EXPLORING FLEXIBLE CONFIGURATIONS OF A CUSTOM MULTIPATH LC –MS FOR SMALL MOLECULES AND PROTEIN ANALYSIS

by

DANANJAYA KALU APPULAGE

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Abstract

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Dananjaya Kalu Appulage, PhD

The University of Texas at Arlington, 2017

Supervising Professor: Kevin A. Schug

There are diseases where both small molecules and proteins are found as disease indicators. In the usual analysis of small molecules, proteins are precipitated out during the sample preparation. In the small molecule quantitation, triple quadrupole mass spectrometers (QqQ- MS) are commonly used even in the clinical lab settings. Even though QqQ is giving only unit resolution, it is highly sensitive and specific specially when it is used in the multiple reaction monitoring (MRM) mode. In the liquid chromatography method development, automation of column and mobile phase screening could save significant amount of operators' time. The use of Shimadzu Nexera Method Scouting Solutions software to automate the column and mobile phase screening was evaluated. The selectivity differences among four reversed phase stationary phases, C18, biphenyl, polar embedded (IBD) and pentafluorophenyl propyl (PFPP) were studied using model analyte set which includes analytes from different classes depending on the functionality and physicochemical properties. It has shown that biphenyl phase shows similar selectivity behavior as C18 when acetonitrile is used as the organic mobile phase. Further, use of Type C silica based phases, (cholesterol, bidentate C18, diol and diamond hydride) for the small molecule separation under both reversed-phase as well as aqueous normal phase were explored. Four analytes, fentanyl, hydrocodone, hydromorphone and matrine showed dual mode retention with the all four phases studied. In our lab, new system was developed to analyze both small molecules and proteins simultaneously using QqQ- MS. Model analyte set which includes over 20 small molecules and five proteins, ubiquitin, myoglobin, cytochrome c, lactalbumin, lysozyme were used to prove that the system is working. Both small molecules and proteins were detected simultaneously on Shimadzu LCMS 8050 QqQ-MS using MRM mode. Restricted access media (RAM) columns were used to trap small molecules

while excluding proteins in the developed multi-channel system. Among the four RAM columns explored, Shim pack MAYI C8 column gave overall the best exclusion percentage for the proteins explored. Further, two-dimensional liquid chromatography mass spectrometry (2DLC-MS) system was developed in our lab on the same LCMS 8050. Six proteins, ubiquitin, myoglobin, cytochrome c, β-casein, carbonic anhydrase, β-lactoglobulin A were used with same mobile phase in both dimension to prove that the system is working. Different reversed-phase stationary phase columns as well as several mobile phases were evaluated to use as the first-dimension column while keeping Restek ARC 18 column and mobile phases with 0.1% formic acid and 0.05% trifluoroacetic acid for the second-dimension. It has shown that the system is working but could not achieved desired separation with RP x RP combination. Therefore, exploring the use of other modes of liquid chromatography such as hydrophilic interaction chromatography (HIC) column for the first dimension is currently undergoing.

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Chapter 1

Introduction

1.1 Development of high performance liquid chromatography

Chromatography, a Greek word meaning “color writing” was first introduced by M. Tswett in his attempt to separate plant pigments in 1900 [1]. The basic principle of chromatography is separation of a mixture of components based on differences in the physicochemical properties of the individual constituents. There are two main phases, the stationary phase and the mobile phase that are used for the separation. The mobile phase carries analytes from an injected mixture across the stationary phase, where differential interactions by the analytes with the phases ideally induces their separation from one another. The two most common chromatographic techniques are gas chromatography and liquid chromatography; the former uses a gaseous stationary phase, whereas the latter features a liquid mobile phase.

This thesis work is mainly focused on liquid chromatography, where the stationary phase is a solid and the mobile phase is a liquid. At the early stages of liquid chromatography, a column was filled with a stationary phase and the liquid mobile phase was passed through the column under gravity. With improvements in the technology, high performance liquid chromatography (HPLC) was introduced, where the mobile phase is forced to pass through the column under high pressure was introduced; this vastly improved separation speed and efficiency. As of today, HPLC is the most common chromatographic technique used in the clinical and pharmaceutical fields. With the fast growth of its applications, analysis is currently being performed under even higher pressures, (9000-20 000 psi) and this concept has been termed ultra-high-performance liquid chromatography (UHPLC) [2]. There are various modes of liquid chromatographic techniques as well as various stationary phases, which have been developed and used in the analysis of various classes of molecules.

1.2 Different types of stationary phases used in the liquid chromatography

Early on, liquid chromatography separations were performed using liquid-liquid partitioning or liquid-solid adsorption. But there were problems with these techniques, including holding the liquid stationary phase in place, and thus, applications were limited. With the development of bonded phases, where a specific functional unit is bonded to a solid support, such as silica, many of the earlier problems were

eliminated. Early forms of bonded phases were mainly used for reversed-phase liquid chromatography (RPLC). With the refinement of bonding chemistry, various ligands with a different functionality such as alkyl ligands, aromatic ligands, and other polar-functionalized units were bonded to silica supports and helped to grow applications of RPLC rapidly.

The most common reversed phase stationary phase is C18, and it induces interactions with the analytes through the hydrophobic effect (also, via London dispersion and van der Waals forces). Other alkyl chain ligands, such as C30, C8, C4, and C2, have been used for the RP stationary phases. Depending on the hydrophobicity of the analyte molecules, one can select the length of the ligand in the stationary phase to be used to provide the desired retention for a particular application. As an improvement for alkyl ligands, polar groups such as amides have been incorporated to induce hydrogen bonding or polar interactions with analytes, in addition to retention based on the hydrophobic effect. As the applications have grown, more and more stationary phases have been developed. Aromatic ligands such as biphenyl, fluorophenyl, phenyl hexyl, hexyl biphenyl, where pi-pi, cation-pi, and induced polarization interactions are the main interaction with the analytes. Further, polar stationary phases such as cyano- and amino-bonded phases have been used for the analysis of more polar analytes.

In order to have better stability of the stationary phases, ligands were bonded via more than one bond to the solid support to obtain polymeric phases. These phases enable the use of harsher mobile phase conditions (wider variation of pH) compared to the traditional phases. In addition to the more common phases, there are special stationary phases have been developed with unique selectivities for specialized applications. Stationary phases such as cholesterol shows properties such as shape recognition due to its structural properties, and these phases can be used in different modes of liquid chromatography in the analysis of multiple classes of analytes at the same time. Figure 1-1 shows a selection of different types of stationary phases that are commercially available [3-6].

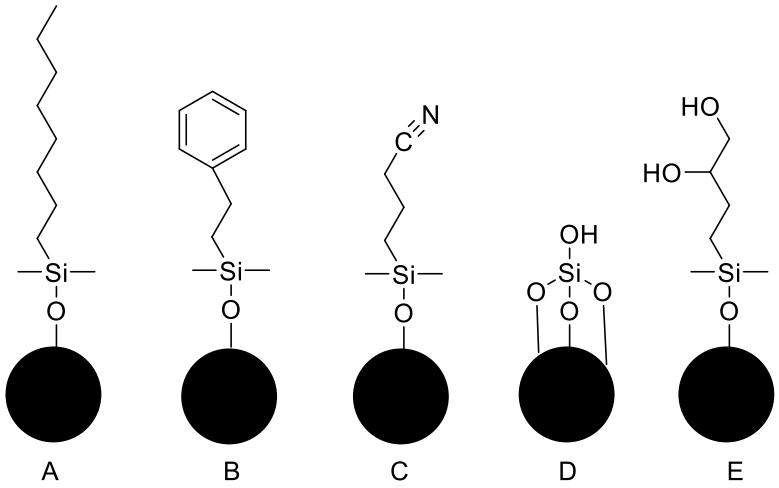

Figure 1-1. A selection of different types of stationary phases commercially available: A) alkyl-; B) phenyl-; C) cyano-; D) silica-; and E) diol-bonded phases are shown as a few examples.

1.3 Different modes of liquid chromatography

Reversed phase liquid chromatography is the most commonly used mode of liquid chromatography, but when the complexity of the samples and the variability in the types of analytes that are needed to be analyzed increases, additional modes have been introduced. Normal phase (NP), aqueous normal phase (ANP), hydrophilic interaction liquid chromatography (HILIC), hydrophobic interaction chromatography (HIC), ion exchange chromatography (IEC), and size exclusion chromatography (SEC) are other common modes of liquid chromatography.

The stationary phases used in the normal phase chromatography are polar and the mobile phases are non-polar. Polar analytes retain due to interaction with the stationary phase. Currently, normal phase chromatography is widely used in organic chemistry for the purification of synthetic products. Bare silica is one of the most common stationary phases used in the normal phase chromatography. Reversed-phase chromatography, so far, the most famous mode uses non-polar stationary phases and mobile phases that are relatively polar. There are various stationary phases, as described earlier, which have been developed for the reversed-phase chromatography and the list is still growing. HILIC uses both polar stationary phases and mobile phases, but the mobile phase, which is dominated by the use of acetonitrile, also contains some percentage of water. Water being the more polar solvent competes for the stationary phase with the polar analytes and helps to elute more polar analytes. Water is considered a

strong solvent in HILIC, whereas it is the weak solvent in RPLC. HIC is similar to reversed-phase, as the stationary phase is non-polar and mobile phases used are more polar. This mode has special use for the separation of large molecules, such as proteins, and uses high salt concentrations in the mobile phases. Under the HIC conditions, the structure of the proteins remains intact without denaturing and more hydrophobic biomolecules retain longer. Salt concentration gradients are used to elute analytes and only under certain conditions can this be coupled to a mass spectrometer [7].

IEC uses either positively or negatively charged ligands bound to a solid support as the stationary phases. When the ligand is positively charged, anions can be exchanged and separated; when the ligand is negatively charged, cations can be exchanged and separated. In both cases, analytes having the same charge as the stationary phase are repelled and therefore elute first, followed by the neutral analytes, but both classes are poorly retained relative to the analyte class, which is complementary to the stationary phases. In order to elute the exchanged or bound analytes either by altering the pH or the ionic strength in the mobile phases.

SEC is quite different from all the other modes because it does not use a ligand bound to a solid support. Instead, it uses a porous material where the size of the pores is in a range, so that depending on the size some analyte molecules could enter the pores whereas some are excluded. [2] Smaller molecules enter the pores, have to pass through a longer path, and elute later compared to the excluded larger molecules, which travel a relatively shorter path.

In addition to these modes there are cases where mixed mode or dual mode of liquid chromatography is used in the method development. Depending on the nature of the ligands and the mobile phase conditions used, some phases can be used in two different modes, such as in reversed-phase as well as in aqueous normal phase modes, depending on the mobile phase present. When the sample mixture has analytes from different classes with vastly different physicochemical properties and need to be analyzed in a single injection, these kinds of multi-mode phases are very useful [8-9].

1.4 Small molecule separation method development

The most common mode of HPLC, reversed-phase is widely used for the separation of small molecules (molecular weight < 2000 Da). Among all the stationary phases available still C18 is the first choice for most

of the small molecule separation method development. Under the RP conditions, both polar and non-polar analytes can be separated. Further, this can be extended to acidic and basic analytes by altering the mobile phase conditions with additives. Most common mobile phases used in the RP are water, methanol, and acetonitrile. As additives, especially for the separations to be compatible with MS detection, formic acid and acetic acid, as well as each of their ammonium salts, are commonly used. In addition, ion pair reagents, such as trifluoroacetic acid, are also used depending on the application. When method development is for ionizable analytes, buffered mobile phases needs to be used, in order to retain the same form of the analyte throughout the analysis. Buffered mobile phases help to ensure good peak shape for the separation of chargeable species in RPLC.

One of the main concerns in the liquid chromatography method development is the number of variables that is needed to be optimized. These variables include mobile phases, gradient rates, modifiers, pH of the mobile phases, temperature of the analysis, and stationary phases, among others. It can be a time-consuming process to address each variable one-at-a-time in order to achieve optimal separation. Information technology has improved during the last couple of decades and have made lot of processes automated. LC instrumentation has also developed and with the help of software programs it can be automate the LC operation. There are several software packages, including Shimadzu Nexera Method Scouting Solutions, Fusion Method Development™ from Waters, and ChromSwordAuto® from Thermo-Fisher Scientific, which are available commercially. These software packages allow operators to automate the mobile phase switching, column switching, and even perform some systematic changes to the gradient rates, in order to save significant amounts of time and operator presence needed in front of the instrument [4,10-11].

1.5 Use of triple quadrupole mass spectrometer in the detection and quantitation of small molecules and proteins

After the introduction of the triple quadrupole mass spectrometer (QqQ) by Enke and Yost in the late 1970s, the applications of mass spectrometry in the detection and quantitation of various kinds of analytes have grown tremendously [12]. A triple quadrupole mass spectrometer as shown in figure 1-2 consists of three quadrupoles in series, and the second quadrupole is used as a collision cell. These

quadrupoles can be operated in scan mode or selected ion monitoring modes. In the multiple reaction monitoring mode first quadrupole selects the given precursor mass/charge (m/z) and transmit to the second quadrupole. Second quadrupole, collision cell is filled with a gas such as nitrogen or argon (pressure range from 17-450 psi for Shimadzu LCMS 8050) There, collision induced fragmentation of the entering ions occur. The third quadrupole filters the given m/z from the product ions formed in the collision cell. The QqQ is not a high-resolution mass spectrometer, and most commercially available instruments have unit resolution. But when the instrument is operated under the multiple reaction monitoring (MRM) mode, where different transitions from precursor ion to product ions are monitored, specificity is very high and background noise is greatly reduced. For quantitative analysis of mass spectrometry, QqQ mass spectrometers play an important role. Currently, analysis of drugs, drug metabolites, and pharmaceuticals in the clinical lab settings, as well as in chemical and pharmaceutical manufacturing settings, these instruments are heavily used.

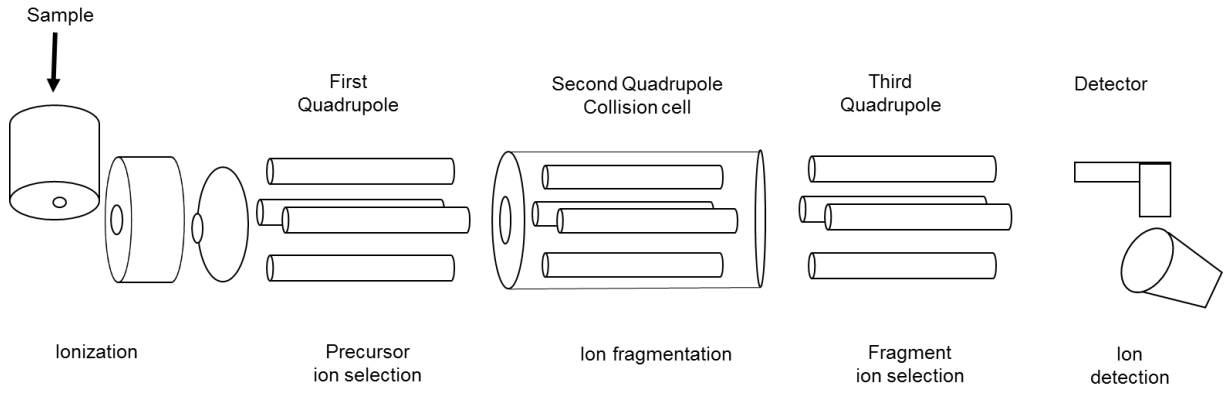

Figure 1-2: Schematic diagram of triple quadrupole mass spectrometer

The use of QqQ is not that common for the analysis of intact proteins. The QqQ is a low-resolution mass spectrometer. Usually one of the most abundant charge states in the distribution of charge states for multiply charged protein is used as the precursor ion. But because of the low resolution of the quadrupole, it can filter various other ions such as adducts, isoforms, isotopes can also be included as a mixture of the ions passed to the collision cell. Recently, Wang *et. al.* has shown that reproducible fragmentation for intact proteins can be performed by carefully adjusting the collision induced gas (CID)

pressure and collision energy in the collision cell [13]. Once reproducible fragmentation is optimized for intact proteins, QqQ can give advantages in quantitation of proteins over the currently available methods. Specially, the less time needed in the sample preparation and can avoid errors due to incomplete digestion.

1.6 Multi-path liquid chromatography mass spectrometry analysis of multiple classes of analytes from single injection

As the applications of liquid chromatography has grown, especially in the fields of bioanalytical chemistry, the complexity of samples has also increased. Further, more attention has been given to reduce the time taken for a single analysis. In clinical laboratories, it is desirable to analyze a maximum number of samples to make more profits.

Restricted access media (RAM) is a special type of trap columns that uses a combination of different modes of chromatography to selectively trap one class of analytes from a sample while excluding the other classes [14-15]. These columns so far have mostly been used for online sample preparation where the excluded molecules are simply send to the waste. With the improvement is the LC MS instrumentation, there is a capability of analyzing these excluded molecules simultaneously with the trapped molecules. In order to do that methods needs to have several paths to route the eluent from trapped column to an analytical column and to elute the trapped molecules from the RAM column both of which later to be coupled and send to the MS for the detection. There is a potential of getting this to even further by having more paths, multi path system where for example use several RAM type columns to tarp small molecules, then send the eluent to second RAM type column to trap say fatty acids and finally couple all together and send to the MS for the detection. These kinds of multipath analysis can be beneficial in many ways for example when the amount of sample is limited single injection can provide most if not all the information that caregivers needed. Moreover, once these methods are well adopted in the clinical lab settings, caregivers can order analysis of multiple classes of molecules when they perform routine checkups and that can help diagnosis of diseases such as cancers at the early stages without patients needing to wait to see actual symptoms of the disease.

1.7 Multi-dimensional liquid chromatography mass spectrometry analysis of proteins

Liquid chromatography has been used for decades for the analysis of various classes of molecules. There has been lot of advancement in the stationary phases used for the liquid chromatography, as well as in the instrumentation. When the sample complexity increases, more resolving power is needed to get obtain the desired separation. Further, there could be analytes with similar physicochemical properties. Therefore, hard to separate using the traditional one-dimensional liquid chromatographic methods. Especially in the case of protein separation, more common RP mode has shown very similar selectivity. Therefore, development of separation methods for proteins using traditional techniques is challenging. In 1970's O'Farrel and Erni *et. al.* has shown methods to improve the peak capacities to get better resolving of complex samples [16-17]. Peak capacity is defined as the maximum number of resolvable peaks in a chromatographic method. When two-dimensional liquid chromatography (2D LC) method is employed with non-similar or orthogonal phases higher peak capacities can be obtained. Theoretically, the peak capacity of the 2D LC method is equal to the product of the peak capacities of first and second dimensions [18]. Even though this theoretical peak capacity is hard to achieve, there is a significant improvement in the peak capacity when two phases are used in a 2DLC method, relative to the peak capacity that can be achieved in 1D LC.

There are mainly two common techniques of 2D LC methods currently used depending on the way the eluent from the 1D column is injected to the 2D column. First, heart-cutting 2D technique (LC-LC) only portions of the eluent from the 1D column is injected to the 2D column for further separation. The more commonly used method known as comprehensive 2D LC (LC x LC) all the eluent from the 1D column is injected to the 2D column for further separation. In the comprehensive either two HPLC systems are connected or one with four pumping units and either one 2-position 10 or 8 port valve or two 2-position 6 port valves is used to inject the eluent from the 1D column to the 2D column. (Figure 1-3) Usually the 1D separation is performed relatively slower. Sampling or the modulation time depends on the size of the fraction collected to inject to the 2D column. The analysis of the second dimension needs to be less than the modulation time. In order to obtain desired separation in the second dimension, it has shown that 3 to 4 modulations per peak is required [19]. These 2D LC methods can be developed to be performed either on-line or off-line. In the case of comprehensive 2D LC, on-line methods, advanced software and

hardware (valves) are used to inject the eluent from the 1D column to the 2D column and sample is collected in small loops. For off-line methods, fractions are collected and if needed can be concentrated and then re-injected to the second-dimension separation system. Off-line methods are relatively simpler but more time consuming and labor intensive. Further, they have problems with contamination, sample loss, and difficulty for automation.

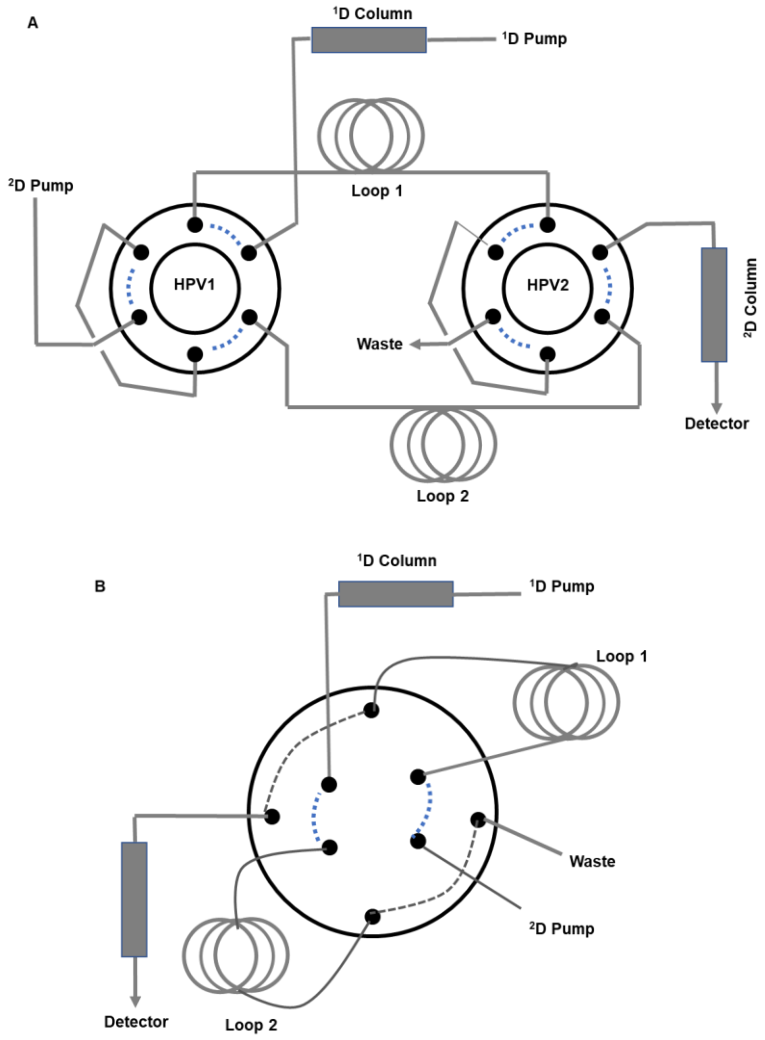

Figure 1-3. Diagram of a typical 2D system showing the injection of 1D eluent to the 2D column. A) Use of two 2 position 6 port valves B) Using one two position 8 port valve

As discussed earlier, there are different modes of LC available but not all of them can be coupled to build a 2D LC system, because in some cases mobile phases will not be miscible or they will not be compatible. When the detector for the system is a mass spectrometer, the limitations are even higher as

at least the mobile phases used in the 2nd dimension needs to be MS compatible. In the literature, it has been shown that many orthogonal phases have been coupled to build successful 2D LC systems and few examples of such combinations are RPLC with Capillary electrophoresis (CE), SEC with RPLC, and IEC with RP [20-22]. Currently, the field of 2D LC method development is growing faster and applications specially in the field of bioanalytical chemistry. One of the main reasons for the popularity of the comprehensive 2DLC is the availability of instrumentation from the commercial instrument manufacturers.

1.8 Towards the development of a multi-channel and multi-dimensional LCMS system for the analysis of small molecules and proteins.

Progress towards the development of a multipath LC MS system and 2D LC MS system for the small molecule and protein analysis is reported here. As discussed above, there are several advantages in using multipath LCMS system for the analysis of multiple classes of molecules from a single injection. In the development of the multi-path system, first, chromatographic separation methods for the analysis of small molecules using different stationary phases and different modes of liquid chromatography were performed, and a detailed discussion is included in chapters 2 and 3. The LCMS separation and detection methods for the protein analytes were developed in parallel by Dr. E. H. Wang [14, 23]. A multipath LCMS system was constructed using a Shimadzu Nexera LC system coupled with a Shimadzu 8050 triple quadrupole mass spectrometer. Various RAM columns were used to optimize the trapping of small molecules while excluding larger molecules. Even though it was not fully successful in developing new method for the simultaneous analysis of small molecules and proteins from a single injection, chapter 4 discuss the attempts towards the development of new multipath system. Chapter 5 focus on the attempts taken towards developing comprehensive 2D LC system for the analysis of proteins using a top-down strategy with the same instrument used for the multi-path method development. There has no literature on the separation of proteins by using RP x RP on comprehensive 2D LC system and chapter 6 discuss the efforts that have been planned to make the 2D LC system better for the analysis of larger molecules.

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Chapter 2

Automated Screening of Reversed-Phase Stationary Phases for Small Molecule Separations using Liquid Chromatography Mass Spectrometry

2.1 Abstract

There are various reversed-phase stationary phases which offer significant differences in selectivity and retention. In order to investigate different reversed-phase stationary phases (aqueous stable C18, biphenyl, pentafluorophenyl propyl, and polar-embedded alkyl) in an automated fashion, commercial software and associated hardware for mobile phase and column selection were used in conjunction with liquid chromatography and a triple quadrupole mass spectrometer detector. A model analyte mixture was prepared using a combination of standards from varying classes of analytes (including drugs, drugs of abuse, amino acids, nicotine, and nicotine-like compounds). Chromatographic results revealed diverse variations in selectivity and peak shape. Differences in the elution order of analytes on the polar-embedded alkyl phase for several analytes showed distinct selectivity differences compared to the aqueous C18 phase. The electron rich pentafluorophenyl propyl phase showed unique selectivity towards protonated amines. The biphenyl phase provided further changes in selectivity relative to C18 with a methanolic phase, but it behaved very similarly to a C18 when an acetonitrile-based mobile phase was evaluated. This study shows the value of rapid column screening as an alternative to excessive mobile phase variation to obtain suitable chromatographic settings for analyte separation.

2.2 Introduction

Liquid chromatography is used extensively in a variety of research laboratories, as well as in routine analysis. Among the available liquid chromatographic separation modes, reversed-phase liquid chromatography (RPLC) is most common [1]. Early in the development of RPLC column technology, phases were coated on a support; however, the ability to maintain a stable coating was problematic. After the introduction and development of bonded phases, RPLC became more robust and could be used with a wider range of mobile phases [2-4]. The most commonly used reversed-phase stationary phase, octadecylsilyl (ODS) or C18-bonded silica, is sufficient for generic analysis. However, as matrices become more complex and the range of bioactive molecules of interest widens, alternative selectivity is needed to resolve analytes from matrix interferences. Biological matrices, such as urine and plasma can contain high levels of salts and lipids, respectively, which contribute to the complexity of samples. Additionally, in some cases, structurally-similar analytes would make distinguishing molecules based solely on the nonspecific interactions provided by a C18 phase a difficult task. The development of new reversed-phase chemistries has become an active area of academic and commercial research in an effort to provide alternative selectivity for complex mixtures of analytes with varying structural and functional similarities [5-6]. Overall, the use of RPLC may be preferred over other modes of chromatography, such as hydrophilic interaction liquid chromatography (HILIC), because it is more robust and predictable [7].

Many alternatives to standard ODS or C18 phases have been developed. One disadvantage of traditional C18 based phases is phase collapse under high aqueous conditions [8-9]. This greatly reduces retention, and restoration of the collapsed phase to its proper condition can be difficult and time-consuming. To allow for use under extreme aqueous conditions, as well as to improve peak shapes for basic analytes, polar groups have been embedded within or at the base of the bonded alkyl chains[10-11]. Additionally, stationary phases have been developed with bonded groups, such as cyano, phenyl, biphenyl, and fluorinated ligands, at the end of alkyl chains of various lengths to provide alternative selectivities [12]. When different ligands are included in the alkyl chain, those groups can interact with the analytes and thereby give changes in selectivity relative to standard C18 phases. These different non-covalent interactions include hydrogen bond acceptance and donation, pi-pi interactions, and dispersion

interactions. Thus, a wide range of RPLC phases are available and the selectivities they provide are enhanced by mixed mode interactions.

Method development in RPLC can be difficult due to varying parameters that require investigation and optimization, such as mobile phases, modifiers, gradient rates, and stationary phases. Therefore, the time needed to develop new methods, which provide high sensitivity and optimal resolution of target analytes can increase dramatically. Efforts have been made to automate the method development process to create robust methods in a reduced amount of time. There are several software programs available for automating the method development process, including Nexera Method Scouting Solutions [13] from Shimadzu, ChromSwordAuto® [14] from Thermo-Fisher Scientific, and Fusion Method Development™ from Waters[15]. Even though a liquid chromatograph may be required to have certain hardware configurations (e.g. multi-channel pumps and selection valves) to use these software programs, the evaluation of multiple mobile and stationary phases in an automated fashion reduces method development time and allows efficient exploration of separation parameters. For example, using the Nexera Method Scouting Solutions software, up to 96 combinations of column and mobile phase compositions can be screened. The software creates the batch with minimal user input and then controls the method and column screening without need for operator attendance. Following this, various other functionalities are available for compiling and analyzing results.

Many column manufacturers provide selected sets of RPLC column chemistries that are designed to be combined with method development efforts to provide a range of selectivities to achieve separations in different applications. Column chemistries can vary between types of bonded phase support, degrees of porosity, and chemical nature of the bonded phases. Even though some specification about the applicability of these phases for different classes of analytes is usually provided, one still needs to evaluate their performance for a given application. The most challenging applications include those with multiple classes of target compounds in a sample mixture. Thus, experimental investigation is inevitable to find the column chemistry that provides optimal separation of all desired analytes [16-18].

The aim of this study was to combine liquid chromatography – triple quadrupole mass spectrometry, Nexera Method Scouting Solutions software, a custom library of small molecules, and the Restek USLC® Method Development Toolbox to investigate selectivity in an automated liquid

chromatography – mass spectrometry (LC-MS) method development work flow. The differences between four reversed-phase stationary phases, with respect to different analytes classes, were investigated using a generic liquid chromatography method with gradient mobile phase compositions. The effects of the mobile phases on the selectivity and retention behavior of the analytes with the four stationary phases were studied. Each of the phases provided unique selectivities towards the range of model analytes included in the study. Ultimately, the experiments and results demonstrated the value of rapid column screening under generic RPLC mobile phase conditions to achieve the separation of various target analyte classes. Such an approach can be expected to reduce overall method development time when compared to a generic one-column-at-a-time approach where more focus is often placed on appropriate mobile phase optimization.

