Separation and characterization of polycyclic aromatic hydrocarbons and azaarenes using normal phase liquid chromatography and electrospray ionization mass spectrometry

by

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Abstract

Normal phase liquid chromatography (NPLC) is widely used for separating petroleum into compound classes, such as polycyclic aromatic hydrocarbons (PAHs) and polar nitrogen containing compounds. The charge-transfer and hypercrosslinked polystyrene NPLC phases provide unique selectivities for PAHs. However, their retention mechanisms are not well understood.

In this thesis, I evaluate various methods for the determination of the fundamental parameter, void volume, on charge-transfer phases: pycnometry; the minor disturbance method based on injection of weak solvent; the tracer pulse method; the hold-up volume based on unretained compounds; and the accessible volume based on Martin's rule and its descendants. The accurate measurement of void volume assures accurate retention and selectivity calculation for further understanding of the retention mechanism. Based on accurate measurement of void volume, Chapter Three studies the retention, selectivity, and thermodynamic behavior of PAHs on charge-transfer and hypercrosslinked polystyrene phases under NPLC conditions. The retention of PAHs on these phases is mainly caused by π-π interaction and dipole-induced dipole interactions. Charge-transfer columns possess weaker π-π interaction and greater planarity selectivity than hypercrosslinked polystyrenes for PAHs. This suggests that the charge-transfer phase interacts with PAHs through a disordered phase arrangement, while hypercrosslinked polystyrene behaves as an ordered adsorption phase.

Electrospray ionization mass spectrometry (ESI-MS) has been used widely for characterization of mixtures. But ESI-MS alone cannot differentiate molecular isomers and suffers from uneven response for different analytes. Richer molecular composition

information for petroleum can be obtained by coupling NPLC to ESI-MS. However, it is challenging to couple NPLC to ESI-MS due to the incompatibility of the NPLC eluent with ESI ion generation. The latter half of this thesis explores techniques that enable hyphenation of NPLC with ESI-MS, using azaarenes analysis as an example. Chapter Four reviews the strategies for both off-line and on-line NPLC-ESI-MS. The application of post column solvent addition, sheath liquid interface, and ambient ionization for on-line NPLC-ESI-MS are discussed and compared. Chapter Five compares post column solvent addition and ambient ionization for ionization of azaarenes in non-ESI solvent. Both methods enhance ionization of azaarenes in hexane, but neither method eliminates the ion suppression caused by the compounds within the azaarenes class. Thus, azaarenes are separated on charge-transfer phase with NPLC mobile phase prior to ESI-MS analysis in Chapter Six. A 30 min NPLC separation coupled to ESI-MS through post column solvent addition of isopropanol provides detection limits for individual azaarene in the range of 0.8 – 120 ng/mL, which is low enough for general environmental monitoring.

Preface

This thesis consists of seven chapters including an introduction (**Chapter 1**), four main body chapters for four complete projects (**Chapters 2, 3, 5** and **6**), a brief review (**Chapter 4**) and **Chapter 7** for conclusion, preliminary results and future work. **Chapter 2**, **3** and **5** have been published. **Chapters 4** and **6** are in preparation for publication. Dr. Charles A. Lucy was the supervisor for all the works described below. He actively involved in guiding the direction of research and manuscript revision.

A revised version of **Chapter 2** has been published as Ping Jiang, Di Wu, Charles. A Lucy. Determination of void volume in normal phase liquid chromatography, *Journal of Chromatography A*. 2014, 1324: 63-70. I performed all the experiments, data collection and manuscript preparation. Ms. Di Wu was involved in the initial stages of the project. Dr. Lucy helped with data analysis and manuscript revision.

Chapter 3 has been published as Ping Jiang, Charles. A Lucy. Retentivity, selectivity and thermodynamic behavior of polycyclic aromatic hydrocarbons on charge-transfer and hypercrosslinked stationary phases under conditions of normal phase high performance liquid chromatography. *Journal of Chromatography A*. 2016, 1437:176-182. I was responsible for designing the research project, performing the experiments and analyzing the data. The manuscript was drafted by me and assisted by Dr. Lucy as indicated above.

Chapter 4 will be submitted to *Analytical Methods* as an invited review article. I performed the literature search and wrote the manuscript. Dr. Lucy helped with defining the scope of the review and revising the manuscript.

Chapter 5 has been published as Ping Jiang, Charles. A Lucy. Enhancement of

ionization efficiency of mass spectrometric analysis from non-electrospray ionization friendly solvents with conventional and novel ionization techniques. *Analytica Chimica Acta*. 2015, 897: 45-52. I performed all experiments, data collection and analysis, and wrote the manuscript. Dr. Lucy and I were both involved in the concept formation and he helped in manuscript revision.

A version of **Chapter 6** is in preparation for submission to *Analytical and Bioanalytical Chemistry*. I was responsible for the experimental design, data collection, data analysis, and wrote the manuscript. Dr. Lucy assisted in manuscript revision.

The preliminary results in **Section 7.2.5** has been presented as Matthew Fung, Ping Jiang and Charles A. Lucy, Effect of Basic Solvent Additives on Ionization of Petroleum Analogues by Electrospray-Ionization Mass Spectrometry at the Western Canadian Undergraduate Chemistry Conference, 2014. Dr. Lucy and I were responsible for the concept formation. I designed the experiments. Under my guidance, undergraduate student Matthew Fung collected the data shown in **Fig. 7-4**. I prepared the figure.

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List of Symbols and Abbreviations

2D	Two dimensional
5HGN	Polymeric hypercrosslinked polystyrene stationary phase
A	Acceptor
A	Absorbance
A	Empirical regression parameter in Eq.2-6
AC	Alternating current
ACN	Acetonitrile
AI	Ambient ionization
APCI	Atmospheric pressure chemical ionization
APPI	Atmospheric pressure photoionization
A_s	Peak asymmetry factor
b	Cell pathlength
B	Average ln α between successive homologs in Eq. 2-6
c	Sample concentration
C_{18}	Octadecyl
C_8	Octyl
CE	Cholesterol esters
CF-EDESI	Continuous flow extractive desorption electrospray ionization
C_{inj}	Concentration of the sample injected
d	Distance
D	Donor
Da	Dalton

DAGs	Diacylglycerols
DBE	Double bond equivalence
DC	Direct current
DCM	Dichloromethane
DESI	Desorption electrospray ionization
DNAP	3-(2,4-Dinitroanilino)propyl stationary phase
d_p	Particle size
EESI	Extractive electrospray ionization
ELDI	Electrospray-assisted laser desorption ionization
ENFB	Ethoxynonafluorobutane
ESI-MS	Electrospray mass spectrometry
EtOH	Ethanol
FT-ICR-MS	Fourier transform ion cyclotron resonance mass spectrometry
GC-MS	Gas chromatography-mass spectrometry
h	Reduced plate height
H	Plate height
HC-Tol	Hypercrosslinked polystyrene with toluene group
HILIC	Hydrophilic interaction liquid chromatography
HOAc	Acetic acid
HPLC	High performance liquid chromatography
I.D.	Inner diameter
IE	Ionization energy
IEX	Ion exchange chromatography

IPA	Isopropanol
IR	Infrared
k	Retention factor
K	Distribution coefficient
L	Column length
L/B	Length-to-breadth ratio
LC	Liquid chromatography
LC-MS	Liquid chromatography-mass spectrometry
L_f	Loading factor
LIAD	Laser-induced acoustic desorption
LOD	Limit of detection
LOQ	Limit of quantification
m/z	Mass to charge ratio
mAu	Milli absorbance unit
MeDAG	Monoalkylether diacylglycerols
MeOH	Methanol
M_n	Molecular weight
MP	Polarizability
MS	Mass spectrometry
MTBE	Methyl tert-butyl ether
MWD	Molecular weight distribution
N	Column efficiency
n	Ring number

n	Carbon number
n_m	Moles of analyte in mobile phase
NPLC	Normal phase liquid chromatography
n_s	Moles of analyte in stationary phase
O.D.	Outer diameter
P	1-Octanol/water partition coefficient
PAHs	Polycyclic aromatic hydrocarbons
PANHs	Polycyclic aromatic nitrogen hydrocarbons
PEEK	Polyether ether ketone
PTFE	Polytetrafluoroethylene
QM	Quadrupole moment
R	Ideal gas constant
R	Correlation coefficient
RPLC	Reversed phase liquid chromatography
R_s	Resolution
RSD	Relative standard deviation
S/N	Signal-to-noise ratio
SAESI	Solvent-assisted electrospray ionization
SARA	Saturates, Aromatics, Resins, Asphaltenes
T	Temperature
t_0	Void time
TAGs	Triacylglycerols
TBAOH	Tetrabutylammonium hydroxide

TEAOH	Tetraethylammonium hydroxide
THF	Tetrahydrofuran
TIC	Total ion current
TLC	Thin layer chromatography
TMAOH	Tetramethylammonium hydroxide
t_p	Time during injection
t_R	Retention time
t_r	Retention time of the fronting peak maximum
TTBB	1,3,5-tri-tert-butylbenzene
U	Voltage of direct current
u_0	Velocity of mobile phase
UV	Ultraviolet
V	Voltage of alternating current
V_0	Void volume
$V_{0,weight}$	Void volume determined by pycnometry method
V_0^*	Accessible volume
V_i	Interstitial volume
V_{inj}	Volume of the sample injected
V_{IS}	Hold-up volume
V_M	Mobile phase volume
V_n	Retention volume for analyte with n carbon number
V_{n-1}	Retention volume for analyte with (n-1) carbon number
V_p	Pore volume

V_R	Retention volume
w	Peak width
W	Weight
$w_{0.1}$	Peak width at 10% peak height
ZDV	Zero dead volume
α	Selectivity
Å	Angstrom
ΔG	Change in Gibbs free energy
ΔH	Change in enthalpy
ΔS	Change in entropy
ε	Molar absorptivity
ρ	Density
φ	Ratio of stationary phase volume to mobile phase volume

Chapter One: Introduction

1.1 Overview and motivation

When first invented in the 1900s, liquid chromatography (LC) used polar adsorbents as the stationary phase and organic solvents with low polarity as the mobile phase [1, 2]. This format of LC is termed as normal phase liquid chromatography (NPLC) or adsorption chromatography. Adsorbents such as alumina, silica and polar bonded phases have been widely used for various naturally occurring compounds under normal phase conditions since then [3-5]. However, the development of reversed phase LC (RPLC) in the 1970s reduced the popularity of NPLC. RPLC uses non-polar stationary phases and aqueous mobile phases, which are more compatible with the largely biological and medical-related samples separated by HPLC [6-9]. But NPLC is still highly useful in cases such as the separation of: (1) very polar compounds that have weak retention in RPLC; (2) samples that are sparingly soluble in RPLC mobile phases or samples that are instable in aqueous phase [10]; (3) isomers, especially stereoisomers [3]; and (4) complex samples such as lipids [11] and petroleum [12-14] into groups based on their characteristic chemical functionalities or molecular structures.

Petroleum is one of the complex samples that are more suited to separation by NPLC. Petroleum consists mostly of non-polar hydrocarbons, with small amounts of polar heteroatom (N, S, O, etc.) containing compounds. Although existing as minor constituents, polar heteroatom containing compounds affect the upgrading and refining process significantly. For example, nitrogen (N) containing compounds cause catalyst deactivation [15, 16] during refining process. Methods of analyzing heteroatoms (especially N) containing compounds are needed to guide the refining. NPLC is useful for

separating petroleum into various groups [12-14], such as hydrocarbons and the polar N-containing compounds [14], but NPLC is limited in characterizing petroleum when using typical optical spectrometric detectors.

Mass Spectrometry (MS) is a more powerful technique and widely used for the analysis of petroleum samples [17-20] because MS resolves petroleum constituents based on their mass-to-charge ratio (*m*/*z*). With development of high resolution MS, thousands of molecular compositions for hydrocarbons, N and S containing compounds with molecular weight up to 800 amu have been identified accurately in crude oil or petroleum products [21]. However, ESI-MS is still limited in identification of compounds in asphaltenes due to the higher complexity and aggregation within asphaltenes [17]. Electrospray ionization MS (ESI-MS) is especially specific for polar N-containing compounds in petroleum [18, 19].

Off-line mode coupling of NPLC with ESI-MS has been developed for nitrogen compounds analysis [22]. Group-type separation for N-containing compounds on hypercrosslinked polystyrene phases (quasi-normal phases) and commercial charge-transfer stationary phases using NPLC have been studied [23]. After HPLC separation, the N-containing fractions were collected. Then the fractions were evaporated down and reconstituted in an ESI-MS compatible solvent. The reconstituted fractions were analyzed by Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS). Rich information about the fate of N-containing compounds after hydrotreatment and adsorptive denitrogenation treatment of heavy gas oils was obtained [22].

However, there remain problems in using NPLC-ESI-MS for petroleum analysis. Firstly, stationary phases (mainly charge-transfer phases and quasi-normal phases)

exhibit different selectivity for polycyclic aromatic hydrocarbons (PAHs), and the mechanism of the retention behavior on these phases is not clear. Secondly, NPLC focuses on group-type separation for petroleum instead of "within class" separation. More selectivity needs be explored for compounds with similar functionality. Thirdly, for MS ionization, electrospray ionization (ESI) shows specific suitability to N-containing compounds, but the mobile phase used in NPLC separations is not directly compatible with ESI-MS. Thus, most NPLC-ESI-MS for petroleum has to be performed in off-line mode. An on-line NPLC-ESI-MS is necessary to reduce the tedious sample re-preparation and reduce the possibility of the fractions being contaminated [24]. Fourthly, for MS analysis, the ionization efficiency varies with different types of additives in the solvent [25]. Also, ionization efficiency of different types of compounds in petroleum is inconsistent [18, 25]. The low ionization efficiency and unevenly response factor for different compounds may result in misinterpretation of compounds distribution in petroleum samples.

My thesis focuses on many of the challenges noted above for NPLC-ESI-MS analysis of petroleum. To study the retention mechanism of N-containing compounds on different stationary phases, fundamental research on retention within the column is necessary. One important fundamental parameter in chromatography is the *void volume* of the column, V_0. Accurate determination of V_0 is needed to get the accurate information about retention and thermodynamic behavior. In **Chapter 2**, various methods to estimate the void volume are compared: pycnometry; the minor disturbance method based on injection of weak solvent; the tracer pulse method; the hold-up volume based on unretained compounds; and accessible volume based on Martin's rule and its

descendants. These methods are applied to the determination of *void volume* on silica and charge-transfer columns under NPLC conditions.

In **Chapter 3**, the retentivity, selectivity and thermodynamic behavior of PAHs on hypercrosslinked polystyrene phases (HGN and HC-Tol) and a commercial charge-transfer phase (DNAP) are explored to unravel the selectivity differences observed on these columns.

The second half of my thesis focuses on the coupling of NPLC to electrospray ionization MS (ESI-MS). **Chapter 4** reviews the prior literature on coupling NPLC to ESI-MS. The discussion includes off-line NPLC-ESI-MS and on-line NPLC-ESI-MS through use of post-column solvent addition, a sheath liquid interface, or an ambient ionization source. In **Chapter 5**, the enhancement of the ionization efficiency in ESI-MS for model compounds in non-ESI compatible solvents such as hexane is demonstrated. Post-column solvent addition of ESI-compatible solvents is compared to the recently developed technique of continuous flow extractive desorption electrospray ionization (CF-EDESI) method **[26]**. **Chapter 6** illustrates the effectiveness of both techniques for characterization of azaarenes, which are a group of N-containing compounds widely found in petroleum related products.

Chapter 7 summarizes the conclusions for the work of NPLC and NPLC-ESI-MS in this thesis. Preliminary results and perspectives for future work are also presented in **Chapter 7**.

1.2 Brief history of liquid chromatography

Liquid chromatography (LC) is an important separation technique for sample purification and simplification [1]. The word "chromatography" is derived from the Greek word "chroma", which means "color" and the word "graphein", which means, "to write". In Tswett's pioneering studies of the pigments in plants, the column was calcium carbonate [1]. Such columns separate based on an adsorption mechanism (Section 1.3.3.1) which means the solute sticks to the polar sites on the surface of the adsorbent [27]. Subsequently, in the 1940s A.J.P. Martin developed paper-based LC or thin-layer based LC [28], and partition chromatography (Section 1.3.3.2), referring to solutes dissolving into stationary phase [27]. A.J.P. Martin [28] received the Nobel Prize in Chemistry in 1952 for the invention of partition chromatography.

Over the past half century, the need for greater separation capability and more integrated instrumentation has lead to the development of high performance liquid chromatography (HPLC). Since the introduction of HPLC instrumentation [29], researchers are now able to perform sampling, elution and detection of a separation process with an automated system. Fast and high efficiency separation has been achieved by the improvement on the various stationary phases [30]. HPLC has become an indispensable technique for qualitative and quantitative analysis for analytical chemistry.

1.3 Theory of liquid chromatography [31]

Modern liquid chromatography (LC) is a column-based technique. **Fig. 1-1** shows a scheme for a general LC system that integrates pumping, injection, elution, detection

and data analysis. The output of the instrument is a chromatogram, which consists of several separated peaks that are recorded. The stationary phase is housed in a column that is a cylindrical jacket made of stainless steel or polyether ether ketone (PEEK*). The stationary phase usually consists of either bare particles (*e.g.*, silica, alumina) or a liquid-

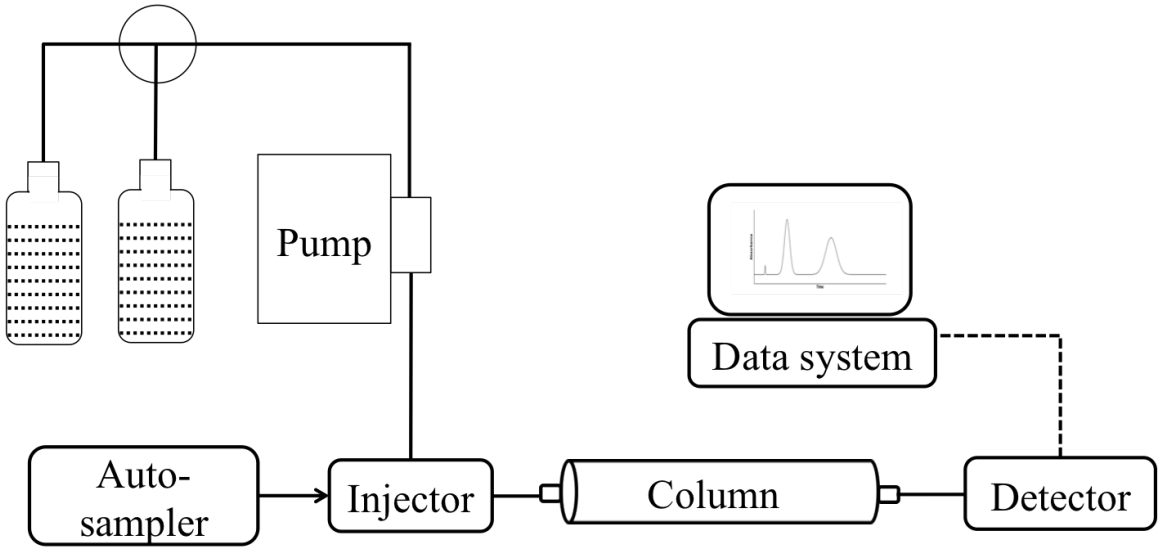

Fig. 1-1 Configuration of a modern HPLC system.

like phase that is covalently bonded on a solid porous particle support (*e.g.*, bonded phases). A mobile phase (liquid) is pumped at constant velocity through the column. If the mobile phase flows at a velocity of u_0 (cm/min) and the column length is L (cm), then the *void time* t_0 (min) that the mobile phase takes to travel through the column is:

$$t_0 = \frac{L}{u_0} \qquad \text{Eq. 1-1}$$

The corresponding volume of mobile phase that passes through the column during t_0 is V_0 (mL), which is the aforementioned *void volume*. The void volume is an important

* PEEK (polyether ether ketone) cannot be used with chlorinated hydrocarbons such as dichloromethane (DCM) which are commonly used in NPLC.

fundamental parameter in liquid chromatography. **Chapter 2** examines the accuracy of various methods to measure V_0 in NPLC.

An HPLC separation starts when a sample containing several analytes is loaded (injected) onto the column. As the analyte is carried through the column by the mobile phase, a portion of the solute distributes onto the stationary phase. The fraction of the solute that distributes onto the stationary phase depends on the strength of interaction with the stationary phase. As a result, different analytes (*e.g.*, X, Y and Z) move along the column at different rates and are separated from each other as illustrated in **Fig. 1-2**. In this case, the elution speed follows the order of X < Y < Z. As the compounds elute from

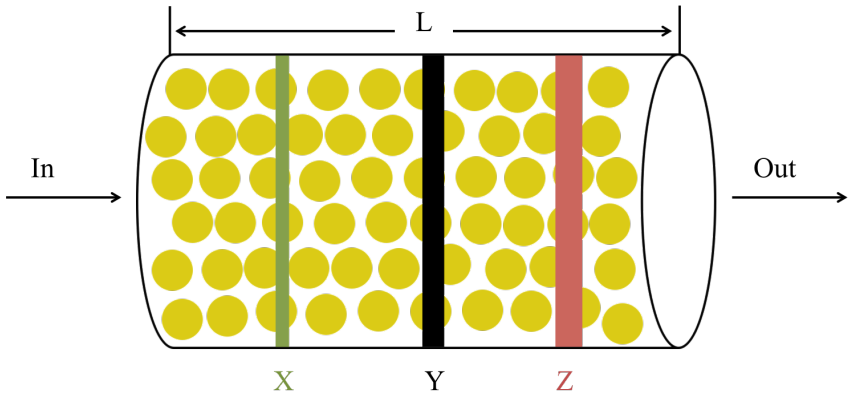

Fig. 1-2 Scheme of separation process in LC.

the column, they are detected by an on-line detector. The output is a chromatogram, such as shown in **Fig. 1-3**, which plots the detector response (*e.g.*, absorbance) versus the time since sample injection.

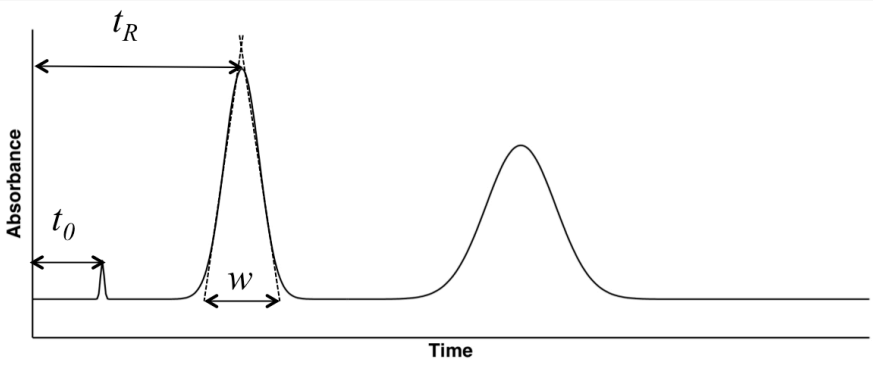

Fig. 1-3 Schematic representation of elution profile (Gaussian peaks).

1.3.1 Retention and selectivity

When an analyte is loaded to the column, it equilibrates with the stationary phase. The time needed for an analyte to elute from the column is its retention time, t_R, as shown in the chromatogram in **Fig. 1-3**. The retention factor, k, quantifies the distribution of analyte between the stationary and mobile phase. The retention factor is the ratio between the amounts of analyte in stationary phase (n_s) to that in the mobile phase (n_m).

$$k = \frac{n_s}{n_m} \qquad \text{Eq. 1-2}$$

This ratio also equal to the ratio of the time that the analyte spends in the stationary phase to its time in the mobile phase. Within the column, the analyte is either retained (stationary) on the stationary phase or moving with the mobile phase with velocity u_0. Because the total time of the analyte spends in the column is t_R and the time in the mobile phase is t_0, then the time in stationary phase is ($t_R - t_0$). Thus, a practical way to express k is:

$$k = \frac{t_R - t_0}{t_0} \qquad \text{Eq. 1-3}$$

Thus, from the retention time t_R and void time t_0, we are able to determine the retention factor k. The retention factor is related to the distribution coefficient K by the phase ratio φ, which is the ratio of stationary phase volume to mobile phase volume.

$$k = K \cdot \varphi \qquad \text{Eq. 1-4}$$

The distribution coefficient can be related to thermodynamic parameters, *e.g.*, Gibbs free energy, ΔG, enthalpy ΔH, and entropy ΔS by:

$$\Delta G = \Delta H - T\Delta S = -RT ln\, K \qquad \text{Eq. 1-5}$$

Thus, measurement of void time (**Chapter 2**) and retention time not only provides the retention factor, but also provides thermodynamic information.

Selectivity (α) is the relative retention of adjacent compounds in the chromatogram. Selectivity is the ratio of the retention factor of one compound (k_2) to the other (k_1) ($k_2 > k_1$).

$$\alpha = \frac{k_2}{k_1} \qquad \text{Eq. 1-6}$$

This expression is used for the relative retention of PAHs on different columns in **Chapter 3**. Selectivity is an important parameter to evaluate the ability of a column to resolve two compounds for specific separation purpose. Changes in the stationary phase properties change the selectivity between two compounds. For the same stationary phase, different mobile phase results in different selectivity as well.

1.3.2 Efficiency and resolution

Fig. 1-3 also shows that the injected analyte band broadens as it migrates through the column. Ideally, the peak is Gaussian (symmetrical). The peak width, *w*, is typically measured using the tangent method, as illustrated in **Fig. 1-3**. The sharpness of the peak is described by the column efficiency which may be expressed either as using the plate

number (N) or plate height (H, height equivalent to a theoretical plate). Mathematically, for an ideal Gaussian peak, N and H are:

$$N = 16\left(\frac{t_R}{w}\right)^2 \quad \text{Eq. 1-7}$$

$$H = \frac{L}{N} \quad \text{Eq. 1-8}$$

Column efficiency depends on the particle size (d_p) of the stationary phase. The reduced plate height, h, factors out this dependence:

$$h = \frac{H}{d_p} \quad \text{Eq. 1-9}$$

The reduced plate height has been used to evaluate the column efficiency in **Chapter 6**, as the columns studied possessed different particle sizes.

In practice, chromatographic peak shapes deviate from the ideal Gaussian peak into asymmetrical peak. For example, tailing peaks such as shown in **Fig. 1-4** are often observed for basic solutes on silica columns. The peak asymmetry factor A_s is used to characterize the peak tailing:

$$A_s = \frac{B}{A} \quad \text{Eq. 1-10}$$

A and B values at 10% peak height are determined as in **Fig. 1-4**. For a tailing peak, A_s is greater than 1. The higher the A_s is, the more tailing the peak is. Eq. 1-10 is used to calculate A_s in **Chapter 6**.

When peak tailing occurs, Eq. 1-7 is not suitable for calculation of the plate number as the peak is not Gaussian. For tailed peaks, the Foley-Dorsey equation (Eq. 1-11) [32] provides a more accurate estimation of the plate number.

$$N = \frac{41.7\cdot\left(\frac{t_R}{w_{0.1}}\right)^2}{\frac{B}{A}+1.15} \quad \text{Eq. 1-11}$$

where $w_{0.1}$ is the peak width at 10% peak height, *i.e.*, $w_{0.1}$ = A+B (**Fig. 1-4**).

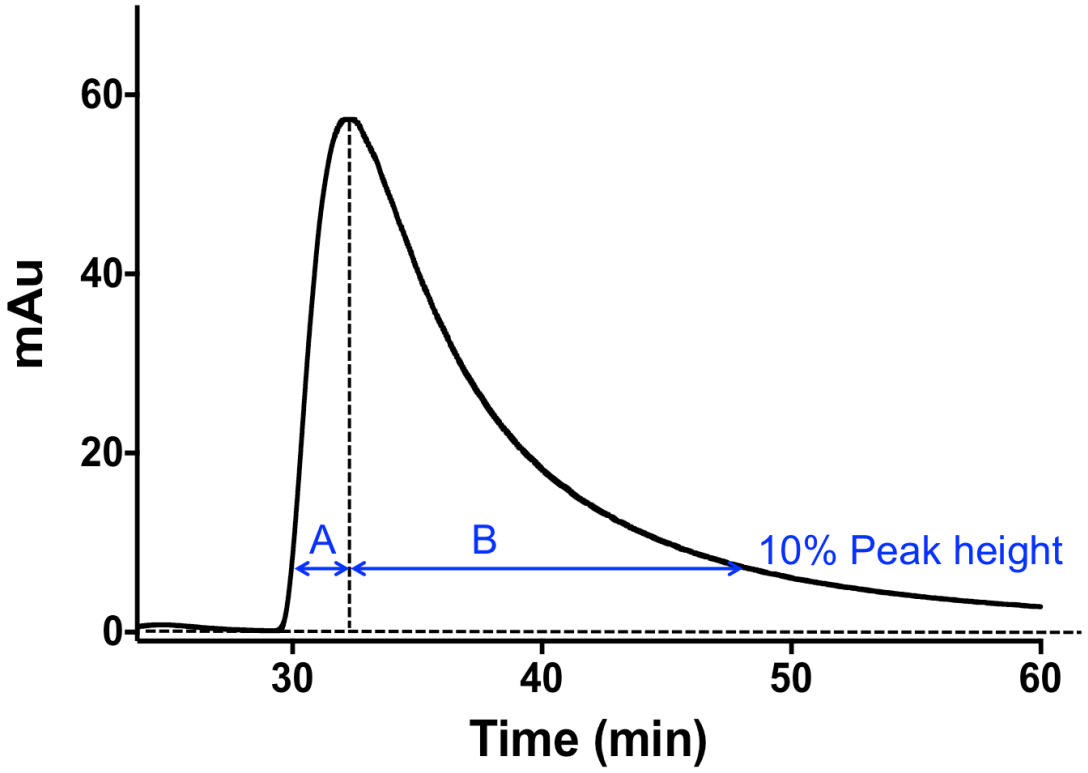

Fig. 1-4 Measurement of peak asymmetry factor on a tailing peak. Conditions: sample, acridine; column, 5HGN; mobile phase, 20/80 IPA/hexane.

The resolution (R_s) between two peaks defines how well two analytes are separated. R_s is related to retention factor, selectivity (Section 1.3.1) and efficiency through:

$$R_s = \frac{\sqrt{N}}{4} \cdot \frac{k_2}{k_2+1} \cdot \frac{\alpha-1}{\alpha} \qquad \text{Eq. 1-12}$$

Where k_2 is the retention factor of the more retained solute of the two adjacent peaks. For a separation for multiple analytes, the resolution between adjacent peaks is calculated based on the retention time and peak widths within the chromatogram by:

$$R_s = \frac{2(t_2 - t_1)}{w_1 + w_2} \qquad \text{Eq. 1-13}$$

where t_1 and t_2 and w_1 and w_2 are the retention times and peak widths of the two adjacent peaks. Eq. 1-13 is also used for R_s calculations in **Chapter 6**. When R_s is greater than 1.5, two peaks are considered baseline resolved. The adjacent peaks with the lowest R_s within a chromatogram are considered as the critical pair of the separation, *i.e.*, the most important peaks to resolve during method development.

1.3.3 Liquid chromatography modes

Dependent on the chemical properties of the stationary phase and the mobile phase, LC can be performed in various modes.

1.3.3.1 Reversed phase liquid chromatography (RPLC)

The most widely used HPLC mode is RPLC. RPLC uses a non-polar stationary phase and a polar mobile phase to separate compounds with no or low polarity. The stationary phase usually consists of an alkane chain bonded to porous silica particles (**Fig. 1-5**). The alkyl length of commercial RPLC bonded phases varies from C_1 to C_{30}, with C_8 and C_{18} being most common.

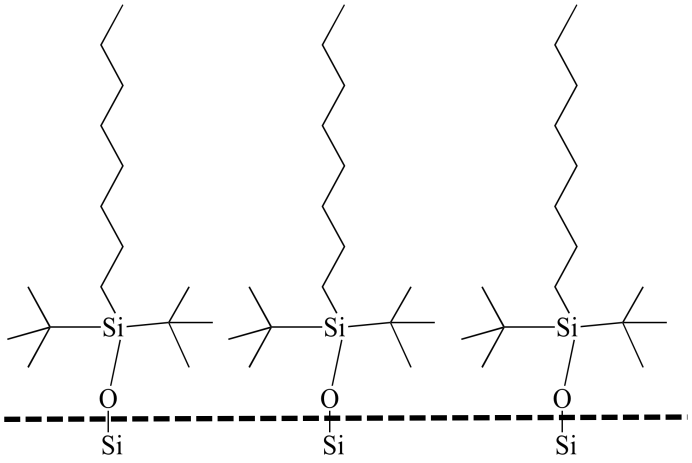

Fig. 1-5 Scheme for bonded phase in RPLC

The retention of solutes is dominated by the partition mechanism. As shown in **Fig.1-6(a)**, the solutes either dissolve into the bonded stationary phases or are dissolved in the mobile phase. With a polar mobile phase, the more polar solutes interact more with the mobile phase, and thus elute earlier.

An RPLC mobile phase commonly consists of a polar organic modifier and water. Methanol (MeOH) and acetonitrile (ACN) are commonly used modifiers in RPLC because they are fully miscible with water, are low viscosity, have low surface tension (making them compatible with ESI-MS), and are transparent in the ultraviolet (making them compatible with absorbance detection, UV cutoff for MeOH is 205 nm and for ACN is 190 nm). The higher the percentage of organic modifier, the stronger the mobile phase. Selectivity in RPLC can be altered by using a hydrogen bonding organic modifier such as MeOH rather than a dipolar modifier such as ACN.

However both MeOH and ACN are toxic, and ACN is considered non-sustainable. Recently, Welch and co-workers proposed ethanol as a sustainable organic and less toxic modifier for RPLC [33]. They even demonstrated that domestic sources of ethanol (EtOH) such as vodka and rum could be used as an RPLC mobile phase. During my doctoral studies, I participated in a brief examination of *cocktail chromatography*. In a resultant Letter to the Editor [34], we pointed out that Welch and co-workers had failed to note the increased back pressure caused by ethanol. We also demonstrated that UV impurities in vodka caused ghost peaks in gradient chromatograms, and that purification of the vodka using a Britta filter (as demonstrated by MythBusters [35]) eliminated these

ghost peaks. While this work was done during my doctoral studies, I will not discuss this work further in this thesis.

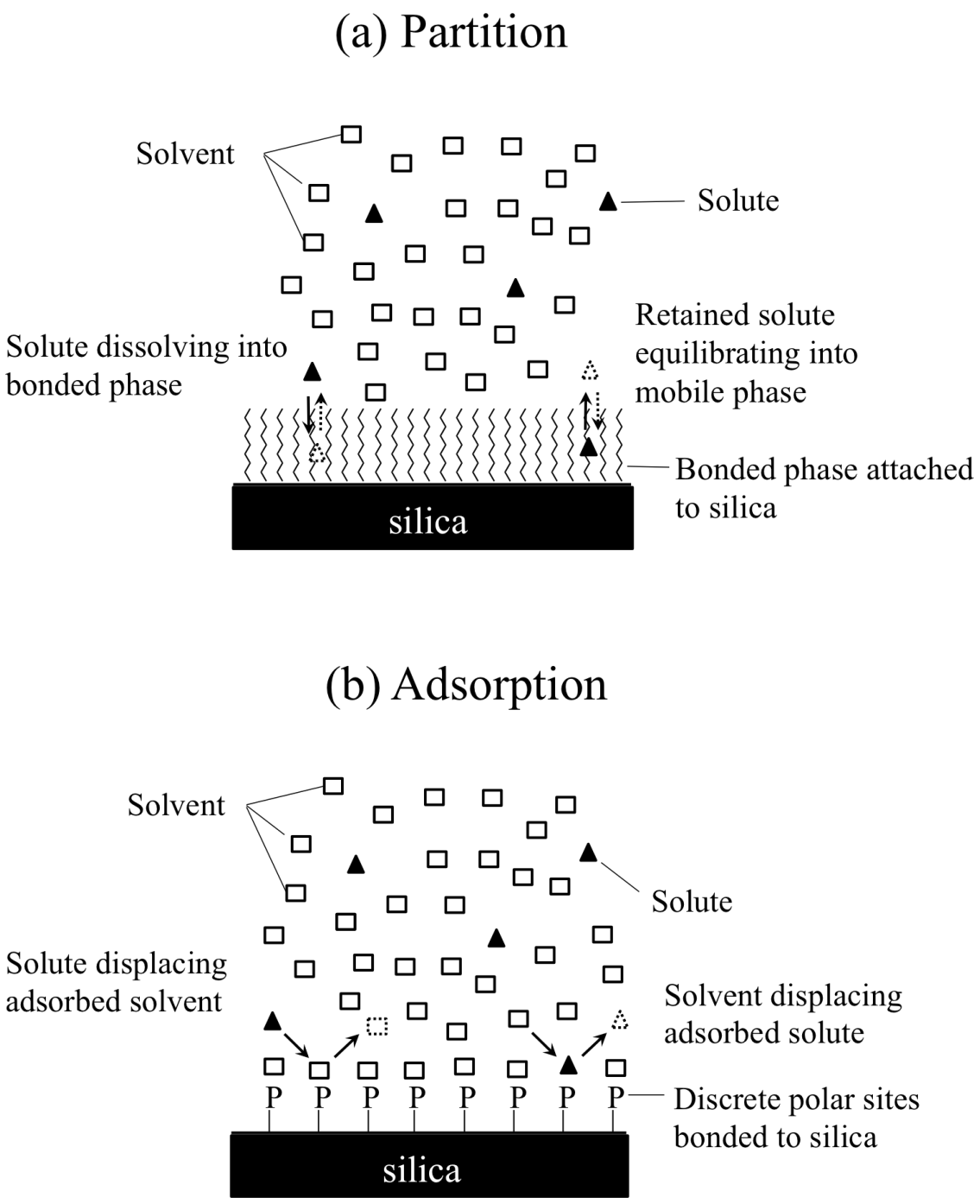

Fig. 1-6 Partition and adsorption mechanism. Adapted from C.A. Lucy [27].

1.3.3.2 Normal phase liquid chromatography (NPLC)

In contrast to RPLC, NPLC uses a polar stationary phase in conjunction with low-polarity mobile phase (*e.g.*, hexane, dichloromethane) for separation. The first LC experiment by Tswett [1] and the later developed thin-layer chromatography (TLC) [28] are examples of NPLC separations. For column chromatography, the conventional NPLC stationary phases are bare silica (**Fig. 1-7**) or alumina. These phases possess discrete polar groups (*e.g.*, silanols in **Fig. 1-7**). Solutes adsorb onto the surface by forming dipole-dipole or hydrogen bond interaction with these polar sites. This process is called *adsorption*, and is shown in **Fig. 1-6(b)**. The more polar the compounds are, the more they are retained on the column. The solvent molecules compete with solutes for the adsorption sites to displace the solutes from the stationary phase (**Fig. 1-6(b)**). Thus, the more polar the solvent is, the stronger its elution ability. NPLC is widely used for separations of polar compounds and for chiral separations [3-5]. Also, NPLC is useful for resolving compounds that barely dissolve in water (e.g., petroleum) [12-14] or samples that are unstable in aqueous phases [10].

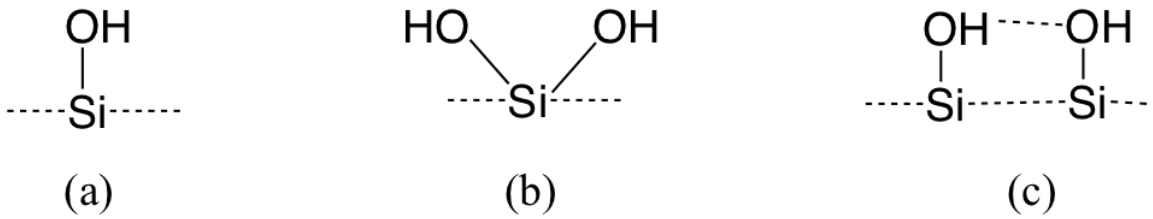

Fig. 1-7 Various types of polar silanols on silica stationary phases (a) Free silanols; (b) Geminal silanols; and (c) Associated silanols.

1.3.3.2.1 Conventional NPLC stationary phases

However, conventional stationary phases are sensitive to the water content in the mobile phase [36]. This sensitivity makes the retention of analytes quite irreproducible. Bonded normal phases on silica were developed to lessen this problem [31] and to provide adsorption sites with different polarities. Among the bonded NPLC phases, amino and cyano columns are the most frequently used. The bonded polar phase improves the water compatibility, but does not change the adsorption mechanism of the separation. That is, both the bare normal adsorbents and bonded phases possess distinct adsorption sites on the surface of the adsorbent.

Besides distinct dipole-dipole interaction or hydrogen bonding, other interactions between stationary phase and the solutes have been investigated in NPLC separations. In this thesis, I study two types of these modes. One is charge-transfer liquid chromatography [37] and the other is quasi-normal phase liquid chromatography [38].

1.3.3.2.2 Charge-transfer liquid chromatography

A charge-transfer complex is formed when an electron partially or completely transfers from an electron rich donor (D) molecule to an electron deficient acceptor (A) molecule [37]. The formation process of a complete charge-transfer can be expressed by:

$$D+A \leftrightarrows (D, A) \leftrightarrows D^{+}: A^{-}$$

A charge-transfer type stationary phase is obtained when one of the components of the charge-transfer complex is bonded onto a solvent-compatible support. The resulting stationary phase can be applied for separation of compounds that can form charge-transfer complexes with the ligand [39-41]. The π-π interaction between conjugated systems was once considered as a type of charge-transfer complex formation.

Thus, phases that use a π electron conjugation system, *e.g.*, aromatic systems, as ligands are referred to as charge-transfer phases. Nitro substituted aromatic ligands bonded to a silica base is an example of such a charge-transfer phase [40, 41]. However, the nature of π-π interaction is not truly charge-transfer. A π-system can be treated as a sandwich, which consists of positively charged σ-framework and negatively charged π-electron orbitals. When two π-systems (e.g., molecules M and N) interact with each other, the π-electrons of molecule M is attracted by the σ-framework of N and repelled by the π-electrons of N. When the π-σ attraction overcomes the π-π repulsion, there is a favorable π- π interaction for molecules M and N [42].

The initial interest in charge-transfer chromatography was for biochemical separations, *e.g.*, nucleosides and nucleotides [37]. Later applications of charge-transfer LC expanded to polycyclic aromatic hydrocarbon analysis and petroleum separations [39, 40, 43]. One of charge-transfer phases explored during my doctoral study is 3-(2,4-dinitroanilino) propyl (DNAP). The DNAP phase is synthesized by bonding electron acceptor 2,4-dinitrofluorobenzene to the primary amino group on silica surface [39]. **Fig. 1-8** shows the nucleophilic aromatic substitution reaction for the synthesis of DNAP phase. The N atom in the amino group is a nucleophile that attacks the electron deficient benzene ring in 2,4-dinitrofluorobenzene. One ambiguity in this field has been that the primary commercial source of DNAP columns (ES Industries) has changed the nature of the linker between the DNAP and silica. The nature of the linker is believed to affect the selectivity of the DNAP column [44]. However, ES Industries have not been forthcoming with information about this linker nor are they clear about the nomenclature of the

columns (see **Chapter 2** for details). The separation for PAHs or PANHs are performed on DNAP phase with NPLC mobile phases in **Chapter 3** and **Chapter 6**.

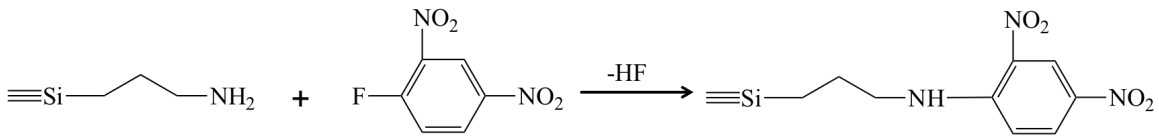

Fig. 1-8 Synthesis scheme for 3-(2,4-dinitroanilino)propyl (DNAP)

1.3.3.2.3 Quasi-normal phase liquid chromatography

The term *quasi-normal phase liquid chromatography* was coined by Davankov and co-workers to describe hypercrosslinked polystyrene phases that are run with normal phase solvents [45-47]. The term *hypercrosslinked* indicates a high percentage (40% to 100%) of crosslinking between the polymer chains. The hypercrosslinked polystyrene developed initially by Davankov et al. is a pure polymeric phase [38]. A hypercrosslinked polystyrene phase was synthesized through post-crosslinking polystyrene chains using agents such as dimethylformamide. In contrast to conventional poly(styrene-divinylbenzene), a hypercrosslinked polystyrene phase contains methylene $-CH_2-$ bridges between phenyl groups (**Fig. 1-9**). Hypercrosslinked polystyrene also contains both micropores and macropores, and has an extremely high apparent specific surface area [38]. In addition, the hypercrosslinked polystyrene is mechanically rigid, once it swelled, its volume changes less significantly with the solvent type than conventional poly(styrene-divinylbenzene) phase. Thus hypercrosslinked phases are compatible with

various solvents and different separation modes ranging from RPLC to quasi-normal phase [45, 46].

Of interest in my studies is that while hypercrosslinked polystyrene shows special selectivity for polar compounds separation, it does not have discrete adsorption sites on the surface. The quasi-normal stationary phases investigated in **Chapter 3** for PAHs separation are 5HGN and HC-Tol. 5HGN is a column that is packed with hypercrosslinked polystyrene beads with particle size of 5 μm. H means hypercrosslinked, G means gel-type and N means non-activated. HC-Tol phase consists of a hypercrosslinked aromatic network modified with toluene group (**Chapter 3**).

1.3.4 HPLC instrumentation

HPLC systems consist of solvent pumps, a sample injector, a column and a detector. **Fig. 1-1** is a schematic diagram of the HPLC instrument used through this thesis. The mobile phase (HPLC grade solvents) are stored separately in 1 L reservoirs. HPLC grades solvents pass through 20 μm solvent inlet filter to remove any particulate matter. The solvents that are driven by a pump from individual reservoir are mixed. The proportion of solvents is controlled by commercial software. A degasser is installed before the pump head to remove any air in the solvents. Typically HPLC pumps use graphite-filled PTFE piston seals. PTFE is compatible with most organic solvents. However for the NPLC solvents used in this thesis, polyethylene piston seals (Agilent, Part No. 0905-1420) are recommended by the manufacturer and were installed in my HPLC. Such seals are limited to 5000 psi, meaning that NPLC cannot be performed under ultra-high performance liquid chromatography conditions, regardless of the instrument used.

Samples are prepared in advance. Each sample is filtered through 0.5 μm PTFE filter and distributed into 2 mL glass vials. Samples are at room temperature in the sample tray and injected onto the column by an auto-sampler. To avoid broadening caused by injection, less than 50 μL of sample dissolved in mobile phase should be injected onto a column with dimension of 50 × 4.6 mm and packed with 5 μm particles [31]. In this thesis, the injection volume is 2 μL. The loaded sample is then carried through the column by the mobile phase. As the analyte elutes from the column, it is monitored by an on-line detector. In **Chapter 2, 3** and **6**, a UV detector was used, as indicated in **Fig. 1-1** (Section 1.3.5). In **Chapter 5 and 6** an electrospray ionization-mass spectrometer (Section 1.3.6) was used. All the waste solvents generated are disposed and recycled by the Department of Chemistry in University of Alberta.

