Chromatographic Behavior of Therapeutic Monoclonal Antibodies in Hybrid Hydrophobic Interaction Chromatography (HHIC)

By:
Madhaviprasanna Srikoti

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DISSERTATION COMMITTEE APPROVALS

We certify that we read this dissertation and that in our opinion it is adequate to scientific scope and quality as a dissertation for the degree of Doctor of Philosophy.

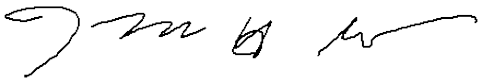
APPROVED BY:

4/27/2020

Dr. Yuri Kazakevich
Research Mentor, Department of Chemistry and Biochemistry, Seton Hall University

Mark S Bolgar 4.26.2020

Dr. Mark S Bolgar
Co-Research Mentor, Bristol-Myers Squibb, New Brunswick



Dr. Nicholas Snow
Member of Dissertation Committee, Department of Chemistry and Biochemistry, Seton Hall University

David Sabatino 4/27/2020

Dr. David Sabatino
Member of Dissertation Committee, Department of Chemistry and Biochemistry, Seton Hall University

Stephen K. Kelty 4.27.2020

Dr. Stephen P. Kelty
Chair, Department of Chemistry and Biochemistry, Seton Hall University

Abstract

A hybrid mode of hydrophobic interaction chromatography (HHIC) is an emerging chromatographic technique for the separation of biomolecules under non-denaturing conditions. This technique separates biomolecules in their native form where the difference in surface hydrophobicity is maximized. Hybrid mode of HIC (HHIC) methodology uses HIC stationary phases made with poly (alkyl aspartamide) silica columns, which function as a hybrid form of conventional HIC and reversed-phase chromatography (RPLC). This research provided fundamental knowledge about the impact of chromatographic parameters on the separation of co-formulated mAbs. The influence of mobile phase parameters such as salt concentration, pH and the role of organic modifier and as well as stationary phase parameters on the separation were evaluated. This research demonstrated that an adequate chromatographic separation of mAbs and related biomolecules was achieved using a low ammonium acetate concentration which was not achieved with model proteins previously. The study showed the analytical utility of low ammonium acetate condition (0.5M) to widen the chromatographic elution window by eluting early eluters faster and late eluters later and also an increase in peak capacity. In addition, low ammonium acetate concentration showed a five-fold increase in ESI intensity, which is an advantage for online MS.

In Chapter 1, a brief review of the theory, principles of the methodology and the details about the poly (alkyl aspartamide) silica columns is provided. In addition, the area of interest and the scope of the research is presented. In Chapter 2, the impact of salt concentration and organic modifier on the separation of therapeutic monoclonal antibodies and related biomolecules is presented. Data demonstrated that using these columns, with low concentrations of ammonium acetate, a small portion of organic solvent is required to elute biomolecules in a reasonable time frame. The research showed chromatographic separation is achievable under low ammonium acetate conditions and also helps to widen the chromatographic window. Assessment of conformational changes in the presence of moderate organic content using orthogonal methods is also presented. This chapter demonstrates the impact of linear flow velocity and gradient time on chromatographic efficiency using these columns. Chapter 3 presented how pH of the mobile phase can help tune the chromatographic separation along with the impact of temperature on the separation.

In the literature it was clearly discussed that to obtain adequate separation using ammonium acetate, a pentyl alkyl chain is required. This fact indicates that hydrophobicity of the stationary phase has a great influence on retention. In Chapter 4, the effect of alkyl chain length and the impact of hydrophobicity on selectivity along with the interdependencies of mobile phase parameters and the stationary phase is presented. HHIC chromatographic parameters such as organic modifier can induce conformational changes in biomolecules. Intrinsic fluorescence was used to gain basic knowledge on the conformational changes of a biomolecule. Chapter 5 provides the experimental results of conformational changes which were obtained using an offline batch mode of intrinsic fluorescence and provided fundamental knowledge about possible conformational changes in the presence of poly (alkyl aspartamide) silica columns using hybrid HIC mobile phases.

Dedication to

My Parents Late Mr. Babu Rao and Late Mrs. Krishnaveni

My Husband Manohar and My Daughter Sindhu

My In-Laws Mr. Gangadhar and Mrs. Narsubai

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List of Abbreviations

HIC	Hydrophobic Interaction Chromatography
HHIC	Hybrid mode of Hydrophobic Interaction Chromatography
RPLC	Reversed-phase liquid chromatography
SEC	Size exclusion chromatography
IEX	Ion exchange chromatography
LC	Liquid chromatography
HILIC	Hydrophilic interaction chromatography
MS	Mass spectrometry
LC-MS	Liquid chromatography-mass spectrometry
ELISA	Enzyme linked immunosorbent assay
SPAC	Salt-promoted adsorption chromatography
QToF	Quadrupole Time-of-Flight
ESI	Electrospray ionization
iFRET	Intrinsic Fluorescence Förster resonance energy transfer
CD	Circular Dichroism
NMR	Nuclear magnetic resonance
SLS	Static Light Scattering
DLS	Dynamic Light Scattering
HDX	Hydrogen deuterium exchange
LSS	Linear solvent strength
mAb	Monoclonal antibody
ADC	Antibody drug conjugate
HIB/R	Hydrophobic-interaction binding/release
PTMs	Post-translational modifications
CDRs	Complementarity-determining regions
HOS	Higher Order Structure