2.3 Materials and Methods

2.3.1 Materials, reagents, and sample preparation

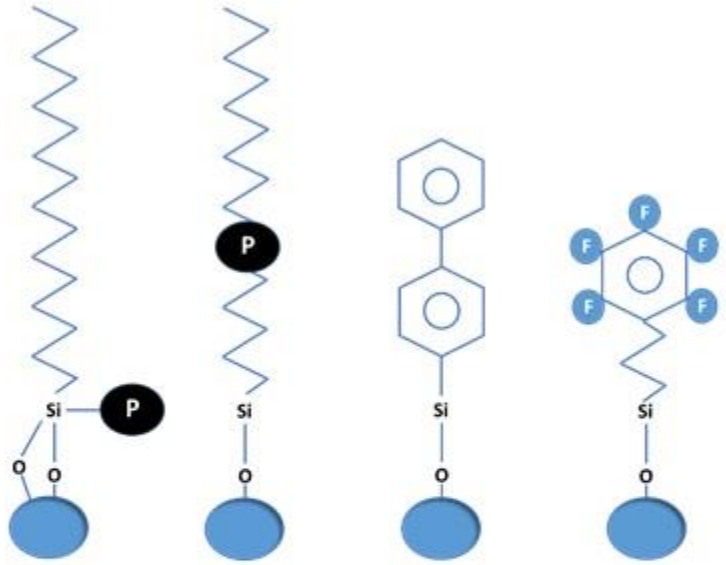
Standard solutions (1 mg/mL in methanol) of hydrocodone, hydromorphone, methamphetamine, phenobarbital, warfarin, oxymorphone, diazepam, acetaminophen, and fentanyl were purchased from Cerilliant Corporation (Round Rock, TX, USA). A formylated peptide (formyl-Met-Leu-Lys-Ile-Ile-OH) was synthesized and purified (>98%) by Johns Hopkins University Synthesis and Sequencing Facility (Baltimore, MD, USA). Neat, solid standards for nicotine, anabasine, matrine, cotinine, catechin, bentazon, diclofenac sodium, reserpine, adenosine monophosphate, thiamine hydrochloride, estrone sulfate, estrone glucuronide, and histidine were purchased from Sigma-Aldrich (St. Louis, MO, USA). HPLC-MS grade acetonitrile, water, methanol, and formic acid were purchased from J.T. Baker (Phillipsburg, NJ, USA). Ammonium formate (99.995%) was purchased from Sigma-Aldrich.

All solid standards were dissolved in water or methanol, according to their solubility, to prepare 1 mg/mL stock solutions. Methamphetamine and fentanyl were diluted to prepare 100 µg/mL stock solutions. The final mixture of analyte standards was prepared by diluting with water: methanol mixture (95:5; v/v) to a final working concentrations of 1 µg/mL for all the analytes, except for methamphetamine and fentanyl which were 100 ng/mL.

2.3.2 Instrumentation and settings

The HPLC system consisted of two Nexera LC-20ADXR pumps, a SIL-20ACXR auto-sampler, and a CTO-20AC column oven (Shimadzu Scientific Instruments, Inc., Columbia, MD). The column oven was equipped with one FCV-12AH two position six port valve and a six position column switching valve. All tubing was either stainless-steel or PEEK (0.010 in id). Analytical columns used were from the Restek USLC® Method Development Toolbox, which included four columns (100 x 2.1 mm, 3 µm d_p): Ultra Aqueous C18; Ultra Biphenyl; Ultra IBD (polar embedded); and Ultra PFPP (pentafluorophenyl propyl) (Restek Corporation, Bellefonte, PA). Figure 2-1 shows a generic depiction of these phases and lists primary and secondary interactions imparted by each phase, according to the manufacturer. The temperature of the oven that houses the column selection valve and columns, was held at 30 °C. Aqueous mobile phases explored included water with 0.5% formic acid (Pump A; Line 1), water with 5 mM ammonium formate (Pump A; Line 2) and water with 5mM ammonium formate and 0.5% formic acid (Pump A; Line 3). Methanol, acetonitrile, methanol with 0.5% formic acid, and acetonitrile with 0.5% formic acid (Pump B; Lines 1-4, respectively) were designated as the organic mobile phases. A solution of 5:95 water: isopropanol (v/v) was used as the needle wash solution. Sample injection volumes were 5 µL. A scouting gradient of 5% to 99% B over nine minutes at 0.2 mL/min was used for all analyses.

In order to determine the dead time (t_0), a combination of experimental and theoretical methods was used. The volume of the column was estimated using the formula $V_m = 0.5\ L\ d_c^2$ where L is the length of the column and d_c is the diameter of the column [19]. The calculated volume was divided by the flow rate to obtain the dead time for the column. In order to calculate the dead time for all the other components (i.e. tubing, connectors, and valves, etc.), retention time analysis was performed using uracil while replacing the column with a zero-dead volume adaptor. Finally, to obtain t_0, the calculated dead time for the column and the experimentally determined dead time for all the other components were summed. This value was used for all column tested, given that they had equivalent dimensions and solid support materials.



	Aqueous C18	Polar embedded (IBD)	Biphenyl	Fluorophenyl propyl (PFPP)
Main interaction	Dispersion	Hydrogen bonding	Polarizability	Cation-exchange
Secondary interaction	Hydrogen bonding	Dispersion	Dispersion	Polarizability

Figure 2-1: Four different RPLC phases and the type of interactions they provide.

2.3.3 Mass spectrometry parameters

The MS system was a Shimadzu LCMS-8050 triple quadrupole mass spectrometer with an electrospray ionization (ESI) source. The MS was operated under multiple reaction monitoring (MRM) conditions with 5 msec polarity switching to detect both positively and negatively charged ions. The interface voltage was ±4.5 kV; drying gas and nebulizing gas flow rates (nitrogen) were 15 and 3 L/min, respectively; the heat block temperature and desolvation line temperatures were 400 and 300 °C, respectively. The collision gas (argon) pressure was set to 270 kPa. The optimized MRM transitions used to detect the analytes are given in Table 3-1.

2.4 Results and Discussion

Selectivity is the most influential parameter (among selectivity, efficiency, and retention) for generating resolution between analytes in chromatography. The chemistry of the stationary phase is the parameter that most affects selectivity. To investigate method development on a series of RPLC stationary phases for small molecule separation, we compiled a mixture of analytes that vary in their functionality and physicochemical character. The analytes in this study include: drugs, such as acetaminophen and diclofenac sodium; drugs of abuse, such as methamphetamine and hydrocodone; herbicides, such as bentazon and nicotine; bioactive chemicals, such as estrone and catechin; metabolites, such as cotinine, estrone glucuronide, and estrone sulfate; and biological molecules, such as histidine, adenosine monophosphate, and a formylated pentapeptide. The structures of analytes are shown in Figure 2-2. Scouting gradients with variable modifiers and organic solvents were used to explore selectivity differences, as detected by electrospray ionization – triple quadrupole mass spectrometry.

The C18 phase used in this study was slightly different from the common C18 phases available in the market. It is modified with a polar group attached to the silane unit bonded to the particle surface. This allows for use of 100% aqueous mobile phase conditions without phase collapse. Further, the polar group (exact functionality is not reported by the manufacturer) can undergo hydrogen bonding interactions, thereby altering selectivity relative to typical ODS phases. The biphenyl phase is rich with electrons and highly polarizable. In addition to interactions driven by the hydrophobic effect, the biphenyl phase can also enable π-π and cation-π interactions with appropriately functionalized analytes.[20] The third phase is a polar embedded alkyl phase (IBD). Typically, these incorporate an amide or carbamate group midway along the alkyl chain; this unit can provide increased retention of acids, and the phase is also amenable for use in highly aqueous mobile phases. The PFPP phase has polar fluorine atoms on the phenyl ring and, because C-F bonds are more polarizable than C-H bonds, this phase can provide ion-exchange type interactions. This is advantageous for retention of basic analytes that are protonated when chromatographed under acidic mobile phase conditions.

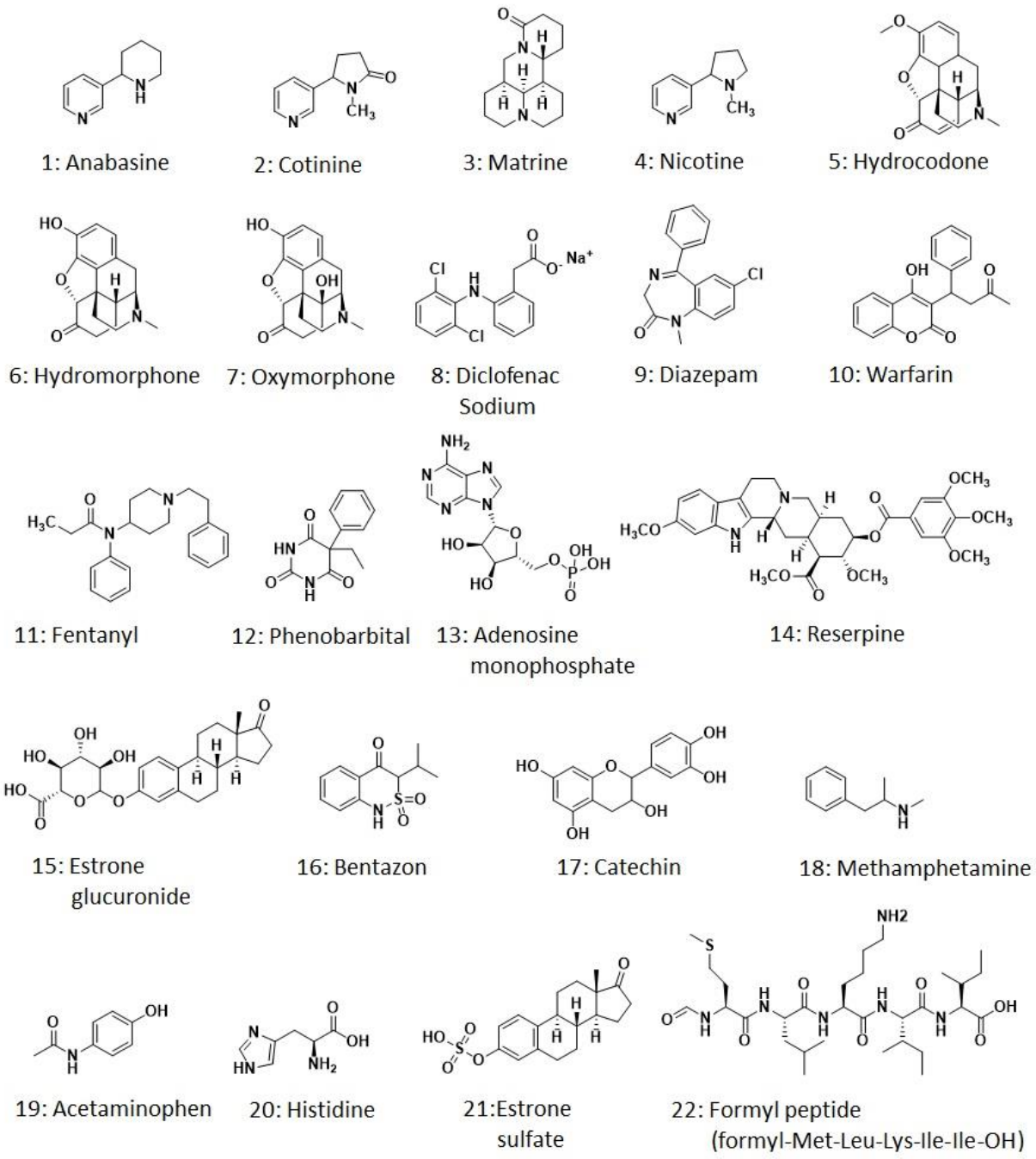


Figure 2-2: Structures of model analytes.

2.4.1 Overall performance of the phases

A representative comparison of the four phases under typical LC-MS mobile phase conditions (0.5% formic acid in water and methanol) is shown in Figure 2-3. All analyses were performed in triplicate, retention times were tabulated, and their standard deviations and percent relative standard deviation (RSD) calculated. Results showed that retention was highly reproducible (%RSD was less than or equal to 5.0% for all the analytes, except adenosine monophosphate and hydromorphone (RSD ≤ 10.0%) on the four phases). This data is shown in Tables S2-S5 in the supplementary electronic supporting information for this article.

In the methanol-based mobile phase, the biphenyl phase retained most analytes longer compared to the other three phases. The majority of the analytes in the sample mixture possess either aromatic groups or pi electrons and therefore stronger interactions with the biphenyl phase were expected. Another noticeable feature was that nicotine (4) and anabasine (1) co-eluted on all four phases, even though their retention was significantly changed from phase to phase. Anabasine is a metabolite of nicotine, and their structures are very similar. Previous work has shown that they can be effectively resolved using hydrophilic interaction liquid chromatography, [21] but under the generic RPLC conditions shown here, virtually no selectivity was exhibited by the phases for these analytes. Further manipulation of mobile phase composition and gradient could potentially provide for improved selectivity, but this was not specifically explored in this study. In contrast, while the formyl peptide (22) and phenobarbital (12) co-eluted on the biphenyl and IBD phases, they were well separated by the PFPP phase. This finding exemplifies the advantages of the automated method scouting, since analytes retained longer with PFPP phase, such as the formyl peptide (22), diclofenac (8), and warfarin (10) are all nitrogen-containing compounds which are protonated under acidic conditions. The IBD phase exhibited the least retention for almost all analytes out of the four phases tested and many of the analytes had broader peak shapes. This behavior could be due to several reasons. With mobile phases that can hydrogen bond with the polar group, the polar group may be shielded from analytes resulting in diminished interactions. Alternatively, the relatively short alkyl chain length on this phase offers less retention through the hydrophobic effect. Finally, when the analytes interact with the phase through multiple interaction modes, significant peak broadening can result.

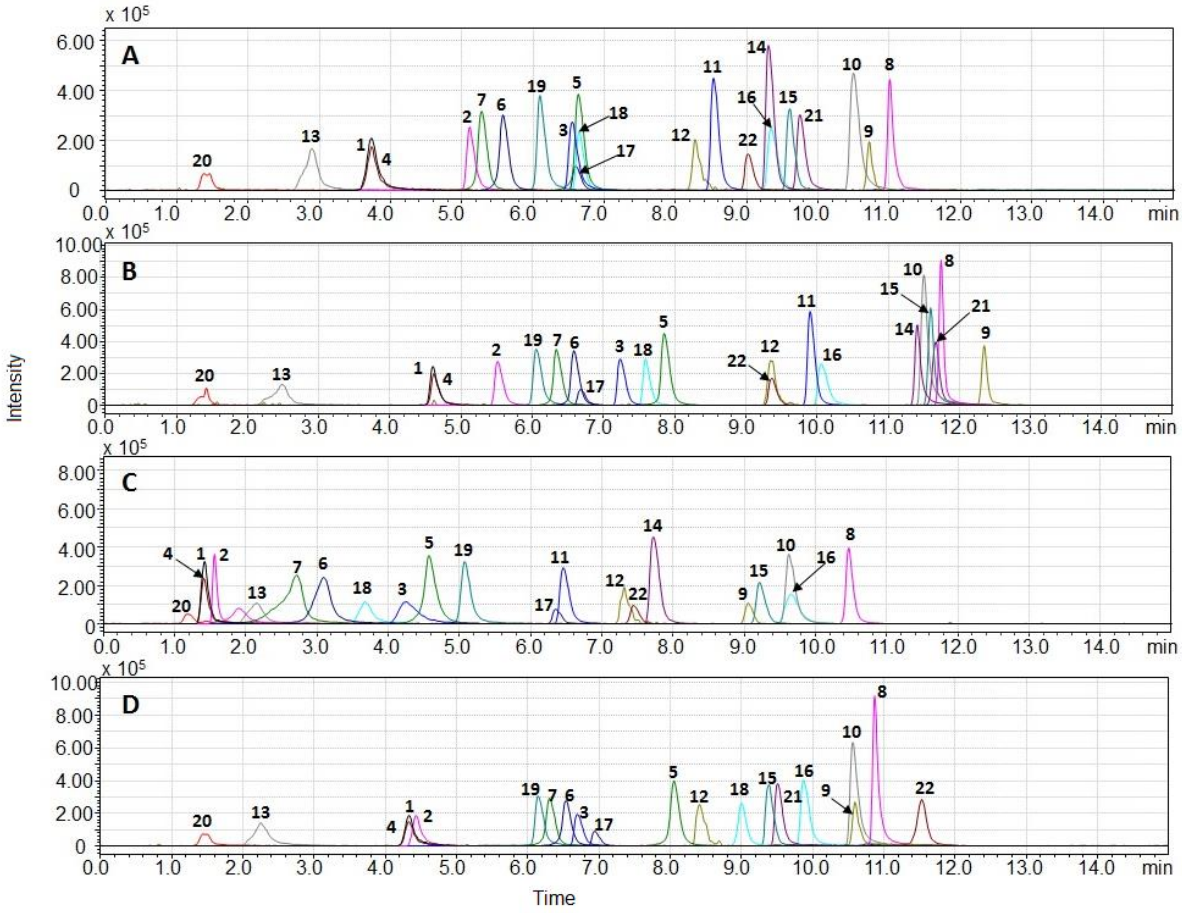


Figure 2-3: Comparison of the four reversed-phases used in the study: A) Aqueous C18; B) Biphenyl; C) Polar embedded; and D) PFPP (Mobile phase A: 0.5% formic acid in water, Mobile phase B: Methanol).

2.4.2 Biphenyl phase behavior

The hydrophobic effect is typically the main driver for retention in RPLC. The biphenyl phase also features two linked aromatic rings, which provide a large electron cloud that can strongly polarize analyte molecules. As depicted in Figure 2-4, there are four possible resonance forms for the biphenyl group and the polarizability of its aromatic π faces provides potential for additional retention and selectivity. Polarizability, cation-π, and π-π interactions are all strongly mediated by the mobile phase. With methanolic mobile phases, electrons from the biphenyl ligands are readily available for interactions with analyte molecules and therefore retention is increased. When the mobile phase is switched to acetonitrile, retention is strongly diminished, as shown in Figure 2-5. Acetonitrile has the strongest π character of the

organic solvents investigated, and thus it effectively shields the phase from such interactions with many analytes. Shown in Figure S1, a closer look reveals that the biphenyl phase in the presence of acetonitrile as the organic modifier behaves very similarly to the aqueous C18 phase. Retention is diminished and a comparison of capacity factors obtained for analytes on each phase shows a high degree of correlation.

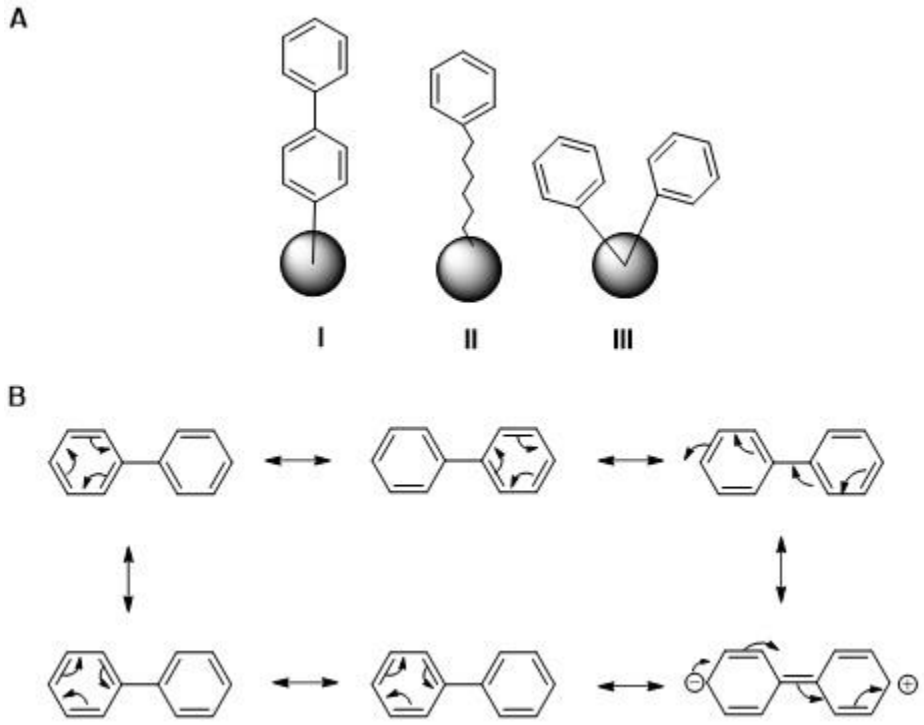


Figure 2-4: Phenyl Phases: A) Different forms of phenyl phases; B) different resonance forms of a biphenyl ligand.

Figure 2-6 shows that under methanolic mobile phase conditions, the biphenyl phase exhibits some unique selectivities. For example, matrine (3), hydrocodone (5), and methamphetamine (18) all co-eluted on the aqueous C18 phase but were well separated on the biphenyl phase. Reserpine (14) and bentazon (16) also coeluted on the C18 but were resolved on the biphenyl phase. Another important feature is for diazepam (9), which was retained much longer with the biphenyl phase. Diazepam has two aromatic rings plus two more pi bonds; therefore, it interacts more strongly with the biphenyl phase and is retained longer [22]. It has also been shown in literature that when analytes contain sulfoxide groups and aromatic groups, such as that for bentazon, longer retention on biphenyl phases can be expected due to the increased dipole moment on the molecule [23].

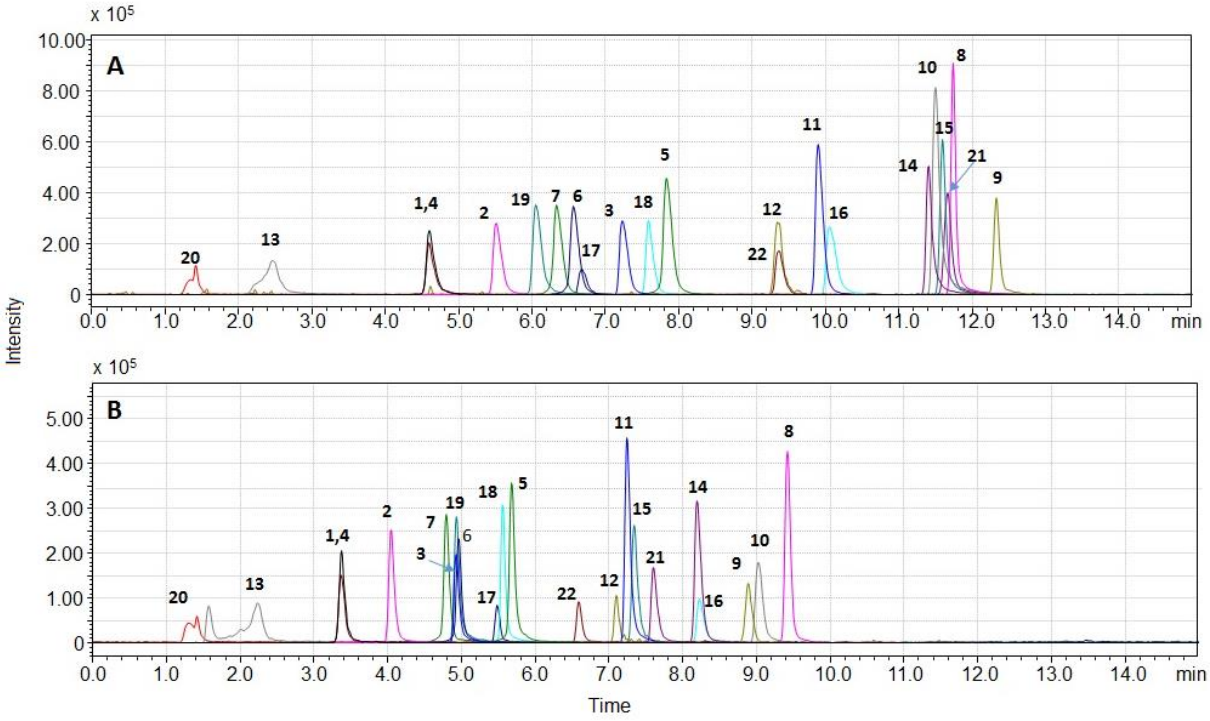


Figure 2-5: Changes in retention as a function of organic modifier for the biphenyl phase using A) 0.5% formic acid in water with methanol and B) 0.5% formic acid in water with acetonitrile.

2.4.3 Significant selectivity differences with polar embedded alkyl phase

Even though ODS phases are the most common, some literature claims that alkyl chains of 14 carbons in length can provide better overall performance[2]. This performance can be due to several reasons, including the ability to re-equilibrate the column faster when shorter alkyl chains are used and increased resistance towards phase collapse in the presence of higher aqueous content. As for traditional long alkyl chain-based phases, it is difficult for them to be solvated in high aqueous content mobile phases. For the IBD phase, there is a polar group embedded in the alkyl chain, which helps the phase to be solvated under high aqueous conditions [24]. Further, this polar group can help shield interactions between analytes and the silica surface and improve peak shapes [25].

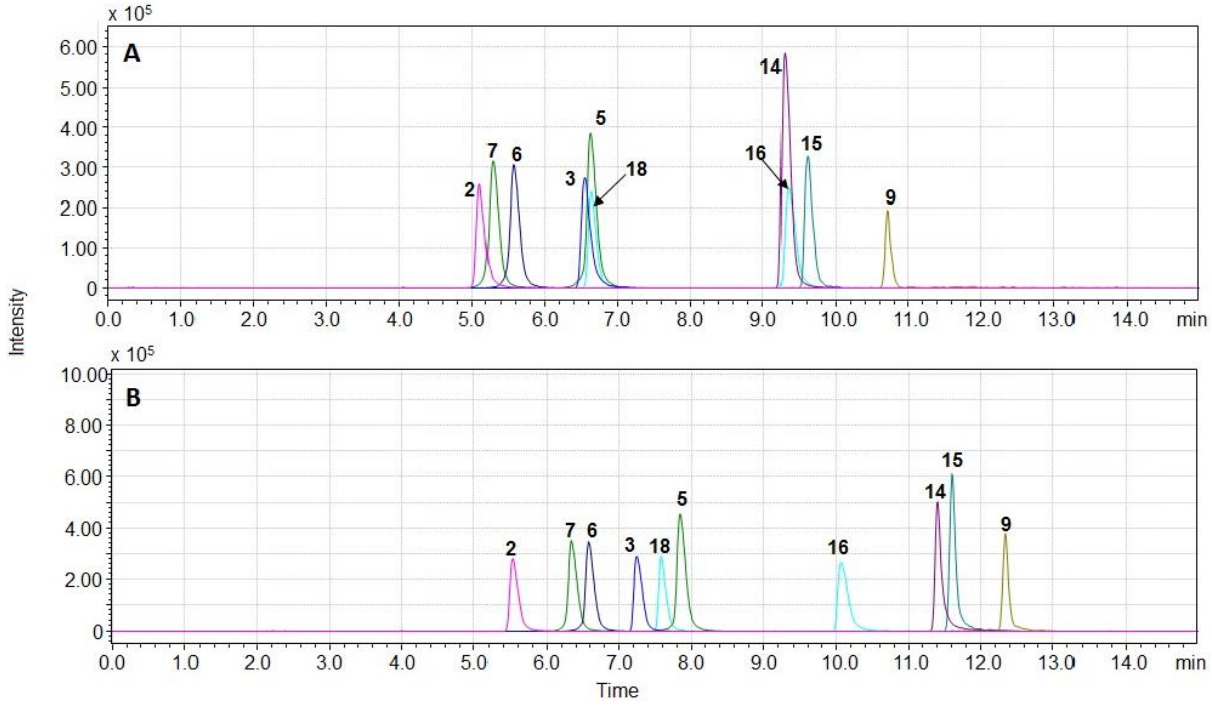


Figure 2-6: Significant selectivity and retention differences between phases: A) Aqueous C18 and B) biphenyl (Mobile phase A: 0.5% formic acid in water, Mobile phase B: Methanol).

A comparison of model analyte retention on the IBD phase with that of the aqueous C18 shows that many unique selectivities and changes in elution order could be observed. In the case of the aqueous C18 phase, the analytes anabasine (1), cotinine (2), and nicotine (4) eluted after adenosine monophosphate (AMP, 13). In contrast, on the IBD phase, all three analytes eluted before AMP. This is highlighted in Figure 2-7. AMP and other nucleotides are usually separated using ion-exchange chromatography or hydrophilic interaction liquid chromatography [26]. Charged analytes, when subjected to reversed-phase conditions, are less retained because they interact less with the non-polar stationary phase. The polar group embedded in the alkyl chain of the IBD allows highly hydrophilic analytes like AMP to be retained. Therefore, the availability of alternative RPLC phases, like the IBD phase, allows chromatographers to continue to operate in the reversed-phase mode to achieve separations of hydrophilic analytes, without resorting to HILIC mode or other alternate modes. For HILIC in particular, it is hard to predict retention and phases take a longer time to equilibrate; this can cause problems with

reproducibility [27-28]. A more extensive study is still needed to understand the full scope of retention possible for hydrophilic analytes on polar embedded phases, such as the IBD.