1.3.5 UV-Visible detector [31]

The most common detector used in HPLC is the ultraviolet (UV) absorbance detector. UV-visible detectors provide several advantages. Firstly, UV detection provides high sensitivity for compounds with π-π bonds and other UV absorbing chromophores. Secondly, UV detectors are easy to operate and compatible with various mobile phases used with HPLC. Thirdly, the results generated with UV absorbance detector are more predictable and reproducible than other detection method, such as mass spectrometer. Lastly, UV absorbance detectors can provide selective response when different wavelengths are used for different classes of compounds. Comparing their retention times to those of standards identifies compounds. This procedure was used to identify neutral and basic nitrogen containing compounds within petroleum in our lab [22, 48].

Quantification is based on Beer's law:

$$A = \varepsilon bc \qquad \text{Eq. 1-14}$$

where ε is the molar absorptivity of the sample, *b* is the cell pathlength in cm, and *c* is the sample concentration.

However, UV-vis detection provides little information for untargeted analysis. Firstly, the UV detector is inefficient for an analyte that has low or no UV absorbance. Secondly, UV absorbance provides little structure or molecular information about unknown analytes.

1.3.6 Mass spectrometry

Mass spectrometry (MS) is a technique that measures the mass-to-charge ratio (*m*/*z*) of the analytes or analyte fragments [27]. Mass spectrometry provides richer qualitative information than UV detector about the analytes eluting from an HPLC column. A mass spectrometer is composed of: a sample introduction system; an ionization source; a mass analyzer; an ion detector; and a data system. In LC-MS, the LC system acts as the sample introduction component. In the ionization source, a charge is introduced to the analyte. A variety of ionization sources are available, with the best choice being dependent on the chemical and physical properties of the analytes. For example, electrospray ionization (ESI) is more favorable for polar compounds. ESI has been widely used for biological samples [49, 50] and petroleum [18, 20, 21, 51], with or without LC separation. ESI-MS is the primary technique reviewed in **Chapter 4** and used in **Chapters 5** and **6** to characterize polycyclic aromatic nitrogen hydrocarbons (PANHs). ESI-MS is the only ionization technique used in this thesis, and so only it is discussed below.

1.3.6.1 ESI-MS principles

ESI is a "soft" ionization technique because the ions are generated without fragmentation during ESI process. **Fig. 1-9** is a schematic diagram that shows the ionization mechanism for ESI in the positive mode [27, 52].

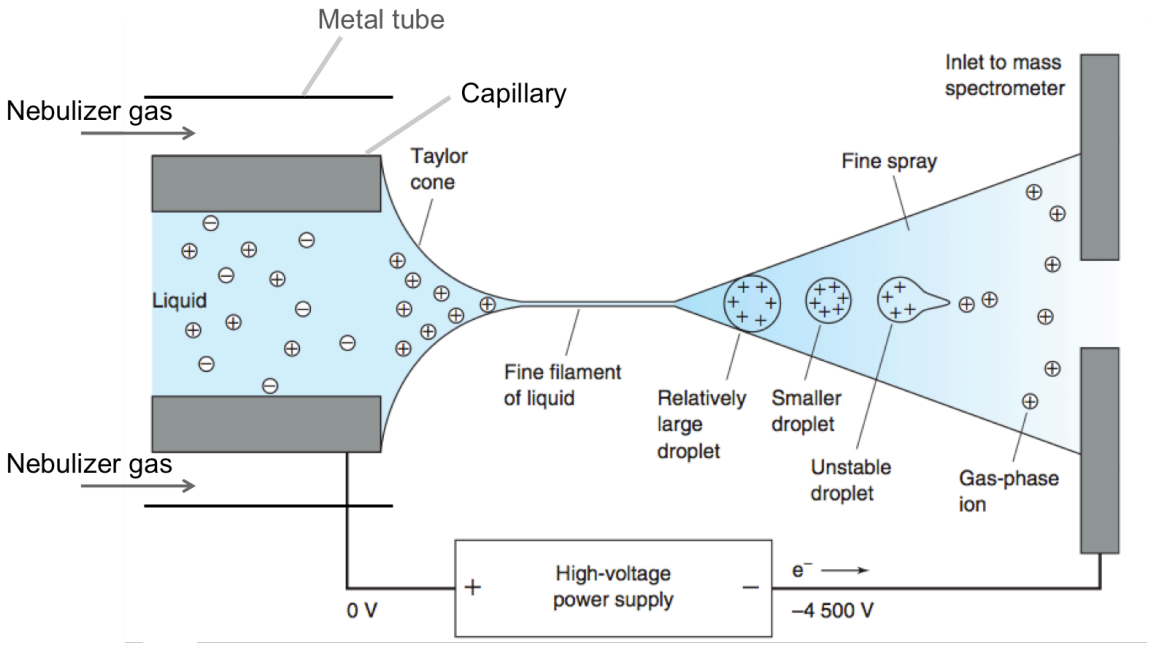

Fig. 1-9 Electrospray ionization process in positive mode. Adapted from Daniel C. Harris [27].

The analyte is introduced into the ionization chamber as a solution through a metal capillary. The capillary is centered in a stainless steel tube through which a nebulizer gas is introduced (**Fig. 1-9**). A high voltage (2~4 kV) is applied between the tip of the capillary and the mass analyzer orifice. Under the high voltage, positive and negative ions in the solution are partly separated due to electrochemical reactions. In positive mode ESI, the positive ions in the solution move towards the surface of the

solution. When the repulsion between the charges overcomes the surface tension of the bulk liquid, a cone shape called the Taylor cone (**Fig. 1-9**) is formed at the tip of the capillary. The tip of the Taylor cone is unstable and breaks into a thin filament. From the filament, the first generation of droplets is created.

Within the ionization chamber, the first generation droplets shrink into smaller droplets. Gas-phase ions are generated from these small droplets either through the ion evaporation or charge residue mechanism [52, 53]. At the end, the ions with single charge or multiple charges are transferred to the orifice of the mass analyzer. In this thesis, all compounds were detected as singly charged ions. That is, the analytes are either protonated to form $(M+H)^+$ in the positive mode or deprotonated to form $(M-H)^-$ in the negative mode. In this thesis, positive ESI has been used for the characterization of PANHs in **Chapter 5** and **6**. Negative ESI has been used in the preliminary studies of the effect of basic additives on the ionization efficiency of petroleum standards in **Chapter 7**.

1.3.6.2 Mass analyzer

The mass analyzer is the component of a MS that separates the ionized analytes based on their mass-to-charge ratio. Mass spectrometers are commonly classified based on their mass analyzer. The mass analyzer also determines the resolution and mass range of a MS. For complex petroleum samples, a high-resolution mass analyzer is able to provide rich molecular compositions [17, 18]. Thus, ultra-high resolution FT-ICR mass spectrometer has been widely used for molecular composition specification for various petroleum products [12, 18, 19]. However, this thesis focuses on coupling of NPLC to MS and optimizing ESI ionization (**Chapters 5** and **6**). For these purposes, a lower resolution and less mass-accurate MS met the needs of our studies. Thus, all the

experiments in **Chapters 5** and **6** are performed on a single quadrupole mass spectrometer.

Quadrupoles are relatively simple and cost effective mass analyzers. A typical quadrupole is composed of four (two pairs) parallel cylindrical electrodes as shown in **Fig. 1-10** [54]. A combination of direct current (DC) and alternating current (AC) voltages are applied to each pair of electrodes. The form of the applied voltage is $\Phi_0 = U + V\cos(\omega t)$, where U is the DC voltage and $V\cos(\omega t)$ is the AC voltage with a frequency of $\omega/2\pi$ [55]. The amplitude of the voltage applied to each electrode pair is the same but with opposite sign (**Fig. 1-10**). Thus, a hyperbolic electrical field is created. With a particular $U + V\cos(\omega t)$, ions with a narrow range of *m*/*z* can travel through the quadrupole rods with a stable trajectory, and thus are transmitted to the detection unit (trace (a) in **Fig. 1-10**). Ions of other *m*/*z* values follow an unstable path and are rejected by hitting one of the electrodes (trace (b) in **Fig.1-10**). The resolution of a quadrupole mass spectrometer is 1000 to 4000 and the mass accuracy is 0.1 to 0.2 Da. The resolution and accuracy is more than enough for characterization of the relatively less complex sample in this thesis.

A quadrupole mass analyzer is sometimes referred to as a mass filter, because if the U/V ratio is held constant, only the targeted *m*/*z* is transmitted to the ion detector. Thus a quadrupole mass analyzer can be used as selected ion monitoring (SIM) for a given *m/z*. In practice, U and V are increased simultaneously (sweeping) while the U/V ratio is kept constant. This transmits different m/z to the ion detector one by one [56]. The sensitivity of sweeping quadrupole is less than that of SIM since the quadrupole only spends a portion of time on a given *m/z*.

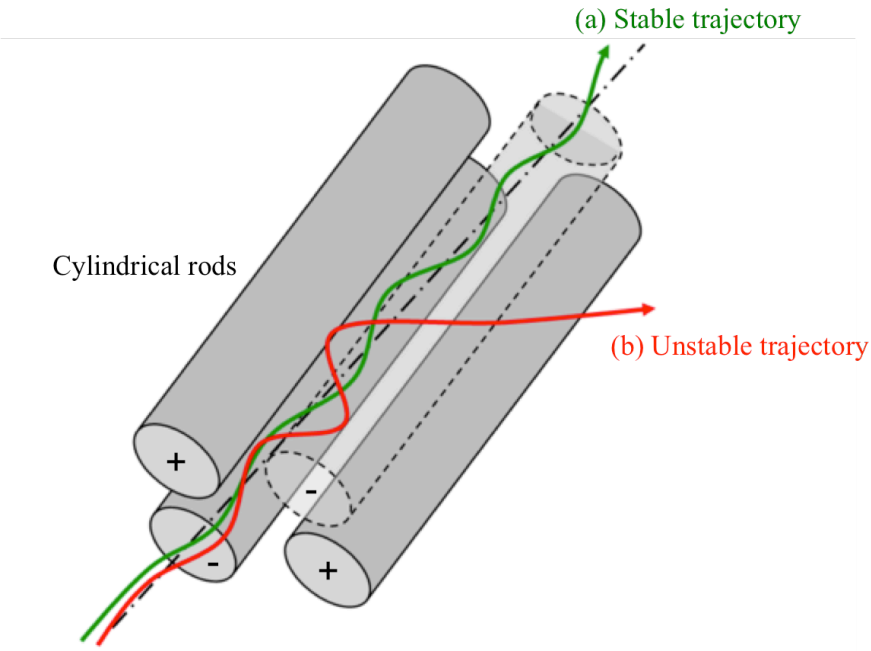

Fig. 1-10 Quadrupole electrodes and ion trajectories. Adapted from the University of California Davis Chem Wiki [54].

1.4 Summary of thesis

Charge-transfer and hypercrosslinked polystyrene phases provide unique selectivities for polycyclic aromatic hydrocarbons (PAHs) and polycyclic aromatic nitrogen hydrocarbons (PANHs) in normal phase liquid chromatography (NPLC) separations. Thus, these phases hold potential for petroleum separations. To study the retention mechanism of PAHs and PANHs on different stationary phases, fundamental studies focused on NPLC were carried out. In **Chapter 2**, the fundamental parameter, *void volume* V_0 is determined with various methods. The appropriateness of these methods for practical use is discussed and compared. In **Chapter 3**, the size, shape selectivity, planarity selectivity, and thermodynamic behavior for PAH standards on charge-transfer (DNAP column) and hypercrosslinked polystyrene (HC-Tol and 5HGN columns) phases in NPLC are investigated.

The latter half of this thesis focuses on coupling NPLC separation with ESI-MS. **Chapter 4** describes literature strategies to hyphenate NPLC with ESI-MS. For on-line NPLC-ESI-MS, the strategies include post-column solvent addition, sheath liquid interface, and ambient ionization. However, limited information is available for the comparison between those strategies. Thus, in **Chapter 5**, post-column solvent addition and ambient ionization are used and optimized for ionization of PANHs in non-ESI friendly solvents. Lastly in **Chapter 6**, the performance of on-line NPLC-ESI-MS using post-column solvent addition or ambient ionization is compared for PANHs separated on a charge-transfer phase under NPLC conditions.

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Chapter Two: Determination of void volume in normal phase liquid chromatography[†]

2.1 Introduction

In liquid chromatography (LC), the most commonly used fundamental parameter is the retention factor *k*:

$$k = \frac{V_R - V_M}{V_M} \quad \text{Eq. 2-1}$$

where V_R is the retention volume of a pure compound and V_M is the volume of the mobile phase that is truly moving within the column. With an accurate *k*, the retention behavior of a specific compound on a specific column can be characterized, which in turn enables optimization of a separation [1]. In addition, the ratio of two accurate *k* values yields the selectivity (α, Eq. 1-6), which allows comparison of different types of columns and guides column selection [2]. Last but not least, accurate determination of *k* allows for determination of thermodynamic parameters (Eq. 1-5) that provide insight into the retention mechanism [3-5], as will be done in Chapter 3.

To accurately determine *k*, an accurate V_M is required. V_M has been defined as a kinetic void volume and reported to be constant in a specific column [6]. The truly freely moving mobile phase volume is hard to measure. In practice, other void volume concepts such as the *total void volume* (V_0), the *hold-up volume* (V_{IS}) and the *accessible volume* (V_0^*) are used as substitutes for V_M. The total void volume is a thermodynamic void volume [6]. To further confuse matters, some papers use the terms void volume and hold-up volume interchangeably [7, 8], while in others the terms are considered different [9].

[†] A version of this chapter has been published as Ping Jiang, Di Wu and Charles A. Lucy, Journal of Chromatography A, 1324 (2014) 63-70. DOI: 10.1016/j.chroma.2013.11.019. I performed all the experiments and wrote the manuscript.

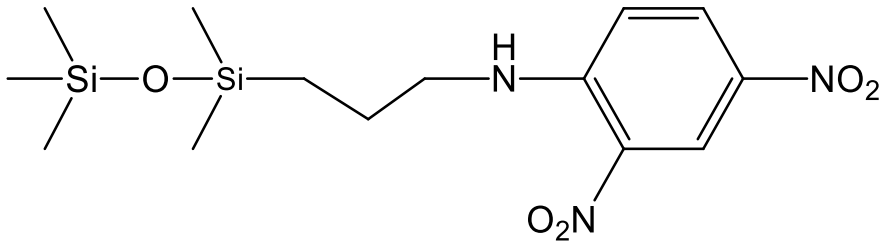
Numerous reviews have discussed methods for the measurement of "void volume" in reversed phase chromatography [10, 11]. However there is some ambiguity in the terms and methods used, and it is unclear whether these methods are applicable to normal phase liquid chromatography (NPLC). In this chapter, we explore the determination of void volume in NPLC. In particular, our discussion focuses on the traditional silica normal phase packing and charge-transfer columns such as the RingSep (trade mark) and dinitroanilinopropyl (DNAP) columns (ES Industries) that are widely used for petroleum analysis [12-16].

The RingSep column is packed with a 3-(2,4-dinitroanilino)propyl stationary phase (**Fig. 2-1(a)**). This column shows retention for aromatic hydrocarbons due to formation of charge-transfer complexes [16]. As implied by its brand name, a common application of the RingSep column is the separation of polycyclic aromatic hydrocarbons (PAHs) based on their ring numbers [12, 13, 15] under NPLC conditions. The RingSep column has also been used for LC-MS analyses of polar compounds in petroleum [17]. The DNAP (**Fig. 2-1(b)**) phase is similar to the RingSep phase but lacks the amino group in the linker group. It also exhibits PAH retention via charge-transfer interactions.

The structures presented in **Fig. 2-1** were based on Ref. 14 and personal communications [18] with ES Industries in 2010. Personal communications with Dr. Winston K. Robbins of Ohio University suggested that the structure of these columns has not been consistent over time [19]. ES Industries has altered the bonded phase over recent years. We were able to confirm with ES Industries [20] that our DNAP column is packed with materials of structure in **Fig. 2-1(a)**. However, ES industries would only state that the RingSep column is packed with multi-nitro groups substituted aromatic phase.

Fortunately, the two structures of the two phases did not directly affect the investigation and the conclusions in this chapter since our aim was to measure the void volumes of these two columns, and not to investigate their the chemical difference.

(a) RingSep column



(b) DNAP column

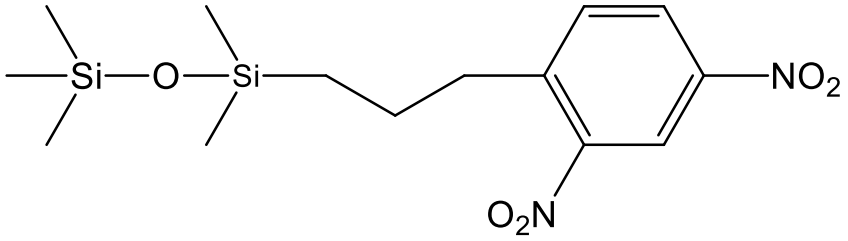


Fig. 2-1 Structure of bonded phase of the (a) RingSep and (b) DNAP columns

Regardless, both the DNAP and RingSep columns possess an nitro- substituted aromatic system. Both columns are able to separate PAHs into groups based on ring numbers. However, the DNAP column provides different selectivity from RingSep column for polar petroleum compounds. Specifically on the RingSep column, the polar compounds can only be eluted with alcohol (*e.g.*, methanol) containing mobile phases [19]. However, column bleeding, which means degradation of the stationary phase, is

observed for RingSep phase in the presence of methanol. In contrast, on the DNAP column nitrogen containing compounds can be resolved from other classes of polar components with either DCM [14] or alcohols [21]. In the course of trying to understand the substantial selectivity differences that result from the subtle differences in the functionality on these two columns, we required accurate retention factors and selectivity factors. We were surprised to find essentially no discussion in the literature about the determination of accurate void volumes in NPLC. This chapter provides a review of the literature on determination of void volume and then compares the reported methodologies on a variety of NPLC phases.

2.2 Background

The literature presents several different methods to determine the void volume [7, 22, 23]. To provide clarity to the present discussion, these terms and how they are determined are summarized in **Table 2-1**. The total void volume V_0 is defined as the total volume of the mobile phase in the column. It is a thermodynamic definition for void volume. V_0 consists of the interstitial volume (V_i) between particles and the pore volume (V_p) within the particles [22]. V_0 can be measured by methods such as pycnometry (weight difference method), the minor disturbance method and the tracer pulse method. In pycnometry, the mass of the column is measured when filled with two different solvents, each of a unique density. The $V_{0,weight}$ is obtained by calculating the ratio of the mass difference over the density difference between the two solvents [24]. Pycnometry provides a maximum value for the total void volume [24]. This maximum value is an indisputable quantity that reflects the maximum volume accessible to a single solvent.

Thus pycnometry has been recommended as the only meaningful total void volume measurement [10, 25]. However, pycnometry requires that the column be repeatedly removed from the chromatograph, which is inconvenient and increases the chance that extra column voids will be introduced into the flow path. In addition, pycnometry assumes that there is no sorption of mobile phase onto the stationary phase. This is rarely the case for LC [24, 26], and even rarer in NPLC where the strong mobile phase component adsorbs onto the stationary phase [27, 28].

Table 2-1 Glossary of volume definitions, symbols and measurements

Nomenclature (symbols)	**Definition**	**Measurement methods**
Interstitial volume (V_i)	Volume of mobile phase between the particles	Total exclusion volume determined by size exclusion chromatography [32-33]
Pore volume (V_p)	Volume of mobile phase within the pores inside the particles	
Total void volume (V_0)[a]	Total volume of mobile phase in the column, $V_0 = V_i + V_p$	Pycnometry [10, 21, 45], Minor disturbance method [10, 19, 26], Tracer pulse method [19, 32]
Hold-up volume (V_{IS})	Elution volume of an unretained solute	Elution of unretained solutes [7, 29, 41-42]
Mobile phase volume (V_M)[a]	Volume of mobile phase truly move through the column	
Accessible volume (V_0^*)	Physical meaning is uncertain	Linearization of homologous series [7, 9, 10, 42]

[a]The symbols for total void volume and mobile phase volume follow the convention of Wang and co-authors [13]. Other symbols have been used for the same terms in older literature such as [19].

In the minor disturbance method, the total void volume V_0 is estimated by injecting a solvent mixture that is slightly different than the mobile phase. V_0 determined in this manner can vary dramatically with the mobile phase composition [29]. For instance, in RPLC the V_0 measured using the baseline disturbance method ranged from 3.86 mL to 2.70 mL upon changing the mobile phase from 10% ACN to 70% ACN [29]. Thus, the minor disturbance method also does not consider the volume occupied by the adsorbed strong solvent. Kazakevich and McNair dealt with this variation by integrating the retention volume observed for the disturbance over the entire range of mobile phase composition [29]. The resultant *integrated* V_0 was independent of the column temperature and the type of mobile phase modifier.

Usually, injection of mobile phase causes multiple disturbance peaks in the chromatogram **(Fig. 2-2)**. Two approaches can be used to simplify the disturbance pattern. First, injection of a solvent that is close in composition to the mobile phase will reduce the number of peaks observed. However, injection of a *near mobile phase solvent* will also reduce the magnitude of the baseline disturbances, such that in some instances no obvious baseline disturbance is observed. Alternately, to elicit a greater solvent-induced disturbance a pure mobile phase component can been injected [30]. In RPLC, the less-retained solvent in a binary mobile phase is recommended [31]. The elution volume of the pure solvent is considered as the total void volume, V_0. However even the injection of a pure solvent can cause multiple peaks, making the identification of the disturbance peak that correlates with V_0 difficult [32, 33]. Also, the literature is vague as to precisely which disturbance feature should be used to determine V_0. For instance, in **Fig. 2-2** the V_0

would vary by 4% depending on whether the negative or positive disturbance peaks were used.

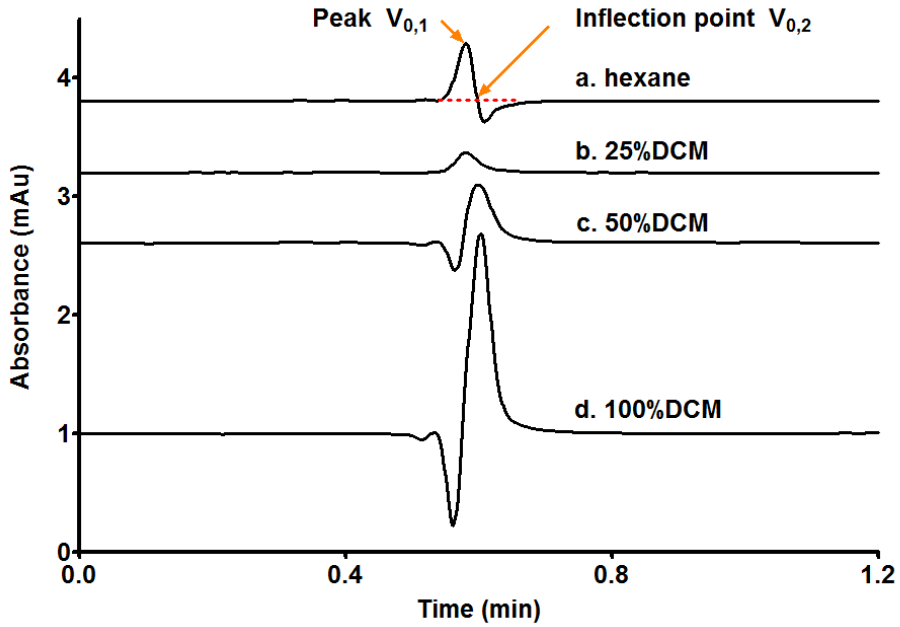


Fig. 2-2 Disturbance peaks upon injecting various compositions of hexane and DCM onto a DNAP column equilibrated with 25:75% DCM:Hexane. Other conditions: injection volume, 2 µL; column temp., 35°C; flow rate 1.0 mL/min; detection, 254 nm. The extra-column delay was subtracted from all data prior to plotting.

For the tracer pulse method, isotopically labeled eluent components are injected. The total void volume is determined by:

$$V_0 = \sum^i V^*_{R,i}\theta_i \qquad \text{Eq. 2-2}$$

where $V_{R,i}^*$ is the elution volume of each isotopically labeled eluent component i and θ_i is the fraction of component i in the eluent. The tracer pulse method was originally used for determination of gas-liquid or liquid-solid sorption isotherms [34]. The tracer pulse method has also been applied to the determination of total void volume [22, 35].

However, as with the previous two methods, the tracer pulse method incorrectly assumes that there is no sorption of mobile phase by the stationary phase.

In some literature, *hold-up volume* (V_{IS}) is defined as the retention volume of an unretained compound [7, 9, 36]. The volume determined in this manner could range from the interstitial volume (V_i) to the total void volume (V_0) depending on the degree to which the unretained compound can access the pores. Various neutral compounds or salts have been used as unretained markers in RPLC [7, 10, 11, 37]. The ideal solute to measure V_0 should be small enough to penetrate all of the pores and not be retained by the stationary phase. In reality, such a solute is actually impossible to find [11, 37]. One suggestion in the literature is to use isotopically labeled mobile phase to determine the V_{IS} [22, 24]. Regardless, great caution must be exercised when using an unretained compound to measure the *hold-up volume*.

The *accessible volume* (V_0^*) is provided by the linearization of retention of a homologous series of compounds. The general method is based on Martin's rule derived in 1950 [38]. In liquid chromatography, the Gibb's free energy required to transfer a given group (*e.g.*, CH_2) is described by $\Delta G(CH_2)$. Thus, for a compound containing a functional group X and n methylene groups, the total free energy for transferring the molecule is:

$$\Delta G^\circ = \Delta G(X) + n\Delta G(CH_2) \qquad \text{Eq. 2-3}$$

where $\Delta G(X)$ and $\Delta G(CH_2)$ are the free energy required to transfer group X and each methylene, respectively. The Gibbs free energy is related to the equilibrium constant (K) by:

$$\Delta G^\circ = -RT\ln K \qquad \text{Eq. 2-4}$$

where R is the ideal gas constant, T is the temperature in Kelvin, and K is the equilibrium constant. The equilibrium constant is related to the retention factor k through the phase ratio φ:

$$k = K\varphi \qquad \text{Eq. 2-5}$$

Based on Eq. 2-4 and 2-5, the natural logarithm of k should increase linearly with the carbon number (n) for a homologous series:

$$\ln k_n = \ln \frac{(V_R - V_0^*)}{V_0^*} = A + Bn \qquad \text{Eq. 2-6}$$

where A and B are empirical regression parameters, with the V_0^* defined as that value which yields the best linearity for a plot of ln k *vs*. n. Thus, multiple regressions must be performed to determine a V_0^* value using Martin's rule. This V_0^* value has been termed the *accessible volume*, and is sometimes assumed to be V_M in Eq. 2-1 [9, 23]. Martin's rule has been used extensively in both GC and LC [10, 39-41].

To bypass the iterative process (*i.e*., varying V_0^* until Eq. 2-6 is linear), Berendsen et al. made use of the fact that B in Eq. 2-6 is equal to the average ln α between successive homologs (α is the selectivity) [7]. If the selectivity is assumed to be constant for any successive homologs:

$$\ln k_n - \ln k_{n-1} = B \qquad \text{Eq. 2-7}$$

$$\frac{(V_n - V_0^*)}{(V_{n-1} - V_0^*)} = e^B \qquad \text{Eq. 2-8}$$

$$V_n = e^B V_{n-1} - (e^B - 1)V_0^* \qquad \text{Eq. 2-9}$$

where V_n and V_{n-1} are the retention volumes for solutes with n and n-1 carbon numbers respectively.

Trathnigg and coworkers further generalized Eq. 2-9 as [42]:

$$V_n - V_0^* = e^B V_{n-1} - e^B V_0^* = -e^B(V_n - V_{n-1}) + e^B(V_n - V_0^*) \quad \text{Eq. 2-10}$$

$$\gamma = \frac{e^B}{(e^B - 1)} \quad \text{Eq. 2-11}$$

This simplifies to the relationship:

$$V_n = V_0^* + \gamma \Delta V \quad \text{Eq. 2-12}$$

where $\Delta V = V_n - V_{n\text{-}1}$. In this *extrapolation method*, the intercept of a plot of V_n *vs.* ΔV yields the V_0^* value. Since the *accessible volum*e is easy to measure and is *similar* to V_M, it has been used extensively in studies of RPLC [9, 23, 42]. However, the true physical meaning of the accessible volume is still unknown [35].

In addition to the theoretical concern whether the homologous series methods (Eq. 2-6 and 2-12) will give a reliable void volume, there are also some practical considerations. Choices of the proper homologous series, the consistency of the different linear regression methods, and the consistency between different methods have all been subjects of research [7, 9-11, 37, 43, 44].

These various methods have been discussed in the literature to measure "void volume" in RPLC [7, 9-11, 22], but with no clear conclusion as to which method is most appropriate. There has been much less discussion of the appropriateness of these methods in NPLC [36, 45]. Engelhardt et al. [36] observed large variation (2% to 11%) in *k* when V_{IS} in NPLC was measured using a variety of unspecified "unretained compounds" including deuterated solvents [36]. Recently, Kim et al. investigated the retention behavior of polystyrene oligomers on silica under NPLC conditions [45]. Using Eq. 2-12, they determined the accessible volume, which was smaller than the V_{IS} determined by injection of solvent (the specific solvent and baseline feature were not specified) [45]. To

the best of our knowledge, no work has been done on other normal phase columns, including the widely used petro-columns mentioned previously.

The objective of this chapter is to explore the appropriateness of the above methods for determining void volume on a variety of NPLC phases, specifically silica, RingSep and DNAP columns. Efforts have been made to minimize the ambiguity from the concept, definition and measurement of "void volume". Pycnometry, the minor disturbance method and the tracer pulse method are used to measure the total void volume. Unretained compounds are carefully chosen to determine the hold-up volume. The accessible volume is obtained by linearization of linear polycyclic aromatic hydrocarbons (PAHs) and bent PAHs on all columns. Direct comparisons are made to illustrate the suitability of these methods in NPLC systems, especially for the petro-columns.

2.3 Experimental

2.3.1 Materials

HPLC grade hexane, dichloromethane (DCM) and tetrahydrofuran (THF) were from Fisher Scientific (Fairlawn, NJ, USA). Hexane-d_{14} (99% purity), dichloromethane-d_2 (DCM-d_2) (99.9% purity), polystyrene (average $M_n \approx 1020$ g/mol), benzene, anthracene, tetracene, phenanthrene (>90% purity) and 1,3,5-tri-*tert*-butylbenzene (TTBB, 97% purity) were from Sigma-Aldrich (St. Louis, MO, USA). The trace impurities in hexane-d_{14} were determined by gas chromatography-mass spectrometry (GC-MS, Zebron ZB-5MS (Phenomenex), 30 m x 250 μm x 0.25 μm; 40°C for 3.5 min then 20°C/min to 250 °C). Naphthalene (>90% purity) was from Fisher Scientific. Chrysene and picene (>90%

purity) were from K & K Laboratories (Carlsbad, CA, USA). Polystyrene A-300 (average $M_n \approx 453$ g/mol), A-500 (average $M_n \approx 500$ g/mol) and A-2500 (average $M_n \approx 2980$ g/mol) were from Tosoh (Tokyo, Japan).

The 5 cm × 0.46 cm I.D. column was packed in-house with 5 μm diameter (80 Å pore size) silica particles (Agilent, Santa Clara, CA, USA). A 3 g SiO_2/17 mL isopropanol slurry was packed using a constant pressure Haskel pump (DSF-122-87153, Burbank, CA, USA) driven with N_2 gas (Praxair Inc., Edmonton, AB, Canada). Packing was performed in the downward direction at 6000 psi (1 bar = 14.5 psi) for about 25 minutes with isopropanol as the driving solvent. The RingSep (packed on 95th day of 2012) and DNAP (packed on 250th day of 2010) columns (ES Industries, West Berlin, NJ, USA) were both 5 cm x 0.46 cm ID packed with 5 μm particles of 60 Å pore size.

2.3.2 Apparatus

All experiments were performed on an Agilent 1260 Infinity Quaternary LC system (Agilent). A G1311B pump was used to pump hexane/DCM or hexane/THF at 1.0 mL/min. An online degasser was used. All tubing and fittings were stainless steel (0.17 mm ID). The length of all connecting tubing was minimized. Sample injections of 2 μL were performed with an Agilent G1329B autosampler. The column was maintained at 35 °C using an Agilent G1316A thermostatted column compartment. Detection was at 254 nm unless otherwise indicated, as UV is the most common detection mode in NPLC. Data acquisition was achieved using Agilent Chemstation at a rate of 10 Hz and detector response time of 1 s. Other detectors such as refractive index (RI), evaporative light scattering (ELSD) and mass spectrometry are also used for NPLC. However, the RI

responds to all of the artifacts, which complicates the identification of the accurate void volume. For other detectors, the results in this chapter are expected to be similar.

2.3.3 Procedures

2.3.3.1 Extra column volume

The extra column volume was determined by removing the column and connecting the connecting tubing from the injector directly to the detector using a ZDV fitting (Agilent). Injection of 2 μL of pure deuterated hexane into a pure hexane mobile phase at 35 °C and 1 mL/min resulted in an elution time of 0.107±0.03 min. This value was subtracted from all retention times before calculations or plotting were performed.

2.3.3.2 Pycnometry

The silica, RingSep and DNAP columns were each equilibrated at room temperature (~23.5°C) with 100% hexane (0.668±0.001 g/cm^3) for 1 h at 1 mL/min. After the prime valve was open to release the pressure, the column was detached from the system and capped immediately with finger-tightened nuts. No solvent was observed to flow out of the column during the detachment. Then the column was weighed. The column was then flushed for an additional 30 min with hexane and weighed again. This was repeated until the mass of the column filled with solvent reached a constant value (±1 mg). Then the column was flushed with 100% DCM (1.322±0.001 g/cm^3) for 1 h at 1 mL/min, and then weighed. The column was flushed for an additional 30 min with DCM and re-weighed. This was repeated until a constant weight (±1 mg) was achieved. The overall process of equilibrating with DCM, weighing, equilibrating with hexane and weighing was repeated once more. The void volume for each column was calculated by $V_0 = (W_1 - W_2) / (\rho_1 - \rho_2)$. Pycnometry was also performed using hexane and THF (0.887 ±

0.001 g/cm^3). The uncertainty in $V_{0,weight}$ for each solvent pair is based on duplicate pycnometric measurements for each column. The densities of solvents used were measured manually at the corresponding room temperature and conditions.

2.3.3.3 Minor disturbance method

To cause a baseline disturbance at the void volume, various mobile phase compositions were injected into all columns. Generally, the disturbance showed two peaks of different direction (one positive and the other negative) with an inflection point in the middle **(Fig. 2-2)**. The retention volume at the apex of the first visible peak ($V_{0,1}$) and the inflection point ($V_{0,2}$) were measured manually. To determine the inflection point, the baseline was extended under the disturbance peaks. Where this baseline intersected with the chromatogram between the apex of the positive peak and the apex of the negative peak was defined as $V_{0,2}$ **(Fig. 2-2)**. The injections were done in at least duplicate for each mobile phase condition. Replicate measurements of $V_{0,1}$ and $V_{0,2}$ made over periods of months were reproducible within the errors noted in **Table 2-3.**

2.3.3.4 Unretained compounds

Pure hexane, deuterated hexane and 1,3,5-tri-*tert*-butylbenzene (TTBB) were tested as "unretained compounds" for all columns. Each sample was filtered through a 0.2 μm syringe-driven filter (PTFE, Millipore, Billerica, MA, USA) before injection. All retention data were expressed in volume units and are the average of triplicate injections. The volume value of the first visible peak was identified as the elution volume of each compound.

2.3.3.5 Tracer pulse method

For this method, deuterated hexane and deuterated dichloromethane were injected into all columns. All retention data were expressed in volume units and are the average of triplicate injections. The elution volume of each isotopically labeled component was identified as the first visible peak on the corresponding chromatogram. For each mobile phase composition, V_0 was calculated as the weighted average of the two elution volumes for the isotopically labeled solvents, based on Eq. 2-2.

2.3.3.6 Linearization of homologous series

Linear PAHs (benzene, naphthalene, anthracene and tetracene) and bent PAHs (naphthalene, phenanthrene, chrysene and picene) series were used as homologous series on all columns. All of the samples were prepared in mixtures of DCM and hexane with the same composition as the corresponding mobile phase. The analyte concentration ranged from 0.1 mg/mL to 1 mg/mL, dependent upon each compound's molar absorptivity. Each sample was filtered through a 0.2 μm syringe-driven filter before injection. The compounds were injected in a random order to minimize systematic errors. All of the retention data were expressed in volume units and are the average of three measurements. For each mobile phase composition, the accessible volume was calculated both by iterative regression of Martin's rule (Eq. 2-6) and by extrapolation of line V_n *vs.* ΔV to $\Delta V = 0$ (Eq. 2-12). Regression analysis was performed in Microsoft Excel 2011 with a personal computer as discussed below and in **Appendix A**.

To perform Martin's rule (Eq. 2-6), log k for each compound were calculated using the measured V_R (extra-column volume subtracted) and a series of estimates of V_0^* ranging from well below the expected V_M to well above (*e.g.*, 0.3 to 0.6 mL) in 0.002 mL

increments. For each V_0^* estimate, linear regression was performed for log *k vs*. *n*, where *n* is the ring number [46, 47] (*i.e*., 1 for benzene, 2 for naphthalene, etc.) The correlation coefficient (*R*) was calculated for each plot using the "CORREL" function in Excel. The accessible volume (V_0^*) was defined as the value that yields the best linearity of line log *k vs. n*. The uncertainty for V_0^* was calculated using the "LINEST" function in Excel.

To determine the accessible volume (V_0^*) using the extrapolation method (Eq. 2-12), ΔV was calculated as the difference between each pair of consecutive homologs. Then by plotting V_n (extra column volume subtracted) *vs.* ΔV, a linear line was obtained. The intercept of the line is the accessible volume (V_0^*). The uncertainty for V_0^* was calculated using the "LINEST" function in Excel.

2.3.3.7 Student *t*-test

The Student *t*-test was used to compare the void volume measured by one method with that determined by another method to determine whether they are significant different. Because we are comparing two sets of measurements by two different methods, paired *t*-test is applied. First, the difference (d_i) is calculated for each measurement by two different methods. Then the mean ($\bar{d}$) and the standard deviation (s_d) of all the differences are computed. Then the *t* value (t_{calc}) can be calculated by Eq. 2-13.

$$t_{calc} = \frac{|\bar{d}|}{s_d}\sqrt{n} \qquad \text{Eq. 2-13}$$

where *n* is the number of measurements by each method. After t_{calc} is obtained, it was compared with tabulated *t*-values [48]. If t_{calc} is less than the table t-value at 95% confidence interval, then we conclude that the two sets measurements are not significantly different.

2.4 Results and Discussion

The void volume of a chromatographic column is a fundamental parameter necessary to understand the retention and selectivity of LC phases. Many methods have been described in the literature for estimation of the void volume [7, 11, 22, 29, 39]. However, these studies have primarily focused on RPLC [7, 10, 11, 35], and in only a few cases have the methods been directly compared with one another [7, 10, 37]. For normal phase LC, there has been little discussion of the measurement of void volume [36, 45]. The definitions for void volume in RPLC and NPLC are similar (as summarized in **Table 2-1**). However, the suitability of application of the different methods for measuring various void volume concepts in NPLC is not clear.

This chapter compares a variety of methods for the determination of void volume in NPLC including pycnometry, minor disturbances, tracer pulse, unretained compounds and linearization of homologous series. These methods are applied to silica, which is a commonly used NPLC stationary phase, and to charge-transfer columns **(Fig. 2-1)**, which are widely used for petroleum analyses [12-17].

2.4.1 Pycnometry

As discussed in Section 2.2, in pycnometry the void volume is determined by the weight difference when the column is filled with two solvents of different density (hexane and DCM or hexane and THF, in this work). **Table 2-2** summarizes the total void volumes determined by pycnometry ($V_{0,\text{weight}}$). The $V_{0,\text{weight}}$ measured by hexane and DCM or hexane and THF are in good agreement with each other for each column. The relative standard deviations (RSD) were 0.3%, 0.4% and 1.1% for the silica, RingSep and DNAP columns, respectively. In comparison, an overall 1.5% RSD was previously

reported for the determination of $V_{0,weight}$ of a chiral column using multiple solvent sets [8]. The average value based on both solvent pairs could be used to represent the total void volume.

Each pycnometric measurement involves extended equilibration of the column and multiple detachments and weighings. Hence the method is slow. A minimum of 5 hours was required for each column. Also, during the whole process, the column had to be disconnected from the instrument at least three times, which caused abrasion to the connecting fittings and could result in extra column voids.

Table 2-2 Determination of total void volume ($V_{0,weight}$) by pycnometry [a]

Solvent System	**$V_{0,weight}$ (mL)**		
	Silica	**RingSep**	**DNAP**
Hexane-DCM	0.586 ± 0.001	0.566 ± 0.001	0.564 ± 0.002
Hexane-THF	0.589 ± 0.001	0.569 ± 0.003	0.573 ± 0.002
Average	0.588 ± 0.002	0.568 ± 0.002	0.569 ± 0.006

[a] Equilibration conditions: flow rate, 1 mL/min; equilibrium under flow conditions for 30 min before each weighing; Weighing was in at least triplicate for each solvent or until constant weight was achieved; room temperature (~23.5^{o}C). In some cases, pycnometry with the second solvent set was performed months after the first set.

Another disadvantage is that the pycnometry method does not consider the solvent distribution between the mobile phase and stationary phase. That is, any solvent adsorbed onto the stationary phase (and thus not moving) is included within the void volume determined by pycnometry. Thus $V_{0,\ weight}$ is usually considered an upper limit for the void volume [7, 10, 11, 24].

In RPLC, $V_{0,weight}$ has been used to evaluate if there are other factors that affect the void volume measured by other more convenient methods [24]. For example, if the elution volume of a "unretained solute" is larger than $V_{0,weight}$, the solute is weakly

retained by the column, and so is not a true unretained compound [24]. Alternately, if an "unretained solute" elutes before $V_{0,weight}$, this means either that the solute is too large to fully explore the pore volume (V_p) within the column [49] or that some space within the column is occupied by solvent adsorbed onto the stationary phase [49]. Thus, although time-consuming, pycnometry should be used to validate whatever more convenient void volume measurement will be subsequently used [8] as is done in our later discussion.

2.4.2 Minor disturbance method

The minor disturbance method is one of the most commonly used methods for determining the void volume in RPLC [11, 22, 29, 31]. In this method, mobile phase is injected and the resultant minor baseline perturbations are used to determine the void volume. For example, **Fig. 2-2** shows the minor disturbance caused by injection of various DCM:hexane mixtures into a 25:75% DCM:hexane mobile phase on the DNAP column. The disturbance is complex with multiple peaks, as has been noted previously in RPLC [32, 33]. Even changing the mobile phase composition (*e.g.*, to 10% DCM) can change the disturbance pattern **(Fig. 2-3)**. Likewise, changing the column type results in further changes to the disturbance pattern.

There are two phenomena that can contribute to the complex disturbance patterns such as observed in **Fig. 2-2** and **2-3**. First, injection of a solvent of a different composition than the eluent results in a transient zone of varying refractive index. This refractive index gradient acts as a dynamic lens within the flow cell, which results in a derivative-shaped signal [50, 51]. The inflection point ($V_{0,2}$ in **Fig. 2-2 and 2-3**) reflects the maximum in the refractive index profile (*i.e.*, where there is no change in refractive index). The magnitude and direction of the derivative response are not predictable based

solely on the absolute refractive index of the solvents, but rather depends on the dynamic focal properties of the solvent lens within the flow cell. Also, the two features of the refractive index pattern may be displaced from one another if volume overload due to injection is significant. Herein, small injection volume of 2 µL is used to minimize this effect.

Second, injection of a solvent which differs in composition from the mobile phase affects the distribution of the mobile phase components on the stationary phase [52]. This will cause system peaks, which may be either positive or negative, and relate to the sorption of each individual component [24, 52, 53].

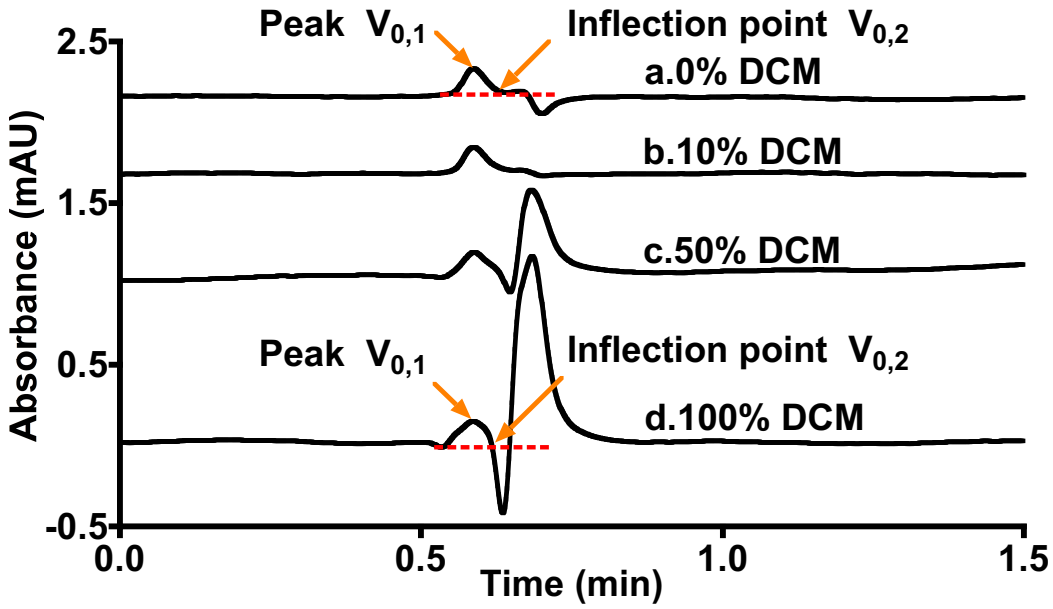


Fig. 2-3 Disturbance peaks upon injecting various compositions of hexane and DCM onto a DNAP column equilibrated with 10:90% DCM:hexane. Other conditions: injection volume, 2 µL; column temp., 35°C; flow rate, 1.0 mL/min; detection wavelength 254 nm. a-d refer to the composition of the sample.

Thus due to both refractive index and solvent displacement effects, complex minor disturbance patterns such as those in **Fig. 2-2** and **2-3** are observed upon injecting various mobile phase compositions. Moreover the pattern of the disturbance depends on the composition of both the mobile phase and the injection solvent. Even injection of hexane to the column equilibrated with hexane generates a disturbance in the baseline. This is presumably caused by an unknown impurity in hexane. This variation in the disturbance patterns in **Fig. 2-2** and **2-3** is significant. While the minor disturbance method has been used extensively in the literature [7, 10, 29], there is no description as to which disturbance feature should be used to determine V_0. Typically in RPLC, the pure less retained solvent in a binary mobile phase is injected to determine the void volume [31]. As described in Section 2.3.3.3 and illustrated in the upper chromatogram of **Fig. 2-2**, we used hexane injections to induce a minor disturbance. For the mobile phase conditions studied (0%-50%DCM), the minor disturbance caused by injection of hexane always showed a positive initial peak (**Fig. 2-4**). We evaluated both the first peak ($V_{0,1}$) and the inflection point ($V_{0,2}$) as estimates of the void volume. These values are tabulated in **Table 2-3**.