IgG	Immunoglobulin G
IgA	Immunoglobulin A
IgD	Immunoglobulin D
IgE	Immunoglobulin E
IgM	Immunoglobulin M
NIST	National Institute of Standards and Technology
BMS	Bristol Myers Squibb
DMF	N,N-dimethylformamide
NH_4OAc	Ammonium acetate
MeCN/ACN	Acetonitrile
IPA	Isopropyl alcohol
MeOH	Methanol
HCl	Hydrochloric acid
Trp	Tryptophan
Met	Methionine
MP	Mobile phase
M	Molar
mM	Milli molar
Mg	Milli grams
mL	Milli Liter
p*I*	Isoelectric point
kDa	Kilo Dalton
ΔH	Change in Enthalpy
ΔS	Change in Entropy
ΔG	Gibbs Free Energy
R	Gas constant
T	Temperature

Chapter 1 : Introduction and Recent Advances in HHIC Using Poly (alkyl aspartamide) Silica Columns

1.1 Overview

For the past three decades, therapeutic monoclonal antibodies (mAbs) and their derivatives have become the most promising and fastest growing therapeutics to treat various diseases such as cancer, autoimmunity, metabolic disorders, and infections [1-2]. The unique pharmacological advantages of mAbs (e.g., target specificity, selectivity, long half-life, and excellent safety profile) and the evolving protein engineering (bispecific antibody, fusion protein, antibody-drug conjugate, and nanobody) continuously drive the development of new mAb-based therapeutics. Therapeutic monoclonal antibodies act through multiple mechanisms, such as blocking of targeted molecule functions, inducing apoptosis in cells which express the target, or by modulating signaling pathways [3-4]. Monoclonal antibodies treat immunotherapeutic diseases, because each type of monoclonal antibody will target a specific targeted antigen in the body. Over 40 therapeutic antibodies have been already approved and more than 450 molecules are currently at different stages of clinical development [5-6]. Similar to antibodies, mAb-based antibody drug conjugates (ADCs) and related products such as Fc fusion proteins also gaining more attention to treat various deceases [7-9]. There are over 60 ADCs currently at the development stage for various indications [10-14]

In recent years, along with mono therapeutics, developing combination drugs became very important due to potential advantages. For example, combination drugs provide an option to use drugs at lower doses, reducing side effects but increasing efficacy, therefore very well established in therapeutic area. Combination drugs present a promising approach for cancer research [15-19], viral diseases [20] and anti-toxins [21]. The combination of two or more human

monoclonal antibodies (mAbs) co-formulated into a single drug product has advantages such as reducing medications errors, easy to use and patient safety [22]. In practice, combination chemotherapy results in a better response and improved survival compared with single-agent therapy. Compared to marketed small molecule combination products, the number of co-formulated biologics are very limited [23-25]. However, the interest is growing. So far only one protein co-formulation containing rituximab (MabThera) and human hyaluronidase is currently marketed to treat [26] and other examples include the combination of Platinol (cisplatin) and Navelbine (vinorelbine) to treat non-small cell lung cancer and TCH (Taxol, carboplatin, and Herceptin) for the treatment of HER2/neu-positive tumors [27-28].

MAbs and their derivatives are considered the most complex biologics due to their complex conformational and structural dynamics, large molecular sizes, and micro heterogeneity caused by various post-translational modifications. Because of their nature these molecules offer great analytical challenges in characterization. To ensure the product quality, comprehensive characterization and quantitation of each intact protein and all its variants is very crucial [29 30]. In addition, co-formulation of therapeutic antibodies increases the complexity of the drug product. Therefore, the characterization and release assay development [31] can be extremely challenging. It gets more complicated when the co-formulated antibodies have similar physicochemical properties and wide disparity in their concentrations. Even though there are robust analytical methods such as reversed-phase (RPLC), size exclusion (SEC), ion exchange (IEX), hydrophobic interaction chromatography (HIC) or affinity chromatography available, it can be highly challenging to characterize biomolecules in combination products due to the ratio of the molecules in the drug product. The reason being each of the co-formulated antibodies can exist in various heterogeneities such as size, charge, and post-translational modifications (PTMs) [32-33]

during manufacturing [34-35]. As combination therapy is a growing strategy, only limited analytical methods have been reported to measure quality attributes in combination biomolecule formulations [22].

