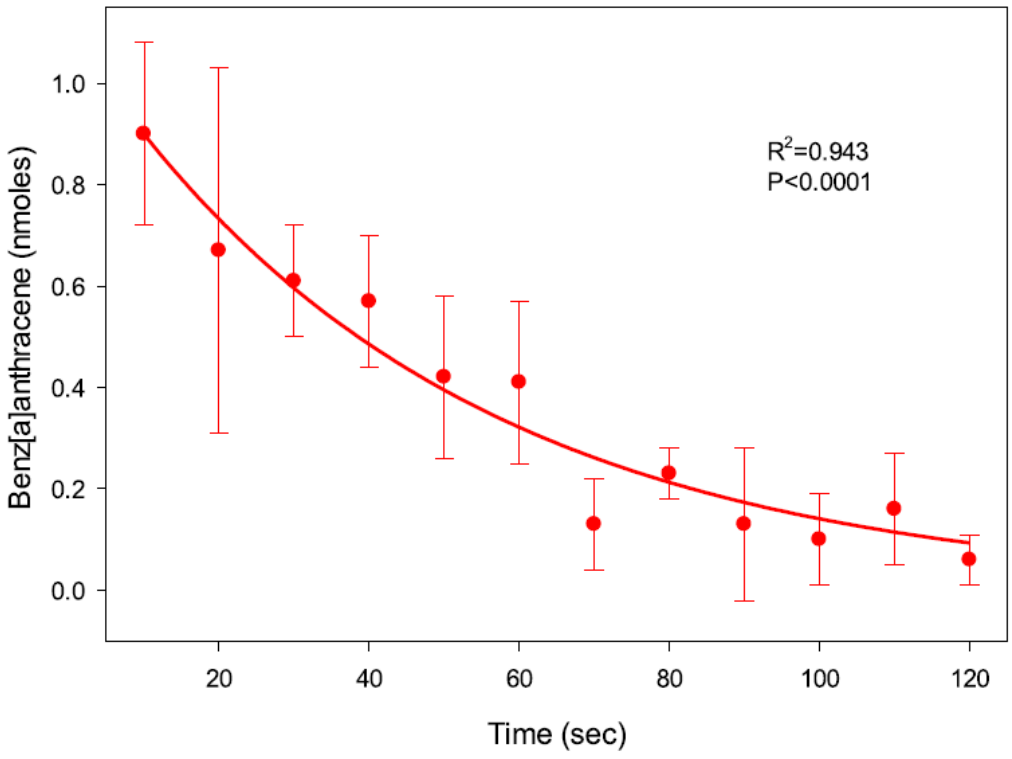

Figure 5.1 *The profile of loss benz[a]anthracene over time at 25 ºC. Each data point represents the arithmetic mean ± SE of 5 replicate measurements.*

The temperature of the organic layer did not have a significant effect on the rates of absorption of the individual PAHs. We speculate that because of the large concentration gradient between the PAHs in hexane and the beads diffusion rates are large and that this effect is greater than the expected change in diffusion rates caused by changes to the temperature of the hexane phase.

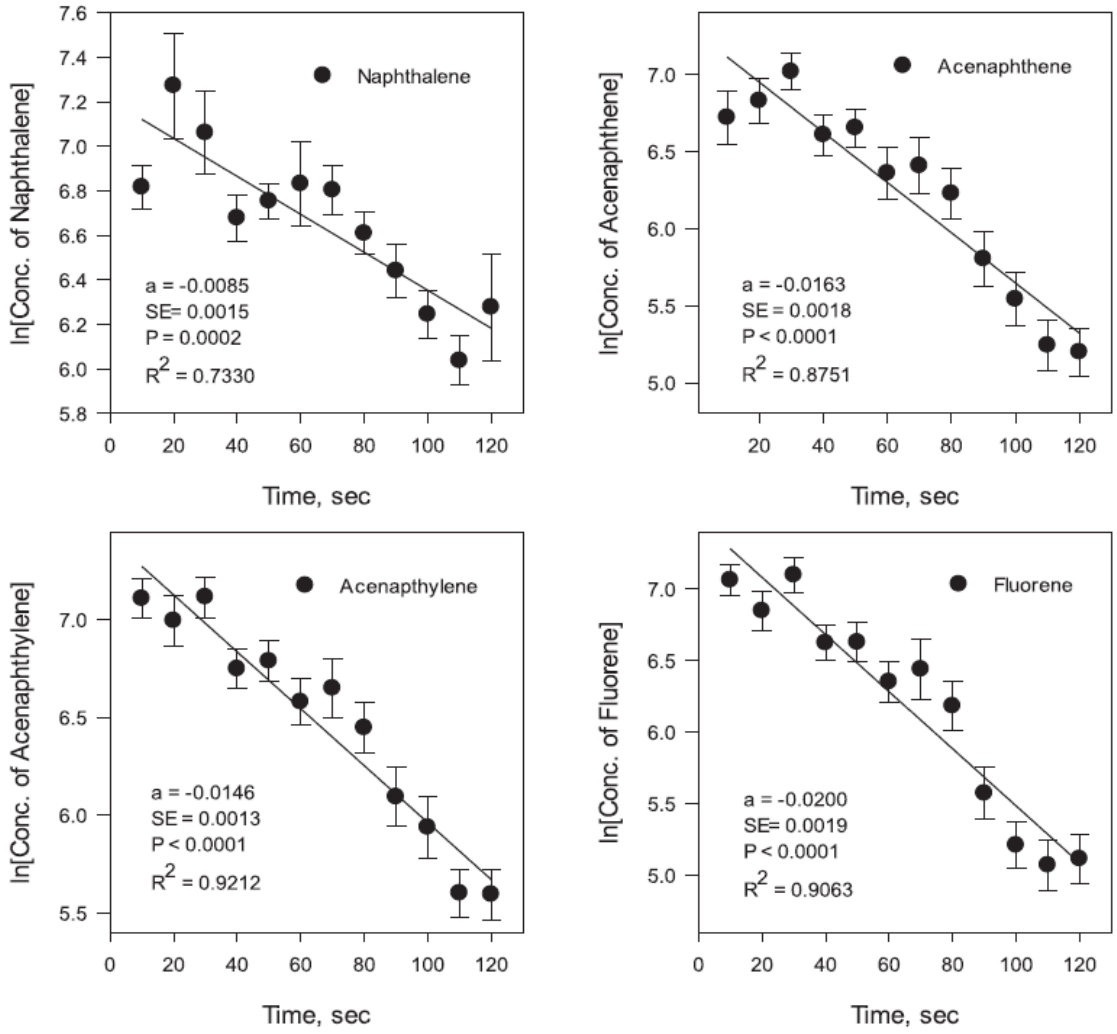

Figure 5.2 *Plot of natural log of concentration of 16 PAHs in organic phase against time. Each data point represents the arithmetic mean ± SE of 35 replicate measurements.*

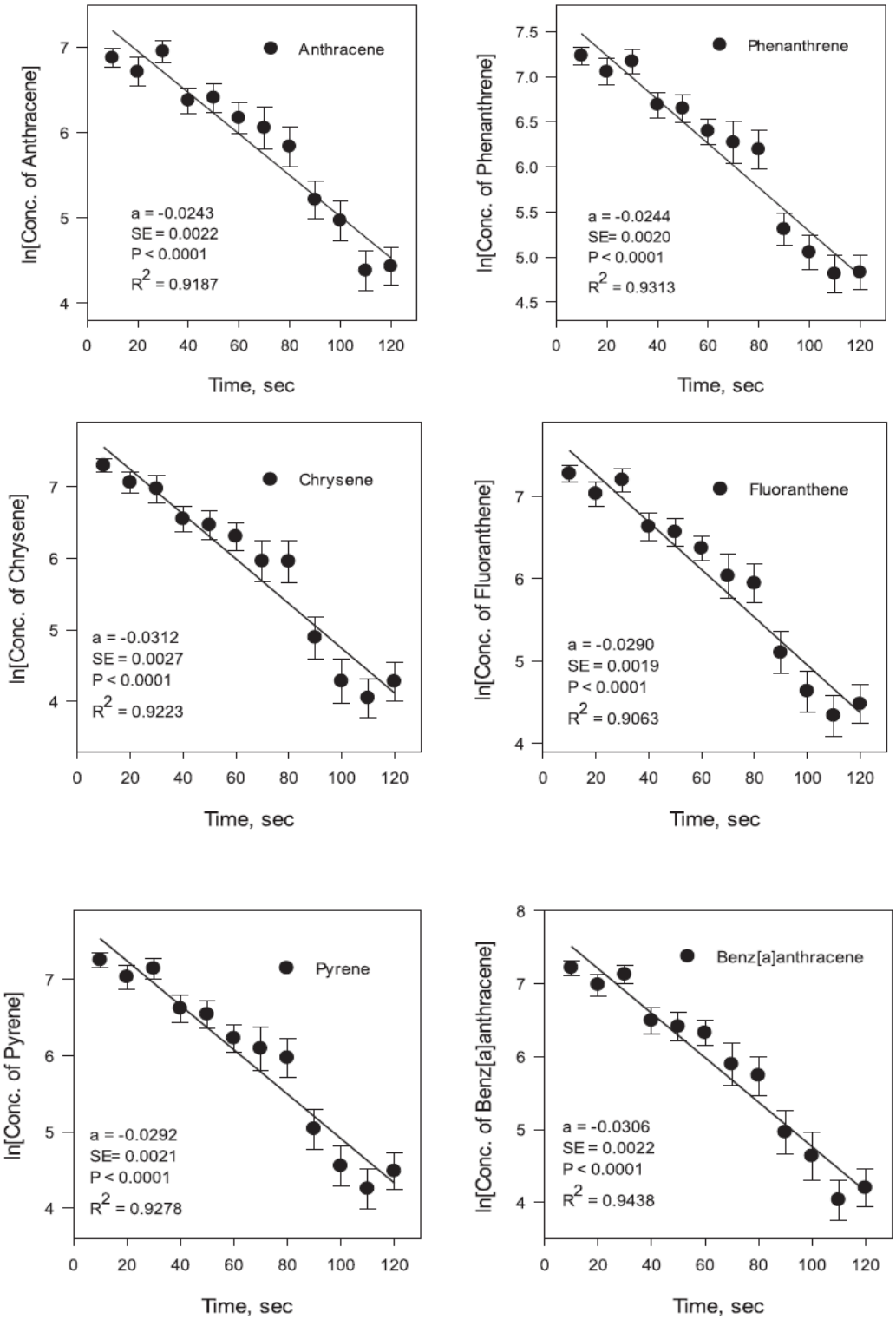

Figure 5.2 *Continued*

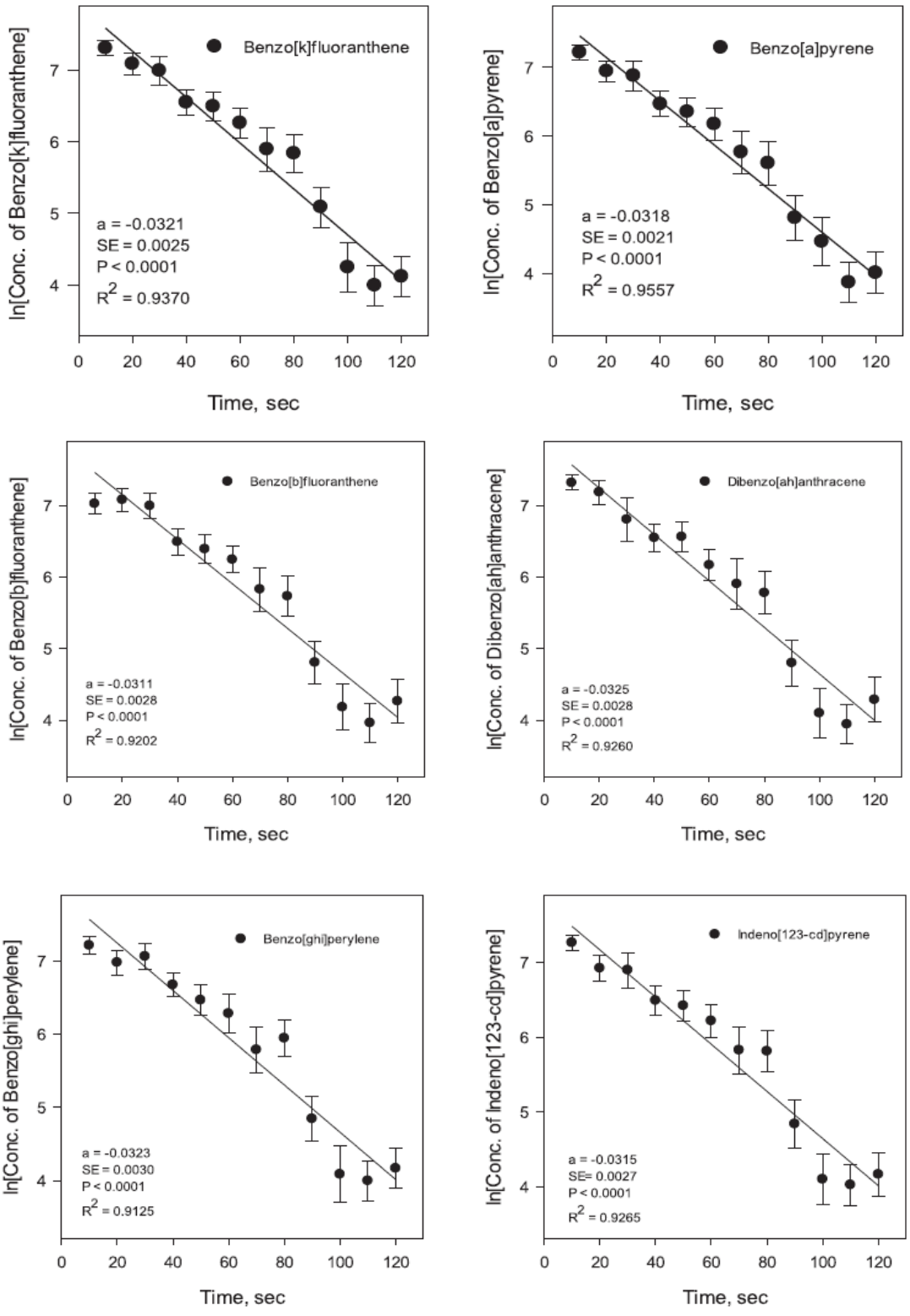

Figure 5.2 *Continued*

Because there were no significant differences in the slopes of first-order uptake rates (z-test, 99% confidence level) at any of the study temperatures all the data from saltwater and freshwater were combined in the determination of uptake rates (s^{-1}). Fig. 5.2 shows the combined data for each individual PAH. Table 5.1 shows the uptake rates of all the PAHs studied. First order uptake rates ranged from 0.0085 (naphthalene) to 0.0325 s^{-1} (dibenzo[a,h] anthracene).

There is some experimental variability in our data which is likely due to the intrinsic nature of the beads themselves. The beads are very granular and while we accurately weighed 25 mg for each exposure experiment into a vial there was always static charging that caused the beads to stick to the sides of the vial. This made it difficult to quantitatively transfer all of the beads from the vial into the exposure vessel. It is likely that this is partly driving some of the imprecision in our data.

Leo, et al. [26] first showed that for non-polar compounds, molecular volume is the main intrinsic driver of hydrophobicity. More recently, the importance of molecular structure and shape of cyclic compounds including some PAHs on hydrophobicity was studied by Sedov and Solomonov [27]. Using thermodynamic models, the authors showed that molecular structure and shape do not significantly affect the hydrophobicity of aromatic compounds and that molecular volume is the main factor that determines compound hydrophobicity. Taken together, this implies that hydrogen bonding and intermolecular interactions between PAHs and water can be assumed to be negligible.

We examined the rate of absorption of PAHs by the beads and compared it to molecular volume of the individual PAHs. Our hypothesis was that fugacity of PAHs would be greater as the molecular volume increased resulting in a linear trend of increasing absorption rates with increasing molecular volume. Fig. 5.3 shows the resulting relationship between rates of absorption and molecular volume at 25 ºC. Molecular volumes for 16 PAHs were derived from Molinspiration [28]. Interestingly, there is a sharp linear increase in absorption rates up until a molecular volume of

ca. 185 A^3 after which the rate levels off and is essentially constant to a molecular volume of ca. 260 A^3. This implies that there is a critical threshold in molecular volume of PAHs in which the change in fugacity for these compounds in water is insignificant. Based on the regression analysis, the critical value was 190 A^3.

The ratio of the mass of PAHs and Imbiber Beads® used in this study was ~1:32 000. At this ratio and under competitive conditions, the absorption of all 16 PAHs from the organic layer was nearly exhaustive. The beads themselves are not limited to absorption of PAHs but to any hydrophobic compound less dense than water. As an exercise in the effectiveness of using the beads for remediation purposes, measurement data made on surface water concentrations of PAHs and their alkylated derivatives as a result of the Deepwater Horizon explosion in the northern Gulf of Mexico were used to assess the mass of beads needed for remediation. Diercks, et al. [29] measured total PAH concentrations in surface waters (sum of alkyl- and parent PAHs) to be 85 μg/L twenty-one days after the explosion and in the vicinity of the wellhead. If we consider a spill area of 1000 x 1000 m and a depth of 1 cm equates to a volume of 10 000 m^3, the total amount of PAHs at the surface and sub-surface of water in this scenario would be approximately 85 kg.

Table 5.1: First-order uptake rates (s^{-1}) of PAHs in saltwater and freshwater

Compound	# of Rings	Molecular Volume (A^3)	Absorption rate(s^{-1})	Standard error	p-value	R^2-value
Naphthalene	2	128.03	0.0085	0.0015	0.0002	0.7330
Acenaphthylene	3	144.61	0.0146	0.0013	<0.0001	0.9212
Acenaphthene	3	150.80	0.0163	0.0018	<0.0001	0.8751
Fluorene	3	161.41	0.0200	0.0019	<0.0001	0.9063
Anthracene	3	172.03	0.0243	0.0022	<0.0001	0.9187
Phenanthrene	3	172.03	0.0244	0.0020	<0.0001	0.9313
Chrysene	4	216.02	0.0312	0.0027	<0.0001	0.9223
Fluoranthene	4	188.60	0.0290	0.0019	<0.0001	0.9063
Pyrene	4	207.19	0.0292	0.0021	<0.0001	0.9278
Benz(a)anthracene	5	216.02	0.0306	0.0022	<0.0001	0.9438
Benzo(k)fluoranthene	5	232.59	0.0321	0.0025	<0.0001	0.9370
Benzo(a)pyrene	5	232.59	0.0318	0.0021	<0.0001	0.9557
Benzo(b)fluoranthene	5	232.59	0.0311	0.0028	<0.0001	0.9202
Dibenzo(a,h)anthracene	5	260.01	0.0325	0.0028	<0.0001	0.9260
Benzo(g,h,i)perylene	6	249.17	0.0323	0.0030	<0.0001	0.9125
Indeno(1,2,3-c,d)pyrene	6	247.17	0.0315	0.0027	<0.0001	0.9265

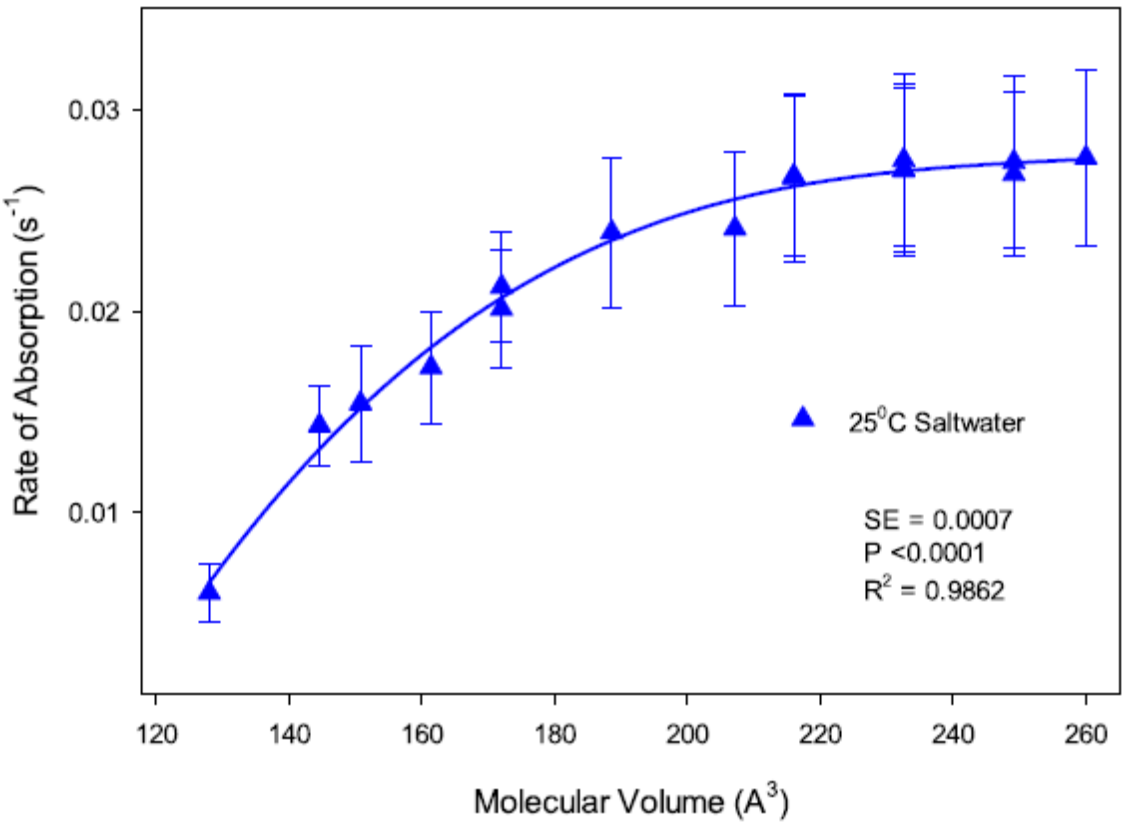

Figure 5.3 *Relationship between rates of absorption at 25 ºC in saltwater for 16 studied PAHs and molecular volume. Each data point represents the arithmetic mean ± SE of 5 replicate measurements.*

Based on the ratio employed in this study and in the current scenario, a mass of approximately 2.5 x 10^6 kg of beads would be needed to achieve near exhaustive removal of PAHs from surface water. Admittedly, our approach is somewhat of an oversimplification and factors like saturation of the beads by other hydrophobic compounds present and potential impact of wave movements on PAH absorption were not considered. However, it does provide some insights into the potential usefulness of the beads in remediation of large spills.

5.4. Conclusions

Our study provides insights into the competitive uptake rates of PAHs by Imbiber Beads® from the surface of water under non-equilibrium conditions to simulate an early oil spill. First order absorption rates were useful in describing the uptake and the efficacy of absorption was considered to be exhaustive. Molecular volume was the main driver of absorption of PAHs by the beads and uptake rates plateaued at molecular volumes greater than 190 A^3. Work to further assess the effectiveness of the beads to absorb the suite of compound classes known to be present in crude oils in a competitive scenario is clearly warranted. The results presented here represent a necessary first step towards that goal.

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Chapter 6

Comprehensive Two-Dimensional Gas Chromatography High Resolution Mass Spectrometry for the Analysis of Substituted and Unsubstituted Polycyclic Aromatic Compounds in Environmental Samples

A version of this chapter has already been published as Ifeoluwa Idowu; Wesley Johnson; Olga Francisco; Terry Obal; Chris Marvin; Philippe J. Thomas; Courtney D. Sandau; Jörg Stetefeld and Gregg T. Tomy. Comprehensive Two-Dimensional Gas Chromatography High Resolution Mass Spectrometry for the Analysis of Substituted and Unsubstituted Polycyclic Aromatic Compounds in Environmental Samples. Journal of Chromatography A, 1579: 106-114, 2018.

This experiment was designed by myself with contribution from my supervisor, Gregg Tomy. I performed the experiments, interpreted the data and wrote the manuscript.

Abbreviations are found in Appendix

Abstract

Polycyclic aromatic compounds (PACs) consists of multiple compounds and the number of theoretically possible isomers can reach into the thousands. Currently each PAC group is quantified collectively as a single group of compounds. However, individual PACs can reveal important information on how the PACs were formed and this information may be used to determine sources of PACs in environmental samples. It is hypothesized that many of the limitations with characterizing alkylated PACs with one dimensional gas-chromatography (1D GC) can be circumvented using GC×GC (two dimensional gas chromatography). Here we apply comprehensive GCxGC coupled to high-resolution time of flight mass spectrometry (GC×GC-HFTOF-MS) to aid in the separation, identification and quantitation of APACs in three environmental matrices: mussel tissue (*Mytilus edulis*), lubricating oil and coal. In the absence of authentic analytical standards, differences in the mass spectral fragmentation pattern of isomers were used to confirm the identity of isomers within a PAC group. The method was validated according to the EURACHEM guidelines and used to quantify a biological standard reference material (SRM 2974a). The method met all the standard method performance requirements such as trueness, precision and measurement of uncertainty and is fit for quantifying these compounds in biota. Furthermore, the method was used to identify and quantify additional PAC compounds in the SRM 2974a material which to date have not been certified. With appropriate statistical analytical tools, the described GC×GC method can be used as a tool for more robust source fingerprinting and source apportionment of PACs in the environment.

6.1 Introduction

Application of gas chromatography coupled with mass spectrometry has been a dominant technique employed for separation, identification and quantification of volatile environmental contaminants present in complex matrices. This technique has been mostly been established using unidimensional column which poses difficulty for separation of closely or co-eluting compounds in complex mixtures.[1] Hence, the common approach of cluster analysis for quantitation of these compounds. Polycyclic aromatic compounds (PACs) are ubiquitous environmental contaminants. They are among the most complex class of environmental compounds known and their complexity is largely source-dependent.[2] The three major sources of PACs include: i) pyrogenic, from pyrolysis of substrates such as fossil fuels and biomass, ii) petrogenic from petroleum-related sources and iii) biogenic which are naturally occurring.[3] Crude oil exploration, accidental spills, urban/municipal run-off and discharge from routine tanker operations can introduce petrogenic PACs into both terrestrial and aquatic environments.[4-5] PACs in environmental samples include the 16 environmental protection agency EPA parent PAHs (polycyclic aromatic hydrocarbons), other unsubstituted and substituted PACs with more focus on '*the alkylated five'* i.e. alkylated (C_1-C_4) homologs of PACs (naphthalene, phenanthrene, dibenzothiophene, fluorene and chrysene).[6] PAHs consist of carbon and hydrogen only while PACs are known to possess carbon, hydrogen and heteroatoms in their chemical structure. Alkylated PACs (APACs) possess vast structural diversity resulting in a large number of isomers that are theoretically possible and also potentially bio-available in the environment. For example, a C_4-pyrene has 225 theoretically possible isomers.[7] These alkylated homologs of PACs are more abundant than their parent compounds in crude oil.[3, 8] Thus, an increased input of petrogenic PACs from crude oil can lead to the presence of APACs in the environment.

Recent studies have shown that some APACs are more bioaccumulative and toxic than their unsubstituted analogs.[9-11] It has also been documented that the positioning of the alkyl substituent(s) can affect the toxicity of these compounds as well as the susceptibility of these compounds to weathering/degradation processes.[12] Like other environmental contaminants (e.g., PCBs and PBDEs) the environmental fate of some PAC isomers can be different and understanding their behaviour in the environment hinges on reliable analytical methods.[13-14] There have been a few studies that have quantified both substituted and unsubstituted PACs using one dimensional gas chromatography (1D-GC).[1, 15-16] The 1D-GC analyses of complex PACs (in particular alkyl PACs) often result in ion chromatograms containing unresolved components. These components present an arduous task during spectral interpretation and data analysis of structural isomers or compounds with significant concentration differences. Two-dimensional GC is commonly employed to circumvent these shortcomings and when coupled to high-resolution mass spectrometry can be used for simultaneous targeted and non-targeted chemical analyses in a single injection.

The introduction of comprehensive multidimensional GC has dramatically improved separation efficiency, trace-level analysis and identification of key components in complex samples[17-18]. The improved peak capacity makes it a natural choice for complex geological and biological samples.[19-20] Although several methods have been employed to identify and quantify analytes using the two-dimensional (2D) system, most of these methods focus on chemical class separation but not individual isomer separation and mostly employed a low resolution instrument[21-22]. Instances of high resolution analysis were carried out on one dimensional set up[23]. Environmental forensic investigations are solely based on the ability to obtain a quantitative measurement of specific compounds or their families [22]. The ability to quantify each APAC isomer enhances

studies on source apportionment[24-25], determination of individual isomer degradation/persistence and potentially characterization of toxicity of samples from the environment[26].

The present study focuses on a comprehensive 2D GC coupled with high-resolution time of flight mass spectrometry (GC×GC/HRTOF-MS). This was applied to separate and identify PACs and their isomers in organic matrices. NIST SRM 2974a and authentic analytical standards were employed to establish method performance characteristics. These characteristics are the measurement uncertainty, the working range, trueness, detection limits, robustness and the precision of the applicable method. The greater peak capacity of the 2D system compared to 1D provided the opportunity to expand the list of quantifiable analytes in NIST material in addition to the compound list included in the certificate of analysis.