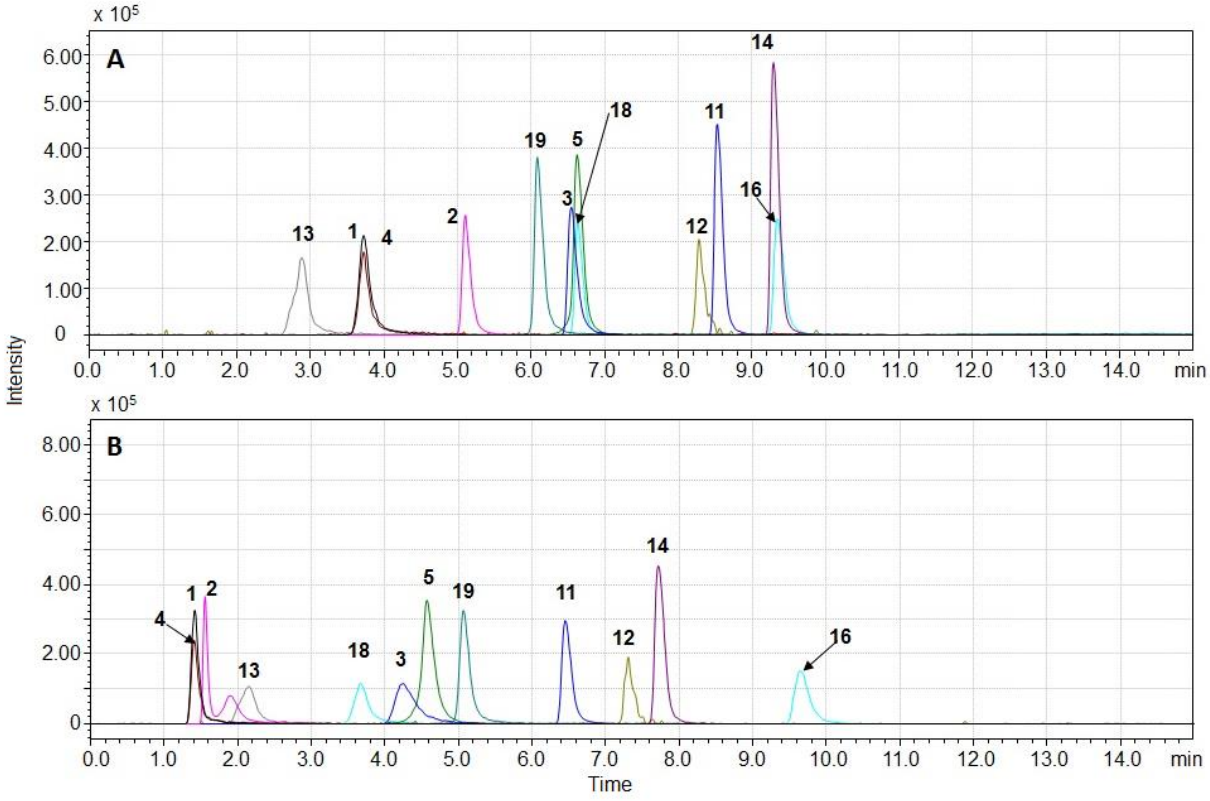


Figure 2-7: Significant selectivity and retention differences between phases: A) Aqueous C18 and B) IBD (Mobile phase A: 0.5% formic acid in water, Mobile phase B: Methanol).

Additional observations could be made. Fentanyl (11) and phenobarbital (12) changed elution order when switching from the aqueous C18 to the IBD phase. This is likely due to increased hydrogen bonding interactions by phenobarbital with the polar group in the IBD phase. Additionally, bentazon (16) exhibited longer retention on the IBD compared to the aqueous C18. This can be attributed to the interaction of lone pairs of electrons on the nitrogen and oxygen on bentazon with the polar group embedded in the IBD phase. These interactions suggest that the polar group (not specified by the manufacturer) might be an amide functionality that has both H-bond accepting (with bentazon) and H-bond donating (with phenobarbital) capabilities. Matrine (3), hydrocodone (5), and methamphetamine (18) co-eluted on the aqueous C18 phase under the generic scouting gradient tested, as did reserpine

(14) and bentazon (16). All of these analytes were well separated when the IBD phase was used without altering the generic 0.5% formic acid in water/methanol mobile phase scouting gradient. It is worth noting, that it would be quite possible to resolve these co-eluting compounds on the aqueous C18 phase; however, significant additional method development would be required to find the proper mobile phase and gradient conditions. However, since column switching is readily accommodated in the method scouting set-up, the need for extensive mobile phase alterations is eliminated. Consequently, the separations were easily achieved by simply altering the stationary phase used.

2.4.4 Pentafluorophenyl propyl phase selectivity

The development and use of fluorinated phases for altered RPLC selectivity has grown in recent years [29-30]. When a C-H bond is replaced with a C-F bond, especially in the case of aromatic functional units, the developed phase can show different retention mechanisms compared to typical alkyl-based phases [31]. Fluorine is a group 17 halogen and is rich in electrons. When fluorine is incorporated into bonded phases, it can promote ion-exchange type interactions with electron-deficient analytes. In the PFPP phase, five fluorines provide additional electrons, and as a result, the phase has the ability to polarize analytes [32-33]. With our model analyte set, it was clear that analytes containing protonated amines were retained longer on the PFPP phase compared to the aqueous C18 phase. Hydromorphone (6), hydrocodone (6), the formyl peptide (formyl-Met-Leu-Lys-Ile-Ile-OH, 22), and methamphetamine (18) all exhibited this effect under the acidic mobile phase conditions tested. Furthermore, three analytes that co-eluted on the C18 phase, matrine (3), hydrocodone (5), and methamphetamine (18), were well separated on the PFPP phase using the generic 0.5% formic acid in water/methanol mobile phase gradient. This comparison is shown in Figure 2-8.

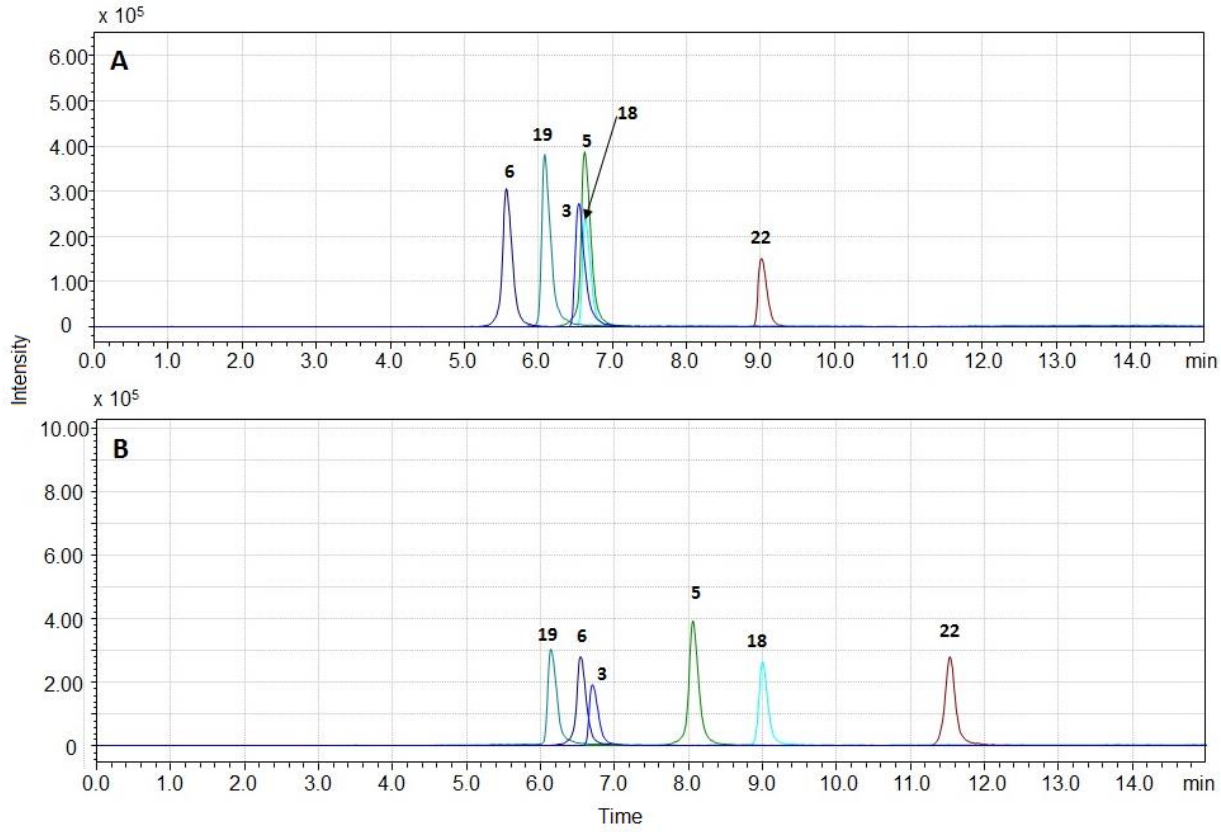


Figure 2-8: Significant selectivity and retention differences between phases: A) Aqueous C18 and B) PFPP (Mobile phase A: 0.5% formic acid in water, Mobile phase B: Methanol).

2.4.5 Effect of acid modifier on retention

When the analysis was performed in two different mobile phase conditions, a) 5 mM ammonium formate in water with methanol and b) 0.5% formic acid in water with methanol, using all four stationary phases, two groups of analytes behaved differently. Five analytes, including warfarin (10), estrone glucuronide (15), estrone sulfate (21), bentazon (16), and diclofenac sodium (8) were less retained with the ammonium acetate modifier compared to the formic acid modifier. On the other hand, all the other analytes less retained in the presence of acid (Supporting information Table 2-S2-2-S9). Differences are attributable generally to changes in the ionization state of the analytes at neutral versus acidic pH conditions. That said, sensitivity was significantly compromised for virtually all analytes when neutral buffer was used without acid. It is for this reason that the majority of attention was placed on evaluating selectivity effects under acidic mobile phase conditions above.

2.5 Concluding remarks

Use of the automated method scouting minimized the time necessary for programming the software for acquisition and readying different mobile phase and stationary phase combinations. This can greatly increase the efficiency of the method development process. In this study, a generic mobile phase scouting gradient was used to compare the retention and selectivity of four RPLC stationary phases. Such a strategy was shown to be very powerful, especially to find conditions appropriate to separate critical pairs of analytes. Because of the model analyte set chosen, it was necessary to use very weak and very strong elution conditions. In practice, the method scouting could be further optimized after an initial scouting run in an effort to target the efficient separation of a fewer number of target analytes. The method used here was relatively fast, but it could be made faster with higher flow rates and higher column temperatures, among other variables. Overall, this approach has been shown to be useful for evaluating multiple phase chemistries in parallel. The diversity of the analyte set revealed information about diverse molecular interactions that were responsible for separations, revealing interesting and useful trends in selectivity.

2.6 Acknowledgements

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2.8 Supporting information

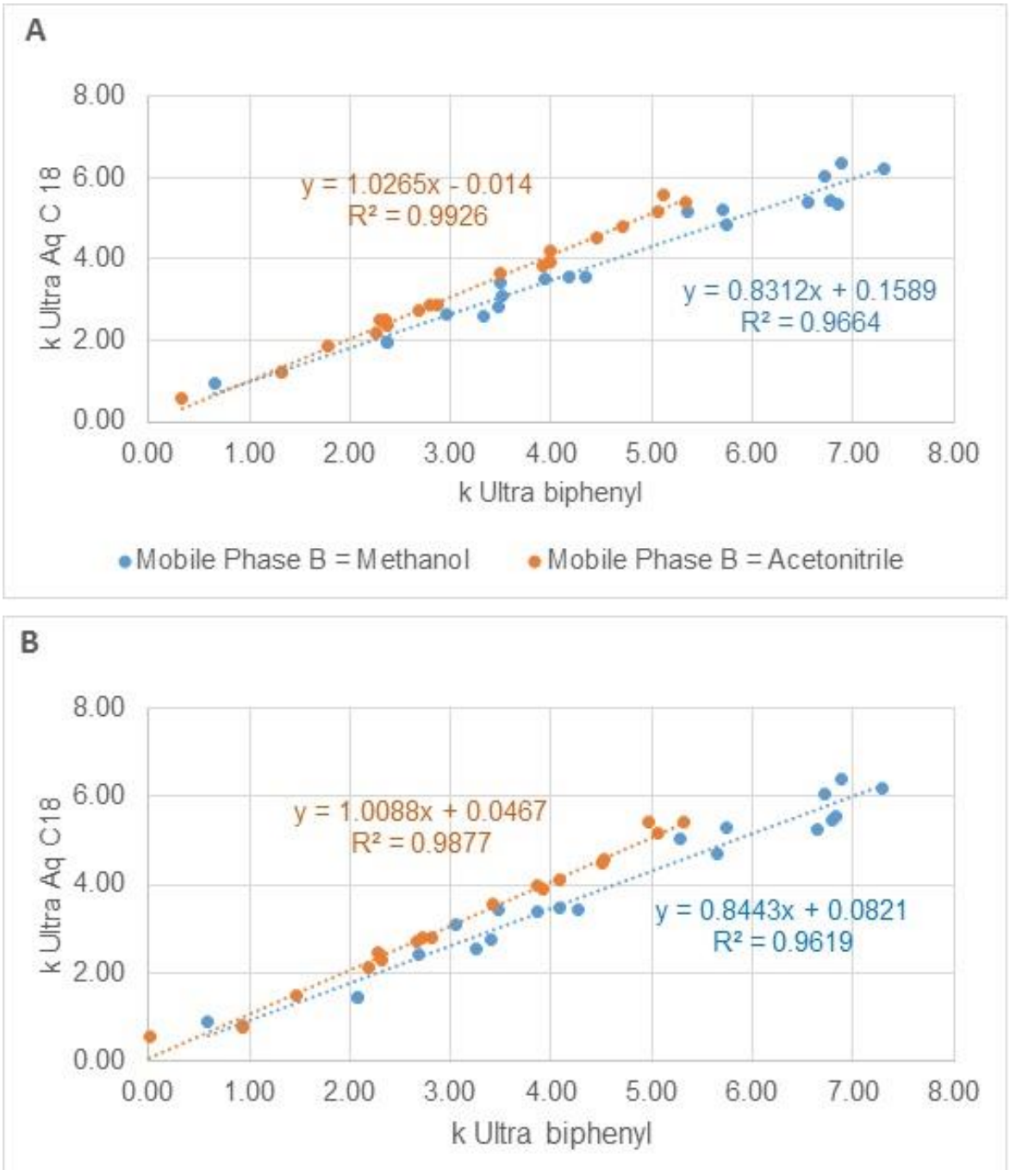

Figure 2-S1. Comparison of retention factors (k) when varying organic mobile phase between methanol and acetonitrile for A) Ultra Aq C18 phase with Ultra Biphenyl phase (Mobile phase A: 0.5% formic acid and 5 mM ammonium formate in water) and B) Ultra Aq C18 phase with Ultra biphenyl phase (Mobile phase A: 0.5% formic acid in water).

Table 2-1. Optimized MRM for the analytes

Number	Compound	Precursor Ion m/z	Product Ion m/z	Dwell time (msec)	Q1 pre Bias (V)	Collision Energy (V)	Q3 pre Bias (V)
1	Anabasine	163.20	118.20	20.0	-20.0	-24.8	-11.0
2	Cotinine	177.20	80.15	20.0	-21.0	-25.6	-30.0
3	Matrine	249.10	148.20	20.0	-30.0	-33.2	-14.0
4	Nicotine	163.20	117.15	20.0	-19.0	-27.6	-19.0
5	Hydrocodone	300.25	199.25	20.0	-20.0	-31.0	-20.0
6	Hydromorphone	286.25	185.20	20.0	-14.0	-30.0	-18.0
7	Oxymorphone	302.10	227.20	20.0	-15.0	-25.0	-14.0
8	Diclofenac Sodium	296.15	214.00	20.0	-20.0	-33.0	-21.0
9	Diazepam	284.90	193.40	20.0	-14.0	-16.0	-13.0
10	Warfarin	307.15	161.10	20.0	14.0	18.6	29.0
11	Fentanyl	337.30	188.20	20.0	-22.0	-23.0	-20.0
12	Phenobarbital	231.15	188.15	20.0	25.0	10.0	13.0
13	Adenosine monophosphate (AMP)	348.00	136.20	20.0	-17.0	-21.6	-13.0
14	Reserpine	609.20	195.20	20.0	-30.0	-38.0	-20.0
15	Estrone glucuronide	445.30	269.20	20.0	20.0	30.4	29.0
16	Bentazon	239.20	132.10	20.0	25.0	26.0	26.0
17	Catechin	289.30	245.20	20.0	30.0	13.8	17.0
18	Methamphetamine	150.20	91.10	20.0	-17.0	-22.0	-15.0
19	Acetaminophen	152.00	110.15	20.0	-17.0	-19.0	-18.0
20	Histidine	156.20	110.20	20.0	-18.0	-15.0	-18.0
21	Estrone Sulfate	349.25	269.15	20.0	16.0	32.2	29.0
22	formyl-Met-Leu-Lys-Ile-Ile-OH	239.20	132.10	20.0	25.0	26.0	26.0

Table 2-2. Retention time data for the analytes. (Column: Ultra aq C18, Mobile phase A: 0.5% formic acid in water, Mobile phase B: Methanol)

Number	Compound	Trial 1 (min)	Trial 2 (min)	Trial 3 (min)	Average	Standard Deviation	RSD (%)
1	Anabasine	3.533	3.725	3.726	3.7	0.1	3.04
2	Cotinine	5.078	5.108	5.114	5.10	0.02	0.38
3	Matrine	6.530	6.549	6.547	6.54	0.01	0.16
4	Nicotine	3.533	3.730	3.733	3.7	0.1	3.13
5	Hydrocodone	6.623	6.629	6.630	6.627	0.004	0.06
6	Hydromorphone	5.557	5.571	5.574	5.567	0.009	0.16
7	Oxymorphone	5.253	5.277	5.279	5.27	0.01	0.27
8	Diclofenac Sodium	10.998	11.003	11.033	11.01	0.02	0.17
9	Diazepam	10.701	10.712	10.710	10.708	0.006	0.05
10	Warfarin	10.501	10.502	10.504	10.502	0.002	0.01
11	Fentanyl	8.524	8.528	8.525	8.526	0.002	0.02
12	Phenobarbital	8.260	8.280	8.297	8.28	0.02	0.22
13	Adenosine monophosphate (AMP)	2.579	2.887	2.904	2.8	0.2	6.56
14	Reserpine	9.297	9.299	9.298	9.298	0.001	0.01
15	Estrone glucuronide	9.596	9.606	9.599	9.600	0.005	0.05
16	Bentazon	9.332	9.344	9.399	9.36	0.04	0.38
17	Methamphetamine	6.630	6.642	6.640	6.637	0.006	0.10
18	Catechin	6.601	6.598	6.604	6.601	0.003	0.05
19	Acetaminophen	6.076	6.091	6.100	6.09	0.01	0.20
20	Histidine[a]						
21	Estrone sulfate	9.737	9.751	9.751	9.746	0.008	0.08
22	formyl-Met-Leu-Lys-Ile-Ile-OH	8.998	9.015	9.006	9.006	0.009	0.09

[a]- Either analyte compound was not detected or the peak is split in to two or more peaks and did not use in the retention time calculation.

Table 2-3. Retention time data for the analytes. (Column: Ultra biphenyl, Mobile phase A: 0.5% formic acid in water, Mobile phase B: Methanol)

Number	Compound	Trial 1 (min)	Trial 2 (min)	Trial 3 (min)	Average	Standard Deviation	RSD (%)
1	Anabasine	4.511	4.607	4.603	4.57	0.05	1.19
2	Cotinine	5.477	5.517	5.517	5.50	0.02	0.42
3	Matrine	7.258	7.237	7.235	7.24	0.01	0.18
4	Nicotine	4.516	4.605	4.606	4.58	0.05	1.13
5	Hydrocodone	7.860	7.842	7.834	7.85	0.01	0.17
6	Hydromorphone	6.579	6.573	6.569	6.574	0.005	0.08
7	Oxymorphone	6.337	6.334	6.331	6.334	0.003	0.05
8	Diclofenac Na	11.741	11.735	11.736	11.737	0.003	0.03
9	Diazepam	12.340	12.339	12.340	12.340	0.001	0.00
10	Warfarin	11.512	11.503	11.503	11.506	0.005	0.05
11	Fentanyl	9.923	9.896	9.880	9.90	0.02	0.22
12	Phenobarbital	9.349	9.348	9.352	9.350	0.002	0.02
13	Adenosine monophosphate (AMP)	2.140	2.470	2.489	2.4	0.2	8.29
14	Reserpine	11.438	11.399	11.369	11.40	0.03	0.30
15	Estrone glucuronide	11.602	11.599	11.602	11.601	0.002	0.01
16	Bentazon	10.056	10.061	10.059	10.059	0.003	0.03
17	Catechin	6.670	6.680	6.674	6.675	0.005	0.08
18	Methamphetamine	7.595	7.584	7.575	7.58	0.01	0.13
19	Acetaminophen	6.014	6.047	6.051	6.04	0.02	0.34
20	Histidine[a]						
21	Estrone sulfate	11.592	11.671	11.760	11.67	0.08	0.72
22	formyl-Met-Leu-Lys-Ile-Ile-OH	9.368	9.360	9.341	9.36	0.01	0.15

[a]- Either analyte compound was not detected or the peak is split in to two or more peaks and did not use in the retention time calculation.

Table 2-4. Retention time data for the analytes. (Column: Ultra PFPP, Mobile phase A: 0.5% formic acid in water, Mobile phase B: Methanol)

Number	Compound	Trial 1 (min)	Trial 2 (min)	Trial 3 (min)	Average	Standard Deviation	RSD (%)
1	Anabasine	4.336	4.402	4.419	4.39	0.04	1.00
2	Cotinine	4.433	4.495	4.509	4.48	0.04	0.90
3	Matrine	6.696	6.691	6.674	6.69	0.01	0.17
4	Nicotine	4.333	4.396	4.412	4.38	0.04	0.95
5	Hydrocodone	8.057	8.053	8.041	8.050	0.008	0.10
6	Hydromorphone	6.539	6.547	6.541	6.542	0.004	0.06
7	Oxymorphone	6.299	6.315	6.312	6.309	0.009	0.13
8	Diclofenac Na	10.872	10.879	10.871	10.874	0.004	0.04
9	Diazepam	10.597	10.599	10.588	10.595	0.006	0.06
10	Warfarin	10.561	10.566	10.558	10.562	0.004	0.04
11	Fentanyl		8.876	8.661	8.8	0.2	1.73
12	Phenobarbital	8.406	8.417	8.416	8.413	0.006	0.07
13	Adenosine monophosphate (AMP)	2.256	2.516	2.522	2.4	0.2	6.25
14	Reserpine[a]						
15	Estrone glucuronide	9.383	9.382	9.379	9.381	0.002	0.02
16	Bentazon	9.869	9.874	9.869	9.871	0.003	0.03
17	Catechin	6.936	6.948	6.940	6.941	0.006	0.09
18	Methamphetamine	9.001	8.983	8.958	8.98	0.02	0.24
19	Acetaminophen	6.141	6.162	6.168	6.16	0.01	0.23
20	Histidine[a]						
21	Estrone sulfate	9.505	9.513	9.507	9.508	0.004	0.04
22	formyl-Met-Leu-Lys-Ile-Ile-OH	11.531	11.510	11.486	11.51	0.02	0.20

[a]- Either analyte compound was not detected or the peak is split in to two or more peaks and did not use in the retention time calculation.

Table 2-5. Retention time data for the analytes. (Column: Ultra IBD, Mobile phase A: 0.5% formic acid in water, Mobile phase B: Methanol)

Number	Compound	Trial 1 (min)	Trial 2 (min)	Trial 3 (min)	Average	Standard Deviation	RSD (%)
1	Anabasine	1.357	1.418	1.416	1.40	0.03	2.48
2	Cotinine[a]						
3	Matrine	4.145	4.251	4.217	4.20	0.05	1.29
4	Nicotine	1.356	1.410	1.419	1.40	0.03	2.44
5	Hydrocodone	4.539	4.572	4.572	4.56	0.02	0.42
6	Hydromorphone	2.724	3.091	3.055	3.0	0.2	6.84
7	Oxymorphone[a]						
8	Diclofenac Na	10.462	10.460	10.465	10.462	0.003	0.02
9	Diazepam	9.062	9.056	9.062	9.060	0.003	0.04
10	Warfarin	9.646	9.628	9.641	9.638	0.009	0.10
11	Fentanyl	6.464	6.455	6.461	6.460	0.005	0.07
12	Phenobarbital	7.333	7.317	7.306	7.32	0.01	0.19
13	Adenosine monophosphate (AMP)	2.002	2.154	2.129	2.10	0.08	3.89
14	Reserpine	7.724	7.717	7.719	7.720	0.004	0.05
15	Estrone glucuronide	9.219	9.216	9.233	9.223	0.009	0.10
16	Bentazon	9.663	9.649	9.675	9.66	0.01	0.13
17	Catechin	6.355	6.355	6.352	6.354	0.002	0.03
18	Methamphetamine	3.429	3.668	3.644	3.6	0.1	3.68
19	Acetaminophen	5.021	5.069	5.067	5.05	0.03	0.54
20	Histidine[a]						
21	Estrone Sulfate[a]						
22	formyl-Met-Leu-Lys-Ile-Ile-OH	7.442	7.435	7.441	7.439	0.004	0.05

[a]- Either analyte compound was not detected or the peak is split in to two or more peaks and did not use in the retention time calculation.

Table 2-6. Retention time data for the analytes. (Column: Ultra AQ C18, Mobile phase A: 5 mM ammonium formate in water, Mobile phase B: Methanol)

Number	Compound	Trial 1 (min)	Trial 2 (min)	Trial 3 (min)	Average	Standard Deviation	RSD (%)
1	Anabasine	8.110	8.243	8.354	8.2	0.1	1.48
2	Cotinine	8.245	8.230	8.214	8.23	0.02	0.19
3	Matrine*						
4	Nicotine	8.100	8.265	8.323	8.2	0.1	1.41
5	Hydrocodone*						
6	Hydromorphone*						
7	Oxymorphone	6.518	6.575	6.588	6.56	0.04	0.57
8	Diclofenac Sodium	9.580	9.457	9.410	9.48	0.09	0.93
9	Diazepam	10.641	10.631	10.631	10.634	0.006	0.05
10	Warfarin	9.370	9.226	9.162	9.3	0.1	1.15
11	Fentanyl	10.635	10.864	10.956	10.8	0.2	1.53
12	Phenobarbital*						
13	Adenosine monophosphate (AMP)	4.311	4.252	4.246	4.27	0.04	0.84
14	Reserpine	11.147	11.275	11.312	11.24	0.09	0.77
15	Estrone glucuronide	8.322	8.273	8.246	8.28	0.04	0.47
16	Bentazon	6.423	6.377	6.349	6.38	0.04	0.59
17	Catechin	6.713	6.670	6.676	6.69	0.02	0.35
18	Methamphetamine	8.458	8.569	8.615	8.55	0.08	0.94
19	Acetaminophen	6.116	6.092	6.067	6.09	0.02	0.40
20	Histidine	1.749	1.766	1.765	1.76	0.01	0.54
21	Estrone sulfate	8.731	8.680	8.652	8.69	0.04	0.46
22	formyl-Met-Leu-Lys-Ile-Ile-OH	9.691	9.677	9.671	9.68	0.01	0.11

Table 2-7. Retention time data for the analytes. (Column: Ultra biphenyl, Mobile phase A: 5 mM ammonium formate in water, Mobile phase B: Methanol)

Number	Compound	Trial 1 (min)	Trial 2 (min)	Trial 3 (min)	Average	Standard Deviation	RSD (%)
1	Anabasine	6.373	6.434	6.45	6.42	0.04	0.63
2	Cotinine	8.849	8.814	8.81	8.82	0.02	0.24
3	Matrine	7.906	8.081	8.165	8.1	0.1	1.64
4	Nicotine	6.38	6.434	6.443	6.419	0.034	0.53
5	Hydrocodone	8.718	8.95	9.05	8.9	0.2	1.91
6	Hydromorphone	7.181	7.363	7.432	7.3	0.1	1.77
7	Oxymorphone	6.585	6.668	6.693	6.65	0.06	0.85
8	Diclofenac Na	10.806	10.666	10.602	10.7	0.1	0.98
9	Diazepam	12.18	12.165	12.16	12.17	0.01	0.09
10	Warfarin	10.789	10.637	10.569	10.7	0.1	1.06
11	Fentanyl	10.688	10.821	10.9	10.8	0.1	0.99
12	Phenobarbital	9.268	9.202	9.207	9.23	0.04	0.40
13	Adenosine monophosphate (AMP)	2.596	2.534	2.496	2.54	0.05	1.99
14	Reserpine	14.051	14.327	14.388	14.3	0.2	1.26
15	Estrone glucuronide	10.349	10.242	10.211	10.27	0.07	0.71
16	Bentazon	7.273	7.202	7.172	7.22	0.05	0.72
17	Catechin	6.581	6.588	6.651	6.61	0.04	0.58
18	Methamphetamine	7.788	7.801	7.816	7.80	0.01	0.18
19	Acetaminophen	5.889	5.89	5.882	5.887	0.004	0.07
20	Histidine	1.329	1.351	1.356	1.35	0.01	1.07
21	Estrone sulfate	10.634	10.559	10.528	10.57	0.05	0.52
22	formyl-Met-Leu-Lys-Ile-Ile-OH	9.678	9.584	9.577	9.61	0.06	0.59

Table 2-8. Retention time data for the analytes. (Column: Ultra PFPP, Mobile phase A: 5 mM ammonium formate in water, Mobile phase B: Methanol)

Number	Compound	Trial 1 (min)	Trial 2 (min)	Trial 3 (min)	Average	Standard Deviation	RSD (%)
1	Anabasine	7.804	8.108	8.397	8.1	0.3	3.66
2	Cotinine	7.238	7.247	7.250	7.245	0.006	0.09
3	Matrine	8.250	8.556	8.856	8.6	0.3	3.54
4	Nicotine	7.799	8.102	8.409	8.1	0.3	3.76
5	Hydrocodone	9.763	10.173	10.490	10.1	0.4	3.59
6	Hydromorphone	7.758	7.980	8.265	8.0	0.3	3.18
7	Oxymorphone	7.349	7.556	7.777	7.6	0.2	2.83
8	Diclofenac Sodium	9.970	9.852	9.673	9.8	0.1	1.52
9	Diazepam	10.379	10.387	10.435	10.40	0.03	0.29
10	Warfarin	9.624	9.490	9.341	9.5	0.1	1.49
11	Fentanyl	14.418	14.224	14.014	14.2	0.2	1.42
12	Phenobarbital	8.151	8.124	8.139	8.14	0.01	0.17
13	Adenosine monophosphate (AMP)	2.852	2.609	2.459	2.640	0.198	7.51
14	Reserpine	13.833	13.577	13.446	13.6	0.2	1.45
15	Estrone glucuronide	8.000	7.938	7.842	7.93	0.08	1.00
16	Bentazon	6.781	6.652	6.506	6.6	0.1	2.07
17	Catechin	6.754	6.770	6.781	6.77	0.01	0.20
18	Methamphetamine	11.688	13.351	14.746	13	2	11.54
19	Acetaminophen	5.873	5.879	5.877	5.876	0.003	0.05
20	Histidine	1.590	1.672	1.702	1.65	0.06	3.50
21	Estrone sulfate	8.527	8.469	8.387	8.46	0.07	0.83
22	formyl-Met-Leu-Lys-Ile-Ile-OH	9.673	9.627	9.638	9.65	0.02	0.25

Table 2-9. Retention time data for the analytes. (Column: Ultra IBD, Mobile phase A: 5 mM ammonium formate in water, Mobile phase B: Methanol)

Number	Compound	Trial 1 (min)	Trial 2 (min)	Trial 3 (min)	Average	Standard Deviation	RSD (%)
1	Anabasine*						
2	Cotinine	5.922	5.921	5.923	5.922	0.001	0.02
3	Matrine*						
4	Nicotine*						
5	Hydrocodone						
6	Hydromorphone	6.903	6.696	6.671	6.8	0.1	1.88
7	Oxymorphone	4.917	4.822	4.845	4.86	0.05	1.02
8	Diclofenac Sodium	11.301	11.162	11.145	11.20	0.09	0.76
9	Diazepam	9.321	9.316	9.319	9.319	0.003	0.03
10	Warfarin	9.197	9.204	9.178	9.19	0.01	0.15
11	Fentanyl	8.702	8.676	8.688	8.69	0.01	0.15
12	Phenobarbital	7.292	7.330	7.316	7.31	0.02	0.26
13	Adenosine monophosphate (AMP)*						
14	Reserpine	9.932	9.951	9.956	9.95	0.01	0.13
15	Estrone glucuronide	8.464	8.483	8.462	8.47	0.01	0.14
16	Bentazon	8.654	8.639	8.601	8.63	0.03	0.32
17	Acetaminophen	5.041	5.037	5.040	5.039	0.002	0.04
18	Methamphetamine	6.102	6.050	6.108	6.09	0.03	0.52
19	Catechin	6.320	6.375	6.316	6.34	0.03	0.52
20	Histidine	1.717	1.646	1.623	1.66	0.05	2.95
21	Estrone sulfate	10.836	10.700	10.654	10.73	0.09	0.88
22	formyl-Met-Leu-Lys-Ile-Ile-OH	8.536	8.543	8.536	8.538	0.004	0.05

*- Either analyte compound was not detected or the peak is split in to two or more peaks and did not use in the retention time calculation.