1.2 Monoclonal Antibodies

MAbs (Figure 1.1) are glycoproteins, produced by a single clone of cells or cell line and consisting of identical antibody molecules. Derivation from a single B-cell clones, having monovalent affinity and subsequent targeting of a single epitope is what differentiates monoclonal antibodies from polyclonal antibodies.

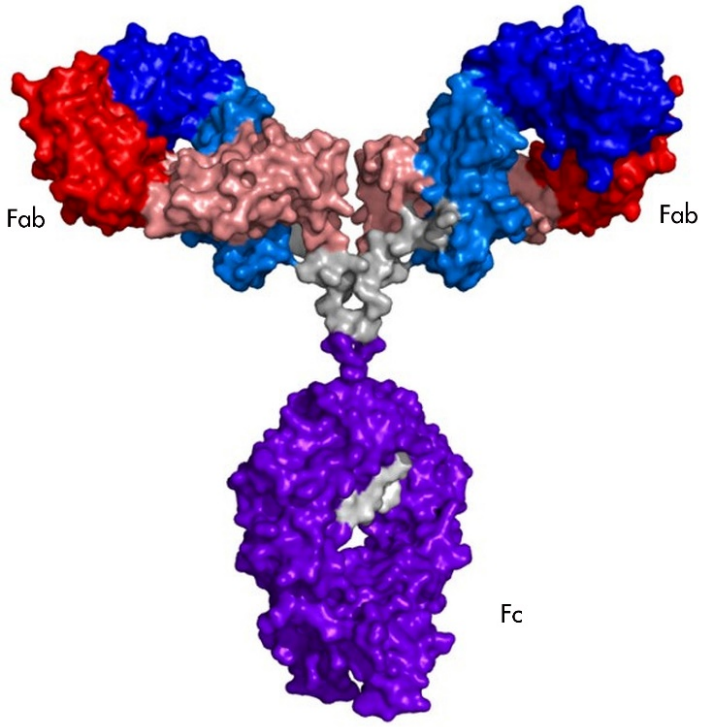

Figure 1.1 NIST mAb
https://www.nist.gov/news-events/news/2018/12/nist-2d-nmr-fingerprinting-study-gives-biopharmaceutical-sector-new-power
Source: www.nist.gov

1.2.1 Classification of antibodies

Humans have 5 classes (Figure 1.2) of antibodies (interchangeably used with Immunoglobulins or IgGs): IgG, IgA, IgD, IgE and IgM. All 5 classes are secreted by activated B cells as glycoproteins. These glycoproteins are produced by the immune system specifically bind to antigen. IgG antibodies are the most common and the most important. As these are the smallest, they can easily move across the cell membranes and circulate in the blood and other body fluids, protecting against bacteria and viruses. They also bind to the antigens to enhance the effectiveness of phagocytosis. Compared to other antibodies IgGs have highest half-life of about 21-23 days [36].

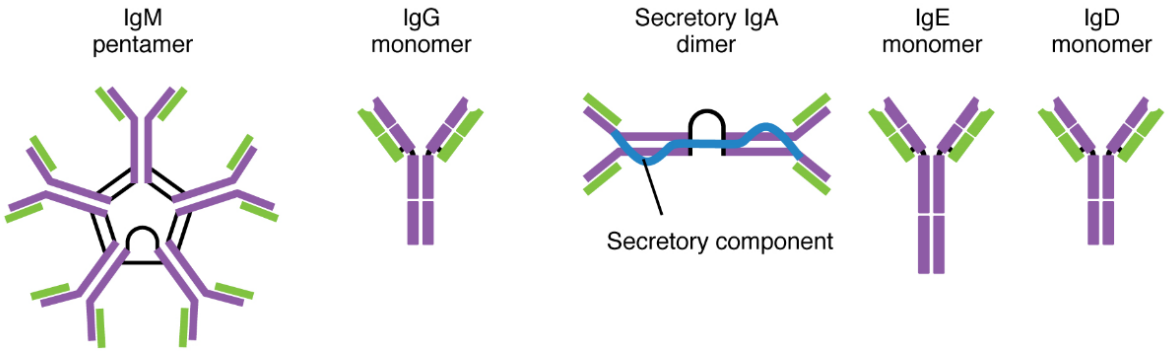

Figure 1.2 Types for Antibodies
https://commons.wikimedia.org/wiki/File:Figure_42_03_02.jpg
Source: commons.wikimedia.org

Through the process of hyper mutation and class switching, high affinity IgGs are produced. Human IgG is further subdivided into IgG1, IgG2, IgG3 and IgG4 isotypes (Figure 1.3), which differ in their heavy chain. They are highly homologous and differ mainly in the hinge region and their function in activating the host immune system. IgG1 and IgG4 contain two inter-chain disulfide bonds in the hinge region where as IgG2 has four and IgG3 has eleven [37-38]. The elongated hinge in IgG3 is also responsible for its higher molecular weight compared to the other subclasses. Even though IgG1 and IgG4 have similar structures, the hinge region of IgG4 is shorter than that of IgG1 and its flexibility is intermediate between that of IgG1 and IgG2. The CH2 domain of IgG4 has its most marked differences with IgG1 in a surface-exposed patch of the C terminal part of the domain, which in the 3D structure is close to the hinge [39]. IgG1, 2 and 4 widely used in therapeutics, however, IgG2 do not cross the placenta as readily as other human IgG isotypes and IgG3, which has a shorter serum half-life, is rarely used [30].