6.2 Experimental Section

6.2.1 Materials

All organic solvents used were of high-purity (Optima grade) and purchased from Fisher Chemicals (Ottawa, Ontario, Canada). Thirty-six (36) individual APAHs including C_1-naphthalene (1-methylnaphthalene; 2-methylnaphthalene), C_2-naphthalene (2,6-dimethylnaphthalene; 1,6-dimethylnaphthalene), C_3-naphthalene (2,3,5-trimethylnaphthalene), C_4-naphthalene (1,4,6,7-tetramethylnaphthalene; 1,2,5,6- tetramethylnaphthalene), C_1-fluorene (1-methylfluorene), C_2-fluorene (1, 7-dimethylfluorene), C_1-chrysene (2-methylchrysene; 3-methylchrysene; 5-methylchrysene; 6-methylchrysene), C_3-chrysene (1,3,6-trimethylchrysene), C_1-pyrene (1-methylpyrene; 4-methylpyrene), C_2-pyrene (4,5-dimethylpyrene) C_1-fluoranthene (1-methylfluoranthene), C_1-phenanthrene (1-methylphenanthrene; 2-methylphenanthrene; 3-methylphenanthrene; 9-methylphenanthrene), C_1-anthracene (2-methylanthracene), C_2-

phenanthrene (1,3-dimethylphenanthrene; 2,6-dimethylphenanthrene; 1,7- dimethylphenanthrene; 1,8- dimethylphenanthrene; 3,6- dimethylphenanthrene), C_3-phenanthrene (1,2,6-trimethylphenanthrene), C_4-phenanthrene (1,2,6,9-tetramethylphenanthrene), retene, C_1-benzo[a]pyrene (7-methylbenzo[a]pyrene); 4 heterocyclic S-based PACs (PASHs) including dibenzothiophene, C_1-dibenzothiophene (4-methyldibenzothiophene), C_2-dibenzothiophene (2,8-dimethyldibenzothiophene) and C_3-dibenzothiophene (2,4,7-trimethyldibenzothiphene) standards were purchased from Accustandard Inc. (New Haven, Cincinnati, USA) and Caledon Laboratory Chemicals (Georgetown, Ontario, Canada). Sixteen (16) unsubstituted PAHs as a native mix and deuterium mass labeled d_{10}-anthracene were purchased from Accustandard Inc. while their deuterated analogs were from Cambridge Isotope Laboratories Inc. (Tewsbury, Massachusetts, USA). All standards were >98% purity. Varying concentration of PAH and APAC standards mixture were prepared as required. The standard reference material, SRM-2974a, organics in freeze-dried mussel tissue (*Mytilus edulis*) was purchased from the National Institute of Science and Technology (NIST: Gaithersburg, Maryland, USA). Diatomaceous earth (DE) dispersant was purchased from Fisher Scientific (Ottawa, ON). The isotope dilution internal standard (IDIS) consisted of d_8-naphthalene, d_8-acenaphthylene, d_{10}-acenaphthene, d_{10}-fluorene, d_{10}-phenanthrene, d_{10}-pyrene, d_{12}-Benz(*a*)anthracene, d_{12}-chrysene, d_{12}-benzo(*b*)fluoranthene, d_{12}-benzo(*k*)fluoranthene, d_{12}-benzo(*a*)pyrene, d_{12}-indeno(*1,2,3-c,d*)pyrene, d_{14}-dibenzo(*a,h*)anthracene, d_{14}-benzo(*g,h,i*)perylene. Labeled anthracene was used as the instrument performance internal standard (IPIS). The C_{18} silica gel powder (HPLC sorbent) was purchased from Sigma Aldrich (St Louis, MO, USA) and the size-exclusion S-X3 Biobeads was purchased from Bio-Rad Laboratories (Mississauga, Ontario, Canada). Silica gel (923 grade, 100-200 mesh), alumina (60-325 mesh), Ottawa sand (a bulk adsorbent which aids extraction) and anhydrous

sodium sulfate were all purchased from Fisher Chemical. An Ultra Grade 19 used lubricating oil (Edwards, Wilmington, USA) and coal samples were used to establish retention time (r_t) windows for the APAHs.

6.3 Sample Preparation

6.3.1 Mussel tissue (SRM 2974a)

6.3.1.1 Accelerated Solvent Extraction (ASE)

One and a half grams of SRM 2974a was spiked with the IDIS (10 ng/µL, 10 µL) and mixed with dispersant in an ASE extraction cell topped with Ottawa sand. Procedural blanks were prepared using a C_{18}- HPLC sorbent matrix. The cells were extracted with dichloromethane for ~ 30 min. The extract was carefully reduced to 2.6 mL and made up to a final extract volume of 5.2 mL with hexane. 0.2 mL of the final extract was transferred to an aluminum boat for lipid determination. Further details can be found in Idowu, et al. [1].

6.3.1.2 Automated Gel Permeation Chromatography

Lipid removal from the sample extracts was done using a gel permeation chromatograph (GPC). The mobile phase was a mixture of DCM and hexane (1:1, v/v) at a flow rate of 5 mL/min at 8 psi. The extract was reduced by rotary evaporator and then finally to 1 mL under a gentle stream of UHP nitrogen for further cleanup. Further details can be found in Idowu, et al. [1].

6.3.1.3 Silica/Alumina Column Chromatography

The GPC extracts were further cleaned up using an open glass column fitted with glass wool. It was filled from bottom-up with 11 g silica gel, 1 g of deactivated alumina and a gram of Na_2SO_4. The elution of compounds was done with solvents of increasing polarity starting with 25 mL of hexane for non-polar/saturate fraction and afterward, 25 ml DCM: Hexane (1:1. v/v) to elute the PAC fraction. The aliquots were evaporated to 1 mL, fortified with IPIS and transferred to GC vials prior to instrumental analyses. Further details can be found in Idowu, et al. [1].

6.3.2 Coal

One gram of subbituminous coal was spiked with IDIS (10 ng/µL, 10 µL) and mixed with dispersant in an ASE extraction cell topped with Ottawa sand. Procedure blanks were prepared using only dispersant spiked with the labeled internal standards. The cells were extracted with dichloromethane for ~ 30 min with conditions described by Idowu, et al. [1]. The extract was carefully reduced to 1 mL for further clean up using open column chromatography. Silica/Alumina column chromatography set up was a described above for mussel tissue sample.

6.3.3 Used lubricating oil

An ultra-grade used Fisher brand 19 mechanical pump fluid (Cat. No.: 01-1840150B) was dissolved in hexane (1:5; v/v) before sample injection. The ultra-grade 19 lubricating oil (Edwards, Wilmington, USA) was used only to establish retention time (r_t) profile for alkyl PACs but was not quantified. Thus, there were no internal or recovery standards added.

6.4 GC×GC/HRTOF-MS Analysis

Analysis of the PAC extracts and standard solutions were performed using a 7890A gas chromatograph (Agilent Technologies, Wilmington, DE, USA) fitted with a split/splitless injector, GC×GC thermal modulator (operated at -80 ºC) and a secondary oven, interfaced with a Pegasus GC-HRT 4D (LECO, St. Joseph, MI, USA) high-resolution time-of-fight mass spectrometer (TOF-MS) operated in positive electron ionization mode and calibrated with perfluorotributylamine (PFTBA). The choice of column combination was based on its applicability in separating most PACs[27] although most other works that adopted the column configuration were done on low resolution system. The column configurations consist of an Rxi -5Sil MS (60 m × 0.25 mm × 0.25 μm) (Restek, Bellefonte, PA, USA) followed by an Rxi-17Sil MS (2 m × 0.25 mm × 0.25 μm) (Restek, Bellefonte, PA, USA). The GC columns were connected using an SGE micro union connector. Standards and samples (2 μL) were injected at 250 º C in splitless mode. The sample was analyzed in both one dimensional and two-dimensional mode. In one dimensional mode, the oven was programmed from 80 ºC (held for 1 min), heated to 210 ºC at 35 ºC/min, further to 260 ºC at 3 ºC/min and finally to 315 ºC at 10 ºC/min (held for 5 min). The temperature ramp in two-dimensional mode was programmed from 55 ºC (held for 2 min) heated to 110 ºC at 10 ºC/min, further to 210 ºC at 3 ºC/min and finally to 310 ºC at 8 ºC/min (held for 15 min). The modulation period was 2 s (hot pulse, 0.7 s; cool time, 0.3 s). The MS transfer line temperature was at 300 ºC, ion source temperature at 250 ºC and Helium as carrier gas supplied at 1.4 mL/min in both modes. The HRTOF-MS was operated at a mass range of *m/z* 50 – 500 with an acquisition rate of 13 spectra/second (1D) and 194.4 spectra/ second (2D) at 70 eV. The MS system was calibrated daily using PFTBA as the mass calibration gas. The resolving power (full width half height) of the system was typically greater than 25 000 based on the peak width of *m/z* 218.9851

of PFTBA. During the chromatographic run, PFTBA was continuously bled into the ion source to serve as an internal standard for instrument mass calibration.

6.5 Method Performance Characteristics

The method developed for the identification of PACs by 2D was then assessed according to its capability to accurately quantify analytes in accordance with the Eurachem guidelines.[28] The limit of detection (LOD) and the limit of quantification (LOQ) in both 1D and 2D modes were determined by analyzing ten (10) replicates of C_{18} matrix fortified with 50 ng of PACs (16 PAHs and 27 APACs) and 500 ng of mass-labeled PAH internal standards. Procedural blanks were prepared with the C_{18}-matrix spiked with only the suite of deuterated PAH internal standard and taken through the same analytical procedure as the samples. The results obtained from the replicate measurements was employed in calculating standard deviation, s_0'.[28]

$$s_0' = s_0 \sqrt{\frac{1}{n} + \frac{1}{n_b}} \qquad \text{..... (6.1)}$$

Where s_0' is the standard deviation used for calculating the LOD and LOQ values, s_0 is estimated standard deviation for single results at or near zero concentration, n is the number of replicate observations averaged when reporting results where each replicate is obtained following the entire measurement procedure, n_b is the number of blank observations used to calculate the blank correction. The limit of detection and quantification were calculated as $3 \times s_0'$ and $10 \times s_0'$, respectively according to the Eurachem guidelines.

The working range and linearity were based on the in-house custom mix of all the PAC standards as stated in the experimental method covering the proposed range of interest. Instrument performance internal standard (IPIS), d_{10}-anthracene, was added to each calibration level to account for any small fluctuations in the response of the instrument between injections. This was

added at a constant final concentration of 100 pg/µL to each calibration standard. The peak area obtained for each APAC analyte was normalized to d_{10}-anthracene (IPIS) and plotted as a function of concentration. The linearity was evaluated by the magnitude of the R^2 (correlation coefficient) value and the level of significance (p-value). Residual plots were also generated and examined to ensure the random distribution about zero to confirm linearity.

6.6 Results and Discussion

6.6.1 Identification and separation of PACs

Individual injections of all available standards (2.5 ng/µL) were carried out to (*i*) build an in-house library to establish their unique fragmentation patterns and establish diagnostic *m/z* values of isomers, (*ii*) to generate spectra to be compared with the NIST spectral library and *(iii)* establish elution time windows of isomer groups.

The first steps involved in generating and interpreting full scan EI mass spectra for comparison with mass spectra reported in previous studies and the NIST library. The end goal was to use the empirical evidence to select diagnostic *m/z* values, and ratios of *m/z* values, selective (if possible) for a particular PAC having different degrees of alkylation *i.e.*, using *m/z* values to distinguish between two C_2-PACs isomers, one with 2 methyl groups *vs* the other with one ethyl substituent. To highlight this high resolution EI mass spectra of some representative compounds are presented in Fig. 6.1.

Table 6.1 shows the list of quantification and confirmation ion fragments used in this study, other significant fragments for each compound class not listed in the table were also considered. The table was compiled from our own data and those from Zeigler, et al. [22], Zeigler, et al. [29], Moustafa

and Andersson [30], and mass spectra obtained from software containing NIST database. In general, the appearance of the mass spectra of isomers within a PAC class has similar fragmentation pattern but varying ion intensities. For example, the base peak for 2, 6-dimethyl naphthalene and 1-ethyl naphthalene is $[M]^{+\cdot}$ (molecular ion) and $[M\text{-}CH_3]^{+}$, respectively. A similar trend is observed for other isomer groups of all PAC classes. The most intense peaks (base peaks) were chosen as the quantitation ion for the analyte of interest. Two or three other prominent peaks as presented in Table 6.1 were chosen as confirmation ions while in some cases more peaks were monitored as confirmation ions. Other isomer peaks identified without a reference standard were done based on evaluation of their mass fragmentation pattern.

The MS used in this study was operated at a resolving power > 25 000 with a scan range from *m/z* 50-500 which reflects the mass range of analytes. High resolution MS depends on exact masses to identify chemical compound and for PACs, this was done within a narrow mass tolerance window (MTW) of ± 3 ppm. The MTW is the mass range around the expected *m/z*. In this study a small mass window of ± 3 ppm was selected to aid accurate mass measurements of our target analytes. To improve the systems mass calibration and accuracy data, injected extracts were acquired with the PFTBA bled directly into the ion source. Concomitant monitoring of ions from PFTBA allows for on the fly instrument drift correction. Typically, the mass accuracy of the ions monitored for PFTBA was less than 1 ppm and root mean square (RMS) was < 1ppm. The HRMS total ion chromatograms (TIC) of the standard solution containing all 68 PACs (16 native PAHs, 16 deuterated PAHs, 32 alkyl PAHs and 4 PASHs) available in our laboratory was analyzed using both 30 m and 60 m columns with the same stationary phases.

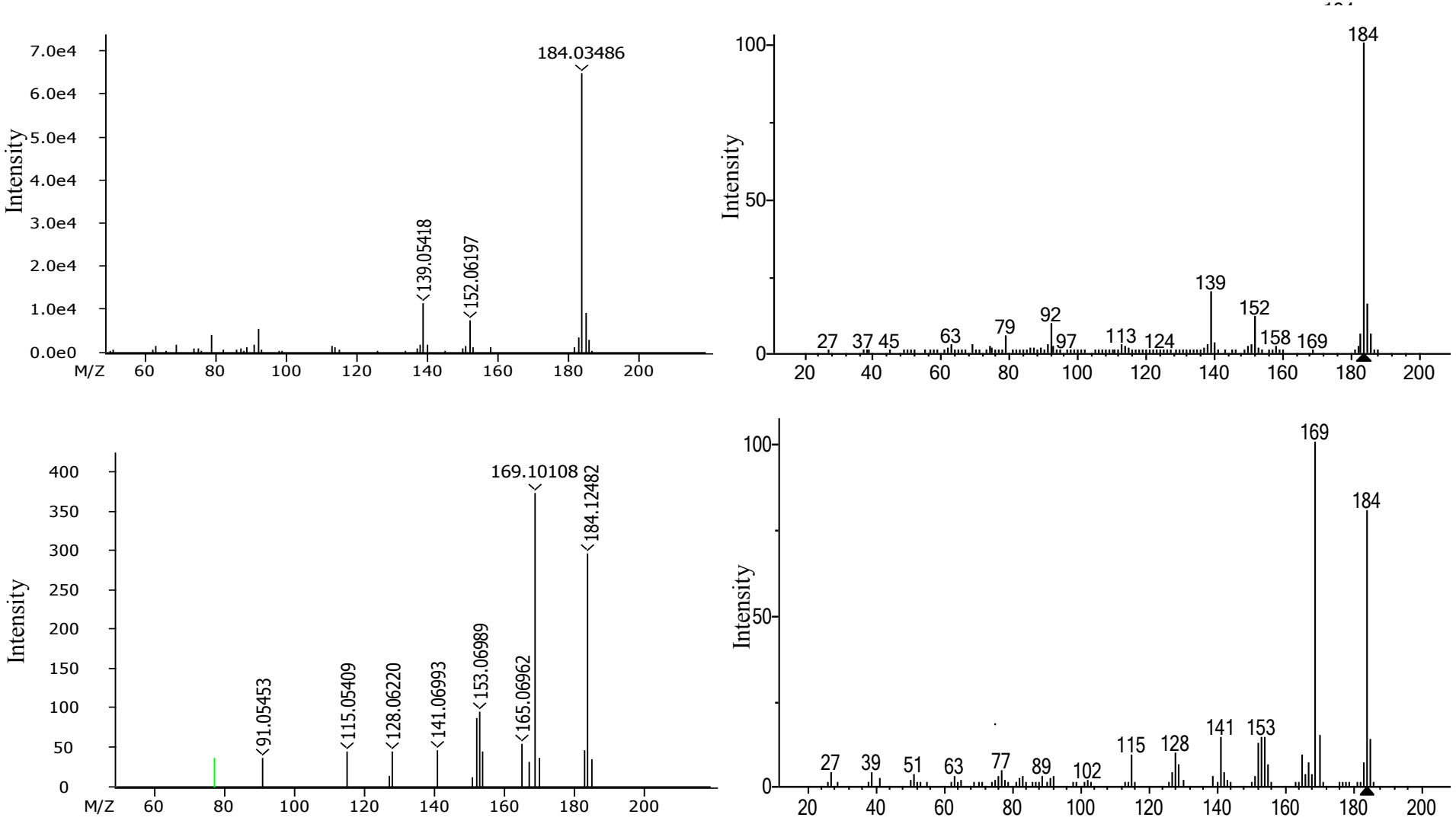


***Figure 6.1a**: Mass spectra of DBT, 1,4,5,6-tetraN, 1,2,3,4-tetraN. Top panel is HRMS of DBT (left); NIST spectra of DBT (right). Bottom panel is HRMS of 1,4,5,6-tetraN (left); NIST spectra of 1,2,3,4-tetraN (right)*

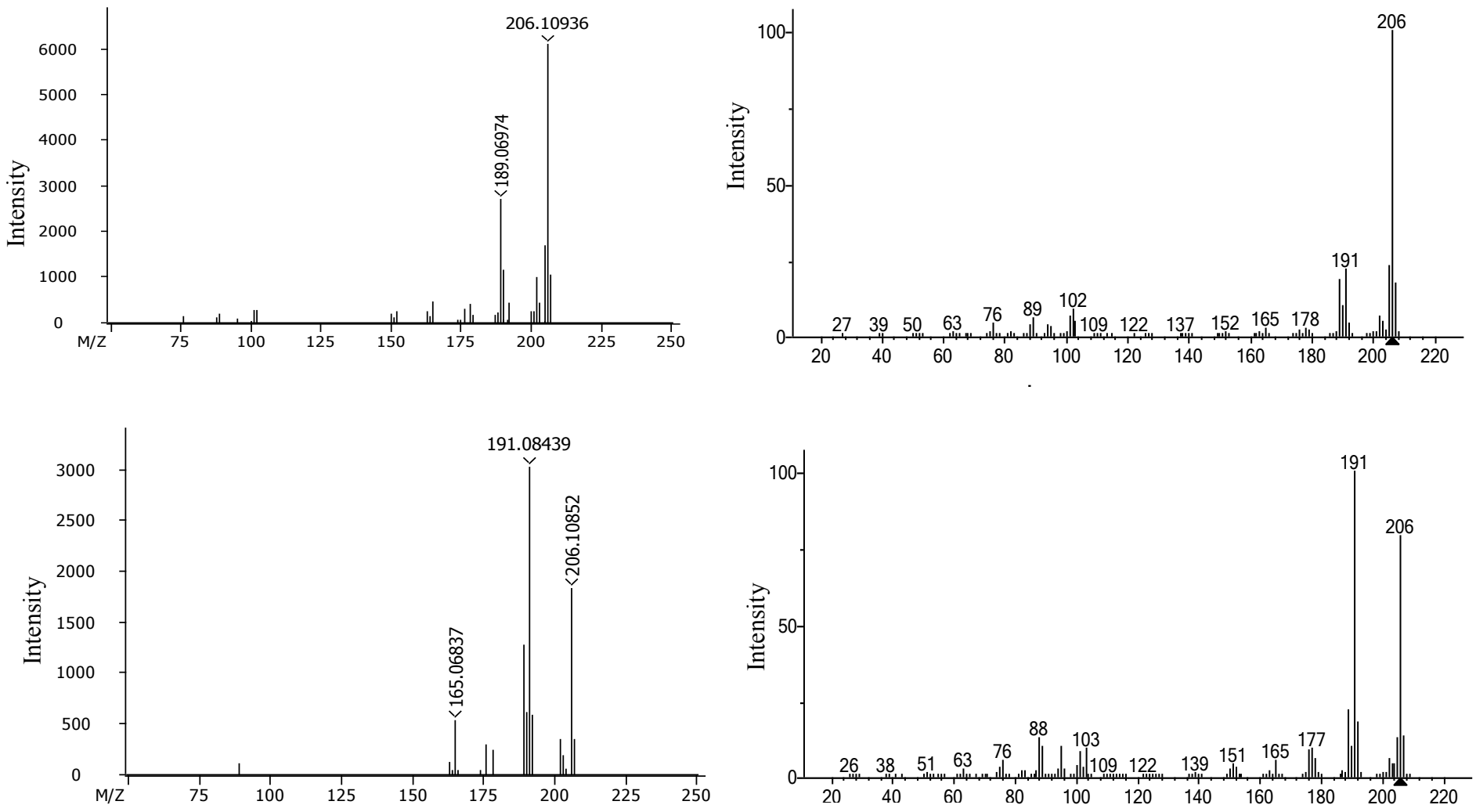


***Figure 6.1b**: Mass spectra of 1,7-DMPh and 2-EPh. Top panel is HRMS of 1,7-DMPh (left); NIST spectra of 1,7-DMPh (right). Bottom panel is HRMS of an EPh (left) from coal extract; NIST spectra of 2-EPh (right)*

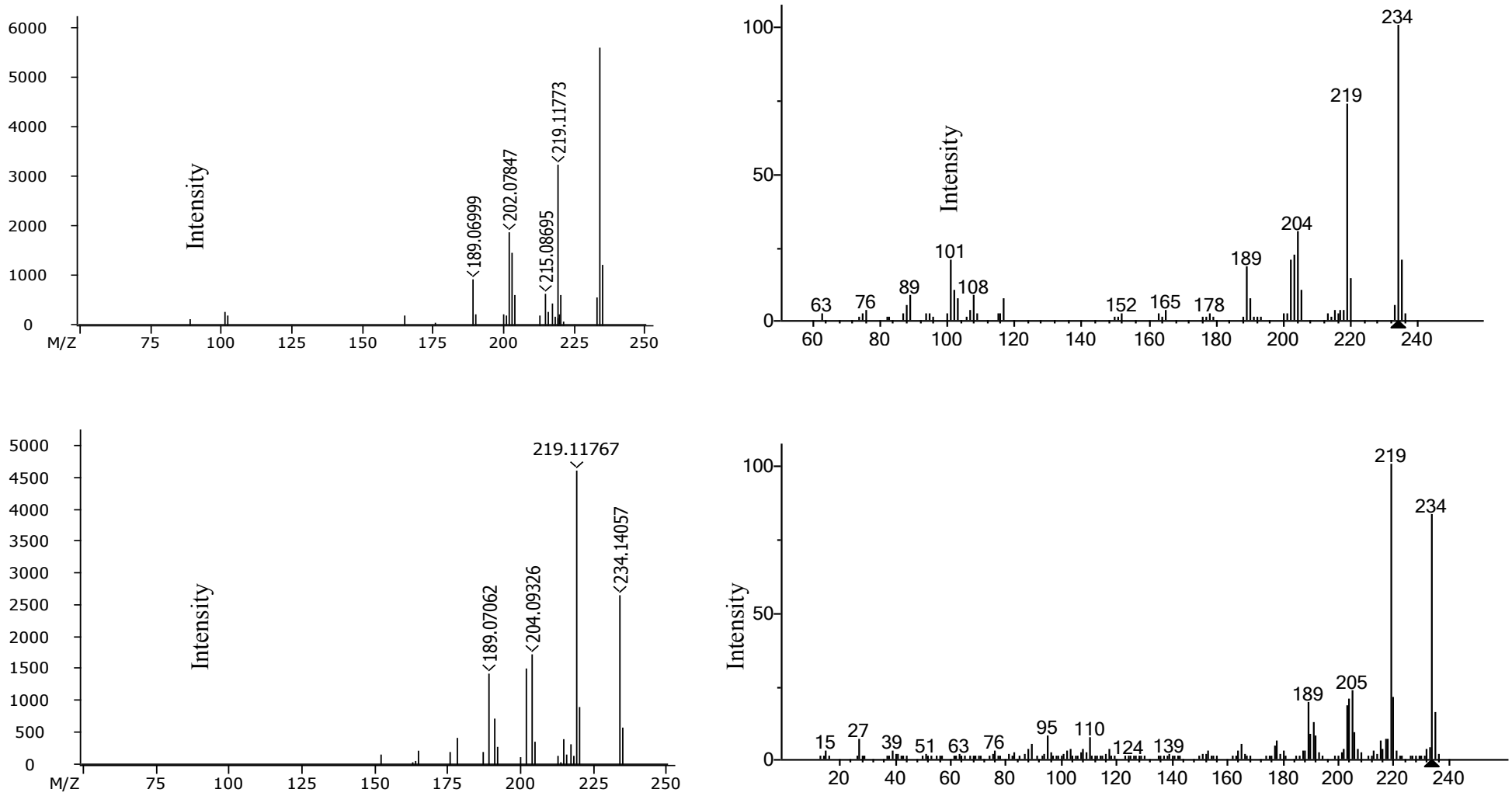


***Figure 6.1c**: Mass spectra of 1,2,6,9-tetraMPh and Retene. Top panel is HRMS of 1,2,6,9-tetraMPh (left); NIST spectra of 1,7-1,2,6,9-tetraMPh (right). Bottom panel is HRMS of an Retene (left) from coal extract; NIST spectra of Retene (right)*

Table 6.1. *Quantitation and confirmation fragment ions and respective m/z values of PAC and deuterated PAH analytes*

Target Analyte	**Target Analyte**	**Quantitation ions**	**Confirmation ions**
NAPHTHALENES			
	Naphthalene-d_8	$[M]^{+\cdot}$ (136.11227)	$[M-D]^{+\cdot}$ (134.09816); $[M-C_2D_2]^{+\cdot}$ (108.08406)
	C_0 Naphthalene	$[M]^{+\cdot}$ (128.06205)	$[M-C_2H_2]^{+\cdot}$ (102.04640); $[M-C_4H_3]^{+\cdot}$ (77.03858)
C_1 Naphthalene	Methyl Naphthalene	$[M]^{+\cdot}$ (142.07770)	$[M-H]^{+\cdot}$ (141.06988); $[M-C_4H_3]^{+\cdot}$ (115.05423)
C_2 Naphthalene	Dimethyl Naphthalene [a]	$[M]^{+\cdot}$ (156.09335)	$[M-CH_3]^{+\cdot}$ (141.06988); $[M-C_3H_5]^{+\cdot}$ (115.05423)
	Ethyl Naphthalene	$[M-CH_3]^{+\cdot}$ (141.06988)	$[M]^{+\cdot}$ (156.09335); $[M-C_3H_5]^{+\cdot}$ (115.05423)
C_3 Naphthalene	Trimethyl Naphthalene	$[M-CH_3]^{+\cdot}$ (155.08553)	$[M]^{+\cdot}$ (170.10900)
	Methyl Ethyl Naphthalene	$[M-CH_3]^{+\cdot}$ (155.08553)	$[M]^{+\cdot}$ (170.10900); $[M-C_2H_5]^{+\cdot}$ (141.06988)
	Propyl Naphthalene	$[M-C_2H_5]^{+\cdot}$ (141.06988)	$[M]^{+\cdot}$ (170.10900); $[M-CH_3]^{+\cdot}$ (155.08553)
C_4 Naphthalene	Tetra methyl Naphthalene [b]	$[M-CH_3]^{+\cdot}$ (169.10118)	$[M]^{+\cdot}$ (184.12465); $[M-C_2H_5]^{+\cdot}$ (155.08553)
	Butyl Naphthalene	$[M-C_3H_7]^{+\cdot}$ (141.06988)	$[M]^{+\cdot}$ (184.12465); $[M-C_2H_5]^{+\cdot}$ (155.08553)
PHENANTHRENES/ ANTHRANCENES			
	Phenanthrene/ Anthracene-d_{10}	$[M]^{+\cdot}$ (188.14047)	$[M-2D]^{+\cdot}$ (184.11227); $[M-C_2D_2]^{+\cdot}$ (160.11227)
	C_0 Phenanthrene/ Anthracene	$[M]^{+\cdot}$ (178.07770)	$[M-2H]^{+\cdot}$ (176.06205); $[M-C_2H_2]^{+\cdot}$ (152.06205)
C_1 Phenanthrene/ Anthracene	Methyl Phenanthrene/ Anthracene	$[M]^{+\cdot}$ (192.09335)	$[M-H]^{+\cdot}$ (191.08553); $[M-3H]^{+\cdot}$ (189.06988)
C_2 Phenanthrene/ Anthracene	Dimethyl Phenanthrene/ Anthracene	$[M]^{+\cdot}$ (206.10900)	$[M-H]^{+\cdot}$ (205.10118); $[M-CH_3]^{+\cdot}$ (191.08553); $[M-CH_5]^{+\cdot}$ (189.06988)
	Ethyl Phenanthrene/ Anthracene	$[M-CH_3]^{+\cdot}$ (191.08553)	$[M]^{+\cdot}$ (206.10900); $[M-CH_5]^{+\cdot}$ (189.06988)
C_3 Phenanthrene/ Anthracene	Trimethyl Phenanthrene/ Anthracene	$[M]^{+\cdot}$ (220.12465)	$[M-CH_3]^{+\cdot}$ (205.10118); $[M-C_2H_7]^{+\cdot}$ (189.06988)
	Methyl Ethyl Phenanthrene/ Anthracene	$[M-CH_3]^{+\cdot}$ (205.10118)	$[M]^{+\cdot}$ (220.12465); $[M-C_2H_7]^{+\cdot}$ (189.06988)
C_4 Phenanthrene/ Anthracene	Tetra methyl Phenanthrene/ Anthracene	$[M]^{+\cdot}$ (234.14030)	$[M-CH_3]^{+\cdot}$ (219.11683); $[M-C_2H_8]^{+\cdot}$ (202.07770); $[M-C_3H_9]^{+\cdot}$ (189.06988)
	Butyl Phenanthrene/ Anthracene	$[M-C_3H_7]^{+\cdot}$ (191.08553)	$[M]^{+\cdot}$ (234.14030); $[M-C_3H_9]^{+\cdot}$ (189.06988)
CHRYSENES			
	Chrysene-d_{12}	$[M]^{+\cdot}$ (240.16867)	$[M-D_2]^{+\cdot}$ (236.14047); $[M-C_9D_6]^{+\cdot}$ (120.08406)