Chapter 3

Silica Hydride Based Phases for Small Molecule Separations using Automated LC-MS Method Development

3.1 Abstract

Silica hydride, or Type C silica, has been developed as an alternative chromatographic support material for liquid chromatography. There are various bonded phases available with this new support. For four such phases (Cholesterol, Bidentate C18, Diamond Hydride, and Diol), retention and selectivity behavior were investigated using liquid chromatography coupled with triple quadrupole mass spectrometry. A set of small molecules from several chemical classes of interest, and varying in their physicochemical properties, were chromatographed under both reversed-phase and aqueous normal phase modes. To screen the columns, column switching was performed using an automated platform controlled by associated software and an additional valve. A typical scouting gradient was implemented. The separation conditions were not further optimized since the goal was simply to evaluate the variable retention behavior of the phases and selectivity under generic conditions. Further, retention of the analytes were evaluated under isocratic conditions with varying percentages of organic phase to visualize the potential for dual retention modes on the same column for certain analytes. Four analytes (fentanyl, hydrocodone, hydromorphone and matrine) showed dual mode retention behavior with all four phases. Especially, fentanyl exhibited dramatic “U-shaped” retention profiles on Cholesterol and Bidentate C18 phases. Overall, changes in the retention order between reversed phase and aqueous normal phases emphasized the potential for altered selectivity. Results showed that the Cholesterol phase provided the highest retention for most analytes compared to the other phases. The more polar Diol phase still provided good retention in reversed phase mode. Retention and selectivity were all highly reproducible.

Keywords: aqueous normal phase; Bidentate C18; column screening; selectivity; Type C Silica

3.2 Introduction

In liquid chromatography (LC), silica has been used as a solid support for chromatographic separations for many years [1]. Initially, silica was used alone, with no chemically bonded groups attached. As regular silica is polar, due to the presence of silanol groups, applications were limited to the separation of low-

polarity and non-polar compounds. With the development of reversed-phase column technology, silica was used as a solid support to bond different phases, mainly octadecylsilyl (ODS) or C18 phases [2-3]. With this development, the popularity of liquid chromatography bloomed rapidly, mainly due to its wider applicability.

These days, even with the most sophisticated manufacturing technologies, including high purity silica, end-capping, and various bonded phase chemistries, significant amounts of free silanol groups still remain in the final materials. These free silanol groups can have undesirable properties towards chromatographic separation, especially for basic analytes, where secondary interactions with free silanols can induce significant peak tailing. In order to avoid these undesirable properties, a new silica support known as silica hydride or type C silica has been developed. [4] These solid supports have significant advantages over regular silica, including the ability to withstand lower pH conditions, compatibility with higher aqueous composition mobile phases, and the reduction of unfavorable secondary interactions.

During the last decade, LC methods have been increasingly used for the analyses of biological samples, especially in cases such as for diagnostic purposes, for evaluation of progress of a particular disease, and for monitoring the efficiency of a particular treatment. Moreover, such LC methods have been used for the quantification of some currently used drugs, such as immunosuppressive agents, which have narrow therapeutic ranges and require precise quantification [5-6]. Many samples are already quite complex, but as the list of analytes of interest, which vary widely in their structure, polarity, and acidity, grows, this places a larger burden on separation technology. As determinations become more comprehensive and detailed, the discovery of new phases with specific retention capabilities and broad selectivity becomes very important. As a means to address this need, phases with multi-mode interactions have been introduced into the market [7-9]. In order to develop methods for the separation of such complex mixtures, a number of columns often need to be screened in order to find the one that provides appropriate performance [10]. If automated screening technology can be used, the time that an operator needs to dedicate to this process can be significantly reduced.

Silica hydride or type C silica based columns were introduced almost two decades ago, but they are currently less utilized compared to the traditional type B silica phases offered by most manufacturers. Silica hydride phases are purported to have greater stability and can handle extremes of both aqueous

and organic mobile phases [11]. Bare or phase-bonded versions of silica hydride phases can be used to perform different modes of separations, including aqueous normal phase (ANP) which possess some of the features of hydrophilic interaction liquid chromatography (HILIC) and reversed phase (RP). HILIC depends on a thick water layer on the surface leading to a partition mechanism where ANP has less than a monolayer of water resulting in adsorption/displacement interactions for retention of polar analytes [12-13]. They can thereby separate multiple classes of analytes with the same column, requiring only a change in mobile phase composition.

The most commonly used reversed phase stationary phase is ODS. Retention of analytes with the ODS phase is mainly based on dispersive forces, influenced by the degree of hydrophobicity of the analyte. As the complexity of analyte mixtures increase, so does the need for phases, which can provide alternate selectivity. [7] One of the phases introduced on the silica hydride support is based on a linked Cholesterol unit. This moiety is capable of both hydrophilic as well as hydrophobic interaction properties. Because of this, the Cholesterol phase can be used in both reversed-phase and aqueous normal phase. Further, it has shown high resolving power and shape selectivity due to its liquid crystalline properties and associated phase transitioning nature [14].

Early in the development of liquid chromatography, the creation of stable stationary phases was a problem. Once bonding chemistry was refined, more stable phases were developed. Along these lines, one of the improvements was the creation of polymeric bonded phases rather than monomeric or link to the backbone via more than one linkage [15]. One such improved phase, Cogent Bidentate C18 provide all the properties of a conventional monodentate C18 phases, but as the name suggests, these bidentate phases are bonded via two linkages. Because of the extra linkage these phases are relatively more stable at lower pH compared to monodentate phases, minimizing degradation by hydrolysis. [16] Even though polymeric stationary phases are stable at low and high pH conditions, it is not easy to get a uniform coverage. In the case of Bidentate C18, a uniform coverage of the stationary phase has been reported to have been attained, improving reproducibility for retention among different production lots [17].

To separate hydrophilic analytes, it is often necessary to use chromatographic modes other than reversed-phase. That is because hydrophilic analytes, such as amino acids, sugars, and many metabolites, are poorly retained under reversed phase conditions. An alternative is aqueous normal

phase (ANP) mode. The phases used in ANP mode have both hydrophilic and hydrophobic interactions with the solutes [18-19]. The Diamond Hydride phase is one such chemistry that can support separations by ANP mode. The phase features approximately 2% coverage of carbon on the silica hydride surface, and therefore, this phase can be quickly equilibrated compared to stationary phases that have longer alkyl chains. The Diamond Hydride phase has been previously featured for use in the separation of hydrophilic compounds that are commonly found in metabolic analysis, namely amino acids, sugars and organic acids [20]. As further improvements to the stationary phases, polar phases such as cyano, diol, amino, and others were introduced. The Diol phase, one of the phases discussed here, has two hydroxyl groups per bonded unit, and it is expected that it will induce hydrogen bonding as the main interaction. One of the interesting applications of Diol phase is that it can be used for the analysis of sugars. It gives advantages such as stability at high pH and it will not react with sugars, as will more common amino acid based phases [21-22].

The aim of this work was to use a model small molecule analyte set for the study of the retention behavior of four different silica hydride based stationary phases under different modes of chromatography. Retention behavior of the phases were studied using generic methods under reversed-phase and aqueous normal phase conditions. Screening of the columns were performed with the use of Nexera Method Scouting Solutions software for the automation of column switching. Further, analysis was performed under isocratic conditions of different organic phase percentages to more closely investigate the dual mode retention behavior of the analytes. Results have shown that these phases can be successfully used in reversed-phase as well as ANP mode for the retention and separation of a wide range of analytes. Further, these results show that the columns studied can be used for the separation of vastly different classes of analyte groups just by changing the mobile phase compositions. Finally, this study demonstrates a rapid means for screening columns in an automated fashion for the analysis of multiple classes of molecules, using different modes of chromatography with minimal user intervention.

3.3 Experimental

3.3.1 Materials, reagents, and sample preparation

Small molecule standards were prepared either using stock solutions of 1 mg/mL in methanol for acetaminophen, diazepam, hydrocodone, hydromorphone, methamphetamine, oxymorphone, phenobarbital, and warfarin (Cerilliant Corporation, Round Rock TX, USA) or using solid standards for adenosine monophosphate, anabasine, bentazon, cotinine, catechin, diclofenac sodium, estrone glucuronide, estrone sulfate, histidine, matrine, nicotine, reserpine, and thiamine hydrochloride (Sigma – Aldrich, St. Louis MO, USA). Formyl peptide (Formyl-Met-Leu-Lys-Ile-Ile-OH) was obtained from Johns Hopkins University (Synthesis and Sequencing Facility, Baltimore MD, USA). All solid standards were dissolved in water to make 1 mg/mL stock solutions. The working solutions for the analyses were prepared as a mixture of all 23 analytes using the stock solutions and concentrations were kept as 100 ng/mL for methamphetamine and fentanyl and 1 µg/mL for all the other analytes. LC-MS grade solvents, water and acetonitrile were purchased from Honeywell Burdick and Jackson (Morristown NJ, USA) and a mobile phase additive formic acid was purchased from Sigma – Aldrich (St. Louis MO, USA).

3.3.2. Instrumentation and settings

The HPLC system consisted of two Nexera LC-20ADXR pumps, a SIL-20ACXR auto-sampler, and a CTO-20AC column oven (Shimadzu Scientific Instruments, Inc., Columbia MD, USA). The column oven was equipped with one FCV-12AH two position six port valve and a six-position column switching valve. All tubing was either stainless-steel or PEEK (0.010 in id). Analytical columns used were from MicroSolv Technology Corporation (Leland NC, USA) and included four stationary phase variants (all 100 x 2.1 mm, 2.2 µm dp): Cogent™ UDC-Cholesterol; Cogent Diamond Hydride™; Cogent Bidentate C18™; and Cogent Diol 2.0™. Figure 3-1 shows a generic depiction of the phases, except the Diamond Hydride phase, which was not available. The Diamond Hydride phase is an unmodified silica hydride with a few unspecified organic moieties attached, according to the manufacturer. The temperature of the oven that houses the column selection valve and columns was set to 30 °C. Water with 0.1% formic acid (Pump A; Line 1) was used as the aqueous mobile phase and acetonitrile with 0.1% formic acid (Pump B; Line 1) was used as the organic mobile phase. A solution of 5:95 water: isopropanol (v/v) was used as the needle

wash solution. Sample injection volumes were 5 µL. A scouting gradient of 5% to 95% B over nine minutes at 0.2 mL/min was used for all reversed phase analyses. In the aqueous normal phase analyses, 0.1% formic acid in water (Pump A, Line 1) and 0.1% formic acid in acetonitrile (Pump B, Line 1) was used and a scouting gradient of 95% to 55% of B over nine minutes at 0.2 mL/min was programmed. All other parameters were unchanged from the reversed-phase analyses. To investigate and visualize the dual retention modes of the stationary phases, water and acetonitrile with 0.1% formic acid were used. These experiments were performed in isocratic mode with different percentages (ranging incrementally from 10% to 90%) of organic phase.

Determination of dead time (t0) for the analysis was performed using a combination of experimental and calculation methods. First, the column dead volume was determined using the formula Vm = 0.5 L dc 2 where L is the length and dc is the diameter of the column. Then, Vm was divided by the flow rate to calculate the dead time due to the column itself (tc). In order to find the dead volume due to all the other components (ta) (connectors, tubing and valves etc.), the column was replaced with a zero-dead volume adaptor and uracil was injected as an analyte. Then total dead volume was calculated by adding tc and ta and this value was used to calculate retention factors for the analytes on the different columns. [23]

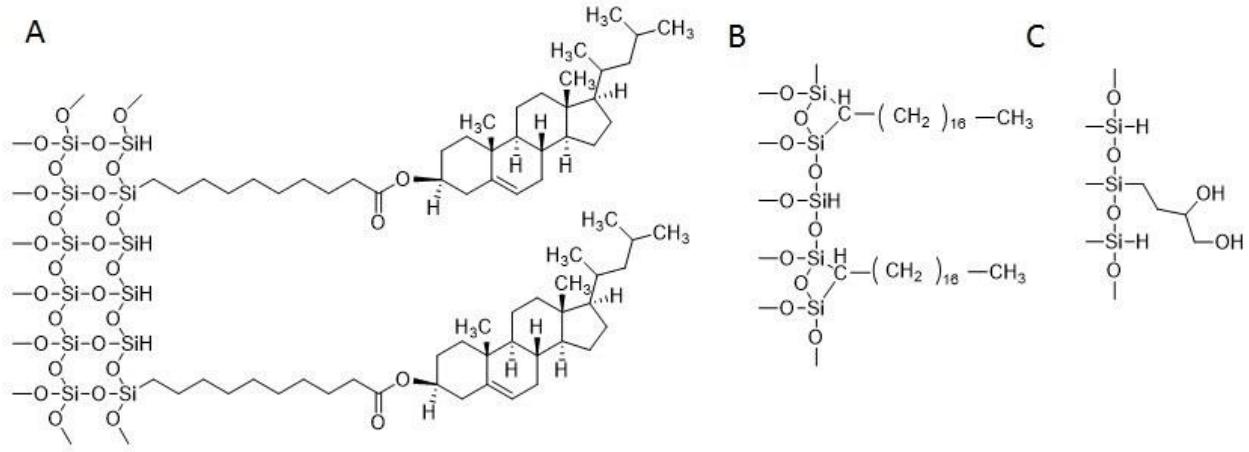

Figure 3-1: Different phases investigated. (A). UDC Cholesterol (B). Bidentate (C). Diol (fourth phase is not shown)

3.3.3. Mass spectrometry parameters

The MS system was a Shimadzu LCMS-8050 triple quadrupole mass spectrometer with a heated electrospray ionization (ESI) source. The MS was operated under multiple reaction monitoring (MRM) conditions with 5 msec polarity switching to detect both positively and negatively charged ions. The interface voltage was ±4.5 kV; drying gas and nebulizing gas flow rates (nitrogen) were 15 and 3 L/min, respectively; the heat block temperature was kept at 400 °C and desolvation line temperature was kept at 300 °C. The collision gas (argon) pressure was set to 270 kPa. The data analysis was performed using Shimadzu LabSolutions Software (v.5.65). The optimized MRM transitions used to detect the analytes are given in Table S1 of the Electronic Supplementary Information document.

3.4 Results and Discussion

3.4.1. Overall performance of the phases

Liquid chromatography column manufacturing is growing rapidly and providing researchers with a wealth of options to address their particular applications. The vast majority are silica-supported phases, but they vary widely in terms of different bonded chemistries to provide a range of selectivities [23-24]. The phases used in this study are different from most others on the market. For one, they are designed to be used in different liquid chromatography modes by changing the mobile phase combinations. Therefore, the applications for which these columns can be used is potentially wider. This is mainly due to the use of type C silica as the particle of choice for bonding phases. Further, the phases that are used have special features. For example, the Cholesterol phase has been reported to have size and shape selectivity due to more ordered structure in the bonded unit with the silica hydride support. This ordered structure is purported to be present because of the liquid crystal nature of the cholesterol ligand [25].
In this study, all the experiments were performed using water and acetonitrile as the mobile phase with formic acid as an additive under 30 °C temperature. Temperature can be used as an important variable to get better resolution and peak shapes in chromatography. There have been several studies using silica hydride phases to see the effects of temperature on different modes of chromatography. Pesek et. al. has shown that for aniline retention decreases as the temperature increases in both reversed-phase and ANP modes whereas for metformin retention decreases under 50 °C and retention increases at higher

temperatures [26]. Further, Soukup et. al. has performed another study using all the phases discussed here to investigate the mobile phase temperature effect on the separation [27]. According to them, retention and peak width decreases when the temperature was increased and it was significant with

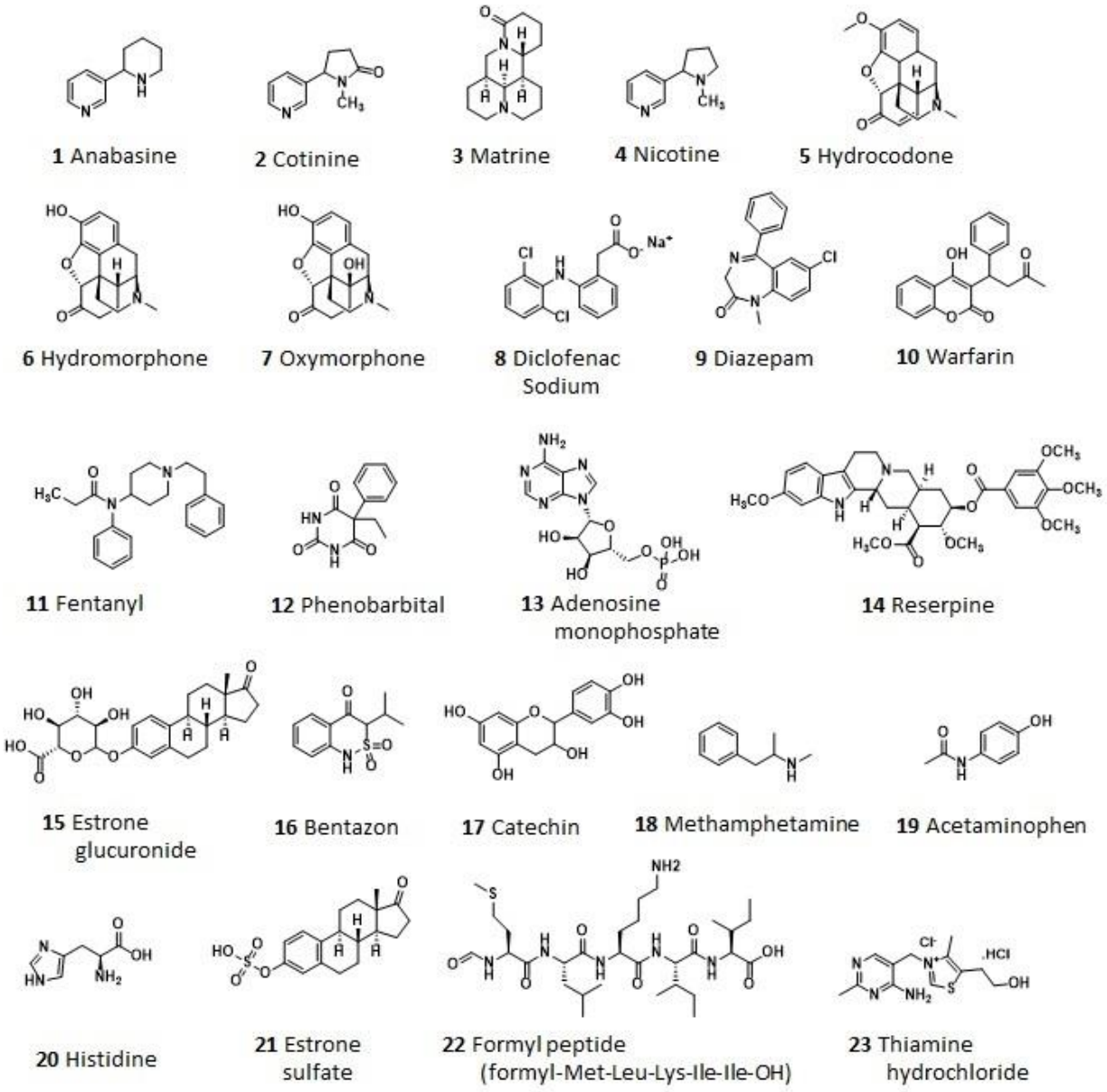

Figure 3-2: Structures of the analytes selected to provide wide range of physicochemical properties

Diamond Hydride phase under the HILIC mode. Further, changes in selectivity has also been observed with these phases at higher temperatures for phenolic acids. Another study performed by Pesek et. al. on

Diamond Hydride phase using amino acids to investigate the effects on temperature under the reversed-phase mode and have found that the retention increases as the temperature was increased [20]. Therefore, temperature control can be used to improve the peak width, selectivity and to get retention increased or decreased depending on the mode of chromatography you want to used. Several studies can be found where effects of the mobile phase additives on silica hydride phases. One such study using Diamond Hydride phase with amino acid analysis has shown that peak shapes can be improved significantly by using small amounts of trifluoroacetic acid (TFA) instead of either formic acid or acetic acid. Further, it has shown that using a stronger TFA has decreased the retention for amino acids significantly [28].

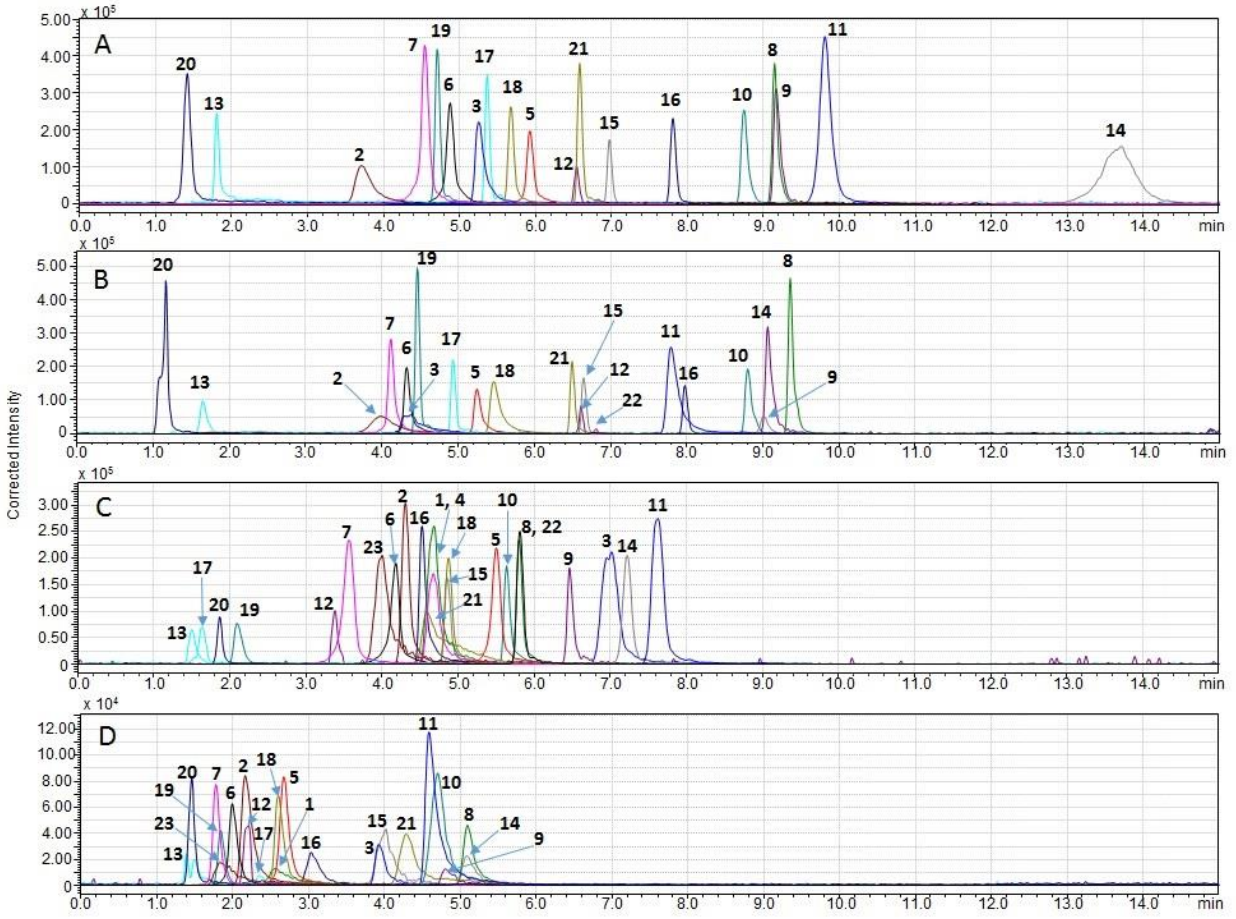

Figure 3-3: Comparison of the four phases in reversed-phase mode for the separation of model analyte set: (A) Cogent™ UDC-Cholesterol; (B) Cogent Bidentate C18™; (C) Cogent Diamond Hydride™; (D)Cogent Diol 2.0™.

When the overall retention of the model analytes was compared (Figure 3-3), it was apparent that the Cholesterol phase retained most of the compounds longer compared to all the other three phases used in the study. As the Cholesterol phase has a relatively high carbon load (13-14%) and both hydrophilic and hydrophobic groups, it can interact with many analytes and can provide increased retention. The Diol phase, being the most polar among the four phases investigated, showed less retention for most of the analytes tested. Less carbon loading (9-10%) in the Diol phase likely contributed to the decreased retention compared to the other phases, as well as the presence of hydroxyl units, which do not support strong interactions through the hydrophobic effect. The bidentate phase showed similar retention to that of the Cholesterol phase, except for a few analytes, such as fentanyl (11) and reserpine (14), which were retained significantly less with the bidentate phase. Analytes histidine (20) and adenosine mono phosphate (13) showed similar retention on all four phases, but their elution order was switched on the Diamond Hydride phase compared to the Cholesterol and Bidentate C18 phases; they co-eluted on the Diol phase. The retention behavior and selectivity of the model analyte set on the different columns were obtained using generic scouting gradient conditions. None of these conditions were optimized for any column and the same scouting method was used for the comparison. Further optimization could be envisioned to improve the separation of any of the analytes of interest, if desired.

3.4.2. Analyte retention on Cholesterol versus Bidentate C18 phases

When the Cholesterol and Bidentate C18 phases were compared (Figure 3-S1) for specific retention differences, it was clear that reserpine (14) exhibited significantly higher retention on the Cholesterol phase. Further, reserpine had a broader peak shape on the Cholesterol phase. Both the longer retention and the broader peak shape could be attributed to the multi-mode interaction that reserpine can have with the Cholesterol phase. The reason for this behavior is that reserpine is large and has many polar groups. Both hydrogen bonding and dispersive interactions can contribute to retention on the Cholesterol phase. Two analytes, diclofenac (8) and diazepam (9) co-eluted on the Cholesterol phase but were well separated using the Bidentate C18 phase. On the other hand, the analytes matrine (3) and hydromorphone (6) were baseline separated with Cholesterol phase, but co-eluted on the Bidentate C18 phase.

3.4.3. Analyte retention on Cholesterol versus Diamond Hydride phases

When comparing the retention between the Cholesterol and Diamond Hydride phases (Figure 3-S2) under reversed- phased conditions, reserpine (14) and matrine (3) showed significant differences in retention. The exact reason for the decreased retention of matrine with the Cholesterol phase is not fully understood. Reserpine showed significantly less retention with the Diamond Hydride phase whereas, matrine was retained significantly longer with the Diamond Hydride compared to the Cholesterol phase. Peak shape is well improved with Diamond Hydride phase for reserpine compared to the Cholesterol phase. These behaviors could be due to the interactions that these analytes can have with the larger Cholesterol ligand compared to low-carbon loaded Diamond Hydride phase. Further, with the Cholesterol phase, more polar analytes anabasine (1) and nicotine (4) exhibited broader peaks (> 4 min) and therefore, had relatively low intensity. These two analytes were co-eluted on the Diamond Hydride phase and had narrower peak widths and higher intensity. These two analytes are very similar in structure and therefore will produce similar interactions with the stationary phases. According to the literature, these two analytes have shown similar behavior under reversed phase conditions in other separation systems [23]. Thiamine hydrochloride (23) was detected with the Diamond Hydride phase but not with the Cholesterol phase. This is again due to the fact that thiamine hydrochloride experienced extensive band broadening (peak width > 4 min) and as a result could be detected with a significant intensity using the standard screening method. Broader peak shapes for thiamine chloride with the Cholesterol phase could be due to multiple types of interactions because of the hydrophobic and hydrophilic groups present in the structure of thiamine hydrochloride.

3.4.4. Analyte retention on Cholesterol versus Diol phases

Under the reversed-phase conditions, the Diol phase showed the least retention for most of the analytes studied. However, the retention is still significant as Diol phases are usually used to retain analytes in normal phase and HILIC chromatography modes. The silica hydride-based Diol phase is less hydrophilic compared to standard silica-based diol phases, due to the fact that solid support silica hydride does not have free silanols. Another feature noted with the Diol phase is the difference observed in peak shape.

Most of the analytes exhibited higher peak asymmetry compared to the Cholesterol phase. (Figure 3-S3) This is likely due to the fact that analytes will experience both hydrophilic interactions with the hydroxyl ligands and hydrophobic interactions with the other carbon groups in the phase, similar to that which might be observed using a type B silica support. Two analytes, phenobarbital (12) and estrone sulfate (21), which co-eluted on the Cholesterol phase were well separated with the Diol phase.