At the start of this work, we had assumed that the inflection point ($V_{0,2}$, **Fig. 2-2**) would be the best estimate of the void volume, as it reflected the maximum of the refractive index peak. However, **Table 2-3** shows that the inflection peak ($V_{0,2}$) is strongly positively biased relative to pycnometry ($V_{0,\text{weight}}$), which should be the maximum estimate for the void volume (Section 2.4.1) [7, 10, 11, 24]. Thus the inflection point is not an appropriate feature to determine the void volume. Instead, the initial

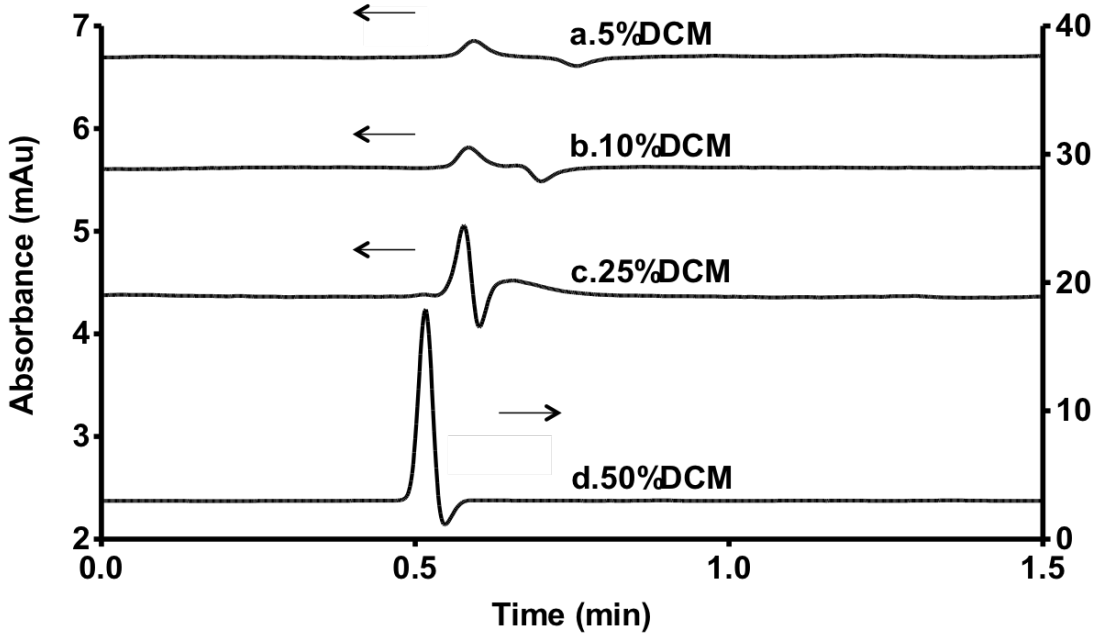


Fig. 2-4 Disturbance peaks upon injecting pure hexane onto a DNAP column equilibrated with various compositions of hexane and DCM as eluent. The arrows indicate the y-axis. a-c, left y-axis. d, right y-axis. Other conditions: injection volume, 2 μL; column temp., 35^{o}C; flow rate, 1.0 mL/min; detection wavelength 254 nm. a-d refer to the composition of the mobile phase.

disturbance peak ($V_{0,1}$) was found to agree better with the pycnometry $V_{0,weight}$ for all columns and all mobile phase compositions.

A second feature evident in **Table 2-3** is that $V_{0,1}$ decreases with increasing %DCM in the mobile phase. This would appear to be in conflict with Kim et al. who stated that the dead volume for a silica column determined by injection of the weak solvent was constant, independent of the composition of the THF/hexane mobile phase [45]. However, closer inspection of their data shows a downward drift (from 3.30 mL for 5% THF to 3.18 mL for 20% THF) in the dead volume as the percentage of the strong solvent (THF) increased. As %DCM increased, either $V_{0,1}$ or $V_{0,2}$ will decrease. This decrease is consistent with the expectation that more DCM would be strongly adsorbed

onto the stationary phase at higher %DCM [27, 28]. Upon changing the mobile phase from 10% to 50% DCM, the increase in the volume of DCM predicted to adsorb onto our silica phase is 0.03-0.15 mL, depending on the isotherm used to describe the DCM adsorption [27, 28]. Thus the change in $V_{0,1}$ observed in **Table 2-3** is consistent with the expected change in dead volume with increasing mobile phase strength.

A final surprising observation in **Table 2-3** is that for low mobile phase strengths, $V_{0,1}$ is greater than the $V_{0,\text{weight}}$ for the RingSep and DNAP columns. This is also true for the silica column under 0% DCM. Pycnometry is theoretically the maximum value for the dead time [7, 11, 24, 41]. Similar behavior has been observed in the literature [24]. The greater value than $V_{0,\text{weight}}$ indicates retention of the solutes. This apparent retention of hexane may be explained by vacancy chromatography or impurities in the solvent [24]. In summary, the disturbance features are complex and hard to identify. Thus, caution must be taken in determining which feature of the disturbance correlates with the void volume. Here the first visible peak was found to be the best marker as it yields total void volume values, which are closest to the weighing method. It is recommended that the weighing method be performed with each new column to validate which disturbance feature best correlates with the total void volume.

2.4.3 Unretained compound

It is challenging to find a completely unretained compound to determine the hold-up volume (V_{IS}) [11, 37]. In NPLC it is generally considered that the weak solvent does not adsorb onto the stationary phase [25, 31]. Hexane is a weaker solvent than DCM, and so has been used as an unretained compound for hold-up volume measurements on silica [27]. Therefore, the weak solvent (hexane) was tested as an unretained compound for our

three columns (*i.e.*, the retention volume of pure hexane ($V_{0,1}$), can also be considered as a V_{IS}). However, as discussed in Section 2.4.2, the baseline disturbance caused by injecting hexane is minor in magnitude and complex in nature. Thus, it may be difficult to identify the correct feature associated with the elution of hexane.

Isotopically labeled isomers have been proposed as hold-up markers in RPLC [7, 10, 32]. Engelhardt and co-authors used deuterated DCM to determine V_{IS} in NPLC on silica [36]. Herein we tested deuterated weak mobile phase component (hexane-d_{14}) as a V_{IS} marker for mobile phases from 0% to 50% DCM on the silica, RingSep and DNAP columns **(Table 2-3)**. Hexane-d_{14} yields more defined features than those for hexane **(Fig. 2-5)**. The first visible peak (absorbance of 1-4 mAu) is due to hexane-d_{14}, and was used to determine V_{IS}. However, care must be taken because a later more prominent peak (25 mAu) is present due to trace perdeuterobenzene (as confirmed by GC-MS). In **Table 2-3** the V_{IS} based on hexane-d_{14} is smaller than either $V_{0,weight}$ or $V_{0,1}$ (at 95% confidence interval) for all mobile phase conditions and all columns studied. Differences in elution between isotopically labeled and unlabeled solvents have been reported in RPLC [22, 24]. Thus, hexane-d_{14} is expensive, contains distracting trace UV absorbing impurities, and results in void volumes that are biased low. For these reasons, hexane-d_{14} is not recommended as a void volume marker in NPLC.

1,3,5-Tri-t-butyl benzene (TTBB) has been proposed [54] and widely used [8, 55] as an unretained compound in chiral chromatography performed under normal phase conditions. The steric bulk of the substituents of TTBB is assumed to prohibit interaction with the stationary phase. To the best of our knowledge TTBB has not been tested as an

Table 2-3 Determination of void volume by different methods on silica, RingSep and DNAP columns

Columns		Pycnometry	Minor disturbance[b,c]		Unretained compound[c]		Linearization of PAHs[c]	
			hexane		hexane-d_{14}	TTBB	Martin's rule	V_n *vs.* ΔV
		$V_{0,weight}$[a]	$V_{0,1}$[d]	$V_{0,2}$	V_{IS}	V_{IS}	V_0*	
Silica	0%DCM	0.588±0.002	0.599±0.001		0.555±0.001	0.898±0.001		
	5%DCM		0.584±0.002	0.717±0.004	0.551±0.001	0.656±0.001	0.474±0.001	0.462±0.029
	10%DCM		0.579±0.002	0.650±0.010	0.554±0.000	0.604±0.000	0.448±0.001	0.441±0.024
	25%DCM		0.570±0.004	0.589±0.009	0.531±0.001	0.552±0.001	0.326±0.000	0.326±0.007
	50%DCM		0.532±0.002	0.555±0.001	0.529±0.000	0.530±0.001		
RingSep	0%DCM	0.568±0.002	0.624±0.001		0.553±0.001	0.597±0.001		
	5%DCM		0.597±0.002	0.683±0.004	0.537±0.004	0.572±0.001	0.540±0.004	0.557±0.019
	10%DCM		0.587±0.001	0.652±0.004	0.531±0.004	0.557±0.001	0.542±0.006	0.561±0.024
	25%DCM		0.579±0.001	0.597±0.006	0.517±0.003	0.524±0.001	0.520±0.002	0.525±0.007
	50%DCM		0.520±0.000	0.538±0.003	0.512±0.000	0.495±0.001	0.510±0.001	0.513±0.003
DNAP	0%DCM	0.569±0.006	0.628±0.001		0.547±0.003	0.600±0.001		
	5%DCM		0.590±0.004	0.696±0.006	0.542±0.001	0.572±0.001	0.538±0.007	0.572±0.036
	10%DCM		0.582±0.003	0.660±0.014	0.533±0.001	0.555±0.000	0.530±0.005	0.551±0.023
	25%DCM		0.570±0.006	0.591±0.003	0.515±0.000	0.521±0.000	0.512±0.003	0.524±0.011
	50%DCM		0.513±0.004	0.534±0.002	0.503±0.000	0.493±0.001	0.506±0.001	0.510±0.005

[a] Average value from Table 2-2.
[b] As illustrated in Fig. 2-1, $V_{0,1}$ was determined as the peak maximum of the first positive peak and $V_{0,2}$ as the inflection point where the detector signal crosses the baseline. The uncertainties of $V_{0,1}$ and $V_{0,2}$ are based on replicate runs, which in some cases were done months apart.
[c] Conditions: flow rate, 1 mL/min; injection volume, 2 μL; column temp., 35°C, detection, 254 nm. Injections are performed on all the columns under various mobile phases.
[d] $V_{0,1}$ is also used as V_{IS} when hexane is considered as an unretained compound.

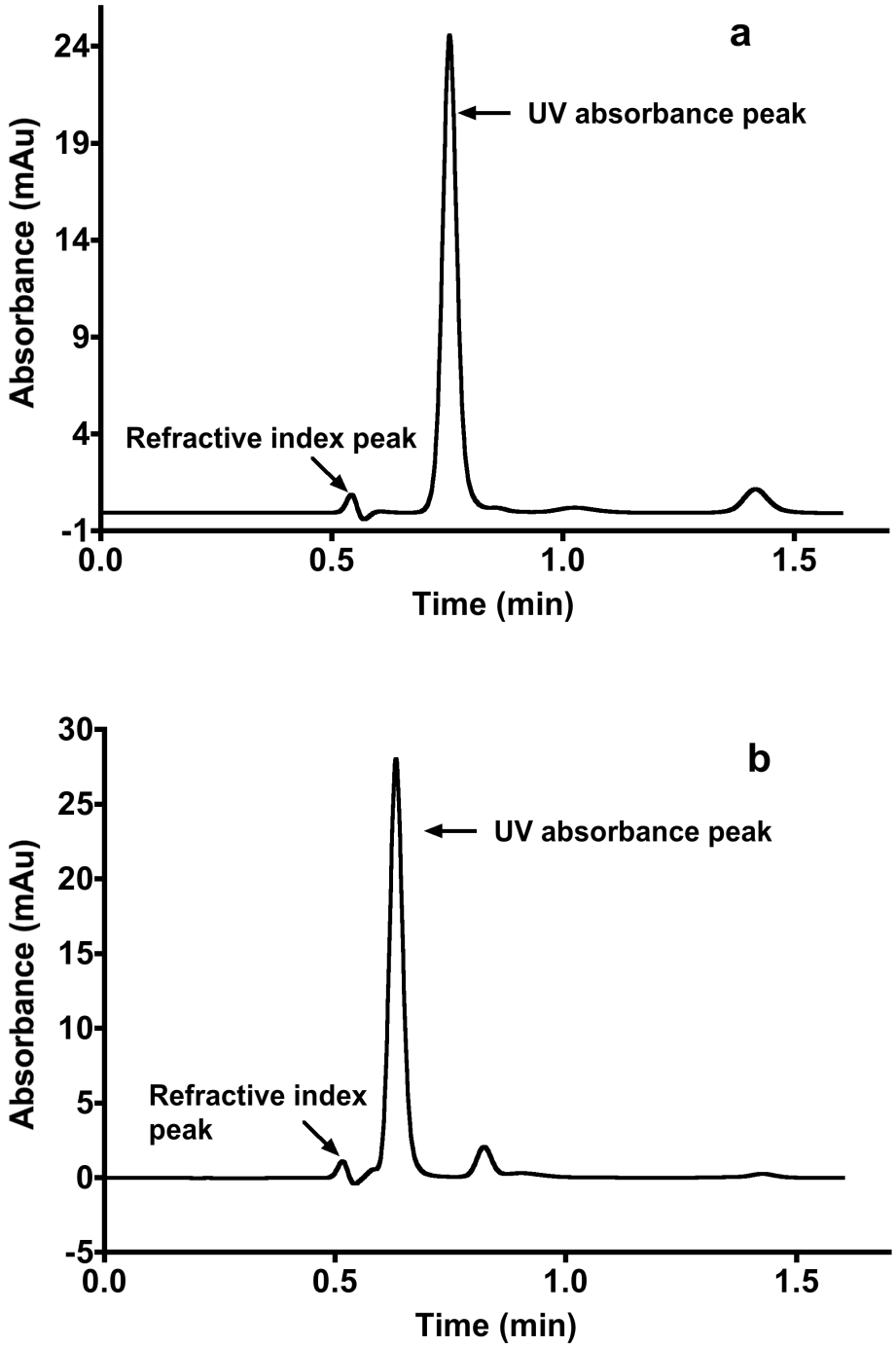


Fig. 2-5 Elution of deuterated hexane on DNAP column with various eluent. a. 5:95% DCM:hexane b. 25:75% DCM:hexane. Other conditions: injection volume, 2 μL; column temp., 35°C; flow rate, 1.0 mL/min; detection wavelength 254 nm. The refractive index peak is caused by hexane-d_{14}; UV absorbance peak is caused by trace perdeuterobenzene impurity within the hexane-d_{14}.

unretained compound for hold-up volume measurement in NPLC. **Table 2-3** compares the V_{IS} determined with TTBB with pycnometry and the disturbance methods. On silica, TTBB exhibits retention (*i.e.*, $V_{IS} > V_{0,weight}$) under weak (<25% DCM) mobile phase conditions, but agrees well with $V_{0,1}$ under stronger mobile phase conditions. For the RingSep and DNAP columns, V_{IS} based on TTBB is biased high relative to $V_{0,weight}$ at 0% DCM indicating retention, and agrees with the $V_{0,weight}$ at 5% DCM at the 95% confidence interval. The $V_{IS,TTBB}$ then decreases as the mobile phase strength increases, as expected based on increased DCM adsorption (Section 2.4.2). Thus, TTBB would be suitable as an unretained marker for silica only under strong eluent conditions, but is an effective unretained compound for the petroleum columns for eluent containing DCM larger than 5%. This limitation of TTBB as an unretained compound is not dissimilar to the restrictions on uracil which is widely used as an unretained marker in RPLC [56]. Uracil has weak retention on C_{18} columns when the RPLC mobile phase contains less than 40% organic component [32].

2.4.4 Tracer pulse method

Our mobile phase system consisted of the binary solvents hexane and DCM. To perform the tracer pulse method (Eq. 2-2), the elution volumes of both hexane-d_{14} and DCM-d_2 must be determined for each mobile phase composition **(Table 2-4)**. Neither of the elution volumes individually gives a good estimation of the total void volume ([22] and **Table 2-4**) V_0 based on the tracer pulse method (Eq. 2-2) for 0%-50% DCM were always biased lower than the $V_{0,weight}$. The tracer pulse method also suffers from the same impurity challenges as discussed in Section 2.4.3. Thus, the tracer pulse method is complex and does not increase the accuracy. Therefore it is not recommended.

2.4.5 Methods based on homologous series

Use of Martin's rule (Eq. 2-6) or its descendants (Eq. 2-12) requires retention data for a homologous series. Commonly, at least four homologs are needed to perform a reliable linearization [37]. Too weakly retained homologs yield unreliable *k*, whereas too strongly retained homologs are difficult to detect and tedious to use. Also, in RPLC nonlinearity have been observed when using too small of homologs [16] or homologs whose dimensions are larger than the bonded phase thickness [40, 43]. Also considerations such as detectability and solubility may limit the options as to which series can be used [11].

Various homologous series have been used for the determination of accessible volume in RPLC [7, 9, 10, 16, 35, 43] and as retention indices in gas chromatography (GC) [39, 46]. However there has been little discussion of the use of homologous series in NPLC [45]. Polystyrene oligomers have been proposed [45], but we observed either weak retention on silica using the same eluent as reference [45] or peak splitting for higher oligomers when using a weaker DCM/hexane mobile phase. Such peak splitting behavior has been observed and explained in the literature [57]. Thus, polystyrene is not a suitable homologous series.

Polycyclic aromatic hydrocarbons (PAHs) possess moderate retention on normal phase columns. Further, PAHs are resolved based on their ring numbers on both the RingSep [12, 58] and DNAP columns [14]. This makes the linear PAHs (detailed in Section 2.3.3.5) an attractive homologous series for determination of accessible volume (V_0^*) in NPLC.

Table 2-4 Determination of total void volume by the tracer pulse method

Columns	Mobile phase	Pycnometry	Elution volume (mL)		V_0 (mL)
		$V_{0,weight}$	Hexane-d_{14}	DCM-d_2	
Silica	0%DCM	0.588±0.002	0.555±0.001	0.839±0.001	0.555±0.001
	5%DCM		0.551±0.001	0.657±0.001	0.556±0.001
	10%DCM		0.554±0.000	0.608±0.001	0.559±0.000
	25%DCM		0.531±0.001	0.534±0.000	0.532±0.001
	50%DCM		0.529±0.000	0.506±0.003	0.517±0.001
RingSep	0%DCM	0.568±0.002	0.553±0.001	0.708±0.001	0.553±0.001
	5%DCM		0.537±0.004	0.606±0.001	0.540±0.004
	10%DCM		0.531±0.004	0.566±0.000	0.535±0.004
	25%DCM		0.517±0.003	0.503±0.000	0.514±0.002
	50%DCM		0.512±0.000	0.466±0.000	0.489±0.000
DNAP	0%DCM	0.569±0.006	0.547±0.003	0.728±0.000	0.547±0.003
	5%DCM		0.542±0.001	0.594±0.003	0.545±0.001
	10%DCM		0.533±0.001	0.584±0.002	0.538±0.001
	25%DCM		0.515±0.000	0.499±0.001	0.511±0.000
	50%DCM		0.503±0.000	0.461±0.002	0.482±0.001

Table 2-5 summarizes V_0^* for all columns under various eluent conditions For Martin's rule (Eq. 2-6), the value of V_0^* in **Table 2-5** is the value which maximizes the correlation coefficient (R) of a plot of log *k vs. n*, where *n* is the ring number. The R-values for the plots are all larger than 0.9999 (**Table 2-5**), with random residuals. The V_0^* values are

Table 2-5 Determination of accessible volume by linear PAHs and bent PAHs

Volumes (mL)		Martin's rule (V_0^*)[a]				V_n *vs.* ΔV (V_0^*)[b]			
		PAHs	**R^2**	**Bent PAHs**	**R^2**	**PAHs**	**R^2**	**Bent PAHs**	**R^2**
Silica	5%DCM	0.474±0.001	0.99996	0.446±0.003	0.99990	0.462±0.029	0.99892	0.482±0.070	0.99807
	10%DCM	0.448±0.001	0.99997	0.422±0.002	0.99984	0.441±0.024	0.99829	0.454±0.072	0.99404
	25%DCM	0.326±0.000	0.99999	0.344±0.001	0.99989	0.326±0.007	0.99974	0.371±0.077	0.97022
RingSep	5%DCM	0.540±0.004	0.99993			0.557±0.019	0.99999		
	10%DCM	0.542±0.006	0.99994	0.494±0.014	0.99988	0.561±0.024	0.99996	0.604±0.131	0.99992
	25%DCM	0.520±0.002	0.99998	0.518±0.014	0.99975	0.525±0.007	0.99997	0.546±0.054	0.99979
	50%DCM	0.510±0.001	0.99998	0.503±0.007	0.99983	0.513±0.003	0.99994	0.522±0.027	0.99929
DNAP	5%DCM	0.538±0.007	0.99995			0.572±0.036	0.99998		
	10%DCM	0.530±0.005	0.99996	0.456±0.010	0.99994	0.551±0.023	0.99997	0.547±0.108	0.99995
	25%DCM	0.512±0.003	0.99996	0.488±0.007	0.99993	0.524±0.011	0.99992	0.521±0.043	0.99986
	50%DCM	0.506±0.001	0.99997	0.474±0.002	0.99998	0.510±0.005	0.99986	0.503±0.023	0.99950

[a] For Martin's rule, R^2 is the correlation coefficient for the line defined by log *k vs. n*.
[b] For V_n *vs.* ΔV, R^2 is the correlation coefficient for the line defined by V_n *vs.* ΔV.

also presented in **Table 2-3** based on the extrapolation method (Eq. 2-12) in which V_0^* was determined as the intercept.

Bent PAHs have been proposed as retention time indices in gas chromatography [46]. Use of bent PAHs with Martin's rule and the extrapolated method yielded poorer linearity and greater uncertainty than using the linear PAHs **(Table 2-5)**. Therefore further discussion of accessible volume will be restricted to the linear PAHs.

Independent of the other methods for determining the void volume discussed in this chapter, a number of observations would suggest that the V_0^* determined by either Martin's rule (Eq. 2-6) or the extrapolation method (Eq. 2-12) are good estimates of the void volume. Firstly, the high correlation coefficients **(Table 2-5)** noted in the last paragraph are alluring. Secondly, consistent values of V_0^* are obtained in **Table 2-3** regardless of whether Martin's rule (Eq. 2-6) or the extrapolation method (Eq. 2-12) is used. Martin's rule and the extrapolation method yielded statistically equivalent values for V_0^* at the 95% confidence interval. Similar agreement between the two mathematical treatments has been reported in both RPLC [35] and NPLC [45]. Thirdly, the V_0^* decreased with increasing %DCM, as would be expected based on adsorption of the strong solvent (Section 2.4.2).

However when the accessible volumes (V_0^*) in **Table 2-3** are compared with void volumes measured by other techniques, significant bias is apparent. The linearization of homologous series methods yield accessible volumes V_0^* that are smaller than the $V_{0,\text{weight}}$ given by pycnometry. While pycnometry is considered to yield an upper limit for the void volume [7, 11, 24, 41], the difference between V_0^* and $V_{0,\text{weight}}$ is greater than the maximum volume estimated for DCM adsorbed on silica (0.15 mL, Section 2.4.2). Also,

the V_0^* in **Table 2-3** are consistently biased low (18%-42% on silica and 2%-10% on RingSep and DNAP) relative to void volumes measured under comparable conditions using either the disturbance method ($V_{0,1}$) or TTBB **(Table 2-3)**. This bias low for the accessible volume is consistent with the literature. For instance, the accessible volume determined using a polystyrene series on silica was 26%-39% lower than V_{IS} [45], while the accessible volume determined using polyethylene glycol on a C_{18} RPLC column was 10%-30% less than that measured by injecting acetone [9]. Indeed, Wang et al. concluded that in RPLC the accessible volume is usually between the total void volume and interstitial volume, but its physical meaning is uncertain [35].

In summary, both Martin's rule and the extrapolation method are tedious to perform, and ultimately do not provide an accurate estimate of the void volume. These methods are not recommended for NPLC.

2.5 Conclusions

Void volumes are of fundamental importance in chromatography. Our work compared measuring void volumes by pycnometry, the minor disturbance method, unretained compounds, the tracer pulse method, and linearization of homologous series. Pycnometry provides an upper limit for the void volume, but does not allow for the volume occupied by strong mobile phase on the stationary phase surface. Hence pycnometry is the recommended method only under weak mobile phase conditions. The minor disturbance method, where the weak mobile phase is injected, can yield a convenient estimate of void volume, but caution must be exercised as to which disturbance feature is measured. 1,3,5-Tri-t-butylbenzene (TTBB) is an effective

unretained compound on charge-transfer columns such as RingSep and DNAP. However 1,3,5-tri-t-butylbenzene shows retention on silica under weaker mobile phase conditions. Therefore it is only recommended as a void volume marker on silica when stronger mobile phases are used. The tracer pulse method and linearization of homologous series method are not recommended in NPLC.

As a whole, either the injection 1,3,5-tributylbenzene or weak solvent is the most convenient and reliable estimates of void volume in NPLC. Validation of void volumes is best done by: comparing the disturbance method *vs.* pycnometry under weak eluent conditions; or the disturbance method *vs*. 1,3,5-tributylbenzene under strong eluent conditions. The authors recommend using the minor disturbance method (first peak) for identification of the void volume in routine analysis due to its simplicity and relatively high accuracy over a wide range of mobile phase composition.

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Chapter Three: Retentivity, selectivity and thermodynamic behavior of polycyclic aromatic hydrocarbons on charge-transfer and hypercrosslinked stationary phases under conditions of normal phase high performance liquid chromatography‡

3.1 Introduction

Polycyclic aromatic hydrocarbons (PAHs) are potential carcinogens derived mainly from incomplete combustion of fossil fuels. The carcinogenicity of PAHs varies with their size, isomeric shape and planarity [1]. Characterization of PAHs have been performed in air particulate matter [2, 3], water [4-6], water sediments [4] and biological samples [7, 8]. Spectroscopic and chromatographic analyses of PAHs have been reviewed in Refs. [9, 10]. High performance liquid chromatography (HPLC) enables characterization of the various PAH isomers in complex matrices [9, 11, 12]. UV absorbance [5], fluorescence [13] and mass spectrometric detection [14-18] have all been used in conjunction with HPLC.

Both reversed phase liquid chromatography (RPLC) and normal phase liquid chromatography (NPLC) are widely used for PAH separations [19, 20]. In RPLC, PAHs can be resolved into alkyl-substituted and non-substituted PAHs [21]. However, the retention of PAHs on RPLC is heavily dependent on the nature of the C_{18} phase, *i.e.* monomeric or polymeric [22, 23]. Also, RPLC retention cannot be used to group PAHs

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of equal number of aromatic carbon atoms or aromatic rings, as alkyl substitution on the PAHs significantly affects retention.

PAHs are grouped based on the number of aromatic carbon atoms or aromatic rings in NPLC [24-27]. However, conventional normal phase stationary phases (*e.g.*, silica, alumina and amine columns) still provide only limited resolution for PAH groups [27]. Charge-transfer phases provide superior group type separation for PAHs [28-30]. In charge-transfer liquid chromatography, retention is mainly based on π-π interactions between the electron accepting bonded phase and the electron donating PAHs [28, 31-33]. But π-π interaction is not a true charge-transfer transition, as stated in Section 1.3.3.2.2. 3-(2,4-Dinitroanilino) propyl (DNAP) is the most widely used charge-transfer bonded phase for PAHs [27, 28, 32] and fuel analysis [14, 15, 34-36]. DNAP columns separate PAHs according to their aromatic ring numbers [27, 28], rather than the aromatic carbon number basis of columns such as silica and alumina [25, 26]. This aromatic ring number dependence yields higher resolution between two and three ring PAHs [20, 28, 37]. DNAP can also resolve saturates and polar compounds in petroleum from PAHs [33]. The retention of PAHs on DNAP is also affected by molecular shape and alkyl substitution [31], as were RPLC and conventional adsorption based phases [27, 32]. However, the retention behavior of PAHs on DNAP has poor correlation ($R^2 < 0.79$) with that in RPLC and adsorption chromatography [31].

Another stationary phase that has interesting selectivity for PAHs is hypercrosslinked polystyrene [38-40]. Hypercrosslinked polystyrene is a new type of porous polymeric phase first developed by Davankov and co-workers [38]. In contrast to conventional poly(styrene-divinylbenzene), hypercrosslinked polystyrene phases are

synthesized by post-crosslinking of polystyrene with a bifunctional linker such as dimethylformamide. The hypercrosslinked polystyrene has high rigidity due to the high percentage of methylene -CH_2- linkers between the phenyl groups. These phases contain both micropores and macropores, and have an extremely high apparent specific surface area [38]. The biporous structure allows hypercrosslinked polystyrene to perform separations of both low molecular weight molecules and macromolecules [41]. The hypercrosslinked polystyrene is mechanically stronger and less prone to swelling or shrinkage than conventional poly(styrene-divinylbenzene). Thus it is compatible with solvents ranging from polar solvents such as methanol and water to non-polar solvents such as hexane [38, 40].

Of the few hypercrosslinked HPLC phases, Chromalite 5HGN has been most used due to its small particle size (5 μm), compatibility with various mobile phases, mechanical stability and a high apparent surface area [42, 43]. 5HGN has been used in both RPLC and NPLC [42, 43]. With normal phase solvents such as hexane, dichloromethane and isopropanol, 5HGN retains aromatic compounds as groups based on the number of aromatic rings [42]. 5HGN can also separate PAHs, sulfur, and oxygen-containing compounds from basic nitrogen-containing compounds for petroleum analysis [33, 44, 45]. The retention mechanism is based mainly on π-π interactions, with minor contributions from dispersion and solvation effects [43]. The retention behavior of aromatic compounds on 5HGN with normal phase solvents is different from traditional NPLC stationary phases since there are no discrete polar sites on 5HGN. Davankov et al. referred to this special retention mode as “quasi-normal phase” [42].

Hypercrosslinked phases on silica have also been developed [46, 47]. To create

the hybrid particle, a bonded dimethyl-(p-chloromethylphenyl) chlorosilane phase was crosslinked using styrene heptamer [46, 47]. After crosslinking, residual benzyl chloride were capped with functional groups such as toluene, the resulting phase is termed as HC-Tol phase [47]. The HC-Tol phase has shown potential for group type analysis of polar compounds in petroleum but low retention for PAHs [33, 45]. The reason for this retention behavior is not known. HC-Tol exhibits localizing adsorption under quasi-normal phase conditions [48]. Whether the localization effect is universal to other quasi-normal phases such as 5HGN is not clear.

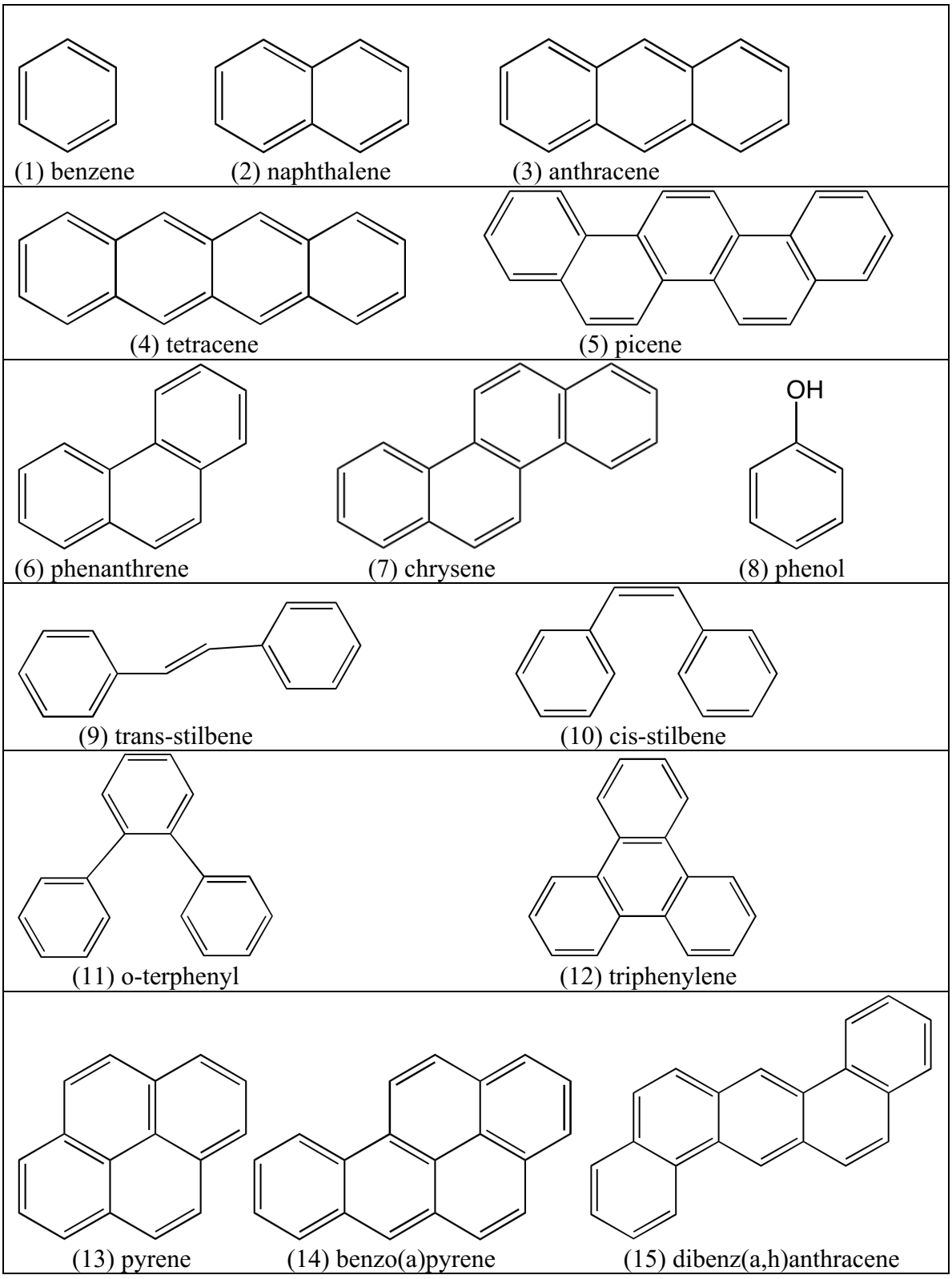
In this chapter, fifteen PAHs (**Table 3-1**) of one to five rings and varying in shape and planarity are used to investigate the retention mechnism and selectivity of charge-transfer and hypercrosslinked phases. DNAP is used as a representative of charge-transfer phases, and 5HGN and HC-Tol are examples of hypercrosslinked phases. Past studies focused solely on the separation of PAHs by ring number [28] or studied the effect of alkyl substituents [27, 29, 42, 43]. The effects of isomer and planarity selectivity have not been investigated. Finally, thermodynamic studies are performed to further elucidate the retention behavior.

3.2 Experimental

3.2.1 Materials

HPLC grade hexane, isopropanol (IPA) and dichloromethane (DCM) were from Fisher Scientific (Fairlawn, NJ, USA). **Table 3-1** shows the structures of the PAH standards. Benzene, anthracene, phenanthrene (90% purity) tetracene, chrysene (95% purity), pyrene, triphenylene, benzo(a)pyrene (>97% purity), dibenzo(a, h)anthracene and

Table 3-1 Structure of standards used in this chapter



phenol were from Sigma-Aldrich (St. Louis, MO, USA). Naphthalene (>90% purity) was from Fisher Scientific. Cis-stilbene, trans-stilbene, picene (>90% purity) and o-terphenyl were from K & K Laboratories (Carlsbad, CA, USA). Each sample was prepared at 0.1 mg/mL in mobile phase and filtered through a 0.2 μm syringe-driven filter (PTFE, Millipore, Billerica, MA, USA) before injection. The retention of each sample was measured in triplicate and in a random order to minimize systematic errors.

Silica particles (5 μm diameter, 80 Å pore size) were from Agilent (Santa Clara, CA, USA). Chromalite 5HGN hypercrosslinked polystyrene particles (4.5 ~ 5.5 μm mean diameter, 1100 ~ 1500 m^2/g apparent surface area) were gifts from Purolite International Limited (Wales, UK and Bala Cynwyd, PA, USA). The term "apparent surface area" was used because the 5HGN phase cannot be considered to contain constant porosity [38]. The HC-Tol particles (5 μm diameter, 180 m^2/g surface area) were synthesized on Type-B Zorbax silica particles [47] and gifted by Peter Carr of the University of Minnesota. The DNAP column (ES Industries, West Berlin, NJ, USA) was 5 x 0.46 cm ID packed with 5 μm particles of 60 Å pore size.

3.2.2 Column packing

The 5 × 0.46 cm ID silica, 5 × 0.46 cm ID HC-Tol, and 25 × 0.46 cm ID HGN columns were packed in-house with the corresponding particles indicated in Section 3.2.1. A 3 g SiO_2/17 mL IPA slurry, 1.8 g HC-Tol/10 mL IPA slurry, and 4 g 5HGN/80 mL IPA slurry were used. Packing was performed using a constant pressure Haskel pump (DSF-122-87153, Burbank, CA, USA) driven with N_2 gas (Praxair Inc., Edmonton, AB, Canada). Packing was in the downward direction at 6000 psi (1 bar = 14.5 psi) for about 25 min with IPA as the driving solvent.

3.2.3 Apparatus

All experiments were performed on a 1260 Infinity Quaternary LC system (Agilent). A G1311B pump with online degasser was used to pump IPA/hexane or DCM/hexane at 1.0 mL/min, unless otherwise indicated. All tubing and fittings were stainless steel (0.17 mm ID). The length of all connecting tubing was minimized to reduce the extra band broadening. Sample injections of 2 μL were performed with a G1329B autosampler. The column was maintained at 35 °C using a G1316A thermostatted column compartment, unless otherwise indicated. Detection was at 254 nm, except for tetracene for which 275 nm was used. Data acquisition was achieved using Agilent Chemstation at a rate of 10 Hz and detector response time of 1 s.

3.2.4 Calculations

3.2.4.1 Retention

Dead time was measured by the minor disturbance in the UV trace caused by injection of hexane as described in Ref. [49]. The retention factors (*k*) and selectivity values were calculated with Microsoft Excel. The relative standard deviation for all retention factors is less than 0.5%.

3.2.4.2 Thermodynamic data

Temperature affects the retention factor through the van't Hoff equation (**Eq. 3-1**).

$$\ln k = -\frac{\Delta H}{RT} + \frac{\Delta S}{R} + \ln \varphi \qquad \text{Eq.3-1}$$

where R is the ideal gas constant and T is temperature in Kelvin. ΔH and ΔS are the enthalpy and entropy change when solute transfers from the mobile phase to the stationary phase. φ is the phase ratio. Over small ranges of T, such as in the van't Hoff

studies herein, ΔH and ΔS can be considered independent of T [50, 51]. Thus, a linear plot is obtained when the ln k value of compound is plotted *vs*. 1/T. The retention factors of one to five rings PAHs were measured on DNAP and 5HGN columns at four temperatures (20-60 ^{o}C). Linear regression was performed for ln k *vs*. 1/T. The intercept values from the linear regression for PAH isomers were compared using Graphpad prism. ΔH is derived from the slope using $\Delta H = -R \times$ slope , and (ΔS+ Rln φ) is calculated based on the intercept using (ΔS+ Rln φ) = $R \times$ intercept.

3.3 Results and discussion

Charge-transfer phases have been widely used for petroleum analysis [14, 15, 34, 35]. Charge-transfer phases group the PAHs based on the number of aromatic rings [14, 15, 34], and separate the PAHs from heteroatom containing compounds [33, 35]. The hypercrosslinked phases have not been as widely used for petroleum separations [44, 52], but show unique selectivity for PAHs and heteroatom containing compounds in petroleum [42-45]. In this study, DNAP is representative of charge-transfer phases and 5HGN and HC-Tol are representatives of hypercrosslinked phases. A silica column is used as a reference column in the normal phase mode.

PAHs are used to study the ring number selectivity (size selectivity) of the columns, while shape selectivity is studied using four-ring PAHs and planarity selectivity is probed using selected compound pairs (cis/trans-stilbene and triphenylene/o-terphenyl). Lastly, thermodynamic data are collected by isocratic separations of homologous PAHs at various temperatures. The enthalpy and entropy values are compared for the charge-transfer and hypercrosslinked phases.

3.3.1 Size selectivity for catacondensed PAHs

The catacondensed PAHs benzene, naphthalene, anthracene, tetracene and picene are selected as homologs to investigate the ring number based separation of PAHs on DNAP, 5HGN and HC-Tol columns. Past studies have observed anomalous behavior for pericondensed PAHs such as the six ring dibenzo[*def,p*]chrysene (dibenzo(a,l)pyrene) [27]. Thus these compounds are excluded from the studies below.

DNAP separates one to four ring PAHs distinctly based on their ring number [31, 34]. Similar separation was observed using DCM/hexane (**Fig. 3-1**). PAHs are also resolved on the 5HGN and HC-Tol columns (data not shown).

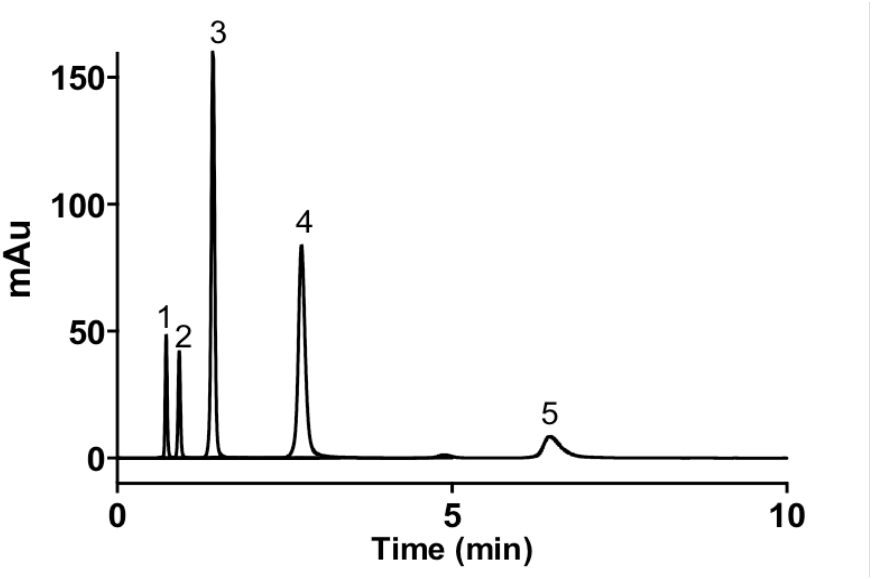


Fig. 3-1 Elution of PAHs on charge-transfer DNAP column. Conditions: 1. benzene; 2, naphthalene; 3, anthracene; 4, tetracene; 5, picene. Mobile phase, 20%DCM:80%hexane. Flow rate: 1 mL/min. Temp., 60 ^{o}C.

Fig. 3-2 shows the relationship between the retention factor (*k*) of PAHs *vs*. ring number on the three columns. Regression parameters are available in **Table 3-2**. For

PAHs with one to five rings, plots of ln *k vs*. ring number are linear ($R^2 > 0.99$) on all columns.

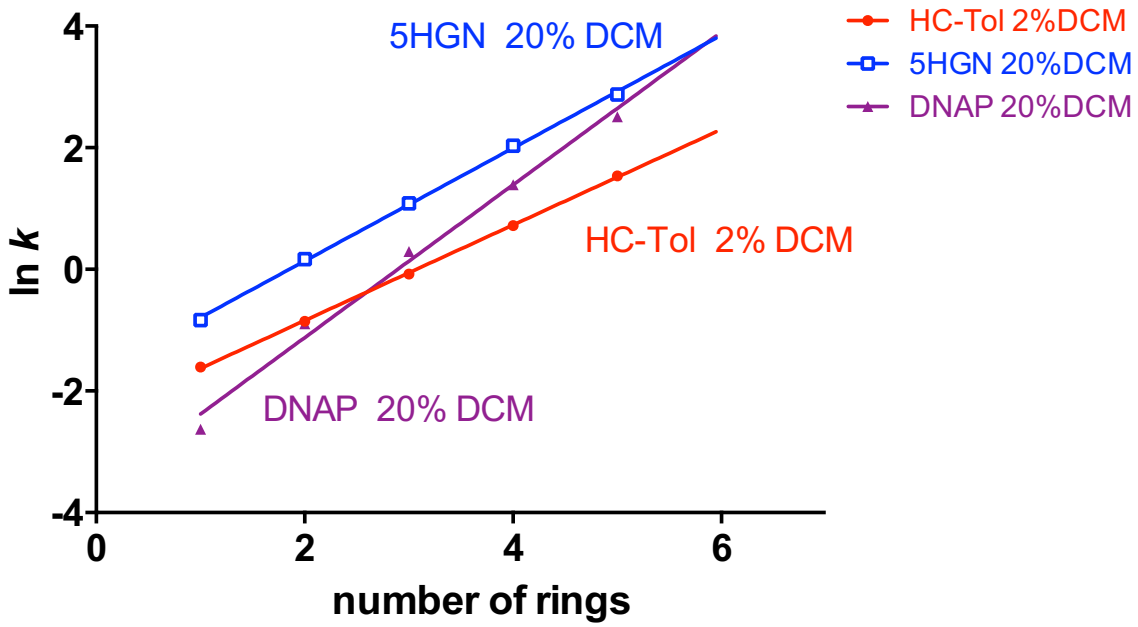


Fig. 3-2 Relationship between ln *k* and number of rings. Conditions: Injection volume, 2 µL. Mobile phases, 20%DCM:80%hexane for DNAP and 5HGN columns; 2%DCM:98%hexane for HC-Tol column. Flow rate, 1 mL/min. Temp., 35 °C. Detection wavelength, 275 nm for tetracene, and 254 nm for others. The standard deviation in ln *k* is smaller than the data symbols.

Table 3-2 Regression parameters of ln *k vs.* ring number (n)

Stationary phases	DNAP	5HGN	HC-Tol
R^2	0.990	0.992	0.997
Slope	1.26 ± 0.07	0.93 ± 0.02	0.786 ± 0.008
Intercept	-3.6 ± 0.2	-1.72 ± 0.05	-2.42 ± 0.03

Comparable linear plots ($R^2 > 0.99$) were obtained for ln *k vs*. number of π electrons (**Fig. 3-3** and **Table 3-3**). These results are consistent with the studies of retention index of catacondensed PAHs *vs*. both ring number and number of π electrons on DNAP, alumina

and amine columns [27]. It should be noted however that retention of pericondensed PAHs on an amine column has been shown previously to be better correlated with the number of π electrons [27].