	C_0 Chrysene	$[M]^{+\cdot}$ (228.09335)	$[M-2H]^{+\cdot}$ (226.07770); $[M-C_9H_7]^{+\cdot}$ (113.03858)
C_1 Chrysene	Methyl Chrysene	$[M]^{+\cdot}$ (242.10900)	$[M-3H]^{+\cdot}$ (239.08553); $[M-CH_4]^{+\cdot}$ (226.07770)
C_2 Chrysene	Dimethyl Chrysene	$[M]^{+\cdot}$ (256.12465)	$[M-CH_3]^{+\cdot}$ (241.10118); $[M-C_2H_6]^{+\cdot}$ (226.07770)
	Ethyl Chrysene	$[M-CH_3]^{+\cdot}$ (241.10118)	$[M]^{+\cdot}$ (256.12465); $[M-C_2H_6]^{+\cdot}$ (226.07770)
C_3 Chrysene	Trimethyl Chrysene	$[M]^{+\cdot}$ (270.14030)	$[M-CH_3]^{+\cdot}$ (255.11683); $[M-C_2H_7]^{+\cdot}$ (239.08553)
	Propyl Chrysene	$[M-C_2H_5]^{+\cdot}$ (241.10118)	$[M]^{+\cdot}$ (270.14030); $[M-C_3H_8]^{+\cdot}$ (226.07770)
C_4 Chrysene	Tetra methyl Chrysene	$[M]^{+\cdot}$ (284.15595)	$[M-CH_3]^{+\cdot}$ (269.13248); $[M-C_3H_8]^{+\cdot}$ (240.09335)
	Butyl Chrysene	$[M-C_3H_8]^{+\cdot}$ (240.09335)	$[M]^{+\cdot}$ (284.15595); $[M-CH_3]^{+\cdot}$ (269.13248)
DIBENZOTHIOPHENES			
	C_0 Dibenzothiophene	$[M]^{+\cdot}$ (184.03412)	$[M-S]^{+\cdot}$ (152.06205); $[M-SCH]^{+\cdot}$ (139.05423)
C_1 Dibenzothiophene	Methyl Dibenzothiophene	$[M]^{+\cdot}$ (198.04977)	$[M-H]^{+\cdot}$ (197.04195); $[M-S]^{+\cdot}$ (166.07770); $[M-SCH_2]^{+\cdot}$ (152.06205)
C_2 Dibenzothiophene	Dimethyl Dibenzothiophene	$[M]^{+\cdot}$ (212.06542)	$[M-CH_3]^{+\cdot}$ (197.04195); $[M-SCH_3]^{+\cdot}$ (165.06988)
	Ethyl Dibenzothiophene	$[M-CH_3]^{+\cdot}$ (197.04195)	$[M]^{+\cdot}$ (212.06542); $[M-H]^{+\cdot}$ (211.05760); $[M-SCH_3]^{+\cdot}$ (165.06988)
C_3 Dibenzothiophene	Trimethyl Dibenzothiophene	$[M]^{+\cdot}$ (226.08107)	$[M-CH_3]^{+\cdot}$ (211.05760); $[M-H]^{+\cdot}$ (225.07325)
	Methyl Ethyl Dibenzothiophene	$[M-CH_3]^{+\cdot}$ (211.05760)	$[M]^{+\cdot}$ (226.08107); $[M-H]^{+\cdot}$ (225.07325)
C_4 Dibenzothiophene	C_4 Dibenzothiophene	$[M]^{+\cdot}$ (240.09672)	$[M-CH_3]^{+\cdot}$ (225.07325); $[M-C_2H_5]^{+\cdot}$ (211.05760)
FLUORENES			
	Fluorene-d_{10}	$[M]^{+\cdot}$ (176.14047)	$[M-2D]^{+\cdot}$ (172.11227); $[M-D]^{+\cdot}$ (174.12637)
	C_0 Fluorene	$[M-1]^{+\cdot}$ (165.06988)	$[M]^{+\cdot}$ (166.07770); $[M-2H]^{+\cdot}$ (164.06205)
C_1 Fluorene	Methyl Fluorene [c]	$[M-CH_3]^{+\cdot}$ (165.06988)	$[M]^{+\cdot}$ (180.09335); $[M-C_2H_4]^{+\cdot}$ (152.06205)
C_2 Fluorene	Dimethyl Fluorene	$[M-CH_3]^{+\cdot}$ (179.08533)	$[M-C_2H_5]^{+\cdot}$ (165.06988); $[M]^{+\cdot}$ (194.10900)
	Ethyl Fluorene [d]	$[M-CH_3]^{+\cdot}$ (179.08533)	$[M-C_2H_5]^{+\cdot}$ (165.06988); $[M]^{+\cdot}$ (194.10900)
C_3 Fluorene	C_3 Fluorene	$[M]^{+\cdot}$ (208.12465)	$[M-CH_3]^{+\cdot}$ (193.10118); $[M-C_2H_6]^{+\cdot}$ (178.07770)
C_4 Fluorene	C_4 Fluorene	$[M-CH_3]^{+\cdot}$ (207.11683)	$[M]^{+\cdot}$ (222.14030); $[M-C_2H_7]^{+\cdot}$ (191.08553)
BENZO(A)PYRENES			
	Benzo(*a*)pyrene-d_{12}	$[M]^{+\cdot}$ (264.16867)	$[M-2D]^{+\cdot}$ (260.14047); $[M-C_{10}D_6]^{+\cdot}$ (132.08406)

	C_0 Benzopyrene	$[M]^{+\cdot}$ (252.09335)	$[M-2H]^{+\cdot}$ (250.07770); $[M-C_{10}H_6]^{+\cdot}$ (126.04640)
C_1 Benzopyrene	Methyl Benzopyrene	$[M]^{+\cdot}$ (266.10900)	$[M-H]^{+\cdot}$ (265.10118); $[M-3H]^{+\cdot}$ (263.08553)
C_2 Benzopyrene	Dimethyl Benzopyrene	$[M]^{+\cdot}$ (280.12465)	$[M - CH_3]^{+\cdot}$ (265.10118)
C_3 Benzopyrene	Trimethyl Fluorene	$[M]^{+\cdot}$ (294.14030)	$[M - CH_3]^{+\cdot}$ (279.11683)
C_4 Benzopyrene	Tetra methyl Fluorene	$[M]^{+\cdot}$ (308.15595)	$[M - CH_3]^{+\cdot}$ (293.13248)
PYRENES/ FLUORANTHENES			
	Pyrene/ Fluoranthene -d_{10}	$[M]^{+\cdot}$ (212.14047)	$[M- D]^{+\cdot}$ (210.12637); $[M - 2D]^{+\cdot}$ (208.11227)
	Pyrene/ Fluoranthene	$[M]^{+\cdot}$ (202.07770)	$[M- H]^{+\cdot}$ (201.06988); $[M - 2H]^{+\cdot}$ (200.06205)
C_1 Pyrene	Methyl Pyrene	$[M]^{+\cdot}$ (216.09335)	$[M- H]^{+\cdot}$ (215.08553); $[M - C_2H_3]^{+\cdot}$ (189.06988)
	Methyl Fluoranthene	$[M- H]^{+\cdot}$ (215.08553)	$[M]^{+\cdot}$ (216.09335); $[M - C_2H_3]^{+\cdot}$ (189.06988)
C_2 Pyrene/ Fluoranthene	C_2 Pyrene/ Fluoranthene [e]	$[M]^{+\cdot}$ (230.10900); $[M-CH_3]^{+\cdot}$ (215.08553)	$[M - C_2H_4]^{+\cdot}$ (202.07770)
C_3 Pyrene/ Fluoranthene	C_3 Pyrene/ Fluoranthene	$[M]^{+\cdot}$ (244.12465)	$[M- CH_3]^{+\cdot}$ (229.10118)
C_4 Pyrene/ Fluoranthene	C_4 Pyrene/ Fluoranthene	$[M]^{+\cdot}$ (258.14030)	$[M- CH_3]^{+\cdot}$ (243.11683)
	Acenaphthylene	$[M]^{+\cdot}$ (152.06205)	$[M-2H]^{+\cdot}$ (150.04640); $[M-H]^{+\cdot}$ (151.05423)
	Acenaphthylene-d_8	$[M]^{+\cdot}$ (160.11227)	$[M-D]^{+\cdot}$ (158.09816); $[M-2D]^{+\cdot}$ (156.08406)
	Acenaphthene	$[M-H]^{+\cdot}$ (153.06988)	$[M]^{+\cdot}$ (154.07770); $[M-2H]^{+\cdot}$ (152.06205); $[M-3H]^{+\cdot}$ (151.05423)
	Acenaphthene-d_{10}	$[M-D]^{+\cdot}$ (162.12637)	$[M]^{+\cdot}$ (164.14047); $[M-2D]^{+\cdot}$ (160.11227); $[M-3D]^{+\cdot}$ (158.09816); $[M-C_6D_6]^{+\cdot}$ (80.05586)
	Benzo(*b)/ (k)* fluoranthene	$[M]^{+\cdot}$ (252.09335)	$[M-2H]^{+\cdot}$ (250.07770); $[M-C_{10}H_6]^{+\cdot}$ (126.04640)
	Benzo(*b*) *)/ (k)* fluoranthene -d_{12}	$[M]^{+\cdot}$ (264.16867)	$[M- 2D]^{+\cdot}$ (260.14047); $[M- C_{10}D_6]^{+\cdot}$ (132.08406)
	Indeno(1,2,3-c,d)pyrene	$[M]^{+\cdot}$ (276.09335)	$[M-2H]^{+\cdot}$ (274.07770)
	Indeno(1,2,3-c,d)pyrene-d_{12}	$[M]^{+\cdot}$ (288.16867)	$[M-D]^{+\cdot}$ (286.15457)
	Dibenz(*a,h*)anthracene	$[M]^{+\cdot}$ (278.10900)	$[M-2H_2]^{+\cdot}$ (274.07770)
	Dibenz(*a,h*)anthracene-d_{14}	$[M]^{+\cdot}$ (292.19688)	$[M-3D]^{+\cdot}$ (286.15457)
	Benzo(*ghi*)perylene	$[M]^{+\cdot}$ (276.09335)	$[M-2H]^{+\cdot}$ (274.07770)

	Benzo(*ghi*)perylene-d_{12}	$[M]^{+\cdot}$ (288.16867)	$[M-D]^{+\cdot}$ (286.15457)
	Benz (a) anthracene	$[M]^{+\cdot}$ (228.09335)	$[M-2H]^{+\cdot}$ (226.07770); $[M-C_9H_7]^{+\cdot}$ (113.03858)
	Benz (a) anthracene-d_{12}	$[M]^{+\cdot}$ (240.16867)	$[M-2D]^{+\cdot}$ (236.14047)**; [**$M-C_9D_6]^{+\cdot}$ (120.08406)

Except [a] 2, 3-dimethyl naphthalene; [b] 1, 4, 5, 8-tetramethylnaphthalene; [c] 9-methyl fluorene; [d] 9-ethyl fluorene; [e] 4, 5-dimethylphenanthrene

Not surprisingly there was better separation of the isomers on the 60 m column compared to the 30 m column.[31] As an example, the appearance of the chromatogram of C_1-phenanthrene/anthracene (C_1Ph/An) analytical standard containing 5 compounds from both columns is shown in Fig. 6.2. Due to the limited number of commercially available standards compared to the number of possible isomers, three environmental samples, used Ultra Grade 19 lubricant, SRM 2974a and a coal sample, were used to further establish elution profiles for the different APACs. The sample extracts illustrated the improved peak capacity and resolution observed in the 2D mode relative to 1D. Chromatographic resolution of closely eluting APACs was evaluated using Giddings [32] proposed formula for 2D resolution. While the chromatography in 1D mode was markedly better on the 60 m column there was equally good separation in the 2D mode on both columns. As the complexity of the sample increases it can be expected that longer columns would be necessary. Here, the chromatographic improvement using the 60m column was pronounced in 2D mode and hence adopted and ideal for the analysis.

$$R = \frac{t_{R(x+1)} - t_{R(x)}}{\frac{1}{2}(W_{(x+1)} + W_{(x)})} \quad \text{..... (6.2)}$$

$$R2D = \sqrt{(R_1^2 + R_2^2)} \quad \text{..... (6.3)}$$

Where R is the resolution in each dimension (either 1 or 2), t_R is the retention time for adjacent compounds x and x + 1, w is the peak width equivalent to four or five modulation periods in the first dimension or the full width at half height of the highest modulated peak for the second dimension for the same pair of compounds.[21] Comparison of the resolution in 1D compared to 2D is presented in Table 6.2 for the isomers of C_1Ph, C_2N, C_3Fl and C_2DBT.

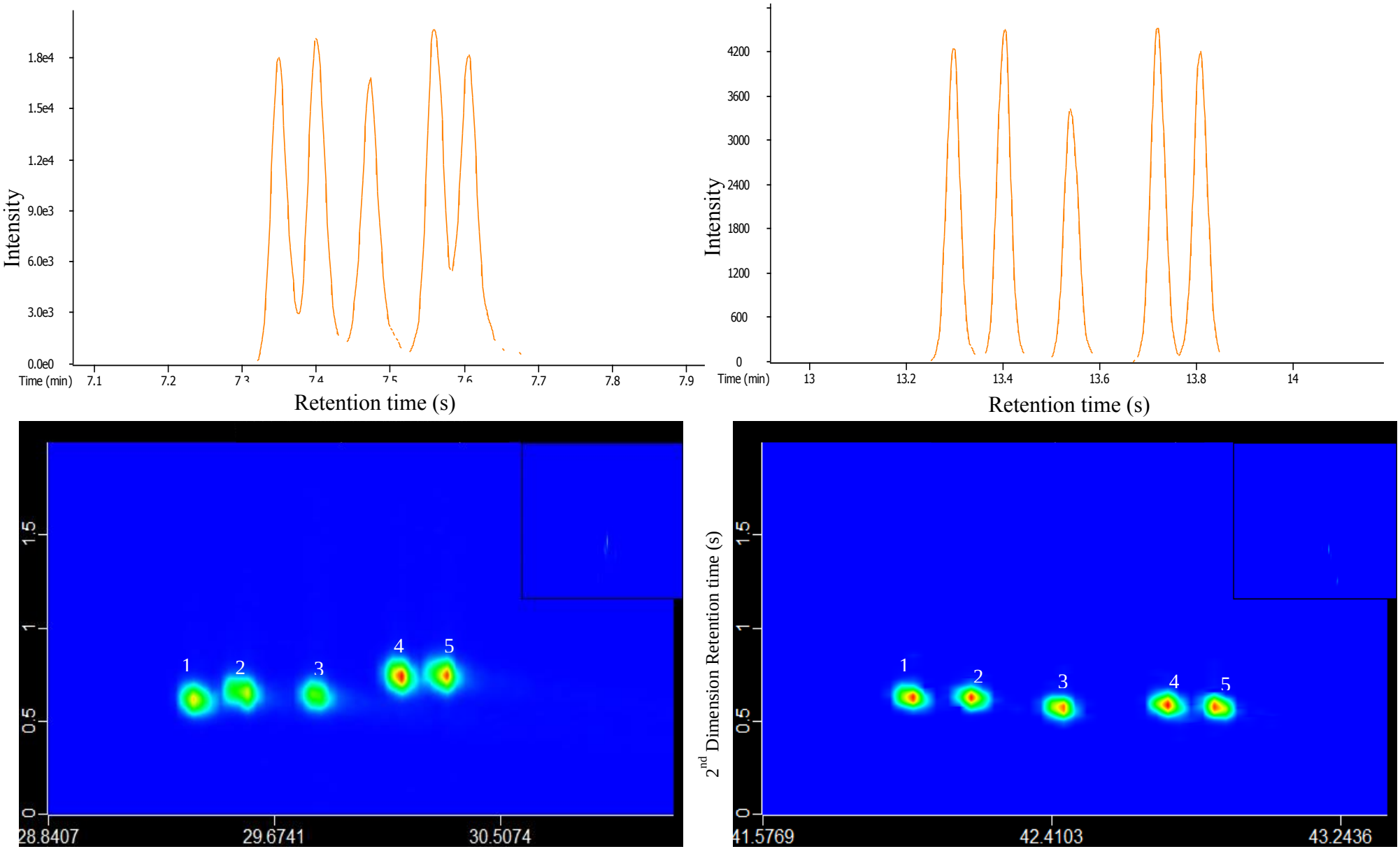


***Figure 6.2**: Elution profiles of C_1Ph/An analytical standard. Top panel is the 1D HRMS TIC on (left) 30m and (right) 60m column. Bottom panel is the 2D HRMS TIC contour plot on (left) 30m and (right) 60 m column (**1**-3MPh; **2**-2MPh; **3**-2MPh; **4**-9MPh; **5**-1MPh)*

With the improved resolution on the longer column, results of the 2D characterization of the three samples are presented in this study using the 60 m column length. Because the focus of our study was to optimize separations in 2D a modified much slower GC-temperature program was adopted for our 2D work. The improved separations using the slower GC-temperature ramp is clearly evident in Figure 6.3a and 6.3b. The effect of modulation time on chromatographic separation and method performance characteristics is addressed later.

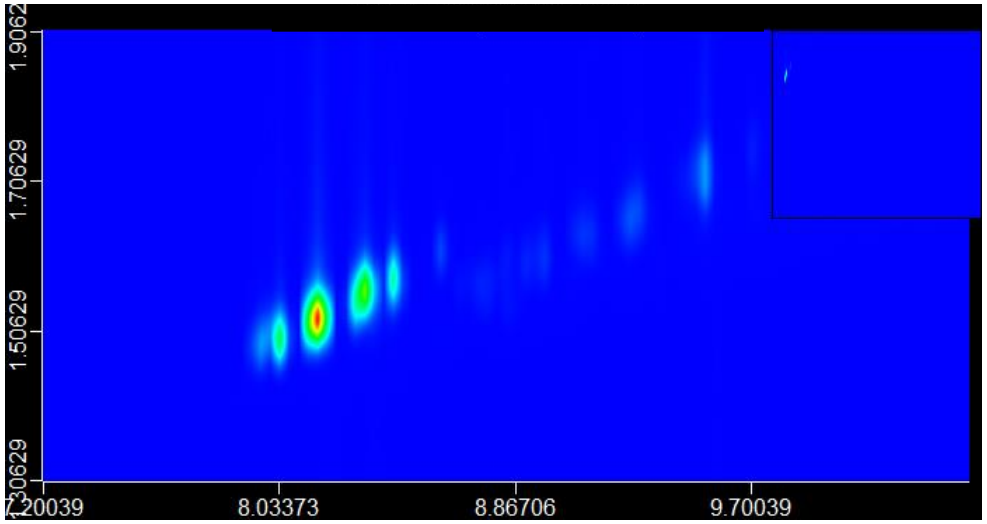
Table 6.2. *Chromatographic resolution (^1R is first dimension resolution; ^2R is second dimension resolution)*

Compound name	1D mode	2D mode		
		^1R	^2R	R_{2D}
9&1- methyl phenanthrene	0.97	1.43	0.32	1.46
1,6-dimethylnapthalene & a coeluting ethylnapthalene	0.60	1.25	0.11	1.26
1,7-dimethylfluorene & coeluting dimethylfluorene	0.82	1	0.04	1

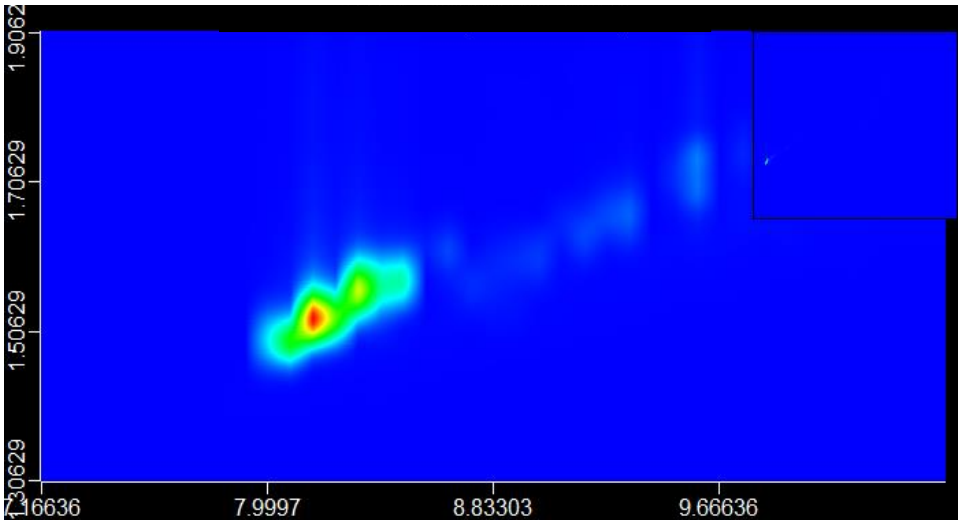
The next sections emphasize the capability of the GC×GC system to detect and resolve the suite of APACs in the three samples.

6.6.1.1 Naphthalene group

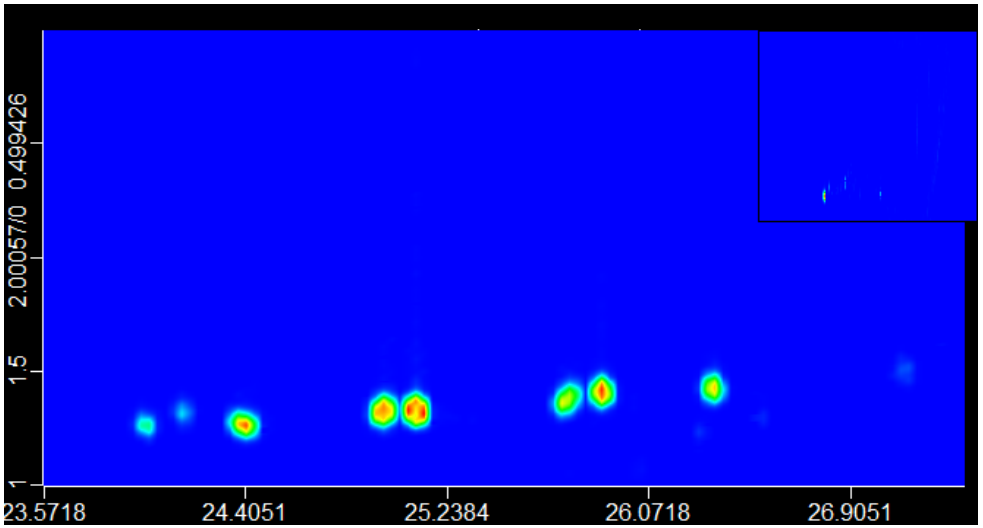
Naphthalene and its alkylated homologs are one of the first group of compounds to elute in PAC class. Diagnostic analysis of these compounds is used for many purposes:



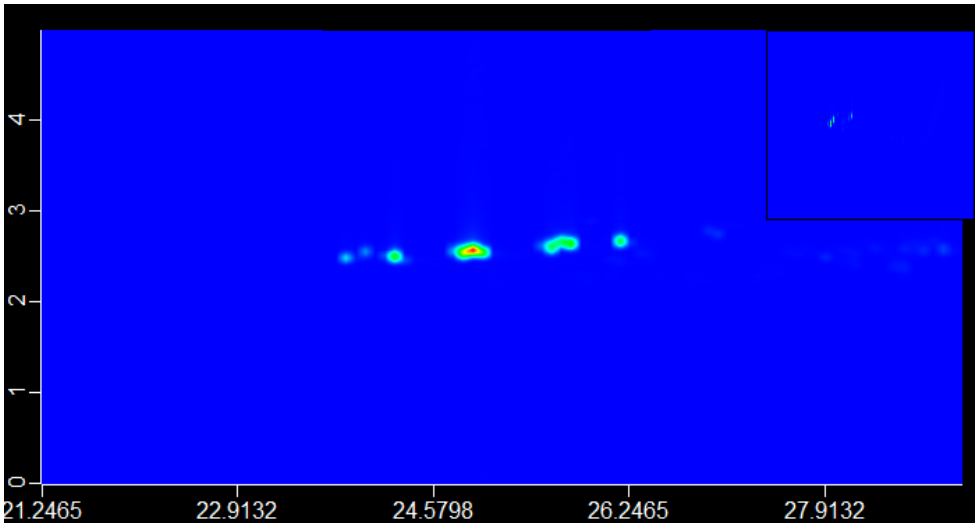
Fast 1D ramp using 2s mod. time



Fast 1D ramp using 5s mod. time



Slow 2D ramp using 2s mod. time



Slow 2D ramp using 5s mod. time

1st Dimension Retention time (min)

Figure 6.3a: *C_2N separation in the two temperature ramps and different modulation periods (2s and 5s).*

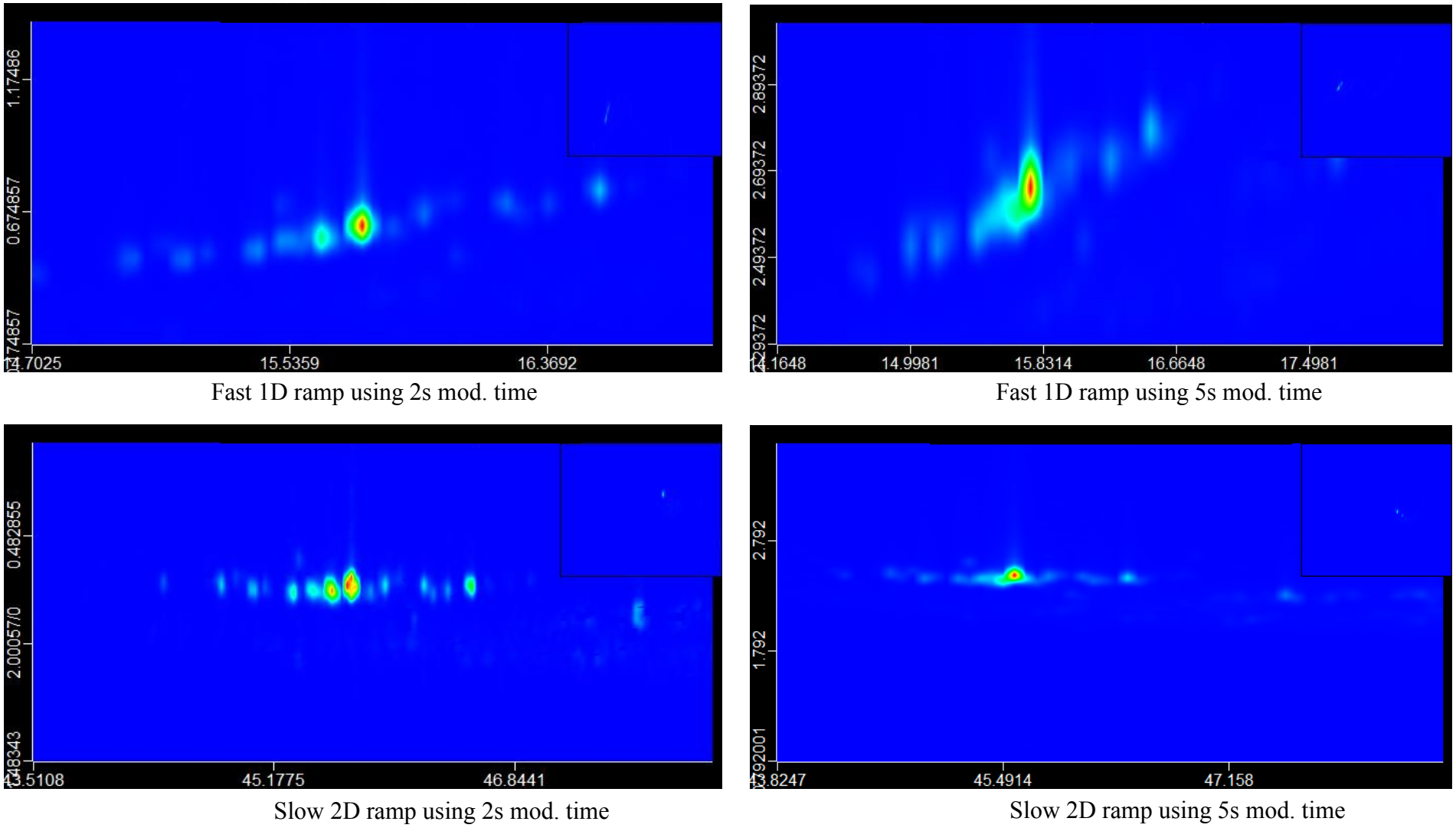


Figure 6.3b: *C_2Ph/An separation in the two temperature ramps and different modulation periods (2s and 5s).*

methylated naphthalenes are abundant constituents of sedimentary organic matter and their distribution is largely controlled by the effects of source thermal maturity or stress and biodegradation[33]. The retention times of the alkyl naphthalene standards determined by monitoring *m/z* 142.0777 (C_1N) and 156.0933 (C_2N) and other prominent fragment ions, were used to help establish the elution window profile of these isomers in the 3 environmental samples. For C_1N, there are only 2 isomers possible[7]; Fig. 6.4 shows these 2 isomers in the three environmental samples.

For C_2N there are 12 theoretical isomers possible.[7] There are 8 clearly resolved peaks in the chromatograms for the SRM 2974 and coal sample extracts while only 7 peaks were detected in the used lubricating oil sample extract (Fig. 6.5). Based on our knowledge of the EI mass spectral fragmentation patterns, we were able to positively identify 5-ethyl- and 3-dimethyl- substituted naphthalenes in the coal while 6-ethyl- and 2-dimethyl- substituted naphthalenes were identified in the SRM 2974a sample. Without authentic analytical standards assigning position of substitution on the aromatic backbone is not possible.

Relative to C_1- and C_2N, there are clearly more peaks in the chromatograms for C_3N and C_4N in the 3 environmental samples (see App Fig. 1 & for C_3N and C_4N. The number of peaks in the SRM 2974a sample is much smaller than the other 2 samples for both C_3 and C_4N with coal extract being the most complex of the 3 matrices. The number of theoretical number of isomers for the C_3N is 32.[7] In the environmental samples, 16, 12 and 14 peaks were observed in the C_3N HRMS contour plot for coal, SRM2974a and used lubricating oil samples, respectively. The peaks corresponded to *m/z* 155.0855 indicating isomers that are trimethyl or methyl ethyl-substituted and we were only able to positively identify the 1, 6, 7-trimethylnaphthalene isomer which was

detected in all the sample extracts. Based on our knowledge of the fragmentation behavior of the isomers, no propyl-substituted isomers of naphthalene were detected in any of the samples.

The peaks in the HRMS TIC of C_4N for the 3 samples all contained the $[M\text{-}CH_3]^+$ ion as their base peak suggesting that only the tetra methyl-substituted isomers of naphthalene were detectable in the samples. There are 110 possible isomers for C_4N[7] and in 2D mode we observe 28 and 17 peaks in the coal and used lubricating oil samples, respectively. 1,4,6,7- and 1,2,6,9-tetramethylnaphthalene were positively identified in all 3 matrices. Two unknown isomers of the C_4N were identified in coal sample having $[M]^{+\cdot}$ as the base peak (see App Fig. 2).

6.6.1.2 Phenanthrene/ Anthracene group

These isomers are found predominantly in crude oil or oil-contaminated samples and are known for their toxicity especially to developing fish[34]. Brinkworth, et al. [35,] Incardona, et al. [36] have suggested that they can influence the appearance of oxidative stress and cardiovascular defects.

The number of theoretical isomers possible for C_1Ph and C_1An are 5 and 3, respectively.[7] All the peaks observed in the 3 samples could be positively identified as their RT (retention time) agreed with those of the authentic standards (see Fig. 6.6). The HRMS contour plots for the SRM2974a and used lubricating oil samples were similar in that 4 peaks are observable all assigned as methyl substituted phenanthrenes. Only the coal sample showed the presence of a methyl substituted anthracene. The increase in the complexity of the HRMS contour plot for the C_2Ph/An is in agreement with the increase in number of possible isomers.