3.4.5. Aqueous normal phase behavior of the phases

When the four phases were compared (Figure 3-4) using an automated scouting gradient under ANP conditions, it could be seen that the analytes were separated into generally two groups. The distribution coefficient (log D) values of a particular compound shows how hydrophobic or hydrophilic that compound is under a given pH. [29] When the log D values at the pH corresponding to the mobile phase conditions (apparent pH of 2.70) were compared for the analytes in these two groups, it shows that early eluting analytes have log D values greater than zero and greater in magnitude than the group of analytes eluting later (Figure 3-S5). When the log D value is greater than zero, that compound is more hydrophobic. Under the conditions used in the ANP mode, we have higher organic mobile phase content at the beginning and therefore those compounds that have log D values greater than zero would elute early. The Bidentate C18 phase showed the least retention for the analytes compared to the other phases, as expected. This is due to the fact that C18 induces mainly hydrophobic interactions with analytes, and under ANP conditions (high acetonitrile content), such interactions are not favored. Reserpine (14) and fentanyl (11) were strongly retained with all four phases; they exhibited similar retention behavior under the reversed phase condition as well. When we looked at the structures of these two analytes, both of them contain hydrophobic ring portions, as well as hydrophilic groups. More specifically, in the case of reserpine, there is an ester group and fentanyl has an amide group. Further, when we looked at the log D values of these two compounds at pH 2.7 (reserpine = 0.96, fentanyl = 0.79) are very close to zero meaning they have intermediate properties of hydrophilicity and hydrophobicity. Analytes such as diclofenac sodium (8) phenobarbital (12), estrone glucuronide (15), bentazon (16), catechin (17), and estrone sulfate (21), among others, were less retained among all the phases studied. When log D vs retention factor (k) was plotted for all the analytes, it clearly shows that analytes with positive log D values

are less retained compared to other analytes (Figure 3-S5). Analytes cotinine (2), hydrocodone (5), hydromorphone (6), and histidine (20) all have negative log D values and they exhibit longer retention in ANP mode on all the phases.

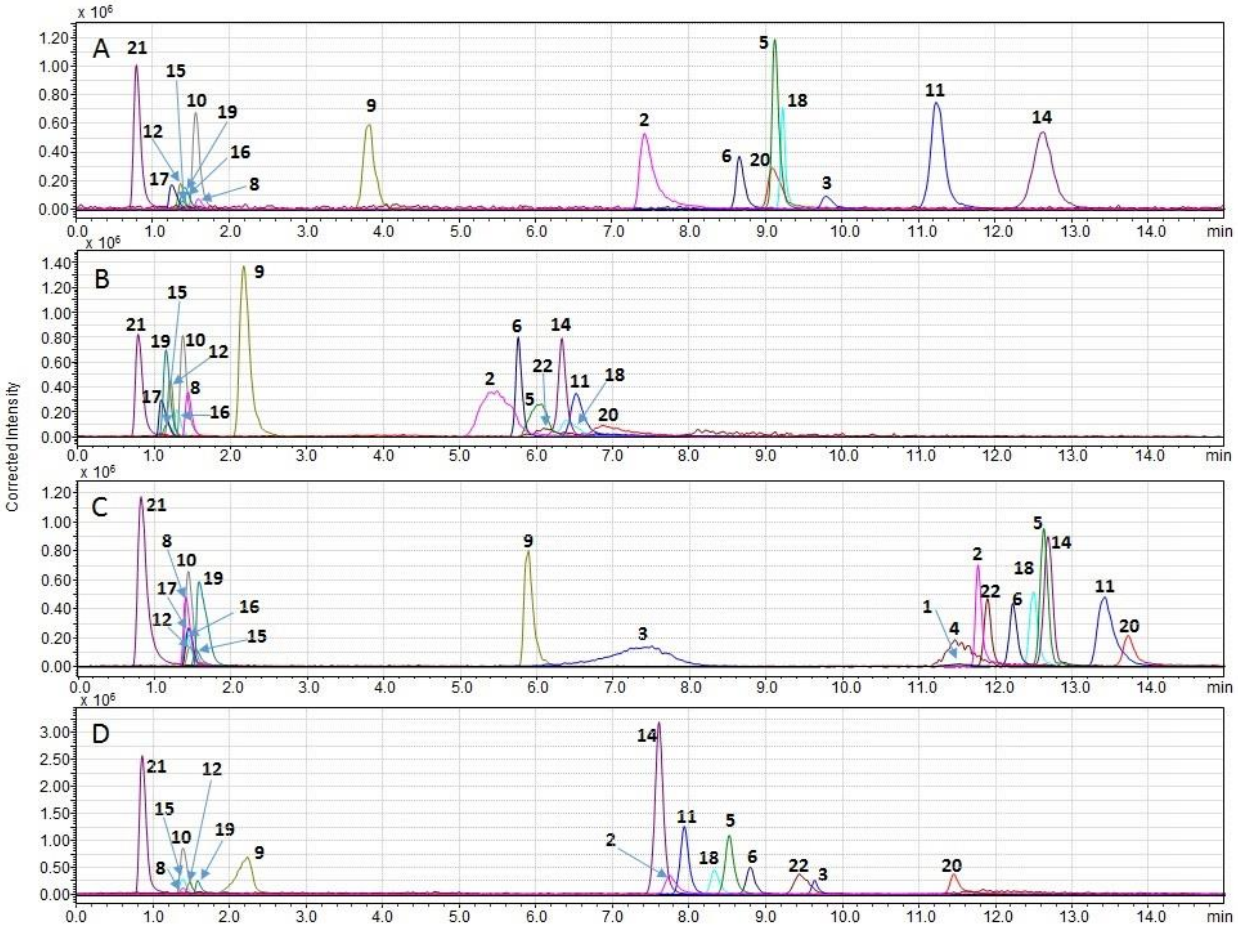

Figure 3-4: Comparison of the four phases in aqueous normal phase mode for the separation of model analyte set: (A) Cogent™ UDC-Cholesterol; (B) Cogent Bidentate C18™; (C) Cogent Diamond Hydride™; (D)Cogent Diol 2.0™.

3.4.6. Dual mode retention behavior of the analytes

Application of liquid chromatography – mass spectrometry in pharmaceutical analysis for drugs and drug metabolites has increased rapidly during the past decade. Most of the analytes involved in these fields have polar functional groups and may therefore be less retained under more commonly used

reversed phase condition [30-31]. According to the literature, more polar compounds are retained longer under ANP conditions [32-33]. The chromatographic stationary phases discussed here have shown that they can be utilized under both reversed phase as well as aqueous normal phase mode conditions. In order to see the dual mode behavior of the analytes with the stationary phases, a set of analyses was performed isocratically using different percentages of acetonitrile. The retention factor for each analyte was plotted against the acetonitrile composition in the mobile phase. Four analytes, matrine (3), hydrocodone (5), hydromorphone (6) and fentanyl (11) exhibited significant retention both at lower and higher acetonitrile concentrations as shown in Figure 3-5. With low acetonitrile content, analytes are retained through a reversed phase mode, and at high acetonitrile, they are retained through an ANP mode. All of the analyses were performed in triplicate and the average retention factor was used for graphing. Raw retention factor data is available in the supporting information in Tables 3-S3 – 3-S4.

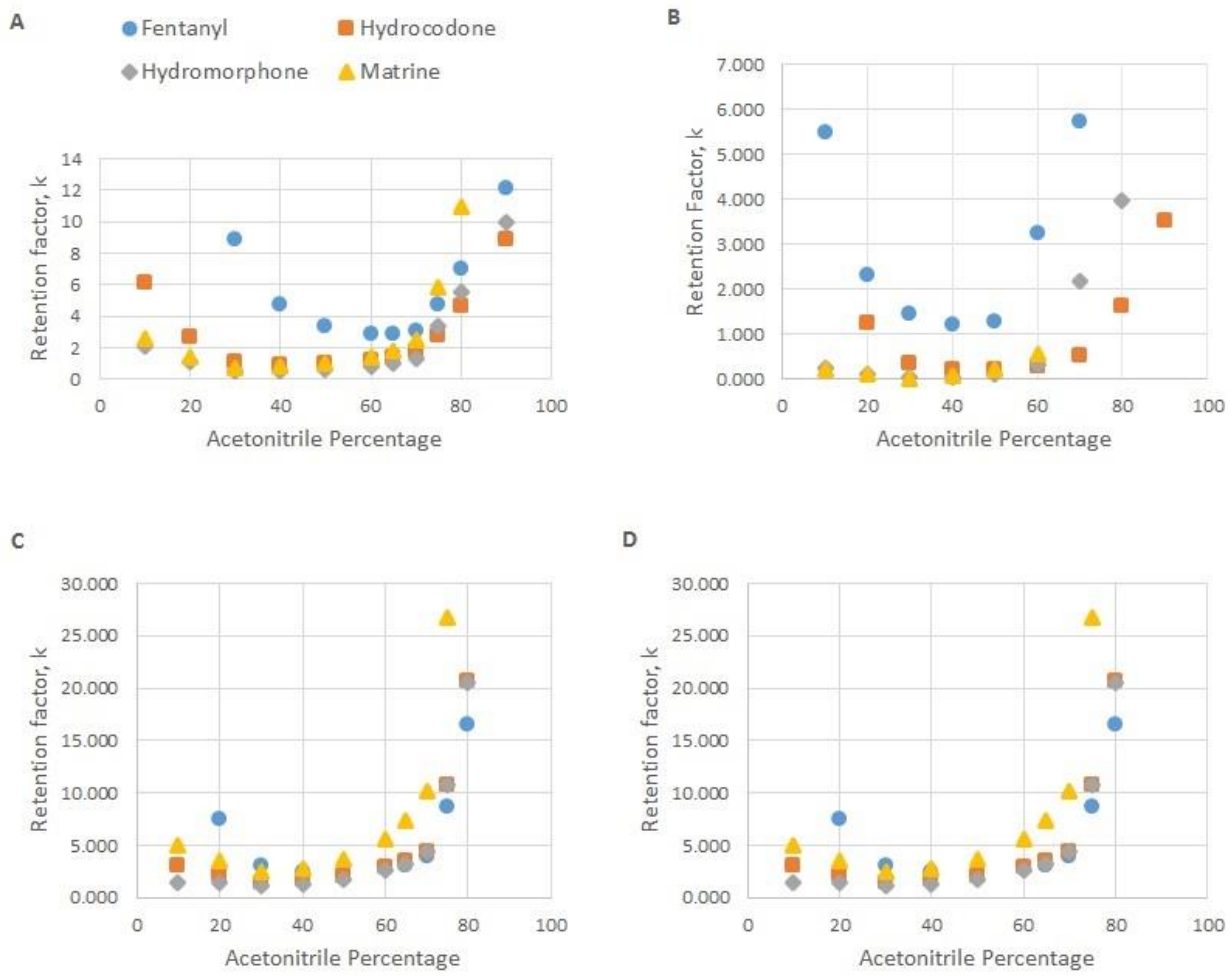

Figure 3-5: Dual mode retention- Change of retention with the acetonitrile content in the mobile phase. (A) Cogent™ UDC-Cholesterol; (B) Cogent Bidentate C18™; (C) Cogent Diamond Hydride™; (D)Cogent Diol 2.0™

With the Cholesterol phase, four analytes exhibited retention in both reversed phase and ANP regions. Fentanyl exhibited the most pronounced dual mode retention behavior (U-shaped curve) as a function of percent of acetonitrile in the mobile phase. Fentanyl (11) has basic properties and has two benzene rings in its structure. Therefore, under the conditions used in the analysis, it exhibited both hydrogen bonding interactions with the cholesterol ligand as well as dispersive interactions between the hydrophobic ring moieties available in both the analyte and the ligand. Other analytes, matrine (3), hydrocodone (5), and hydromorphone (6) contain amine groups that are protonated under the acidic conditions. At the same time, these analytes have non-polar ring structures. As a result of these two

types of structural motifs, both hydrophilic and hydrophobic interactions with the stationary phase can occur. The rings in fentanyl are well spaced and readily available to maximize interaction with the phase relative to the other three analytes. Two analytes, bentazon (16) and warfarin (10) returned retention only in the reversed phase region when the analysis was performed. To see the retention behavior under different acetonitrile percentages, see Figure 3-S4. Bentazon has sulfone and amine group in its structure in addition to the benzene ring. Under the acidified mobile phase conditions, these sulfone and amine group can participate in hydrogen bonding with the stationary phases making hydrophilic interactions more dominant. As a result, it was not expected to have a strong interaction with the stationary phase at higher organic percentages. This is further explained considering the log D value for Bentazon (2.94) is relatively large and positive. Similarly, warfarin, being a less polar analyte (log D = 3.41 at pH 2.7), exhibited less retention under high acetonitrile conditions.

3.5. Conclusions

In the current chromatography field, the complexity of the samples that are desired to be analyzed is always increasing. In order to analyze such complex mixtures, it is necessary to evaluate phases with alternate selectivities. The model analytes set used in this study includes analytes having a broad range of physical and chemical properties and therefore, the separation conditions used designed to be generic in order to study their relative retention on different phases. Further optimization can certainly be performed when specific analytes of interest are targeted. On the other hand, the analyte set can be further expanded to include more analytes where there are different functionalities such as hydroxyl groups, acids, amides and alkyl chains included in their structure to expand the study of retention behavior in reversed-phase mode, ANP mode, and dual mode retention behavior for these phases. According to the results, Cholesterol phase is giving the best retention for most of the analytes tested and it can be used in both RP and ANP mode. Analytes capable of having hydrogen bonding and having ring structures, cholesterol phase will be a good phase to start with. Diol phase being a polar phase still shows significant retention under the RP mode for most of the analytes investigated and it can be used in cases where it is necessary to separate co eluting analytes such as phenobarbital and estrone sulfate. When the retention is compared among the phases it shows that Cholesterol phase has shown

wider range from k =0.244 for AMP and k=9.504 for Reserpine whereas Diamond Hydride showed the smallest distribution of retention factor from 0.144 to 4.548 for the corresponding analytes under the reversed-phase mode. These selectivity ranges were exactly opposite under the ANP mode where Diamond Hydride phase showed significantly larger distribution of retention compared to all the other three phases. The analysis performed here used standard samples and therefore matrix effects were not accounted for. In real samples, this would need to be evaluated. For example, high salt matrices, such as urine, may cause significant interferences in ANP mode. Conversely, for plasma samples, which contain high abundances of lipids, matrix effects may be more readily observed in reversed phase mode. Therefore, method optimization will still be required when these phases are used for the real-world sample analysis.

3.6 Acknowledgements

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3.7 Supporting Information

Table 3-S1: Multiple reaction monitoring (MRM) conditions optimized for the model analyte set

Number	Compound	Precursor Ion m/z	Product Ion m/z	Dwell time /msec	Q1 pre Bias / V	Collision Energy/ V	Q3 pre Bias / V
1	Anabasine	163.20	118.20	20.0	-20.0	-24.8	-11.0
2	Cotinine	177.20	80.15	20.0	-21.0	-25.6	-30.0
3	Matrine	249.10	148.20	20.0	-30.0	-33.2	-14.0
4	Nicotine	163.20	117.15	20.0	-19.0	-27.6	-19.0
5	Hydrocodone	300.25	199.25	20.0	-20.0	-31.0	-20.0
6	Hydromorphone	286.25	185.20	20.0	-14.0	-30.0	-18.0
7	Oxymorphone	302.10	227.20	20.0	-15.0	-25.0	-14.0
8	Diclofenac Sodium	296.15	214.00	20.0	-20.0	-33.0	-21.0
9	Diazepam	284.90	193.40	20.0	-14.0	-16.0	-13.0
10	Warfarin	307.15	161.10	20.0	14.0	18.6	29.0
11	Fentanyl	337.30	188.20	20.0	-22.0	-23.0	-20.0
12	Phenobarbital	231.15	188.15	20.0	25.0	10.0	13.0
13	Adenosine monophosphate (AMP)	348.00	136.20	20.0	-17.0	-21.6	-13.0
14	Reserpine	609.20	195.20	20.0	-30.0	-38.0	-20.0
15	Estrone glucuronide	445.30	269.20	20.0	20.0	30.4	29.0
16	Bentazon	239.20	132.10	20.0	25.0	26.0	26.0
17	Catechin	289.30	245.20	20.0	30.0	13.8	17.0
18	Methamphetamine	150.20	91.10	20.0	-17.0	-22.0	-15.0
19	Acetaminophen	152.00	110.15	20.0	-17.0	-19.0	-18.0
20	Histidine	156.20	110.20	20.0	-18.0	-15.0	-18.0
21	Estrone Sulfate	349.25	269.15	20.0	16.0	32.2	29.0
22	formyl-Met-Leu-Lys-Ile-Ile-OH	239.20	132.10	20.0	25.0	26.0	26.0
23	Thiamine hydrochloride	265.20	122.20	20	-13	-17	-20

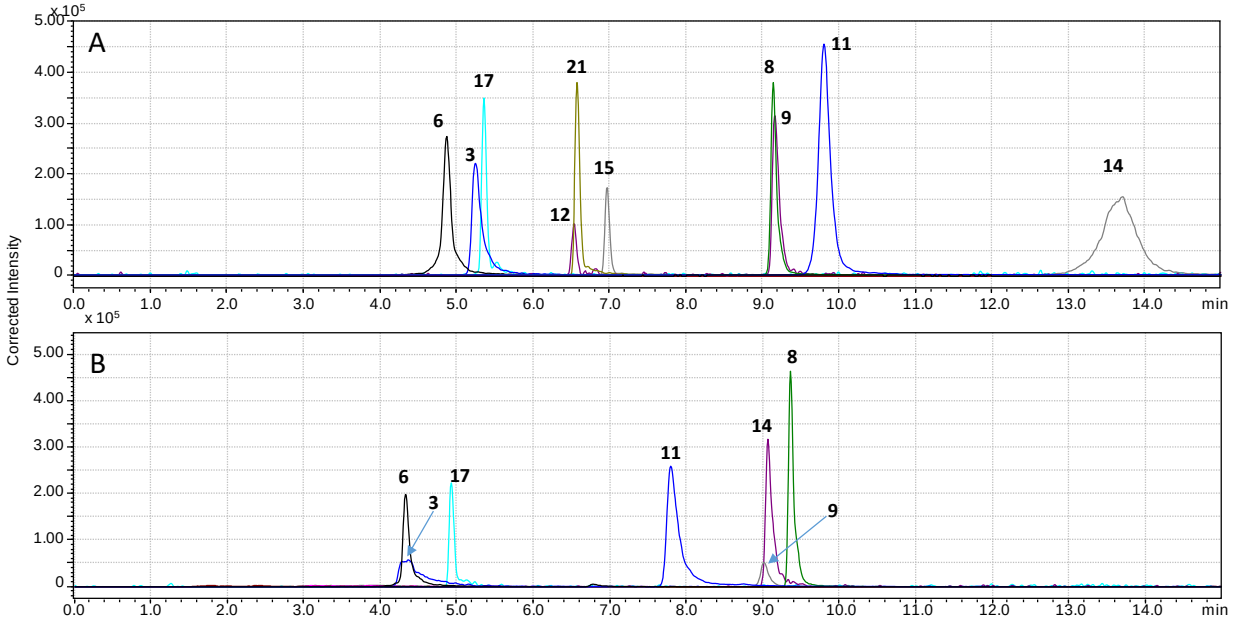


Figure 3-S1. Comparison of selected retention differences on the (A) Cogent™ UDC-Cholesterol and (B) Cogent Bidentate C18™ phases under reversed phase scouting gradient conditions.

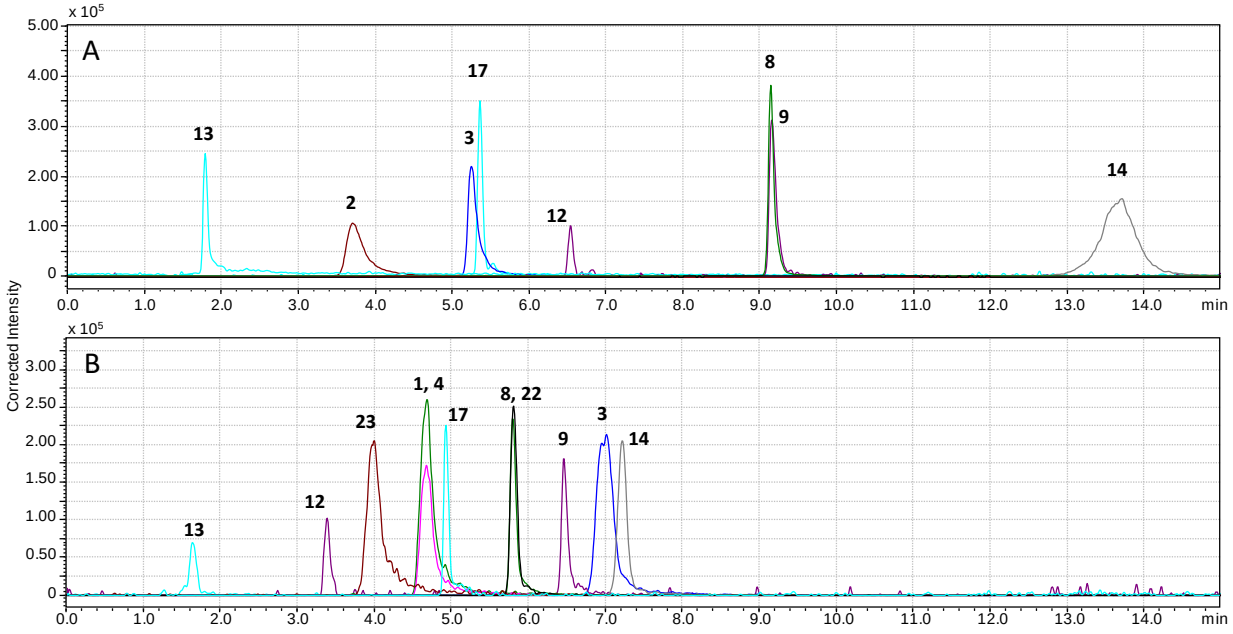


Figure 3-S2. Comparison of the two phases under reversed phase conditions for the specific retention differences. (A). Cogent™ UDC-Cholesterol; (B). Cogent Diamond Hydride™

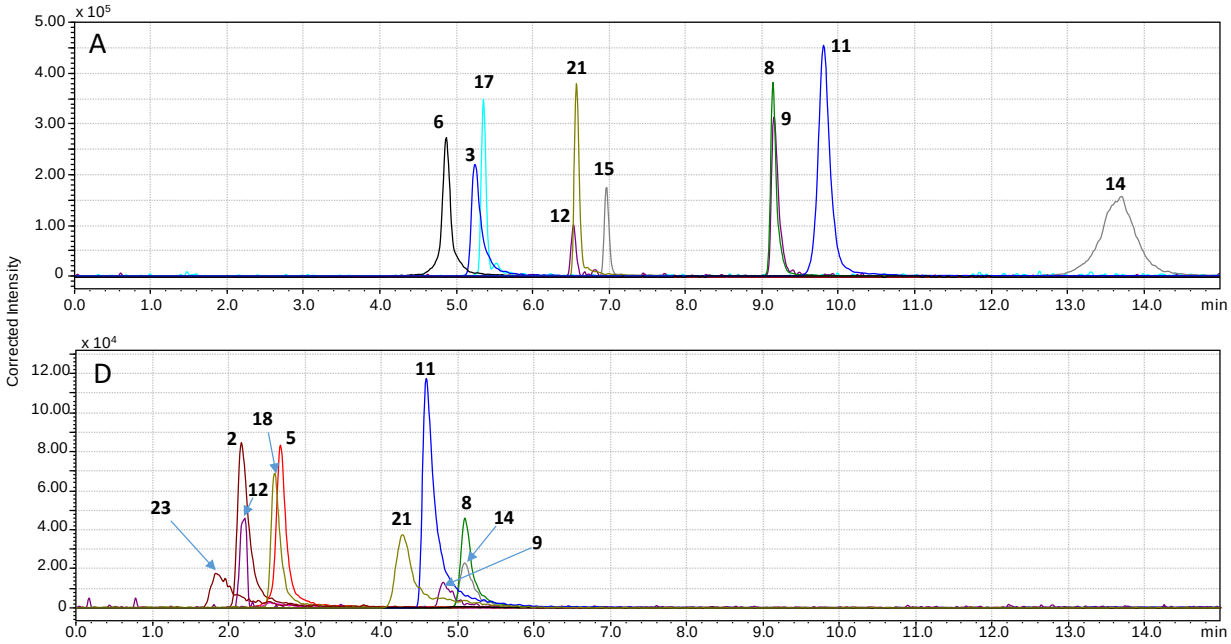
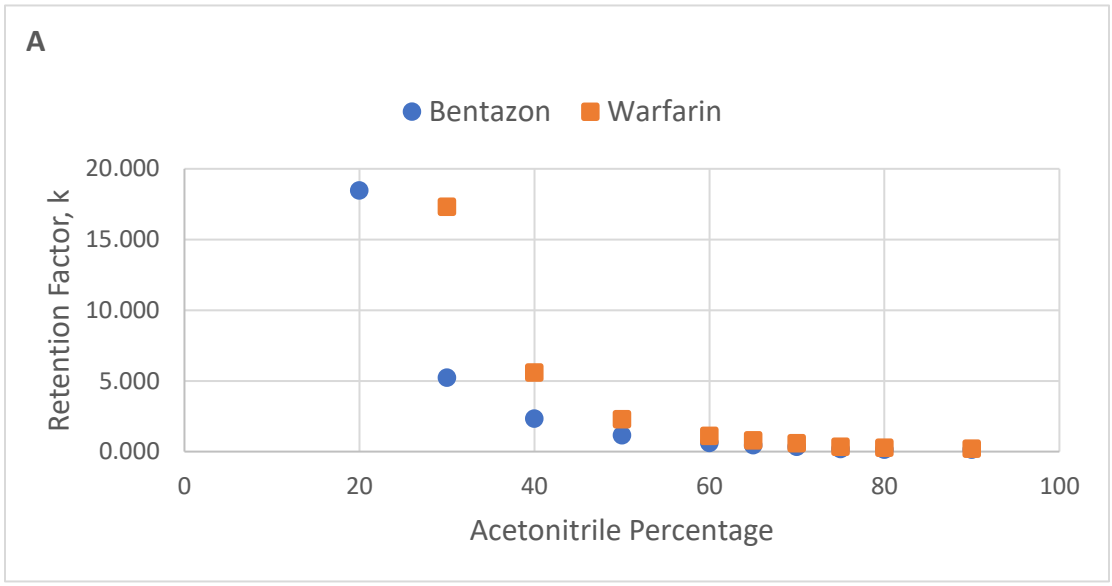
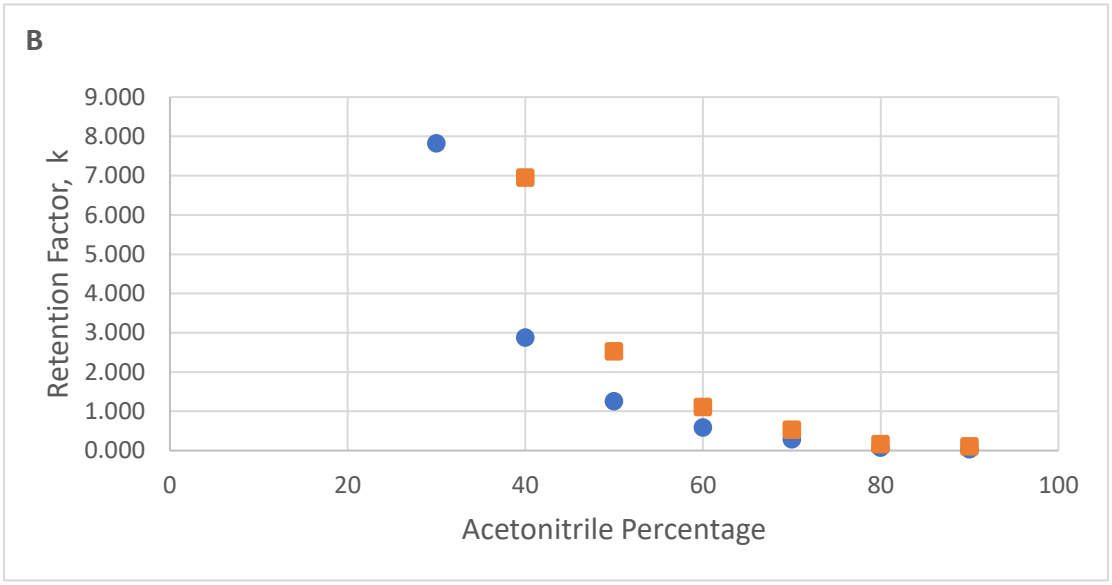


Figure 3-S3. Comparison of the two phases under reversed phase conditions for the specific retention differences. (A). Cogent™ UDC-Cholesterol; (B). Cogent Diol 2.0™


A
Bentazon
Warfarin
20.000
15.000
10.000
5.000
0.000
Retention Factor, k
0
20
40
60
80
100
Acetonitrile Percentage


B
9.000
8.000
7.000
6.000
5.000
4.000
3.000
2.000
1.000
0.000
Retention Factor, k
0
20
40
60
80
100
Acetonitrile Percentage