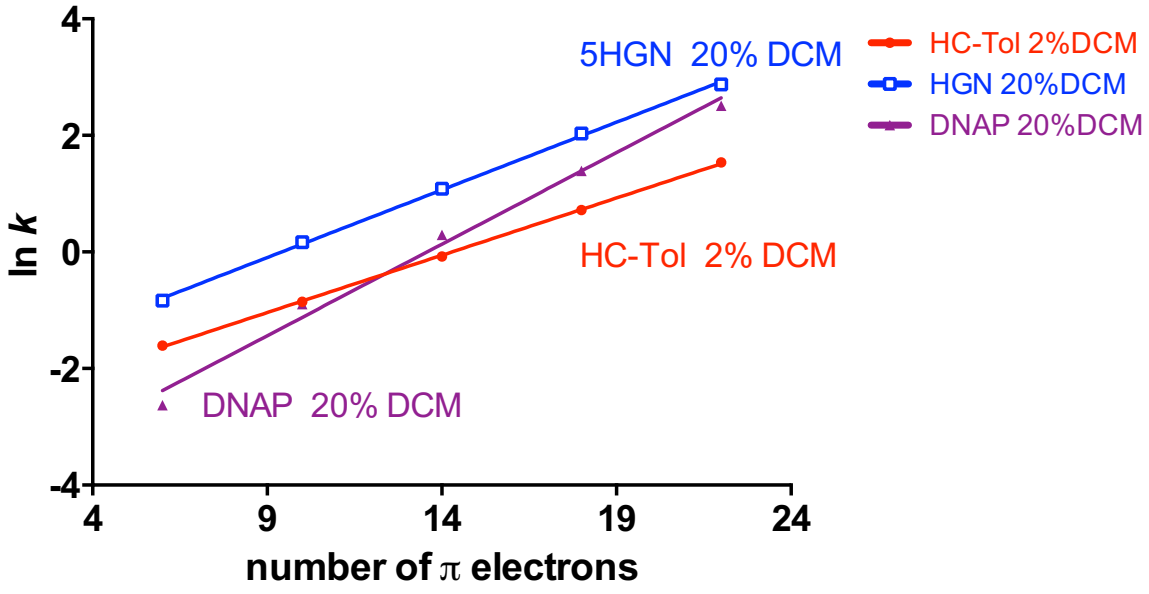


Fig. 3-3 Relationship between retention factors, ln *k* and number of π electrons. Conditions as in **Fig. 3-2**.

Table 3-3 Regression parameters of ln *k vs.* number of π electrons

Stationary phases	DNAP	5HGN	HC-Tol
R^2	0.990	0.992	0.997
Slope	0.31 ± 0.02	0.231 ± 0.004	0.197 ± 0.002
Intercept	-4.3 ± 0.3	-2.19 ± 0.06	-2.81 ± 0.03

With the same mobile phase, 5HGN retains PAHs more than DNAP. However, as the number of rings increases, the retention of PAHs on 5HGN increases more gradually (lower slope in **Fig. 3-2**) than that on DNAP. For PAH molecules with greater than five rings, the retention on 5HGN is projected to be smaller than that on DNAP. HC-Tol

retains PAHs very weakly at 20% DCM [45]. As a consequence, the mobile phase was lowered 2% DCM for HC-Tol to get comparable retention of PAHs (**Fig. 3-2**).

The relative retention of anthracene *vs*. phenol ($\alpha = k_{anth}/k_{phenol}$) has been used to reflect the strength of π-π interactions on the 5HGN column and other NPLC columns [43]. To test the suitability of this compound pair, we first measured the retention of anthracene and phenol on our columns with the previously studied mobile phase (7% IPA:93% hexane). Silica provides almost no retention for anthracene, and so the relative retention for anthracene/phenol is near 0 (**Table 3-4)**, consistent with Ref. [43]. The relative retention for anthracene/phenol is largest on the 5HGN column, which agrees with Ref. [43], albeit they reported nearly irreversible retention of anthracene.

Table 3-4 Relative retention ($\alpha = k_{anth}/k_{phenol}$) of anthracene/phenol pair with IPA/hexane as mobile phase

Stationary phases	**Silica**	**DNAP**	**5HGN**	**HC-Tol**
$\boldsymbol{k_{anth}}$	0.001	1.39	16.39	0.41
$\boldsymbol{k_{phenol}}$	0.312	1.40	7.41	0.41
$\boldsymbol{\alpha = k_{anth}/k_{phenol}}$	0.005	0.993	2.21	1.00

Conditions: mobile phase, 7%IPA:93%hexane; flow rate, 1 mL/min; temp., 35 ^{o}C.

Next, the relative retention ($\alpha = k_{anth}/k_{phenol}$) was measured for 20%DCM: 80%hexane to match the conditions used in **Fig. 3-1**. **Table 3-5** shows that the π-π interaction strength follows the order 5HGN > DNAP > > HC-Tol > silica. This is consistent with the past observation that 5HGN possesses strong π-π interactions with PAHs [43]. The retention of anthracene on DNAP is strong (k = 1.34), as is phenol (k = 20.1), but the α remains small. As the retention of phenol is similar on bare silica and DNAP, the strong retention of phenol on DNAP may be due to residual unfunctionalized

silanols. Similar to the IPA studies, silica retained anthracene very weakly and phenol extremely strongly, due to the polar silanol adsorption sites (**Table 3-5)**. Thus, the k_{anth}/k_{phenol} on silica is close to 0 (**Table 3-5**), which indicates very weak π-π interaction. The retention of anthracene on HC-Tol is similarly weak as on silica, but phenol is more weakly retained.

Table 3-5 Relative retention ($\alpha = k_{anth}/k_{phenol}$) for anthracene/phenol pair

Stationary phases	Silica	DNAP	5HGN	HC-Tol
$\mathbf{k_{anthracene}}$	0.29	1.34	3.18	0.22
$\mathbf{k_{phenol}}$	25.7	20.1	16.3	7.37
$\mathbf{\alpha = k_{anth}/k_{phenol}}$	0.011	0.066	0.195	0.029

Conditions: mobile phase, 20%DCM:80%hexane; flow rate, 1 mL/min; temp., 35 ^{o}C

3.3.2 Shape selectivity

Fig. 3-2 shows that the retention of PAHs increases with aromatic ring number on charge-transfer [27, 31, 33] and hypercrosslinked phases [42, 43, 45]. However, for molecules with the same aromatic ring number but different shape, the shape selectivity on charge-transfer and hypercrosslinked phases has not been previously investigated.

3.3.2.1 Elution of four-ring isomers

Table 3-6 shows the shape selectivity for four-ring planar PAHs. All three isomers have 18 π electrons, but tetracene is a linear molecule, chrysene is a bent PAH, and triphenylene is more disk-like (**Table 3-1**).

Table 3-6 shows that both silica and HC-Tol exhibit weak retention of PAHs, with slightly more retention for the disk-like molecules. This weak shape selectivity on silica agrees with that in Ref. [53]. Secondly, DNAP and 5HGN retain the four-ring PAHs much more strongly than silica. Thirdly, the 5HGN column shows unique

Table 3-6 Retention factor (*k*) of four-ring PAH isomers

Stationary phases	*k* (Silica)	*k* (DNAP)	*k* (5HGN)	*k* (HC-Tol)
tetracene	0.40 ± 0.03	4.30 ± 0.00	7.99 ± 0.02	0.41 ± 0.00
chrysene	0.45 ± 0.05	4.60 ± 0.00	7.54 ± 0.02	0.41 ± 0.00
triphenylene	0.48 ± 0.02	4.92 ± 0.00	7.49 ± 0.01	0.42 ± 0.00

Conditions: mobile phase, 20%DCM:80%hexane; flow rate, 1 mL/min; temp., 35 ^{o}C.

shape selectivity, with the linear isomer (tetracene) being more retained. The linear and bent three-ring PAHs were moderately retained but co-eluted using a weaker mobile phase (50% IPA:20% $CHCl_3$:30% hexane) than here [42]. Finally, DNAP retains the disk-like isomer (triphenylene) more strongly. This shape selectivity has also been observed in Ref. [31].

To better understand these selectivities, they will be compared with shape selectivity studies on other columns. Higher retention of linear *vs*. disk-like isomers has been observed on alumina [54] for three-ring PAHs, and has been attributed to the more ordered adsorption sites on alumina which enables localization with linear molecules [54]. Similarly, the highly ordered crystalline poly(octadecyl acrylate) phase retains linear PAH isomers more than disk-like ones [55]. That the 5HGN column also shows higher retention of linear *vs*. disk-like isomers, is suggestive that ordered adsorption sites exist on 5HGN.

Conversely, higher retention of disk-like isomers has been observed for a number of columns in Ref. [53]. The relative retention of triphenylene *vs*. tetracene decreased in the following order: poly(4-vinylpyridine)-grafted silica (1.55) > polystyrene-grafted silica (1.13) > silica (1.08) [53]. It was proposed that there was hydrogen bonding between the between the pyridine moieties and the silanols on the poly(4-vinylpyridine)-

grafted silica phase. Thus, the ligands alignment was distorted and the mobility of the chain was reduced. As a result, the poly(4-vinylpyridine)-grafted silica phase was believed to have a disordered polymer chain alignment [53]. The disordered structure interacts with disk-like molecules strongly through multiple adsorption sites [53, 56].

Our measure of the triphenylene *vs*. tetracene selectivity on DNAP is 1.14, which lies between that on the poly(4-vinylpyridine)-grafted silica and polystyrene-grafted silica phase. This is suggestive that DNAP is a disordered phase and retains disk-like isomers more through multiple sites interactions. The disordered alignment of the ligands probably formed through the similar mechanism as in poly(4-vinylpyridine)-grafted silica phase.

Thus the differences in shape selectivity observed herein are apparently due to 5HGN behaving as an ordered adsorption phase and DNAP behaving as a disordered phase.

3.3.2.2 Correlation of retention to molecular properties

Correlations between molecular properties and observed retention were explored to further understand the retention mechanism on the DNAP and 5HGN phases. Previously, molecular polarizability (MP) [57], quadrupole moment (QM), length-to-breadth ratio (L/B), 1-octanol/water partition coefficient (log *P*) [19, 53] and ionization energy (IE) [24] were correlated with retention of PAHs on NPLC phases [19, 24, 53]. L/B and log *P* [58] are also properties that affect PAH retention in RPLC [19, 40]. MP is an indication of inductive interaction (dipole-induced dipole interaction) [19, 53]. L/B characterizes the shape selectivity, and log *P* directly reflects the hydrophobic interactions. Ionization energy (IE) has been correlated with charge-transfer interactions

for PAHs [24]. QM has been correlated to the retention of PAHs on poly(4-vinylpyridine)-grafted silica, but has not been related to a particular type of interaction with the stationary phase [53]. Also, QM values are not available for all of the PAHs studied. Therefore, QM was not studied herein.

Two-ring to five-ring isomers are included in correlations of PAH retention *vs*. the molecular properties (**Table 3-7)**.

Table 3-7 Retention factors (*k*) and molecular properties for PAHs

Standards	ln *k* (DNAP)	ln *k* (5HGN)	MP[a]	IE[b]	L/B[b]	log *P*[c]
naphthalene	-0.853	0.190	17.48	0.62	1.27	3.38
anthracene	0.356	1.118	25.93	0.41	1.57	4.68
phenanthrene	0.426	1.104	24.7	0.61	1.46	4.64
tetracene	1.470	2.078	32.27	0.29	1.89	5.71
chrysene	1.518	2.020	33.06	0.52	1.72	5.4
triphenylene	1.594	2.014	33.51	0.68	1.12	5.28
pyrene	1.231	1.546	29.34	0.45	1.27	4.88
benz(a)pyrene	2.316	2.513	38.84	0.37	1.5	6.3
dibenz(a,h)anthracene	2.501	2.817	41.31	0.47	1.72	6.6

[a] Molecular polarizability, data from Ref. [57]
[b] Ionization energy, data from Ref. [53]
[c] Length-to-breadth ratio, data from Ref. [57]
[d] *P*, water/1-octanol partition coefficient, data from Ref. [58]

Table 3-8 shows the correlation coefficients for plots of ln *k vs*. MP, IE, L/B and log *P*. The plots are attached as **Appendix B**. Retention of two to five ring PAHs on both DNAP and 5HGN is highly correlated ($R^2 > 0.98$) to molecular polarizability (MP), indicating inductive interactions [19, 53]. The charge-transfer parameter IE and the molecular shape factor L/B values correlate poorly with the retention of two to five ring PAHs ($R^2 < 0.45$). This indicates that charge-transfer is not the main mechanism for the retention of PAHs on both the DNAP and 5HGN phases.

Table 3-8 Correlation coefficients (R^2) of ln *k vs*. PAH properties

Property	2-5 ring		4 ring	
	R^2 (DNAP)	R^2 (5HGN)	R^2 (DNAP)	R^2 (5HGN)
Molecular Polarizability (MP)	0.99	0.98	0.97	0.93
Ionization Energy (IE)	0.42	0.44	0.95	0.95
Length/Breadth (L/B)	0.21	0.14	0.91	0.54
Log *P*	0.97	0.95	0.94	0.97

Conditions: mobile phase, 20%DCM:80%hexane; flow rate, 1 mL/min; temp., 35 ^{o}C.

Previous principle component analysis (PCA) studies of 5HGN under quasi-normal phase conditions suggested that the second most important interaction after π-π interactions was hydrophobic interactions [42, 43]. Similar high correlations ($R^2 = 0.89$) of log *P* to retention have been observed for PAHs on poly(4-vinylpyridine)-grafted silica under normal phase conditions [53]. These literatures suggest that the retention of PAHs of 5HGN is counter-intuitively affected by hydrophobicity. Indeed, **Table 3-8** shows a high correlation ($R^2 > 0.95$) between two to five rings PAH retention and log *P*. However, even on silica we observed a strong correlation ($R^2 = 0.93$) between log *P* and retention of two to five ring PAHs. We believe this correlation is happenstance, and due to log *P* being strongly correlated ($R^2 = 0.96$) with the molecular polarizability MP.

Table 3-8 also shows correlations for only the four-ring isomers studied in Section 3.3.2.1. Many of the molecular parameters correlate strongly with the retention of the four-ring isomers. However, as discussed above the molecular polarizability correlates strongest with the full set of PAHs, and so is believed to be the causative property.

3.3.3 Planarity selectivity

Section 3.2.1 showed that the selectivity of planar PAHs isomers on charge-transfer phases is different than that on hypercrosslinked phases. The main factor affecting retention is the molecular polarizability, with the charge transfer factor IE and shape affecting the within ring-class selectivity (Section 3.2.2). Steric hindrance also causes differences in retention [27, 30, 43]. Previous studies of the effect of steric hindrance on charge-transfer [27, 29] and hypercrosslinked phases [43] focused on the effect of alkyl substituent length and position. There is only limited discussion of the effect of planarity steric hindrance on separations of PAHs with same number of π electrons [30]. Here we test the planarity selectivity for our charge-transfer and hypercrosslinked phases.

Trans-stilbene/cis-stilbene and triphenylene/o-terphenyl are planar/non-planar pairs used to probe planarity selectivity [53, 59]. Within each pair, the compounds have same number of aromatic carbon and π electrons and would have the same predicted molecular polarizability [60], but differ in their planarity. Cis-stilbene is non-planar due to the steric interaction between the phenyl rings. **Table 3-9** summarizes the retention factors and $k_{planar}/k_{non\text{-}planar}$ selectivities on the DNAP, 5HGN, HC-Tol and silica columns. On all columns, the selectivity is above 1, indicating that the planar molecules are more strongly retained than the non-planar molecules. This is consistent with the literature [53] and the expectation that the non-planarity disrupts the conjugated π system.
As a result, π-π interactions with the stationary phases are reduced and so is retention. Also, there might be size exclusion effect caused by the steric hindrance of the non-

planar molecules. Solvents such as benzene or toluene might be used to shield the π-π interaction sites to investigate the presence the size exclusion effect.

Table 3-9 Selectivity (α) for cis-stilbene/trans-stilbene or o-terphenyl/triphenylene

Compounds	Silica		DNAP		5HGN		HC-Tol	
	k	α	k	α	k	α	k	α
cis-stilbene	0.28	1.3	0.18	3.5	0.98	2.0	0.10	1.6
trans-stilbene	0.35		0.63		1.94		0.16	
o-terphenyl	0.42	1.2	0.23	22	1.27	5.9	0.14	3.1
triphenylene	0.48		4.92		7.49		0.42	

Conditions: mobile phase, 20%DCM:80%hexane; flow rate, 1 mL/min; temp., 35 ^{o}C.

The planarity recognition ability of the phases follows the order: DNAP > 5HGN > HC-Tol > silica. The strong planarity selectivity of the charge-transfer DNAP phase is consistent with other charge-transfer phases [30], and understandable as such π electron deficient phases form stable complex with the π electron rich solutes [29, 61]. 5HGN exhibit the strongest π-π interactions with PAHs (Section 3.1), but has only medium non-planarity recognition. Thus, one cannot only consider π-π interactions (Section 3.1) when explaining the retention of PAHs on quasi-normal phases. Retention of triphenylene and o-terphenyl was weak (k < 0.5) on silica and HC-Tol, and so the planar / non-planar selectivity was not studied.

3.3.4 Thermodynamic data

Thermodynamic information provides additional insight into liquid chromatography retention [62, 63]. The enthalpy for retention of PAHs on the charge-transfer DNAP phase with chloroform/hexane displayed comparable enthalpies to the polynitrobenzene-PAHs charge-transfer complexes [28]. This indicated that the main interaction between PAHs and DNAP can be attributed to charge-transfer interactions

[28]. There have been no thermodynamic studies of PAHs on hypercrosslinked phases. To compare charge-transfer to hypercrosslinked phases, we studied the thermodynamic behavior of PAHs on DNAP, 5HGN and HC-Tol columns.

3.3.4.1 Enthalpy

Retention of both linear and bent PAHs was measured on DNAP, 5HGN and HC-Tol columns. Plots of ln *k vs*. 1/*T* over 20-60 ^{o}C showed good ($R^2 > 0.99$) linearity (data not shown). This linearity indicates that there was no change in retention mechanism over the temperature range studied. **Fig. 3-4** and **Table 3-10** summarize the enthalpies from the van't Hoff plots (Section 3.2.4.2).

Table 3-10 Enthalpy of PAHs with DNAP, 5HGN and HC-Tol column

Standards	DNAP ΔH (kcal/mol)	5HGN ΔH (kcal/mol)	HC-Tol ΔH (kcal/mol)
Naphthalene (2)	-1.73 ± 0.05	-0.61 ± 0.05	-1.4 ± 0.2
Anthracene (3)	-2.31 ± 0.03	-1.02 ± 0.05	-1.8 ± 0.2
Phenanthrene (6)	-2.42 ± 0.03	-0.96 ± 0.02	-1.8 ± 0.2
Tetracene (4)	-3.05 ± 0.03	-1.49 ± 0.05	-2.4 ± 0.2
Chrysene (7)	-3.12 ± 0.02	-1.42 ± 0.09	-2.3 ± 0.2
Picene (5)	-3.97 ± 0.01	-1.8 ± 0.2	-3.0 ± 0.2

Note: The enthalpy information of benzene is not shown due to the poor linearity (R^2 <0.94). The poor correlation probably resulted from too weak retention of benzene.

The enthalpy values in **Fig. 3-4** on all columns were negative, which indicates that the retention of PAHs on the charge-transfer and hypercrosslinked phases is exothermic. For DNAP, the enthalpy value becomes increasingly negative with ring number (**Fig. 3-4(a**)), consistent with Ref. [28]. Similar changes in enthalpy are evident in **Fig. 3-4(a)** for the 5HGN and HC-Tol columns.

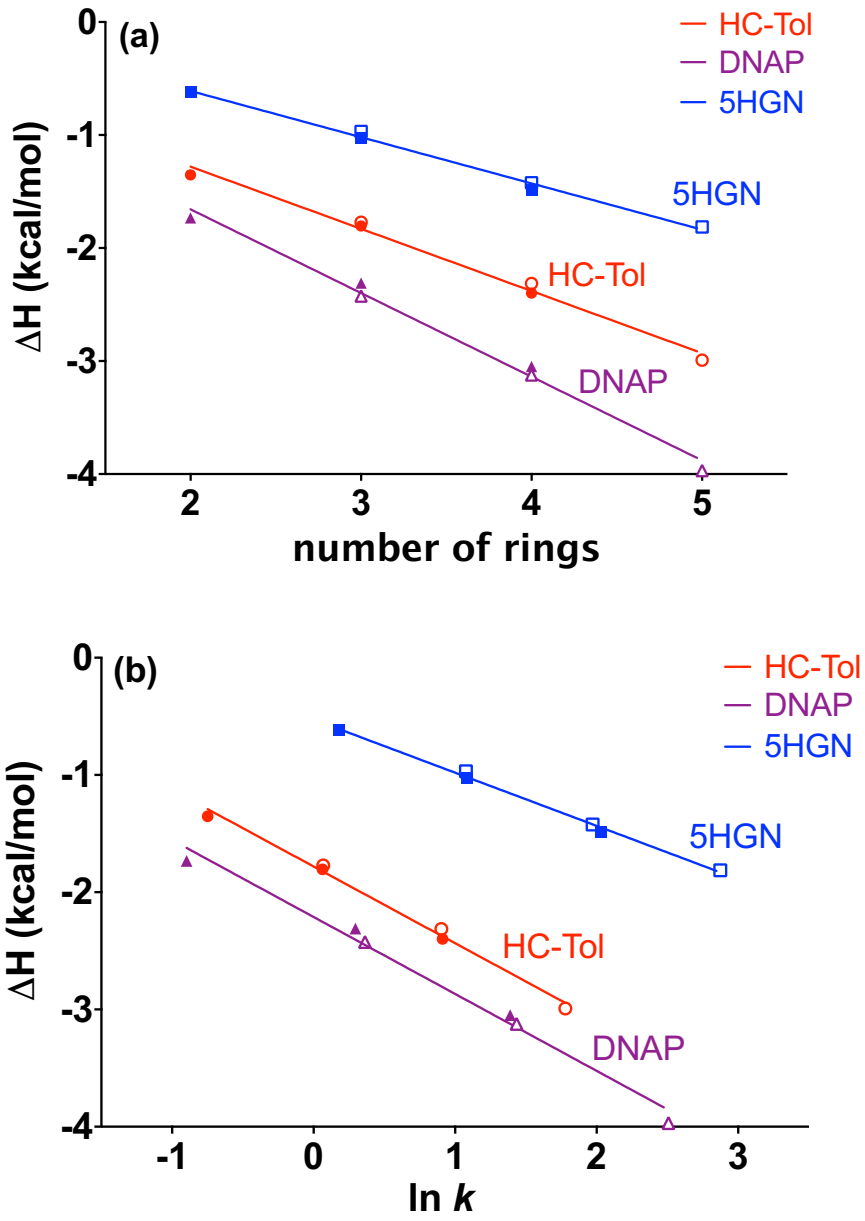


Fig. 3-4 (a) Relationship between ΔH and number of rings; (b) Relationship between ΔH and ln *k* for linear (filled symbols) and bent PAHs (open symbols) on DNAP, 5HGN and HC-Tol columns.

Fig. 3-4(b) plots the enthalpy for the linear and bent PAHs vs. ln *k* at 40 °C for all of the columns. If high correlations for Δ*H vs.* ln *k* are observed, this indicates that the retention mechanisms of the linear and bent PAHs are similar [53]. High correlations (R^2

> 0.98) are observed in **Fig. 3-4(b)**. Thus, the retention mechanism for the linear and bent PAHs are similar, on all columns studied.

3.3.4.2 Entropy

The intercepts of the van't Hoff plots are ($\Delta S + R\ln\varphi$) (Section 3.2.4.2), and are tabulated in **Table 3-11.**

Table 3-11 ($\Delta S + R\ln\varphi$) of PAHs with DNAP, 5HGN and HC-Tol column

Standards	DNAP $\Delta S + R\ln\varphi$ (J/mol·K)	5HGN $\Delta S + R\ln\varphi$ (J/mol·K)	HC-Tol $\Delta S + R\ln\varphi$ (J/mol·K)
Naphthalene (2)	-30.6 ± 0.7	-6.8 ± 0.7	-25.7 ± 3.0
Anthracene (3)	-28.4 ± 0.5	-4.7 ± 0.6	-24.7 ± 2.2
Phenanthrene (6)	-29.4 ± 0.4	-4.0 ± 0.2	-24.2 ± 2.2
Tetracene (4)	-29.2 ± 0.4	-3.0 ± 0.7	-26.0 ± 2.5
Chrysene (7)	-29.8 ± 0.3	-2.5 ± 1.2	-24.9 ± 2.1
Picene (5)	-32.2 ± 0.2	0.2 ± 2.2	-27.1 ± 3.2

Direct comparison of the entropies for PAH retention between the columns is not possible because the measurement of the phase ratio, φ, is challenging. Thus, it is impossible to say whether the difference in the ($\Delta S + R\ln\varphi$) between the phases arises from difference in the phase ratio, or to the presence of residual silanols, or to any other factor. However, for each column itself, the phase ratio is assumed to be independent of the solute [51, 62]. Thus, the intercept values in **Table 3-11** can be used as a proxy of ΔS for each individual column. The dramatic difference in entropy for the PAHs on 5HGN shows that the contribution of entropy to retention is significant. The entropy for PAH retention on 5HGN becomes less negative as the number of rings of in the solute increases (*e.g.*, the ($\Delta S + R\ln\varphi$) of tetracene is less negative than that of anthracene). This

indicates that the process of transferring the PAHs from the mobile phase to 5HGN becomes more entropically favorable with increasing number of rings. However, on the DNAP and HC-Tol column, the entropy becomes slightly more negative (entropically disfavored) as the number of rings increases.

Comparison of the intercept values (*i.e.*, $\Delta S + R\ln \varphi$) from the linear regressions indicates that the intercepts for the PAH isomers are statistically different at the 95% confidence interval (Section 3.2.4.2). Thus, on 5HGN, the entropy change is more negative for linear than for bent isomers (*e.g.*, anthracene is more negative than phenanthrene, and tetracene is more negative than chrysene). When linear PAHs adsorb on 5HGN phase, they will form a more ordered structure. This entropy trend is consistent with the better surface contact of linear PAHs with 5HGN (Section 3.3.2). Likewise, on DNAP, the entropy change is more negative for bent PAHs (Section 3.3.2). This also agrees that DNAP phase will prefer to interact with bent PAHs.

3.4 Conclusions

The similarities and differences for charge-transfer (DNAP) and hypercrosslinked phases (5HGN and HC-Tol) for normal phase retention of PAHs have been investigated. Both phases mainly retain PAHs via π-π interactions and are affected by molecular polarizability. The differences in the columns' retention properties are evident in their shape selectivity, planarity selectivity and thermodynamic parameters. Based on our study, HC-Tol is more like conventional normal phases (*e.g.*, silica), with low retention for PAHs, low ability to differentiate PAH isomers, and low planarity selectivity. 5HGN show comparable retention of PAHs as the charge-transfer DNAP phase. 5HGN and

DNAP phases are effective in class separation based on ring numbers or planarity for petroleum. However 5HGN differs from DNAP in its larger π-π interaction strength, more ordered adsorption site, and lower ability in differentiating planar/non-planar molecules. But the lower planarity selectivity on 5HGN *vs*. on DNAP column is not consistent with the larger π-π interaction. This indicates that other factors affect the retention of PAHs on 5HGN column.

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Chapter Four: Coupling normal phase liquid chromatography with electrospray ionization mass spectrometry: strategies and applications

4.1 Introduction

Normal phase liquid chromatography (NPLC) and mass spectrometry (MS) are complementary to one another. MS offers greater sensitivity and specificity than conventional NPLC detectors such as ultraviolet absorbance or refractive index detection. On the other hand, NPLC can resolve isomers that cannot be differentiated by MS, as isomers have the same mass-to-charge (m/z). Of the commonly used ionization techniques, electrospray ionization (ESI) is more specific to characterization of polar compounds than atmospheric pressure chemical ionization (APCI) or atmospheric pressure photoionization (APPI) because ESI generates less background signals [1, 2]. ESI provides higher a signal-to-noise ratio for example for azaarenes [3] and pharmaceuticals [4] than does APCI or APPI. Furthermore, ESI generates an intact protonated or deprotonated molecular ion, while APCI and APPI yields more fragment ions due to thermal decomposition [5]. For complex samples, the simpler spectrum offered by ESI is a benefit.

However, the coupling of NPLC to ESI-MS is challenging. Common NPLC mobile phases contain large amounts of low polarity solvents such as hexane or heptane, which cannot be easily sprayed to promote analyte ion formation [6, 7]. Thus, several strategies have been developed to combine NPLC with ESI-MS. **Fig. 4-1** summarizes the off-line and on-line methods that have been reported. In an off-line method, fractions of

the NPLC effluent are collected, the ESI-MS-incompatible solvents is removed by evaporation, and the analytes are re-dissolved in an ESI compatible solvent and analyzed by ESI-MS. The off-line mode has been widely used in petroleum analysis [8-11]. On-line coupling of NPLC separations with ESI-MS can be done by direct effluent introduction; use of post-column solvent addition or a sheath liquid interface; or using an ambient ionization source. Each of the strategies in **Fig. 4-1** and their applications are discussed in the following sections.

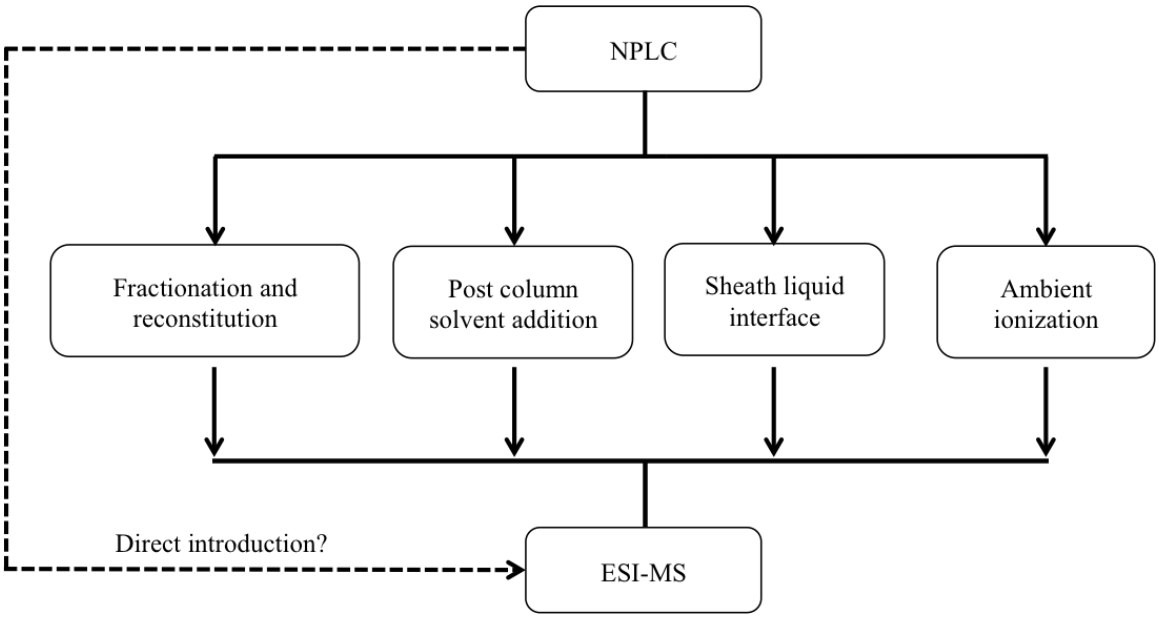

Fig. 4-1 Strategies to couple NPLC to ESI-MS.

4.2 Solvents in ESI-MS

The ESI process involves multiple complex steps: formation of the Taylor cone; generation of charged droplets; shrinkage of the droplets by evaporation; and production of gas phase ions [12], as discussed in Section 1.3.6.1. The ions are formed through four mechanisms: self-dissociation; electrochemical redox reactions caused by the high

voltage; adduct formation; and gas phase proton transfer reactions [13]. Various instrumental parameters (voltage, capillary dimensions and flow rate) and solution characteristics (solvent surface tension, conductivity, dielectric constant, ionic strength and volatility) affect the stability of ESI operation [13, 14]. The formation of a stable spray and stable ESI response is dependent on the balance of these parameters. For example, when the solvent is changed from methanol (MeOH) to water, the onset voltage for Taylor cone formation and charge emission increased from 2.2 kV to 4.0 kV due to the large surface tension of water [12, 15]. Similarly, the onset flow rate had to be reduced five-fold due to the conductivity increase caused by addition of 0.05% HCl to pure isopropanol (IPA) [6, 15]. For positive mode ESI, MeOH, acetonitrile (ACN), IPA, ethanol (EtOH) and their aqueous mixtures (organic composition > 50%) are commonly used [13]. For negative mode, halogenated solvents or MeOH/halogenated solvents provide optimal performance [13].

With some solvent systems, formation of a stable spray is difficult. For example, to form a stable spray for pure water, which has high surface tension, such a high voltage is needed that corona discharge occurs [16]. Pure water is not a good spray solvent also because water is not good for charge separation [12, 13]. Conversely, for non-polar solvents such as hexane, benzene and toluene, it is not possible to form stable spray due to their low conductivity, low surface tension and low dielectric constant [17].

Additives in the solvent also affect the ESI response by affecting one or more of the mechanisms of ion formation, as has been reviewed [13, 14, 18]. First, weakly acidic or basic additives will change the solution pH. In ESI, the charged analyte ions are often formed in solution by dissociation. In this case, acidic analytes are best ionized in basic

solutions and basic analytes in acidic solution [13, 14]. For instance, tetramethylammonium hydroxide is has higher basicity than ammonium hydroxide, and so addition of tetramethylammonium hydroxide to petroleum samples yields protonation of six times more acidic analytes [19]. Second, for polar analytes that possess no acidic or basic functionality, solution phase ions can be generated through adduct formation with additives, *e.g.*, addition of NH_4^+ can generate $[M+NH_4]^+$ [13]. Lastly, for analytes that are neutral in solution, the additives may carry charge that is transferred in the gas phase to the analyte [13, 14]. For example, protonated valine ions $[M+H]^+$ have been observed in ESI-MS for valine in a basic solution adjusted using ammonium hydroxide. During the ESI process, both neutral valine analyte and NH_4^+ are present in the droplets. In the gas phase, protons transfer from NH_4^+ to valine because of valine's stronger proton affinity than NH_3 [20].

4.3 Direct infusion of NPLC eluent to ESI-MS

There are several cases in the literature where NPLC eluent is directly introduced into an ESI-MS. As discussed in Section 4.2, NPLC eluent containing predominantly nonpolar solvents such hexane or heptane are not compatible with ESI-MS because of their low polarity. However, the intensity of analyte ions by direct infusion ESI increases with addition of polar solvents (Section 4.2). For instance as will be shown in **Chapter 5** for acridine, the ESI-MS signal for protonated acridine is observed only when ≥ 20% IPA is present in an IPA/hexane mixture [21]. In some NPLC separations there is sufficient polar solvent in the eluent to enable direct ESI ionization. For example, propranolol enantiomers eluted using 20/80 IPA/hexane can be ionized in ESI-MS without make-up

solvent [22]. Similarly, direct coupling NPLC with ESI-MS for drug enantiomers in 23/77/0.1 EtOH/hexane/trifluoroacetic acid [23] and clevudine in 68/28/10/0.02 MeOH/EtOH/hexane/trifluoroacetic acid [24] has been achieved without make-up solvent.

Alternatively a more ESI-compatible weak solvent can be used in the NPLC mobile phase. Ethoxynonafluorobutane (ENFB, mixture of $CF_3CF_2CF_2OCH_2CH_3$ and $(CF_3)_2CFCF_2OCH_2CH_3$) offers similar separation selectivity for various compounds as hexane in NPLC [25, 26]. But ENFB possesses much lower flammability which would reduce any explosion hazard in ESI-MS [25]. However, NPLC for diphenylmethyl phenylsulfoxide and diaminocyclohexane acrylamide using 10/90 EtOH/ENFB eluent with ESI-MS produced higher detection limit than with UV absorbance detection [25].

Many other papers were found in *ISI Web of Science* when searching "normal phase" in the title and "electrospray ionization " and "mass spectrometry" in the topic. However, the vast majority of these 84 papers used normal phase adsorbents with mobile phases that containing mainly MeOH, ACN, chloroform and 5-25% water [27-33]. These solvent systems are compatible with ESI-MS. Strictly speaking, these mobile phases are better termed as hydrophilic interaction chromatography (HILIC), which is beyond the scope of this review [34].

4.4 Off-line NPLC-ESI-MS

4.4.1 Approaches to Off-Line NPLC-ESI-MS

The solvent incompatibility of most NPLC mobile phases with ESI makes direct introduce of the effluent into an ESI challenging. Thus, for a long time, coupling of

NPLC to ESI-MS was performed in the off-line mode. For off-line NPLC-ESI-MS, effluent fractions are collected, typically using retention time windows trigger by the peaks [9, 11, 35]. The non-ESI compatible solvents in each fraction are evaporated down, and the analytes are redissolved in ESI-compatible solvents, *e.g.*, petroleum samples redissolved in <50% toluene in MeOH [11, 36]. MeOH provides the stable ESI response while toluene assists the dissolution of the petroleum fractions. The reconstituted analytes are then directly introduced into the ESI-MS. The sample reconstitution process concentrates the analytes to yield higher sensitivity, which for example, enhances the signal intensities and makes the composition feature visible in crude oil samples [37]. Also, higher sensitivity has been achieved by combining fractions from 3-5 replicate runs [9, 37]. However, off-line NPLC is time consuming and may cause loss of compositional information or compositional biases. For instance, the molecular composition identified within same retention time range for petroleum analysis by on-line LC-MS was 3 times that identified by off-line NPLC [38].

Alternatively, the collected fractions can be introduced into an ESI-MS after direct addition of an ESI-compatible solvent, such as MeOH/H_2O or MeOH/toluene as will be shown in **Chapter 5** [21]. This strategy is more time effective compared to sample reconstitution, but dilutes the analytes and is more labor intensive than on-line LC-MS (Section 4.5). Our experience is that most stable and higher response are obtained if the ESI-compatible solvent is miscible with the solvent in the fractions [21].

The off-line reconstitution procedure has been used for NPLC-ESI-MS for complex samples such as petroleum [9-11, 36, 39]. Crude oil contains tens of thousands of compounds, from non-polar molecules such as saturates to polar nitrogen, sulfur or

oxygen containing hydrocarbons [40, 41]. The peak capacity of state-of-the-art 2D NPLC × RPLC is 300-1500 [42, 43]. Thus, it is not possible, nor practical, to separate all of the individual compounds in crude oil. More commonly petroleum is isolated with fractionation or class separation, often by NPLC [44]. Nonetheless, the sub-fractions of petroleum are still too complex to be analyzed by low resolution MS. Thus, high resolution MS such as Fourier transform ion cyclotron resonance MS (FT-ICR-MS) are used [45, 46], most commonly with ESI, especially for polar compounds such as nitrogen containing compounds and acids [40, 41, 47].

It should be noted that there is some disagreement as to whether pre-separation is needed for petroleum analysis by ESI FT-ICR-MS. Rodgers and co-workers claim that saturates, resins and asphaltenes do not affect the identification of polar compounds in the aromatics fraction when using positive ESI [8]. Also, ESI FT-ICR-MS can identify basic polar nitrogen compounds in heavy gas oil without interference by other compound classes (*e.g.*, polycyclic aromatic hydrocarbons, sulfur containing compounds) [8]. However, ion suppression has been observed amongst compounds with the same functionality in ESI-MS analysis. For example, the ESI signal intensity for pyridine decreases 10 times in the presence of acridine [21]. Thus, a pre-separation for simplifying the complex petroleum sample is needed [21]. In addition, a much larger number of molecular classes can be identified by FT-ICR-MS with pre-separation than by direct infusion into an FT-ICR-MS [10, 48] indicating the desirability of a pre-separation step.

4.4.2 Applications of Off-Line NPLC-ESI-MS

Off-line NPLC-ESI-MS methods used for petroleum analysis fall into two categories: preparative and analytical NPLC separation for petroleum. First, preparative

NPLC has been widely applied in separating crude oil into sub-fractions. For example, the conventional stationary phases were used in preparative NPLC for crude oil fractionation. SARA was the most straightforward example of such a NPLC separation and usually serves as the first step for petroleum purification before subsequent NPLC classification and ESI FT-ICR-MS analysis [9, 49, 50]. In SARA, Asphaltenes (A) were precipitated from crude oil using pentane, hexane or heptane. The alkane soluble supernatant was then separated on a column consisting of a clay layer atop a mixture of silica and clay. Sequential elution with hexane, toluene/acetone and finally toluene yielded the Saturates (S), Aromatics (A) and Resins (R) fractions, respectively. After SARA fractionation, each fraction was evaporated down to dryness under nitrogen. The total recoveries were usually 92-95% [8, 9]. The SARA fractions were redissolved in 90/10 MeOH/toluene for analysis by ESI-MS [2, 9, 36]. Nitrogen containing compounds in the SARA fractions have been analyzed by ESI FT-ICR-MS in both positive [2, 9] and negative mode [36].

Preparative scale NPLC based on a silica gel open column has also been applied to resolve sub-fractions of various petroleum extracts [39, 51, 52]. Commonly, 6-7 sub-fractions were obtained from each extract by stepwise elution with anhydrous cyclohexane, dichloromethane (DCM), chloroform, toluene, MeOH and EtOH. The sub-fractions were reconstituted in 50/50 MeOH/toluene for ESI-MS analysis. Six known types and three new types ($C_nH_mN_4VO_2$, $C_nH_mN_4VO_3$ and $C_nH_mN_4VO_4$) of petroleum vanadyl porphyrins [39, 51, 52] were detected from the sub-fractions using ESI FT-ICR-MS. In addition, the fractions from preparative NPLC have been further separated by ion exchange chromatography (IEX) for petroleum separation and characterization [53, 54].

For example, pre-fractionation and purification of petroleum by NPLC-IEX concentrated the nickel porphyrins and increased the ESI-FT-ICR-MS signal-to-noise ratio for nickel porphyrins about 100 fold in Ref. [54].

Second, analytical scale NPLC has also been used for class type separation of crude oil or its extracts, followed by off-line ESI FT-ICR-MS of the fractions collected [9, 11, 35]. For instance, charge transfer phases such as 3-(2,4-dinitroanilino)propyl (DNAP) phase separate polycyclic aromatic hydrocarbons based on their aromatic ring number [55] and also can separate polycyclic aromatic hydrocarbons from polar compounds [55, 56]. **Fig. 4-2** shows four fractions that were collected for treated and untreated heavy gas oil using DNAP with a CH_2Cl_2/hexane eluent [11]. The fractions were re-concentrated in 75/25 MeOH/toluene for characterization of nitrogen containing compounds by ESI FT-ICR-MS. Another system used a cyano column and an amino column in series to separate resins into six sub-fractions using 20/80 IPA/hexane [9]. No reconstitution information for the sub-fractions was provided. But the abundance of nitrogen containing compounds was identified with ESI-MS in each sub-fraction. Alumina column with MeOH/chloroform eluent has also been used to generate a nitrogen enriched petroleum fraction. This nitrogen fraction was further separated into neutral and basic nitrogen containing fractions on a silicic acid column using a toluene or toluene/diethyl ether mobile phase. Prior to ESI, MeOH was added to the final fractions without solvent evaporation [57].

Off-line NPLC-ESI-MS has also been applied to procyanidins analysis from apple juice extracts[58] and peanut skins [59]. Separations were performed on silica with a

MeOH/ethyl acetate/hexane gradient mobile phase. Fractions were collected and rotary evaporated down and lyophilized prior to RPLC-ESI-MS analysis. High peak capacity,

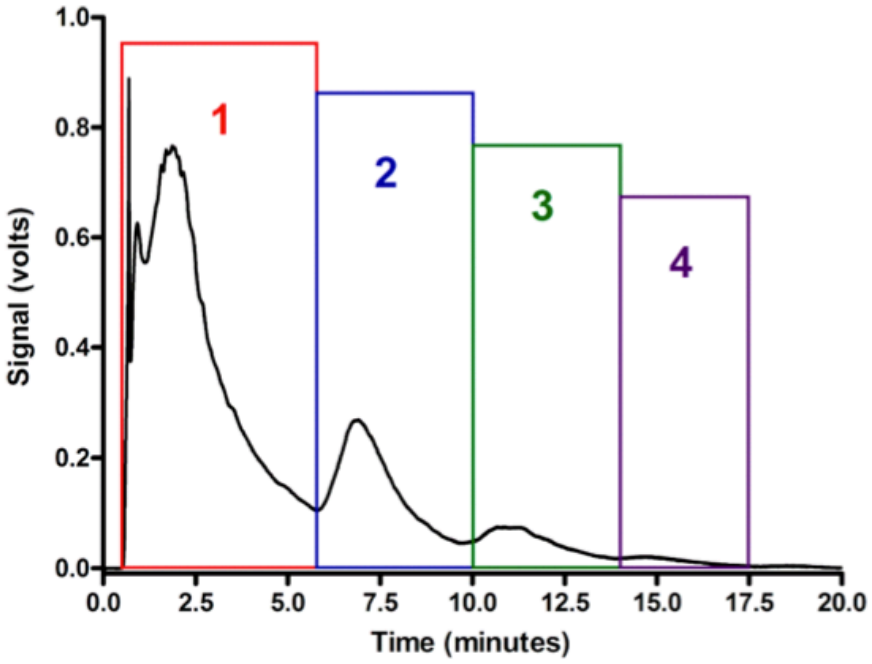

Fig. 4-2 Chromatogram of four petroleum fractions collected on the DNAP column. Reprinted with permission from Ref. [11] Copyright (2013) American Chemical Society.

resolution and specificity were achieved [58, 59]. In another study, crude Ginsenoside-Ro fractions obtained by NPLC on silica were purified by high-performance counter-current chromatography with ethyl acetate/IPA which was directly analyzed by ESI-MS/MS [60].

4.5 Post-column solvent addition for on-line NPLC-ESI-MS

Post-column addition after HPLC separations has been conventionally used to modify the eluent and enhance the detectability of various analytes [61-63]. The addition

of reagent to the effluent may be need to change the chemical properties of the analytes, *e.g.*, post-column derivatization [61, 63]. Alternately, the addition of reagent may merely cause changes to the properties of the effluent. This latter role is the primary purpose of post-column solvent addition for on-line NPLC-ESI-MS [1, 64-66]. **Fig. 4-3** shows that the post-column solvent addition is usually achieved through a zero dead volume (ZDV) T union positioned after the NPLC column and prior to the ESI-MS. The NPLC eluent compromised of ESI-incompatible solvents is connected to one arm of the T union. An ESI-compatible solvent or solvent mixture, called the make-up solvent, is introduced typically at 90° to the eluent, and the mixed stream passes through a short narrow connecting tubing to be introduced into the ESI-MS inlet. This ESI process is usually accompanied by using nitrogen drying gas. Addition of the make-up solvents enables formation of a more stable spray during the ionization process.

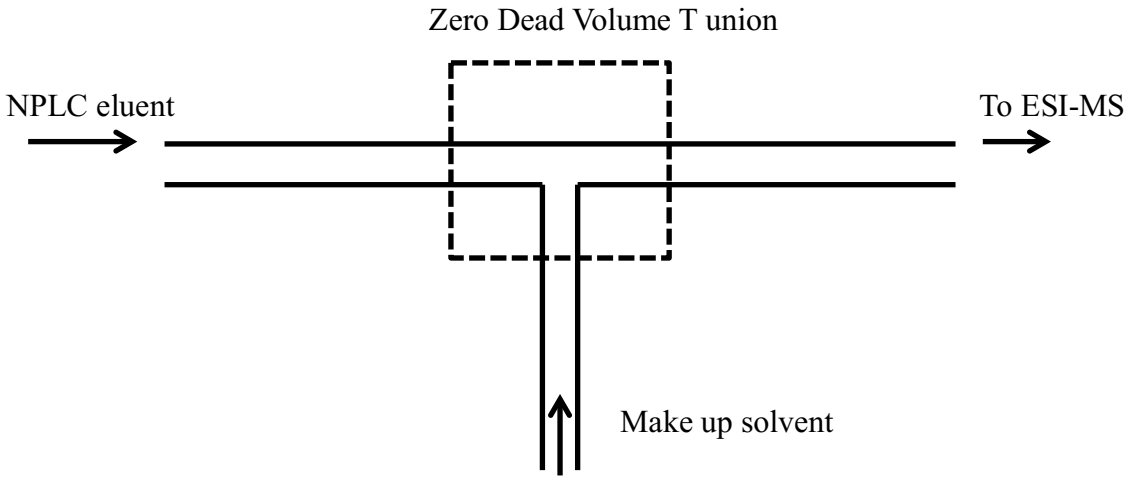

Fig. 4-3 Set-up for coupling of NPLC to ESI-MS through post-column solvent addition.