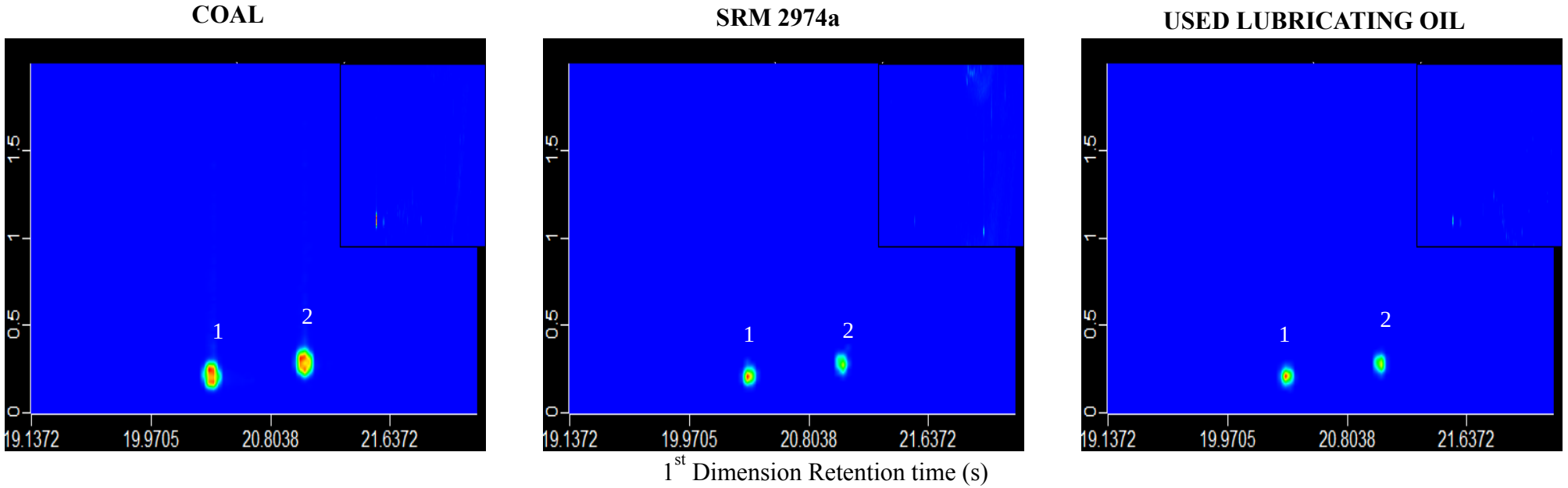


***Figure 6.4**: 2D HRMS contour plots of C_1N in the 3 environmental samples (**1**- 2MN; **2**- 1MN)*

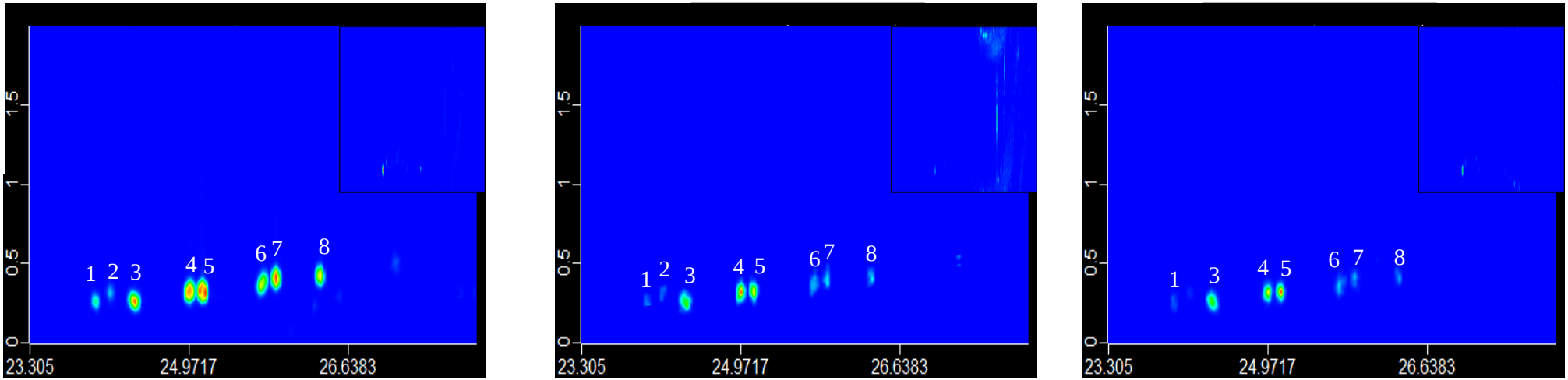


***Figure 6.5**: 2D HRMS contour plots of C_2N in the 3 environmental samples (**1**- eN; **2**- eN; **3**-2,6 -dMN; **4**- eN; **5**-1,6- dMN, **6**- eN, **7**- eN) on (left) coal (middle) SRM 2974a and (right) used lubricating oil.*

The number of isomers possible for a C_2Ph and C_2An are 30 and 18, respectively[7], and the pattern-of detection for the C_2Ph and C_2An were similar for the SRM 2974a and the used lubricating oil sample. Five dimethyl- isomers were confirmed in all SRM 2974a extracts based on the in-house available standards while four were detected in other two extracts. Based on the appearance of the full-scan mass spectra, 3 additional peaks were positively identified in the used lubricating oil as having an ethyl substituent on the phenanthrene or anthracene backbone (i.e., dominant $[M\text{-}CH_3]^+$ ion) and 5 peaks that were identified in the SRM2974a as being substituted with dimethyl groups (dominant $[M]^{+\cdot}$) (See Fig. 6.7). Also, a similar pattern was observed in the second peaks of both coal and used lubricating oil where the spectra showed an ethyl substituted analyte differing from the 3,6-dimethylphenanthrene confirmed in SRM 2974a.

For the C_3Ph/An compounds, the 2D chromatogram showed a group of well-resolved methyl-ethyl-substituted isomers early in the chromatogram and a large number of trimethyl-substituted isomers later in the chromatogram (see App Fig, 3). Only the 1,2,6-trimethyl isomer of phenanthrene could be positively identified. Assuming that a propyl-substituted phenanthrene/anthracene fragments similar to a propyl-naphthalene, then a loss of a C_2H_5 fragment would be the dominant ion in the full scan mass spectra in propyl-phenanthrene/anthracenes. However, this ion was not observed in any of the peaks on the plot suggesting that propyl-phenanthrene/anthracenes were undetectable.

For the coal sample in particular, the 2D HRMS contour plot shows that the C_4Ph/An compounds are dominated by retene (see App Fig. 4) with positive identification of 1, 2, 6, 9-tetramethylphenanthrene in coal and SRM 2974a extracts. There are a group of unknown isomers identified in the coal which all showed a dominant $[M\text{-}CH_3]^+$ ion. However, because there is not

enough mass spectral fragmentation pattern information for the C_4Ph/An compounds (see Table 6.1), it is not possible to assign plausible substitution patterns.

6.6.1.3 Dibenzothiophene (DBT) group

Polycyclic aromatic sulfur heterocyclics (PASHs) consist largely of the thiophene class of compounds. The thiophene groups are the most significant organosulfur compounds in crude oil and sediment extracts[37]. Organic and exploration geochemist have used the presence and distribution of alkylated DBT as molecular markers to establish source rock thermal maturity and oil migration pathways due to the thermodynamic stability of the alkylated substituents on the DBT skeleton [37-38]. Their thermal stability also explains their environmental persistence, a known environmental attribute of PACs.

Fig.6.8 shows the peaks obtained from C_1DBT in the 3 environmental samples by monitoring *m/z* 198.0497 and other fragment ions listed in Table 6.1. The number of isomers possible for C_1DBT is 4^7, however, there are more than 5 peaks in the contour plot of the coal sample extract. Careful examination of the mass spectra of the peaks in 2D mode suggests that only 4 of these peaks have the correct $[M]^{+\cdot}$ ion and ion intensity ratios for their fragment ions. It is possible that the two unidentified peaks are isomers of methylnaptho[b]thiophenes, a compound with the same exact mass and similar fragmentation pattern as C_1DBT. The chromatogram for the SRM2974a and the used lubricating oil samples were much simpler than the coal sample and showed 3 distinct peaks with one peak showing possible co-elution of two or more compounds. In total, 3 methyl DBTs were confirmed in the 3 environmental samples.

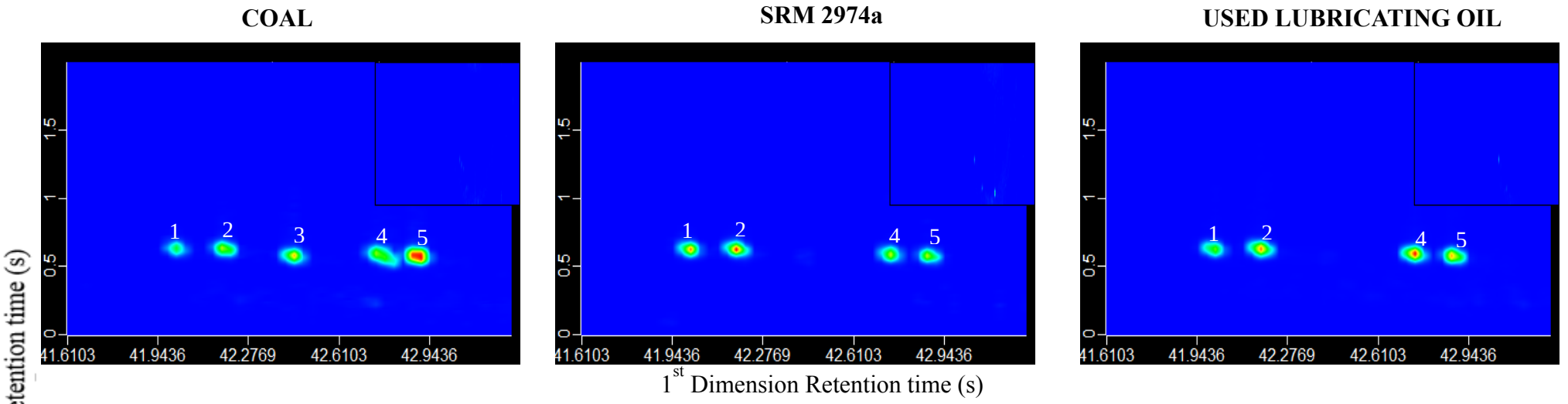


***Figure 6.6**: 2D HRMS contour plots of C_1Ph/An in the 3 environmental samples (**1**- 3-MPh; **2**- 2-MPh; **3**- 2-MAn; **4**- 9-MPh; **5**-1-MPh)*

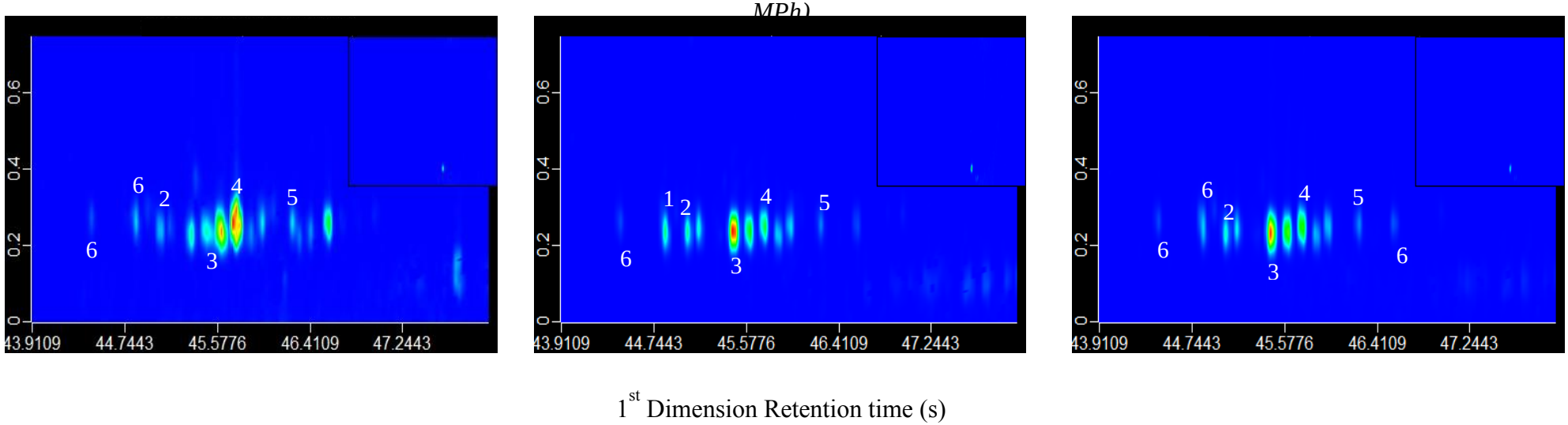


***Figure 6.7**: 2D HRMS contour plots of C_2Ph/An in the 3 environmental samples (**1**- 3,6-DMPh; **2**- 2,6-DMPh; **3**- 1,3-DMPh; **4**- 1,7-DMPh; **5** -1, 8 –DMPh; **6**- ePh; other peaks shown are DMPh) on (left) coal (middle) SRM 2974a and (right) used lubricating oil.*

The rank order of the relative total ion intensities of C_2DBT in the samples is SRM2974a < used lubricating oil < coal (see App Fig. 5). The coal sample contains numerous peaks but only 20 isomers are possible suggesting that interferences are present in the HRMS XIC (extracted ion chromatogram). Based on known mass spectral fragmentation patterns, an ethyl-substituted DBT was positively identified in all 3 samples. 2,8-dimethyl-DBT was also confirmed in the 3 samples; with the exception of the last 2 eluting peaks observed in the 2D chromatogram in all 3 environmental samples, the remaining peaks could be identified as dimethyl-substituted DBTs. The use of 2D analysis for detection and quantification of C_2 DBT is of utmost importance in oil fingerprinting. Diagnostic ratios[39] were been utilized for fingerprinting in conjunction with other alky PACs where an overestimation or underestimation of values of these compounds, typical of 1D analysis, can give rise to false correlations.

All 3 environmental samples contained 2,4,7-trimethyl-DBT (see App Fig. 6) with the coal extract showing the more complex profile in 2D mode relative to the other samples. Because the mass spectral behavior is different for the trimethyl- and methyl ethyl-substituted isomers (see Table 6.1) the additional peaks in the 2D chromatogram were identified to be substituted with methyl groups. The total ion intensities of the isomers in the C_4DBT group were too small for any meaningful diagnostic analysis.

6.6.1.4 Chrysene group

The toxicity of chrysene is not as pronounced as that of its alkylated or its hetero-substituted analogs. It is isobaric with benz[a]anthracene. Methyl substitution such as 5-methyl chrysene has also been well documented to show more toxicity than chrysene.[40-41]

The overall ion intensities of the C_1Ch in the coal and SRM 2974a samples were low and undetectable in used lubricating oil. There are 6 possible isomers for C_1Ch[7] all of which were detected in the coal sample. Of these, 4 C_1Ch could be positively identified. Only the 2-, 3- and 6-methyl-substituted isomers were detected in the SRM 2974a sample (see Fig 6.9).

The total ion intensities of the isomers in the C_2- and C_4Ch group were too small for any meaningful diagnostic analysis. Interestingly, there was a single peak in the C_3Ch HRMS XIC for the coal sample which was identified as a trimethyl substituted isomer (see App Fig 7).

6.6.1.5 Fluorene group

The appearance of the HRMS XIC of the C_1Fl for the 3 environmental samples was similar albeit each of varying total ion intensities (Fig. 6.10). Four of the 5 C_1Fl isomers[7] were detected in all 3 samples with the 1-methyl substituted isomer being the only one that could be identified. For the C_2Fl group, numerous peaks were detected in all 3 samples (see App Fig. 8). Because the fragmentation behavior of the dimethyl and ethyl fluorene isomers are identical (see Table 6.1) it is impossible to discern the peaks in the 2D chromatogram. The total ion intensities of the isomers in the C_3- and C_4Fl groups were too small for any meaningful diagnostic analysis.

6.6.1.6 Pyrene / Fluoranthene group

Similar to the phenanthrene/anthracene group, pyrene and fluoranthene are isobaric and possess the same exact masses. For the C_1Py/Flu group, 4 authentic standards (1-methyl fluoranthene, 3-methyl fluoranthene, 1-methyl pyrene and 4-methyl pyrene) were used to establish the elution profiles of C_1-group and to understand their mass spectral behavior. Despite our best efforts, the

1- and 3-subsituted isomers of fluoranthene were not resolvable in either 1D or 2D modes. Based on the full scan mass spectra, the $[M]^{+\cdot}$ and $[M-H]^{+}$ are the dominant ions for methyl- substituted pyrene and fluoranthene, respectively. Eight isomers in total are possible for C_1Py/Flu and the 2D plot of the 3 environmental samples show as many as 6 methyl-substituted isomers with the 4 authentic standards consistently detected (see Fig. 6.11).

Mass fragmentation patterns for pyrenes and fluoranthenes were observed to be similar although with varying intensities as shown in Fig. 6.12. In light of this, it is difficult to positively identify any of the peaks observed in 2D mode for the alkylated isomers greater than C_1 in this group except with the availability of suites of standards for both compound group. Chromatograms of the 3 environmental samples showing the HRMS contour plots for C_2 and C_3 are shown in Fig. 6.13 & 6.14.

6.6.1.7 Benzo[a]pyrene group

The C_1 and C_2BaP group was detectable only in the coal sample. The appearance of the HRMS XIC shows a number of well resolved peaks. Of the 12 theoretically possible isomers[7], 6 isomers were observed to be of high intensity in the 2D HRMS XIC coal extract with only the 7-methyl BaP positively identified. (see Figure 6.15). One particularly intense peak was observed in the 2D HRMS plot in the C_2BaP group but we were unable to identify its substitution pattern.

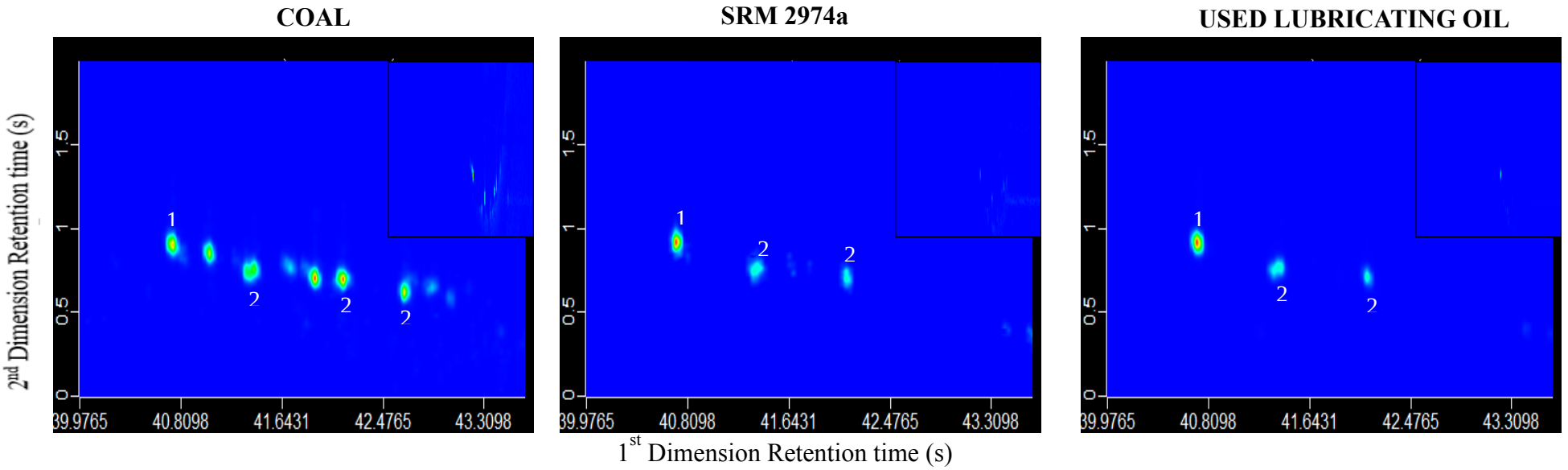


***Figure 6.8**: 2D HRMS contour plots of C_1DBT in the 3 environmental samples (**1**- 4-MDBT; **2**- MDBT)*

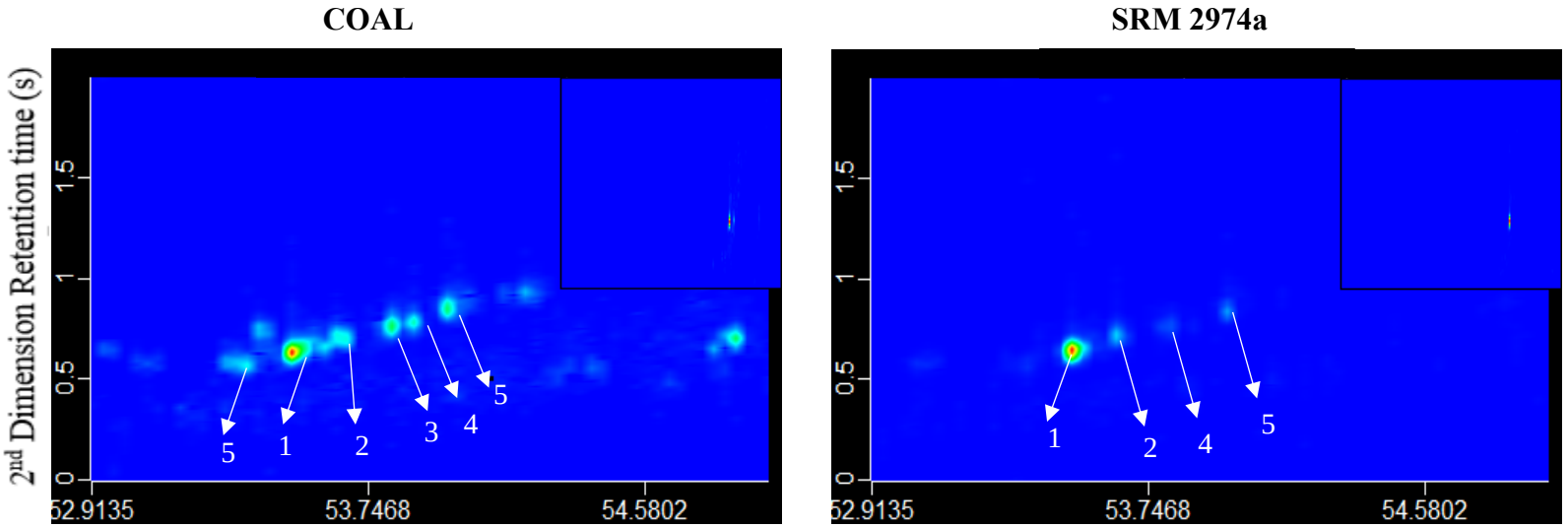


***Figure 6.9**: 2D HRMS contour plots of C_1Ch in 2 environmental samples (**1**- 3- MCh; **2**- 2-MCh; **3**- 5-MCh; **4**- 6-MCh; **5** -MCh) on (left) coal (middle) SRM 2974a and (right) used lubricating oil.*

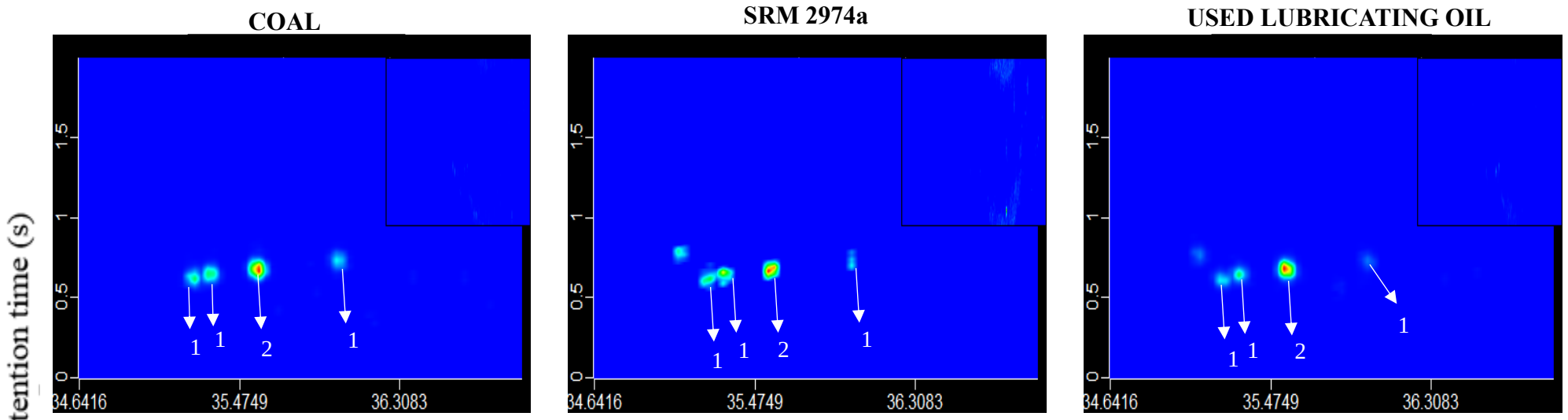


***Figure 6.10**: 2D HRMS contour plots of C_1Fl in the 3 environmental samples (**1**- MethylFluorene; **2**- 1-MethylFluorene)*

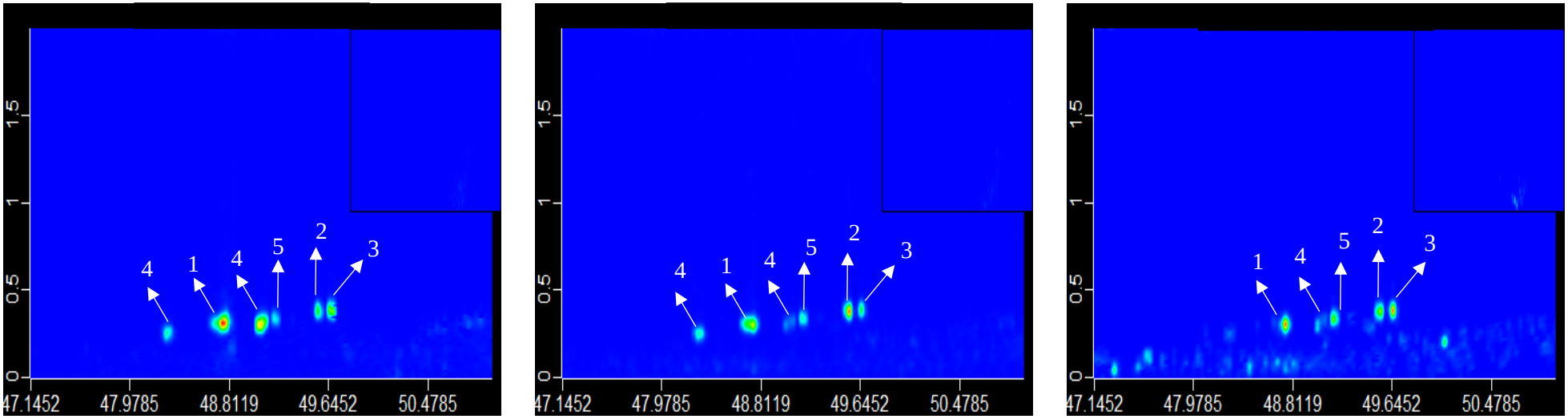


***Figure 6.11**: 2D HRMS contour plots of C_1Py/Flu in 2 environmental samples (**1**- 3- MFlu; **2**- 4-MPy; **3**- 1-MPy; **4**- 6-MFlu; **5** -MPy) on (left) coal (middle) SRM 2974a and (right) used lubricating oil.*

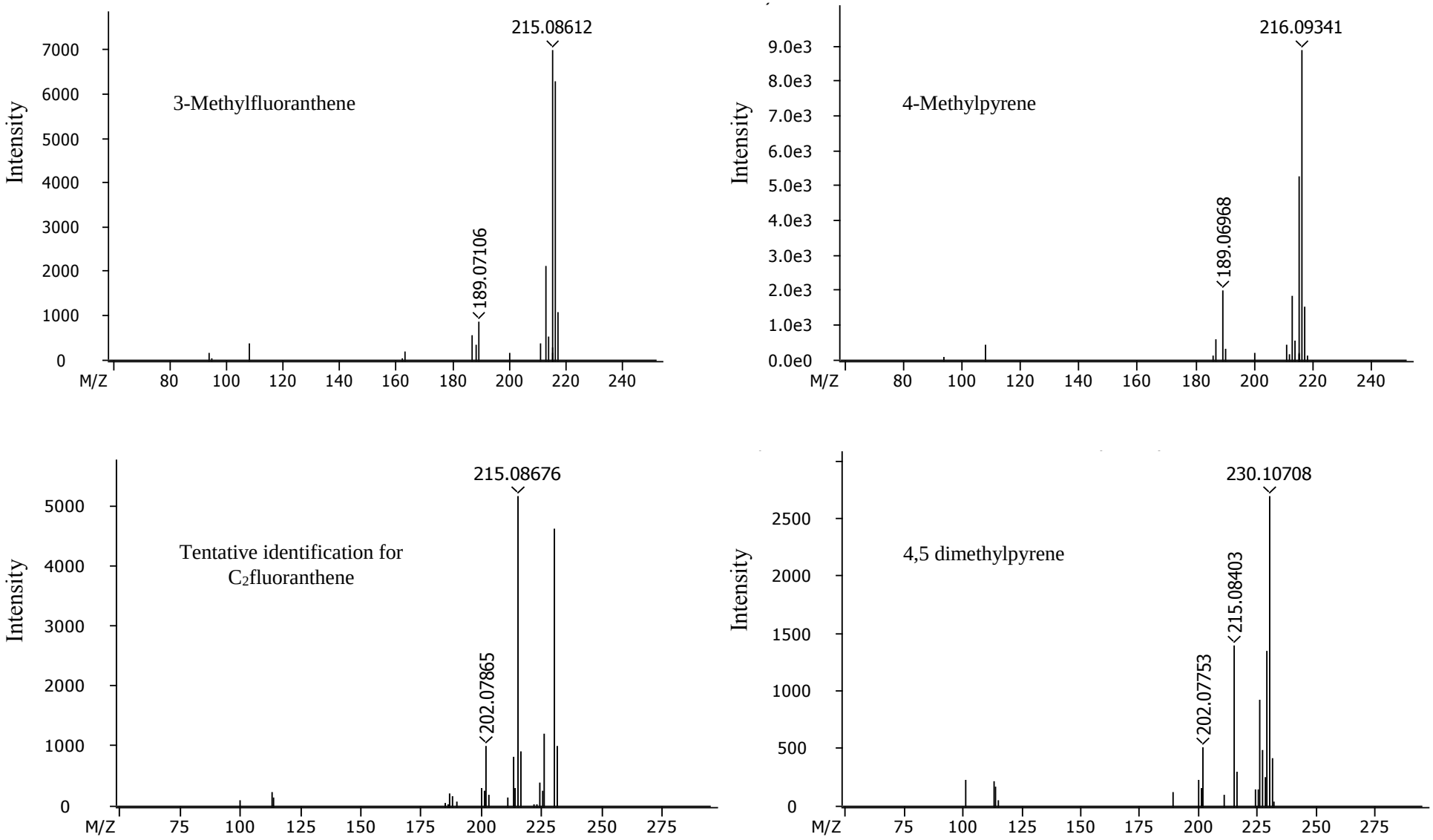


***Figure 6.12**: Mass spectra of C_1 and C_2 pyrenes/fluoranthenes*

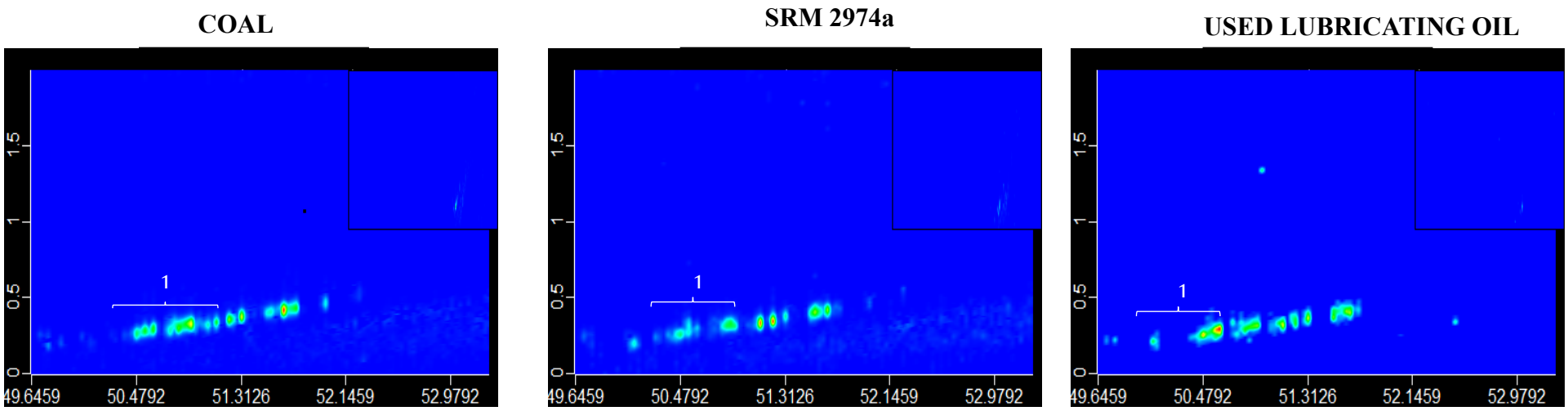


***Figure 6.13**: 2D HRMS contour plots of C_2 Py/Flu in the 3 environmental samples (**1**-suspected to be C_2 Fluoranthenes)on (left) coal (middle) SRM 2974a and (right) used lubricating oil.*