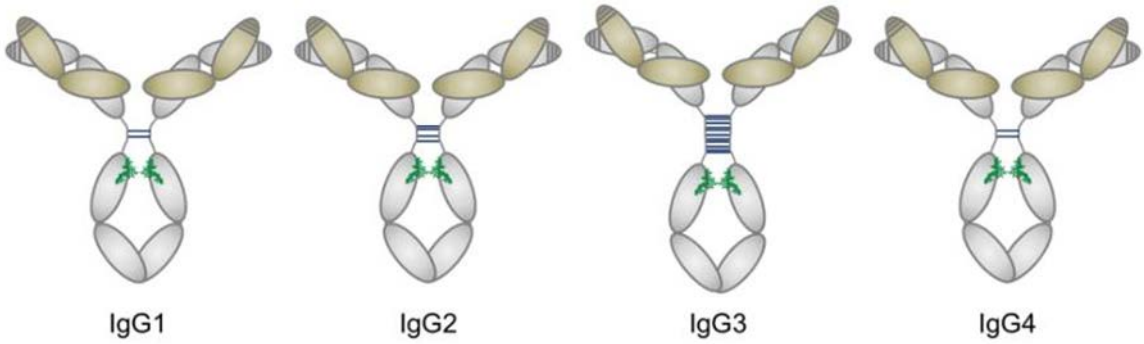

Figure 1.3. Types of IgGs
https://www.burnet.edu.au/projects/229_igg_subclasses_and_immunity_to_malaria
Source: www.burnet.edu.au
(Reprinted with the permission of Burnet Institute)

Table 1.1. IgG Antibody Isotype Comparison

Property	IgG1	IgG2	IgG3	IgG4
Molecular Weight (KDa)	146	146	170	146
Amino acids in hinge region	15	12	62	12
Inter-H chain disulfide bonds	2	4	11	2
Half-life (days)	~21	~21	7	~21
Relative abundance (%)	60	32	4	4

1.2.2 Antibody structure

Antibody molecules are roughly Y-shaped molecules (Figure 1.4) consisting of three equal-sized portions, loosely connected by a flexible chain [40]. Each chain has a tertiary structure consisting of distinct domains. Each domain in an antibody has a very similar structure of two beta sheets packed closely against each other. This whole assembly is finally into a quaternary structure and stabilized by various interactions such as ionic interactions and hydrogen bonds (Refer to section 1.2.3). IgG antibody is composed of one or more units, each containing four polypeptide chains: two identical heavy chains (H) and two identical light chains (L) [37]. Each heavy chain has about 500 amino acids and a molecular weight of ~50 kDa, as each light chain has about 210 amino acids and a molecular weight of ~25 kDa, resulting in a total immunoglobulin monomer molecular weight of approximately 150 kDa. The two heavy chains are each linked to each other and to a light chain by disulfide bonds. The resulting tetramer has two identical halves. Each end of the fork contains an identical antigen binding site, thus, each IgG has two antigen binding sites. The amino terminal ends of the polypeptide chains show considerable variation in amino acid composition and are referred to as the variable (V) regions to distinguish them from the relatively constant (C) regions. Each L chain consists of one

variable domain, VL, and one constant domain, CL. The H chains consist of a variable domain, VH, and three constant domains CH1, CH2 and CH3. CH2 domain is overlaid by an oligosaccharide (N-glycosylation) covalently attached as Asn297 [41]. There are three complementarity-determining regions (CDRs) in each light chain and heavy chain variable region, where antibodies bind to their specific antigen.