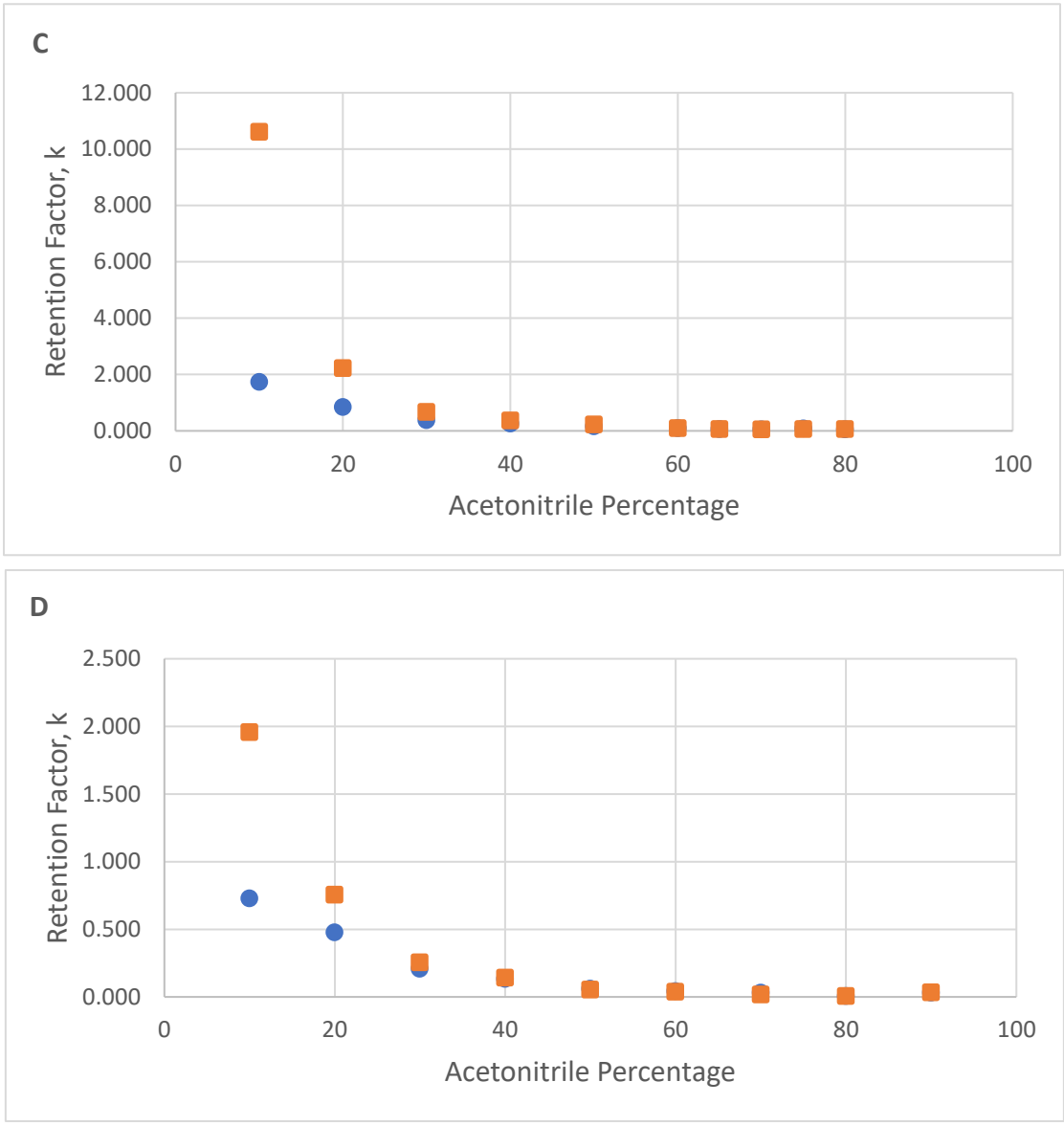
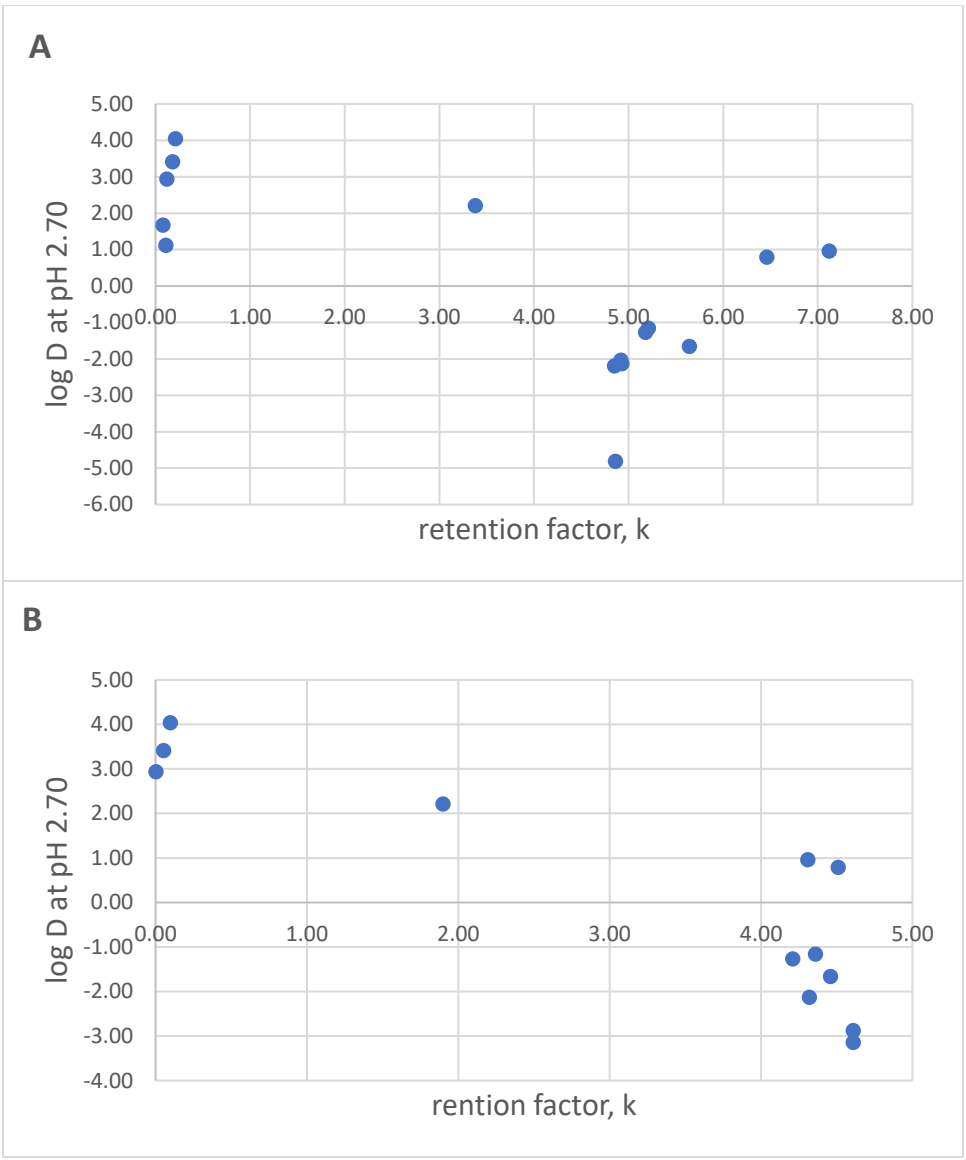
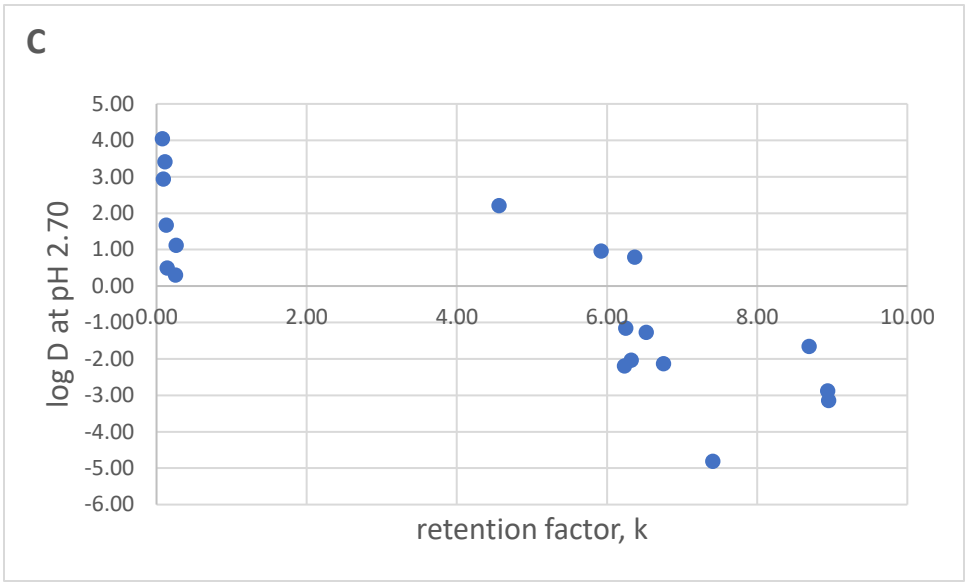


Figure 3-S4: U-shaped retention – change of retention with the acetonitrile content in the mobile phase for selected analytes warfarin and bentazon. (A) Cogent™ UDC-Cholesterol; (B) Cogent Bidentate C18™; (C) Cogent Diamond Hydride™; and (D) Cogent Diol 2.0™.

Table 3-S2: log D values at pH 2.7 for the model analyte set

Number	Compound	log D at pH 2.70
1	Anabasine	-3.14
2	Cotinine	-2.13
3	Matrine	-1.66
4	Nicotine	-2.88
5	Hydrocodone	-1.27
6	Hydromorphone	-2.04
7	Oxymorphone	-2.19
8	Diclofenac Sodium	4.04
9	Diazepam	2.21
10	Warfarin	3.41
11	Fentanyl	0.79
12	Phenobarbital	1.67
13	Adenosine monophosphate (AMP)	-2.04
14	Reserpine	0.96
15	Estrone glucuronide	1.12
16	Bentazon	2.94
17	Catechin	0.49
18	Methamphetamine	-1.16
19	Acetaminophen	0.3
20	Histidine	-4.81
21	Estrone Sulfate	0.0083
22	formyl-Met-Leu-Lys-Ile-Ile-OH	
23	Thiamine hydrochloride	-2.59


A
5.00
4.00
3.00
2.00
1.00
0.00
-1.00
-2.00
-3.00
-4.00
-5.00
-6.00
0.00
1.00
2.00
3.00
4.00
5.00
6.00
7.00
8.00
log D at pH 2.70
retention factor, k
B
5.00
4.00
3.00
2.00
1.00
0.00
-1.00
-2.00
-3.00
-4.00
0.00
1.00
2.00
3.00
4.00
5.00
log D at pH 2.70
rention factor, k



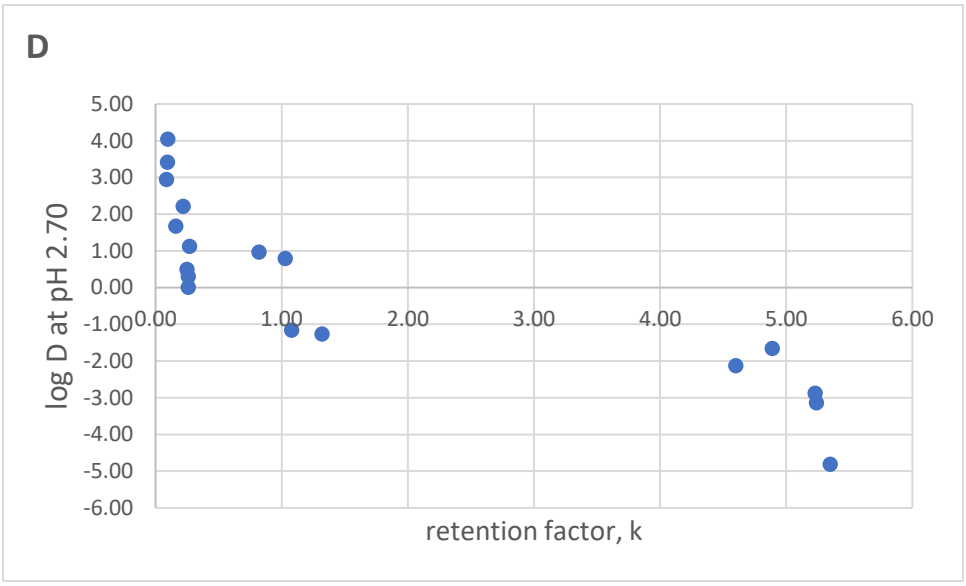


Figure 3-S5: Plot of log D (at pH 2.7) values against retention factor (k)(A) Cogent™ UDC-Cholesterol; (B) Cogent Bidentate C18™; (C) Cogent Diamond Hydride™; and (D) Cogent Diol 2.0™.

Table 3-S3: Calculated retention factor data for all the four phases under reversed-phase mode

		Retention Factor, k			
Number	**Analyte**	**Cogent™ UDC Cholesterol**	**Cogent Diol 2.0™**	**Cogent Bidentate C18™**	**Cogent Diamond Hydride™**
1	Anabasine	*	0.811	0.296	2.488
2	Cotinine	1.566	0.558	1.519	2.197
3	Matrine	2.998	1.789	2.314	4.337
4	Nicotine	*	0.809	0.289	2.489
5	Hydrocodone	3.530	0.873	3.025	3.173
6	Hydromorphone	2.691	0.457	2.270	2.089
7	Oxymorphone	2.427	0.330	1.914	1.586
8	Diclofenac Sodium	6.029	2.902	6.190	3.452
9	Diazepam	6.044	2.666	5.966	3.964
10	Warfarin	5.716	2.565	5.245	3.325
11	Fentanyl	6.529	2.483	5.002	4.845
12	Phenobarbital	4.017	0.681	4.065	1.355
13	Adenosine monophosphate (AMP)	0.287	0.063	0.157	0.144
14	Reserpine	9.504	2.905	5.942	4.548
15	Estrone glucuronide	4.351	2.086	4.098	2.698
16	Bentazon	4.992	1.172	5.121	2.396
17	Catechin	3.104	0.694	2.764	0.234
18	Methamphetamine	3.330	0.827	3.193	2.660
19	Acetaminophen	2.553	0.372	2.366	0.542
20	Histidine	0.087	0.127	-0.103	0.442
21	Estrone Sulfate	4.051	2.195	3.978	2.495
22	formyl-Met-Leu-Lys-Ile-Ile-OH	*	2.847	4.211	3.448
23	Thiamine hydrochloride	*	0.355	0.008	1.983

*- either peak is not detected, peak is so broad or peak is split in to two or more peaks and did not use in the retention factor calculation.

Table 3-S4: Calculated retention factor data for all the four phases under aqueous normal phase (ANP) mode

		Retention Factor, k			
Number	**Analyte**	**Cogent™ UDC Cholesterol**	**Cogent Diol 2.0™**	**Cogent Bidentate C18™**	**Cogent Diamond Hydride ™**
1	Anabasine	*	5.241	4.614	8.950
2	Cotinine	4.930	4.598	4.320	6.748
3	Matrine	5.644	4.888	4.463	8.687
4	Nicotine	*	5.234	4.610	8.941
5	Hydrocodone	5.181	1.324	4.209	6.518
6	Hydromorphone	4.925	*	*	6.320
7	Oxymorphone	4.853	*	*	6.228
8	Diclofenac Sodium	0.208	0.098	0.097	0.079
9	Diazepam	3.375	0.224	1.898	4.558
10	Warfarin	0.185	0.095	0.054	0.108
11	Fentanyl	6.462	1.035	4.512	6.369
12	Phenobarbital	0.078	0.162	-0.051	0.129
13	Adenosine monophosphate (AMP)	*	*	*	*
14	Reserpine	7.124	0.821	4.309	5.917
15	Estrone glucuronide	0.111	0.265	-0.082	0.257
16	Bentazon	0.115	0.087	0.002	0.091
17	Catechin	-0.017	0.251	-0.188	0.145
18	Methamphetamine	5.206	1.078	4.361	6.253
19	Acetaminophen		0.257	-0.112	0.251
20	Histidine	4.857	5.346		7.415
21	Estrone Sulfate		0.260	-0.190	-0.266
22	formyl-Met-Leu-Lys-Ile-Ile-OH	*	4.681	4.346	5.907
23	Thiamine hydrochloride	*	5.721	4.683	*

*- either peak is not detected, peak is so broad or peak is split in to two or more peaks and did not use in the retention factor calculation.

Chapter 4

An Integrated Multi-path LC-MS System for Simultaneous Preparation, Separation and Detection of Proteins and Small Molecules

4.1 Abstract

Methods for the separation, detection and quantitation of small molecules have been well established. Recent progress in the development of biologics as drugs have increased the demand for the method development for the analysis of proteins. Further, involvement of more than one class of molecules for a certain disease as either biomarkers or metabolites of a drug has set the foundation for the analysis of multiple classes of molecules from a single injection. Liquid chromatography mass spectrometry (LC-MS) methods are potentially suitable for the analysis of such multiple classes of molecules from a single injection, with appropriate use of available column technology and instrumentation. Restricted access media (RAM) columns can be used for selectively trapping one class of molecules, while excluding another class of molecules that can be captured downstream. With specific valve and plumbing arrangements, trapped molecules from the RAM column can later be eluted, developed using analytical columns and analyzed with the MS simultaneously with the molecules excluded from the RAM columns. Furthermore, these trap-and-elute based techniques needs minimal or no sample preparation and therefore saved the analysis time is much shorter than the traditional methods. Currently, one of the problems associated with these RAM columns is not excluding proteins quantitatively. This newly built multi- path system is a start point for the simultaneous analysis of multiple classes of analytes from a single injection.

4.2 Introduction

Quantitation methods for small molecule analysis from biological fluids are well established. Often, these include a protein precipitation step prior to analysis. However, this approach not only requires tedious sample preparation, but also disregards the valuable information that can be obtained from proteins. As an example, protein levels in urine samples can provide diagnostic indicator on diseases such as congestive heart failure, kidney diseases, and various cancers [1]. In the fields of biomarker analysis and protein therapeutic development, it would be advantageous to have methods for simultaneous determination of both small molecules (e.g., metabolites, drugs, and small signaling molecules, among others) and macromolecules, such as proteins and antibody-drug conjugates. There are several diseases or conditions, such as oxidative stress, lung cancer, and ricin poisoning, which have been shown to be associated with aberrant levels of multiple classes of compounds [2-3].

In the most common MS based approach for the analysis of proteins, “bottom up approach” proteins are first digested and the resulted fragments are analyzed. Even though current protein digestion methods are robust, the extra protein digestion steps increase the analysis time and uncertainty [4]. Further, fragments with same sequence can be generated from different proteins leading to errors in the analysis. One of the biggest disadvantage in the bottom up approach is losing the important information that can be obtained from the post translational modifications. As an alternative, top down approach where no prior digestion involved can be used. Recently Wang et. al has shown the use of QqQ for the analysis of intact proteins using MRM mode.

There are several methods for intact protein separation presented in the literature. Size exclusion chromatography, affinity chromatography and ion exchange chromatography are few of the methods available for intact protein separations [5-6]. Even though it is not as simple as with the small molecules separation using reversed phase chromatography, proteins still can be separated using currently available stationary phase chemistries. For example, using superficially porous particles and smaller particle sizes enhances protein separation.

Restricted access media (RAM), which features the combined properties of size exclusion with different modes of chromatographic retention can be employed for online sample preparation. There are several advantages of using RAM for online sample preparation, such as reducing the chance for contamination,

sample loses due to repeated handling, and sample preparation time. RAMs as shown (Figure 4-1) can be found in different variations depending on the exclusion function or chromatography mode employed. RAMs are generally packed with porous particles and their outer surface is non-retentive whereas inside of the pores are bonded with functionalized phase [7]. When a mixture of small molecules and larger molecules are flowing through this RAM, as larger molecules cannot enter the pores due to their size and they are simply flushed through. On the other hand, small molecules enter to the pores and can be retained by standard chromatographic principles.

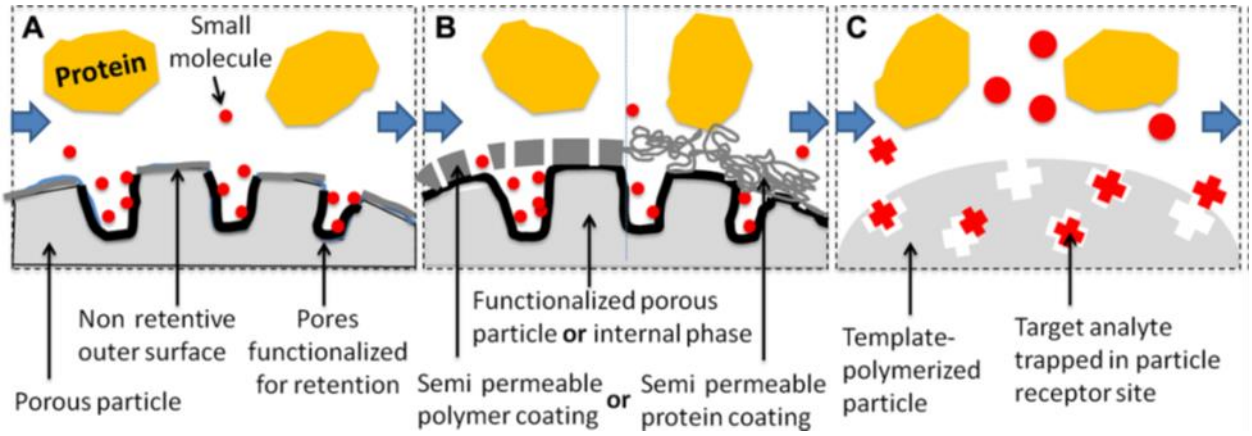


Figure 4-1. Different types of RAM material available (A) Internal surface, (B) Semi-permeable surface (including both polymeric and protein based hybrids), and (C) molecularly imprinted polymer phases. Reproduced with the permission from John Wiley & Sons (Copyright 2013).

The use of RAM material is mainly focused on the trapping of small molecules from biological fluids and the larger molecules such as proteins which are excluded directed to waste. If the excluded larger molecules can be directed to another analytical column and then send to the mass spectrometer for the analysis information for the small molecules as well as for large molecules can be obtained. This is helpful when the analysis of biomarkers for disease where more than one is available.

The aim of this work is to construct and demonstrate a multipath LCMS system for simultaneous analysis of small molecules and proteins. In order to prove that the system is working for the simultaneous analysis for both small molecule and protein analysis, model analyte set including small molecules from various classes with protein that are commonly used in clinical analysis was used. In the analysis of small

molecules and proteins, use of RAM column to selectively trap one class of molecules excluding the other class of molecules was investigated. Different types of RAM columns were used to explore their efficiency of excluding proteins and different flow rates, mobile phase conditions were also investigated to selectively trap one class over the other. Feasibility of the analysis of both small molecules and proteins using the constructed multi-path system has been shown and optimization of the excluding proteins would make the system better for quantitative analysis.

4.3 Experimental

4.3.1 Materials and reagents

Standards of solid proteins ubiquitin (ubiq), myoglobin (myo), lysozyme (lysz), lactalbumin (lact), and cytochrome c (cyt c) were purchased from Sigma Aldrich (St Louis, MO, USA). Solid standards of nicotine, (1) anabasine, (2) cotinine, (3) matrine, (4) nicotine, (8) adenosine monophosphate (AMP), (10) reserpine, (12) histidine, (13) thiamine hydrochloride, (14) diclofenac sodium, (18) catechin, (20) estrone glucuronide, (21) estrone sulfate, and (22) bentazon were purchased from Sigma Aldrich. Standard solutions of 1mg/mL in methanol of (5) hydrocodone, (6) hydromorphone, (7) diazepam, and (9) acetaminophen, (11) methamphetamine, (15) fentanyl, (17) oxymorphone (19) warfarin were purchased from Cerilliant Corporation (Round rock, TX, USA). A formylated peptide (16) (fomyl-Met-Leu-Lys-Ile-Ile-OH) was synthesized by Johns Hopkins University Synthesis and Sequencing Facility (Baltimore, MD, USA). LC-MS grade acetonitrile, methanol, and water were purchased from Honeywell Burdick and Jackson (Muskegon, MI, USA). Formic acid (98%) was purchased from Sigma Aldrich.

4.3.2 Instrumentation

All data were acquired using a custom Shimadzu LCMS 8050 – triple quadruple mass spectrometer (Shimadzu Scientific Instruments Inc., Columbia, MD) equipped with a conventional electrospray ionization (ESI) source. This instrument is equipped with three 2 position 6 port valves, one 1 position 7 port valve (column selection valve), five quaternary pumps, two column ovens, and SIL autosampler. In the construction of the multi-path system as shown in figure 4-2 all the components were used except the column selection valve. Eluents from the small molecule column and protein column were merged using

zero dead volume Tee (). All the chromatographic separations were performed using Restek columns (Restek Corporation, Bellefonte, PA, USA). For the protein separation, a Viva C8 (2.1 x 100 mm, 5 µm) column was used; for the separation of small molecules, an Ultra AQ C18 (2.1 x 100 mm, 3 µm) column was used. To trap small molecules, a Shim pack MAYI -C8 (4.6 x 10 mm, 50 µm, 12 nm pore size) restricted access media trap columns (Shimadzu Corporation, Kyoto, Japan) was used. For further investigation of trapping efficiency, an additional three alternate trap columns and a size exclusion guard column were studied. They were a CAPCELL PAK C8 (4.6 x 35mm, 5 µm, 12 nm pore size) from Shiseido (Tokyo, Japan), a LiChrospher® RP-8 ADS (4 x 25 mm, 25 µm, 6 nm pore size) from Merck (Darmstadt, Germany), and a TSK-GEL SWXL (6 x 400 mm, 7 µm, 12.5 nm pore size) from Tosoh Bioscience (Tokyo, Japan). All the analyses were performed under the multiple reaction monitoring (MRM) mode with the MS. The interface voltage was ± 4.5 kV; drying gas and nebulizing gas (nitrogen) flow rates were set at 15 and 3 L/ min, respectively. Heat block temperature was kept at 400 °C and desolvation line temperature kept at 300 °C. The collision gas (Argon) pressure was set to 270 kPa. MRM conditions were optimized for all the small molecules and protein analytes used and are given in table S1 and S2. The software used for the data collection and analysis is Shimadzu LabSolutions Software (V.5.65)

4.3.3 Sample preparation

For the simultaneous analysis of proteins and small molecules using the multipath system, small molecules phenylalanine, benzylamine, and methamphetamine, along with proteins cytochrome c, myoglobin, and ubiquitin were prepared as a mixture with the concentration of 1 µg/mL in 5% methanol in water. For the investigation of protein recovery from the restricted access media ubiquitin, myoglobin, lysozyme, cytochrome c (bovine), and cytochrome c (equine) stock solutions were prepared in 4 mg/mL. Then working solutions were prepared by diluting the stock solutions to have ubiquitin 100 µg/mL, lysozyme 1000 µg/mL, myoglobin 80 µg/mL, cytochrome c (bovine) 140 µg/mL and cytochrome c (equine) 160 µg/mL in water.

4.4 Results and Discussion

4.4.1 Construction and operation of the multi-path system

Most of the methods available in the LC-MS often focus only on one class of molecules for example either small molecules or proteins. During the recent past, it has shown some diseases involve both small molecules and proteins associated with the diseases. Further, with the increased use of biologics such as proteins as drugs and the analysis of their metabolites make it huge demand on simultaneous analysis of multiple classes of analytes in routine analysis. In the literature, the efforts that have been given for the method development for such analysis is limited. The system discussed here is suitable for the analysis of multiple classes of analytes from single injection with minimal or no sample preparation (figure 4-2).

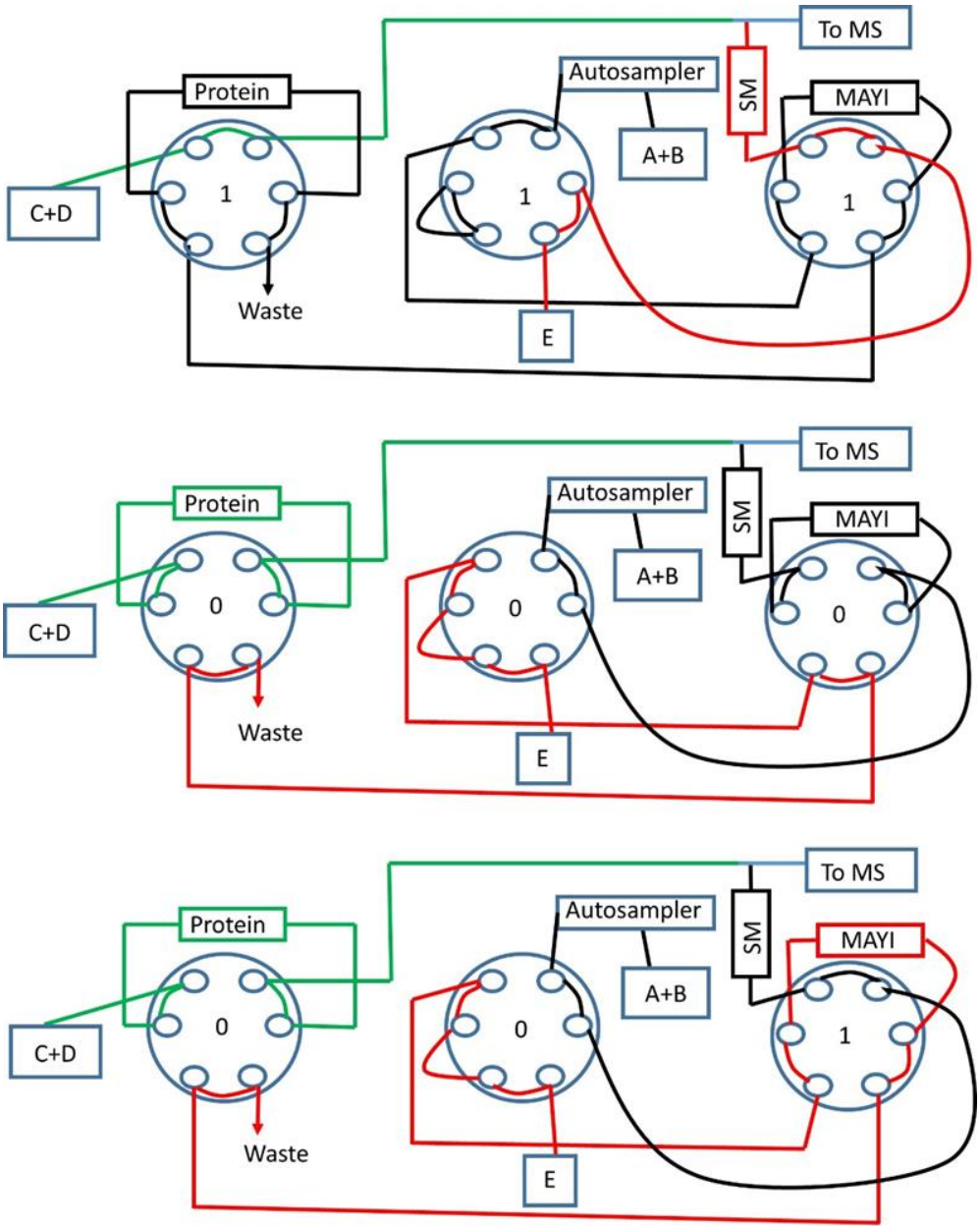


Figure 4-2. Plumbing diagram for the multipath system with the valve position to show the mobile phase flow.