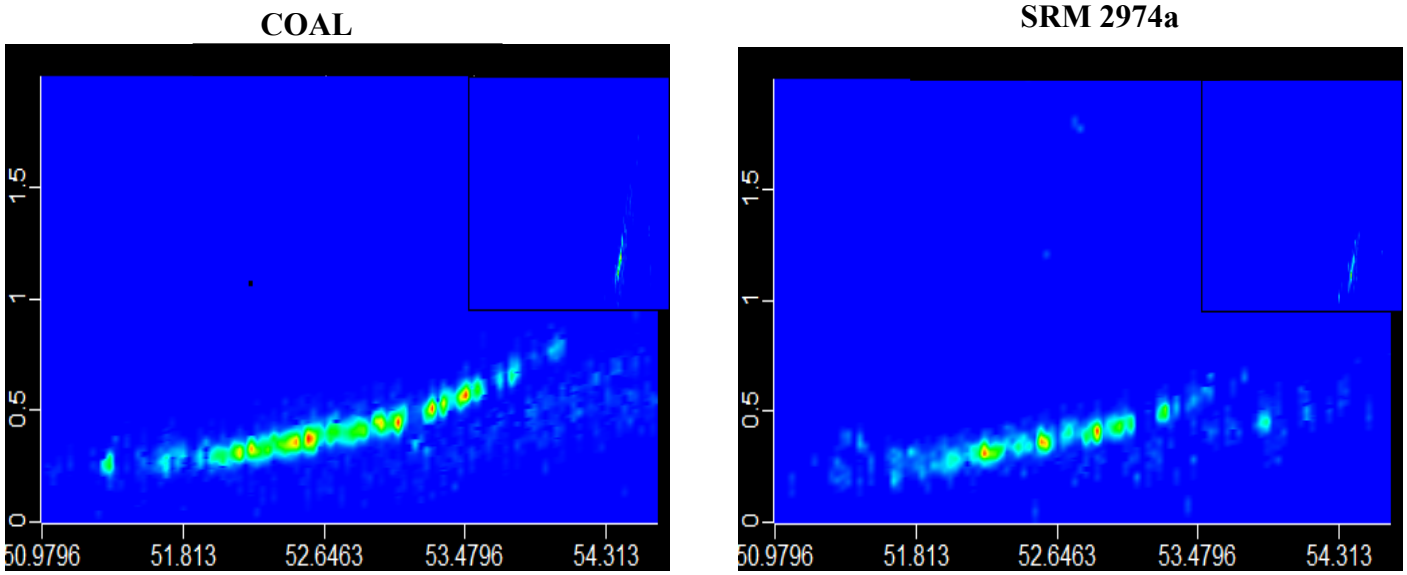


***Figure 6.14**: 2D HRMS contour plots of C_3 Py/Flu in 2 environmental samples (**1**-suspected to be C_2 Fluoranthenes)*

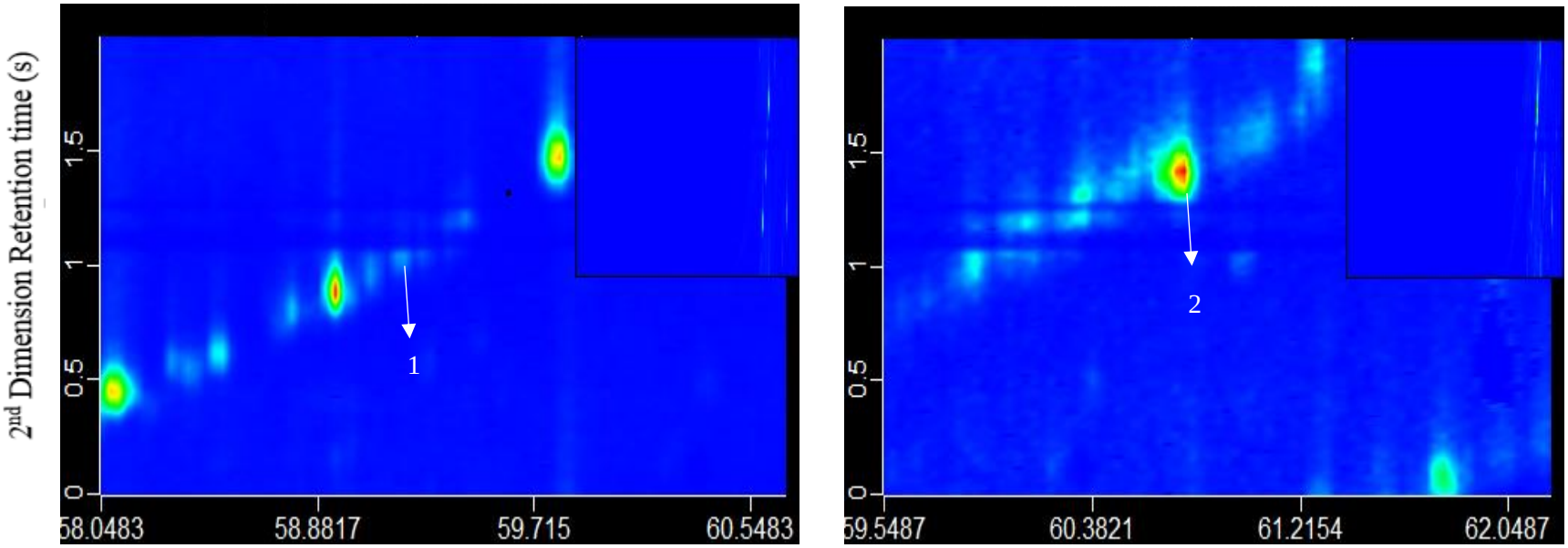


***Figure 6.15**: 2D HRMS contour plots of C_1BaP in coal sample (**1**- 7-methylbenzo(a)pyrene; **2**- C_2 BaP)*

6.6.2 Method Performance Characteristics

Table 6.3 shows the LODs and LOQs (ng/g, lipid weight) for the PACs analyzed in this study in both 1D and 2D mode for biota sample. In 1D mode, the LODs for the PACs ranged from 1.00 ng/g for 6-methyl chrysene to 3.85 ng/g for 1,2,6,9-tetramethyl phenanthrene. LODs in the 2D mode for individual PACs ranged from 1.01 to 4.26 ng/g for 2- methyl anthracene and 7-methylbenzo(a)pyrene respectively.

For the PAHs, the 16 EPA priority pollutants were examined. The LODs in 1D mode ranged from 0.62 to 3.05 ng/g for acenaphthylene and benzo(k)fluoranthene, respectively. For the 2D mode, the LOD ranged from 1.44 to 4.20 ng/g for phenanthrene and benzo(k)fluoranthene, respectively. The LODs/LOQs of these compounds are comparatively lower indicating better detectability than as reported in Lim, et al. [42]

It is not surprising that in some cases the LOD are greater for compounds in 2D compared to 1D. The intensity of peaks observed in 2D is directly related to the duration of the modulation time; shorter modulation times generally leads to improved resolution in 2D but it can also lead to smaller peak intensities i.e., higher LODs. Empirical evidence for this is shown in Figures 6.3a and 6.3b. In our study, we purposely selected a modulation time of 2s (instead of 5s) in order to maximize chromatographic separations.

The correlation coefficients for the calibration standards injected randomly in triplicate were higher than 0.99 with p-values <0.0001 in all cases. The randomness of the distribution around zero observed in the residual plot further confirms linearity.

6.6.3 Quantitation of PACs

The quantitation and confirmation ions used for positive identification of PAHs and PACs for GC×GC/HRTOF-MS is as presented in Table 6.1. The methods of quantitation for PACs are comprehensively described in Idowu, et al. [1]. Quantitation of PAHs by isotope dilution was based on the average relative response factors (ARRFs) of the native PAHs relative to authentic d-PAH surrogates. Prior to calculating ARRFs, the responses of the native and d-PAHs (*i.e.*, electronically integrated peak areas) were normalized to the response of the IPIS, d_{10}-anthracene. The IPIS was added to the calibration solutions and to sample extracts prior to GC-injection at a concentration of 100 pg/μL.

ARRFs for the 16 PAHs (App Table 3a&b) investigated for this analysis ranged from 1.05 ± 0.03 for chrysene to 1.89 ± 0.04 in the one-dimensional run and 1.04 ± 0.09 for anthracene and 1.78 ± 0.09 for benzo*(ghi)*perylene in two-dimensional mode with no statistical difference for all the RRFs at any calibration levels.

For APAHs and PASHs in environmental samples, the two approaches commonly employed and their limitation is highlighted in the recent work of Idowu, et al. [1]. Several approaches have considered using deuterated analogs of parent PAHs to quantify the alkylated isomer but this has sometimes led to an underestimation of the alkyl concentration. The method employed in the quantitation of APAHs and PASHs is by external standard approach.

Table 6.3: *Detection Limits for Polycyclic Aromatic compounds (PACs) studied.*

	One-Dimension		**Two - Dimension**	
Analyte	**LOD (ng/g)**	**LOQ (ng/g)**	**LOD (ng/g)**	**LOQ (ng/g)**
Acenaphthene	1.24	4.12	1.87	6.22
Acenaphthylene	0.62	2.07	2.31	7.69
Anthracene	0.68	2.26	2.91	9.70
Benz[a]anthracene	2.65	8.83	2.16	7.22
Benzo[a]pyrene	1.02	3.40	2.97	9.90
Benzo[b]fluoranthene	3.05	10.16	2.74	9.13
Benzo[g,h,i]perylene	2.47	8.25	4.01	13.38
Benzo[k]fluoranthene	2.86	9.52	4.20	14.01
Chrysene	1.39	4.63	2.74	9.13
Dibenzo[a,h]anthracene	5.00	16.67	2.29	7.65
Fluoranthene	0.81	2.69	2.03	6.77
Fluorene	0.71	2.36	2.32	7.72
Indeno[1,2,3-c,d]pyrene	0.88	2.92	2.42	8.06
Naphthalene	1.19	3.95	2.60	8.65
Phenanthrene	1.01	3.36	1.44	4.79
Pyrene	1.13	3.76	2.54	8.48
1-Methylnaphthalene	1.12	3.74	1.62	5.41
2-Methynaphthalene	1.70	5.66	2.73	9.10
2,6-Dimethylnaphthalene	1.48	4.92	1.19	3.95
1,6-Dimethylnaphthalene	2.69	8.96	2.62	8.74
2,3,5-Trimethylnaphthalene	2.84	9.48	2.19	7.29
1,2,5,6-Tetramethylnaphthalene	2.79	9.30	2.56	8.54
1-Methylphenanthrene	1.38	4.58	2.36	7.86
2-Methylphenanthrene	1.43	4.77	1.87	6.22
2-Methylanthracene	1.66	5.54	1.01	3.37
3-Methylphenanthrene	2.27	7.56	1.40	4.66
9-Methylphenanthrene	1.47	4.89	2.22	7.39

1,7- Dimethylphenanthrene	2.38	7.92	1.99	6.64
1-Methylpyrene	1.96	6.55	1.29	7.67
4-Methylpyrene	1.14	3.82	3.94	4.31
1-Methylfluoranthene	2.29	7.64	1.57	5.24
1,3 – dimethylphenanthrene	1.90	6.34	1.84	6.14
2,6 – dimethylphenanthrene	1.57	5.26	1.58	5.25
1,7 – dimethylphenanthrene	2.10	7.00	1.94	6.48
2,6 – dimethylphenanthrene	1.97	6.56	1.95	6.49
3,6 – dimethylphenanthrene	2.20	7.36	2.00	6.65
3-Methylchrysene	1.34	4.48	2.63	8.77
1,4,6,7- Tetramethylnaphthalene	2.46	8.21	2.67	8.92
1,2,6-Trimethylphenanthrene	3.72	12.44	3.09	10.27
1,2,6,9-Tetramethylphenanthrene	3.85	12.89	3.25	10.81
2,8-dimethyldibenzothiophene	1.99	6.65	2.32	7.73
Dibenzothiophene	1.80	3.99	1.07	3.59
1-MethylFluorene	1.79	5.97	2.08	6.94
4-Methyldibenzothiophene	1.57	5.23	1.73	5.77
2,4,7-trimethyldibenzothiophene	2.48	8.27	3.02	10.08
1,3,6-trimethylchrysene	2.46	8.20	3.20	10.63
4,5-dimethylpyrene	1.66	5.54	2.23	7.43
7-methylbenzo(a)pyrene	3.08	10.26	4.26	14.16
6-Methylchrysene	1.00	3.34	1.62	5.39

Other method performance characteristics as highlighted in the Eurachem guideline including trueness, precision and measurement uncertainty were performed using the SRM2974a extract. App Table 4a&b shows the result from the trueness tests as well as measurement uncertainties. Based on the results and the stated standard method performance requirements the method is fit for its intended use.

It is known that GC×GC/HRTOF-MS provides a full scan for any sample run. It was evident that SRM2974a used for the method validation procedure contains more compounds than listed in the certificate of analysis. For example, C_3Ph isomers were not listed but were present in appreciable concentrations in the SRM2974a material and because of the increased peak capacity of the 2D system there was greater separation of these isomers in 2D. More importantly, the separation of isomers that co-elute in 1D using the 2D system will result in more meaningful quantitative measurements. App Table 5 shows the concentrations of APACs determined using the GC×GC/HRTOF-MS method developed in this study (concentrations lower than the LOQ were also reported). When authentic standards are unavailable, it is tempting to quantify compounds using the RF of isomeric standards with similar alkyl substitution content. However, as shown for the dimethyl isomers of phenanthrene, the average RFs can vary by as much as 30% (see App Table 7) and so we must caution against using this approach.

6.7 Conclusion

The results of this study demonstrate the ability of GC×GC-HFTOF/MS to separate, identify and quantify PACs in range of environmental samples. The method developed exhibits a high degree of linearity over a broad dynamic range with LODs in low ng/g range. Mass spectral fragmentation patterns can be used to identity multiple classes of PAC isomers in the absence of authentic standards. The described methodology enables detailed diagnostic characterization of each PAC grouping. The method was further validated according to EURACHEM guidelines and the performance characteristics of the method clearly demonstrated applicability to quantification of PACs in biological matrices. Additionally, APAC isomers not certified in the NIST material were identified and quantified using authentic standards. Overall, the 2D method represents a step

forward in the ability to measure the occurrence and distribution of PACs and APACs in multiple environmental compartments, compared to techniques based on 1D separation.

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Chapter 7

Analysis of some Halogenated Polycyclic Aromatic Compounds in Biological Samples using Gas Chromatography High Resolution Mass Spectrometry

7.1 Introduction

Halogenated polycyclic aromatic hydrocarbons (HPACs) are a class polycyclic aromatic compounds with one or more halogen substituent attached to the aromatic rings of corresponding parent polycyclic aromatic hydrocarbons.[1] This class of compound includes chlorinated polycyclic aromatic hydrocarbons (ClPAHs) and brominated polycyclic aromatic hydrocarbons (BrPAHs). It was not until the 1980's that halogenated PAHs were found to be present in the environment such as found in urban air, fly ash, coal combustion, soil, automobile exhausts, tap water[2-5] etc. An important source of halogenated PAHs in urban air is waste incineration derived from halogenation of parent PAHs.[6] These parent PAHs are derived from the incomplete incineration of organic matter in waste.[7] Just like other PACs, HPAHs are persistent, bioaccumulative and toxic.[8] Their log Kow are higher than their parent compounds which makes easy their potential to partition into lipids and as a result bioaccumulate and biomagnify in biological organisms thereby inducing toxicity.[6, 9] HPAHs have been detected in biological organisms such as seafood, blue mussels and fish.[9-11] The toxicity of some HPAHs e.g. polychlorinated naphthalenes, have very little information especially for HPAHs with 3-5 aromatic rings in the environments.[8, 12-14] This can be largely attributed to the unavailability of individual analytical standards for purchase resulting in lack of validated analytical methods for the identification and quantitation of these compounds.

This study focusses on samples obtained from the Alberta Oil Sands Region (AOSR). The Canadian oil sands are naturally occurring mixtures of crude oil and bitumen with clay, sand, ultrafine mineral solids and water. Generally, PACs are released naturally from both the oil sands deposits and during bitumen extraction and other oil sands operations.[15] Several studies have shown an increased level of concentrations of substituted PACs and as well as the dibenzothiophenes (a commonly known components of the oil sands) in the soil and sediments,

water, biota etc. domiciled in the AOSR due to their proximity to oil sands operations.[15-18] The geological history of AOSR detailed that the region was covered by sea water which can imply a high concentration of chloride and bromide ions.[19-20] During the deposition of rich organic matter preceding oil formation, a likelihood of organohalogen formation can be attributed to elevated temperatures and pressures associated with diagenesis and catagenesis with the catalytic activation of halides formed with abundant crustal elements aluminium (Al) and iron(Fe) (AlX_3 and FeX_3, X = Cl or Br). Sankoda, *et al*. [21] suggested that the presence of halogen ions and strong sunlight aids the formation of HPAH. The two possible ways of formation gave rise to the hypothesis that, HPAHs resulting from non-combustion sources are present in biological organisms, thus, HPAHs are present in biological samples from the AOSR.

To test this hypothesis, a method to identify and quantify purchased HPAH standards was first established. This was done by using the temperature ramp used for the one dimensional analysis on the GCxGC-HRToFMS system. The high resolution system aids understanding of the mass spectra pattern of the HPAH standards used for the analysis.

7.2 Materials and methods

7.2.1 Reagents and Chemicals

All organic solvents used were of high-purity (Optima grade) and purchased from Fischer Chemicals (Ottawa, Ontario, Canada). Sixteen (16) individual HPACs including 1-chloropyrene, 7-bromobenz[a]anthracene, 7-chlorobenz[a]anthracene, 9-Chloroanthracene, 9-Chlorophenanthrene, 7,12-dichlorobenz[a]anthracene were purchased from Cambridge Isotope Laboratories Inc. (Tewsbury, Massachusetts, USA), 1-bromopyrene, 1-chloroanthracene, 1,5-

dichloroanthracene, 2-bromofluorene, 2,7-dibromofluorene, 3-bromophenanthrene, 5-bromoacenaphthene, 9-bromophenanthrene, 9,10-dibromoanthracene, 9,10-dibromophenanthrene were purchased from Sigma Aldrich (St. Louis, MO, USA). All native PAHs and their deuterated compounds, d_8-naphthalene, d_8-acenaphthylene, d_{10}-acenaphthene, d_{10}-fluorene, d_{10}-phenanthrene, d_{10}-pyrene, d_{12}-benz(*a*)anthracene, d_{12}-chrysene, d_{12}-benzo(*b*)fluoranthene, d_{12}-benzo(*k*)fluoranthene, d_{12}-benzo(*a*)pyrene, d_{12}-indeno(*1,2,3-c,d*) pyrene, d_{14}-dibenz(*a,h*)anthracene, d_{12}-benzo(*g,h,i*)perylene, naphthalene, Aaenaphthylene, acenaphthene, fluorene, phenanthrene, pyrene, benz(*a*)anthracene, chrysene, benzo(*b*)fluoranthene, benzo(*k*)fluoranthene, benzo(*a*)pyrene, Indeno(*1,2,3-c,d*) pyrene, dibenz(*a,h*)anthracene, benzo(*g,h,i*)perylene were also all purchased from Cambridge Isotope Laboratories Inc. (Tewsbury, Massachusetts, USA). The C_{18} powder (HPLC sorbent) was purchased from Sigma Aldrich (St Louis, MO, USA) and the size-exclusion S-X3 Biobeads were purchased from Bio-Rad Laboratories (Mississauga, Ontario, Canada). Silica gel (923 grade, 100-200 mesh), alumina (60-325 mesh), Ottawa sand, Diatomaceous earth (DE) dispersant and anhydrous sodium sulphate were all purchased from Fisher Chemicals (Ottawa, ON, Canada).

7.2.2 Sample Preparation

Fig. 1 shows where all samples were collected. All samples were collected in 2014 and 2015 which include liver samples of otter (*n=6*), northern pike (*n=4*), Lake Whitefish (*n=4*) and snails (*n=3*). Snails were placed in clean water for 24 h to depurate the contents of their guts, then shells were removed and the tissues were pooled and homogenized. Approximately 1.5 g of each sample (wet

weight) was weighed, spiked and extracted according to the procedure presented in Idowu, et al. [22].

7.2.3 GC-MS/MS Analysis

The details of the method adopted for gas chromatography tandem mass spectrometry and quantitation of all compounds are as presented in Chapter 4 of this thesis.[22]

7.2.4 GC-HRTOF-MS Analysis

The analysis of extracts obtained after clean-up was done on a LECO Pegasus 4D HRT (LECO, St. Joseph, MI, USA). This analysis was carried out in one-dimensional mode using a 7890A GC (Agilent Technologies, Wilmington, DE, USA) fitted with a spilt/splitless injector. The column used, temperature programming and flow rate including other parameters adopted for the system was as described in the previous chapter according to Idowu, et al. [23].

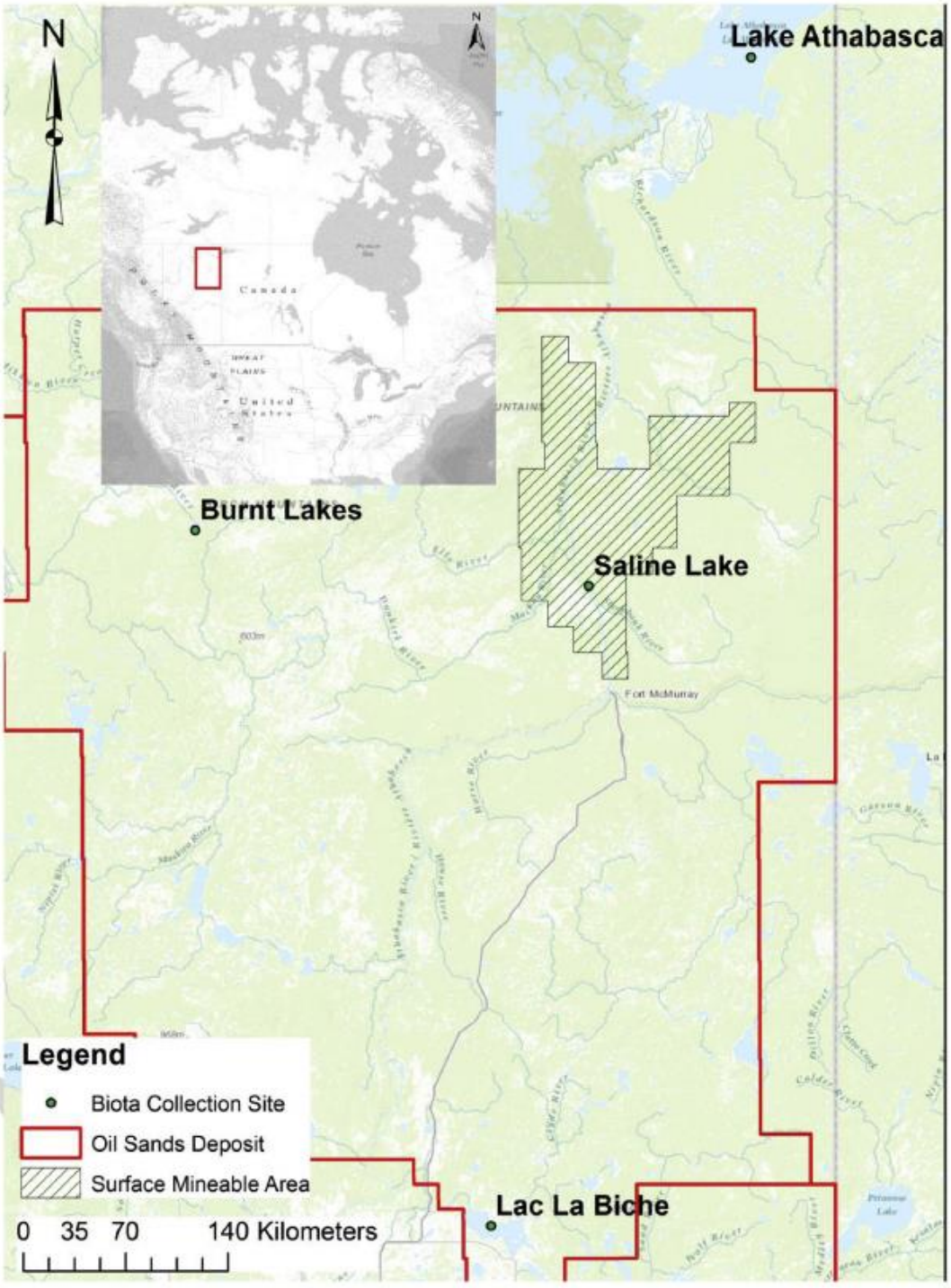

Figure 7.1: *Sample collection sites in the Alberta Oil Sands Region (AOSR)*

7.2.5 Method Verification

A truncated validation study i.e. verification to demonstrate that the validation method conducted for PAHs and APAHs is suited to HPAHs was performed. Since a full validation for PAHs and APAHs has been done in accordance to Eurachem Guidelines,[24] a verification study was only necessary to show that HPACs behave similar to PAHs and APAHs during sample extraction and clean-up steps. The verification study was carried out using a mixture of PAHs and HPACs at 3 different concentration levels: high (500 ng), medium (200 ng) and low (50 ng)] and taken through the entire procedure of extraction.

Limits of detection (LODs) for individual HPACs used for this analysis were obtained by fortification of the ASE cells (*n=10*) containing C_{18}-silica gel with each HPACs (at a final concentration of 50 pg). LODs were calculated using equation as presented in Chapter 4. Calibration curves for each HPACs were obtained by injecting calibration standards prepared in hexanes (1, 5, 10, 25, 50 and 100 pg/ μL) in triplicates.

7.3 Results and discussion

The average recoveries of extraction of HPACs relative to PAHs at any of the fortification levels were examined and it was observed that there were no statistical differences (Student t-test, $p<0.05$) at all three (3) levels. The linear range of the method for HPAC analytes ranged from 1 to 100 pg with the LOD ranged from 1.8 to 5.9 pg. The repeatability of the 16 HPACs and the (16 PAHs) for the 3 spiking levels, high, medium and low are 5.4 (6.7), 7.5 (4.9) and 13.9 (6.4)% respectively. With respect to rules stated in the Eurachem guide for performance characteristics

and its application to ISO-17025 validated PAHs, it can be concluded that the verified method is suited for use with HPACs.[22, 24]

7.3.1 Mass spectra (Electron impact ionization)

Individual purchased standards were used as reference for EI mass spectra. Figure 2 shows the full scan of some mass spectra of analytes available. Just like PAHs and APAHs, the mono- and di-chlorinated PAC molecular ion ($M^{+\cdot}$) were their base peak. The next prominent peak for mono-chloro- isomers arise from the neutral loss of HCl from the $M^{+\cdot}$ ion with the exception of 1-chloropyrene where the loss of a Cl radical from the $M^{+\cdot}$ ion is responsible for the second dominant peak. For di-chlorinated PACs, the next prominent peak after the base peak is attributed to the neutral loss of 2 Cl atoms as observed with the 2 di-Cl standards.

For monobrominated PAH isomer standards available for this study, 3-bromophenanthrene, 9-bromophenanthrenen and 1-bromopyrene have their molecular ions ($M^{+\cdot}$) as base peaks while the base peak for 7-bromobenz(a)anthracene is a result of a neutral loss of HBr from the $M^{+\cdot}$. For 2-bromofluorene and 5-bromoanthracene, the base peak resulted from a free radical loss of Br from the $M^{+\cdot}$. For the dibromo- analytes, the base peak for 9, 10-dibromophenanthrene and 9, 10-dibromoanthracene are as a result of the loss of 2 Br atoms from their parent ion or the concomitant loss of $Br^{\cdot}$ while the loss of HBr is responsible for the base peak of 2,7-dibromophenanthrene.

7.3.2 Instrument verification

To verify the performance of our high resolution instrument, 5 replicate injections were done for the 16 HPACs standard solution. Here, the mean experimental m/z values of all 5 injections were compared with their theoretical exact m/z values for the abundant ions characteristic to each HPAC compound. The mass accuracy of the instrument was lower than ±3.5 ppm and the repeatability of replicate measurement of the standards ranged from 0.1 to 0.6 mmu. These results were evidence that the GC/HRTOF-MS is suitable for identifying chemical compounds in environmental samples.