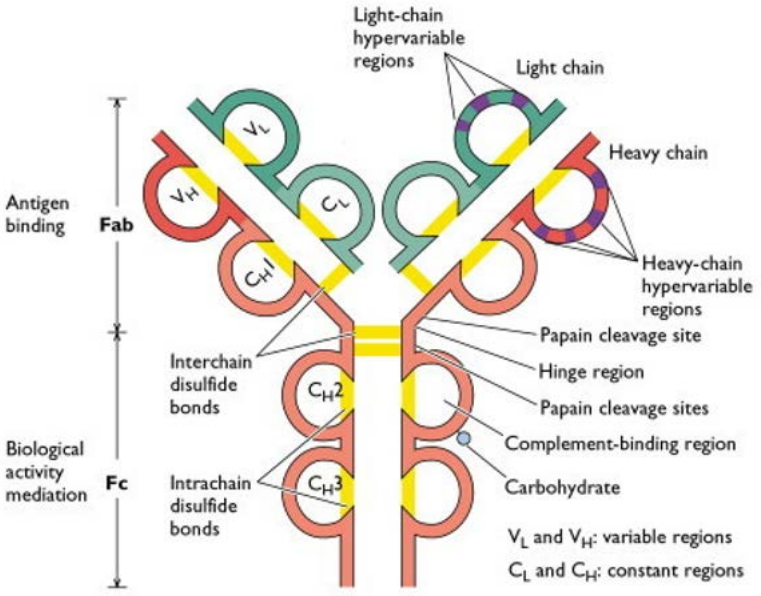

Figure 1.4. Structure of an Antibody
www.bing.com/images/search?view=detailV2&ccid=QWoTyXet&id=772E1BA31E4E8C7AE88C19125374F346613EB920&thid=OIP.QWoTyXetrFsRrLFSEC0OfAAAAA&mediaurl=http%3a%2f%2fi.stack.imgur.com%2f2DGzI.jpg&exph=357&expw=459&q=structure+of+an+antibody&simid=608045000885865324&selectedIndex=19&qft=+filterui%3alicense-L2_L3
Source: www.bing.com

1.2.3 Chemical bonds involved in antibody structure

The function of the mAb depends on the quaternary structure of the molecule. The structure is stabilized with non-covalent interactions between CH3 domains and the inter chain disulfide bonds at the hinge region. The tertiary structure (Figure 1.5) of proteins is determined

by a variety of attractive forces, including hydrophobic interactions, ionic bonding, hydrogen bonding, and disulfide linkages. Individual amino acids are bonded together in a polypeptide chain to make the backbone. The bonding interactions that are present in a mAb are hydrogen bonding, ionic bridges (a salt bridge is a combination of two noncovalent interactions: hydrogen bonding and electrostatic interactions), disulfide bonds, and hydrophobic intermolecular interactions. Hydrogen bonds form between the oxygen of the C=O of each peptide bond in the strand and the hydrogen of the N-H group of the peptide bond. Salt bridges, ionic interactions between positively and negatively charged sites on amino acid side chains, will also help stabilize the tertiary structure of a molecule. Cysteine will contribute to form disulfide bridges to stabilize the tertiary structure, allowing different parts of the molecule chain to be held together covalently. The alkyl groups of non-polar amino acids form hydrophobic interactions between one-another. MAbs consist completely of beta sheets, which are stabilized with the help of hydrogen bonds. The three-dimensional shape of the molecule and its function will be determined based on all of these interactions.

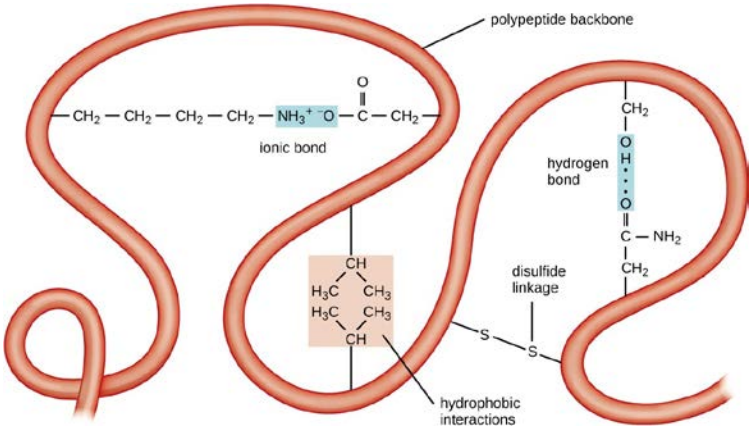

Figure 1.5. Chemical bonds involved in tertiary structure
https://commons.wikimedia.org/wiki/File:OSC_Microbio_07_04_tertiary.jpg
Source: Courses.lumenlearning.com

1.3 Challenges in Analytical Characterization of Antibody and Related

Therapeutic mAbs are produced using living organisms and manufactured using complex processes. As these biomolecules are very sensitive to process conditions, synthesis can cause various post- and co-translational modifications introducing intrinsic heterogeneity [42], which may affect biological activity resulting in molecular variability. Some of the structural differences are glycoforms, charge variants, cysteine-related, oxidized amino acid side chains, formation of aggregates, deamidation products as well as amino and carboxyl terminal amino acid additions and low level point mutation variants [29, 43]. These small structural changes can affect preclinical stability and process optimization in addition to therapeutic product potency, bioavailability and immunogenicity. The development of a successful therapeutic mAb needs the identification and manufacture of a selective and potent molecule that performs the required task, humanization of sequences, affinity maturation, Fc engineering to modulate effector functions. In addition, the development also requires proper engineering to address biophysical liabilities that would negatively impact manufacturability and/or patient effectiveness.