4.5.1 Common NPLC eluents and make-up solvents

Common NPLC mobile phases contain large amounts (>50%) of n-alkanes such as n-hexane or heptane. To overcome the incompatibility of the eluent with the ESI process, post-column solvent addition of polar solvents has been used. **Table 4-1** lists common make-up solvents used. For a given NPLC eluent, the composition of the make-up solvent affects the degree of ESI signal enhancement [22]. For example, for propranolol in 20/80 IPA/hexane, the use of IPA as make-up solvent enhances the signal-to-noise ratio 2-times that achieved using 1/1 IPA/water and 4-times that using pure water [22]. The higher enhancement with IPA was attributed to better distribution of analyte in the IPA due to greater miscibility between the eluent and IPA [22].

However, the composition of the make-up solvent has generally not been optimized nor compared. For example, MeOH [1], IPA/MeOH [66], and MeOH/chloroform [67] have all been used as make-up solvent for ionization of different analytes in IPA/hexane eluent (**Table 4-1**). Similarly, make-up solvents consisting of MeOH/chloroform have been used to promote generation of analyte ions [67, 68] for lipids in either methyl tert-butyl ether (MTBE)/hexane or IPA/hexane eluent. Whether MeOH/chloroform mixture provides universal enhancement for analytes in NPLC eluent is not known.

Table 4-1 shows that the choice of make-up solvent depends on the analyte type and the NPLC eluent. However, some general conclusions can be made. First, a good make-up solvent commonly contains an alcohol (such as MeOH, EtOH and IPA). Often this alcohol is also a component of the mobile phase [22]. For example, a make-up

Table 4-1 Common make up solvents and the relative flow rate used in post-column solvent addition

Analytes	Mobile phase	Flow rate (mL/min)	Make-up solvent	Additives	Flow rate (mL/min)	Ref.
Triacylglycerols	MTBE/hexane/HOAc	0.1 or 0.5	MeOH/chloroform	2.3% NH_4OH	0.6	[68]
Triacylglycerols	MTBE/hexane/HOAc	0.1 or 0.5	MeOH/chloroform	2.3% NH_4OH	0.6	[69]
Diacylglycerol	IPA/hexane	0.7	MeOH/chloroform	0.15% NH_4OH	0.6	[67]
Triacylglycerols	MTBE/hexane/HOAc	0.1 or 0.5	MeOH/chloroform	2.3% NH_4OH	0.6	[70]
Neutral lipids	MTBE/hexane/HOAc	0.1 or 0.5	MeOH/chloroform	2.3% NH_4OH	0.6	[65]
Neutral lipids	MTBE/hexane/HOAc	0.1	IPA/ACN/DCM/H_2O	10 mM NH_4Ac	0.03	[71]
Methyl oleate	IPA/hexane	NA	MeOH/IPA	40 mM NH_4Ac	0.025	[66]
Petroleum	IPA/hexane	1.0	MeOH	2% HCOOH	NA	[1]
Drug	THF/heptane	0.05	MeOH/ACN	10 mM NH_4Ac	~0.01	[72]
Epoxyalcohols	Hexane-IPA	0.3	IPA/Water	_	0.2	[73]

HOAc: acetic acid; THF: tetrahydrofuran; NA indicates not available; "-" indicates no additives was added. Note: All the separation listed above used silica columns except in Ref. [1], where a aminocyano-bonded silica column was used. Positive mode in ESI has been used except in Ref. [73].

solvent consisting of IPA enables more stable ionization than MeOH for propranolol in 20/80 IPA/hexane [22]. Second, a make-up solvent that is miscible with the NPLC mobile phase generates a higher and more stable signal-to-noise ratio, as will be demonstrated in **Chapters 5** [21] and **6**. Thirdly, only in very rare cases does the make-up solvent consists of only solvents [73]. More commonly, a good make-up solvent contains minor additives, such as formic acid (0.1%-2% by vol) [74, 75], ammonia (0.15-2.5% by vol) [68] or ammonium acetate (NH_4Ac) (2-40 mM) [72, 76, 77], as was discussed in Section 4.2. The performance of different types of additives has not usually been optimized. But six times higher ionization intensity was obtained with addition of NH_4Ac than with formic acid for determination of felodipine in NPLC-ESI-MS [78]. This probably explains why that NH_4Ac is the most frequent used additive in **Table 4-1**. **Table 4-1** also lists the relative flow rate between the sample and the make-up solvent. Both higher [68, 77] and lower [72, 74-76] flow rates of make-up solvent *vs*. NPLC eluent have been applied to real sample analysis, but it is not apparent that the flows used were optimized. In the few cases where the effect of the relative flow rate on ionization efficiency was studied systematically, an optimum flow rate of make-up solvent relative to the effluent flow was observed [21, 73, 79]. For example, 2-10 times higher signal intensity of jasmonic acid in 5/95 IPA/hexane was obtained when the ratio of eluent flow rate to make-up solvent (IPA with 0.1% ammonia) was 1:1 [79]. Similarly, optimal signal intensity for progesterone in pure hexane was achieved with addition of 49/49/2 MeOH/H_2O/HOAc at a relative flow rate of 1:1 [21]. Finally, for Prostaglandin $F_{1\alpha}$ in 0.3 mL/min 3/97 IPA/hexane, a study of 0 to 0.4 mL/min of 3/2 IPA/H_2O make-up solvent observed optimal ionization at 0.2 mL/min make-up solvent [73].

Overall, if the make-up solvent flow is too low, it will not be enough to stabilize the non-polar component of the NPLC eluent. Under such conditions, low signal-to-noise results presumably due to less stable ionization [21]. Too high of a make-up solvent flow will unduly dilute the effluent and at extreme make-up flows will cause instable spray in ESI [13, 79].

4.5.2 Applications of NPLC-ESI-MS using post-column solvent addition

On-line NPLC-ESI-MS using post-column solvent addition has been successfully applied to lipids analysis [68-70], enantiomer differentiation for drugs [80], petroleum characterization [1], and detection of reaction intermediates [66].

4.5.2.1 Lipids

Lipids are a diverse group of natural occurring small molecules including fatty acids (FA), glycerolipids, glycerophospholipids, sterol lipids, phenol lipids, saccharolipids and polyketides that exist in various concentration ranges in cells, tissues, and bio-fluids. Lipids are usually hydrophobic or amphiphilic molecules which constitute the membrane, store energy and are involved in cell signaling [71]. Typically LC-MS is used for lipids analysis [81, 82]. RPLC provides separations based on the hydrophobicity of the FA chain. In contrast, NPLC resolves lipids into classes based on the polarity of the function group [28, 83]. For instance, silica with a MTBE gradient in hexane separates neutral lipids into cholesterol esters (CE), monoalkylether diacylglycerols (MeDAG), diacylglycerols (DAGs), and triacylglycerols (TAGs) within 30 minutes [71]. The post-column addition of 10 mM NH_4OH in 45/45/5/5 IPA/ACN/DCM/H_2O facilitates the formation of ammonium ion $[M+NH_4]^+$ of the neutral lipids allowing ESI-MS analysis. Within each class, some individual molecular species are resolved,

identified and quantified, especially within the cholesterol esters. The ionization enhancement achieved by make-up solvent addition enabled identification of a previously unknown neutral lipid, ubiquinone-9, in cells [71].

NPLC-ESI-MS and MS/MS has also been applied for regioisomeric [68-70] and diastereomeric [67] analysis of triacylglycerols (TAGs). The regioisomerism of TAGs affects their physico-chemical and nutritional properties. Regioisomers of short-[68] medium- or long-chain TAGs in butterfat are separated on a series of two or three silica columns with a MTBE/hexane gradient [68-70]. Addition of 20/10/3 chloroform/MeOH/(25% ammonia in water) after the NPLC separation yields strong ammonium adduct ions $[M+NH_4]^+$. Based on the relative intensity of adduct ions, the position of FA chain in TAGs could be identified [69]. Diastereomers of TAGs can also be resolved on silica with 0.37/99.63 IPA/hexane and analyzed by ESI-MS through post-column solvent addition of 24.5/75/0.5 MeOH/chloroform/(30% ammonia in water) [67].

NPLC-ESI-MS is also used to separate and detect intermediates and products of the transformation of lipids [66]. For example, the ozonolysis of model lipids [66] was monitored using NPLC on silica with an IPA/hexane gradient elution. Addition of a make-up solvent consisting of 40 mM NH_4Ac in 1/3 IPA/MeOH charged the secondary ozonide by formation of adducts with NH_4^+ in ESI-MS. Thus, the accurate identification of intermediates and products is achieved by successful coupling of NPLC to ESI-MS. Another example is to separate and detect keto fatty acids, epoxyalcohols, and allylic epoxyalcohols derived from linoleic and α-linolenic acids in negative ESI [73]. Better separation of epoxyalcohols was observed with silica under NPLC than with RPLC. Post-

column solvent addition of 3/2 IPA/H_2O provided a stable spray for MS and MS/MS analysis.

4.5.2.2 Enantiomer separation and impurities mapping in pharmaceutical process

Differentiation and quantitation of individual enantiomers for drug or drug metabolites is essential during drug discovery and development process. Most enantiomeric separation are performed with eluents used in NPLC mode [84]. Post-column solvent addition is the most common method used to connect chiral separation with ESI-MS [80]. **Table 4-2** summarizes the mobile phases and make up solvents used in chiral-ESI-MS. The typical NPLC-like mobile phase for chiral separation consists of an alcohol (*e.g.*, EtOH or IPA) and an alkane (*e.g.*, hexane or heptane). The make-up solvent is generally NH_4Ac in the same alcohol as in the mobile phase. The final two rows in **Table 4-2** are examples of detection of impurities during pharmaceutical activities. Particularly, the impurities detected in Zotarolimus (codenamed ABT-578) are reactive in aqueous environments. A non-aqueous separation like in NPLC is required to identify these impurities [72]. With THF/hexane gradient, aqueous reactive impurities are separated from ABT-578 on silica column. With post-column addition of 10 mM ammonium acetated in MeOH/ACN to the effluent, impurities are identified through the formation of adduct ion $[M\text{-}H+CH_3COOH]^-$ in negative ESI. Similarly, de-fluorinated analogue of Casopitant mesylate (impurity) has been separated from Casopitant mesylate on Chiralpak AD-H column with 95/5 EtOH/hexane. But the sensitivity of UV detector is insufficient to meet the regulated value, which demands the use of ESI-MS. Addition of MeOH containing 0.1% formic acid to the effluent overcomes the incompatibility between NPLC solvent and ESI-MS and enables detection of the impurity.

Table 4-2 Representatives of chiral-ESI-MS with post-column solvent addition for drug analysis

Pharmaceutical	Chiral column	Mobile phase	Make-up solvent	Ref.
Omeprazole	Chiralpak AD	EtOH /ACN/isohexane	EtOH/HCOOH	[74]
Felodipine	Chiralcel OJ-R	IPA/isohexane	EtOH /H_2O/NH_4Ac	[78]
Terazosin	Chiralpak AD	EtOH /IPA/DEA	IPA/ NH_4Ac	[77]
Lercandipine	Chiralpak AD	EtOH /hexane/DEA	EtOH / NH_4Ac	[76]
Verapamil	Chiralpak AD	IPA/hexane/DEA	IPA/ NH_4Ac	[85]
Sotalol	Chiralpak AD	EtOH /IPA/DEA	IPA/ NH_4Ac	[85]
Doxazosin	Chiralpak AD	IPA/hexane/DEA	IPA/ NH_4Ac	[85]
Oxybutynin	Chiralpak AD	IPA/hexane/DEA	IPA/ NH_4Ac	[85]
Jasmonic acid	Cellulose tris(4-methylbenzoate) coated silica	IPA/hexane	IPA/ NH_4OH	[79]
Propranolol	Chiral Art SB	IPA/hexane/NH_4OH	IPA	[22]
Mexiletine	Chiralpak AD	EtOH /IPA/hexane/TFA	Ethanol/ NH_4Ac	[86]
ABT-578	YMC-Pack SIL	THF/heptane	MeOH/ACN/ NH_4Ac	[72]
Impurities in ABT-578	YMC-Pack SIL	THF/heptane	MeOH/ACN/NH_4Ac	[72]
Casopitant Methylate	Chiralpack AD-H	EtOH/heptane	MeOH/HCOOH	[75]

DEA: diethylamine. Note: The identification of ABT-578 is performed in negative ESI, while the others are performed in positive ESI.

4.5.2.3 Petroleum characterization

As stated in Section 4.2, coupling of NPLC with ESI-MS for petroleum analysis is mostly performed in the off-line mode. Nonetheless, there are examples where petroleum or petroleum subclasses have been analyzed by NPLC-ESI-MS with on-line post-column solvent addition.

High resolution ESI-FT-ICR-MS contributes a better understanding of petroleum composition [45, 46]. On-line NPLC-FT-ICR-MS provides faster, better structure separation, and more molecular composition identification than off-line NPLC-FT-ICR-MS [38]. An extra challenge to on-line NPLC with FT-ICR-MS is the balance between the time available when the NPLC eluent leaves the column and the scanning time required for sensitive detection [87]. For NPLC-ESI-FT-ICR-MS analysis of petroleum, the crude oil was baseline resolved into two peaks on an aminocyano-bonded silica column with a gradient of IPA/hexane mobile phase. Addition of formic acid in IPA after elution promoted the generation of protonated analytes ions $[M+H]^+$ in positive ESI, but the ESI-MS trace is very noisy. More nitrogen containing compounds in crude oil were detected with ESI compared to other ionization techniques (APCI and APPI) [1]. This on-line NPLC-ESI-MS also allowed two-dimensional analysis of the nitrogen containing isomers. Two peaks for molecular composition $C_{29}H_{43}N$ appear in the extracted ion chromatogram. The first peak was identified as alkylated carbazoles and the second peak was identified as polar quinoline or carbazole derivatives by comparing with the retention time of standard compounds [1].

Low resolution ESI-MS has also been used for simplified petroleum analogs. As will be shown in **Chapter 6**, nine azaarenes are baseline resolved on a 2,4-

dinitroanilinoylpropyl column and combined to ESI-MS through post-column solvent addition. With addition of 98/2 IPA/HOAc to the 20/80 IPA/hexane eluent, the protonation of azaarenes in ESI has been enhanced. The detection limits were ten times than those with UV detection. The fast separation on relatively simple instruments is potential for azaarenes analysis in petroleum related products.

4.6 Sheath liquid interface for on-line NPLC-ESI-MS

A sheath liquid interface is another strategy for on-line NPLC-ESI-MS. The sheath liquid interface was originally developed for post-column derivatization of carbohydrates for ESI-MS [88], and has subsequently been widely used for capillary electrophoresis-ESI-MS [89-91]. The sheath liquid interface uses a triaxial flow scheme (**Fig. 4-4**) to allow the separation eluent to mix with a sheath liquid at the tip of an ESI nebulizer. The effluent from separation is introduced into a capillary sit in the center of a metal tube (red tube in **Fig. 4-4**). An appropriate sheath liquid, also called make-up liquid

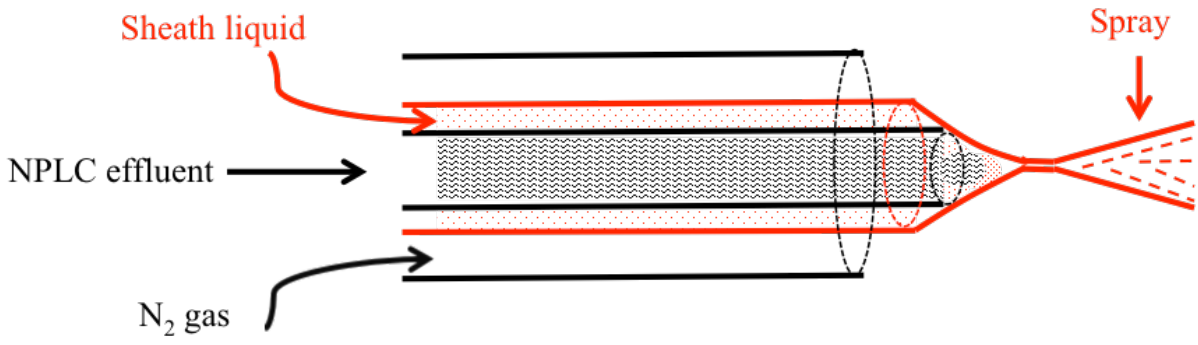

Fig. 4-4 Scheme for sheath liquid interface for coupling of NPLC to ESI-MS.

is added via a syringe pump through the metal tube. N_2 gas is then delivered through a third concentric tube to assist ionization. The sheath liquid forms an external layer around the separation eluent during spray formation [92]. Analytes in an effluent consisting of a solvent that is incompatible with ESI-MS are ionized at the outer layer of the spray [92, 93].

The sheath liquid usually consists of ESI-compatible solvents (mainly MeOH) with minor additives (*e.g.*, 3-60 mM NH_4Ac). In the absence of a sheath liquid or with a sheath liquid without additives, there is no signal. The signal intensity increases significantly with the addition of any amount of NH_4Ac to the sheath liquid [92]. The signal intensity is also increases with sheath flow rate to an optimum, and then decreases with further increases of sheath flow [92, 93] presumably due to dilution effects. Thus, the dependence of ionization efficiency on sheath flow is similar to that in post-column solvent addition (Section 4.5).

However, some differences exist between post-column (Section 4.5) and sheath liquid solvent addition for on-line NPLC-ESI-MS. First, in post-column solvent addition, the use of a make-up solvent that is miscible (*e.g.,* IPA) with the IPA/hexane NPLC eluent enhanced ionization more than using an immiscible make-up solvent (*e.g.*, $MeOH/H_2O$) (**Chapter 5** and **6**) [21]. The opposite dependence has been observed with the sheath liquid interface, where MeOH was the optimal sheath liquid [92]. Second, in a direct comparison (**Fig. 4-5**) post-column solvent addition generated higher ionization efficiency for inophyllum P in ethyl acetate/isooctane than sheath liquid interface when using 60 mM NH_4Ac in MeOH as make-up solvent [92].

The lower ionization enhancement is probably why the sheath liquid interface has not been widely used for on-line NPLC-ESI-MS. The sheath liquid interface has been applied for neoflavonoids [92] and amitrole [93] NPLC analysis. Separation of neoflavonoids on silica with a IPA/isooctane gradient observed optimal ionization with a sheath liquid consisting of 60 mM ammonium acetate in MeOH [93]. Optimal analysis of amitrole was observed using an amine column with 60/40 EtOH/hexane and a sheath liquid of 3 mM NH_4Ac in MeOH [93].

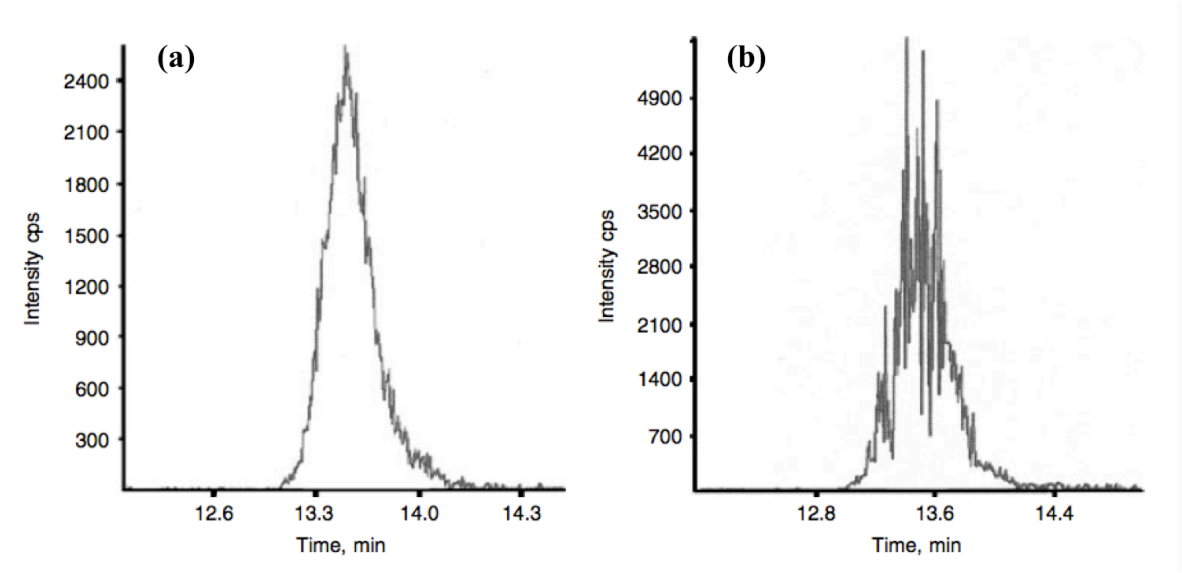

Fig. 4-5 Chromatograms for 1 µg/mL inophyllum P standard (a) With sheath liquid interface (60 mM ammonium acetate in MeOH at 5 µL/min) (b) With post-column solvent addition (60 mM ammonium acetate in MeOH at 20 µL/min). Reprinted with permission from Ref. [92] Copyright (2004) Wiley-VCH.

4.7 Ambient ionization (AI) for ESI-MS-incompatible eluents

Another pathway in **Fig. 4-1** for on-line ESI-MS from ESI-incompatible eluents are the ambient ionization (AI) techniques [94]. AI is a group of ionization techniques that require little or minimum sample preparation. AI usually consists of: a desorption

step; followed by an ionization step. The desorption can be done by nebulization, laser desorption, thermal evaporation, or direct desorption. Common ionization methods are ESI and APCI [94, 95]. Thus, various ESI-based AI systems have been built over the past decade [94].

4.7.1 Background on ambient ionization methods

Desorption electrospray ionization (DESI) was the first and the most popular AI technique [96]. In DESI (**Fig. 4-6(a)**), charged solvent emitted from an electrospray nebulizer impinges on the sample surface or on samples that are deposited on an insulating surface [96]. Direct desorption and ionization occurs simultaneously, and the generated ions are sampled with MS analyzer. DESI is most suitable for direct analysis of both solid and liquid samples, particularly for samples on flat surfaces [97, 98] or biological tissue imaging [99].

Two other techniques similar to DESI isolate the desorption step from the ionization step. Electrospray-assisted laser desorption ionization (ELDI) [102] uses UV- or IR-laser irradiation to desorb samples deposited on flat surfaces [102]. Laser-induced acoustic desorption (LIAD) [103] focuses a laser pulse at the back of the surface where sample are deposited. The resultant acoustic and shock waves cause sample desorption [103]. The desorbed samples from both techniques then enter the electrospray solvent emitted from a nebulizer positioned above the sample surface [102, 103]. The analytes are ionized in electrospray solvent and ionized by mass spectrometer.

Solvent-assisted electrospray ionization (SAESI, **Fig. 4-6(b)**) is a recent ambient ionization technique that utilizes two electrospray nebulizers that are touching [100]. This means that there is no distance between the tips of the two nebulizers. The intimacy of

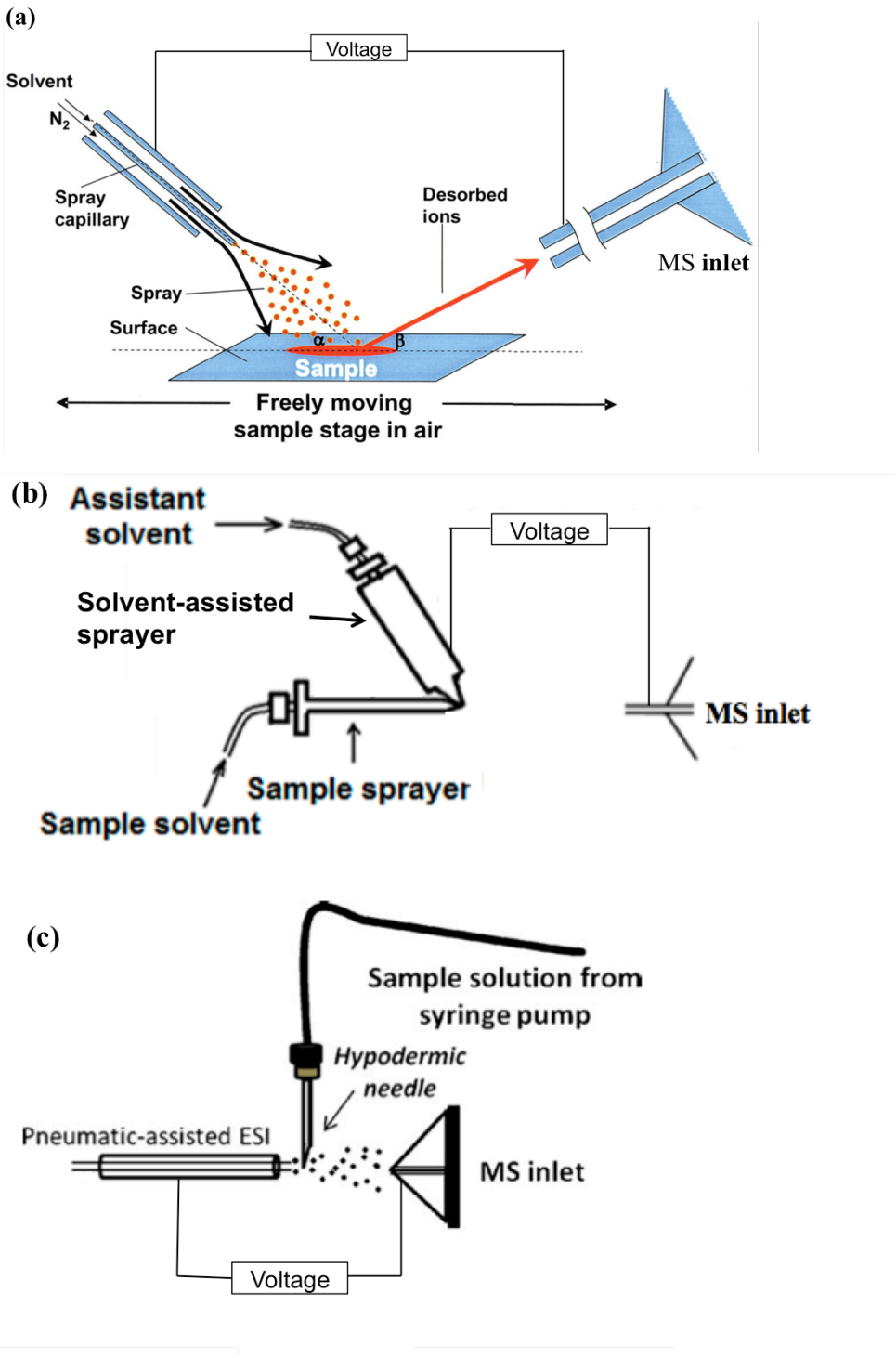

Fig. 4-6 Configuration for (a) DESI. Reprinted with permission from Ref. [96]. Copyright (2004) American Association for the Advancement of Science. (b) SAESI. Reprinted with permission from Ref. [100]. Copyright (2014) American Chemical Society. and (c) CF-EDESI. Reprinted with permission from Ref. [101]. Copyright (2013) Elsevier B.V.

the electrosprays allows immediate contact of the charged solvent droplet and the sample [100]. The percentage of ESI compatible solvents such as ACN in electrospray solvent has little effect on the ionization of fatty acids, indicating extraction is not the dominate mechanism in SAESI [100]. Continuous flow-extractive desorption electrospray ionization (CF-EDESI, **Fig. 4-6(c)**) is a comparable technique which was recently reported by Schug and coworkers [104]. Instead of using a sprayer to nebulize the samples as in SAESI, CF-EDESI utilizes a needle to provide a continuous flow of the sample. In CF-EDESI there is some distance between the tips of the sprayer and the needle. Thus, the position and time that the sample flow and electrospray solvent flow meet are different from those in SAESI. Charged solvent (MeOH/water mixture) droplets produced by an electrosprayer assists desorption and extraction of analytes in the continuous flow of sample. **Fig. 4-6(c)** is the original scheme of CF-EDESI, which is based on an on-axis ESI-MS system. A modified CF-EDESI source based on off-axis ESI-MS instrument has recently been reported (**Chapter 5**) [21].

All the above ESI-based ambient ionization techniques have been reported for ionization of samples in non-ESI compatible solvents, either in normal phase TLC plate or with NPLC-ESI-MS.

4.7.2 Online normal phase TLC-ESI-MS with ambient ionization

AI methods have been predominantly used with thin layer chromatography. Thin layer chromatography is a simple, fast, inexpensive and low solvent consumption separation and purification technique for chemical and biological samples. TLC also allows analysis of multiple samples simultaneously [105]. Resolved bands on TLC plates are usually visualized using optical or spectroscopic methods. Ambient ionization

techniques such as DESI, ELDI and LIAD allow direct sample analysis on planar surfaces, and so serve as ideal interfaces for TLC with MS. A comprehensive discussion on combining TLC with MS is beyond the scope of this review, and but can be found in Refs. [106]. Here we focus on on-line normal phase TLC-ESI-MS through ambient ionization.

Silica gel pre-coated plates developed with normal phase solvents have been used for the separation of *Salvinorin* species in *Salvia divinorum* leaves [107] and small molecules in *Excedrin tablets* [108]. Direct and rapid scanning of the separated bands using 3/1 MTBE/hexane or 99/1 ethyl acetate/HOAc by DESI-MS using 80/20 ACN/H_2O or pure MeOH resulted in comparable resolution and quantification information to that in optical detection. The resolution and sensitivity are highly dependent on the spray to tip distance, solvent flow rate and scanning rate [109]. On-line TLC-ESI-MS has also been observed with ELDI [102] using 1/1 MeOH/H_2O for characterization of drug extracts on TLC plate developed using 98/1/1 ethyl acetate/DCM/HOAc. Similarly, LIAD using 1/1 MeOH/H_2O was applied for rosemary essential oil separated on silica gel TLC plate using 9/1 ethyl acetate/toluene [103]. The various ambient ionizations expand ESI-MS's application for normal phase TLC.

4.7.3 Online NPLC-ESI-MS with SAESI or CF-EDESI

SAESI (**Fig 4-6(b)**) has been used for on-line chiral-NPLC-ESI-MS analysis of benzoin [100] and propranolol [22]. Enantiomers in the IPA/hexane effluent were ionized with high efficiency using an immiscible ESI compatible electrospray solvent [22, 100]. **Fig. 4-7** indicates that SAESI-MS using electrospray solvent 0.1% formic acid in water provided 200-400 folds higher response than using direct NPLC-ESI-MS [22]. This

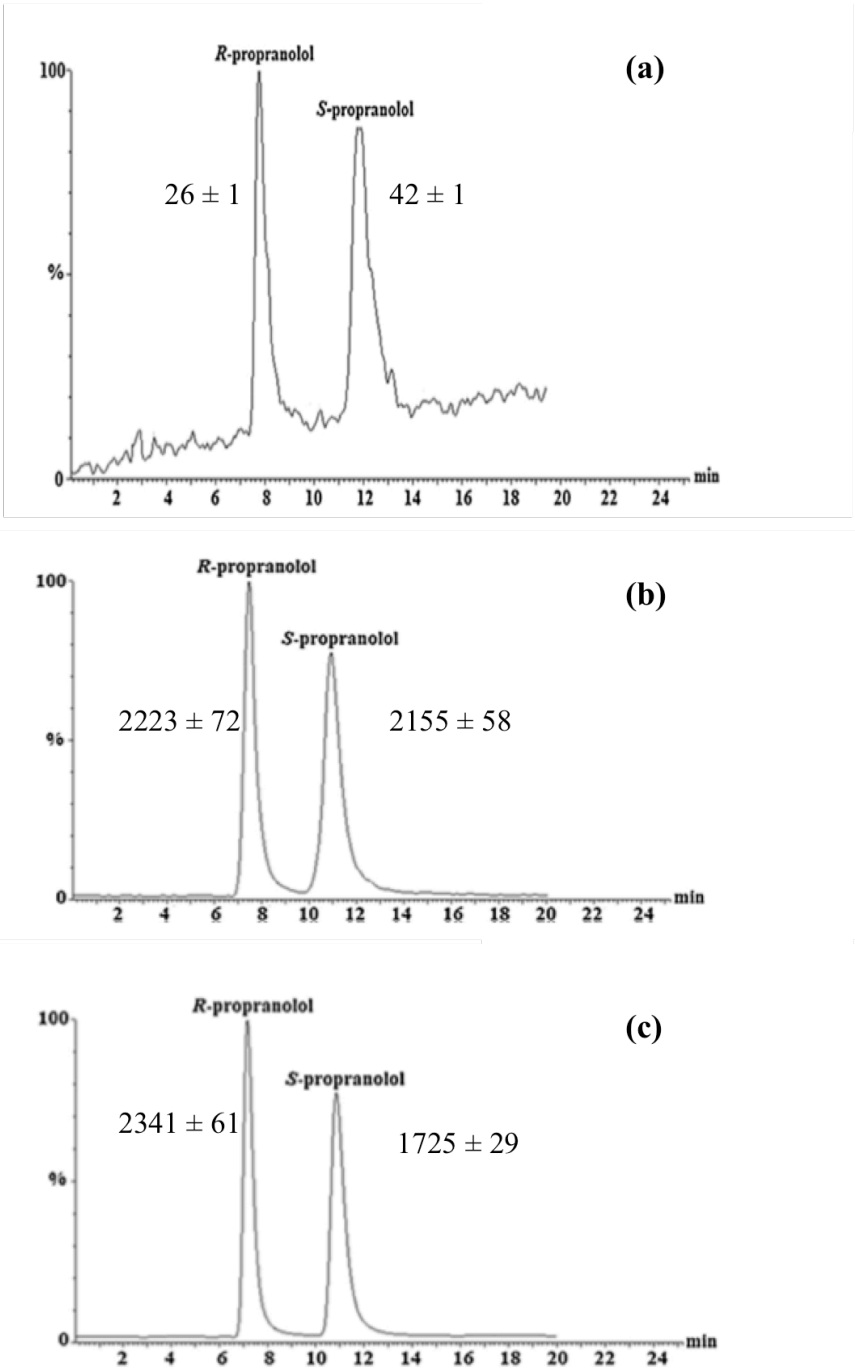

Fig. 4-7 Chromatograms of 1 μg/mL R- and S- propranolol in 20/80 IPA/hexane (a) direct NPLC-ESI-MS (b) with SAESI (electrospray solvent: 0.5% formic acid in H_20) (c) with post-column solvent addition (make-up solvent: 0.5% formic acid in IPA). Reproduced with permission from Ref. [22] Copyright (2004) Elsevier B.V.

SAESI-MS technique also provided higher specificity than direct NPLC-ESI-MS and comparable sensitivity to post-column solvent addition ESI for propranolol enantiomers in 20/80 IPA/hexane [22].

CF-EDESI (**Fig 4-6(c)**) is another ambient technique that is able to improve ionization for analytes in ESI-incompatible solvents [21, 101]. For direct infusion of progesterone in hexane, CF-EDESI with 49/49/2 MeOH/H_2O/HOAc as the electrospray solvent generated the highest ion intensity [101] comparing to 98/2 MeOH/HOAc or 98/2 H_2O/HOAc. The proportion of electrospray solvent is also optimized and 1/1 was found to be the best ratio [21]. The on-line NPLC-ESI-MS through CF-EDESI has been used for chiral separation and identification of amine-containing compounds [110]. Limits of detection (0.02-0.1 ng/mL) comparable to UV absorbance were achieved. CF-EDESI with NPLC of azaarenes exhibited an irregular response which made analysis either only semi-quantitative for low molecular weight azaarenes such as quinoline and isoquinoline or impossible for later eluting azaarenes such as benz(a)acridine and benz(c)acridine (**Chapter 6**).

4.8 Conclusions

Normal phase liquid chromatography is widely used for separations of polar compounds, water labile compounds and stereoisomers for analysis of lipids, plant extracts, drug enantiomers, and petroleum. Electrospray ionization-mass spectrometry or tandem mass spectrometry enables rich molecular composition and structure information. Coupling NPLC with ESI-MS provides a sensitive and specific method for qualitative and quantitative analysis. However, the incompatibility of the nonpolar NPLC solvents

with ESI make the combination a challenging task. There are five possible pathways to couple NPLC with ESI-MS. In limited cases, where the NPLC eluent contains significant quantities of polar solvents, the eluent can be directly introduced into ESI-MS. More commonly, the NPLC effluent fractions have been collected off-line reconstituted in an ESI-compatible solvent. Finally, there are emerging on-line methods that enable direct coupling of NPLC and ESI-MS. A make-up solvent that is ESI compatible has been added through a T union or sheath liquid interface to NPLC effluent to generate analyte ions. Alternatively, analytes separated by normal phase TLC plates can be directly sampled into ESI-MS by ambient ionization, such as DESI, ELDI and LIAD. Also, ambient ionization, such as SAESI and CF-EDESI enhances ionization of analytes in ESI-incompatible solvents by introducing electrospray solvent and NPLC effluent in two different channels.

Our analysis of the NPLC-ESI-MS literature provides some conclusions. First, post-column solvent addition is a simpler, more time efficient and more sensitive method compared to other methods of coupling NPLC to ESI-MS. It is promising to perform the on-line NPLC-ESI-MS analysis by the simple post-column solvent addition to gain rich information from complex samples, such as petroleum. Second, there is an increasing use of ambient ionization techniques, predominantly for TLC separations. The success of these methods warrants further exploration of ambient ionization methods for NPLC separations. Standardization in the configuration of ESI-based ambient ionization source and optimization in the performance are needed for further exploration.

4.9 References

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Chapter Five: Enhancement of ionization efficiency of mass spectrometric analysis from non-electrospray ionization compatible solvents with conventional and novel ionization techniques[§]

5.1 Introduction

Since its introduction in 1984 [1], electrospray ionization (ESI) combined with mass spectrometry (MS) has been widely used for analysis of biomolecules [2-7], pharmaceuticals [8], and elemental speciation [9, 10]. The ESI ion generation process has been described in Section 1.3.6.1 and reviewed in Ref. [11]. With the development of various high resolution MS analyzers, the application of ESI-MS has been expanded to complex samples such as in proteomics [2, 12, 13] and petroleomics [14-16].

ESI-MS experiences some limitations when applied to such complex samples. High resolution ESI-MS can differentiate thousands of chemicals in a mixture [14, 15], but it cannot distinguish between isomers of the same mass to charge ratio. In addition, when using ESI-MS to analyze complex samples, matrix components may suppress analyte ionization [17, 18]. Ion suppression makes the identification and quantification of chemicals of interest difficult, especially when the matrix compounds are present in significant excess [19]. To compensate, a pre-separation is helpful for the analysis of complex samples.

Combining high performance liquid chromatography (HPLC) with ESI-MS has

[§] A version of this chapter has been published as Ping Jiang and Charles A. Lucy, Analytica Chimica Acta, 2015, 897, 45-52. DOI:10.1016/j.aca.2015.09.035

been widely used for biomolecules [20-24], pharmaceuticals [20, 21, 25] and environmental samples [26-28]. However, the sensitivity and selectivity is highly dependent on the separation technique. Reversed phase liquid chromatography (RPLC) is most frequently used prior to ESI-MS analysis, because of the high polarity and low surface tension of the common organic solvents (*e.g.* methanol and acetonitrile) used. Ion exchange (IEX) [29, 30], ion pair [31-33] and size exclusion chromatography [34] have also combined with ESI-MS. These separation techniques are most suitable for samples can be separated with polar mobile phases such as methanol and acetonitrile.

For polar and isomeric compounds, normal phase liquid chromatography (NPLC) conditions and solvents are most suitable. NPLC uses mobile phase solvents with low polarity, low conductivity and low dielectric constant (*e.g.*, hexane or heptane with a small percentage of isopropanol). Such NPLC solvents are not compatible with the electrospray ion generation, and so NPLC cannot be readily coupled with ESI-MS. **Chapter 4** summarized the approaches to couple NPLC with ESI-MS. In this chapter, I focused on using make-up solvent addition (Section 4.5) and continuous flow-extractive desorption electrospray ionization (CF-EDESI) to ionize analytes in non-ESI compatible solvents. Coupling of NPLC with ESI-MS through addition of a make-up solvent has been used for analysis of chiral drugs [35, 36], plant lipids [37], and petroleum [38]. Methanol [39], isopropanol [36, 40] and ethanol/water [41] with acidic or basic additives are common make-up solvents (Section 4.5.2). Reported flow rates of the make-up solvent have been both higher [36] and lower [37, 39] than the flow rate of the eluent. In many cases, the flow rates of the make-up solvent and eluent are not stated [38, 40]. In some cases, make-up solvent is not needed, for instance when the NPLC separation uses

a mobile phase containing isopropanol (IPA) [42, 43]. However, the literature is not clear when a make-up solvent is needed or how much make-up solvent should be added to the eluent to achieve optimal ionization.

Also, there is literature that compares different ambient ionization techniques and their mechanism [44-46]. However, no comparison of the performance of the CF-EDESI *vs.* conventional make-up solvent addition has been reported. Our objective in this chapter is to address the gap in knowledge with regard to the performance of the conventional (make-up solvent addition) and the novel (CF-EDESI) technique. We have optimized the ionization of directly infused analyte in a non-ESI compatible solvent with both make-up solvent addition and a CF-EDESI source. The suitability of both techniques for samples in non-ESI compatible solvent is investigated and the ionization efficiencies are compared. We also studied the suitability of both techniques for petroleum type standards analysis with ESI-MS. Matrix effects are also studied.

5.2 Experimental

5.2.1 Materials

HPLC grade hexane, isopropanol (IPA) and dichloromethane (DCM) were from Fisher Scientific (Fairlawn, NJ, USA). LC-MS grade methanol (MeOH), toluene, progesterone, pyridine, quinoline (99.8%), acridine (99.8%), phenanthrene, and dibenzothiophene were from Sigma-Aldrich (St. Louis, MO, USA). Acetic acid (HOAc) (glacial, >99.7%, HOAc) was from Caledon (Georgetown, ON, Canada). Deionized water (>18.0 MΩ) was from a Barnstead E-pure system. Carbazole was from Kodak (Rochester, NY, USA).

5.2.2 Instrumentation

An Agilent 1100 series G1946A single quadrupole mass spectrometer with a conventional ESI source was used. The mass spectrometer was run in positive mode with: mass range, m/z 500-800 Da; capillary voltage, 4 kV; fragmentor, 160 V (optimal); cycle time: 1.04 s/cycle; drying gas flow, 7-13 L/min; nebulizer pressure, 10-30 psi; and drying gas temperature, 350 °C. The drying gas flow and nebulizer pressure were selected as recommended by the instrument manual based on the flow rate entering the ESI source. The analysis of analytes dissolved in hexane, hexane/IPA or hexane/DCM with ESI-MS was achieved either with make-up solvent addition through a commercial stainless steel T union (tubing O.D. 1/16 in, bore 0.25 mm, Valco Instruments, Houston, TX, USA) or with a home-built CF-EDESI source. **Caution**: Flammable solvents as hexane are a potential explosion hazard when introducing into the high voltage and high temperature MS [47]. However no issues have been observed under our conditions or those in the literature [47].

5.2.2.1 Make-up Solvent addition through T union

Fig. 5-1(a) shows the T-union configuration used for introduction of a polar make-up solvent to a sample in a non-ESI compatible solvent prior to introduction into the MS inlet. The sample in non-ESI compatible solvents was introduced into a horizontal arm of the T-union using a syringe pump (CAT#55-1199, Harvard Apparatus, Holliston, MA, USA). The make-up solvent MeOH/H_2O/Acetic acid (HOAc) = 49/49/2 was added at 90° to the sample flow using an Agilent HPLC G1312A pump. The sample and the make-up solvent mixed within 105 mm of 0.17 mm I.D. stainless steel tube (Agilent) prior to entering the MS inlet

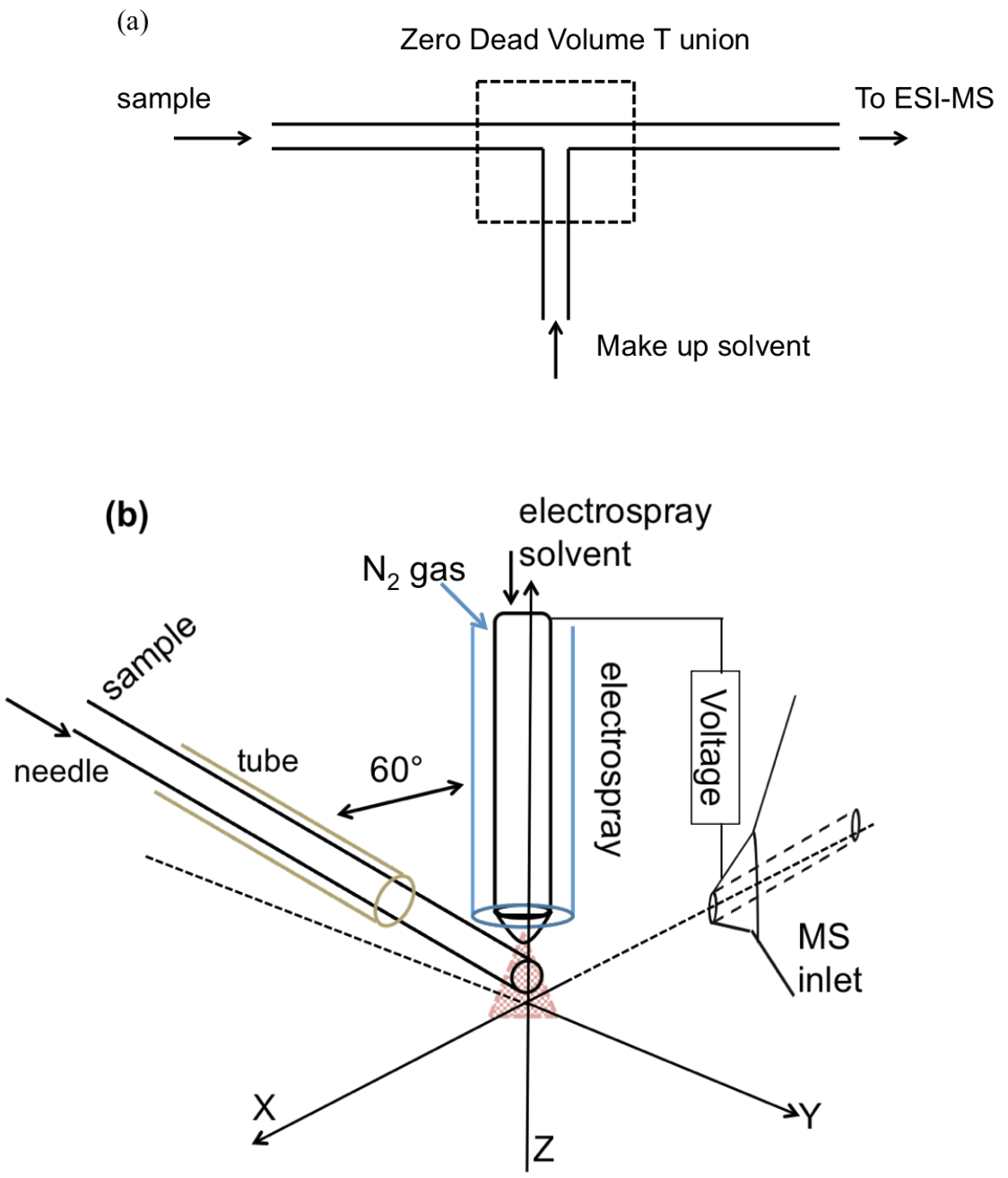

Fig. 5-1 Configuration for (a) make-up solvent addition through T union and (b) home-built CF-EDESI source with needle distance equal to 0.