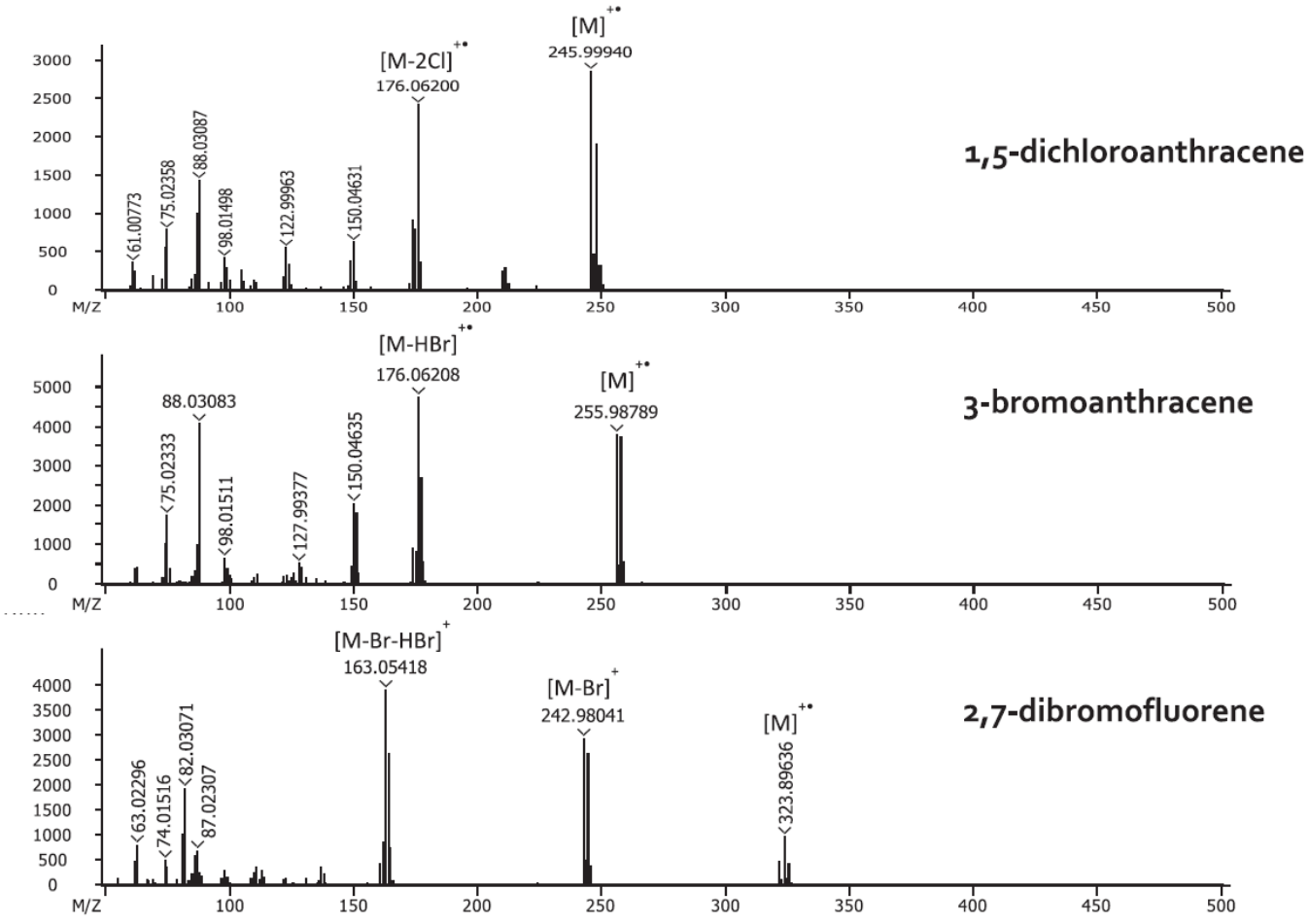

Figure 7.2: *Full scan mass spectra of some HPACs*

7.3.3. Compound identification in biota extract

To identify HPACs in the 4 biota extracts, an exact mass extracted ion chromatogram (XICs) for the two most prominent ions characteristic was constructed by deconvolution of total ion chromatograms (Fig. 3). The ions used are as presented in App Table 8 and these ions are obtained from replicate ($n=5$) injections of standard solution. As examined in the instrument verification step, the experimentally measured m/z values of the 2 most prominent peaks in the sample extracts were compared with the theoretical expected m/z values. Mass accuracy was evaluated and a limit for acceptance of ±5 ppm is often used as a criterion to retain high level of confidence in compound identification or assigning correct formulae.[25]

A further attempt at compound detection and identification was to examine the experimentally measured abundances of ions in the isotopic cluster of any identified analytes in the extract to their theoretical ones. However, this was unsuccessful as the mass of material extracted and the concentrations of the analytes present were small.

Finally, retention time, t_r, method was considered in the identification of HPACs in the samples extract. This approach has been adopted by some previous research work.[26-27] The approach ensure that compounds identify elute closely to the t_r of analytical standard of similar halogenated content and PAH back bone.

After successful identification, concentrations of HPACs in the samples were estimated by comparing the electronically integrated area of peaks in the exact mass XIC in the samples to the area od HPAC external standard of similar halogenated type, content and similar PAH backbone. Mass labelled PAHs were utilized as internal standard to access recoveries. All recoveries were >80% although no recovery correction was applied to the analysis of the data. It was also ensured

that concentrations reported were greater than the method detection limit values. A major setback is the limited availability of isomer standards which made it impossible to predict the substitution positions of the halogen atoms in the HPACs identified in our samples extract.

7.4 Identification of HPACs

Top panel of fig. 7.3 shows the elution profile of all 16 HPACs analytes evaluated in this study. The middle panel and bottom panel shows the 3 HPACs tentatively identified in our biological samples. In Lake Whitefish, an isomer of dichloroanthracene/phenanthrene was identified in all 4 samples. The identification was premised on the agreement of the t_r window of the compound peak with the elution time of 1, 5-chloroanthracene standard observed to be approximately 18.0 min. The knowledge of the constitutional isomers of substituted polycyclic aromatic compounds as reported by Johnson, et al. [28] calculated the possible congeners of dichloro-anthracene and dichloro-phenanthrene to be 15 and 25 respectively. Hence, it can be inferred that although the t_r of the identified compound peak in the samples does not necessarily overlap with that of 1, 5-dichloroanthracene, it is an isomer. The mean experimental measured masses (*n = 4*) of the 2 characteristic ions in the EI mass spectra of this compound were 245.9995 ± 0.0007 and 176.0619 ± 0.0003 amu. When compared with the theoretically exact mass, this corresponds to difference of -1.10 ± 1.50 and -0.94 ± 0.84 ppm respectively. Using the response factor of 1, 5-dichloranthracene, the mean concentrations of dichloro-anthracenes/phenanthrenes were estimated to be 16.3 ± 11.4 ng/g, lipid weight (lw, gravimetrically measured, Table 7.1).

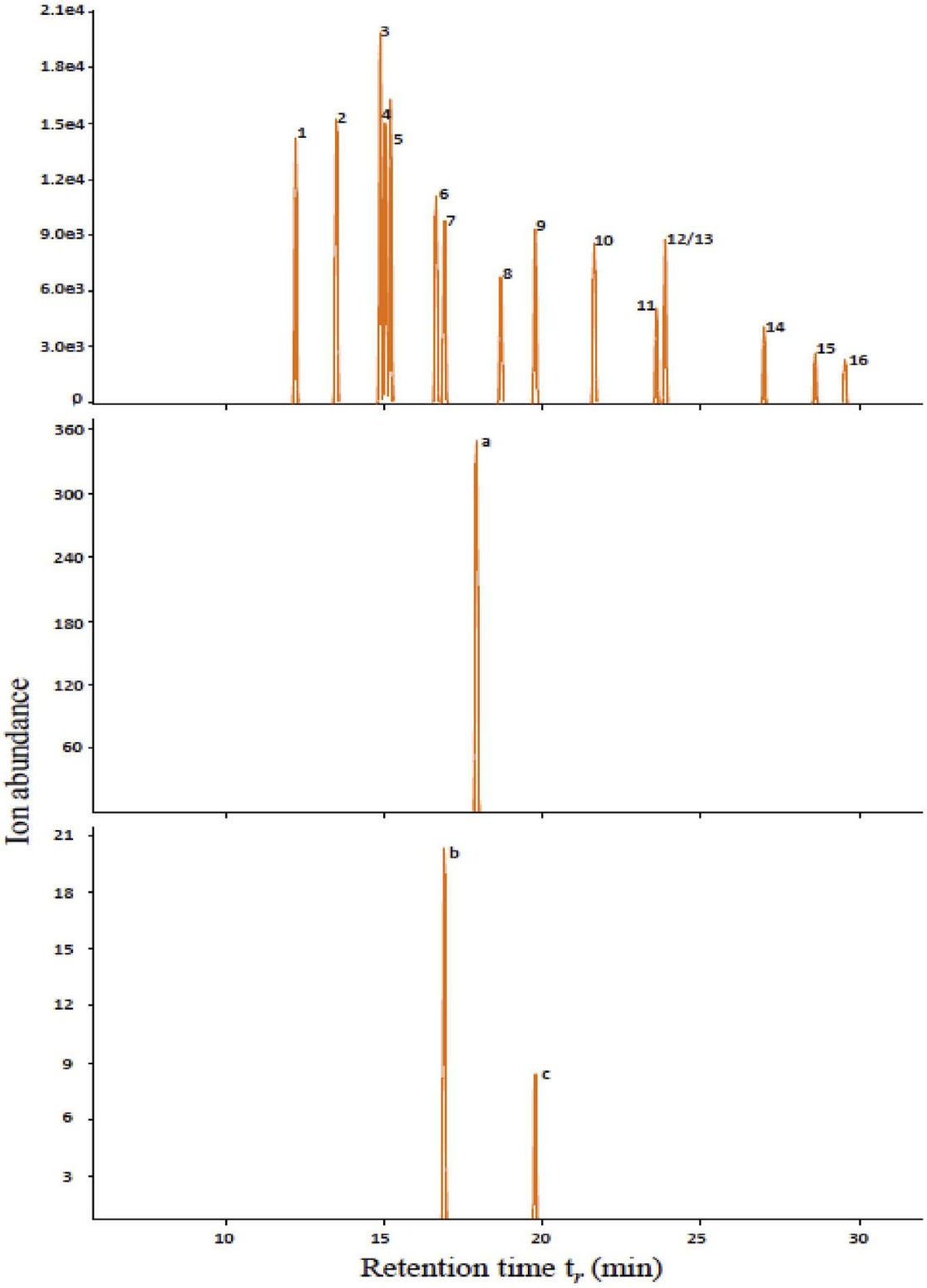

Figure 7.3: *XIC of 16 HPACs standard mixture(top panel), a lake whitefish extract (middle panel; a = dichloro anthracene/phenanthrene) and a snail extract (bottom panel; b =bromo anthracene/phenanthrene, c = dibromofluorene). Refer to Appendix 9.*

In one of the river otter samples, a dichloro-anthracene/phenanthrene was also identified. Although the concentration of the compound detected was ~3 times smaller than as found in Lake Whitefish, the mass spectra of the observed peak has an experimental measured mass of 245.9993 $[M]^{+\cdot}$ and 176.0625 $[M\text{-}2Cl]^{+\cdot}$ with Δm of -1.75 and 2.73 ppm, respectively.

Also tentatively identified in Northern pike and snail samples were two brominated compounds (see Fig.7.3). A monobrominated anthracene/phenanthrene in northern pike and a dibrominated fluorene in a snail (see Table 7.1). The t_r of the monobrominated anthracene/phenanthrene overlaps with that of 9-bromophenanthrene analytical standard, thus, it can be inferred that the identified peak is 9-bromophenanthrene. However, it should be noted that there are 3 and 5 constitutional isomers of monobrominated anthracene and phenanthrenes respectively.[28] The inference can be further premised on comparison of the experimentally measured masses of the 2 characteristic ions of bromo-anthracene/phenanthrene to their theoretical exact masses. In the northern pike samples, it was observed to be 255.9876/176.0621 which correspond to a Δm of -2.38/ 0.51 ppm, respectively while for the snail sample, Δm values of 0.82 and 1.99 ppm. Based on the response factor of 9-bromophenanthene, the lipid concentration was calculated. Lipid-normalized concentrations of identified bromo-anthracene/phenanthrene were estimated to be 12.5 and 170.5 ng/g in northern pike and snail, respectively.

For dibromofluorene detected, the experimentally measured *m/z*-values for its 2 prominent ions in the northern pike and snail samples were 323.8971/163.0567 and 323.8959/163.0541, respectively. Premised upon the knowledge of the t_r of our analytical standard, 2,7-dibromofluorene (~ 19,8 min), the t_r of this compound identified in the extracts falls within the time window of the standard. As earlier pointed out, it will be difficult to identify this peak as 2, 7-dibromofluorene with great certainty due to the numerous constitutional isomers. Using the

response factor of 2,7-dibromofluorene, the concentration of dibromofluorene in northern pike and snail were normalized and estimated to be 26.7 and 111.4 ng/g, lipid weight, respectively.

Table 7.1: *Concentrations of 3 HPAHs and APAHs in biota samples from AOSR.*

Species	HPAH Detected	Measured Mass	Mass Accuracy (Δm, ppm)	Concentrations (ng/g. lw)[a]	
				HPAHs	APAHs[b]
Lake Whitefish	Dichloro-Anthracene/ Phenanthrene	245.9995 176.0619	-1.0976 -0.9372	16.3 ± 11.4[c]	7.3 ± 3.9[c,d]
Snails	Bromo-Anthracene/ Phenanthrene	255.9884 176.0624	0.8203 1.9879	170.5	529.9[e]
	Dibromo- fluorene	323.8959 163.0541	-2.4390 -0.6746	111.4	316.5[f]
River Otter	Dichloro-anthracene/ Phenanthrene	245.9993 176.0625	-1.7480 2.7263	5.5	-
Northern Pike	Bromo-anthracene/ Phenanthrene	255.9876 176.0621	-2.3829 0.5111	12.5	-
	Dibromo- fluorene	323.8971 163.0547	1.1114 2.7598	26.7	3.2

[a] lw = lipid weight.
[b] Alkylated PAHs.
[c] mean ± SD measured in 4 samples.
[d] C_2-Phenanthrene.
[e] Sum of 2-, 3- and 4/9-methyl-Phenanthrene.
[f] C_2-Fluorene.

The study also went further to evaluate the possibility of a relationship between concentration of HPACs identified in our sample extract with PAHs and alkylated PAHs. To do this, two options were considered; i) that the total body burden of HPACs in organisms examined in this study is probably underestimated because there are likely other HPACs that remain undetected attributed to the availability of limited sample mass and analytical standards. This made detection of additional HPACs challenging. ii) that the external standard approach to quantitation whereby the

response factor of one standard is used to quantify suite of detected compounds in our extract may likely introduces bias in our measurements. With these options, a better structured comparison was done by comparing the concentrations of halogenated substituted PACs detected to those of alkyl substituents possessing the same alkyl-substitution content. An example of this will be a comparison of the concentrations of dibromofluorene with C_2-Fluorene and bromo-anthracene/phenanthrene with C_1 anthracene/phenanthrene.

Generally, concentrations of PAHs were below detection limits in the samples, hence, a comparison could not be established. But for the comparison of HPACs to alkylated PAHs, the mean concentrations of dichloro-anthracene/phenanthrene were ~2 times greater than C_2-anthrancene/phenanthrene in Lake Whitefish. Conversely, the concentrations of APAHs in snails were ~3 times greater than estimated HPACs values. Also in northern pike, dibromofluorene concentrations were 8 times greater than that of C_2-Fluorenes. However, for dichloro-anthracene/phenanthrene in river otter and bromo-anthracene/phenanthrene in northern pike, a comparison could not be made as APAHs of similar alkyl-content were below detection limits in the samples.

To further evaluate results obtained, the concept of biomagnification was accessed. Biomagnification factor (BMF) indicates the extent of trophic enrichment or dilution of a compound between a particular predator/prey feeding relationship.[29] This intuitively provides insight into the relative susceptibility of compounds to biotransformation/ clearance in biota. Using the river otter/lake whitefish feeding relationship, the liver-based BMF for the dichloro-PAH congener was 0.34. Although the elution times of the dichloro-PAHs congener in both the river otter and Lake Whitefish were identical, it is possible that the substitution position of the chlorine atoms could be different. We further acknowledge that this BMF represents a single predator/prey

feeding relationship but it does suggest that this HPAH congener is susceptible to metabolism and/or clearance in aquatic organisms.

Interestingly, the concentrations of HPACs and APAHs were greatest in snails relative to the other pelagic organisms. This can be attributed to the fact that snails are benthic feeders and can possibly accumulate HPAHs (and APAHs) from direct interaction with sediments. This further suggests that the concentrations of HPAHs in sediments are elevated and/or the metabolic activity of snails to expel these compounds from their system is low. It is also relevant to note that snails lack the metabolic enzyme system (CYP) necessary to clear PAH type compounds. Further work on characterizing concentrations of HPAHs in sediments is clearly warranted.

Analogous to PAHs and APAHs, it is believed that organism exposure to HPAHs in the AOSR is likely to be from continuous chronic inputs of these compounds into the receiving environment. By definition, bioaccumulation of environmental contaminants in biological organisms occurs when the rate of chemical uptake and storage exceeds the rate of clearance (i.e., depuration and/or metabolism). It is quite plausible then that clearance of HPAHs is rapid but that continuous exposure leads to detection in organism. While this study was not specifically designed to address this issue, it is clear that further work is needed to understand the toxicokinetics of these compounds in biological organisms.

7.5 Conclusion

Generally, environmental research with focus on PACs has over the years focused more on non-halogenated PACs for monitoring activities in polluted areas like the AOSR. The detection of halogenated PACs in this region suggests their possibility of environmental impact. One of the major challenges to adequately evaluate their impact is the lack of authentic standards which limits

the ability to comprehensively identify and quantify compounds in sample extracts. This could lead to an underestimation of their concentrations. It is believed that commercial availability of authentic standards will facilitate thorough understanding of the mass fragmentation of these compounds and aid accurate quantitation.

Although our study identified few HPACs, it is conceivable that other HPACs even halogenated derivatives of APAHs that are of environmental relevance may be present in the AOSR considering that the APAHs are detected in greater concentrations than their parent PAHs. A larger sample mass may help to identify more of these novel compounds in samples from the AOSR. This may further help to assess the efficacy by which these compounds are biotransformed in biological organisms. The identification of 3 HPACs from the extract of the samples obtained from the AOSR is the first report of these compounds from this region.

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Chapter 8

Conclusion and Future Directions

8.1 Conclusion

This thesis sought to develop a method to identify, separate and quantify complex PACs and their numerous isomers in various environmental matrices. Undoubtedly, the most studied class of polycyclic aromatic compounds are the parent PAHs with a primary focus on the 16 US/EU EPA established back in the 1970's. Some of the reasons most studies are limited to only that of the PAH compound class is attributed to, for instance, lack of synthesis of authentic analytical standards of similar compounds, inadequacies in technology to separate the many compounds that co-elute. However, there is a continuous increase in the demand to understand the occurrence, fate and behavior of PACs different to the 16 EPA PAHs.

To date, there is no stand-alone analytical method to simultaneously quantify most of the PACs currently studied after extraction from environmental samples. As well, it should be noted that no fully validated analytical method that includes the entire suite of PACs is currently available. My research was able to address this major scientific gap by establishing a validated method following EURACHEM guideline in an ISO 17025 accredited laboratory to measure a larger suite of PACs.

To successfully develop the method on the GC/MS, the number of PACs isomers theoretically possible was initially evaluated. This highlighted the inherent complexity of alkyl-substituted PACs and helped confirm how many of the theoretically possible isomers were present in my samples.

With the knowledge of the current PACs monitored for in environmental samples, a novel analytical method that incorporates these compounds was developed and validated in biological samples. This method development was done using both GC/MS/MS and 2D-GC-HR-TOF/MS. The 2D analysis owing to its greater peak capacity provided significantly better separation of co-eluting or closely eluting compounds observed in the chromatogram relative to the 1D system. The high resolution technique available on the 2D system was subsequently used to detect previously unreported compounds (Halogenated Polycyclic Aromatic Hydrocarbons –HPAHs) in biological samples from the Alberta Oil Sands Region (AOSR).

While my research study was able to address the lack of a validated method for a larger suite of PACs, one of the major drawbacks of the research is the unavailability of authentic analytical standards. This drawback limits the identification of all the compounds detected in environmental samples. It also limited a full evaluation of the mass spectra fragmentation behavior of these compounds. As such, it was not possible to deduce the site of substitution of alkyl groups on the cyclic backbone. Furthermore, identification of new analytes becomes even more challenging due to this limitation.

Another drawback is the resolution of the instrument intended to aid separation of co-eluting or closely eluting compounds. Although, GCxGC HRToF MS significantly improves the separation of these challenging compounds compared GC-MS/MS, some compounds still prove difficult to separate and identify.

The main strength of this research lies in the innovative method developed to simultaneously separate, identify and quantify most of the commercially available PACs that are of environmental concern in comparison to other methods developed to address only few of these compounds. This method was used as a framework to extract and quantify PACs in different matrices. The use of

the multiple reaction monitoring (MRM) technique as against the single ion monitoring (SIM) also provides an accurate quantitation and reduction of false positives in sample analysis.

8.2 Future Direction

Future work will be aimed at addressing some of the shortcomings of the current method developed and extending the analysis to other relevant PACs that may be present in environmental matrices.

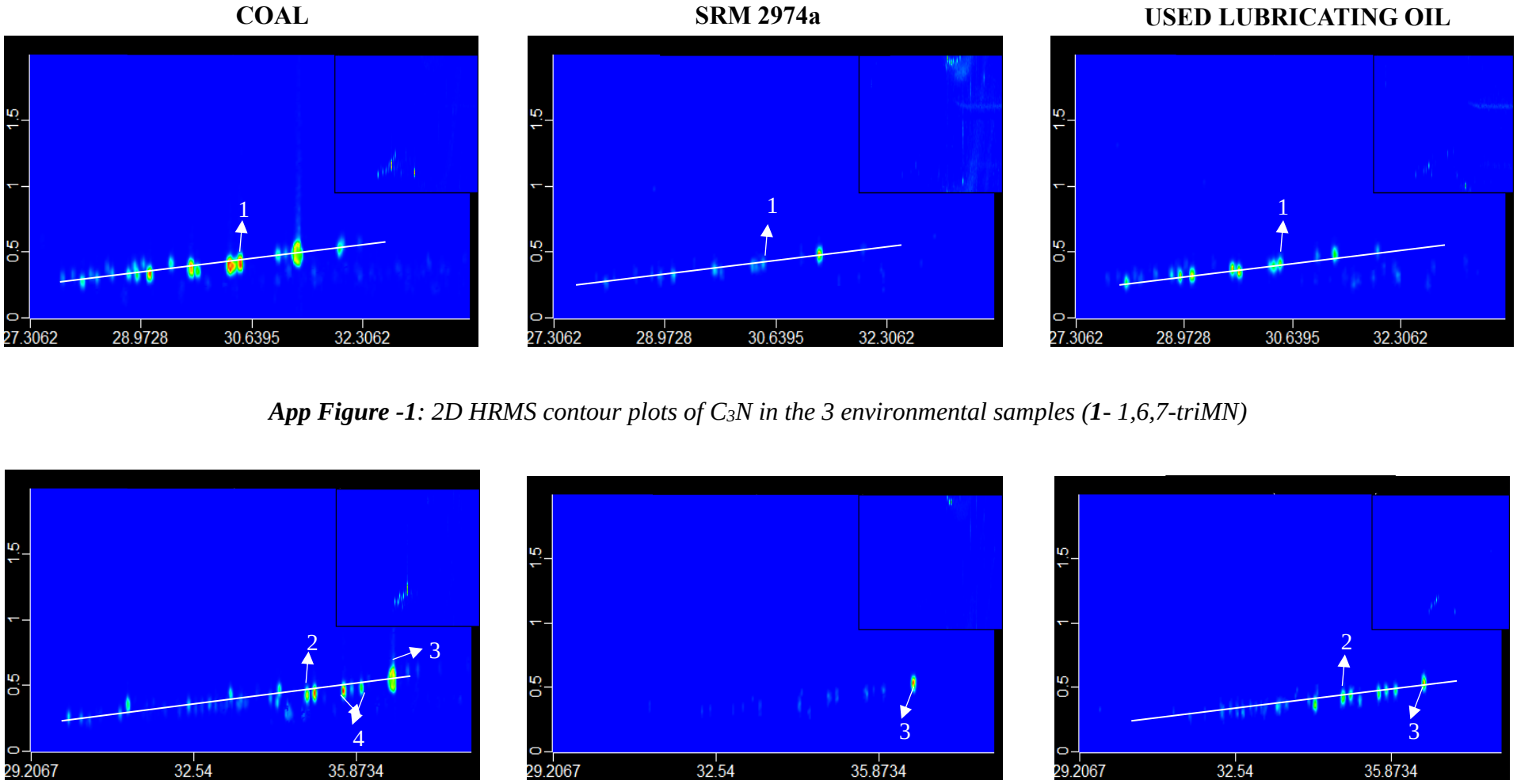
Since it has been previously demonstrated that there exist numerous constitutional isomers, an extensive collaborative work with researchers in the area of organic synthesis is required. This will enable us to determine the chemical and environmental stability of individual isomers. This will lead to targeted synthesis of more compounds that are not commercially available and of possible environmental toxicity. Eventually, an in depth understanding of the mass fragmentation behavior can be gained which will aid in the identification of these compounds in extracts. It is important to note that this limitation if addressed will help increase available compounds in libraries/databases.

The importance of a high resolution system as regards to identification of new compounds if present in an environmental extract is key. There exist suites of other PACs not explored in this study that include other APAHs and S, N, and O-based PAHs. In as much as these compound groups have been researched and proven to exist, other possibilities of substituents on the ring are endless. It is highly conceivable that other PACs with more than one group of substituent may exist. These could be a halogenated derivative of APAHs, S-based PACs or N-based PACs even a halogenated derivative of an APAHs having O-atom embedded in its ring system.

Also, employing the use of other standard reference materials (SRM) with individually quantified substituted PACs. Presently, some SRM such as found in coal tar has its PACs quantified as group class.

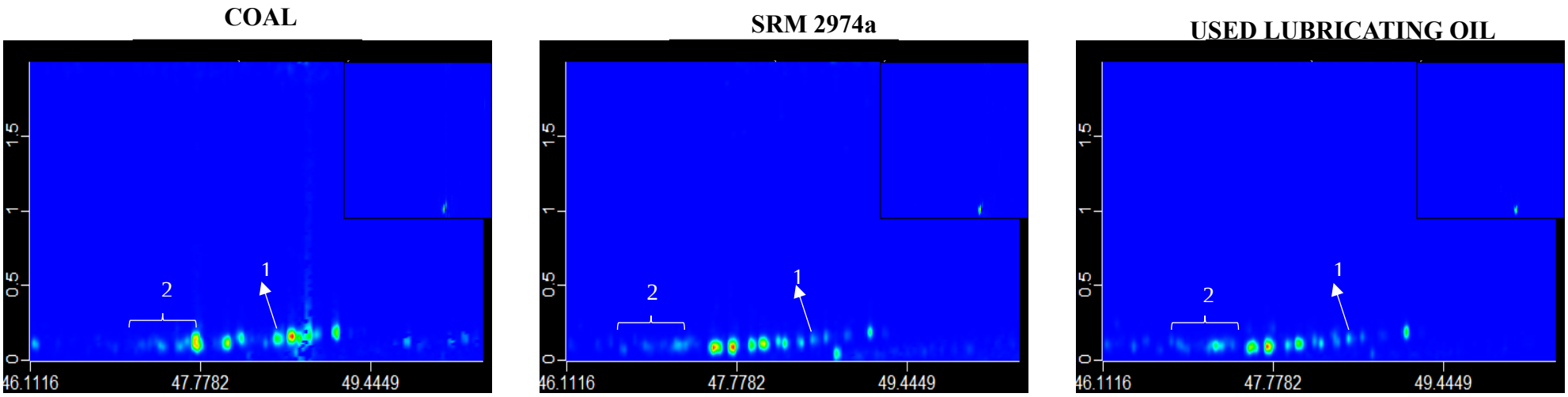
It is hoped that these methods become widely adopted by the environmental community as standard methods of testing to aid accurate quantitation of these compounds in environmental samples. Overall isomer specific APACs measurements will drive much needed ecotoxicity studies and field monitoring activities.