Developing therapeutic biomolecules is a highly complex process and there are many factors that can challenge the manufacturing, processing and storage, which can in turn cause physical and chemical degradation of the product. The most common causes of physical degradation are excessive temperature, mechanical, and freeze/thaw stresses. Chemical degradation affects the primary sequence and may also lead to significant changes in the higher order structure (HOS). Examples of chemical degradation include deamidation, oxidation, isomerization, hydrolysis of a peptide bond, clipping/fragmentation, and cross-linking. The reason that both physical and chemical degradation are so critical is that the efficacy of mAb-based therapeutics is closely tied to their structural, conformational, and chemical stability. To

deliver a therapeutic drug with a greater patient's safety, characterization to understand structural differences, physical degradation and chemical degradation are extremely important.

In addition to mAbs, Fc fusion proteins and ADCs which are manufactured by either joining two or more genes that originally coded for separate biomolecules or an addition of the drug payload to an already structurally-complex antibody via a linker molecule, characterization of ADCs presents a substantial challenge from an analytical development perspective [44] [45]. Combining these complex molecules and making co-formulation of therapeutic antibodies increases the complexity of the drug product. Therefore, the characterization and release assay development [31], can be extremely challenging. It gets more complicated when the co-formulated antibodies have similar physicochemical properties and significant difference in their concentrations. Even though there are robust analytical methods available, it can be highly challenging to characterize biomolecules in combination products due to the ratio of the molecules in the drug product and each of the co-formulated antibodies can exist in various heterogeneities in size, charge, and post-translational modifications (PTMs) [32-33] during manufacturing [34-35]. As combination therapy is a growing strategy, only limited analytical methods have been reported to measure quality attributes in combination biomolecule formulations [22].

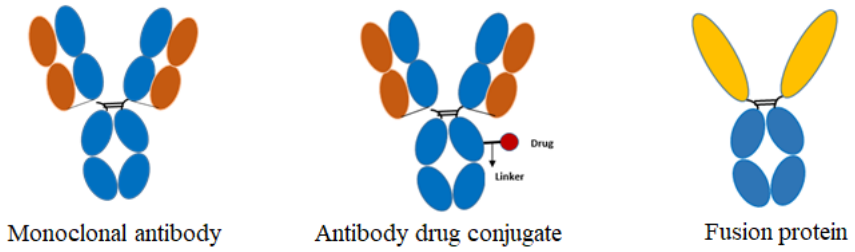

Figure 1.6. Mab and related biomolecules

There are various methods available to analyze biomolecules. However, many challenges remain for top-down proteomics, including the challenges to separate biomolecules in their native intact conformation [46-47]. There are several liquid chromatographic techniques that are well established to characterize biomolecules, such as reversed-phase liquid chromatography (RPLC), size exclusion chromatography (SEC), ion exchange chromatography (IEX) and hydrophobic interaction chromatography (HIC). However, the ideal method is a liquid chromatography (LC) which can use volatile mobile phases to separate complex mixtures. These methods offer the advantage of an online separation with direct coupling to a mass spectrometer and is compatible for automation where fraction collection process can be avoided to achieve high-throughput analysis [48]. However, the number of chromatography methods that are compatible with online MS capability is limited [49]. Hydrophilic interaction chromatography (HILIC) is one of the techniques which can be directly coupled with MS, but HILIC uses solutions which contain high concentrations of organic solvent, which can then denature the biomolecule [50]. The separation in Size Exclusion Chromatography (SEC) and Ion Exchange Chromatography (IEC) is performed using a volatile buffer containing ammonium acetate. Even though these techniques are compatible for MS [51], SEC is not yet a high-resolution method [51]. While IEC is a high-resolution method, complex protein mixtures often require more than one

dimension of fractionation [52]. Reversed-phase Chromatography (RPC) is the most commonly used chromatographic method which is MS compatible [53-58], and has been used in the purification and analysis of biological molecules. Nevertheless, chromatographic conditions such as high temperature, and mobile phase components such as high organic content and stronger hydrophobic stationary phases can denature and expose numerous hydrophobic moieties of the biomolecule [59-61]. This can cause adverse effects such as loss in biological activity, peak broadening, low protein recovery, and also adsorbed on to highly hydrophobic stationary phase resulting in failure to elute from the column [59]. Therefore, the use of RPLC methods is uncommon for large-scale protein separations [62] and there is a need for additional methods for online LC-MS that can provide high-resolution separation of a wide range of proteins with minimal denaturation.

HIC is an alternate liquid chromatographic techniques for RPLC where the separation of biomolecules is based on hydrophobicity. To overcome some of the RPLC challenges, HIC has been used as an alternative method to separate proteins. The main advantage of HIC is that separation can be achieved under protein native conditions where the differences in the surface hydrophobicity will be maximized for molecules with high degree of sequence homology. Although both HIC and RPLC separation mechanisms are based on hydrophobicity, HIC separation is based on native surface hydrophobicity [63] and RPLC separation is based on the hydrophobicity of either partially denatured protein or fully denatured protein. In addition, HIC selectivity can be improved by modifying the column matrix surface, the alkyl chain bonding density and mobile phase variables [64].