5.2.2.2 CF-EDESI source

Fig. 5-1(b) illustrates the configuration of the CF-EDESI source. The CF-EDESI source was based on an Agilent conventional ESI source (G1948A). Use of the Agilent source necessitated altering the angle between the nebulizer and the needle from that in Ref. [46]. A stainless steel tube (72 mm long type 316 stainless steel, 1.59 mm O.D., 0.76 mm I.D.) was mounted 60° from the ESI nebulizer needle. A metal hub needle (76.2 mm long, 22 gauge, Hamilton, NV, USA) was inserted into the stainless steel tube. Our ESI nebulizer was orthogonal to the MS orifice, rather than in-line with the orifice as in Ref. [46]. The position of the tube in the ESI source was adjusted so that the tip of the needle touched the extension line of the ESI nebulizer needle. The position of the stainless steel tube was fixed. The distance between the tip of the needle and the extension line of the ESI nebulizer metal capillary was controlled through adding or removing stainless steel shims (~0.1 mm each) or short tubes (1.0 mm) below the metal tub of the needle to adjust the needle position. **Fig. 5-2** is a photograph of the CF-EDESI source. The sample in non-ESI compatible solvents was infused through the needle to the single quadrupole MS orifice. The make-up solvent (MeOH/H_2O/HOAc = 49/49/2), based on Ref. [46], was introduced at 10 to 500 μL/min using an Agilent HPLC pump (G1312A).

5.3 Results and discussion

Normal phase liquid chromatography (NPLC) prior to electrospray ionization mass spectrometry (ESI-MS) enables separation of isomers and allows analysis of hydrophobic compounds that are not soluble in aqueous-organic mixtures. NPLC also

handles analytes with high boiling point where gas chromatography is not applicable. However, common mobile phases in NPLC such as hexane or heptane are not directly

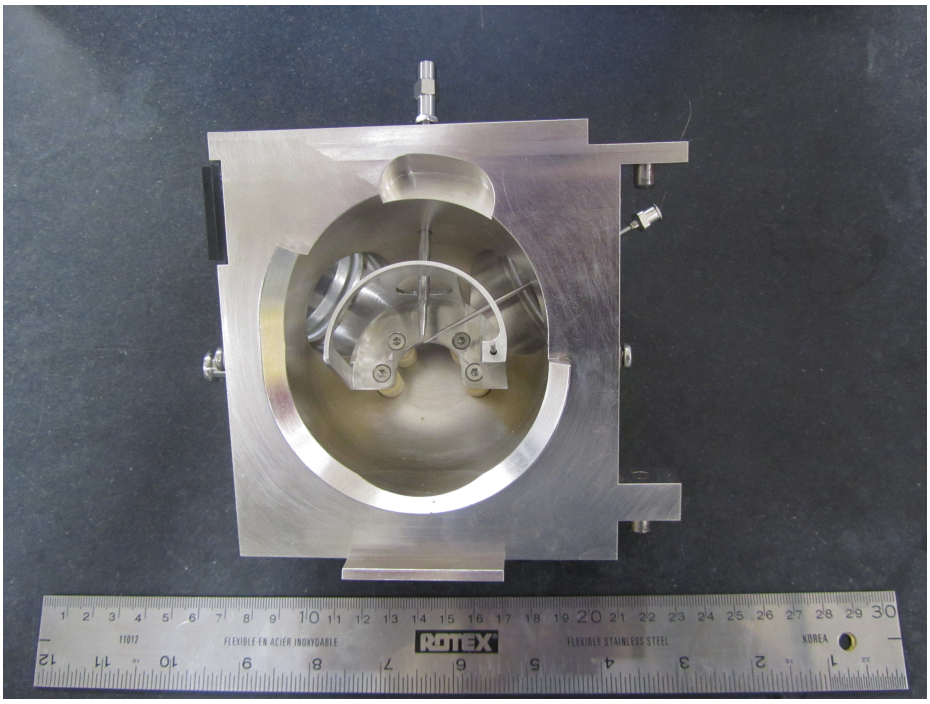

Fig. 5-2 CF-EDESI source

compatible with electrospray ionization, due to their low conductivity and dielectric constant. Addition of polar organic solvents [35-38, 40] and continuous flow extractive desorption electrospray ionization (CF-EDESI) [46, 48] have been used to address this compatibility issue. However there has been little discussion of the optimization or comparison of the techniques for ESI-incompatible solvents. This contribution addresses these gaps to better guide the coupling of NPLC with ESI-MS.

5.3.1 Optimization of make-up solvent addition

Addition of polar organic make-up solvents through T union has enabled the hyphenation of NPLC and chiral chromatography with ESI-MS for the analysis of biomolecules, pharmaceuticals and crude oils [36, 38-40, 42, 43, 47]. The eluents in these

separations are commonly hexane/ethanol or hexane/IPA. Hexane or heptane containing mobile phases are incompatible with ESI. Thus, addition of a polar organic solvent (methanol or isopropanol) is needed to improve the spray conditions [38, 39]. However, there has been no systematic study of the effect of the amount of make-up solvent on the ESI signal.

For these studies we monitored progesterone, which has been used previously to characterize CF-EDESI [46]. Direct infusion of 10 μM progesterone in hexane did not yield any analyte signal at m/z = 315.2, or even any background signal. The low conductivity and low dielectric constant of hexane do not favor formation of a stable spray [49, 50], and thus no ionization occurs. To promote protonation of progesterone in hexane under positive mode, a polar solvent that is compatible with ESI-MS can be added. Here we use MeOH/H_2O/HOAc = 49/49/2 to allow comparison with the CF-EDESI source [46]. Addition of 49/49/2 MeOH/H_2O/HOAc make-up solvent yielded a spectrum dominated by the protonated progesterone ion at m/z = 315.2 (**Fig. 5-3**). The peak at 629.4 amu is a protonated ion for the non-covalent progesterone dimer $[2M+H]^+$, which is common in ESI-MS. The ion at 145.0 amu may be a fragment from progesterone or a contaminant from the ESI-MS chamber [51].

The progesterone signal varied 5% over 2 min at a sample flow of 50 μL/min and make-up flow of 50 μL/min. This relative standard deviation (RSD) is comparable with the typical RSD of ESI-MS. Thus the added make-up solvent promotes formation of a stable a spray in ESI and the generation of ions. Similar improvements in the ionization of drugs and petroleum compounds were observed upon addition of methanol with formic

acid or isopropanol to an alkane (hexane or heptane)/alcohol (ethanol or isopropanol) mobile phase [36, 38, 39].

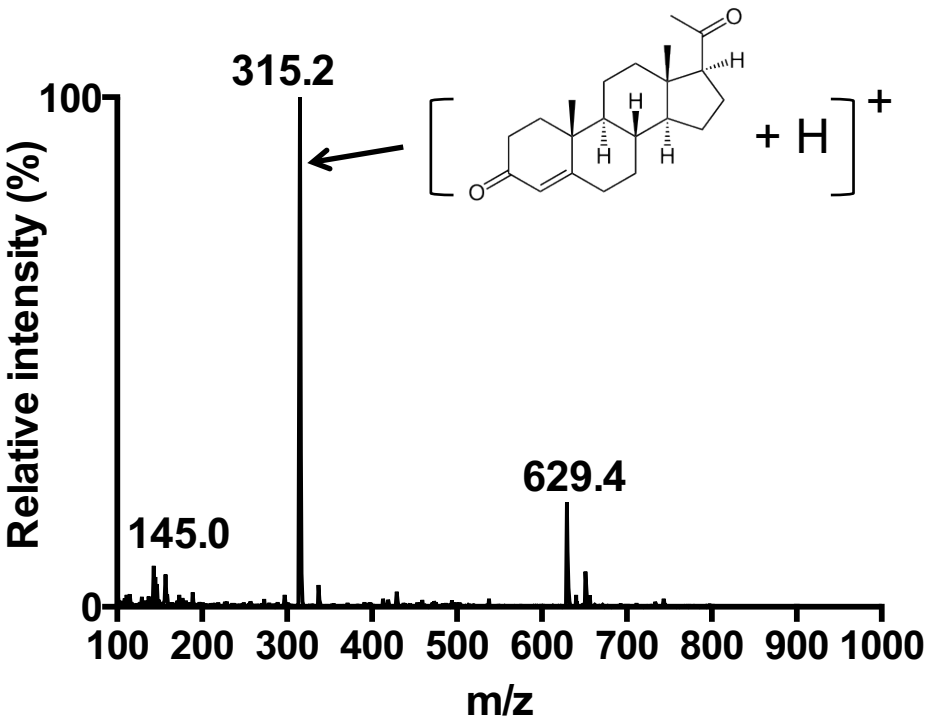

Fig. 5-3 Positive ion mass spectrum of progesterone in hexane with make-up solvent addition. Other conditions: progesterone, 10 μM in hexane; , sample flow, 50 μL/min; make-up solvent, MeOH/H_2O/HOAc=49/49/2; make-up solvent flow, 50 μL/min.

The literature suggests that the relative flow rates of the polar make-up solvent to nonpolar eluent affects the ionization efficiency, but is contradictory as to whether a greater [36] or lesser [37, 39] proportion of make-up solvent favors ionization efficiency. The relative flow rate is probably dependent on the properties of the analyte. In practice, the sample flow rate is typically 10 -500 μL/min. **Fig. 5-4(a)** shows the effect of varying the flow rate of 10 μM progesterone in hexane, while keeping the make-up solvent (MeOH/H_2O/HOAc = 49/49/2) flow constant at 50 μL/min. As the sample flow rate

increases, the corresponding response of protonated progesterone increases. The increased intensity results from the increased molecules that enter the surface of the spray. This increased signal intensity with increased sample flow rate is consistent with theory [52].

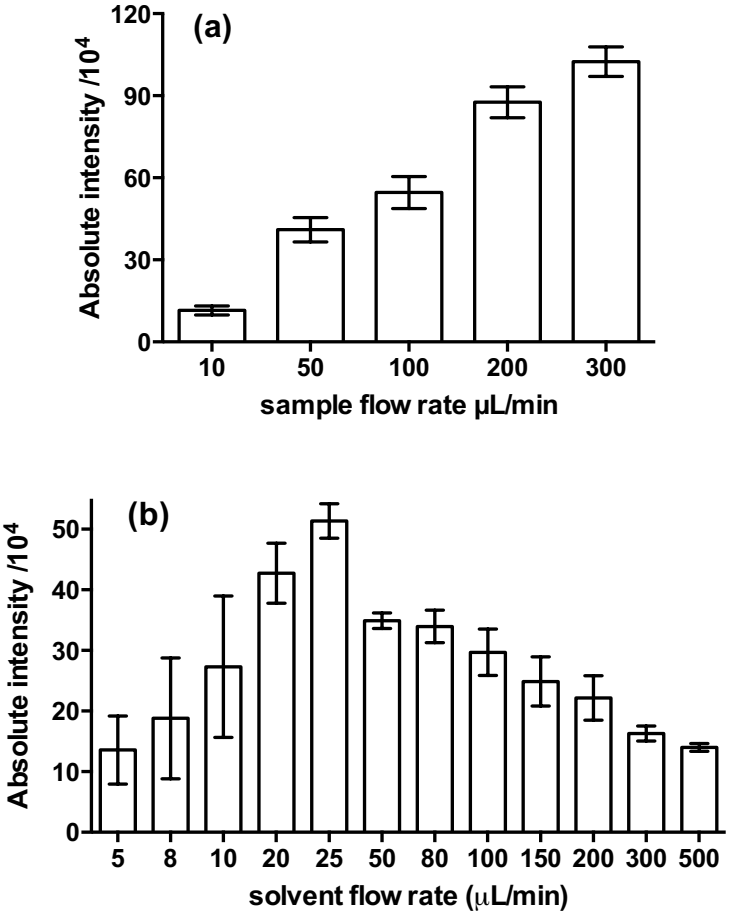

Fig. 5-4 Effect of sample flow and make-up solvent flow with make-up solvent addition on ionization of progesterone in hexane. Make-up solvent: MeOH/H_2O/HOAc=49/49/2. (a) Effect of sample flow. Other conditions: make-up solvent flow, 50 µl/min. (b) Effect of make-up solvent flow. Other conditions: sample flow, 50 µL/min.

Addition of a make-up flow of an acidic polar organic solvent has been reported to assist the formation of a stable spray of non-ESI compatible solvents [38, 39]. **Fig. 5-4(b)** shows the effect of the flow rate of the make-up solvent at a constant 50 µL/min of

sample in hexane. From 10 to 25 μL/min of make-up solvent, the ionization efficiency increased and the RSD decreased from 52% to 5%, the latter of which is typical for ESI-MS. These improvements are due to stabilization of the spray by the polar make-up solvent. Above a make-up solvent flow of 50 μL/min, the protonated progesterone ion intensity decreased more than two-fold with further increases in the make-up solvent flow. This decreased signal intensity can be attributed to dilution due by the excess make-up solvent [53]. In all, addition of ESI compatible make-up solvent enabled ionization of target samples in an ESI-incompatible solvent. However, due to the dilution effect, the sensitivity is expected to be less than that for direct analysis of samples in ESI favorable solvents [38].

5.3.2 Optimization of CF-EDESI source

Make-up solvent addition is a simple and easy-to-design tool to assist in the analysis of samples in NPLC favorable solvents with ESI-MS. However, when using it for combination of NPLC with ESI-MS, it has been suspected to be highly dependent on instrument, change the sample conditions and compromise the separation efficiency [46]. Thus there has been recent interest in using ambient ionization techniques for on-line NPLC-ESI-MS. Continuous flow extractive desorption electrospray ionization (CF-EDESI) has been demonstrated to enhance the ionization efficiency of progesterone in non-ESI compatible solvents [46, 48]. Optimization of the distance between the spray needle and the hypodermic needle has not previously been detailed. Here we fabricated a CF-EDESI source based on an Agilent source and optimized the configuration and performance. In addition, the performance of CF-EDESI has not been compared with conventional approaches to deal with non-ESI compatible solvents such as the addition of

a make-up solvent. Herein, we aim at comparing the performance of our CF-EDESI source with the make-up solvent addition technique discussed in Section 5.3.1.

5.3.2.1 Effect of distance for CF-EDESI performance

Fig. 5-1(b) is a schematic of our CF-EDESI source, based on an Agilent ESI source. Compared to the original CF-EDESI source [46], the angle between the spray needle and the hypodermic needle is 60° rather than 90° due to the constraints of the Agilent ESI source. Also, our ESI nebulizer is orthogonal to the MS orifice, instead of parallel to the orifice as in the original CF-EDESI source [46]. When the spray needle and the hypodermic needle are orthogonal to each other, a distance of 1.5 mm between the tips was used [46, 54]. However, there are no studies of the effect of this distance on the ionization. In our case, the distance between the tip of the hypodermic needle to the extension line of spray needle is defined as in **Fig. 5-5**.

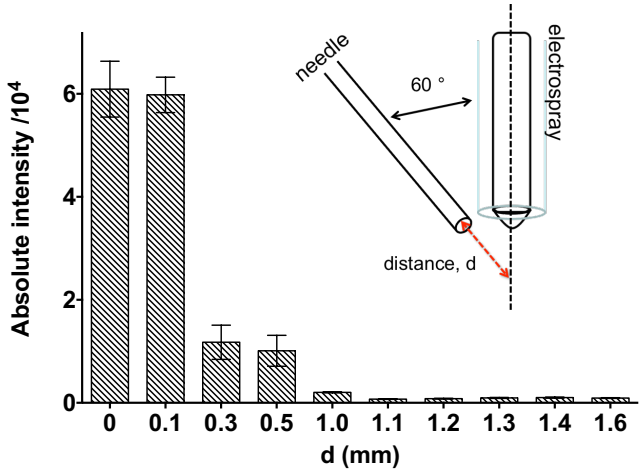

Fig. 5-5 Effect of distance between needle tip and extension line of the nebulizer center on ionization of progesterone in hexane. Other conditions: sample flow, 50 μl/min; electrospray solvent, MeOH/H_2O/HOAc=49/49/2. Electrospray solvent flow, 50 μl/min.

Fig. 5-5 also shows the effect of varying this distance from 0.0 to 1.6 mm on the ionization efficiency. Positioning the tip of the hypodermic needle on the extension line

of the spray needle (d = 0 mm) resulted in maximum ionization of the 10 μM progesterone in hexane. Surprisingly, the wall of the needle did not hinder progesterone ionization. Retracting the needle even 0.3 mm dramatically decreased the signal. So we kept the distance d = 0 mm for our later experiments.

Our optimized CF-EDESI source yielded a lower signal intensity (6.0×10^4) than obtained for the same concentration of progesterone in hexane in the literature (6.5×10^5) [46]. Absolute signal intensity was used for a direct comparison to the performance of the original CF-EDESI. The LCQ Deca XP quadrupole ion trap mass spectrometer used for the original CF-EDESI study [46] commonly produces 10 to 100 times higher signal intensity that the single quadrupole instrument used in our study [55]. Thus, we believe our modified CF-EDESI ionizes analytes in non-ESI compatible solvents comparably to the original CF-EDESI [46]. The orthogonal geometry (off-axis) of our CF-EDESI source (**Fig. 5-2**) reduces background signals from either the sample or the MS orifice [56]. Although the off-axis geometry may cause sample signal loss relative to the original on-axis CF-EDESI, the signal to noise ratio with the off-axis geometry matches that of the on-axis one. More importantly, our orthogonal geometry is more consistent with the modern source design, which will enable more widespread use of CF-EDESI for MS.

The effect of capillary voltage, nebulizer gas flow rate, sample temperature, sample flow rate and electrospray solvent on the ionization of progesterone in hexane was studied with the original CF-EDESI [46]. Their factorial design experiment indicated that the signal intensity increased with an increase in the capillary voltage. The signal intensity also decreased when the sample temperature was above 20 °C [46]. Thus, for our modified CF-EDESI, the capillary voltage was kept at a high value (4 kV) but not so

high as to cause discharge. Our sample was introduced at room temperature (20 °C) to maintain optimal ionization and for convenience. The flow rates of the sample and the electrospray solvent also affect the CF-EDESI ionization efficiency [46]. The impact of the flow rates on the CF-EDESI has been studied in Ref. [46] but only over a limited range. The effect of a wider range of flow rates has been studied as in following sections.

5.3.2.2 Effect of flow rates on CF-EDESI

Fig. 5-6 presents the flow rate dependence of our CF-EDESI source. As the

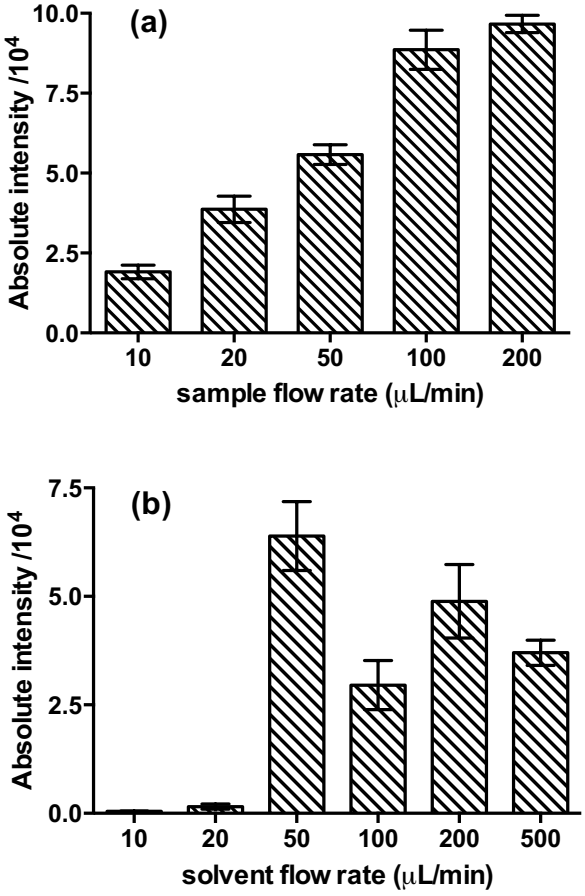

Fig. 5-6 Effect of sample flow and electrospray solvent flow with CF-EDESI on ionization of progesterone in hexane. Electrospray solvent: MeOH/H_2O/HOAc=49/49/2. (a) Effect of sample flow. Other conditions: electrospray solvent flow, 50 μl/min. (b) Effect of electrospray solvent flow. Other conditions: sample flow, 50 μL/min.

sample flow rate is increased (**Fig. 5-6(a)**), greater signal intensity results as more sample passes through the interaction zone per unit time. This is consistent with the observations of [46]. Li and coworkers also observed increased standard deviation for the signal intensity at higher sample flows. In contrast, the standard deviations in **Fig. 5-6(a)** are nearly independent of sample flow.

Li et al. observed increased ionization of progesterone in hexane as the electrospray solvent flow rate was increased from 5 to 50 μL/min at all sample flow rates studied (10 μL/min to 75 μL/min) they studied [46]. **Fig. 5-6(b)** shows our investigation of a wider range of electrospray solvent flow rate. The ionization efficiency dramatically increased at an electrospray solvent flow rate of 50 μL/min, and then gradually decreased as the electrospray solvent flow was further increased. CF-EDESI is in part an extraction-based process [46]. Thus, at low electrospray solvent flow rates there is probably not enough solvent to extract the analyte. At high make-up solvent flows, the extracted sample becomes diluted.

5.3.3 Comparison between using make-up solvent addition and CF-EDESI

The above sections demonstrate that both the make-up solvent addition and CF-EDESI enable ionization of samples in ESI-incompatible solvents. The strong protonated progesterone signal with both the T union (**Fig. 5-3**) and CF-EDESI (data not shown) means that fewer sample preparation steps are needed for samples in hexane analyzed by ESI-MS. The reduced sample preparation and enhanced sensitivity avoids the potential for contamination associated with having to evaporate down the non-ESI compatible solvent and re-constitute the sample in an ESI compatible solvent [19]. Also, both techniques generate reproducible enhancement for analyte in hexane. Experiments

performed one month apart yielded equivalent ionization efficiency within each technique for progesterone solution in hexane at a concentration of 10 μM (**Fig. 5-7**).

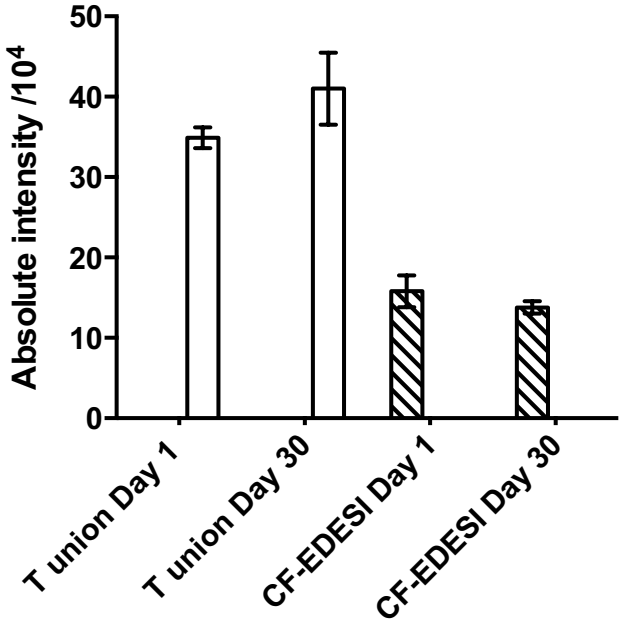

Fig. 5-7 Reproducibility and comparison of performance of using make-up solvent addition and CF-EDESI for progesterone in hexane at two different days. Other conditions as in **Fig. 5-5**.

Fig. 5-8 summarizes other experiments performed to study the stability and reproducibility of the progesterone signal across a range of concentrations in hexane. With either make-up solvent addition (filled triangle) or CF-EDESI (open triangle), the RSD of the progesterone signal decreases as the concentration increases. In **Fig. 5-8** the RSD changes from 54% to 7% when progesterone concentration decreases from 20 nM to 10 μM with make-up solvent addition. Similarly, RSD for progesterone signals changes from 40% to 8% when the concentration varies from 1 μM to 10 μM with CF-EDESI), but the standard deviation increases. The concentration was not lowered to the

limit of detection. However, Fig. 5-8 indicated that make-up solvent detected about two orders of magnitude lower concentration as CF-EDESI.

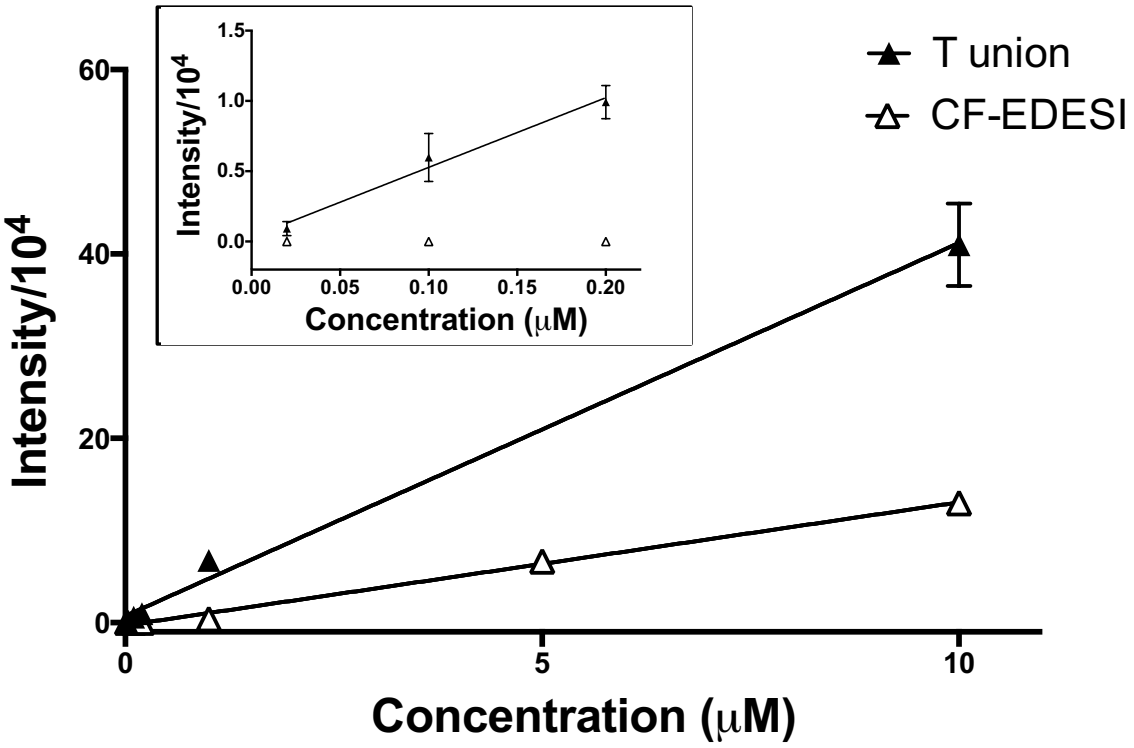

Fig. 5-8 Ionization efficiency of 20 nM to 10 μM progesterone across a range of concentrations in hexane with make-up solvent addition and CF-EDESI. Filled triangle: make-up solvent addition with the make-up solvent flow and sample solvent flow both at 50 μL/min. Open triangle: CF-EDESI with the electrospray solvent flow and sample solvent flows both at 50 μL/min. **Note**, the concentration axis was not drawn to scale. Also, no measurement was made for 5 μM progesterone using make-up solvent addition. Inset plot is an expanded plot of the signal for 20 to 200 nM progesterone in hexane.

Comparing the two ionization techniques reveals some differences. Firstly, both **Fig. 5-7** and **Fig. 5-8 (inset)** demonstrate that under optimized conditions the T union provides higher signal intensity. The sensitivity for progesterone is higher with make-up solvent addition. In **Fig. 5-8 (inset)**, a concentration of progesterone in hexane down to 20 nM can be detected with make-up solvent addition. However, with CF-EDESI, > 1

μM is needed to differentiate the progesterone signal from the background noise. Secondly, when using the make-up solvent addition, there are less restrictions on the choice of make-up solvent. In CF-EDESI, the electrospray solvent cannot be fully miscible with the sample solvent [46]. In our CF-EDESI, using MeOH/toluene (3/1) as the electrospray solvent did not yield ionization of progesterone in hexane. MeOH/toluene (3/1) is miscible with hexane, and so was not able to extract progesterone from hexane. In contrast, addition of a make-up solvent of MeOH/toluene (3/1) through the T union increased ionization of progesterone in hexane, to a three fold greater extent than MeOH/H_2O/HOAc.

5.3.4 Ionization efficiency for samples in different non-ESI compatible solvents

Both the make-up solvent addition and CF-EDESI enhance ionization of analytes in pure hexane. However, in NPLC the eluent will contain polar modifiers such as alcohols in addition to the pure hexane studied above. The make-up solvent addition through a T union has previously been used with hexane/isopropanol (IPA) eluent [39], and the CF-EDESI with heptane/ethanol eluents [48].

Fig. 5-9 summarizes the performance of the both methods for the ionization of acridine, a model nitrogen compound in petroleum, in various compositions of hexane/IPA. One μM acridine was used in the make-up solvent addition studies, while 10 μM had to be used with CF-EDESI. **Fig. 5-9** shows that without make-up solvent or electrospray solvent (open circle and open triangle), there is no ionization for acridine when the sample contains less than 20% IPA. As the %IPA in the sample increases beyond 20%, the protonated acridine signal becomes detectable. Thus, in cases where a NPLC eluent contains a large amount of IPA (>35%), direct ESI-MS is possible [42, 43].

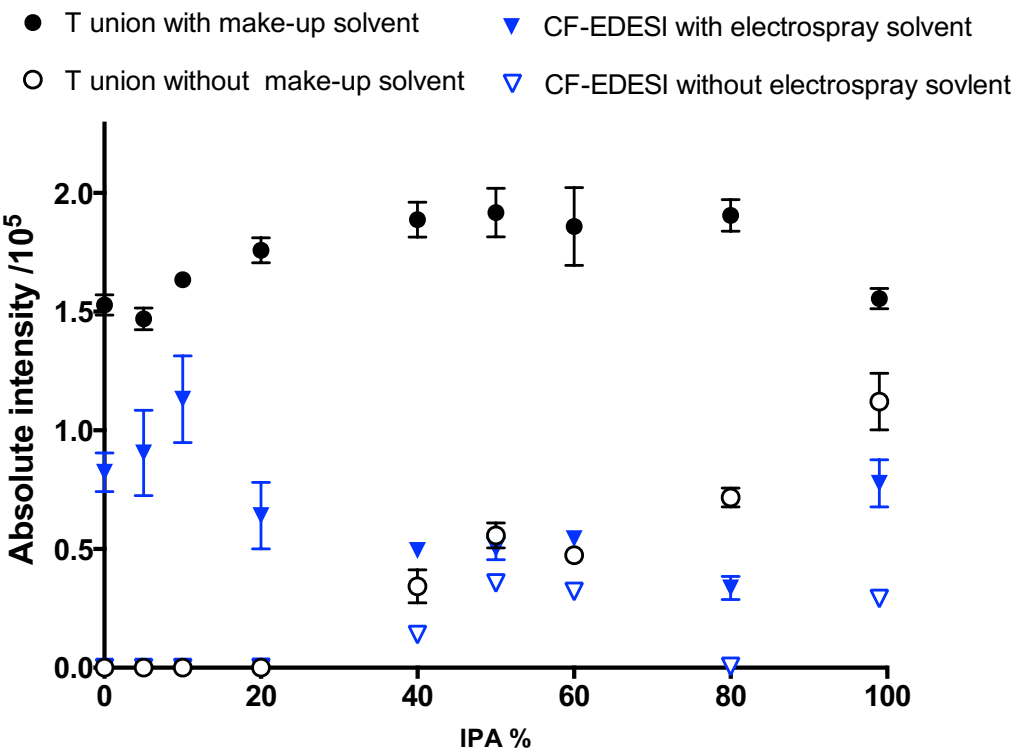

Fig. 5-9 Ionization efficiency of acridine in different composition of hexane/IPA with make-up solvent addition or CF-EDESI. Make-up solvent or electrospray solvent: MeOH/H_2O/HOAc=49/49/2. Other conditions: acridine is prepared at 1 μM in different composition of hexane/IPA when using make-up solvent addition; Filled circle (make-up solvent addition with make-up solvent flow at 50 μL/min); Open circle (make-up solvent addition without make-up solvent); Acridine is prepared at 10 μM in different composition of hexane/IPA when using CF-EDESI; Filled triangle (CF-EDESI with electrospray solvent flow at 50 μL/min); open triangle (CF-EDESI without electrospray solvent).

In **Fig. 5-9**, the filled symbols show the effect when make-up solvent was added through the T union or an electrospray solvent was used with CF-EDESI. There is a dramatic enhancement of the ionization of acridine (filled symbols). The enhancement with the make-up solvent addition (filled circle) is more significant than with CF-EDESI (filled triangles). Note that the make-up solvent addition yields greater signal, even though the acridine concentration was ten-fold higher in the CF-EDESI experiment. These results again support that make-up solvent addition performs better than the CF-

EDESI source. Make-up solvent addition also improves the ionization of acridine in hexane/DCM (**Fig. 5-10**) but with higher standard deviation than in hexane/IPA, presumably due to less stable spray.

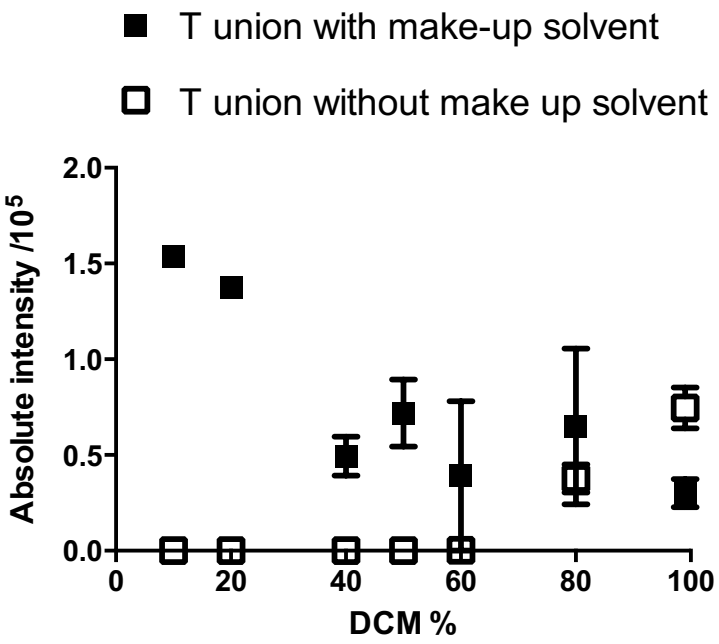

Fig. 5-10 Ionization efficiency of acridine in different compositions of hexane/DCM with make-up solvent addition. Make-up solvent: MeOH/H_2O/HOAc = 49/49/2. Filled square: with make-up solvent flow at 50 μL/min. Open square: without make-up solvent. Samples: acridine is prepared at 1 μM in different composition of hexane/DCM. Sample flow: 50 μL/min.

5.3.5 Suppression effects

The above discussion illustrates that ESI-MS with make-up solvent addition has advantages over using CF-EDESI in the ionization efficiency of individual compounds dissolved in non-ESI compatible solvents. For complex samples, such as petroleum, different components compete for ionization [18, 57, 58].

In direct infusion of nitrogen containing petroleum compounds, positive mode ESI preferably ionizes basic nitrogen containing compounds *vs.* other types of compounds [14]. Given the preferential sensitivity of the basic nitrogen compounds as a class, it has been claimed that a pre-separation is not required for their analysis in petroleum [14]. In our work, protonated acridine ion (m/z at 180.1) also dominated the spectrum when a sample containing an equimolar solution of phenanthrene (poly aromatic hydrocarbon), dibenzothiophene (sulfur compound), carbazole (non-basic nitrogen), pyridine, quinoline and acridine (three basic nitrogen compounds) in hexane was infused into the ESI-MS with addition of make-up solvent (**Fig. 5-11**). Similar behavior was observed for the six compounds in hexane with CF-EDESI source (data not shown). This agrees with the literature that other classes of compounds did not interfere with the characterization of basic nitrogen containing compounds [14].

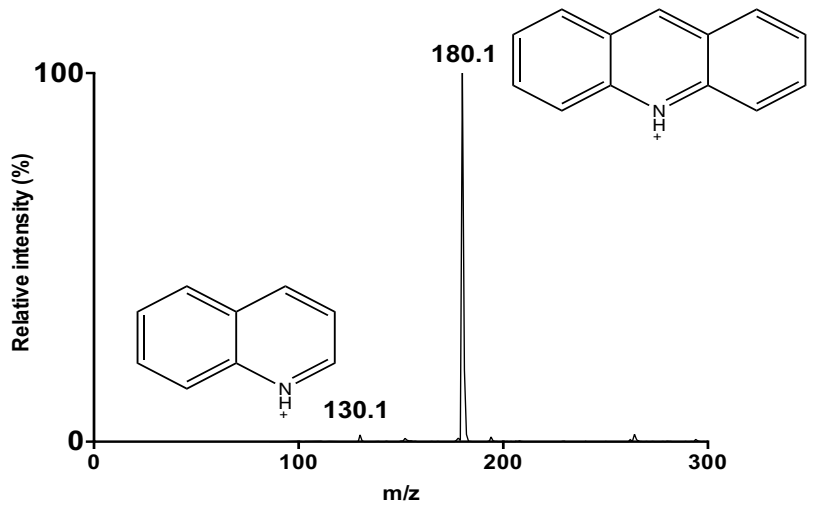

Fig. 5-11 Positive ion mass spectrum of equimolar phenanthrene, dibenzothiophene, pyridine, quinoline, acridine and carbazole in hexane with make-up solvent addition. Other conditions: the six compounds are prepared at 10 μM in hexane; sample flow, 50 μL/min; make-up solvent, MeOH/H_2O/HOAc=49/49/2; make-up solvent flow, 50 μL/min.

However, in this equimolar solution, pyridine and quinoline intensities were near background (**Fig. 5-11**). To further investigate whether there is a suppression effect between the basic nitrogen compounds, solutions were prepared with a constant 10 μM pyridine, and various concentrations of acridine.

Fig. 5-12 showed that pyridine in hexane can be detected very well in the absence of acridine with either the make-up solvent addition or CF-EDESI. As the concentration of acridine increases, the ionization of pyridine was heavily suppressed. Thus, neither the T union nor CF-EDESI eliminates suppression amongst compounds of the same functionality. NPLC separation technique before ESI-MS would be helpful to simplify samples to get more information from petroleum [38]. To achieve online-NPLC-ESI-MS, make-up solvent addition and CF-EDESI would contribute significantly.

Current NPLC separations tend to cluster compounds of the same class (*i.e.*, same functionality) into individual regions of the chromatogram [38, 59]. However, given the ionization suppression within a class of compounds observed above for the basic nitrogen compounds, compounds of a given class should ideally be distributed across the chromatogram, for example, using 2D separation to avoid ionization suppression.

5.4 Conclusions

Make-up solvent addition and CF-EDESI are both useful techniques for sample ionization in non-ESI compatible solvents. Our work characterized and compared the ionization ability of make-up solvent through T union and CF-EDESI for nitrogen containing compounds in hexane. Sample flow rate and make-up solvent flow rate are important factors affecting the performance of both techniques. High sample flow rate

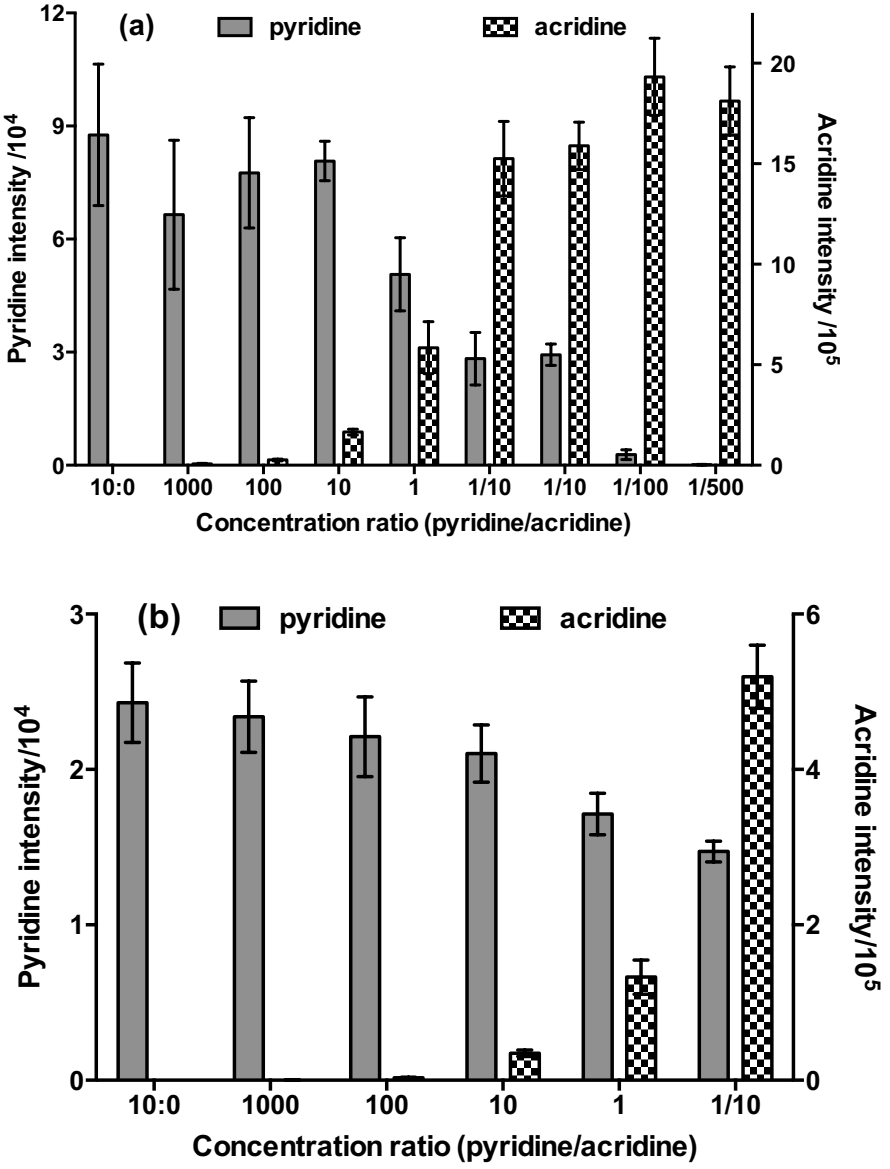

Fig. 5-12 Ion suppression of acridine to pyridine with make-up solvent addition or CF-EDESI. (a) suppression effect of using make-up solvent addition (b) suppression effect of using CF-EDESI. Other conditions: make-up solvent or electrospray solvent, MeOH/H_2O/HOAc=49/49/2, flow rate, 50 μL/min; pyridine is prepared at 10 μM in hexane; acridine is prepared at various concentrations (0-5.0 mM); sample flow, 50 μL/min.

and medium make up solvent flow rate are preferred for optimum performance. After optimization, reproducible and stable ionization was achieved for samples in non-ESI

compatible solvents system with either the make-up solvent addition or CF-EDESI technique. Although both techniques significantly improved the ionization efficiency of samples in hexane with MS, make-up solvent addition has advantages of higher ionization efficiency, solvent compatibility and simplicity.

Neither the T union nor CF-EDESI prevented ion suppression of analytes caused by compounds of similar structure. However, both techniques are promising for combining NPLC with ESI-MS for analysis of complex petroleum samples.

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Chapter Six: Electrospray ionization mass spectrometry coupled to normal phase liquid chromatography using post column addition and ambient ionization for azaarenes analysis

6.1 Introduction

Azaarenes are a group of nitrogen containing polycyclic aromatic compounds that exist widely in air particulate matter [1, 2], lake sediments [3], automobile exhaust [4], tobacco smokes [5], fried food products [6, 7] and shale oil/coal tar processing [8, 9]. Azaarenes are also referred as polycyclic aromatic nitrogen hydrocarbons (PANHs). Azaarenes possess similar structures to polycyclic aromatic hydrocarbons (PAHs), and both arise mainly from incomplete combustion of fuels [8]. But the chemical properties of azaarenes and PAHs are quite different [8, 10]. Azaarenes are more polar than PAHs, and thus have higher bioavailability [11]. Also, azaarenes are present at 1-10% of the concentration of PAHs in the environment [10, 12], which makes their characterization challenging. Some azaarenes are carcinogenic and mutagenic. For example, quinoline is carcinogenic to rats [13] and humans [14]. Azaarenes with three/four fused aromatic rings are also potent carcinogens. The carcinogenicity and mutagenicity are dependent on the size of the azaarenes and the position of the nitrogen atom in the isomers [8, 10]. For example, benz(c)acridine is more mutagenic than benz(a)acridine for the *Salmonella* bacteria [15]. Various methods including UV absorbance [16, 17], fluorescence [1], NMR [18] and mass spectrometry (MS) [19-23] have been explored for the identification of azaarenes in environmental samples and fuels. With the exception of mass

spectrometry [3, 19, 20, 22, 24, 25], these methods are either insensitive or only applicable to relatively simple sample matrices [8]. Electrospray ionization (ESI) [19, 20, 25] and atmosphere pressure chemical ionization (APCI) [22, 25] are the most common ionization sources used in MS. ESI is more sensitive for azaarenes than APCI [25]. Coupled with high resolution mass analyzers such as Fourier transform ion cyclotron resonance MS (FT-ICR-MS), ESI-MS provides molecular fingerprints for azaarenes in complex samples such as petroleum [26]. However, the application of ESI-MS for azaarenes analysis faces limitations. Firstly, there are various isomers for azaarenes with same ring numbers, which MS alone cannot differentiate. Secondly, when multiple components are introduced into an ESI, the compounds compete the surface of the droplet for ionization [27]. Less ionizable analytes and more dilute analytes are suppressed by the easily ionizable analytes and more concentrated analytes, as observed in Refs. [28] and [29].