APPENDIX

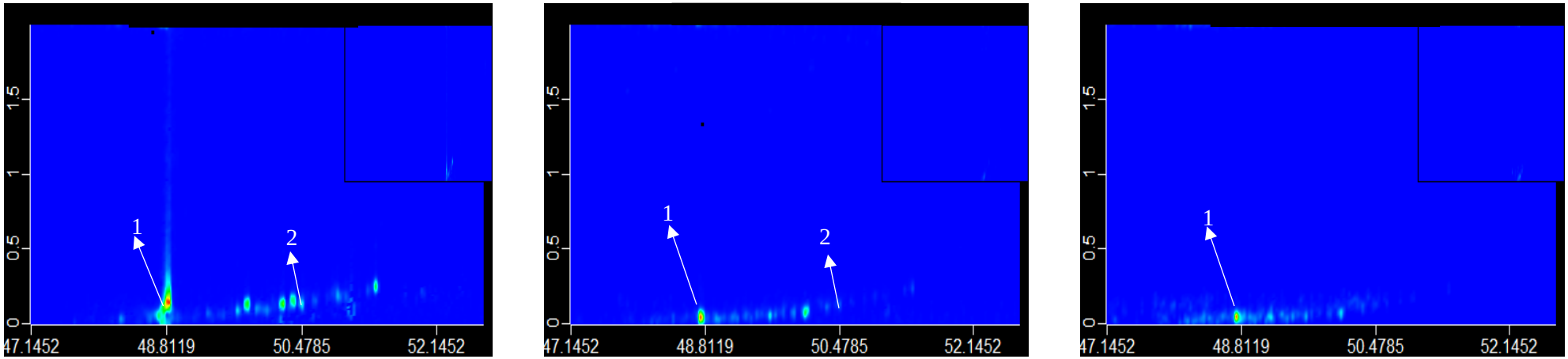


App Figure -1*: 2D HRMS contour plots of C_3N in the 3 environmental samples (**1**- 1,6,7-triMN)*

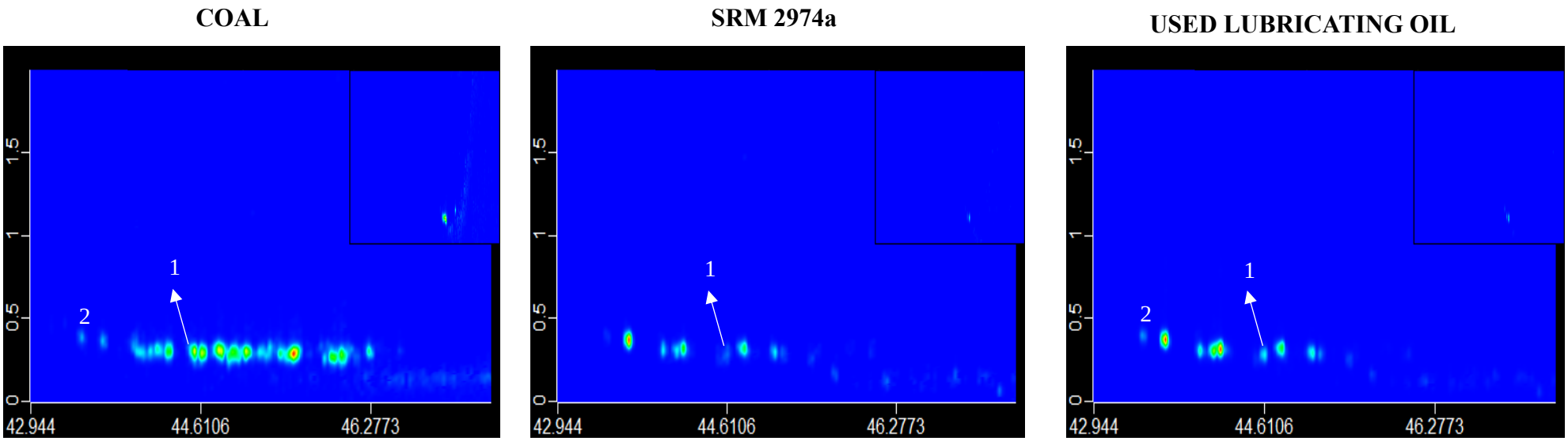
App Figure 2*: 2D HRMS contour plots of C_4N in the 3 environmental samples (**2**- 1,4,6,7-teMN; **3**-1,2,5,6-teMN; **4**- Unknown C_4 Isomer; other peaks are teMN) on (left) coal (middle) SRM 2974a and (right) used lubricating oil.*



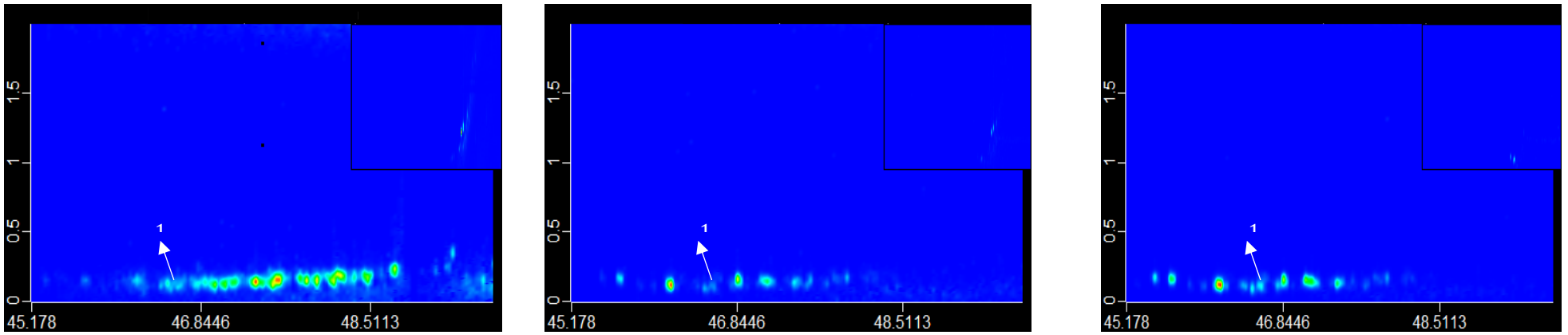
***App Figure 3**: 2D HRMS contour plots of C_3Ph in the 3 environmental samples (**1**- 1,2,6-triMPh; **2**-methylethyl Ph/An; other peaks shown are triPh/An.*



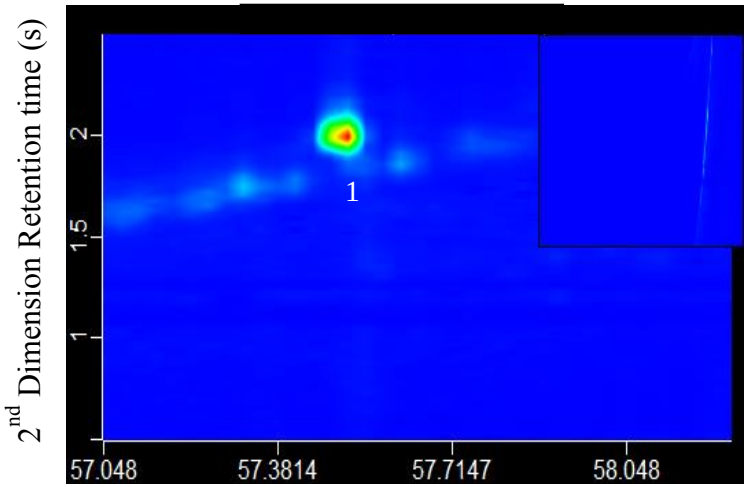
***App Figure 4**: 2D HRMS contour plots of C_4Ph/An in the 3 environmental samples (**1**- Retene **2**-1,2,6,9-teMPh) on (left) coal (middle) SRM 2974a and (right) used lubricating oil.*



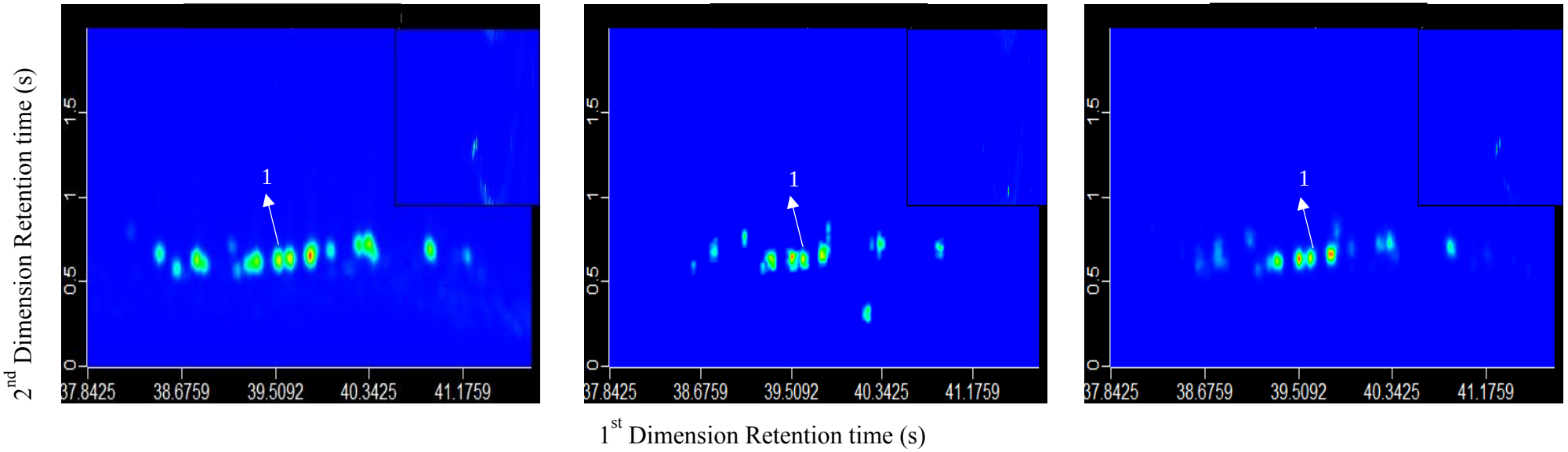
***App Figure 5**: 2D HRMS contour plots of C_2DBTin the 3 environmental samples (**1**- 2,8-dMDBT **2**-ethylDBT)*



***App Figure 6**: 2D HRMS contour plots of C_3 DBT in the 3 environmental samples (**1**- 2,4,7 triMDBT) on (left) coal (middle) SRM 2974a and (right) used lubricating oil.*



***App Figure 7**: 2D HRMS contour plots of C_3Ch in coal samples (**1**- triMCh).*



***App Figure 8**: 2D HRMS contour plots of C_2 Fl in the 3 environmental samples (**1**- 1,7-dimethylFluorene; other peaks are either dimethyl or ethylfluorenes) on (left) coal (middle) SRM 2974a and (right) used lubricating oil.*

App Table 1*: Amount of nmoles of PAHs (arithmetic mean ± SE, n=5) remaining in salt- and freshwater after exposure to 25 mg Imbiber beads.*[a]

Compound	Temp (^{0}C)	Amount of PAH (nmol)											
		10s	**20s**	**30s**	**40s**	**50s**	**60s**	**70s**	**80s**	**90s**	**100s**	**110s**	**120s**
Acenaphthene	**5**	1.25 ± 0.57	1.52 ± 0.45	1.31 ± 0.64	0.38 ± 0.18	0.46 ± 0.17	0.59 ± 0.26	0.92 ± 0.42	0.49 ± 0.30	0.85 ± 0.82	0.20 ± 0.18	0.24 ± 0.25	0.36 ± 0.14
	10	0.69 ± 0.07	0.78 ± 0.17	0.99 ± 0.35	0.81 ± 0.26	0.58 ± 0.21	0.86 ± 0.63	1.03 ± 0.36	0.73 ± 0.49	0.56 ± 0.51	0.47 ± 0.31	0.12 ± 0.13	0.06 ± 0.01
	15	0.65 ± 0.43	0.75 ± 0.31	0.58 ± 0.27	0.85 ± 0.26	0.92 ± 0.28	0.48 ± 0.32	0.60 ± 0.38	0.29 ± 0.14	0.30 ± 0.14	0.27 ± 0.22	0.12 ± 0.03	0.10 ± 0.06
	20	0.58 ± 0.17	0.47 ± 0.27	1.08 ± 0.11	0.29 ± 0.07	0.92 ± 0.59	0.21 ± 0.11	0.66 ± 0.18	0.57 ± 0.02	0.21 ± 0.14	0.07 ± 0.00	0.10 ± 0.02	0.08 ± 0.03
	25	1.08 ± 0.30	0.75 ± 0.39	0.78 ± 0.24	0.84 ± 0.16	0.58 ± 0.16	0.65 ± 0.26	0.22 ± 0.13	0.50 ± 0.12	0.27 ± 0.20	0.22 ± 0.13	0.39 ± 0.19	0.19 ± 0.19
	25[b]	0.60 ± 0.12	0.32 ± 0.14	0.47 ± 0.22	0.23 ± 0.08	0.26 ± 0.17	0.34 ± 0.20	0.12 ± 0.03	0.15 ± 0.09	0.12 ± 0.04	0.12 ± 0.04	0.12 ± 0.03	0.12 ± 0.05
Acenaphthylene	**5**	1.37 ± 0.60	1.65 ± 0.48	1.45 ± 0.64	0.43 ± 0.16	0.52 ± 0.16	0.64 ± 0.29	1.06 ± 0.40	0.55 ± 0.32	0.94 ± 0.86	0.63 ± 0.76	0.28 ± 0.25	0.42 ± 0.14
	10	0.78 ± 0.05	0.86 ± 0.17	1.02 ± 0.35	0.88 ± 0.27	0.63 ± 0.06	0.91 ± 0.62	1.10 ± 0.35	1.01 ± 0.79	0.65 ± 0.51	0.52 ± 0.31	0.19 ± 0.17	0.27 ± 0.23
	15	0.70 ± 0.42	1.10 ± 0.46	0.60 ± 0.26	0.88 ± 0.25	0.94 ± 0.27	0.63 ± 0.25	0.64 ± 0.39	0.34 ± 0.14	0.34 ± 0.13	0.31 ± 0.21	0.16 ± 0.03	0.14 ± 0.06
	20	0.77 ± 0.24	0.50 ± 0.26	1.18 ± 0.11	0.38 ± 0.04	1.03 ± 0.55	0.25 ± 0.10	0.83 ± 0.18	0.97 ± 0.43	0.29 ± 0.17	0.12 ± 0.00	0.14 ± 0.03	0.11 ± 0.03
	25	1.15 ± 0.27	0.79 ± 0.35	0.84 ± 0.21	0.86 ± 0.14	0.65 ± 0.16	0.72 ± 0.28	0.32 ± 0.13	0.53 ± 0.12	0.32 ± 0.19	0.26 ± 0.11	0.36 ± 0.16	0.23 ± 0.07
	25[b]	0.66 ± 0.08	0.40 ± 0.13	0.57 ± 0.21	0.30 ± 0.08	0.32 ± 0.16	0.38 ± 0.19	0.19 ± 0.04	0.24 ± 0.11	0.19 ± 0.06	0.18 ± 0.04	0.17 ± 0.03	0.17 ± 0.05
Anthracene	**5**	0.92 ± 0.44	1.15 ± 0.33	0.93 ± 0.51	0.30 ± 0.08	0.28 ± 0.11	0.43 ± 0.21	0.55 ± 0.31	0.36 ± 0.26	0.62 ± 0.65	0.16 ± 0.16	0.17 ± 0.20	0.30 ± 0.04
	10	0.63 ± 0.09	0.65 ± 0.13	0.63 ± 0.25	0.62 ± 0.16	0.53 ± 0.39	0.62 ± 0.51	0.73 ± 0.29	0.68 ± 0.61	0.29 ± 0.30	0.32 ± 0.23	0.02 ± 0.00	0.02 ± 0.01
	15	0.55 ± 0.32	0.62 ± 0.20	0.45 ± 0.24	0.57 ± 0.18	0.66 ± 0.23	0.34 ± 0.21	0.42 ± 0.28	0.18 ± 0.09	0.18 ± 0.09	0.18 ± 0.17	0.05 ± 0.02	0.04 ± 0.04

	20	0.34 ± 0.07	0.31 ± 0.15	0.99 ± 0.02	0.19 ± 0.09	0.75 ± 0.58	0.11 ± 0.07	0.55 ± 0.17	1.13 ± 1.00	0.11 ± 0.09	0.02 ± 0.00	0.03 ± 0.02	0.03 ± 0.01
	25	0.88 ± 0.24	0.64 ± 0.42	0.63 ± 0.11	0.60 ± 0.12	0.46 ± 0.17	0.45 ± 0.17	0.20 ± 0.09	0.32 ± 0.09	0.16 ± 0.16	0.13 ± 0.10	0.21 ± 0.14	0.09 ± 0.05
	25[b]	0.46 ± 0.10	0.26 ± 0.14	0.73 ± 0.42	0.13 ± 0.10	0.18 ± 0.14	0.36 ± 0.23	0.04 ± 0.01	0.06 ± 0.04	0.05 ± 0.02	0.05 ± 0.02	0.06 ± 0.02	0.05 ± 0.02
Benz(a)anthracene	**5**	0.86 ± 0.39	1.02 ± 0.23	0.86 ± 0.48	0.32 ± 0.10	0.28 ± 0.12	0.40 ± 0.18	0.51 ± 0.31	0.32 ± 0.22	0.53 ± 0.56	0.11 ± 0.11	0.14 ± 0.17	0.17 ± 0.11
	10	0.60 ± 0.11	0.63 ± 0.13	0.69 ± 0.26	0.60 ± 0.20	0.44 ± 0.37	0.29 ± 0.10	0.64 ± 0.24	0.47 ± 0.32	0.25 ± 0.28	0.30 ± 0.23	0.02 ± 0.02	0.01 ± 0.00
	15	0.69 ± 0.38	0.62 ± 0.37	0.48 ± 0.22	0.52 ± 0.15	0.59 ± 0.22	0.54 ± 0.41	0.35 ± 0.24	0.16 ± 0.09	0.15 ± 0.09	0.14 ± 0.15	0.03 ± 0.02	0.03 ± 0.03
	20	0.57 ± 0.21	0.41 ± 0.20	0.79 ± 0.03	0.14 ± 0.08	0.56 ± 0.42	0.12 ± 0.08	0.45 ± 0.11	0.28 ± 0.07	0.07 ± 0.07	0.00 ± 0.00	0.02 ± 0.02	0.03 ± 0.02
	25	0.90 ± 0.18	0.67 ± 0.36	0.61 ± 0.11	0.57 ± 0.13	0.42 ± 0.16	0.41 ± 0.16	0.13 ± 0.09	0.23 ± 0.05	0.13 ± 0.15	0.10 ± 0.09	0.16 ± 0.11	0.06 ± 0.05
	25[b]	0.44 ± 0.08	0.26 ± 0.11	0.30 ± 0.22	0.12 ± 0.10	0.13 ± 0.14	0.23 ± 0.19	0.02 ± 0.01	0.03 ± 0.03	0.03 ± 0.01	0.04 ± 0.02	0.02 ± 0.01	0.04 ± 0.04
Benzo(a)pyrene	**5**	0.81 ± 0.38	1.01 ± 0.23	0.82 ± 0.47	0.29 ± 0.11	0.24 ± 0.11	0.36 ± 0.17	0.43 ± 0.30	0.30 ± 0.20	0.50 ± 0.53	0.10 ± 0.10	0.12 ± 0.15	0.21 ± 0.03
	10	0.57 ± 0.10	0.61 ± 0.13	0.63 ± 0.25	0.57 ± 0.20	0.41 ± 0.36	0.27 ± 0.10	0.60 ± 0.23	0.38 ± 0.26	0.22 ± 0.24	0.28 ± 0.21	0.01 ± 0.01	0.01 ± 0.00
	15	0.61 ± 0.36	0.43 ± 0.17	0.42 ± 0.20	0.45 ± 0.13	0.50 ± 0.20	0.68 ± 0.76	0.29 ± 0.21	0.13 ±0.08	0.13 ± 0.08	0.12 ± 0.13	0.03 ± 0.02	0.04 ± 0.03
	20	0.50 ± 0.19	0.33 ± 0.16	0.68 ± 0.04	0.11 ± 0.07	0.48 ± 0.37	0.09 ± 0.06	0.36 ± 0.09	0.28 ± 0.05	0.06 ± 0.06	0.00 ± 0.00	0.02 ± 0.01	0.02 ± 0.01
	25	0.76 ± 0.17	0.56 ± 0.31	0.51 ± 0.08	0.47 ± 0.11	0.38 ± 0.10	0.35 ± 0.13	0.09 ± 0.08	0.24 ± 0.08	0.10 ± 0.12	0.08 ± 0.07	0.13 ± 0.09	0.05 ± 0.04
	25[b]	0.42 ± 0.07	0.23 ± 0.12	0.23 ± 0.24	0.11 ± 0.09	0.11 ± 0.12	0.20 ± 0.18	0.01 ± 0.01	0.02 ± 0.03	0.03 ± 0.02	0.03 ± 0.02	0.17 ± 0.31	0.02 ± 0.01
Benzo(b)fluoranthene	**5**	0.83 ± 0.38	1.02 ± 0.24	0.84 ± 0.48	0.31 ± 0.11	0.25 ± 0.12	0.37 ± 0.17	0.45 ± 0.30	0.30 ± 0.21	0.51 ± 0.54	0.08 ± 0.09	0.12 ± 0.15	0.30 ± 0.09
	10	0.58 ± 0.10	0.62 ± 0.12	0.62 ± 0.25	0.58 ± 0.19	0.42 ± 0.35	0.27 ± 0.10	0.60 ± 0.23	0.43 ± 0.25	0.05 ± 0.01	0.02 ± 0.00	0.01 ± 0.01	0.01 ± 0.00

	15	0.60 ± 0.35	1.10 ± 1.02	0.49 ± 0.22	0.45 ± 0.14	0.50 ± 0.20	0.44 ± 0.30	0.29 ± 0.21	0.13 ± 0.08	0.13 ± 0.08	0.12 ± 0.13	0.03 ± 0.02	0.04 ± 0.03
	20	0.58 ± 0.22	0.35 ± 0.17	0.72 ± 0.03	0.12 ± 0.07	0.50 ± 0.39	0.10 ± 0.07	0.39 ± 0.11	0.30 ± 0.07	0.06 ± 0.07	0.00 ± 0.00	0.01 ± 0.01	0.03 ± 0.01
	25	0.76 ± 0.19	0.56 ± 0.31	0.51 ± 0.09	0.48 ± 0.11	0.37 ± 0.11	0.34 ± 0.12	0.09 ± 0.08	0.24 ± 0.07	0.10 ± 0.12	0.09 ± 0.08	0.13 ± 0.09	0.05 ± 0.05
	25[b]	0.40 ± 0.06	0.22 ± 0.10	0.26 ± 0.22	0.10 ± 0.09	0.11 ± 0.12	0.19 ± 0.16	0.02 ± 0.01	0.02 ± 0.02	0.03 ± 0.01	0.03 ± 0.02	0.02 ± 0.01	0.02 ± 0.01
Benzo(ghi)perylene	**5**	0.80 ± 0.37	1.03 ± 0.23	0.81 ± 0.46	0.31 ± 0.12	0.24 ± 0.12	0.34 ± 0.17	0.48 ± 0.29	0.28 ± 0.19	0.47 ± 0.51	0.07 ± 0.09	0.12 ± 0.14	0.21 ± 0.03
	10	0.57 ± 0.07	0.62 ± 0.13	0.63 ± 0.25	0.61 ± 0.21	0.55 ± 0.37	0.57 ± 0.50	0.62 ± 0.24	0.52 ± 0.30	0.05 ± 0.00	0.02 ± 0.01	0.01 ± 0.00	0.01 ± 0.00
	15	0.61 ± 0.35	0.66 ± 0.61	0.39 ± 0.21	0.44 ± 0.13	0.49 ± 0.21	1.08 ± 1.61	0.26 ± 0.18	0.12 ± 0.07	0.12 ± 0.07	0.12 ± 0.13	0.03 ± 0.02	0.10 ± 0.16
	20	0.53 ± 0.19	0.33 ± 0.16	0.71 ± 0.05	0.11 ± 0.07	0.48 ± 0.37	0.09 ± 0.06	0.32 ± 0.13	0.30 ± 0.07	0.06 ± 0.06	0.00 ± 0.00	0.02 ± 0.01	0.03 ± 0.01
	25	0.67 ± 0.15	0.48 ± 0.26	0.45 ± 0.10	0.41 ± 0.08	0.30 ± 0.14	0.29 ± 0.11	0.08 ± 0.07	0.21 ± 0.07	0.09 ± 0.09	0.07 ± 0.06	0.11 ± 0.07	0.05 ± 0.04
	25[b]	0.31 ± 0.16	0.14 ± 0.02	0.16 ± 0.08	0.16 ± 0.14	0.13 ± 0.13	0.14 ± 0.08	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.02	0.07 ± 0.01	0.02 ± 0.01	0.02 ± 0.01
Benzo(k)fluoranthene	**5**	0.84 ± 0.39	1.04 ± 0.23	0.84 ± 0.47	0.31 ± 0.11	0.25 ± 0.12	0.37 ± 0.18	0.46 ± 0.28	0.30 ± 0.20	0.53 ± 0.57	0.08 ± 0.10	0.13 ± 0.15	0.22 ± 0.03
	10	0.60 ± 0.10	0.64 ± 0.14	0.66 ± 0.28	0.59 ± 0.21	0.51 ± 0.34	0.53 ± 0.46	0.62 ± 0.24	0.49 ± 0.28	0.05 ± 0.01	0.02 ± 0.01	0.01 ± 0.01	0.01 ± 0.00
	15	0.67 ± 0.42	0.68 ± 0.30	0.49 ± 0.21	0.49 ± 0.14	0.54 ± 0.24	0.35 ± 0.14	0.32 ± 0.23	0.14 ± 0.08	0.14 ± 0.08	0.13 ± 0.14	0.03 ± 0.02	0.03 ± 0.03
	20	0.65 ± 0.23	0.38 ± 0.18	0.81 ± 0.05	0.13 ± 0.08	0.53 ± 0.40	0.11 ± 0.07	0.43 ± 0.12	0.25 ± 0.12	0.07 ± 0.07	0.00 ± 0.00	0.02 ± 0.02	0.03 ± 0.01
	25	0.84 ± 0.22	0.63 ± 0.34	0.54 ± 0.10	0.51 ± 0.12	0.39 ± 0.15	0.37 ± 0.13	0.10 ± 0.09	0.27 ± 0.08	0.11 ± 0.13	0.09 ± 0.07	0.15 ± 0.10	0.06 ± 0.05
	25[b]	0.43 ± 0.07	0.26 ± 0.11	0.27 ± 0.28	0.11 ± 0.10	0.13 ± 0.14	0.11 ± 0.06	0.02 ± 0.01	0.03 ± 0.03	0.04 ± 0.01	0.04 ± 0.02	0.02 ± 0.01	0.02 ± 0.01
Chrysene	**5**	0.89 ± 0.41	1.09 ± 0.23	0.88 ± 0.50	0.33 ± 0.11	0.28 ± 0.13	0.42 ± 0.19	0.54 ± 0.30	0.33 ± 0.22	0.55 ± 0.58	0.09 ± 0.10	0.14 ± 0.17	0.20 ± 0.07

	10	0.64 ± 0.12	0.68 ± 0.14	0.71 ± 0.28	0.64 ± 0.21	0.47 ± 0.39	0.60 ± 0.52	0.67 ± 0.25	0.58 ± 0.33	0.06 ± 0.01	0.02 ± 0.01	0.01 ± 0.01	0.01± 0.00
	15	0.70 ± 0.39	0.61 ± 0.17	0.50 ± 0.22	0.54 ± 0.16	0.60 ± 0.24	0.36 ± 0.16	0.35 ± 0.25	0.16 ± 0.09	0.15 ± 0.09	0.15 ± 0.16	0.03 ± 0.02	0.04 ± 0.03
	20	0.72 ± 0.25	0.44 ± 0.21	0.86 ± 0.04	0.14 ± 0.09	0.61 ± 0.45	0.12 ± 0.08	0.48 ± 0.13	0.36 ± 0.07	0.08 ± 0.08	0.00 ± 0.00	0.02 ± 0.02	0.03 ± 0.01
	25	0.96 ± 0.22	0.67 ± 0.36	0.62 ± 0.11	0.57 ± 0.13	0.43 ± 0.15	0.42 ± 0.15	0.12 ± 0.10	0.30 ± 0.09	0.13 ± 0.15	0.11 ± 0.09	0.16 ± 0.11	0.06 ± 0.05
	25[b]	0.48 ± 0.06	0.28 ± 0.13	0.27 ± 0.28	0.13 ± 0.11	0.15 ± 0.16	0.11 ± 0.06	0.02 ± 0.01	0.03 ± 0.03	0.03 ± 0.02	0.03 ± 0.02	0.03 ± 0.01	0.03 ± 0.01
Dibenz(ah)anthracene	**5**	0.82 ± 0.39	1.04 ± 0.23	0.83 ± 0.48	0.29 ± 0.12	0.24 ± 0.11	0.36 ± 0.17	0.47 ± 0.29	0.28 ± 0.19	0.50 ± 0.54	0.07 ± 0.08	0.12 ± 0.14	0.18 ± 0.04
	10	0.57 ± 0.11	0.61 ± 0.13	0.60 ± 0.24	0.56 ± 0.20	0.40 ± 0.35	0.26 ± 0.09	0.60 ± 0.23	0.37 ± 0.27	0.05 ± 0.00	0.02 ± 0.01	0.01 ± 0.00	0.00 ± 0.00
	15	0.60 ± 0.37	1.15 ± 0.78	0.40 ± 0.20	0.45 ± 0.14	0.49 ± 0.21	0.30 ± 0.13	0.28 ± 0.20	0.12± 0.07	0.12 ± 0.08	0.12 ± 0.13	0.03 ± 0.02	0.06 ±0.07
	20	0.60 ± 0.22	0.34 ± 0.17	0.75 ± 0.03	0.12 ± 0.07	0.50 ± 0.36	0.10 ± 0.07	0.37 ± 0.10	0.53 ± 0.34	0.06 ± 0.06	0.00 ± 0.00	0.01 ± 0.01	0.02 ± 0.01
	25	0.76 ± 0.16	0.56 ± 0.29	0.50 ± 0.09	0.47 ± 0.12	0.49 ± 0.21	0.33 ± 0.12	0.09 ± 0.07	0.22 ± 0.07	0.10 ± 0.11	0.08 ± 0.07	0.13 ± 0.08	0.05 ± 0.04
	25[b]	0.37 ± 0.06	0.21 ± 0.08	0.21 ± 0.25	0.10 ± 0.08	0.11 ± 0.11	0.09 ± 0.05	0.01 ± 0.01	0.02 ± 0.02	0.03 ± 0.01	0.02 ± 0.02	0.02 ± 0.01	0.03 ± 0.03
Fluoranthene	**5**	1.01 ± 0.47	1.14 ± 0.28	0.98 ± 0.54	0.59 ± 0.36	0.34 ± 0.15	0.48 ± 0.21	0.60 ± 0.34	0.39 ± 0.26	0.64 ± 0.66	0.11 ± 0.13	0.17 ± 0.20	0.24 ± 0.08
	10	0.70 ± 0.12	0.73 ± 0.15	0.76 ± 0.28	0.70 ±0.24	0.55 ± 0.43	0.35 ± 0.13	0.74 ± 0.27	0.58 ± 0.32	0.07 ± 0.01	0.03 ± 0.01	0.01 ± 0.00	0.01 ± 0.00
	15	0.75 ± 0.40	0.78 ± 0.24	0.50 ± 0.27	0.60 ± 0.18	0.68 ± 0.25	0.36 ± 0.20	0.41 ± 0.28	0.20 ± 0.10	0.18 ± 0.11	0.18 ± 0.18	0.05 ± 0.03	0.04 ± 0.04
	20	0.81 ± 0.29	0.47 ± 0.23	0.98 ± 0.04	0.18 ± 0.09	0.70 ± 0.52	0.14 ± 0.09	0.55 ± 0.17	0.41 ± 0.08	0.10 ± 0.09	0.01 ± 0.00	0.03 ± 0.02	0.04 ± 0.02
	25	1.04 ± 0.25	0.77 ± 0.42	0.70 ± 0.12	0.68 ± 0.15	0.51 ± 0.18	0.48 ± 0.18	0.14 ± 0.11	0.35 ± 0.10	0.15 ± 0.17	0.14 ± 0.11	0.22 ± 0.14	0.09 ± 0.06
	25[b]	0.52 ± 0.04	0.30 ± 0.15	0.68 ± 0.27	0.14 ± 0.12	0.18 ± 0.15	0.37 ± 0.21	0.03 ± 0.01	0.04 ± 0.04	0.05 ± 0.02	0.05 ± 0.02	0.04 ± 0.02	0.04 ± 0.01