HIC is one of the most widely used techniques for determining the relative hydrophobicity of mAbs, to separate the different populations of ADC molecules that differ in

their number of drugs per antibody (drug-to-antibody ratio) species and also to separate and characterize their positional isomers [65-66]. The separated proteins can be collected for further activity measurements (such as cell based potency, receptor binding, cell proliferation assay, enzyme assay, functional enzyme linked immunosorbent assay (ELISA) and many more). HIC has been a valuable tool in downstream purification process where it is frequently used for protein purification based on the apparent hydrophobicity of impurities [63, 67-68]. Due to the nature of the technique, it gained a lot of importance in analytical separation of micro-heterogeneity in mAbs caused by post translational modifications and the analysis of antibody drug conjugates. Methionine and Tryptophan oxidation are common chemical modifications which affects the activity loss in biomolecules [69]. Oxidized mAb microvariants are generally characterized using a bottom up (peptide mapping) approach and techniques such as HIC and RPLC [70]. In a case study Boyd et al [71] showed that HIC was able to isolate oxidized Trp IgGs from a basic peak hence enabling the monitoring of Trp oxidation. In addition, HIC demonstrated the capability of separating oxidized Met and deamidation products that coelute with another basic peak under the same conditions.

As previously described [44] conventional HIC may not be efficient enough to separate positional isomers at protein level. However, HIC under low salt concentration with online MS compatibility may extend capabilities to monitor isomerization.

1.4 Commercially Available HIC Columns to Characterize Antibodies and Related Molecules

HIC is a growing separation technique to separate biological molecules and only limited number of columns with different matrices are available to achieve adequate separation. Columns with solid phase description including known applications are indicated in Table 1.2. Below are the commercially available columns for conventional HIC chromatography [66].

Table 1.2. HIC-HPLC Columns and Analytical Applications for mAbs and related molecules [66].

Column	**Solid phase**	**Particle/pore size**	**Application**
Dionex Propac	proprietary ethyl/amide based chemistry on non-endcaped silica	5 µm, 300Å pore size	Trp oxidation, Asp isomerization, succinimide in mAbs, Carboxy terminus processing in Fc, serine O-fucosylation
TSKgel butyl-NPR	butyl on polymethacrylate base material	2.5 µm, (non-porous)	Proteolytic cleavage aggregates, misfolded domains.
TSKgel phenyl- 5PW	phenyl on polymethacrylate base material	10 µm, 1000Å pore size	Asp isomerization in mAbs, Fab N-glycosylation, free thiol in Fab
TSKgel ether-5PW	poly ethyl ether on polymethacrylatebase material	10 µm, 1000Å pore size	(Fab)2 purification, Antibody drug conjugates
Sepax Proteomix HIC Butyl	Spherical, highly cross-linked PS/DVB	1.7, 5 and 10 µm, non-porous	Proteins, mAbs, ADCs, oligonucleotides and peptides
MAbPac HIC-Butyl	Hydrophilic polymer-based	5 µm non Porous	mAbs and ADCs
POROS P2/20	Phenyl on polystyrenedivinylbenzene particles	20 µm, 500–10000Å pore size	Preparative applications
PolyLC ethyl Aspartamide	ethyl/aspartamide on silica	5 µm, 1000Å pore size	Antibodies, polypeptides and proteins
PolyLC methyl Aspartamide	methyl/aspartamide on silica	5 µm, 1000Å pore Size	Isolation of integral membrane proteins and their complexes
PolyLC propyl Aspartamide	propyl/aspartamide on silica	5 µm, 1000Å pore size	Antibodies, Antibody minor variants, polypeptides and proteins
PolyLC butyl Aspartamide	propyl/aspartamide on silica	2 and 3 µm, 1000Å and 1500 Å pore size	Antibodies
PolyLC pentyl Aspartamide	propyl/aspartamide on silica	2 and 3 µm, 1000Å and 1500 Å pore size	Antibodies and proteins

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1.5 Theoretical Aspects of HIC

Since RPLC technique operates under protein denaturing conditions such as mobile phase with acidic additive, high percentages of organic modifier, elevated temperatures and stationary phases with high hydrophobicity, it is very difficult to separate the biomolecules with minor hydrophobicity differences. In those circumstances, HIC will be a valuable tool [72] where it can separate molecules with very minor differences in overall hydrophobicity and significant difference in surface hydrophobicity. In 1948, for the first time Tiselius [73] described the separation concept of HIC based on the protein salting-out principle. Later on, in 1973 Hjertén called this mode of separation hydrophobic interaction chromatography [67]. Due to the unique characteristic nature of this technique, HIC gained a significant interest in the industry. In 2016, Fekete [74] clearly showed in Figure. 1.7, the difference in chromatographic profiles of a reduced mAb obtained by both RP and HIC. In HIC, the original "Y" shape of the mAb is maintained even after the disulphide bridges have been reduced. Whereas in RPLC, the heavy- and light chains (Hc, Lc) are well separated.