A separation technique can resolve the azaarenes isomers prior to introduction into the ESI, and thus will reduce ion suppression. A large number of systems have been developed for the separation of azaarenes [22, 24, 30-32]. Reversed phase liquid chromatography (RPLC) is widely used with ESI-MS for azaarenes analysis [3, 20, 21, 33]. In RPLC the retention of azaarenes increases with the number of aromatic rings [30, 32]. Three-ring isomeric azaarenes, such as benzo(f)quinoline and acridine can be resolved in RPLC [32]. However, some azaarenes-enriched samples, such as petroleum, are barely soluble in RPLC eluents. Secondly, isomeric resolution of the smaller azaarenes is limited when using RPLC [8]. Normal phase liquid chromatography (NPLC), on the other hand, provides better resolution of azaarene isomers than RPLC

due to the specific polar interaction between the nitrogen within the azaarenes and NPLC stationary phases [30, 32, 34]. The more exposed the nitrogen atom is, the stronger the polar interaction [34]. In contrast, in benz(c)acridine the nitrogen atom is not accessible, and so it is weakly retained [32, 34]. However, azaarenes with different numbers of aromatic rings may co-elute [32]. For example, amino phases can resolve azaarenes from other classes of aromatic nitrogen containing compounds, but can not separate individual molecule [30]. Despite the limited resolution offered by conventional NPLC adsorbents, there have been no literature studies of azaarene separations on hypercrosslinked or charge-transfer phases.

Coupling NPLC with ESI-MS can enhance the ability to identify isomeric azaarenes [19, 25]. However, the mobile phase in NPLC cannot be directly introduced into ESI-MS due to its low polarity and low dielectric constant, particularly when there is high percentage of hexane/heptane. Post column solvent addition [35] has been used conventionally for on-line NPLC-ESI-MS (Section 4.5). Alternatively, ambient ionization [36-39] including solvent assisted electrospray ionization (SAESI) [37, 38] and continuous flow-extractive desorption electrospray ionization (CF-EDESI) [36, 39] have been reported for on-line NPLC-ESI-MS (Section 4.7). Comparison of the performance of post column solvent addition with SAESI for on-line NPLC-ESI-MS indicates that both techniques provide comparable enhancement to signal to noise ratio (S/N) [38]. For direct infusion of azaarenes in hexane to ESI-MS studied in **Chapter 5**, post column solvent addition generates 10-fold high ionization efficiency than CF-EDESI [29]. However, the performance of post column solvent addition and CF-EDESI for on-line NPLC-ESI-MS has not been compared.

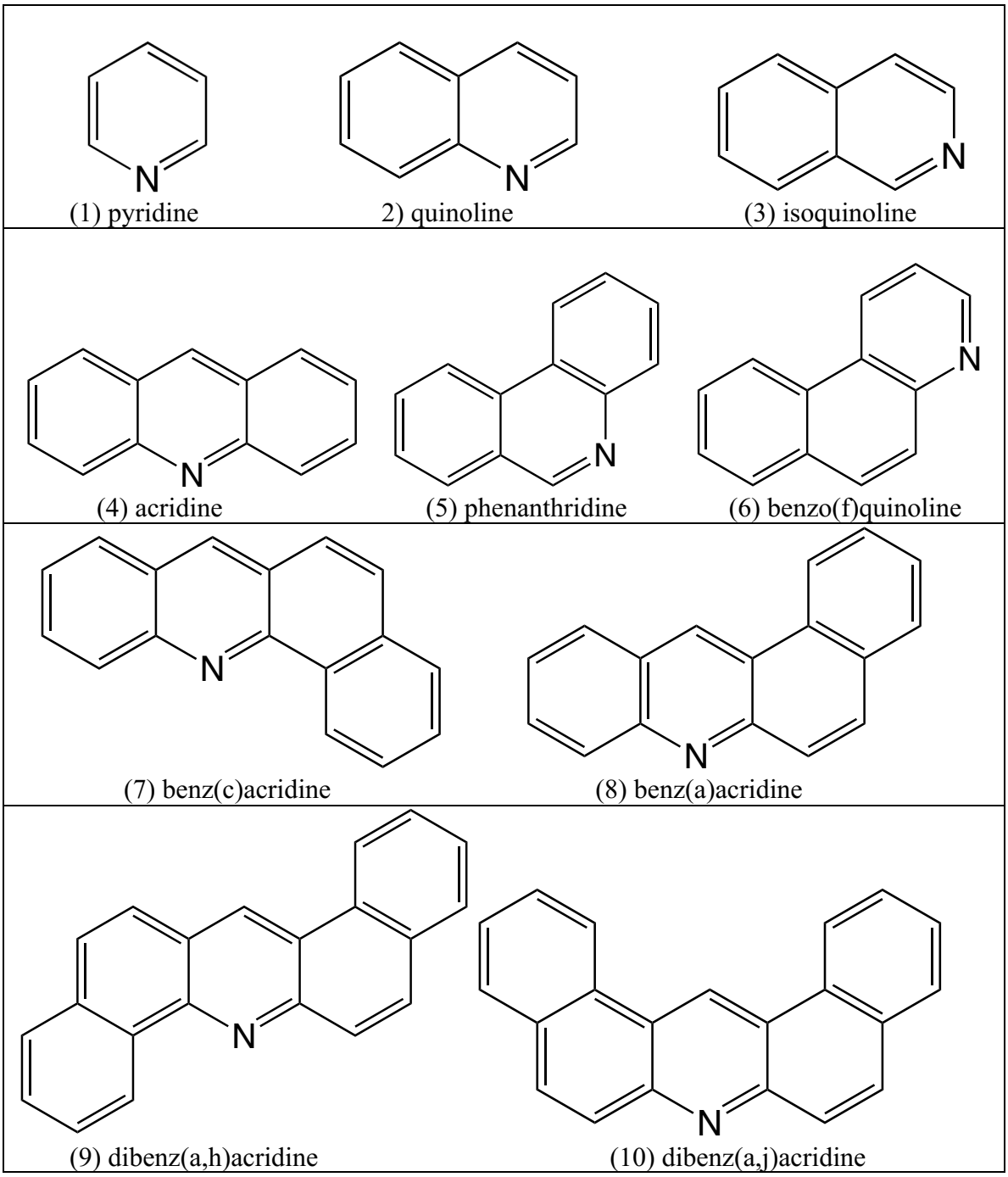
In this chapter, azaarenes are used as a model system to compare approaches of coupling NPLC to ESI-MS. The variety of azaarene isomers and their low abundance in the environment make their separation and characterization challenging. Nine azaarenes **(Table 6-1)** with 2-5 aromatic rings were selected for study based on their toxicity and presence in air particulates [1], water sediments [3] and fried meats [6, 40]. Both linear and bent azaarenes were selected. The chromatographic systems we studied are the hypercrosslinked polystyrene 5HGN phase and a 3-(2,4-dinitroanilino) propyl (DNAP) charge-transfer phase. Both 5HGN and DNAP can separate polycyclic aromatic hydrocarbons (PAHs) by ring numbers and shape [41]. Considering the similarities in structure for azaarenes and PAHs, the 5HGN and DNAP columns are of interest for the separation of azaarenes.

6.2 Experimental

6.2.1 Materials

HPLC grade hexane and isopropanol (IPA) were from Fisher Scientific (Fairlawn, NJ, USA). LC-MS grade methanol (MeOH) and toluene, acridine (99.8%), isoquinoline, phenanthridine, benzo(f)quinoline, benz(a)acridine, benz(c)acridine, and dibenz(a,j)acridine (97%) were from Sigma-Aldrich (St. Louis, MO, USA). Quinoline (99.8%) was from Acros Organics (NJ, USA). Dibenz(a,h)acridine was from K & K Laboratories (Carlsbad, CA, USA). Acetic acid (glacial, >99.7%, HOAc) was from Caledon (Georgetown, ON, Canada). Deionized water (>18.0 MΩ) was from a Barnstead E-pure system.

Table 6-1 Molecular structures of azaarenes



6.2.2 NPLC separation

Separation was performed on an Agilent HPLC (Santa Clara, CA, USA) consisting of a binary pump with online degasser (G1312A), an autosampler (G1329B), a thermostatted column compartment (G1316A). The eluent was directed to an Agilent G1314F UV absorbance detector or an Agilent 1100 series G1946A single quadrupole mass spectrometer for detection. Two columns were investigated; a 5HGN column (250 × 4.6 mm, 5 μm) which was packed in the lab [41]; and a DNAP column (50 × 4.6 mm, 5 μm, ES Industries, West Berlin, NJ, USA). The eluent was 1 mL/min IPA/hexane, unless otherwise indicated. Samples were prepared in the corresponding mobile phase and filtered through a 0.2 μm syringe-driven filter (PTFE, Millipore, Billerica, MA, USA) before injection of 2 μL. The detection wavelength was 254 nm. The sampling rate was 10 Hz with a response time of 1 s.

6.2.3 ESI-MS Conditions

The Agilent 1100 series G1946A single quadrupole mass spectrometer was run in positive mode with: mass range, m/z 50-800 Da; capillary voltage, 4 kV; fragmentor voltage, 160 V; cycle time: 1.04 s/cycle; Drying gas flow, 7 L/min; nebulizer pressure, 10 psi; and drying gas temperature, 350 °C. Based on Ref. [20], the protonated molecules $[M+H]^+$ monitored in SIM mode were: 130.1 (2-ring); 180.1 (3-ring); 230.1 (4-ring); and 280.1 (5-ring). Data was acquired with Agilent Chemstation software. The obtained chromatogram was transferred to MassHunter software, and automated integration was used to identify the retention time and peak area.

6.2.3.1 Post column solvent addition

A home built splitter was made manually using a zero dead volume T union, a long polyether ether ketone (PEEK) tube (493 mm, 0.17 mm I.D.) and a short PEEK tube (20 mm, 0.17 mm I.D.). A split ratio of 1:20 was achieved based on this configuration. That is, when 1 mL/min of IPA/hexane flow was introduced to the splitter, 50 μL/min of the flow would pass through the long PEEK tube. The effluent from the long PEEK tube was introduced into the horizontal arm of another T union (tubing O.D. 1/16 in, bore 0.25 mm, Valco Instruments, Houston, TX, USA) (**Fig. 5-1(a)**). A make-up solvent of 49/49/2 MeOH/H_2O/HOAc or 98/2 IPA/HOAc was added through the perpendicular arm of the T union at 50 μL/min using an Agilent G1312A pump. The sample and the make-up solvent mixed within 105 mm of 0.17 mm I.D. stainless steel tube (Agilent) prior to entering the MS inlet.

6.2.3.2 CF-EDESI

The CF-EDESI source was fabricated based on an Agilent conventional ESI source (G1948A) (**Fig. 5-1(b)**) [29]. Fifty μL/min of the IPA/hexane NPLC effluent was introduced into a hypodermic needle mounted on the CF-EDESI source through the homemade splitter described in Section 6.2.3.1 [29]. Fifty μL/min of an electrospray solvent of 49/49/2 MeOH/H_2O/HOAc or 98/2 IPA/HOAc was introduced to the nebulizer by an Agilent G1312A pump. The sample and the electrospray solvent mixed in the CF-EDESI chamber and the ions were detected by the MS.

6.2.3.3 Miscibility test

The miscibility of solvent mixtures was determined in test tubes by mixing the mobile phase (20/80 IPA/hexane) with the make-up solvent (49/49/2 MeOH/H_2O/HOAc

or 98/2 IPA/HOAc) at 1:1 (v/v). The results show that MeOH/H_2O/HOAc is not miscible with the NPLC mobile phase. In contrast, IPA/HOAc dissolves in NPLC mobile phase completely.

6.2.4 Calculations

The dead time was measured using the minor disturbance in the UV trace caused by injection of hexane [42]. The retention time of each peak was based on the peak maximum. The retention factors (*k*) were calculated with Microsoft Excel. The relative standard deviation for all retention factors was less than 0.5%. The column efficiency was calculated using the Foley-Dorsey equation [43] for tailing peaks.

6.3 Results and discussion

The hyphenation of LC with ESI-MS is a powerful technique for azaarenes analysis. But limited selectivity for azaarenes has been observed on conventional NPLC phases [30, 34]. Novel phases such as the hypercrosslinked polystyrene 5HGN and charge-transfer DNAP phases show strong retention for PAHs through π-π interaction and provide separation of PAHs based on aromatic ring numbers [41]. In this manuscript, 5HGN and DNAP are investigated for azaarenes separation under normal phase conditions. To achieve coupling of NPLC to ESI-MS, post column solvent addition [35] and CF-EDESI [36, 39] techniques are investigated. The performance of both techniques for on-line analysis of nine azaarenes standards are compared from the perspectives of the solvent compatibility, signal stability, and detection limits.

6.3.1 Separation of azaarenes

Azaarenes have been resolved from other nitrogen containing hydrocarbons on conventional NPLC phase [30]. Also, 3-5 rings azaarenes isomers have been resolved in thin layer chromatography on silica gel [34] with low efficiency. However, little isomer selectivity for azaarenes has been reported in NPLC. 5HGN and DNAP separate PAHs based on the ring number [41]. DNAP also resolves azaarenes from PAHs [44]. Thus, 5HGN and DNAP columns are investigated for the isomeric separation of azaarenes.

6.3.1.1 Separation of azaarenes on 5HGN

Table 6-1 shows the model azaarenes studied. Using 20/80 IPA/hexane pyridine, quinoline and acridine are eluted on 5HGN in order of ring number with retention factors (*k*) of 6.1~23.0 (**Fig. 6-1**).

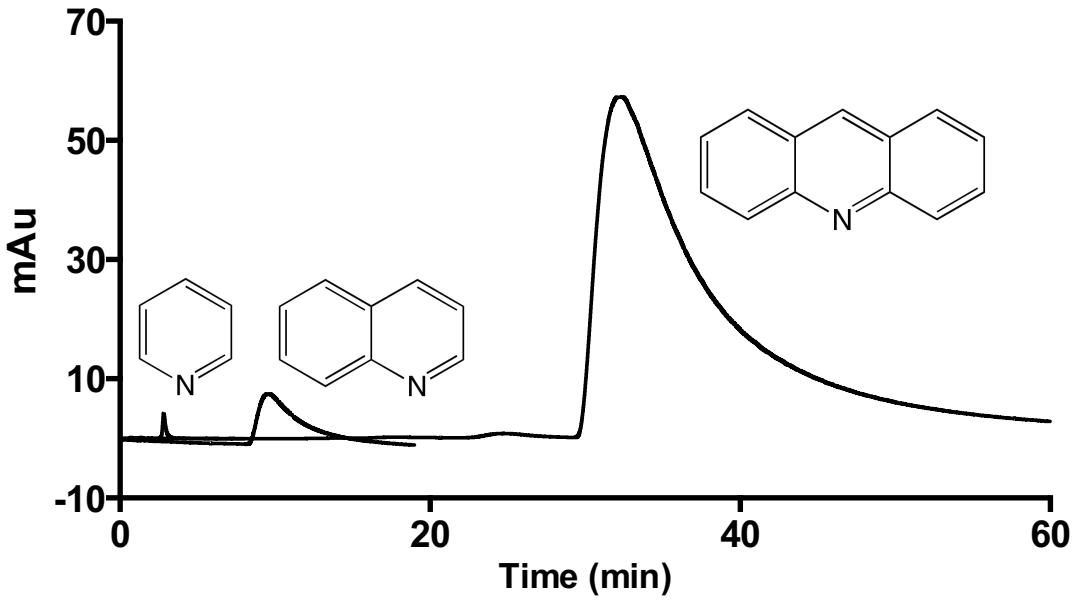


Fig. 6-1 Elution of 1-3 rings azaarenes on 5HGN column Conditions: mobile phase, 20/80 IPA/hexane; flow rate, 1.5 mL/min; temp, 50 °C; Detection wavelength, 254 nm.

The increasing retention behavior with number of rings is in agreement with retention behavior of PAHs on 5HGN in Ref. [41], but azaarenes are stronger retained than their corresponding PAHs. Larger azaarenes (such as dibenz(a,j)acridine) are irreversibly retained (k >50) on 5HGN irreversibly even when 50/50 IPA/hexane and 65 °C were used. Thus, 5HGN is not suitable for analysis of azaarenes with widely different ring numbers.

6.3.1.2 Separation of azaarenes on DNAP

Fig. 6-1 shows that the ln *k* for the nine azaarenes on DNAP decreases linearly with ln %IPA, as expected for an adsorption retention mechanism [45]. However, the mobile phase composition affects benz(c)acridine and dibenz(a,h)acridine less significantly than the other azaarenes. This is probably due to the steric hindrance around the nitrogen atom in benz(c)acridine and dibenz(a,h)acridine. This steric hindrance may reduced the hydrogen bonding with possible residual silanols and charge-transfer interaction with the DNAP phase of these two molecules [46]. Thus, **Fig. 6-1** also shows that the azaarenes behave as an irregular sample, where the relative retention of azaarenes changes with the mobile phase strength. For example, when 10%IPA is used, acridine and dibenz(a,h)acridine co-elute. Whereas, when %IPA is increased to 40%, isoquinoline and benz(c)acridine overlap. The irregular response to the mobile phase composition makes peak tracking challenging using a less specific detector such as UV absorbance,highlighting the necessity of using MS to identify the analytes.

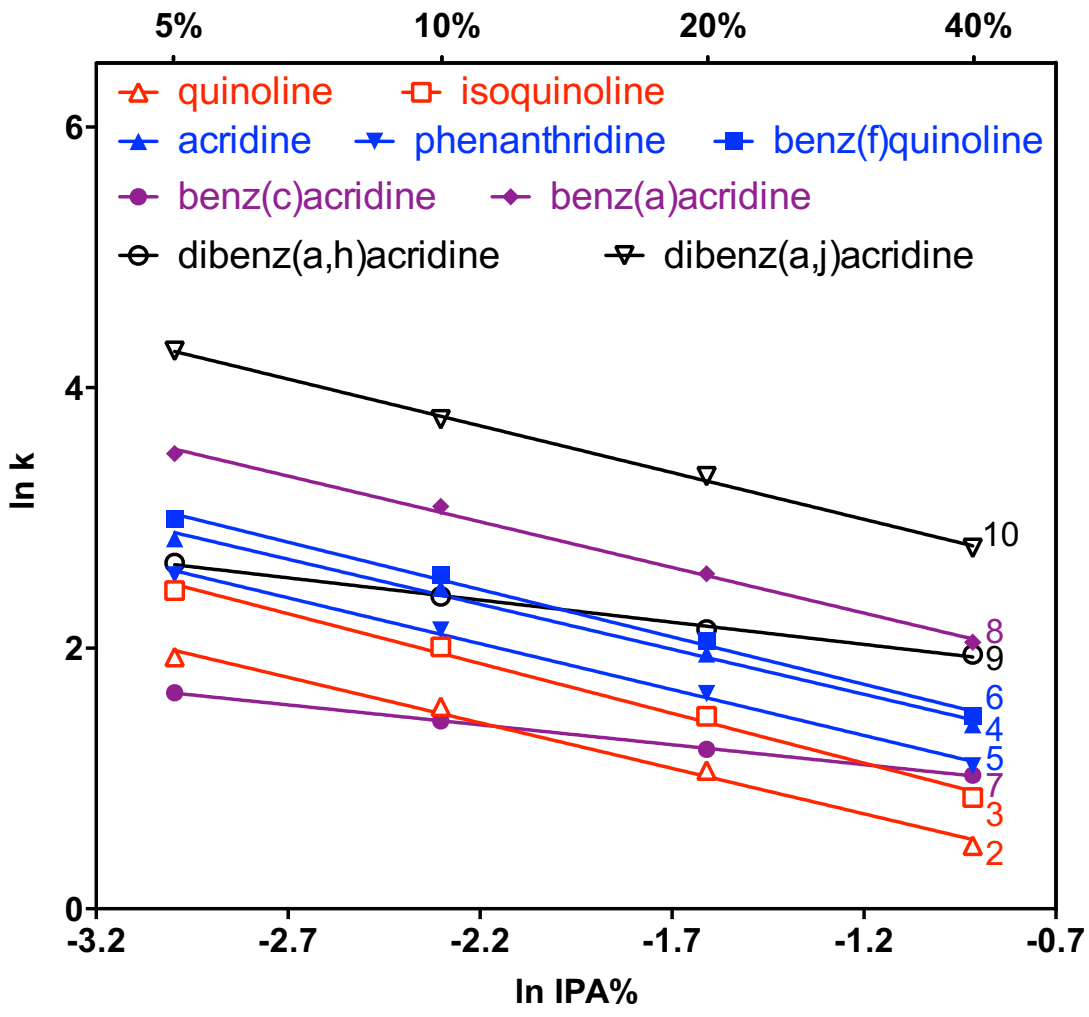


Fig. 6-2 ln *k vs.* ln %IPA on DNAP for azaarenes: 2) quinoline; 3) isoquinoline; 4) acridine; 5) phenanthridine; 6) benz(f)quinoline; 7) benz(c)acridine; 8) benz(a)acridine; 9) dibenz(a,h)acridine; and 10) dibenz(a,j)acridine. Red symbols indicate 2-ring azaarenes, blue are 3-ring, purple are 4-ring, and black are 5-ring.

Comparable optimal resolution for all nine azaarenes are achieved at 5% or 20% IPA on DNAP, but the elution time was reduced by half by using 20%IPA rather than 5% IPA. Thus 20/80 IPA/hexane was selected as the optimal mobile phase for the separation of azaarenes for all following work. **Fig. 6-3** shows the separation of nine azaarenes ranging from 2 to 5 rings (**Table 6-1**) on DNAP under 20/80 IPA/hexane ($2.9 < k < 27.7$). For azaarenes with same aromatic ring number, retention is stronger for azaarenes with a

more exposed nitrogen. For example, benz(a)acridine (peak 8) elutes much later than benz(c)acridine (peak 7), and dibenz(a,j)acridine (peak 10) elutes much later than dibenz(a,h)acridine (9). Similar isomer selectivity has been observed on silica gel in thin layer chromatography [32] and amino column [46].

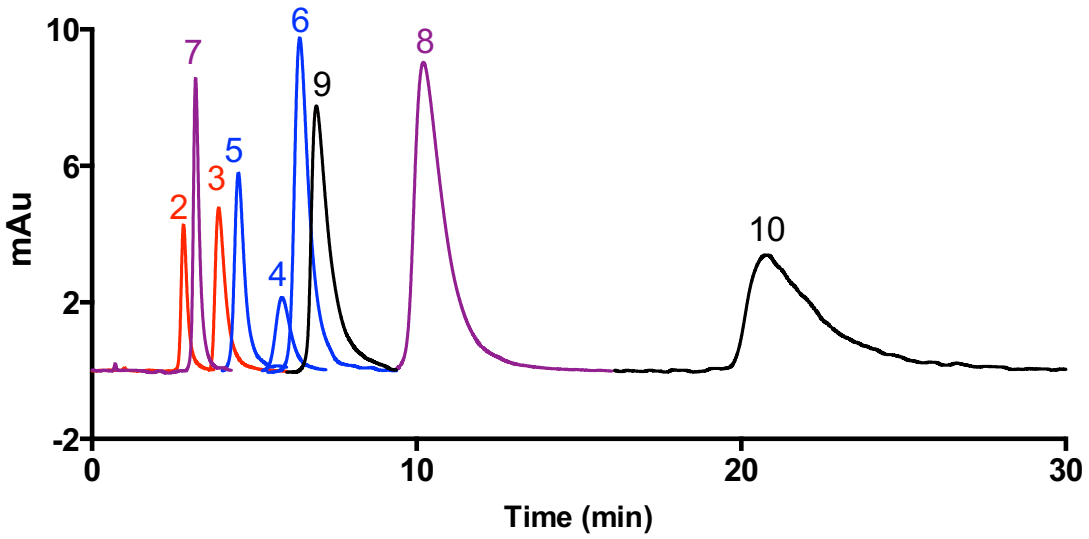


Fig. 6-3 Elution of azaarenes on DNAP with UV absorbance detection. Conditions: eluent, 1 mL/min 20/80 IPA/hexane; temp., 35°C; detection, 254 nm. Analytes: 2) quinoline (53 µg/mL); 3) isoquinoline (95 µg/mL); 4) acridine (5.6 µg/mL); 5) phenanthridine (9.1 µg/mL); 6) benzo(f)quinoline (59µg/mL); 7) benz(c)acridine (17 µg/mL); 8) benz(a)acridine (13 µg/mL); 9) dibenz(a,h)acridine (515 µg/mL); and 10) dibenz(a,j)acridine (501 µg/mL). Color code as in **Fig. 6-2**.

For linear azaarenes, the retention of azaarenes increases with increased aromatic ring number. This is similar with the retention behavior of PAHs on DNAP [41]. However, the azaarenes retain much stronger on DNAP than the PAHs with same aromatic ring number. For example, the retention factors of acridine and benzo(f)quinoline (3-ring azaarenes) are 6.8 and 8.0, while the retention factor for

anthracene (3-ring PAH) is 1.3. Only a 5-ring PAH benz(a)pyrene (k = 8.6) matches the retention of a 3-ring azaarenes on DNAP column.

The peak efficiency is comparable for azaarenes and PAHs that retained comparably but poorer for azaarenes than the PAHs with same aromatic ring number for a given concentration. For example, the reduced plate height h_p is 22 for acridine (3-ring azaarene) in **Fig. 6-3** and 20 for benz(a)pyrene (5-ring PAH). For equimolar anthracene (3-ring PAH), h_p is 6.0. Similar reduced plate heights (4 ~ 11) have been reported for PAHs using benzene peak on charge transfer phases [47, 48]. Thus, the DNAP column used in this chapter functions properly for small size PAHs (ring number <3). The efficiency for large size azaarenes (ring number ≥ 3) was low (H = 0.1-0.3 mm) and the peaks were tailed at all flow rates studied (**Fig. 6-4)**.

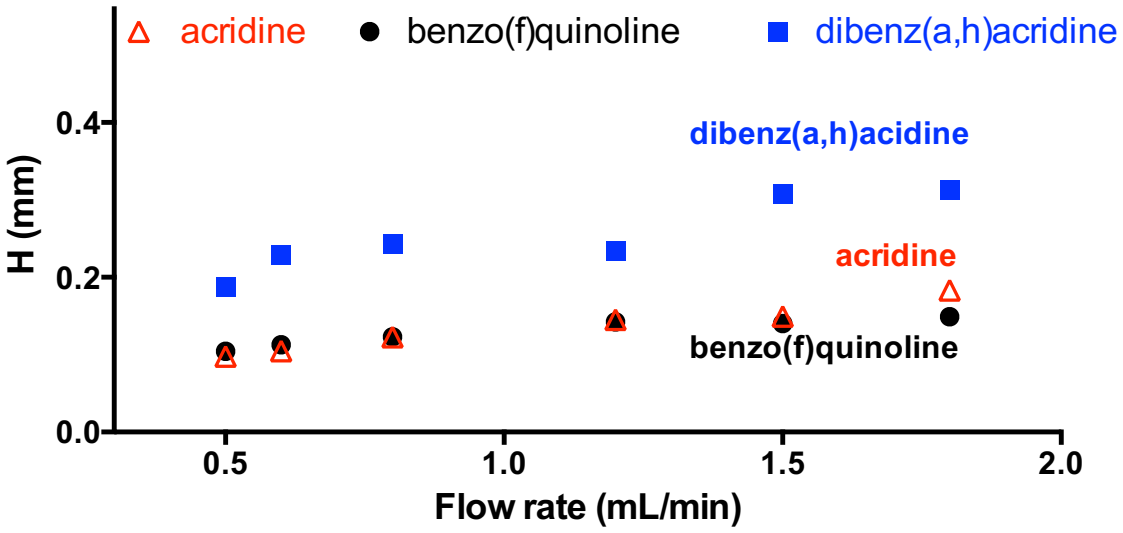


Fig. 6-4 Van Deemter curve for acridine (5.25 µg/mL), benzo(f)quinoline (58.8 µg/mL) and dibenz(a,h)acridine (515 µg/mL). Red triangles are acridine, black circles are benzo(f)quinoline, and blue squares are dibenz(a,h)acridine. Other conditions as in **Fig. 6-2**.

Overloading of the DNAP by the azaarenes is suspected to be the cause of the peak tailing. Injection of increasing concentrations of acridine, benzo(f)quinoline and dibenz(a,h)acridine resulted in an increase in peak tailing and a decrease in the retention time of the peak maximum (**Table 6-2** and **Fig. 6-5)**, which are typical features of convex isotherm overloading [49].

Table 6-2 Peak asymmetry factors A_s for acridine, benzo(f)quinoline and dibenz(a,h)acridine in **Fig. 6-5**

Acridine		**Benzo(f)quinoline**		**Dibenz(a,h)acridine**	
Conc. (mM)	**A_s**	**Conc. (mM)**	**A_s**	**Conc. (mM)**	**A_s**
0.29	2.00	1.6	3.00	0.74	3.83
0.12	1.83	0.66	2.71	0.37	3.31
6.0×10^{-2}	1.78	0.33	2.28	0.18	3.16
3.0×10^{-2}	1.69	0.16	2.26	0.09	1.73
6.0×10^{-3}	1.61	6.6×10^{-2}	2.26		

Conditions as in **Fig. 6-3.**

The column capacity may be estimated from the peaks in **Fig. 6-5** by [49, 50]:

$$capacity = \frac{C_{inj} \cdot V_{inj}}{L_f} \quad \text{Eq. 6-1}$$

where C_{inj} and V_{inj} are the concentration and volume of the sample injected. L_f is the loading factor, *i.e.*, the ratio of the sample injected to the sample that saturates the column.

The loading factor L_f can be measured using [49, 50]:

$$L_f = [1 - (\frac{t_r - t_0 - t_p}{t_{R,0} - t_0})^{\frac{1}{2}}]^2 \quad \text{Eq. 6-2}$$

where t_r is the retention time of the peak maximum; t_0 is the void time, which was determined based on the time of the first peak with minor disturbance method [42] in this work; t_p is the time during injection, which is very short and can be considered as 0; and

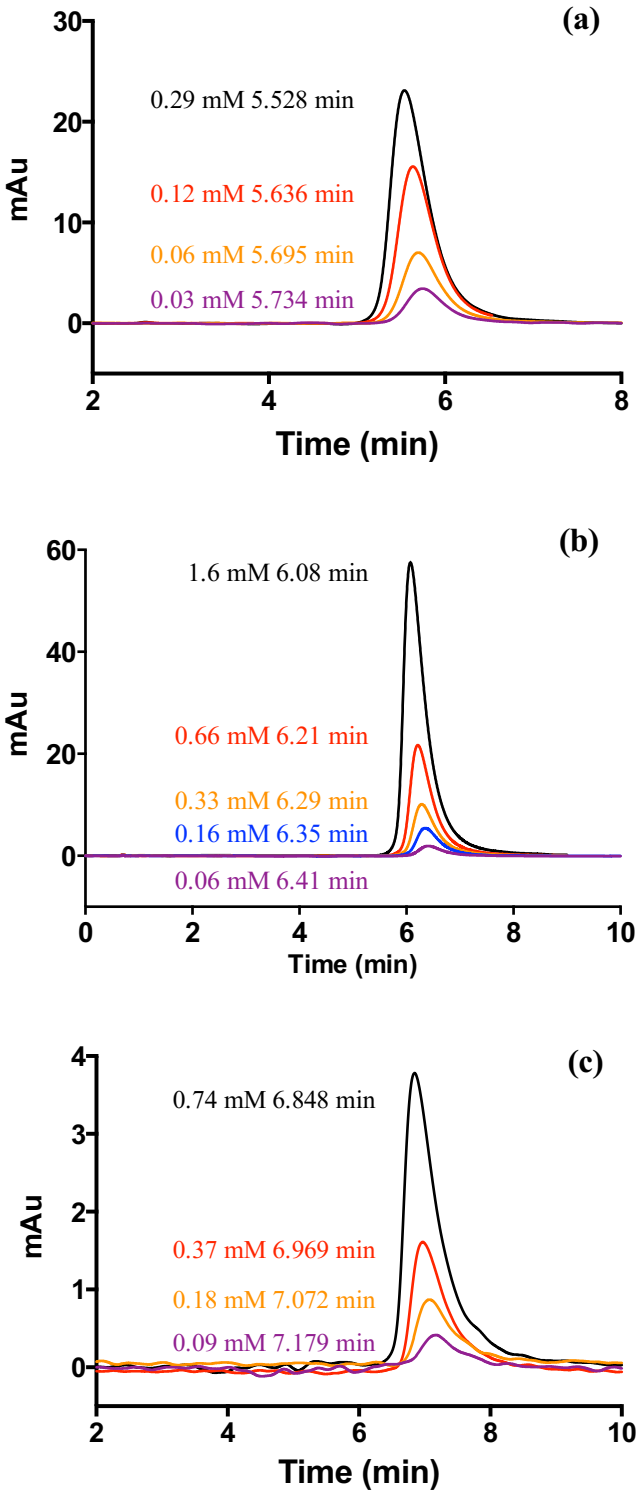


Fig. 6-5 Elution of benzo(f)quinoline at various concentration. (a) acridine (b) benzo(f)quinoline (c) dibenz(a,h)acridine. Conditions as in **Fig. 6-3**.

$t_{R,0}$ is the retention time of the analyte under analytical (trace) conditions [49, 50]. We initially estimated $t_{R,0}$ as the retention time of the lowest concentrated sample in **Fig. 6-5**. Then a series of $t_{R,0}$ were estimated ranging from the initial estimate to 7.5 min (near the back of the tailing peaks). For each $t_{R,0}$, L_f and capacity were calculated until a consistent capacity was obtained based on all of the concentrations studied for the analyte. Similar capacity measurements are performed with PAHs (*e.g.*, anthracene and benz(a)pyrene).

Table 6-3 Capacity of DNAP column for azaarenes and PAHs

Analyte	Retention factor	Injected (mg/10^5)	Column capacity (mg)
Acridine	6.8	10.5	0.14
		4.20	0.12
		2.10	0.13
		1.05	0.14
		average	0.13 ± 0.01
Benzo(f)quinoline	8.0	58.9	0.43
		23.6	0.34
		11.8	0.36
		2.36	0.40
		average	0.38 ± 0.04
Dibenz(a,h)acridine	9.8	11.5	0.07
		5.75	0.06
		2.88	0.06
		1.44	0.07
		average	0.07 ± 0.01
Anthracene	1.3	79.8	5.9
		7.98	6.6
		3.99	7.4
		0.80	6.0
		average	6.5 ± 0.7
Benz(a)pyrene	8.6	50.5	0.19
		25.2	0.17
		5.05	0.16
		2.50	0.20
		average	0.18 ± 0.02

Table 6-3 shows that the capacity of DNAP column for azaarenes is 0.07 to 0.38 mg. For azaarenes and PAHs that have similar retention factor, the capacity is comparable. However, the capacity is one order of magnitude higher for less retained PAHs (*i.e.*, anthracene) than the strongly retained analytes. This is understandable because for certain amount of sample loading, a higher proportion of analytes adsorb to adsorption sites on the DNAP surface for strongly retained analytes.

In **Fig. 6-3**, some compound pairs are overlapped and may cause ion suppression in ESI-MS. For example, benzo(f)quinoline (6) *vs.* dibenz(a,h)acridine (9) has the lowest resolution ($R_s \approx 0.6$), and so would seem to be the critical pair. However, the acridine (4) *vs.* benzo(f)quinoline (6) has only slightly greater resolution ($R_s \approx 0.7$), but are of greater concern as these compounds are isomers and so cannot be differentiated by ESI-MS. **Fig. 6-5** indicates that as the concentration of the azaarenes decreases, the efficiency improves, and so the resolution would be expected to improve. However, the limit of detection with UV absorbance for acridine and benzo(f)quinoline were estimated as 2.6 μM (4.6×10^2 ng/mL) and 4.7 μM respectively (8.6×10^2 ng/mL). These concentrations are of the same order of magnitude as the limit of detection achieved with LC-diode array detecting in Ref. [20]. At 3.9 μM acridine and 8.2 μM benzo(f)quinoline, the resolution between acridine and benzo(f)quinoline increased to 1.2. These concentrations are the upper limit used in the following studies of NPLC-ESI-MS.

6.3.2 NPLC-ESI-MS by post column solvent addition

Both post column solvent addition and CF-EDESI are able to enhance the ionization of azaarenes in non-ESI friendly solvents. Thus both are potential methods for coupling NPLC to ESI-MS [29, 39]. Post column solvent addition has been used widely

for on-line NPLC-ESI-MS (Section 4.5). However, there is no discussion about the factors, such as miscibility, that are important for make-up solvents. CF-EDESI is a novel technique that enabled analysis of progesterone and fatty acids in none-ESI friendly solvents [39]. CF-EDESI has also been reported to couple NPLC-ESI-MS for chiral analysis, but no chromatograms were shown [36]. The limits of detection for NPLC-CF-EDESI-MS were comparable to NPLC-UV detection [36]. However, there is no direct comparison between the performances of post column solvent addition and CF-EDESI for on-line NPLC-ESI-MS. These two methods are directly compared in the following sections for the NPLC-ESI-MS analysis of azaarenes.

6.3.2.1 Solvent compatibility in post column solvent addition

The NPLC conditions for the separation of azaarenes are stated in Section 6.3.1.2. About 50 μL/min NPLC eluent is directed to ESI-MS inlet through a homemade splitter. As discussed in **Chapter 4** there is no consistency in the literature regarding the type of make-up solvent used for NPLC-ESI-MS. **Chapter 5** showed that addition of a make-up solvent containing MeOH/H_2O/HOAc dramatically enhanced the electrospray ionization efficiency of azaarenes in hexane [29]. Solution containing MeOH/H_2O have also been used as make-up solvent in post column solvent addition for NPLC-ESI-MS analysis of lipids [51, 52] and drugs [53]. IPA is another common make-up solvent used in post column solvent addition [54, 55]. Thus, the performance of make-up solvents of MeOH/H_2O/HOAc and IPA/HOAc are compared. The miscibility test of the make-up solvents with NPLC mobile phase (IPA/hexane) in test tube shows that MeOH/H_2O/HOAc is not miscible with the NPLC mobile phase. On the contrary, IPA/HOAc dissolves in NPLC mobile phase completely.

Azaarenes cannot be directly detected in 20/80 IPA/hexane (Section 5.3.4) [29]. **Fig. 6-6** shows the total ion current (TIC) chromatograms for 47 μg/mL isoquinoline in 20/80 IPA/hexane with post column addition of either MeOH/H_2O/HOAc or IPA/HOAc. Addition of the immiscible MeOH/H_2O/HOAc (**Fig. 6-6a**) results in poor signal enhancement and a noisy trace. Noisy peaks have also been observed for other larger azaarenes studied (data not shown). The noise may be caused by the inappropriate mixing due to the immiscibility. In contrast, use of the miscible IPA/HOAc as make-up solvent (**Fig. 6-6b**) results in greater signal enhancement and less noise. The higher and more stable intensity generated with IPA/HOAc is consistent with direct infusion studies in **Chapter 5** [29]. These studies show the importance of the miscibility of the make-up solvent with the NPLC eluent.

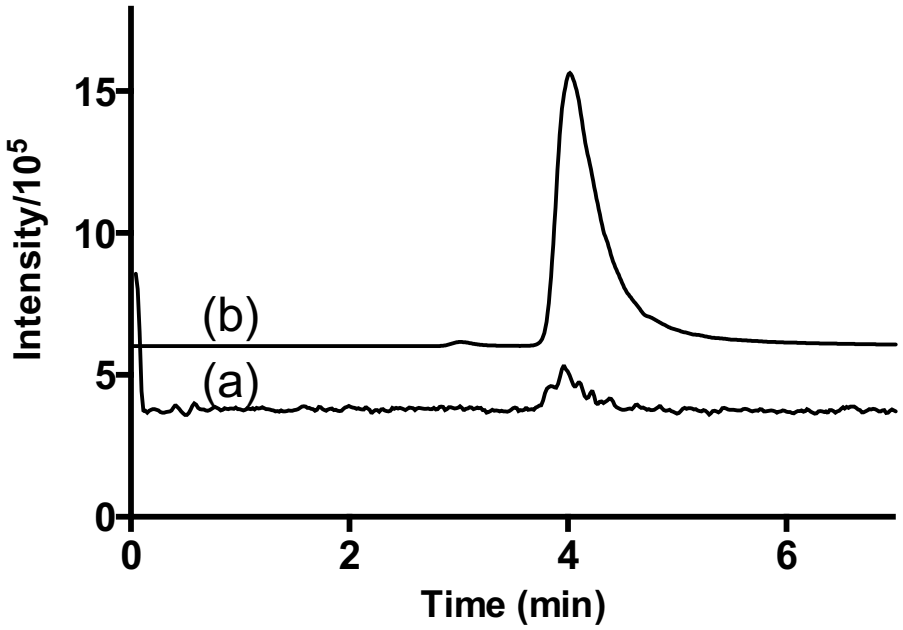


Fig. 6-6 Total ion current chromatogram of isoquinoline (47.2 μg/mL) under 20/80 IPA/hexane in NPLC-MS through post column solvent addition using: a) 49/49/2 MeOH/H_2O/HOAc as make-up solvent, and b) 98/2 IPA/HOA as make-up solvent. Conditions: temp., 35°C; NPLC effluent flow, 50 μL/min; make-up solvent flow, 50 μL/min.

6.3.2.2 Quantitative analysis

Fig. 6-7 is the TIC chromatogram for NPLC-ESI-MS analysis of nine azaarenes using IPA/HOAc as make-up solvent. The azaarene concentrations in **Fig. 6-7** are 10-

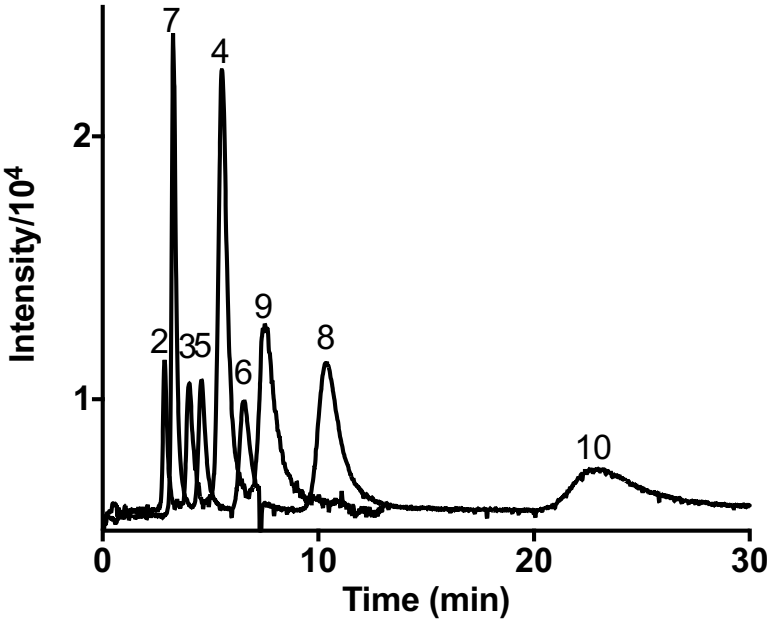


Fig. 6-7 TIC of nine azaarenes in NPLC-MS with IPA make-up solvent. Analytes: 2) quinoline (0.497 μg/mL); 3) isoquinoline (0.536 μg/mL); 4) acridine (0.630 μg/mL); 5) phenanthridine (0.228 μg/mL); 6) benzo(f)quinoline (0.25 μg/mL); 7) benz(c)acridine (0.208 μg/mL); 8) benz(a)acridine (0.246 μg/mL); 9) dibenz(a,h)acridine (0.590 μg/mL); and 10) dibenz(a,j)acridine (0.204 μg/mL). Other conditions and MS settings are in Section 6.2.3.

2000-times lower than in **Fig. 6-3**, which was monitored by UV absorbance. The peaks in **Fig. 6-7** appeared to be more symmetrical than in **Fig. 6-3**, and were independent over the concentration range of 0.05 ~ 2.5 μg/mL (3.5×10^{-4} ~ 1.4×10^{-2} mM). Thus column overload does not appear to be an issue at the lower azaarenes concentrations detectable by ESI-MS. However, the efficiencies in **Fig. 6-7** are only comparable to those in **Fig. 6-3**, presumably due to extra-column band broadening caused by the make-up solvent addition. The extra column band broadening did not deteriorate the separation

significantly. For example, the resolution between acridine(4) and benzo(f)quinoline(6) is 1.0 in **Fig. 6-7**, while optimal resolution for this compound pair is 1.2 in NPLC with UV absorbance detector.

Calibration curves for the nine azaarenes for peak area *vs.* concentration (0.05 ~ 2.5 μg/mL) were linear ($R^2 > 0.996$). LOD and LOQ are summarized in **Table 6-4**.

Table 6-5 compares he LOD achieved in this work to literature results [1, 20-23].

Table 6-4 Quantitative analysis results for selected azaarenes

Compounds	[a]LOD (ng/mL)	[b]LOQ (ng/mL)	R^2
(2) quinoline	1.2×10^2	$3.9*10^2$	0.998
(3) isoquinoline	$0.8*10^2$	$2.6*10^2$	1.00
(4) acridine	1.5	5.0	0.999
(5) phenanthridine	0.8	2.5	0.998
(6) benzo(f)quinoline	1.0	3.2	0.997
(7) benz(c)acridine	1.2	3.8	0.998
(8) benz(a)acridine	4.3	14.3	0.997
(9) dibenz(a,h)acridine	8.3	27.7	0.998
(10) dibenz(a,j)acridine	4.6	15.5	0.996

a. LOD is determined at S/N=3. b. LOQ is determined at S/N=10.

The LOD achieved with UV absorbance detection in Section 6.3.1.2 and in Ref. [20] is two orders of magnitude higher than achieved with NPLC-ESI-MS using IPA/HOAc post column solvent addition. However, compared to fluorescence [1], our LOD is 1-2 orders of magnitude higher.

Comparing to tandem MS analysis with various ionization methods, the LOD in this work generally appears to be one order of magnitude higher than that obtained in other methods for most azaarenes (**Table 6-5**). These differences are consistent with the

Table 6-5 Limit of detection comparison with literature (ng/mL)

Method	Quinoline	Acridine	Benz(a)acridine	Benz(c)acridine	Dibenz(a,j)acridine
LC-Fluorescence [1]	-	1.3	0.21	0.25	0.07
RPLC-DAD [20]	4.3×10^{2}	2.3×10^{2}	1.9×10^{2}	2.3×10^{2}	1.9×10^{2}
RPLC-ESI-MS/MS [20]	1.6	0.2	0.1	0.1	0.1
RPLC-APCI-MS/MS [23]	-	-	<0.28	<0.70	<0.12
RPLC-APCI-MS/MS [22]	-	-	0.8×10^{-2}	0.8×10^{-2}	0.8×10^{-2}
RPLC-APPI-MS/MS[21]	-	0.43	0.19	0.36	0.17
NPLC-UV (this work)	-	4.6×10^{2}	-	-	-
NPLC-ESI-MS (this work)	1.2×10^{2}	1.5	4.3	1.2	4.6

Note: LOD of NPLC-UV is estimated as (blank + 3×SD). LOD of NPLC-ESI-MS is based on S/N=3.

2-10 fold greater sensitivity expected for the triple quadrupole mass spectrometer used in ESI-MS/MS [56] and APPI-MS/MS [21], and the 10-100 higher signal intensity expected for the LCQ Fleet ion trap mass spectrometer used in APCI-MS/MS [22, 23] than for the single quadrupole spectrometer used in our method [57]. Thus, we believe the sensitivity achieved in this work is comparable to other MS/MS based methods. In addition, compare to the literature methods [1, 20-23] that separating the azaarenes by RPLC, our NPLC method provides better resolution of the isomers.

6.3.3 NPLC-ESI-MS by CF-EDESI

Chapter 5 demonstrated that post column solvent addition was superior to CF-EDESI for direct infusion of azaarenes in non-ESI friendly solvent [29]. However, there is no comparison between the performance of post column solvent addition and CF-EDESI for on-line NPLC-ESI-MS. Thus, the NPLC separation of azaarenes standards was coupled to ESI-MS by CF-EDESI to evaluate the performance. Conditions for the coupling is adapted from those in **Chapter 5** [29].