Fluorene	**5**	1.12 ± 0.51	1.35 ± 0.38	1.14 ± 0.57	0.39 ± 0.13	0.40 ± 0.15	0.54 ± 0.25	1.06 ± 0.48	0.44 ± 0.27	0.75 ± 0.74	0.17 ± 0.15	0.22 ± 0.23	0.38 ± 0.05
	10	0.69 ± 0.03	0.76 ± 0.13	0.86 ± 0.31	0.76 ± 0.25	0.61 ± 0.32	0.76 ± 0.59	0.90 ± 0.33	0.83 ± 0.45	0.14 ± 0.03	0.07 ± 0.00	0.04 ± 0.01	0.04 ± 0.01
	15	0.66 ± 0.42	0.67 ± 0.29	0.65 ± 0.26	0.72 ± 0.22	0.80 ± 0.27	0.42 ± 0.26	0.51 ± 0.33	0.24 ± 0.12	0.24 ± 0.12	0.23 ± 0.18	0.09 ± 0.03	0.08 ± 0.05
	20	0.73 ± 0.22	0.45 ± 0.23	1.08 ± 0.04	0.27 ± 0.08	0.87 ± 0.58	0.19 ± 0.10	1.24 ± 1.21	0.51 ± 0.06	0.17 ± 0.12	0.05 ± 0.00	0.08 ± 0.02	0.07 ± 0.03
	25	1.07 ± 0.28	0.75 ± 0.43	0.77 ± 0.15	0.79 ± 0.15	0.57 ± 0.18	0.58 ± 0.22	0.21 ± 0.12	0.43 ± 0.10	0.23 ± 0.19	0.20 ± 0.13	0.34 ± 0.19	0.16 ± 0.05
	25[b]	0.53 ± 0.09	0.30 ± 0.14	0.63 ± 0.28	0.20 ± 0.09	0.22 ± 0.15	0.24 ± 0.05	0.08 ± 0.02	0.12 ± 0.07	0.09 ± 0.03	0.10 ± 0.03	0.10 ± 0.02	0.09 ± 0.04
Indeno(123-cd)pyrene	**5**	0.80 ± 0.37	0.99 ± 0.23	0.84 ± 0.50	0.28 ± 0.11	0.24 ± 0.11	0.36 ± 0.17	0.49 ± 0.29	0.28 ± 0.19	0.50 ± 0.54	0.07 ± 0.09	0.12 ± 0.15	0.21 ± 0.03
	10	0.56 ± 0.11	0.60 ± 0.13	0.61 ± 0.26	0.56 ± 0.19	0.42 ± 0.36	0.51 ± 0.46	0.58 ± 0.22	0.68 ± 0.53	0.18 ± 0.20	0.02 ± 0.01	0.01 ± 0.01	0.00 ± 0.00
	15	0.57 ± 0.35	0.53 ± 0.68	0.43 ± 0.19	0.43 ± 0.13	0.49 ± 0.20	0.30 ± 0.14	0.28 ± 0.20	0.13 ± 0.07	0.12 ± 0.07	0.12 ± 0.13	0.03 ± 0.02	0.04 ± 0.03
	20	0.54 ± 0.19	0.31 ± 0.14	0.65 ± 0.07	0.11 ± 0.07	0.45 ± 0.34	0.09 ± 0.06	0.34 ± 0.09	0.21 ± 0.07	0.06 ± 0.06	0.00 ± 0.00	0.01 ± 0.01	0.02 ± 0.01
	25	0.69 ± 0.16	0.50 ± 0.27	0.46 ± 0.09	0.44 ± 0.09	0.35 ± 0.08	0.31 ± 0.10	0.08 ± 0.07	0.22 ± 0.07	0.10 ± 0.11	0.08 ± 0.07	0.12 ± 0.08	0.05 ± 0.04
	25[b]	0.36 ± 0.07	0.22 ± 0.08	0.20 ± 0.22	0.09 ± 0.09	0.11 ± 0.11	0.10 ± 0.5	0.02 ± 0.01	0.02 ± 0.03	0.03 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.03 ± 0.01
Naphthalene	**5**	1.25 ± 0.61	1.48 ± 0.63	1.29 ± 0.59	0.36 ± 0.14	0.57 ± 0.14	0.79 ± 0.47	0.88 ± 0.31	0.47 ± 0.19	0.91 ± 0.74	0.34 ± 0.18	0.34 ± 0.22	0.67 ± 0.03
	10	0.40 ± 0.18	0.77 ± 0.21	1.03 ± 0.36	0.78 ± 0.26	0.52 ± 0.13	0.94 ± 0.37	1.00 ± 0.28	0.87 ± 0.45	0.68 ± 0.27	0.60 ± 0.20	0.24 ± 0.03	0.27 ± 0.01
	15	0.66 ± 0.26	1.08 ± 0.00	0.66 ±0.28	1.13 ± 0.33	1.01 ± 0.30	0.79 ± 0.20	0.92 ± 0.55	0.54 ± 0.19	0.54 ± 0.17	0.51 ± 0.29	0.34 ± 0.02	0.33 ± 0.09
	20	0.78 ± 0.19	0.53 ± 0.21	1.30 ± 0.26	0.53 ± 0.10	1.17 ± 0.33	0.35 ± 0.08	1.05 ± 0.11	1.07 ± 0.09	0.83 ± 0.68	0.28 ± 0.01	0.23 ± 0.04	0.19 ± 0.04
	25	1.04 ± 0.23	0.70 ± 0.08	0.77 ± 0.19	0.89 ± 0.34	0.64 ± 0.06	0.82 ± 0.35	0.76 ± 0.70	0.72 ± 0.15	0.43 ± 0.15	0.65 ± 0.48	0.73 ± 0.51	0.44 ± 0.15

	25[b]	0.73 ± 0.16	0.52 ± 0.10	0.45 ± 0.25	0.46 ± 0.06	0.52 ± 0.16	0.57 ± 0.20	0.42 ±0.08	0.46 ± 0.16	0.35 ± 0.08	0.31 ± 0.05	0.51 ± 0.39	0.38 ± 0.20
Phenanthrene	**5**	1.14 ± 0.52	1.31 ± 0.33	1.10 ± 0.58	0.53 ± 0.20	0.40 ± 0.16	0.55 ± 0.23	0.73 ± 0.37	0.45 ± 0.29	0.75 ± 0.76	0.17 ± 0.14	0.21 ± 0.23	0.36 ± 0.05
	10	0.77 ± 0.12	0.82 ± 0.16	0.84 ± 0.29	0.79 ± 0.26	0.68 ± 0.47	0.75 ± 0.61	0.87 ± 0.31	0.84 ± 0.48	0.11 ± 0.01	0.05 ± 0.01	0.03 ± 0.01	0.03 ± 0.01
	15	0.81 ± 0.46	1.15 ± 0.62	0.54 ± 0.31	0.70 ± 0.22	0.80 ± 0.28	0.42 ± 0.25	0.48 ± 0.31	0.24 ± 0.11	0.22 ± 0.11	0.22 ± 0.19	0.07 ± 0.03	0.06 ± 0.05
	20	0.89 ± 0.29	0.52 ± 0.24	1.17 ± 0.02	0.24 ± 0.10	0.84 ± 0.60	0.17 ± 0.10	0.65 ± 0.20	0.91 ± 0.62	0.14 ± 0.11	0.03 ± 0.00	0.06 ± 0.02	0.06 ± 0.02
	25	1.08 ± 0.25	0.85 ± 0.50	0.79 ± 0.12	0.78 ± 0.15	0.57 ± 0.20	0.54 ± 0.19	0.17 ± 0.14	0.41 ± 0.12	0.20 ± 0.19	0.18 ± 0.12	0.29 ± 0.18	0.13 ± 0.05
	25[b]	0.53 ± 0.09	0.30 ± 0.16	0.93 ± 0.52	0.18 ± 0.11	0.21 ± 0.17	0.41 ± 0.24	0.06 ± 0.01	0.07 ± 0.05	0.07 ± 0.02	0.07 ± 0.02	0.08 ± 0.02	0.06 ± 0.02
Pyrene	**5**	1.00 ± 0.46	1.14 ± 0.28	0.95 ± 0.39	0.61 ± 0.39	0.34 ± 0.15	0.47 ± 0.21	0.64 ± 0.32	0.38 ± 0.25	0.62 ± 0.64	0.11 ± 0.13	0.17 ± 0.20	0.25 ± 0.08
	10	0.71 ± 012	0.74 ± 0.15	0.75 ± 0.28	0.70 ± 0.14	0.54 ± 0.43	0.66 ± 0.55	0.74 ± 0.27	0.59 ± 0.32	0.07 ± 0.01	0.03 ± 0.01	0.01 ± 0.00	0.01 ± 0.00
	15	0.74 ± 0.41	1.12 ± 1.02	0.68 ± 0.25	0.60 ± 0.18	0.50 ± 0.25	0.33 ± 0.18	0.40 ± 0.28	0.19 ± 0.10	0.18 ± 0.11	0.18 ± 0.18	0.04 ± 0.03	0.05 ± 0.04
	20	0.80 ± 0.29	0.46 ± 0.22	0.97 ± 0.03	0.17 ± 0.09	0.70 ± 0.53	0.14 ± 0.09	1.02 ± 0.98	0.69 ± 0.40	0.10 ± 0.09	0.01 ± 0.00	0.03 ± 0.02	0.04 ± 0.02
	25	1.00 ± 0.22	0.75 ± 0.41	0.68 ± 0.11	0.66 ± 0.14	0.50 ± 0.18	0.47 ± 0.18	0.13 ± 0.11	0.33 ± 0.09	0.15 ± 0.16	0.13 ± 0.10	0.21 ± 0.14	0.09 ± 0.06
	25[b]	0.50 ± 0.07	0.28 ± 0.14	0.66 ± 0.28	0.14 ± 0.11	0.17 ± 0.14	0.26 ± 0.13	0.03 ± 0.01	0.04 ± 0.09	0.04 ± 0.02	0.04 ± 0.02	0.04 ± 0.02	0.03 ± 0.01

[a] Our kinetic data was fitted to both a zero- and first order reaction rates. By definition, for a zero-order reaction, the rate constant is independent of the concentration and has units of M s^{-1}. To determine if absorption obeyed zero-order kinetics we plotted the change in the number of moles of each PAH against time (s). For a first-order reaction, the rate is proportional to the concentration and the rate constant has units of s^{-1}. If the plot of the natural log of concentration against time (s) produces a straight-line then the reaction is first-order.

[b] Freshwater data

App Table 2: *Zero-order uptake rates (nmoles s^{-1}) parameters for PAHs studied in saltwater and freshwater.*

Compound	Slope (nmoles s^{-1})	Standard error	p-value	R^2-value
Naphthalene	0.0043	0.0008	0.0002	0.7313
Acenaphthylene	0.0065	0.0007	<0.0001	0.8836
Acenaphthene	0.0064	0.0006	<0.0001	0.9064
Fluorene	0.0066	0.0009	<0.0001	0.8333
Anthracene	0.0054	0.0007	<0.0001	0.8379
Phenanthrene	0.0076	0.0007	<0.0001	0.9097
Chrysene	0.0063	0.0005	<0.0001	0.9387
Fluoranthene	0.0070	0.0005	<0.0001	0.9386
Pyrene	0.0071	0.0007	<0.0001	0.9111
Benz[a]anthracene	0.0058	0.0004	<0.0001	0.9493
Benzo[k]fluoranthene	0.0059	0.0005	<0.0001	0.9369
Benzo[a]pyrene	0.0051	0.0004	<0.0001	0.9495
Benzo[b]fluoranthene	0.0057	0.0005	<0.0001	0.9181
Dibenzo[ah]anthracene	0.0056	0.0007	<0.0001	0.8595
Benzo[ghi]perylene	0.0052	0.0004	<0.0001	0.9261
Indeno[123-cd]pyrene	0.0050	0.0004	<0.0001	0.9293

App Table 3a. *Relative response factors (RRF), average relative response factors (ARRF) and SD for native PAHs in 1D.*

1D

Analyte	**RRF**						**ARRF**	**SD**
	10	**200**	**400**	**600**	**800**	**1000**		
Acenaphthene	1.25	1.29	1.32	1.30	1.30	1.37	1.31	0.03
Acenaphthylene	1.18	1.19	1.20	1.19	1.22	1.27	1.21	0.03
Anthracene	1.05	1.09	1.05	1.05	1.06	1.08	1.06	0.01
Benz(*a*)anthracene	1.26	1.41	1.28	1.32	1.39	1.39	1.34	0.05
Benzo(*a*)pyrene	1.83	1.87	1.85	1.92	1.83	2.02	1.89	0.04
Benzo(*b*)fluoranthene	1.51	1.49	1.51	1.51	1.53	1.51	1.51	0.01
Benzo(*ghi*)perylene	1.22	1.64	1.29	1.32	1.34	1.35	1.36	0.02
Benzo(*k*)fluoranthene	1.22	1.21	1.26	1.21	1.24	1.25	1.23	0.02
Chrysene	1.04	1.02	1.03	1.09	1.03	1.10	1.05	0.03
Dibenz(*a,h*)anthracene	1.77	1.46	1.49	1.59	1.70	1.69	1.62	0.08
Fluoranthene	1.24	1.23	1.32	1.28	1.33	1.36	1.29	0.04
Fluorene	1.40	1.49	1.46	1.50	1.48	1.55	1.48	0.03
Indeno(*1,2,3-c,d*)pyrene	1.42	1.40	1.44	1.45	1.42	1.41	1.42	0.01
Naphthalene	1.25	1.21	1.22	1.22	1.26	1.24	1.23	0.01
Phenanthrene	1.18	1.21	1.18	1.22	1.30	1.33	1.24	0.05
Pyrene	1.49	1.55	1.52	1.58	1.58	1.65	1.56	0.04

App Table 3b. *Relative response factors (RRF), average relative response factors (ARRF) and SD for native PAHs 2D.*

2D

Analyte	**RRF**						**ARRF**	**SD**
	10	**200**	**400**	**600**	**800**	**1000**		
Acenaphthene	1.36	1.40	1.44	1.39	1.36	1.33	1.38	0.03
Acenaphthylene	1.23	1.26	1.31	1.27	1.33	1.34	1.29	0.03
Anthracene	0.85	1.05	1.10	1.09	1.09	1.07	1.04	0.09
Benz(*a*)anthracene	0.96	1.35	1.34	1.35	1.37	1.34	1.28	0.13
Benzo(*a*)pyrene	1.05	1.65	1.73	1.89	1.89	1.91	1.69	0.20
Benzo(*b*)fluoranthene	1.34	1.61	1.68	1.97	1.94	2.01	1.76	0.15
Benzo(*ghi*)perylene	1.01	1.63	2.23	1.95	1.88	2.01	1.78	0.09
Benzo(*k*)fluoranthene	1.11	1.26	1.53	1.37	1.65	1.63	1.42	0.15
Chrysene	0.69	1.21	1.26	1.28	1.27	1.26	1.16	0.20
Dibenz(*a,h*)anthracene	0.90	1.27	1.57	1.62	1.60	1.63	1.43	0.20
Fluoranthene	1.27	1.33	1.44	1.44	1.41	1.46	1.39	0.05
Fluorene	1.38	1.61	1.54	1.50	1.61	1.62	1.54	0.06
Indeno(*1,2,3-c,d*)pyrene	0.88	1.06	1.21	1.20	1.24	1.28	1.14	0.13
Naphthalene	1.16	1.21	1.21	1.15	1.22	1.09	1.17	0.04
Phenanthrene	1.20	1.38	1.41	1.45	1.44	1.44	1.39	0.07
Pyrene	1.37	1.87	1.89	1.84	1.87	1.82	1.78	0.11

App Table 4a. *Certified and Measured APAH and PAH concentrations reported to include Measurement Uncertainty for SRM 2974a (1D)*

Analyte	**Measured SRM 2974a Mass Fraction (ng/g)**	**U_{IP}[1]**	**Bias[2]**	**U_{bias}[3]**	**95% Confidence interval for Bias[4]**		**Combined MU (U_c)[5]**	**Certified SRM 2974a Mass Fraction (ng/g)**	**Certified MU for SRM 2974a[6]**
					lower limit	**upper limit**			
1-Methylphenanthrene	17.28	0.88	-0.32	0.89	-0.21	1.46	± 1.2	17.6	± 1.6
2-Methylphenanthrene	25.36	1.97	-2.84	1.57	-5.98	0.30	± 2.5	28.2	± 2.6
3-Methylphenanthrene	22.18	0.91	-1.92	0.81	-3.54	-0.30	± 1.2	24.1	± 1.4
9-Methylphenanthrene	16.33	0.58	0.43	0.70	-0.97	1.83	± 0.9	15.9	± 1.3
1-Methylpyrene	9.96	0.95	-0.73	0.59	-1.92	0.46	± 1.1	10.69	± 0.83
4-Methylpyrene	17.52	1.39	-2.25	0.76	-3.78	-0.73	± 1.6	19.77	± 0.89
Fluoranthene	203.60	12.95	-83.40	17.96	-119.32	-47.48	± 22.1	287	± 34
Phenanthrene	60.821	3.08	-13.59	2.72	-19.04	-8.14	± 4.1	74.4	± 4.7
Pyrene	117.50	5.59	-48.50	10.79	-70.08	-26.91	± 12.2	166	± 21
Benz(*a*)anthracene	16.45	2.11	-14.65	2.17	-18.98	-10.32	± 3.0	31.1	± 3.9
Benzo(*b*)fluoranthene	30.75	2.44	-10.75	1.34	-13.44	-8.07	± 3.2	41.5	± 2.6
Benzo(*k*)fluoranthene	21.45	2.44	2.50	1.09	0.32	4.69	± 2.9	18.95	± 0.54
Benzo(*a*)pyrene	6.35	0.67	-3.38	0.37	-4.12	-2.64	± 0.8	9.73	± 0.43
Benzo(*ghi*)perylene	14.88	1.34	-8.82	1.04	-10.90	-6.74	± 1.70	23.7	± 2.2

App Table 4b. *Certified and Measured APAH and PAH concentrations reported to include Measurement Uncertainty for SRM 2974a (2D)*

Analyte	Measured SRM 2974a Mass Fraction (ng/g)	U_{IP}[1]	Bias[2]	U_{bias}[3]	95% Confidence interval for Bias[4]		Combined MU (U_c)[5]	Certified SRM 2974a Mass Fraction (ng/g)	Certified MU for SRM 2974a[6]
					lower limit	upper limit			
1-Methylphenanthrene	16.46	2.50	-1.14	1.38	-3.89	1.61	± 2.9	17.6	± 1.6
2-Methylphenanthrene	27.14	3.05	-1.06	1.88	-4.83	2.70	± 3.6	28.2	± 2.6
3-Methylphenanthrene	26.77	3.70	2.67	1.79	-0.92	6.26	± 4.1	24.1	± 1.4
9-Methylphenanthrene	17.49	1.34	1.59	0.88	-0.17	3.36	± 1.6	15.9	± 1.3
1-Methylpyrene	8.35	2.35	-2.34	1.13	-4.60	-0.08	± 2.6	10.69	± 0.83
4-Methylpyrene	18.83	3.35	-0.94	1.56	-4.07	2.18	± 3.7	19.77	± 0.89
Fluoranthene	216.80	18.52	-70.20	18.91	-108.03	-32.38	± 26.5	287	± 34
Phenanthrene	58.66	1.21	-15.74	2.41	-20.56	-10.92	± 2.7	74.4	± 4.7
Pyrene	109.43	2.92	-56.57	10.58	-77.73	-35.41	± 11	166	± 21
Benz(*a*)anthracene	17.26	0.64	-13.84	1.97	-17.78	-9.90	± 2.1	31.1	± 3.9
Benzo(*b*)fluoranthene	31.67	2.17	-9.83	1.01	-11.84	-7.81	± 2.4	41.5	± 2.6
Benzo(*k*)fluoranthene	17.56	0.86	-1.39	0.39	-2.16	-0.62	± 1.0	18.95	± 0.54
Benzo(*a*)pyrene	3.44	0.66	-6.29	0.37	-7.02	-5.55	± 0.8	9.73	± 0.43
Benzo(*ghi*)perylene	10.18	1.29	-13.52	1.03	-15.57	-11.46	± 1.7	23.7	± 2.2

[1] The uncertainty from intermediate precision was estimated through the use of the one-way ANOVA table (refer to Equation 10)

[2] Bias was calculated as the difference between laboratory obtained value for the SRM2974a and the certified SRM2974a value

[3] The uncertainty of the bias was determined by combining the uncertainty from the laboratory estimate of the bias and the uncertainty from the certified reference number (refer to Equation 13)

[4] 95% confidence interval for the bias includes lower and upper limits which were estimated as Bias $\pm 2 \times U_{bias}$

[5] The uncertainty listed with each compound for SRM 2974a is the expanded uncertainty about the mean with the coverage factor of 2. The combined uncertainty includes uncertainty of the bias and intermediate precision.

[6] The uncertainty listed with each value is an expanded uncertainty about the mean, with coverage factor 2 (95% confidence), calculated by combining a between-method variance incorporating inter-method bias with a pooled within method variance following ISO/NIST Guide to Expression of Uncertainty in Measurement (SRM 2974a CoA).

App Table 5. *Between day and within day repeatability for target analytes.*

Analyte	**Repeatability (% RSD) (One-D)**		**Repeatability (% RSD) (Two-D)**	
	Between day	**Within day**	**Between day**	**Within day**
1-Methylphenanthrene	5.07	5.07	10.28	0.11
2-Methylphenanthrene	4.21	6.51	4.34	10.35
3-Methylphenanthrene	4.11	4.29	6.83	11.99
9-Methylphenanthrene	3.57	3.10	7.68	7.68
1-Methylpyrene	7.48	5.93	22.62	16.73
4-Methylpyrene	7.06	3.59	7.10	16.30
Fluoranthene	6.36	6.36	8.54	8.54
Phenanthrene	4.18	2.87	1.42	1.49
Pyrene	4.54	1.43	0.81	2.54
Benz(*a*)anthracene	5.89	11.39	1.89	3.16
Benzo(*b*)fluoranthene	6.01	7.42	3.10	6.12
Benzo(*k*)fluoranthene	11.93	11.93	4.91	4.91
Benzo(*a*)pyrene	10.62	10.62	19.29	14.58
Benzo(*ghi*)perylene	7.27	5.32	5.79	11.23

App Table 6*: Concentrations of APACs (ng/g) in SRM 2974a identified in this study*

	1D	**2D**
Compound name	**Conc. (ng/g)**	**Conc. (ng/g)**
2-methyl naphthalene	3.96 ± 0.60	5.02 ± 1.09
1-methyl naphthalene	2.75 ± 0.37	3.32 ± 0.73
2,6-dimethyl naphthalene	3.70 ± 0.32	3.63 ± 1.55
1,6-dimethyl naphthalene	6.26 ± 0.71	4.64 ± 1.51
2,3,5/1,6,7-trimethylnaphthalene	1.58 ± 0.41	1.22 ± 0.13
1,4,6,7-tetramethylnaphthalene	1.93 ± 0.25	1.39 ± 0.30
1-methylfluorene	2.31 ± 0.38	2.68 ± 1.26
1,2,5,6-tetramethylnaphthalene	1.00 ± 0.17	1.08 ± 0.33
Dibenzothiophene	3.26 ± 0.56	2.16 ± 1.95
1,7-dimethylfluorene	1.82 ± 0.29	0.96 ± 0.62
4-methyldibenzothiophene	6.65 ± 0.76	5.75 ± 0.95
2,8-dimethyldibenzothiophene	2.37 ± 0.38	1.53 ± 0.47
3,6-dimethylphenanthrene	12.20 ± 1.71	11.10 ± 1.12
2,6-dimethylphenanthrene	7.76 ± 1.08	8.74 ± 1.88
1,3-dimethyl phenanthrene	51.36 ± 5.09	50.49 ± 5.36
1,7-dimethyl phenanthrene	10.80 ± 1.61	10.11 ± 1.12
1,8-dimethyl phenanthrene	5.20 ± 0.73	4.49 ± 0.82
2,4,7-trimethylbenzothiophene	2.19 ± 0.58	1.11 ± 0.05
1,2,6-trimethylphenanthrene	4.66 ± 0.82	3.18 ± 0.16
1/3-methyl fluoranthene	13.83 ± 1.53	8.98 ± 2.56
1,2,6,9-tetramethylphenanthrene	1.31 ± 0.11	0.91 ± 0.67
3-methyl chrysene	16.02 ± 2.02	14.93 ± 1.26
2-methyl chrysene	5.39 ± 0.33	4.92 ± 0.36
6-methyl chrysene	1.41 ± 0.07	1.35 ± 0.15

App Table 7: *Response factor of five dimethylphenanthrene standards.*

Compound Name	Response Factor
3, 6- dimethylphenanthrene	1.14
2, 6- dimethylphenanthrene	1.33
1, 3- dimethylphenanthrene	1.05
1, 7- dimethylphenanthrene	1.10
1, 8- dimethylphenanthrene	1.01

ABBREVIATIONS

N-Naphthalene; Ph/An- Phenanthrene/Anthracene; MPh- Methyl phenanthrene; DMPh- dimethyl phenanthrene; EPh- ethylphenanthrene; DBT- Dibenzothiophene; MN- Methylnaphthalene; Py/Flu – Pyrene/Fluoranthene ;Fl – Fluorene; Ch – Chrysene

App Table 8*. Nomenclature, EI fragmentation patterns, mean measured experimental mass and mean mass accuracy of the HPAC standards determined using GC-HRTOF-MS.*

Compound	r_t (min)[a]	Fragmentation pattern	DBE[b]	Theoretical mass (amu)[c]	Mean measured mass ± SD (amu)[d]	Mean mass accuracy ± SD (ppm)
5-Bromo-anthracene	12.21	$M^{+\bullet}$ $[M–Br]^+$	8	231.9882 **153.0699**	231.9880±0.0001 153.0698±0.0001	-0.8046±0.3193 -0.6141±0.2697
2-Bromo-fluorene	13.49	$M^{+\bullet}$ $[M–Br]^+$	9	243.9882 **165.0699**	243.9879±0.0001 165.0698±0.0001	-1.3935±0.5249 -0.6179±0.1931
9-Chloro-Phenanthrene	14.88	$M^{+\bullet}$ $[M–HBr]^{+\bullet}$	10	212.0387 176.0621	212.0386±0.0001 176.0620±0.0001	-0.6052±0.4402 -0.1515±0.5212
1-Chloro-Anthracene	15.04	$M^{+\bullet}$ $[M–HCl]^{+\bullet}$	10	**212.0387** 176.0621	212.0386±0.0001 176.0620±0.0001	-0.5502±0.4075 -0.1515±0.4827
9-Chloro-Anthracene	15.20	$M^{+\bullet}$ $[M–HCl]^{+\bullet}$	10	**212.0387** 176.0621	212.0386±0.0001 176.0620±0.0001	-0.7310±0.4380 -0.3029±0.2757
3-Bromo Phenanthrene	16.63	$M^{+\bullet}$ $[M–HBr]^{+\bullet}$	10	255.9882 **176.0621**	255.9880±0.0001 176.0620±0.0001	-0.7617±0.5278 -0.0568±0.3921
9-Bromo Phenanthrene	16.90	$M^{+\bullet}$ $[M–HBr]^{+\bullet}$	10	255.9882 **176.0621**	255.9880±0.0001 176.0620±0.0001	-0.7292±0.4217 -0.3029±0.1958
1,5-Chloro Anthracene	18.68	$M^{+\bullet}$ $[M–2Cl]^{+\bullet}$	10	**245.9998** 176.0621	245.9995±0.0001 176.0619±0.0001	-0.9350±0.3736 -0.8520±0.2927
2,7-dichloro Fluorene	19.76	$M^{+\bullet}$ $[M–Br-HBr]^+$	9	321.8987 **163.0542**	323.8962±0.0002 163.0542±0.0001	-1.7289±0.5996 0.1838±0.2877
1-Chloro-Pyrene	21.63	$M^{+\bullet}$ $[M–Cl]^+$	12	**236.0387** 201.0699	236.0386±0.0001 201.0698±0.0001	-0.6849±0.5021 -1.8319±0.2287
9,10-dibromo-Anthracene	23.59	$M^{+\bullet}$ $[M–2Br]^{+\bullet}$	10	335.8967 **176.0621**	335.8964±0.0001 176.0621±0.0001	-1.1015±0.2909 0.2130±0.2389
9,10-dibromo-phenanthrene	23.86	$M^{+\bullet}$ $[M–2Br]^{+\bullet}$	10	335.8967 **176.0621**	335.8964±0.0001 176.0621±0.0001	-1.1015±0.2909 0.2130±0.2389
1-Bromo	23.87	$M^{+\bullet}$	12	279.9882	279.9880±0.0002	1.0715±0.7805

Pyrene		$[M–Br]^+$		**201.0699**	201.0696±0.0001	-1.2433±0.4813
7-Chloro-benz (a)anthracene	26.99	$M^{+\bullet}$ $[M–HCl]^{+\bullet}$	13	**262.0544** 226.0777	262.0541±0.0002 226.0778±0.0001	-0.9387±0.4739 0.4512±0.1910
7-Bromo-benz (a)anthracene	28.60	$M^{+\bullet}$ $[M–HBr]^{+\bullet}$	13	306.0039 **226.0777**	306.0034±0.0001 226.0776±0.0002	-1.3970±0.4726 -0.5861±0.7284
7,12-dichloro-benz (a)anthracene	29.53	$M^{+\bullet}$ $[M–2Cl]^{+\bullet}$	13	**296.0154** 226.0777	296.0150±0.0006 226.0777±0.0002	-1.5033±1.9878 -0.0111±0.4327

[a]r_t = retention times (mins.); [b]DBE=double bond equivalence; [c]m/z value in bold signifies the base peak in the EI full scan mass spectra; [d]mean and standard deviation (SD) is of 5 replicate injections of the standard.

App Table 9: *List of analytes as indicated in Figure 7.3*

	IUPAC Name	Acronym	Source
1	1-chloropyrene	1-Cl-Pyr	Cambridge Isotope
2	7-bromobenz[a]anthracene	7-Br-BaA	Cambridge Isotope
3	7-chlorobenz[a]anthracene	7-Cl-BaA	Cambridge Isotope
4	9-Chloroanthracene	9-Cl-Ant	Cambridge Isotope
5	9-Chlorophenanthrene	9-Cl-Phe	Cambridge Isotope
6	7,12-dichlorobenz[a]anthracene	7,12-Cl_2-BaA	Cambridge Isotope
7	1-bromopyrene	1-Br-Pyr	Sigma Aldrich
8	1-chloroanthracene	1-Cl-Ant	Sigma Aldrich
9	1,5-dichloroanthracene	1,5-Cl_2-Ant	Sigma Aldrich
10	2-bromofluorene	2-Br-Fle	Sigma Aldrich
11	2,7-dibromofluorene	2,7-Br_2-Fle	Sigma Aldrich
12	3-bromophenanthrene	3-Br-Phe	Sigma Aldrich
13	5-bromoacenaphthene	5-Br-Ana	Sigma Aldrich
14	9-bromophenanthrene	9-Br-Phe	Sigma Aldrich
15	9,10-dibromoanthracene	9,10-Br_2-Ant	Sigma Aldrich
16	9,10-dibromophenanthrene	9,10-Br_2-Phe	Sigma Aldrich