The multipath system is constructed using Shimadzu Nexera LC system. As shown in the figure 4-2, three 2-position 6 port valves were used to direct the mobile phase flow. Further, five LC pumps were used to flow the mobile phase. The way this system works is, when the sample is injected using the auto-sampler, pumps A and B load the sample to the trap column. Theoretically, small molecules are trapped in the RAM trap column, and the larger molecules are excluded from the trap column and loaded to the

protein separation column. At the same time pump C and D is pumping the mobile phases that will eventually be used for the protein separation directly to the MS, and Pump E is conditioning the small molecule separation column. After a pre-determined time, the valve position is changed so that pump A and B backflush the trap column to release the trapped small molecules from the RAM and transfer them to the small molecule separation column. A gradient mobile phase program is applied for the separation of small molecules and they are directed to the MS for MRM-based detection. At the same time, the protein separation column is developed using Pumps C and D; the eluent is merged together with the eluent from the small molecule separation column and directed to the MS for simultaneous analysis using a zero dead-volume tee connection. Again, after pre-determined time valve switching is done so that pump E can be used to wash the trap column prepare for the next injection.

In the method development for the simultaneous analysis of small molecules and proteins, a model sample mixture was prepared and injected. The small molecules were selected from various analyte classes with different functional groups so that they exhibit different retention behaviors (Figure 4-S1). In the case of proteins, inexpensive, widely-available, and commonly-used protein standards were used. The analysis of small molecules and proteins were performed using multiple reaction monitoring and the optimized conditions are listed in Table 1.

As preliminary results for the use of the developed multipath system, both small molecules and proteins were detected simultaneously. But there were several issues with respect to trapping one class of molecules selectively. When the Shim pack MAYI trap column is used for the analysis some of the proteins also got trapped with the small molecules and trapping of more hydrophilic small molecules were difficult. In the applications where trap columns have mostly used is to concentrate small molecules or trap the small molecules to separate them from the matrix such as proteins. In those cases, if the small molecules are hydrophobic enough to be trapped, then the trap column is directed to waste and proteins and other molecules were not analyzed. Even though, eluting of small molecules were performed in similar manner, as proteins were not analyzed they might have not seen the trapping of proteins in the trap columns.

To optimize the conditions for excluding proteins from the trap column several things were investigated. First, 5% organic phase was used with water under 0.05 mL/min flow rate providing enough

time for the small molecules to get through the size exclusion membrane and interact with the bonded phases. Then, flow rate gradient was used to facilitate the exclusion of the larger molecules using the same organic phase composition. Further, 5 mM ammonium formate is used in the mobile phases with formic acid to improve the exclusion of large molecules for the trap columns. With each of the case, there was slight difference in the exclusion of the larger molecules but it was not possible to exclude them completely. First, small molecules and protein mixture was injected without using the protein or small molecule analytical columns to show that the system works as designed. Figure 4-03 A, chromatogram for simultaneous detection of small molecules and proteins shows that the multipath system constructed using the system available in our lab can run and analyzed both classes of molecules simultaneously. (Figure 4-3A) In the meantime separation and detection conditions for the small molecules and proteins were developed separately with the help of Nexera Method Scouting Solutions [8-9]. Then, protein and small molecule separation was performed with the use of analytical columns and mixture of 22 small molecules and 5 proteins were injected and analyzed. According to the results obtained, as shown in figure 03 B, it shows that the system is working for the separation and detection of both classes of molecules. (Figure 4-3B)

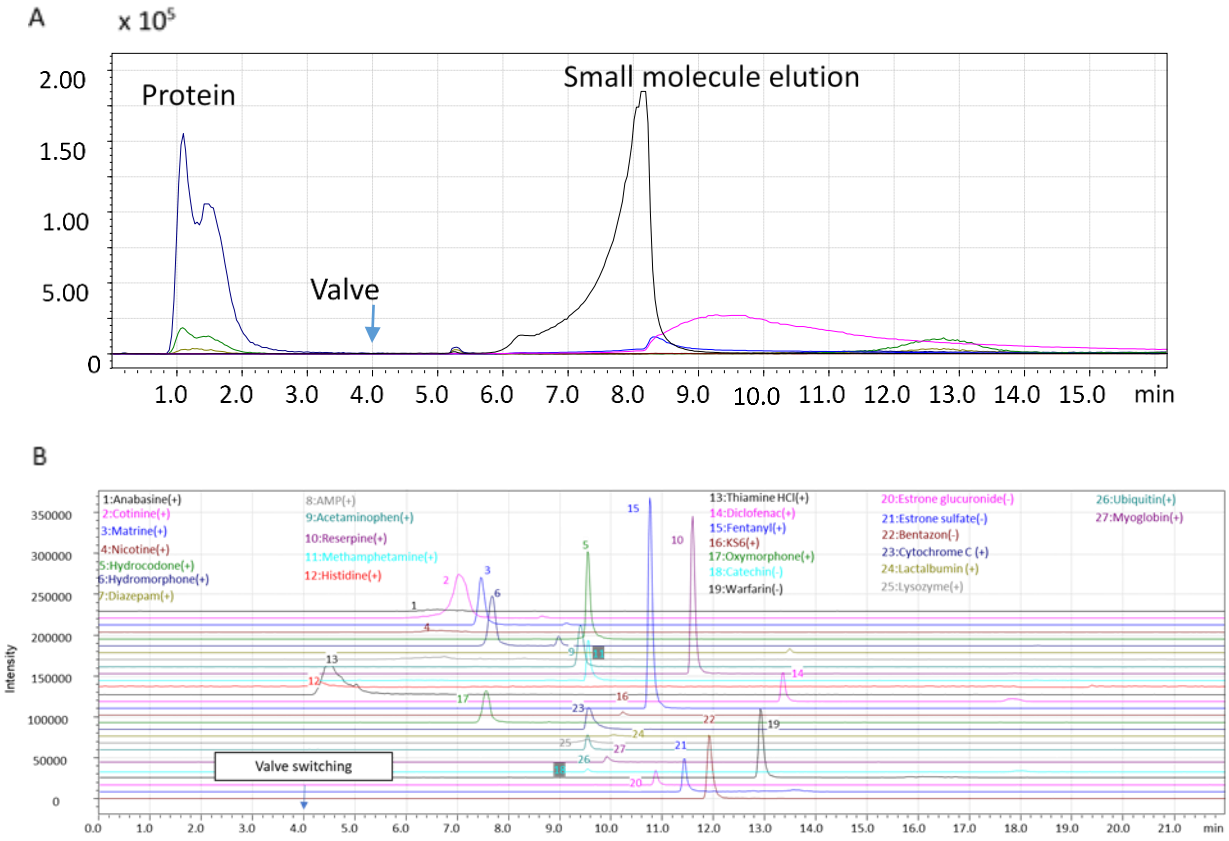


Figure 4-3. Chromatogram showing the analysis of small molecules and proteins with the integrated system A) Using RAM column only (Separation is not optimized with analytical columns) B) Using analytical columns for separation of a mixture of small molecules and proteins

4.4.2 RAM protein trapping efficiency

Once the separation and detection of the proteins and small molecules were performed with the multi-path system, next thing we wanted to investigate is the quantitation of these molecules. To see complete exclusion of the proteins from the trap column, mixture of proteins was injected and continue to run without switching the valves. Then, instead of seeing one peak for each protein, which should be the case if proteins were completely excluded from the trap column two peaks were detected. (Figure 4-4) It shows that some of the proteins get trapped in the trap column and later eluted. One of the possible things that can happen is, under the conditions we used, proteins can get denatured and they can have rod like

shape. When they have rod like shape, those molecules still, at least partially, access the pores of size exclusion membrane and can interact with the bonded phase such as C4 and get trapped.

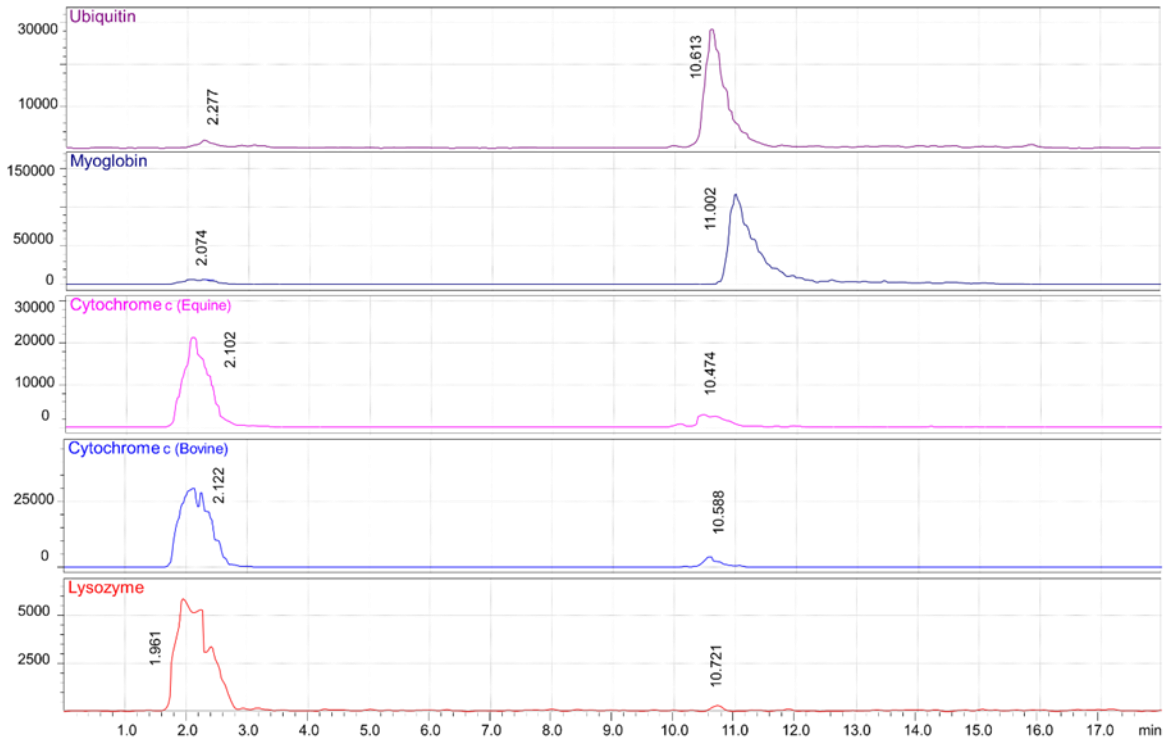


Figure 4-4. Chromatograms showing two peaks for the same analyte using Shim-pack MAYI trap column. First peak is for exclusion and second peak for trap and elute.

In the investigation of excluding proteins from the trap columns three different trap columns along with size exclusion guard column were used. Quantification of the proteins that were excluded from the trap column were analyzed as a percentage of total protein detected when the same analysis performed without a trap column. Results showed that the trapping was protein dependent, meaning out of the five proteins analyzed the amount of proteins excluded from the trap column is different for different proteins. In order to show the amount of proteins excluded from the trap column a bar graph was constructed. When there is no column is used, the area of the peak for each protein is obtained and let that to be P1. Then, same analysis was performed with each trap column and the area of the first peak (for the excluded protein from the trap column) was obtained (P2). Then the percentage of the excluded protein

was calculated using the formula (P2/P1) *100% and plotted against each protein for all three columns. MAYI trap was excluding over 75% of cytochrome c bovine and equine and Tosoh SEC was showing over 50% exclusion for ubiquitin in addition to cytochrome c. Even though the protein exclusion with the Shiseido column is poor compared to the other two columns, it showed the best exclusion for myoglobin (over 50%) (Figure 4-5). Lysozyme exclusion was below 50% for all the columns investigated and more importantly with the Shiseido column exclusion was not detectable. Fourth column used in the study, LiChrospher RP-8 retained all the protein (Figure 4-S2) and therefore not included in the protein exclusion comparison. (Figure 4-5)

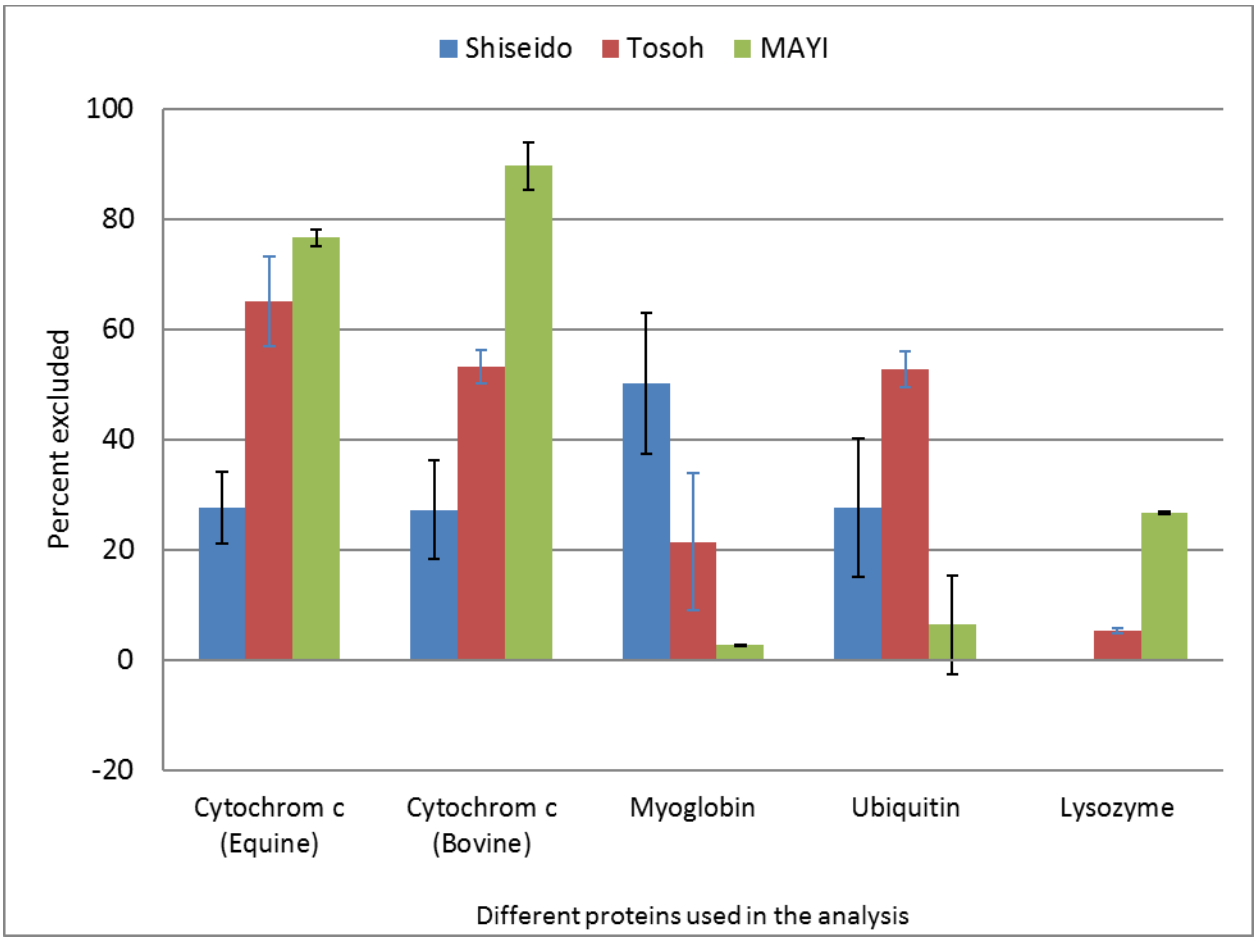


Figure 4-5. Protein exclusion comparison with different trap columns

4.5 Conclusion

Evaluation of the feasibility for the analysis of multiple classes of molecules from single injection with minimal sample preparation with the use of RAM columns was performed in this work. According to the results, analysis of small molecules as well as proteins using the instrument set up discussed is possible but there is a room for improvement in the quantitation. All the RAM columns used are not completely excluding proteins and therefore it needs to improve for the use of quantitation. Among the different types of RAM columns used, MAYI trap gives the best performance in excluding proteins. In order to get better exclusion of the proteins from the trap columns, preserving the tertiary structure of the proteins would be beneficial. Therefore, as a future work, analysis of proteins with different classes of RAM columns with high salt concentration and without acid would be better option. As there are no methods available for the analysis of multiple classes of analytes from single injection, this work would be a starting point for such analysis with further improvement for many different classes of molecules such as small molecules, proteins, fatty acids etc. using integrated multi-path system.

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The authors have declared no conflict of interest.

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4.7 Supporting Information

Table 4-S1. Optimized multiple reaction monitoring (MRM) conditions for the small molecule analytes

Number	Compound	Precursor Ion m/z	Product Ion m/z	Dwell time (msec)	Q1 pre Bias (V)	Collision Energy (V)	Q3 pre Bias (V)
1	Anabasine	163.20	118.20	20.0	-20.0	-24.8	-11.0
2	Cotinine	177.20	80.15	20.0	-21.0	-25.6	-30.0
3	Matrine	249.10	148.20	20.0	-30.0	-33.2	-14.0
4	Nicotine	163.20	117.15	20.0	-19.0	-27.6	-19.0
5	Hydrocodone	300.25	199.25	20.0	-20.0	-31.0	-20.0
6	Hydromorphone	286.25	185.20	20.0	-14.0	-30.0	-18.0
7	Diazepam	284.90	193.40	20.0	-14.0	-16.0	-13.0
8	Adenosine monophosphate (AMP)	348.00	136.20	20.0	-17.0	-21.6	-13.0
9	Acetaminophen	152.00	110.15	20.0	-17.0	-19.0	-18.0
10	Reserpine	609.20	195.20	20.0	-30.0	-38.0	-20.0
11	Methamphetamine	150.20	91.10	20.0	-17.0	-22.0	-15.0
12	Histidine	156.20	110.20	20.0	-18.0	-15.0	-18.0
13	Thiamine hydrochloride						
14	Diclofenac Sodium	296.15	214.00	20.0	-20.0	-33.0	-21.0
15	Fentanyl	337.30	188.20	20.0	-22.0	-23.0	-20.0
16	formyl-Met-Leu-Lys-Ile-Ile-OH (KS 6)	239.20	132.10	20.0	25.0	26.0	26.0
17	Oxymorphone	302.10	227.20	20.0	-15.0	-25.0	-14.0
18	Catechin	289.30	245.20	20.0	30.0	13.8	17.0
19	Warfarin	307.15	161.10	20.0	14.0	18.6	29.0
20	Estrone glucuronide	445.30	269.20	20.0	20.0	30.4	29.0
21	Estrone Sulfate	349.25	269.15	20.0	16.0	32.2	29.0
22	Bentazon	239.20	132.10	20.0	25.0	26.0	26.0

Table 4-S2: Optimized MRM conditions for the proteins

Number	Compound	Precursor Ion m/z	Product Ion m/z	Dwell time (msec)	Q1 pre Bias (V)	Collision Energy (V)	Q3 pre Bias (V)
23	Myoglobin						

24	Lactalbumin						
25	Lysozyme						
26	Ubiquitin						
27	Cytochrome C (bovine)						
28	Cytochrome C (equine)						

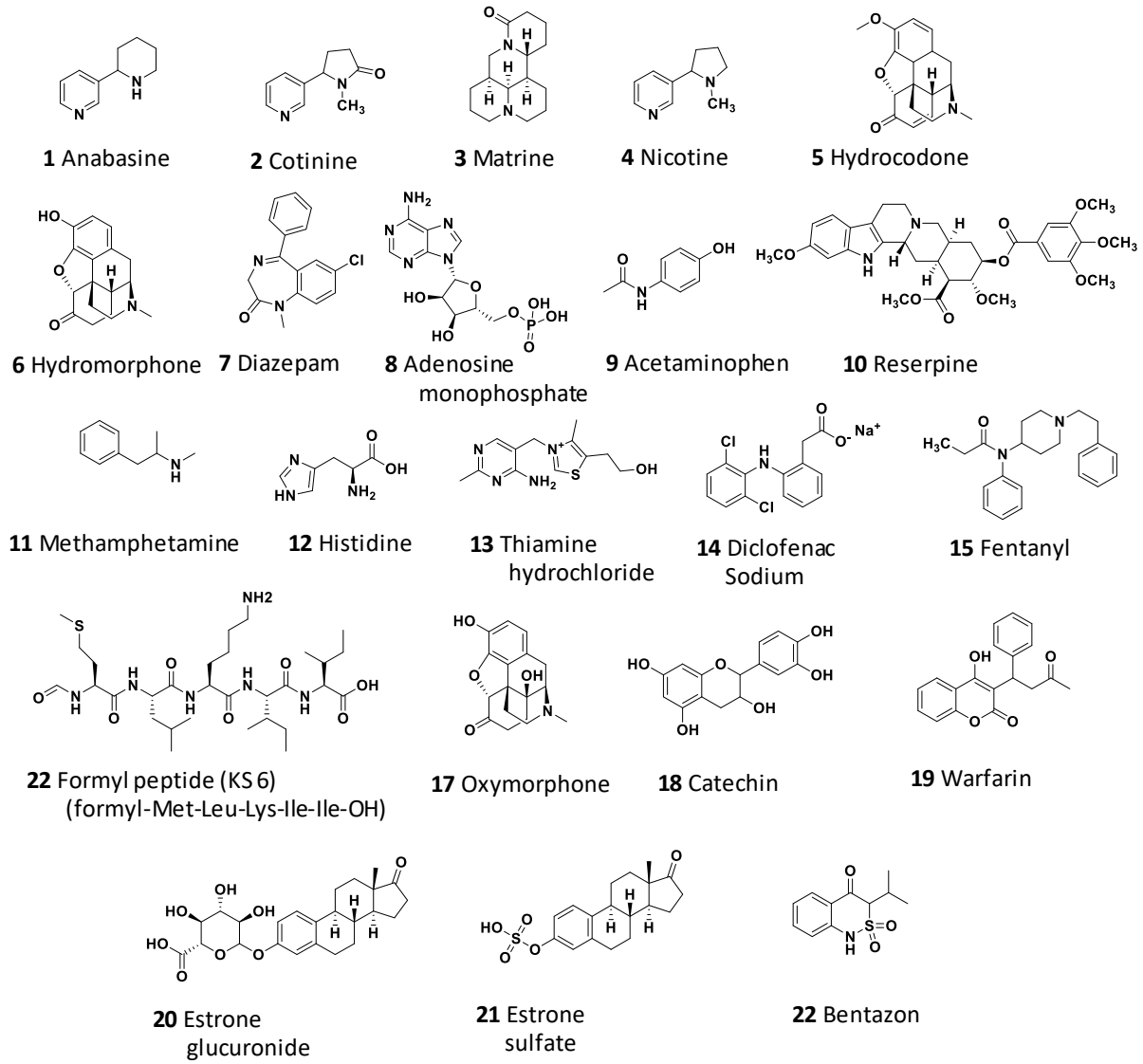

Figure 4-S1: Structures of the small molecules used in the model sample mixture

Chapter 5

Method Development for Comprehensive Two-Dimensional Liquid Chromatographic Separation and Triple Quadrupole Mass Spectrometric Quantitation of Intact Proteins.

5.1 Introduction

Human proteome, the collection of all the proteins expressed by the cells, tissues and organs in the human body is an interesting field of study due to the massive amount of information that can be obtained. It is estimated that there are around 50,000 different proteins exists in the human body [1]. The proteins in the body interact with various body organs and involved in various cellular functions. Further, these proteins have shown their association with various diseases such as cancer, heart diseases, Alzheimer's disease. Because of finding proteins associated with diseases, such proteins can be used as disease indicators or biomarkers. Therefore, study about the proteins can give valuable information regarding diseases which can help early diagnosis. Proteins are produced by the cells and these proteins have complex structures. There could be slight difference such as methylation in their structure but having vastly different function. When there are only slight differences with complex structures, it makes the separation of these proteins challenging. Further, the abundance of different proteins in biological fluids such as blood varies so much. For example, albumin, most abundant protein in human blood has concentration of 35-50 g/L and 99% of the total mass of human proteome in blood leaving just 1% for all the other proteins. Therefore, in the analysis of less abundant proteins it is necessary to either remove the most abundant once by sample preparation methods such as precipitation or resolve different proteins by using chromatography [2].

Gel based methods have been used for the analysis of proteins in the past. In the case of the gel based methods proteins are separated based on the molecular weight and this is still not suitable for more complex mixtures due to the low resolution of the analytes. As an improvement in the gel based methods, two-dimensional (2D) gel electrophoresis was developed. There, proteins first separated based on the isoelectric points and then in the second dimension separated based on the molecular weight [3-5]. Even though separation is better with the 2D gel based analyses there are several problems associated with these methods such as the time taken for development of new methods, difficulty in

automation, difficulty in applying for acidic, basic and membrane proteins and method being labor intensive are among others [6-7]. During the recent past, improvements in the technology specially in the field of mass spectrometry have changed the protein analysis technique and now most common method for the protein analysis is mass spectrometry. Even with the high sensitivity of the mass spectrometers, the analysis of proteins is a difficult task due to the complexity of the biological samples. Therefore, hyphenated techniques such as liquid chromatography coupled with mass spectrometry is very helpful. There, chromatography can be used to separate out proteins as well as enrich low abundant proteins [8].

In the late 20th century, protein therapeutics started emerging as a new class of drugs and since then the growth in the field is exponential. As of 2014 antibody therapeutic market is over $1.3 billion and it is expected to have a compound annual growth rate of 44% in the following five years. In the year 2015, itself United State food and drug administration(FDA) have approved 13 new biologics [9]. There are advantages of using proteins as drugs such as due to the specificity of the protein interactions the side effects that can arise from protein based drug in relatively less compared to small molecule drugs. In addition to the use of proteins as pharmaceutical drugs, proteins have gained attention due to their discovery as biomarkers for various diseases. Therefore, the quantitation of these proteins found as biomarkers is also important.

Triple quadrupole mass spectrometers have been used in the clinical laboratories for the quantification of small molecule analytes such as drugs, metabolites, Because of the specificity of the MRM mode, it is preferable method in the quantitation and with the current technology in the instrumentation it is possible to quantify several analytes from single injection whenever needed. Use of triple quadrupole mass spectrometer for the analysis of intact proteins using multiple reaction monitoring mode have been previously reported from our lab [10]. At the same time, it is worth to note that the separation of intact proteins using the more common reversed-phase chromatography was not easy since all the proteins showing similar selectivity with the different stationary phases available such as alky phases (C4, C8, C18) as well as aromatic phases such as biphenyl and fluorophenyl [11]. Therefore, to get good resolution of the proteins, either different modes of chromatography such as size exclusion(SEC), ion exchange(IE), hydrophobic interaction (HIC)or different mobile phases needs to be explored.

Alternative approach to get high resolving power is to use multi-dimension liquid chromatography methods for the analysis of highly complex samples. In the multidimension analysis such as comprehensive two-dimensional liquid chromatography (2DLC) high resolving power can be obtained because the peak capacities of the 2DLC method is a multiplicative of the peak capacities of the first and second dimension peak capacities. Under the ideal conditions where the first and second dimension separations are fully orthogonal the total peak capacity of the 2DLC method ($n_{c,2D}$) can be give as the product of peak capacities of the 1st dimension (^1n_c) multiplied by the peak capacity of the 2nd dimension (^2n_c) [12].

$$n_{c,2D} = {}^1n_c \times {}^2n_c$$

In the 2DLC of protein different chromatographic methods for the two dimensions have been reported. For example, SEC in the first dimension coupled with RP in the second dimension, IE coupled with RP, affinity chromatography (AC) coupled with RP are several of the reported orthogonal combinations. Opiteck and coworkers have shown the use of comprehensive two dimensional LC system for the analysis of protein mixtures using IEC coupled with RP [13]. Further, Xiang and coworkers have shown an application of 2DLC for the analysis of membrane proteins associated with breast cancer cell lines using strong cation exchange coupled with RP [14]. Comprehensive 2DLC with RP x RP is not that common because when the two dimensions have similar phases, the full advantage of the 2D system cannot be obtained. But as Donato and coworkers have shown the use of comprehensive 2DLC for the analysis of protein samples with the trypsin digestion. There in order to get some orthogonality between the two dimensions, they have shown the use of different pH mobile phases [15]. The main reason is because RP x RP will not give any orthogonality in that combination as the stationary phases going to be similar but if the mobile phases can be changed by altering the pH there is still possibility to get significant orthogonality.

The aim of this study was first to construct the 2D LCMS system as shown in the figure 5-1 and show that the full system is working for the analysis of proteins. Then, evaluated the possibility of RP x RP for the protein separation using different mobile phases and stationary phases. There, for the first-

dimension high pH mobile phases with additives such as triethyl amine is explored. Further, low pH (<2) mobile phase conditions is also investigated for the first dimension. In addition to the different mobile phases, several reversed-phase stationary phases such as C8, fluorophenyl, tri-n-butyl, tripropyl, n-butyldimethyl, tri-isobutyl were also explored. In order to optimize the sampling of the peak eluting after the first dimension, two different sizes of loops,100μL and 50 μL were also used. In addition to the reversed phase mode in the first dimension, SEC mode also used coupled with RP in the second dimension. Finally, to use HIC mode for the 1st dimension with RP for the to investigate the resolution of protein analytes. In all the analyses, reversed phase is used with the pre-optimized mobile phase conditions (water and acetonitrile with 0.1% formic acid + 0.05% trifluoro acetic acid) [16].

5.2 Materials and Methods

5.2.1 Materials, reagents and sample preparation

Neat solid standards for ubiquitin (Ubi), myoglobin (Myo), cytochrome c (Cyt c), carbonic anhydrase (CAH), β-casein (β-Cse), α-lactoglobulin A (Lac. A) were purchased from Millipore Sigma (St. Louis MO, USA). MS grade water and acetonitrile were purchased from J. T. Baker (Phillipsburg, NJ, USA). Formic acid (> 98.0%) was purchased from TCI America (Portland, OR, USA) and trifluoro acetic acid (>99.0%), acetic acid, triethyl amine and ammonium formate (99.995%) were purchased from Millipore Sigma (St. Louis, MO, USA).

All solid standard proteins were dissolved in water to prepare 5 mg/mL stock solutions. The final test mixture was prepared by 10-fold dilutions of the stock solutions to get final working concentrations of 0.5 mg/mL for all the proteins analytes. 1 μL of formic acid was added to the final working solutions. In the HIC experiments, working protein standard mixture was prepared in 1 M ammonium formate solution instead of water.