The eluent from NPLC was split using a homemade splitter and introduced into the ESI-MS front by a hypodermic needle as shown in **Fig. 5-1(b)**. No ionization was observed using IPA/HOAc as the electrospray solvent, because CF-EDESI is an extraction process which requires immiscibility between the electrospray solvent and NPLC eluent [29, 39]. In contrast, detectable ionization was observed (**Fig. 6-8**) when MeOH/H_2O/HOAc was introduced as electrospray solvent. However, the signal intensity for CF-EDESI is about 20 times lower than that from post column solvent addition. In **Fig. 6-8**, the two isomers are well separated, but the peaks are asymmetric and noisy.

Similarly noisy signals were observed for CF-EDESI for direct infusion of analytes in non-ESI friendly solvents (**Chapter 5**) [29].

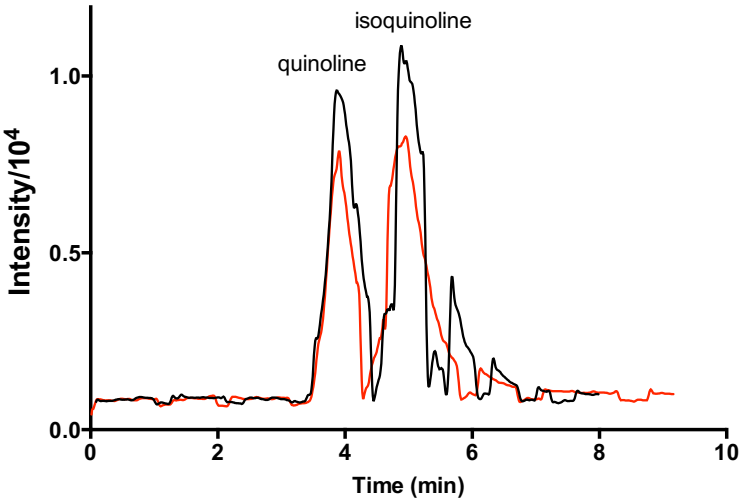


Fig. 6-8 Duplicate TIC chromatograms of quinoline and isoquinoline under 20/80 IPA/hexane in NPLC-ESI-MS by CF-EDESI. Analytes: quinoline (9.94 μg/mL), isoquinoline (10.7 μg/mL). Conditions: NPLC effluent flow, 50 μL/min; electrospray solvent, 49/49/2 MeOH/H_2O/HOAc; electrospray solvent flow, 50 μL/min. Other conditions and MS settings are in Section 6.2.3.

Quantification is possible, as the calibration curves (**Fig. 6-9**) are linear (R^2 >0.95), but RSD for replicate injections is 7% ~ 50% over the concentration range of 1.0-20 μg/mL.

Fig. 6-10 shows the NPLC-CF-EDESI-MS chromatogram for the larger azaarenes. Each peak is divided into several bumps without any consistent pattern. Thus it is challenging to identify the peak area for each peak and no quantitative information can be obtained. Whether the observation here is unique due to the system or not is not clear. Previous studies of CF-EDESI for online NPLC-ESI-MS reported peak areas but showed no chromatograms [36].

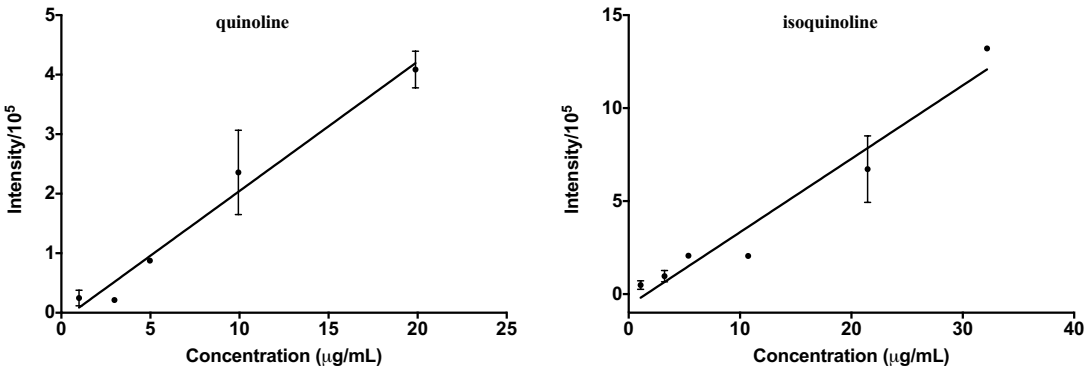


Fig. 6-9 Calibration curve for quinoline and isoquinoline in NPLC-ESI-MS by CF-EDESI. Conditions: DNAP column, mobile phase: 20/80 IPA/hexane; Conditions: NPLC effluent flow, 50 μL/min; electrospray solvent, 49/49/2 MeOH/H_2O/HOAc; electrospray solvent flow, 50 μL/min. Other conditions and MS settings are in Section 6.2.3. Where triplicate injections were performed, the error bars were indicated one standard deviation for duplicate measurements.

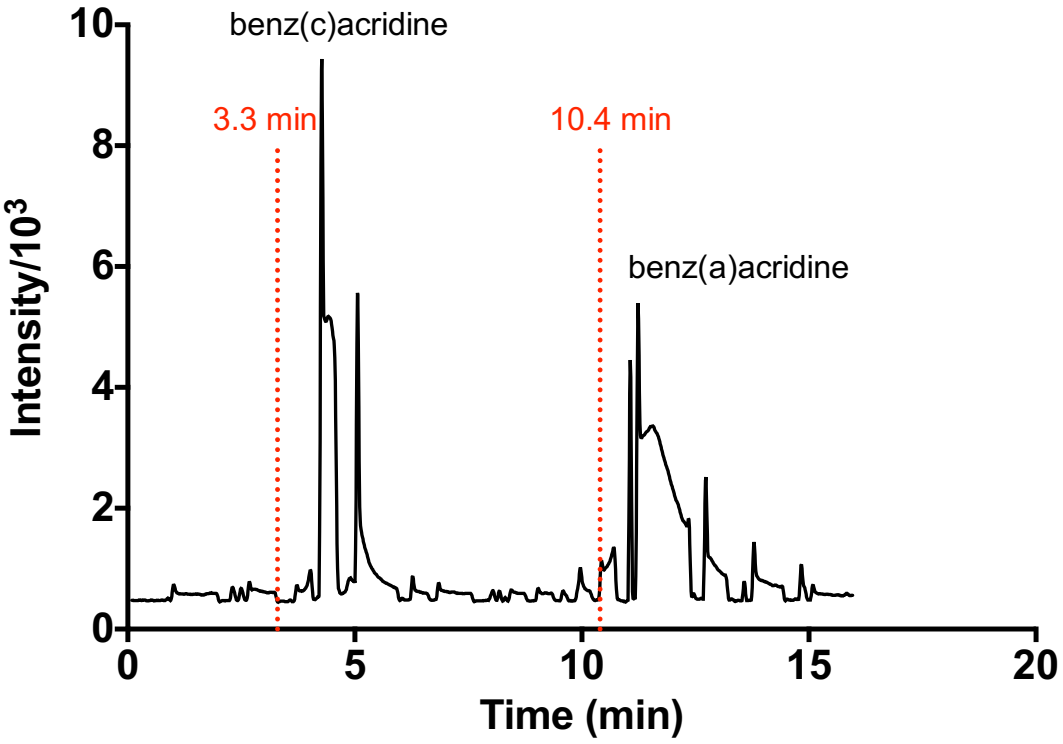


Fig. 6-10 TIC chromatograpm of benz(a)acridine (0.615 μg/mL) and benz(c)acridine (0.520 μg/mL) for NPLC-ESI-MS with CF-EDESI. The red dotted lines indicate the retention time for benz(c)acridine and benz(a)acridine in **Fig. 6-7**. Conditions as in **Fig. 6-8**.

In summary, CF-EDESI provides lower sensitivity than post column solvent addition, and only semi-quantitative analysis of small size azaarenes. Thus, post column solvent addition is the preferred method for coupling NPLC with ESI-MS.

6.4 Conclusions

The charge transfer DNAP column is able to resolve azaarenes with 2-5 aromatic rings under isocratic NPLC conditions within 30 min. However, the DNAP column is easy to be overloaded by azaarenes, particularly when a low sensitivity detector such as UV absorbance is used. Coupling ESI-MS with NPLC by post column solvent addition yields 100-fold greater sensitivity, achieving LOD of 0.8 – 120 ng/mL for azaarenes than that by NPLC-UV. The make-up solvent (*e.g.*, IPA in this work) must be miscible with the NPLC eluent to obtain stable and reproducible ionization. The coupling of NPLC-ESI-MS by CF-EDESI yielded only semi-quantitative information for small size azaarenes. Further investigation is needed for more accurate and reproducible quantitation of azaarenes with NPLC-CF-EDESI-MS.

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Chapter Seven: Conclusions and future work

7.1 Conclusions

This thesis has two main themes. Firstly, I explored the fundamentals and application of normal phase liquid chromatography (NPLC) for the separation of polycyclic aromatic hydrocarbons (PAHs) or polycyclic aromatic nitrogen hydrocarbons (PANHs). Secondly, I coupled NPLC to electrospray ionization mass spectrometry (ESI-MS) through various strategies for PANHs characterization.

7.1.1 NPLC fundamentals and application

I studied five methods for determining void volume estimation on silica, DNAP and RingSep columns under NPLC conditions in **Chapter 2**. The approaches studied had all previously been proposed in the literature, but had never been compared or validated. The methods for V_0 determination included: pycnometry; the minor disturbance method based on injection of weak solvent; the tracer pulse method; the hold-up volume based on unretained compounds; and the accessible volume based on Martin's rule and its descendants. Pycnometry provides a theoretically maximum value for the total void volume and should be performed at least once for each new column. However, pycnometry does not reflect the volume of adsorbed strong solvent on the stationary phase, and so only yields an accurate void volume for weaker mobile phase conditions. 1,3,5-Tri-t-butyl benzene (TTBB) results in hold-up volumes that are convenient measures of the void volume for all eluent conditions on charge-transfer columns (DNAP and RingSep), but is weakly retained under weak eluent conditions on silica. Injection of the weak mobile phase component (hexane) may be used to determine void volume, but care must be exercised to select the appropriate disturbance feature. If this technique is

used, the particular baseline disturbance feature used to determine V_0 should be stated. Accessible volumes, that are determined using a homologous series, are always biased low, and are not recommended as a measure of the void volume.

Based on the accurate measurement of void volume, **Chapter 3** illustrated the retentivity, selectivity and thermodynamic behavior of PAHs under high performance NPLC conditions. The size, shape, and planarity selectivity for PAH standards on charge-transfer (DNAP column) and hypercrosslinked polystyrene (HC-Tol and 5HGN columns) phases are different under NPLC. The HC-Tol column behaves like a conventional NPLC column with low retention of PAHs. Retention of PAHs on the DNAP and 5HGN are strong and increase with the number of aromatic rings. The main retention mechanism is through π-π interaction and dipole-induced dipole interaction. The thermodynamics indicate that the retention mechanism of PAHs does not change over the temperature range 20-60 °C. In addition, on either DNAP or 5HGN column, both linear and bent PAHs are retained through the same mechanism. But DNAP possesses smaller π-π interaction and higher planarity selectivity than 5HGN for PAHs. This is suggestive that DNAP interacts with PAHs through a disordered phase arrangement, while 5HGN behaves as an ordered adsorption phase.

7.1.2 Coupling of NPLC-ESI-MS

The latter half of this thesis focused on coupling NPLC to ESI-MS for azaarenes analysis. The strategies for hyphenating NPLC to ESI-MS and the general application have been summarized in **Chapter 4**. **Chapter 5** investigated the ionization efficiency of ESI-MS for analytes in ESI-incompatible solvents. Both addition of a make-up solvent through a T union and electrospray solvent through continuous flow extractive desorption

electrospray ionization (CF-EDESI) significantly enhanced ionization of analytes in non-ESI friendly solvents. Under optimized conditions, make-up solvent addition provides higher ionization efficiency than CF-EDESI. Neither the make-up solvent addition nor the CF-EDESI eliminates ionization suppression of nitrogen-containing compounds caused by compounds of the same chemical class. To reduce the ion suppression effect and resolve azaarenes isomers in ESI-MS, a pre-separation is required.

Chapter 6 explored the separation of azaarenes on 5HGN and DNAP columns under normal phase conditions. A simple and fast isocratic separation of nine azaarenes on DNAP was developed. Azaarenes were characterized by coupling NPLC to ESI-MS through post column solvent addition and CF-EDESI. NPLC-ESI-MS coupled through post column solvent addition of isopropanol provides detection limits for individual PANH in the range of 0.8 – 120 ng/mL, which is enough for general environmental monitoring. However, NPLC-ESI-MS coupled through CF-EDESI is only applicable for semi-quantitative analysis of small size azaarenes. Comparing the two techniques for online NPLC-ESI-MS indicates that the post column solvent addition is superior due to its simplicity, stable and high signal intensity, and high solvent compatibility for azaarenes analysis.

In summary, this thesis addresses the challenges of using NPLC with high resolution ESI-MS for petroleum characterization. Both charge-transfer DNAP and hypercrosslinked polystyrene 5HGN are effective in separating PAHs based on aromatic ring number. The charge-transfer DNAP also provides better planarity selectivity for PAHs and isomer selectivity for PANHs. The NPLC separation has been coupled to ESI-MS for PANHs monitoring through post-column solvent addition and ambient ionization

techniques such as CF-EDESI. Post-column solvent addition is more effective for on-line NPLC-ESI-MS due to its simplicity and higher ionization efficiency. Thus, post column solvent addition should be chosen for on-line NPLC-ESI-MS for petroleum analysis.

7.2 Future work

7.2.1 NPLC with ESI-MS for polystyrene characterization

In **Chapter 2**, polystyrene oligomers are not used as a homologous series for the determination of accessible volume due to the peak splitting or weak retention on silica. However, **Fig. 7-1** polystyrene was separated into distinct multiple peaks based on the degree of polymerization on the charge transfer DNAP column under NPLC conditions.

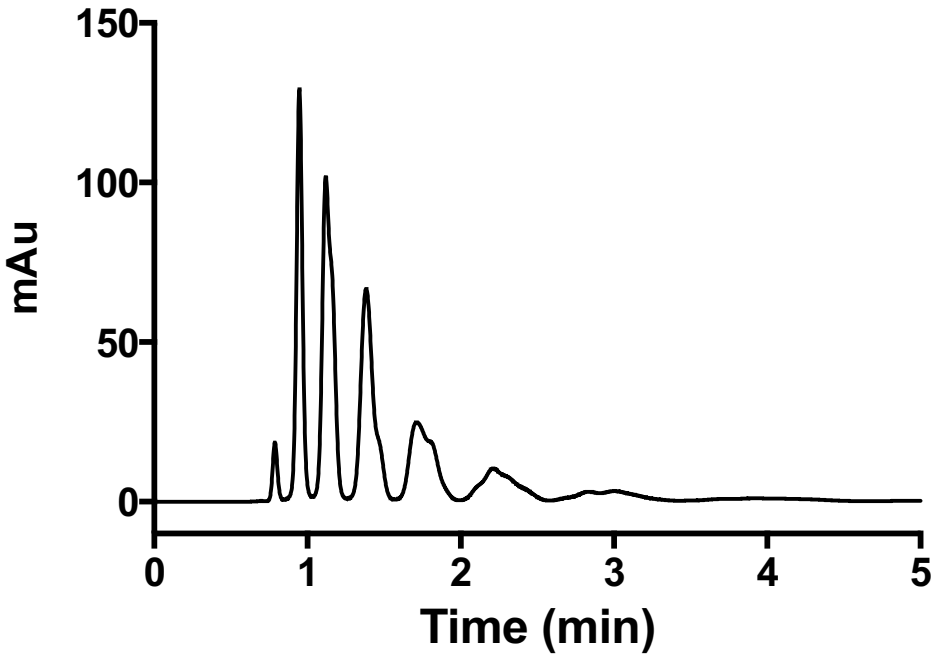


Fig. 7-1 Separation of polystyrene on DNAP column. Conditions: Mobile phase, 5%DCM/95%hexane; flow rate, 1 mL/min; Temp., 35 °C; detection wavelength: 254 nm; Sample, polystyrene A300 (MW = 453 g/mol; TOSOH, Tokyo, Japan) in 5%DCM/95%hexane at concentration of 10.6 mg/mL.

The resolution achieved is much better than that on gel permeation chromatography (GPC) which is the most commonly used method for polymer molecular weight distribution (MWD) determination [1-3]. GPC coupled with ESI-MS through post column solvent addition [1] or solvent assist ESI-MS [4] has been used to characterize polymers. Different salts including sodium iodide [1] and silver(I) tetrafluoroborate [4] have been added after GPC separation to assist ionization of polystyrene in ESI-MS. However, previous GPC separations lack resolution of the polystyrene oligomers. Also, comparison between the performances of different salts addition is absent. In addition, only one ambient ionization technique (solvent assist ESI-MS) was used in the literature, and has not been compared with post column solvent addition.

Thus, based on the separation developed in this thesis for polystyrene and the techniques developed for coupling normal phase liquid chromatography with ESI-MS, systematic studies should be performed for determination of chemical composition and MWDs for polystyrene in NPLC-ESI-MS.

7.2.2 Retention of PAHs and PANHs on charge transfer and quasi normal phases

As demonstrated in **Chapters 3** and **6**, within class separation of PAHs (non-polar) or PANHs (polar) can be achieved on both DNAP and 5HGN column. However, The PAHs are more weakly retained than PANHs on DNAP column. Second, the peak efficiency on DNAP column for anthracene (3-ring PAH) is 3 times as that for equimolar acridine (3-ringPANH) and benz(a)pyrene (5-ring PAH). The DNAP column is easier to overload by more retained compounds. The capacity of DNAP column for PANHs is 0.07~ 0.38 mg, for anthracene is 6.5 mg, and for benz(a)pyrene is 0.18 mg as extrapolated in **Chapter 6**.

The different capacity along different types of solutes on same column has been observed in RPLC [5]. Greater than 250 fold smaller capacities have been observed for charged solutes than for uncharged solutes at low pH on either type A silica based or highly inert silica based C_{18} columns [5]. Similar varied capacity has also been reported on pure polymeric phases in RPLC [6]. The rapid overloading for uncharged solutes is explained by existence of two kinds of sites (neither site is silanols) on the stationary phase or by the ionic repulsion between the ionized solutes [5].

In normal phase mode separation on DNAP column, ionization is not expected. Thus, the hypothesis of ionic repulsion can be excluded. But whether the changed capacity for PAHs and PANHs on DNAP is caused by silanols still needs exploration. On the other hand, the hypercrosslinked polystyrene 5HGN column is a pure polymeric phase without any silica support. Nonetheless, similar retention of PAHs and PANHs was achieved on 5HGN as on DNAP. Thus, the cause of the capacity difference for PAHs and PANHs on DNAP should be studied.

7.2.3 LC-ESI-MS through post column solvent addition for petroleum characterization

Improved ionization for compounds in non-ESI friendly solvent was achieved by addition of ESI compatible make-up solvents in **Chapter 5**. On-line LC-ESI-MS for PANHs has been demonstrated in **Chapter 6**. A single quadrupole mass spectrometer has been used in quantitative analysis of nine PANHs that are separated on DNAP column. However, such a mass spectrometer provides limited resolution for petroleum sample which contains tens of thousands of nitrogen containing compounds and other molecules. Currently, Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS) offers the highest mass resolution for petroleum. But most applications of FT-ICR-MS in

petroleum are performed by direct infusion of sample without separation [7-10]. More molecular components in petroleum can be identified by FT-ICR-MS if there is a pre-separation [11]. However, the limited prior work on LC-ESI-MS for petroleum analysis, petroleum is either characterized by off-line LC with ESI-MS [12, 13] or on-line LC-ESI-MS with separation of petroleum using a conventional normal stationary phase [14]. The off-line modes are time consuming and have the potential for extra contaminants [15] and sample loss. The on-line modes improve the speed of petroleum analysis but the conventional normal stationary phases only provides limited selectivity for petroleum [14]. Thus, it is necessary to explore the petroleum separation on other stationary phases and couple the separation to ESI-MS in the on-line mode.

The charge-transfer phase DNAP is able to separate PAHs from nitrogen containing compounds [16] with a DCM/hexane mobile phase of. Non-basic and basic nitrogen containing compounds are also separated into groups on DNAP [16]. In this thesis, nine basic nitrogen containing compounds are resolved individually on DNAP with mobile phase of IPA/hexane (**Chapter 5**). Thus, DNAP is a potential candidate for petroleum separation prior to ESI-FT-ICR MS characterization.

Specifically, fractions of petroleum can be collected after separation on DNAP column as described in Ref. [12]. Instead of reconstituting the fractions in ESI compatible solvents, the fractions can be directed introduced into ESI nebulizer with the addition of make-up solvents as stated in **Chapter 5** and **6**. The resulting molecular composition information can be compared to that with sample re-constitution to explore if more components in petroleum can be identified or if there is fewer contaminants peak.

Alternatively, the eluent from separation of petroleum on DNAP can be directly connected to an ESI-FT-ICR-MS. This on-line mode would require the balance between the time available for the analytes to elute from the NPLC column and the scanning time required for a sensitive detection in MS [17]. Total ion chromatograms and spectra will be collected. Molecular compositions can be extracted from the resulted spectra and compared to results from off-line mode NPLC-ESI-MS.

7.2.4 Data analysis for high resolution mass spectrometry for petroleum analysis

There are several important factors to consider for application of on-line LC-ESI-FT-ICR-MS to petroleum analysis. First, correct interpretation of the mass spectrum assures accurate reflection of the quantitative information and distribution of certain compound types in petroleum, which lead to better management of refining processes [18]. The petroleum composition should be easily transferable to straightforward graphs such as van Krevelen plots (H/C *vs.* O/C), abundance *vs.* compound types, abundance *vs*. double bond equivalence (DBE), and double bond equivalence *vs.* carbon numbers. However, there is limited discussion in the literature of the data analysis for FT-ICR-MS spectrum. The only commercial software that converts mass spectrometric data into molecular compositions is Composer (Sierra Analytics, USA) [9, 12, 14]. Composer also generates various graphs, such as abundance *vs.* compound types, abundance *vs*. double bond equivalence (DBE), and double bond equivalence *vs.* carbon numbers. However, Composer requires a high level of operator expertise to yield the correct interpretation of the spectrum. The Composer software also has many system bugs, limited graph options and a high licence cost (~$10,000/year) [19]. Alternatively, data analysis has also been accomplished on raw data files by home-written macros with Microsoft Excel [10, 13, 20,

21]. With the home-written macros, various plots including contour plots, bar graphs, and dot plots are displayed [10, 13, 20, 21].

Flexibility for displaying data and low cost data analysis would be obtained if we can write our own files for interpretation of mass spectrum. We can combine reliable commercial software and home-written file to achieve fast and accurate analysis of mass spectrum. *Dataanalysis* software (Bruker Daltonics, Bremen, Germany) usually comes at low price with the FT-ICR-MS instrument. *Dataanalysis* is able to directly generate van Krevelen diagrams, which plots the hydrogen:carbon (H/C) *vs.* oxygen:carbon (O/C) and is widely used for displaying composition of petroleum [22-24]. *Dataanalysis* software is also able to calibrate the spectrum with external standard peak list, extract peak list, and generate molecular formula for each peak. However, the generated peak list cannot be easily transposed into the various graphs used in petroleomics. To compensate this, we can import the generated peak list with molecular composition to Microsoft Excel. Micros that is able to sort peak lists based on the number of carbon atoms, the number of heteroatoms, or the number of DBE needs to be written. Then, the abundance of each group needs to be plotted in various formats as aforementioned and compared with the results generated using the commercial Composer software.

7.2.5 The effect of additives for petroleum analogs ionization in ESI-MS

A second factor that affects the identification of petroleum by ESI-MS is the additives (*e.g*. acetic acid in **Chapter 5** and **6**) used [25]. Commonly, the best ionization is achieved for basic compounds by using acidic additives and for acidic compounds by using basic additives [26]. Occasionally basic compounds are protonated with basic additives [27] and acidic compounds are deprotonated with acid additives [28]. This is

called “wrong-way-round ionization”, which probably ionizes analytes through gas phase proton transfer or onset of discharge [29]. In positive mode, wrong-way-round ionization in ESI-MS has been observed for PANHs. **Fig. 7-2** is the preliminary results and shows

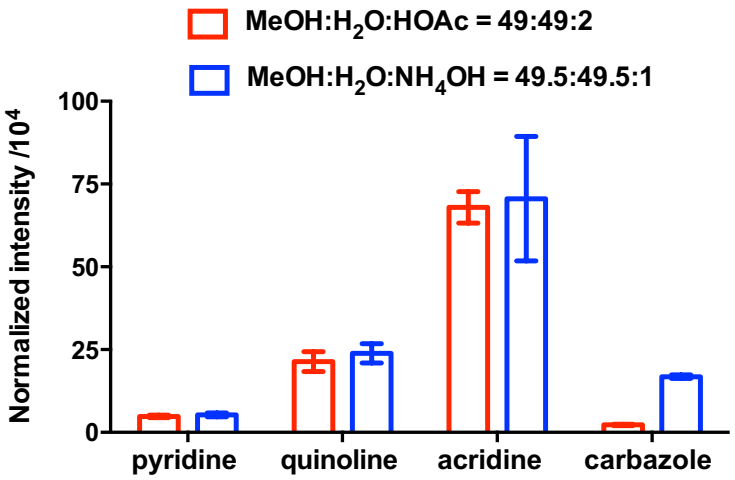


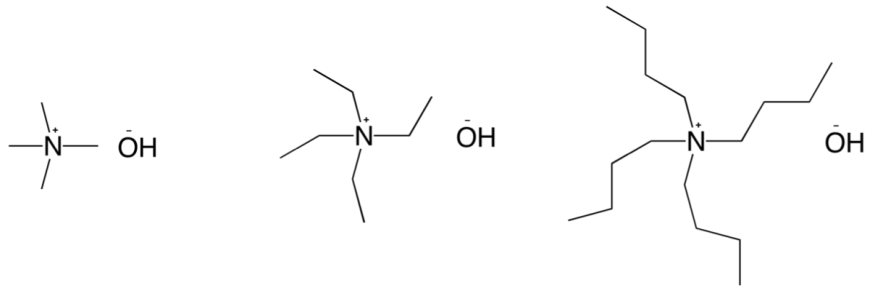
Fig. 7-2 Ionization efficiency with acidic or basic additives for basic and non-basic nitrogen containing compounds. Conditions: pyridine, quinoline and acridine were prepared in hexane at the concentration of 10 μM; carbazole was prepared in hexane at the concentration of 100 μM. All the experiments were performed on an Agilent 1100 series G1946A single quadrupole mass spectrometer. Positive mode for pyridine, quinoline and acridine; negative mode for carbazole. The addition of make-up solvent containing either acidic or basic additives was achieved through a T union as described in Chapter 5. Sample flow, 50 μL/min. Make-up solvent flow, 50 μL/min.

that comparable ionization of nitrogen containing compounds is achieved with either acidic (acetic acid) or basic (ammonium hydroxide) additive. However, whether this wrong-way-round ionization is true for the various compounds in petroleum is unknown. Also, in previous literature formic acid has been used more widely than acetic acid as the additive in ESI-MS for petroleum analysis [13, 19, 30]. There is no comparison between different acidic additives for ionization of petroleum. It is necessary to optimize the

ionization by using different acids in the solvents to explore whether more molecular composition can be identified from petroleum.

In negative ESI-MS, **Fig. 7-2** shows that wrong-way-round ionization exists for carbazole but higher ionization is observed with basic additives (ammonium hydroxide). This probably explains why ammonium hydroxide is the most widely used additive for petroleum characterization under negative mode [10, 12, 13, 31-33].

However, recent exploration indicates that the addition of *tetra*-methyl ammonium hydroxide (TMAOH) allows the expression of more peaks for a petroleum sample compared to the ammonium ion hydroxide (NH_4OH) in negative ESI-MS [34]. Also, the addition of TMAOH favors the ionization of hydrocarbons and less acidic heteroatom containing compounds [34]. But there has not been a study on why such enhancement takes place. Also, the literature lacks investigation of the effect of other additives on ionization efficiency of petroleum type compounds. To explore these issues, I did some preliminary studies with a second year undergraduate student in 2014. Ammonium type basic additives (**Fig. 7-3**) were investigated in negative ESI-MS for the ionization of different types of analytes (**Table 7-1**) with various heteroatoms contents.



(a) (b) (c)

Fig. 7-3 (a) Tetramethylammonium hydroxide (TMAOH) (b) Tetraethylammonium hydroxide (TEAOH) (c) Tetrabutylammonium hydroxide (TBAOH)

Table 7-1 Chemical structures of standards

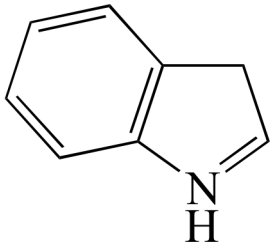
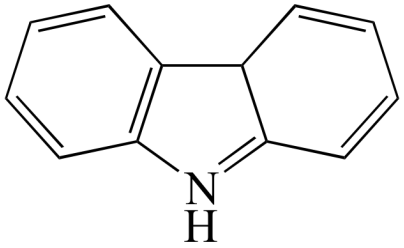
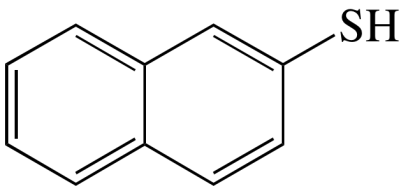
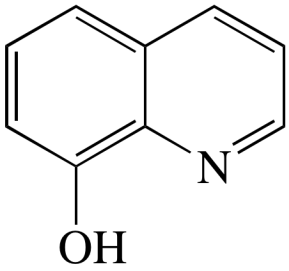
 Indole	 Carbazole
 2-Naphthalenethiol	 8-Quinolinol

Fig. 7-4 shows that enhanced ionization is obtained for all the standards with TMAOH comparing to with ammonium hydroxide, which agrees with the literature [34]. The addition of TEAOH leads to a three-fold increase in signal intensity over TMAOH for most of the standards. More compounds might be identified with TEAOH for petroleum characterization in ESI-FT-ICR-MS.

Future research can be focused on the effect of additives on the ionization of petroleum in ESI-FT-ICR-MS in both positive and negative mode. The types and concentration of additives should be changed to see whether more coverage of compounds in petroleum could be achieved.

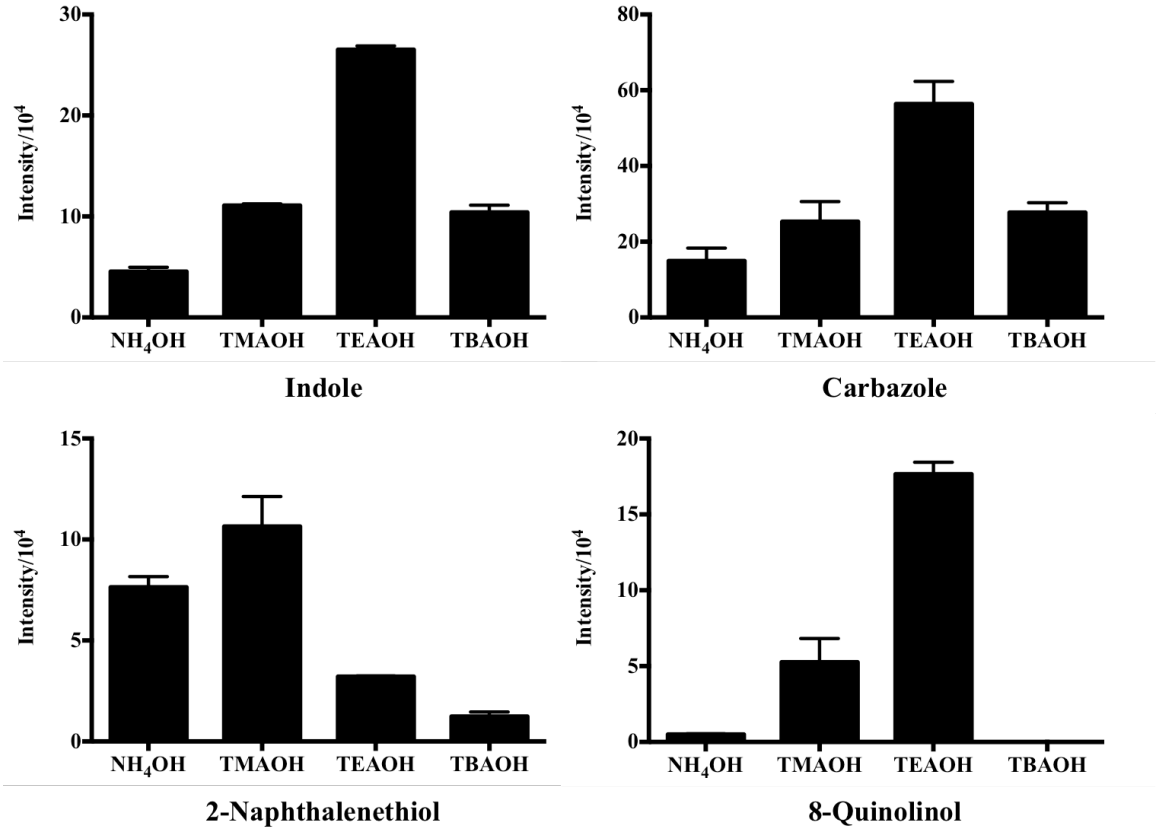


Fig. 7-4 Effects of various basic additives on ionization of standards. Conditions: the standards were prepared in a methanol/toluene = 3:1 (v/v) matrix to form a 10 mM solution with 0.01% (v/v) of basic additive. Each sample was directly infused into ESI quadrupole mass spectrometer at a flow rate of 10 μL/min in negative mode. Triplicate was performed for each sample.

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APPENDIX A. Spreadsheet for measuring accessible volume based on Martin's rule and the extrapolation method

A.1 Martin's rule

- Obtain retention time from Agilent Chemstation for each peak (*e.g.* retention time for benzene, naphthalene, anthracene and tetracene on DNAP column with 5/95 DCM/hexane)
- Calculate retention volume using: Retention volume = Retention time × flow rate (1 mL/min)
- Input retention volume into spreadsheet for the triplicate measurements and calculate the average
- Subtract the extra column volume as determined in Section 2.3.3.3 from the retention volume

	A	B	C	D	E	F	G	H
1	**Calculate retention volume based on retention time obtained from Chemstation**							
2		Retention volume (mL)						Extra column volume (mL)
3		replicate 1	replicate 2	replicate 3	average	Retention volumn – extra column volumn		0.107
4	benzene	0.852	0.854	0.853	0.853	0.746		
5	naphthalene	1.486	1.495	1.494	1.492	1.385		
6	anthracene	3.949	3.983	3.987	3.973	3.866		
7	tetracene	14.388	14.525	14.535	14.483	14.376		
8								
9	Formulas							
10	E4 = AVERAGE (B4:D4)							
11	G4 = E4 - $H$3							

- Calculate log *k* based on measured retention volume (extra column volume subtracted) and a series estimates of accessible volume (full range from 0.3 – 0.6 mL has been studied but only range from 0.532 to 0.544 mL is shown in the spreadsheet below)
- Perform linear regression of log *k vs*. number of rings (Column J in the spreadsheet) using "CORREL" function

	A	B	C	D	E	F	G	H	I	J
1	**Calculate log k based on estimated accessible volume**									
2			0.544	0.542	0.540	0.538	0.536	0.534	0.532	Number of rings
3	benzene	0.746	-0.430	-0.424	-0.419	-0.413	-0.407	-0.401	-0.395	1
4	naphthalene	1.385	0.189	0.192	0.194	0.197	0.200	0.202	0.205	2
5	anthracene	3.866	0.786	0.788	0.790	0.791	0.793	0.795	0.797	3
6	tetracene	14.376	1.405	1.407	1.409	1.410	1.412	1.414	1.415	4
7										
8	**Calculate correlation coefficient of log k vs. ring number**									
9		R	0.99997258	0.99997402	0.99997411	0.9999729	0.9999704	0.99996665	0.9999617	
10		R^2	0.99994516	0.99994804	0.99994823	0.99994579	0.9999408	0.99993331	0.9999234	
11										
12	Formulas									
13	Cells from C2 to I2 are the estimated values of accessible volume at an interval of 0.002									
14	Cells from B3 to B6 are the values of (retention volume - extra column volume)									
15	C3 =log ((B3-$C$2)/$C$2)									
16	C9 = CORREL ($J$3:$J$6,C3:C6)									

- Identify 0.540 mL as the accessible volume that generates the best linearity for the line of log *k vs*. number of rings and the R^2 is 0.99995

- The uncertainty of the accessible volume was calculated based on the uncertainty of the intercept from the linear regression

	A	B	C	D	E	F	G	H
1	**Calculate uncertainty**							
2								
3	Highlight cells B10:C14	number of rings	log k		Retention time	log k	log k + S_b	log k – S_b
4	Type "= LINEST (C4:C7,B4:B7,1,1)	1	-0.419		0.746	-0.419	-0.410	-0.427
5	For PC, press CTRL + SHIFT + ENTER	2	0.194		1.385	0.194	0.203	0.186
6	For Mac, press COMMAND + RETURN	3	0.790		3.866	0.790	0.798	0.781
7		4	1.409		14.376	1.409	1.417	1.400
8								
9		LINEST output:				k	0.389	0.374
10	slope, m	0.60766355	-1.025683907	intercept, b			1.595	1.534
11	S_m	0.003091698	0.008466962	S_b			6.281	6.040
12	R^2	0.99994823	0.006913246				26.126	25.127
13		38630.68188	2					
14		1.846274949	9.55859E-05			accessible volume	0.537	0.543
15							0.534	0.546
16							0.531	0.549
17							0.530	0.550
18						average	0.533	0.547
19						uncertainty for the accessible volume	-0.007	0.007
20								
21	Formulas							
22	Cells E14 to E17 are values of retention time (extra column volume subtracted)							
23	Cells F4 to F7 are values for log k wih accessible volume at 0.540							
24	Cells G4 to G19 are values for log k with the consideration of the upper uncertainty S_b							
25	G4 = F4+$C$11							
26	Cells H4 to H19 are values for log k with the consideration of the lower uncertainty S_b							
27	H4 = F4–$C$11							
28	Cells G9 to H12 are values calculated based on cells G4 to H7							
29	G10 = 10^G4							
30	Cells G14 to H17 are values calculated based on the k values in cells G9 to H12							
31	G14 = E4/(1+G9)							
32	G18 = AVERAGE (G14:G18)							
33	G19 = G18–0.540							
34	H19 = H18+0.540							

A.2 Extrapolation method

- Obtain the retention volume (extra column volume subtracted) for benzene, naphthalene, anthracene and tetracene as described in **Section A.1**. Input the data in cells B3 to B6 as shown in the spreadsheet below
- Calculate ($V_n - V_{n-1}$) based on V_n values
- Use LINEST function to perform linear regression. The intercept is the accessible volume and s_b is the uncertainty

	A	B	C	D	E	F	G
1		Retention volumn (V_n)	$V_n - V_{n-1}$				
2				slope, m	1.31397262	0.57244149	intercept, b
3	benzene	0.746		s_m	0.00581756	0.03633347	s_b
4	naphthalene	1.385	0.639	R^2	0.9999804	0.04318161	
5	anthracene	3.866	2.481		51014.1092	1	
6	tetracene	14.376	10.510		95.1235319	0.00186465	
7							
8	Formulas						
9	Highlight cells E3:F7						
10	Type "= LINEST (B4:B6,C4:C6,1,1)						
11	For PC, press CTRL + SHIFT + ENTER						
12	For Mac, press COMMAND + RETURN						
13	C4 = B4-B3						

APPENDIX B. Correlation of the retention of PAHs to molecular properties

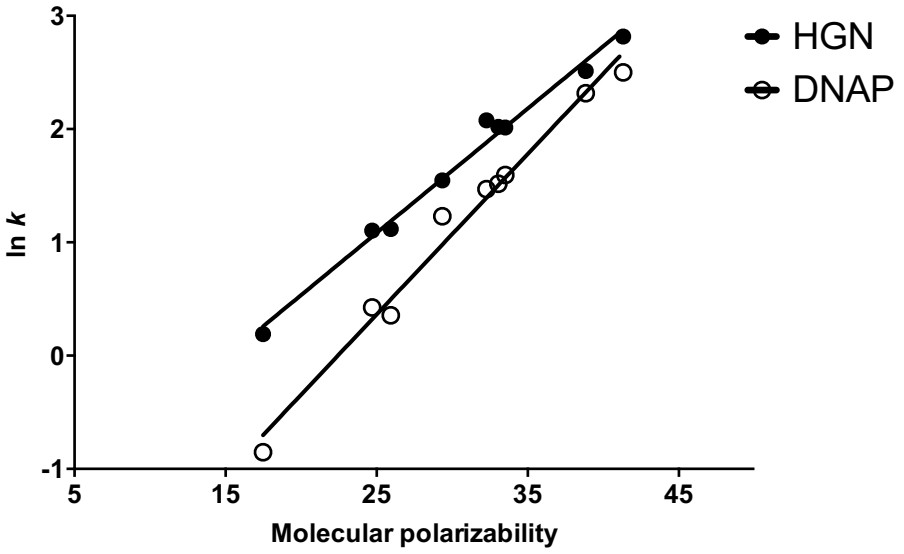

Fig. B-1 Linear regression for *ln k* vs. molecular polarizability

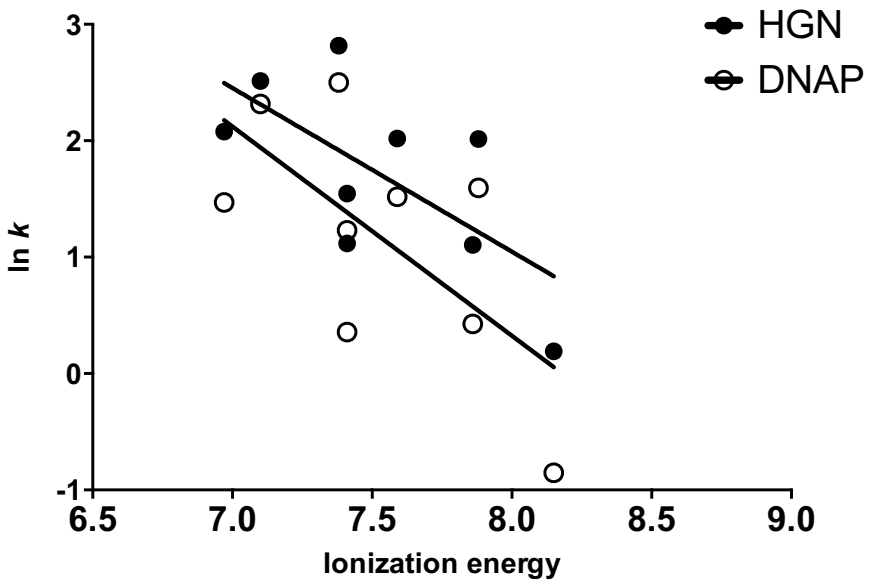

Fig. B-2 Linear regression for *ln k* vs. ionization energy

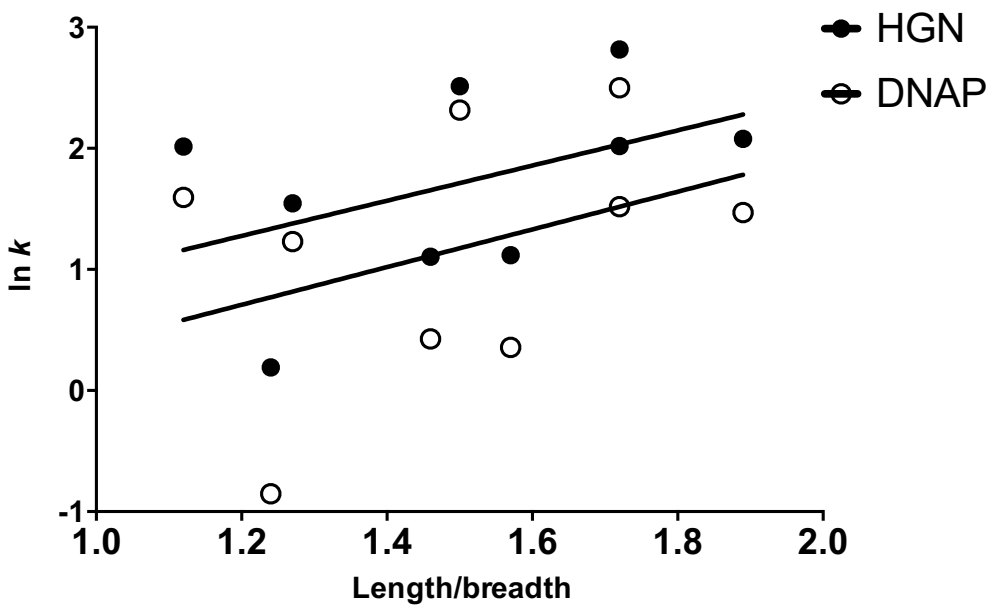

Fig. B-3 Linear regression for *ln k* vs. molecular polarizability

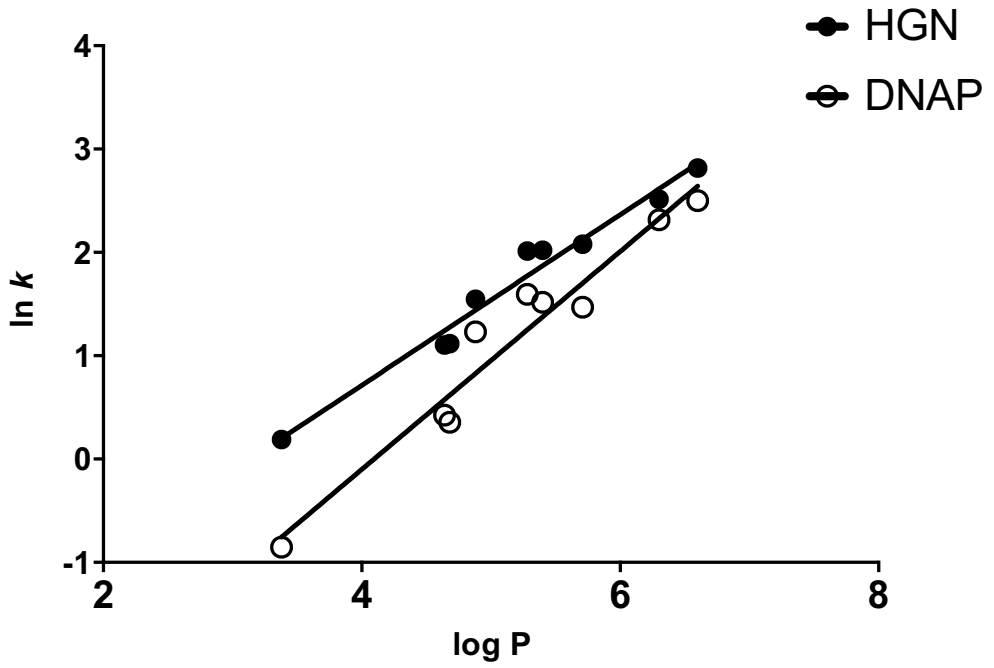

Fig. B-4 Linear regression for *ln k* vs. molecular polarizability