5.2.2 Instrumentation and settings

The HPLC system used consists of four Nexera LC-20ADXR pumps, a SIL-20ACXR auto-sampler, and a CTO-20AC column oven (Shimadzu Scientific Instruments, Inc., Columbia MD). The column oven is equipped with two FCV 12-AH two position six ports valves. All the tubing used were either stainless steel

or PEEK (0.010 in id). For the sample collection and injection to the second dimension 100 µL, 50 µL stainless steel samples loops and Viva C4 guard columns were used. Different analytical columns were used for the first dimension including Viva PFPP (100 x 2.1 mm, 5 µm d_p), Viva C4 (100 x 2.1 mm, 5 µm d_p) and for the second-dimension Raptor® ARC-18 (50 x 3.0 mm, 5 µm d_p) (Restek Corporation, Bellefonte, PA) were used. In addition to four beta phases tri-n-butyl, tripropyl, n-butyldimethyl and tri-isobutyl (100 x 1.00 mm, 3.4 µm d_p) (Restek Corporation, Bellefonte, PA) with 400 Å pore size is also used as first dimension column. In the case of HIC analysis PolyHEXYL A (100 x 1.0 mm, 3.0 µm d_p) with pore size of 1500 Å (PolyLc Inc., Columbia MD) was used. Size exclusion column, QC-PACK GFC 200 (150 x 7.8 mm, 5 µm d_p) from Tosoh bioscience (Tokyo, Japan) was also used as first dimension column. All the analysis was performed at 30 °C unless otherwise noted and HIC analysis was performed at ambient temperature. Figure 5-1 shows the diagram of valve switching and mobile phase flow in the two-dimensional LC system. Different mobile phases were explored for the first dimension including water with 0.1% formic acid and 0.05% trifluoro acetic acid (TFA) in water, acetonitrile with 0.1% formic acid and 0.05% TFA, water with 0.3% TFA and acetonitrile, water with 0.1% triethylamine (TEA) with acetonitrile. In the HIC analyses, 1M ammonium formate in water with acetonitrile was used as 1D mobile phases. For the second dimension, water with 0.1% formic acid and 0.05% trifluoro acetic acid (TFA) in water, acetonitrile with 0.1% formic acid and 0.05% TFA was used as the mobile phases. A gradient of 5% to 85% B over 70 minutes at 0.05 or 0.025 mL/min flow rate for 100 µL and 50 µL loops respectively were used for all the analyses except for the HIC. In the case of HIC analysis 0% B to 85% B over 15-minute gradient followed by 5-minute isocratic hold at 85% B and 10-minute re-equilibration at 0% B was used. Water was used as the needle wash solution and sample injection volume was kept at 5 µL.

5.2.3 Mass spectrometry parameters

The MS system used was a Shimadzu 8050 triple quadrupole mass spectrometer with an electrospray ionization (ESI) source. All the analyses were performed under the multiple reaction monitoring (MRM) mode with the MS. The optimized MRM conditions for intact protein analytes were given in the table 1. The interface voltage was ± 4.5 kV; drying gas and nebulizing gas (nitrogen) flow rates were set at 15 and 3 L/ min, respectively. Heat block temperature was kept at 400 °C and

desolvation line temperature kept at 300 °C. The collision gas (nitrogen) pressure was set to 270 kPa. MRM conditions were optimized for all the protein analytes used and are given in table S1. The software used for the data collection is Shimadzu LabSolutions Software (V.5.65) and data analysis were performed using ChromSquare (V. 2.2).

Table 5-1. Optimized MRM conditions for the protein analytes

Number	Protein	Precursor Ion	Product ion	Q1/V	Collision energy/ V	Q3/V
1	Ubiquitin	1071.4	1084.8	-34.0	-53.0	-34.0
2	Myoglobin (Equine Heart)	998.1	1049.6	-24.0	-35.0	-36.0
3	Myoglobin (Equine Skeleton)	942.7	933.2	-28.0	-33.0	-34.0
4	Cytochrom C (Bovine)	874.0	881.0	-20.0	-40.0	-34.0
5	Cytochrom C (Equine)	884.0	890.0	-38.0	-31.0	-32.0
6	Cytochrom C (Saccharomyces)	978.6	908.0	-38.0	-39.0	-20.0
7	β-Casein	1091.1	1204.9	-34.0	-31.0	-32.0
8	Carbonic anhydrase	807.1	881.6	-32.0	-24.1	-36.0
9	β-lactoglobulin A	1413.6	1558.0	-46.0	-44.4	-40.0
10	β-lactoglobulin B	1406.9	1576.6	-34.0	-45.0	-38.0

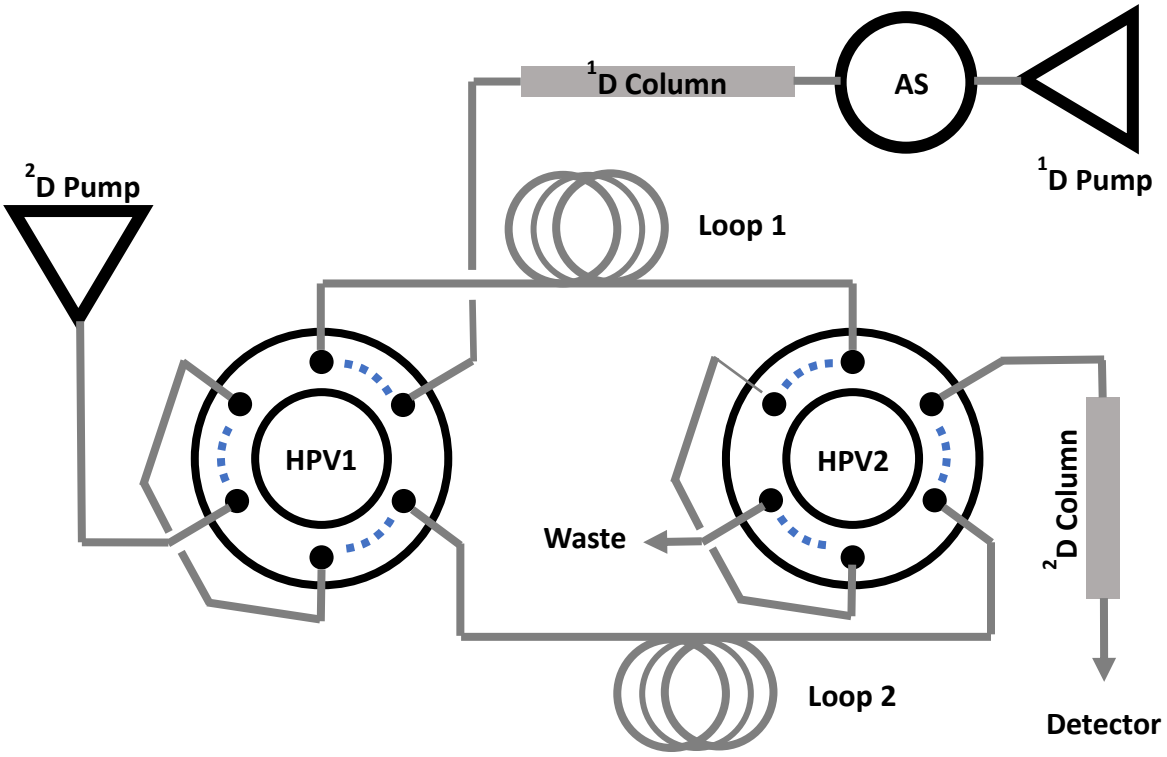

Figure 5-1: Plumbing diagram and the mobile phase flow path for the two-dimensional LC system.

5.3 Results and Discussion

Proteins present in various concentrations in the biological fluids and most abundant proteins can mask out the low abundant proteins in the mass spectrometric analysis. Therefore, resolving these different proteins using chromatography is important. In contrast to the small molecules, proteins have similar physicochemical properties and smaller diffusion coefficients and that leads the protein separation method development harder. Currently, most common method for the analysis of proteins is the analysis of peptides release after the enzymatic digestion of a protein and it is called as “bottom up” approach. But with this approach, important information such as post translational modifications (PTM) cannot be obtained. Further, errors in the identification of proteins can occur due to miss cleavages and having similar peptides from different proteins after digestion. The analysis of intact proteins, known as “top down” approach can be used as an alternative method where tandem mass spectrometry is used for detection. When the molecular weight of the protein is small enough and protein gives reproducible fragments, top down approach is faster and more informative method.

As the complexity of the protein samples are high, the separation methods use needs to have higher resolving power. Reversed-phase liquid chromatography is most common method for the small molecule analysis is one of the more common methods used for the protein analysis as well. Due to the similar physicochemical properties of the proteins, the selectivity difference among different stationary phases is minimal. Wang et. al has shown that when reversed-phase stationary phases C18, C8, C4, biphenyl and fluorophenyl is used for the protein separation method, all the phases show very similar selectivity except the fluorophenyl where slightly different selectivity is shown. In order to obtain resolving power required to separate complex protein mixtures, combinations of different orthogonal modes of liquid chromatography coupled with mass spectroscopy has been developed in the recent past. Even though most of the available chromatographic techniques have coupled in the two dimensions, RP x RP combination is not reported for protein separation. In the selection of RP for the both dimension, it is not orthogonal but by changing the pH of the mobile phases significant difference in the interaction of protein with the phases can be obtained.

5.3.1 Construction of the 2D LCMS system

In the development of the comprehensive 2D-LC method for the protein analysis, as shown in the figure 5-1, two 2-position 6 port valves were used with two loops for the sample introduction to the second dimension. First, Viva C4 with the mobile phase water and acetonitrile with 0.1% formic acid and 0.05% trifluoro acetic acid were used in the one-dimensional LC. As shown in figure 5-2 for six proteins, there is not much difference in selectivity observed. Specially, β-Cas, Myo, Lactog A and CAH were co-eluting while Cyt c and Ubi are resolved from others. In order to show the 2D system is working and protein MRM signals can be detected with MS compatible mobile phases, 0.1% formic acid and 0.05% trifluoroacetic acid with water and acetonitrile in the both dimensions. Restek Viva C4 column with Restek ARC-18 column were used for the 1^{st} and 2^{nd} dimension respectively and the results as shown in the figure 5-3 showed that the system constructed is working and protein signals for the MRMs can be detected. But the resolution of the proteins was not good. This is because of the non-orthogonality in the two phases, two reversed phases and same acidic mobile phases used in the analysis.

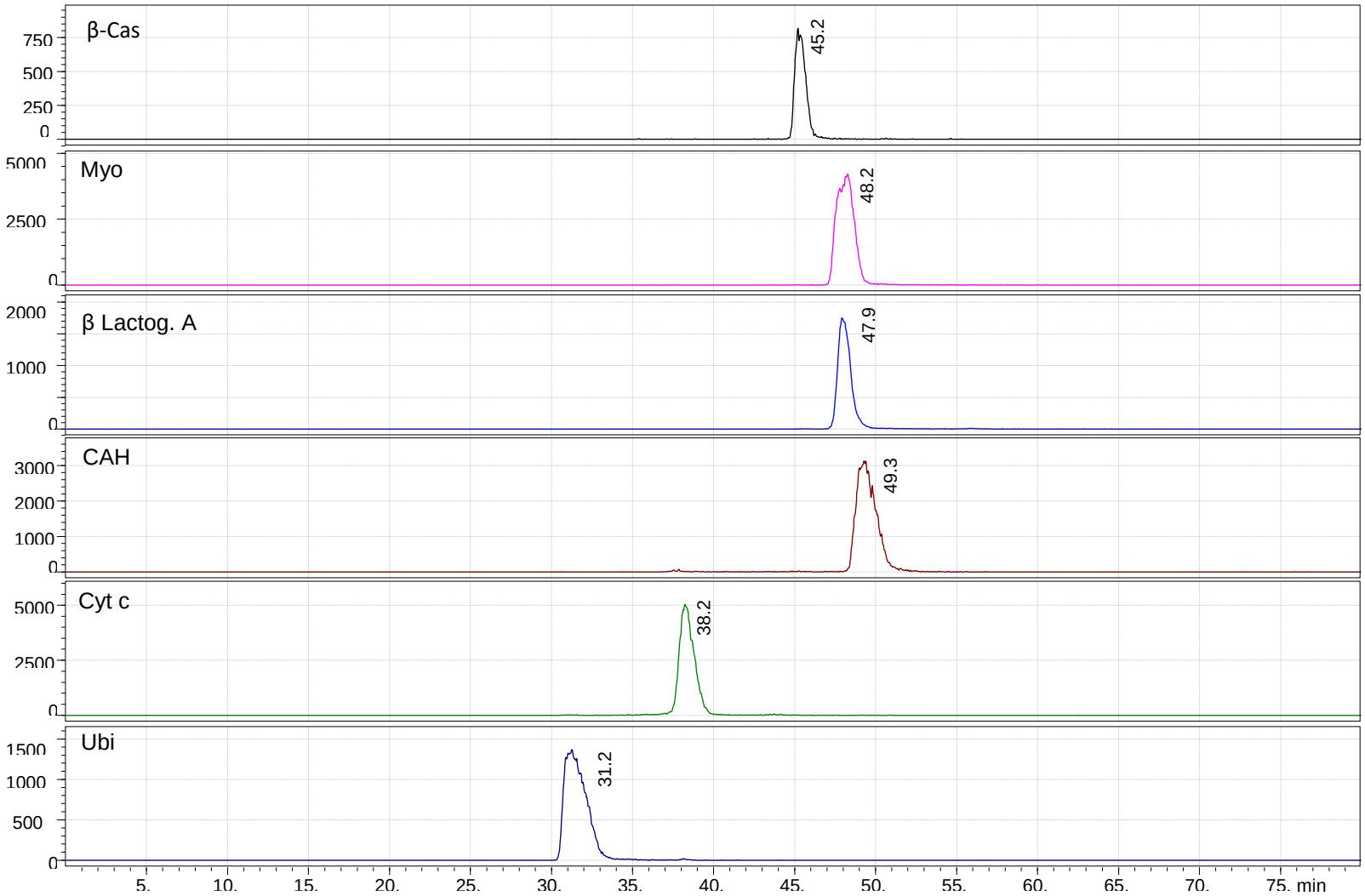

Figure 5-2. Chromatogram showing 1D separation of proteins using Viva C4 column.

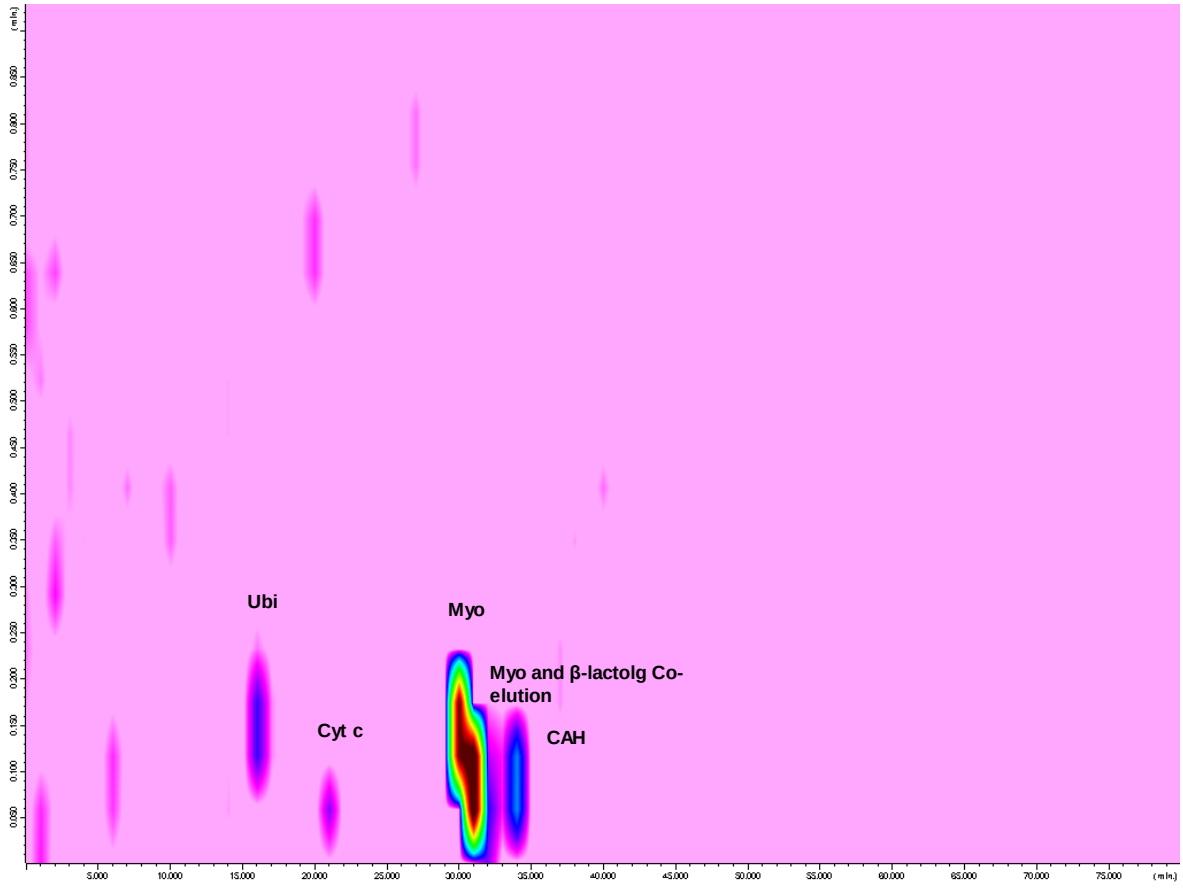

Figure 5-3. 2D chromatogram showing the separation of protein with the 2DLC MS system

5.3.2. Investigation of different additives in the mobile phases for the 1st dimension

When the system is working with the MS compatible mobile phases, more basic mobile phases were used in the first dimension. There, first, 0.1% triethyl amine in water with acetonitrile were used as the 1st dimension mobile phases while keeping the 2nd dimension mobile phases as the previously used MS compatible mobile phases. When the 1st dimension mobile phases are not acidic, protein signals were not detected. There could be various reasons for not detecting the signals. The charge state distribution of the proteins can get altered when the sample is in basic mobile phases (0.1% TEA) and it might not have been changed even though acidic mobile phases were used in the 2nd dimension. MRM conditions have been optimized for the most intense charge state of the protein which was obtained under the acidic condition and under the basic condition used in the 1st dimension, intensity of that charge state could be not that intense. In the triple quadrupole mass spectrometer after the ion generation at the ESI source

signal can be lost due to the loss of ions in the transmission through the quadrupoles. Collectively all these factors might have contributed for the loss of sensitivity in the MRM signals under the basic mobile phase conditions [17-18].

Once the basic mobile phase conditions used for the 1st dimension was not successful different acidic mobile phases with 0.1% acetic acid, 0.3% formic and 60% formic acid were evaluated. In all these analyses, previously used acidic mobile phases were used for the 2nd dimension. Still, there was no detectable signal for the protein MRM under any of these conditions and the reason for this behavior is not fully understood. In order to minimize the effect of the dilution of the sample injected to the second dimension as well as to minimize the effect of the 1st dimension mobile phases 50 µL loops were used instead of 100 µL loops that were previously used. Even under these conditions there we no significant improvement in the protein MRM signal detection.

5.3.3. Investigation of different columns for the 1st dimension

Once the different mobile phases and mobile phase additives were not successful different reversed phase columns, size exclusion as well as HIC column was used for the 1st dimension. First, Restek Viva C4, Restek Viva fluorophenyl columns with 300 Å pores were used in the 1D system to see how the separation of the proteins used. When the fluorophenyl phases is used, there was a better separation compared to the Viva C4. Ubiquitin, cytochrome C were always co-eluting with the conditions used. When the flow rates of 50 µL/min is used for the whole 2D system with the 100 µL loops (2-minute modulation) the sampling of the 1D peaks were not great. Therefore, flow rates were reduced to 25 µL/min and 50 µL loops were used with 1-minute modulations. There was software limitation where it limits the 2D LC time program not to have more than 400 lines and therefore changes were made to the 1D LC time program. The results obtain does not show huge improvement in the separation. Then five different beta columns with alkyl ligand were used with four proteins Ubi, Myo, Cyt c in the 1D to see the selectivity differences and as shown in the table 2 retention time were not significantly different among the columns. That means there is no significant differences in the selectivity when the ligand is changed. The same experiments were repeated using 0.1% acetic acid instead of formic acid and still there were no significant differences in the retention was observed between columns. It shows that all these four phases

except the Viva C4 is having very similar selectivity for the separation of proteins that is used in the analysis. As the RP x RP is not performing well, attention was given to other modes of chromatography. First, SEC column was used for the 1D. There, when the flow rates were 0.2 mL/min or under peaks were drastically broader (peak widths of 10-15 minutes). This is because of the diameter of the column (id 7.8) being so big and proteins having slow diffusion. In order to get better peak shapes, flow rates needed to be at least 0.4 mL/min or higher. When the 1D flow rates are that high it needed to have very fast modulations with faster separations in the 2nd dimension. Chen and coworkers have used HIC mode for the analysis of proteins in the top down approach. [18] Finally, HIC mode is used as the 1st Dimension and the separation for the proteins when this mode is used in 1D is showing better results and further studies on going.

Table 5-2. Retention times for 1D separation of Ubi, Myo and Cyt c. A) with 0.1% formic acid B) with 0.1% acetic acid

Analyte	**Viva C4**	**Triisopropyl**	**Tri-N-butyl**	**N-butyldimethyl**	**triisobutyl**
Ubi	5.686	4.553	4.553	4.512	4.343
Myo	8.005	6.410	6.452	6.452	6.074
Cyt c	5.623	4.523	4.490	4.490	4.406

A.

Analyte	**Viva C4**	**Triisopropyl**	**Tri-N-butyl**	**N-butyldimethyl**	**triisobutyl**
Ubi	5.684	4.889	4.823	4.637	4.889
Myo	7.963	6.699	6.662	6.578	6.662
Cyt c	5.581	4.867	4.783	4.623	4.951

B

Table 2. Retention times for 1D separation of Ubi, Myo and Cyt c. A) with 0.1% formic acid B) with 0.1% acetic acid

5.4 Conclusions

Pharmaceutical industry is growing rapidly on using biologics as therapeutic agents and the analysis of these agents in biological fluids has been challenging due to the complexity of the samples. Attention has been given to development of multidimensional methods as it can give higher resolving power. The work presented here shows the attempts towards constructing a 2D LC system using triple quadrupole mass spectrometer for the analysis of proteins. The attempts to use RP x RP showing there is no significant difference in the selectivity and getting orthogonality by changing the pH of the mobile phase was problematic. When the mobile phases were not acidic detection of analytes were possible. Various reversed phases used or the different mobile phase conditions were also not able to give the desired resolution of the analytes. The use of size exclusion could give better resolution if columns with smaller inner diameter can be investigated. HIC mode has shown to resolve proteins better as and HIC x RP could be a better option as the conditions used are vastly different in the two modes. But still needs to work on how to avoid high salt concentrations getting in to the mass spectrometer or use salts such as ammonium tartrate which are mass spectrometer compatible. In conclusion, the system discussed here is working for the analysis of proteins in the two dimension but needs to work on finding the correct mode of chromatography to be used in the 1st dimension.

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Chapter 6

Summary and Future Work

Liquid chromatography is one of the most extensively used chromatographic techniques and the stationary phases used in LC has improved tremendously over the years. After the introduction of bonding ligands to solid supports, wider range of stationary phases have been introduced commercially. Octadecylsilane(ODS) C18, being the most popular reversed phase stationary phase is giving enough selectivity for simpler samples [1]. With the growth of the liquid chromatography applications, especially in the clinical laboratories, samples needed to be analyzed became more complex and the biological matrices are making the analysis even more challenging. In the LC, there are many variables such as mobile phases, selection of stationary phase, pH, mobile phase additives and temperature. Therefore, method development takes relatively longer time compared to gas chromatography [2].

Faster screening of stationary phases needed in order to get the advantage of the various stationary phases that are commercially available. At the same time if method development process can be automated, it will reduce the time that operator needs to sit in front of the instrument changing either columns or mobile phases. With the improvements in the information technology, use of the software programs have made the method development process automated at least partially. Screening of different stationary phases performed using one such software, Nexera Method Scouting Solutions for small molecule separation [3-4]. Use of model analytes set which includes analytes from various classes such as drugs, drugs of abuse, metabolites, amino acids, peptides and nicotine like molecules with the reversed phases would help to choose the stationary phase for the analysis structurally similar analytes. It has shown that biphenyl phase behaves similar to C18 when acetonitrile is used as the organic mobile phase and there is a significant selectivity difference with the pentafluoro phenyl phase compared to the other stationary phases, C18, biphenyl and polar embedded.

Free silanol group left behind after bonding of ligands sometimes can give undesirable effects on separation such as peak tailing. End capping, bonding the silanol group left behind with trimethylsilyl group is done to get the column performance improved [5-6]. It has shown that end capping help to get better peak shapes specially with the basic analytes. But depending on the size of the ligand used for

bonding, and the amount of coverage from the ligand can affect the end capping reaction and it will not reduce the concentration of free silanols. As an improvement to solid supports specially to avoid silanol effects, silica hydride was introduced. Silica hydride surface does not have any silanols instead it has silica hydride surface and therefore, there is no surface activity. There are four stationary phases where the solid support is silica hydride (Type C Silica) were used in the small molecule separation method development [7-9]. Results have shown those phases can be used in both reversed phase and aqueous normal phase. Further, cholesterol phase has shown a wider range for selectivity factor for the analytes used compared to the other three phases, diamond hydride, diol and bidentate C18 [10].

In both cases of method development for the small molecule separation, conditions were not optimized for each column. Instead same generic gradient condition was used for comparison purposes. Analyte set is composed of molecules from different classes of molecules with vastly different physicochemical properties. Therefore, in some cases the peak shapes are not great for all the analytes and in order to get better peak shapes for a particular analyte the condition needs to be optimized. The use of silica hydride phases would help to use one mode or the other depending on the application.

Methods for the analysis of small molecules using the triple quadrupole mass spectrometers (QqQ) are widely available. Use of triple quadrupole mass spectrometer for the top down protein analysis using MRM mode was introduced by Wang *et al*. Analysis of both small molecules and proteins from a single injection is advantageous [11]. Especially when the amount of sample is scarce and to get more precise information regarding a diagnosis of disease with multiple disease indicators. With the new improvements in the hardware of LC systems building new systems with multiple paths for the analysis of multiple classes of analytes is possible. Since the use of triple quadrupole mass spectrometers in the clinical laboratories is more common compared to the high-resolution mass spectrometers, it is important to develop methods which uses QqQ. Moreover, the discovery of protein biomarkers and protein therapeutic agents has increased significantly during the recent past. Including such protein analysis panel along with the currently analyzing small molecules will give the care givers chance to diagnose diseases at early stages which would benefit the patients significantly. One of the problems associated with the QqQ in protein analysis is the difficulty of getting the higher molecular weight proteins within the operational m/z range (for shimadzu 8050 QqQ m/z is from 2 to 2000). For example, mono clonal

antibodies (mAb) which are used as protein therapeutics have higher molecular weights (>100 kDa) and under normal conditions the charge state envelop goes beyond the operational mass range. The investigation of getting proteins supercharged by using additives such as dimethyl sulfoxide (DMSO), sulfolane, *m*-nitrobenzyl alcohol is currently undergoing to see the feasibility of getting the charge state distribution to operational mass range [12-15].

One of the major issues in the analysis of biological samples is the sample complexity. Biological samples for example blood plasma consists of proteins, metabolites, hormones and fatty acids. When quantitation methods are developed for example for hormones, other classes of molecules either needs to be removed during the sample preparation or chromatographically separate from the analytes of interest. When more steps involved in the sample preparation, it adds more time to the analysis and add uncertainty to the results. Further, it makes it difficult to automate the analysis. Therefore, more convenient and desirable way is to resolve analytes of interest from other molecules chromatographically. This is challenging task specially for the field of proteomics. As the resolving power of 1D chromatography is not sufficient to get enough separation for protein analytes, the focus is towards the multi-dimension separation methods. This study has focused on developing new system for 2D analysis of proteins using LC MS. In the 2DLC system RP x RP is explored with different mobile phase conditions but desired resolution was not observed. It has shown that most proteins have similar selectivity with reversed-phase stationary phases and when the same mobile phase conditions used there is no orthogonality in the RP x RP case. As HIC mode has shown better separation in the 1D mode, it is worth to use HIC x RP to get a better resolution [16-17]. In the HIC mode, high salt concentrations are used in the mobile phases and careful attention should be given to select mass spectrometer compatible salts such as ammonium tartrate. Further, when collecting fractions from the 1D to inject to the 2D column, guard columns can be used in place of loops. This is advantageous as it will help to preserve the separation obtained from 1D. Further, in cases where the mobile phases in the 1D is not matching with 2D, these guard columns can be helpful to adjust the mobile phase conditions. Therefore, investigation of using guard columns in place of loops could give better resolving of analytes [18].

This work on developing a system for the analysis of small molecules and proteins simultaneously using QqQ has potential applications in the bioanalytical field. Further, this system has the

potential to expand further where multiple channels with multiple trap columns to analyze multiple classes of anlaytes simultaneously. As more and more mAbs are developed as protein therapeutic agents the direct quantitation methods on biological samples would be necessary and the use of 2DLC with QqQ would greatly help the new drug development as well as monitoring of the protein therapeutics in the bioanalytical chemistry world.

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Appendix 1

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Biographical Information

Dananjaya Kalu Appulage obtained his Bachelor of Science degree in Chemistry from the University of Peradeniya, Sri Lanka in May 2006. Soon after graduating, he joined the department of chemistry, faculty of science, University of Peradeniya as a teaching assistant. While working as a teaching assistant he involved in research work on separation of cobra venom proteins under the supervision of Dr. Senerath B. Athauda at the department of biochemistry, faculty of medicine. Then, he joined the Wayne State University as a chemistry graduate student in August 2007 and joined with Andreana group in December 2007. He performed research on the carbohydrate based vaccine synthesis and one of the major contributions of his research work is the synthesis of Tn1 antigen. He graduated with M.A. in chemistry from the Wayne State University in 2011.

After taking a break due to his health conditions, he decided to get his Ph.D. degree and joined the department of chemistry and biochemistry at the University of Texas at Arlington in August 2013. Then, he joined Schug group in January 2014 and started working on liquid chromatography mass spectrometry. He studied the use of different reversed phased stationary phases for the analysis of small molecules using liquid chromatography mass spectrometry. Further, he worked on developing new method for the analysis of proteins and small molecules from a single injection using multi-dimensional liquid chromatography system. In 2016, he went to Restek Corporation as a summer intern and he worked in the research and development laboratory and application development laboratory. After coming back from the internship, he started working on the comprehensive two-dimensional liquid chromatography mass spectrometry method development for proteins.

He graduates with his Ph.D. degree in Analytical Chemistry from the University of Texas at Arlington in July 2017. He plans to start a career in the academia.