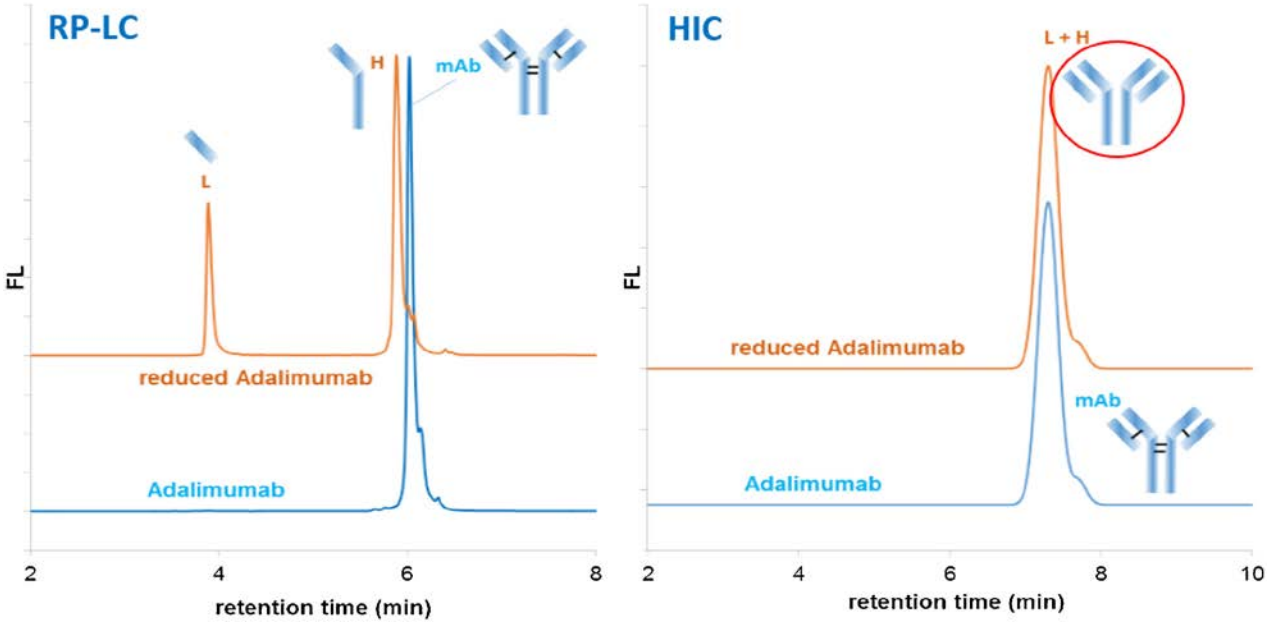

Figure 1.7. Analyzing intact and reduced mAb (adalimumab) in RPLC and HIC [74]
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HIC retention model is a complex mechanism because multiple gradients play a role in the separation. Throughout past few decades, there have been many fundamental studies which led to the different retention models and various theories such as hydrophobic interaction, hydrophobic effects, solvophobic theory, salting-out effect and dehydration of proteins to explain the retention in HIC. Based on these theories and experimental results it was clear that protein retention in HIC is driven by multiple parameters.

1.5.1 Salting-out effect

The concept of protein chromatography is based on hydrophobic interactions. In protein chromatography, the mobile phases are salt solutions, so Tiselius used the term "salting-out chromatography". A salting-out effect is the interaction of mobile phase – protein in HIC, which means nonelectrolyte proteins become less soluble in an electrolyte mobile phase which is in

high salt concentration. In aqueous solutions, proteins fold and the hydrophobic amino acids form protected hydrophobic areas, whereas hydrophilic amino acids interact with the surrounding water molecules to form hydrogen bonds. If the hydrophilic surface of the protein is large enough, then the protein can be dissolved in water. When salts are added to the solution, most of the water molecules will solvate salt ions and the number of water molecules available to interact with the hydrophilic amino acids will decrease. As a result, the protein–protein intermolecular interactions become stronger due to the decreased amount of surrounding water molecules. In the end, the protein molecules can self-associate (aggregate) by forming hydrophobic interactions with each other [74]. Because the separation is highly dependent on salt gradient, this mode separation was also called "salt mediated separations of proteins". In 1986, Porath proposed that it be called "salt-promoted adsorption" or "salt-promoted adsorption chromatography (SPAC)" as alternative names for HIC [75].

1.5.2 Hydrophobic effects, formation of cavity

In general, a hydrophobic effect is defined as the tendency of nonpolar molecules to self-associate in water rather than to dissolve individually**.** Hydrophobicity is defined as the repulsion between a non-polar moiety of the protein and of the polar environment such as water [63]. This effect is responsible for the low solubility of proteins [75-76]. On the same token, the term "hydrophobic interactions" is frequently used to describe the forces resulting in the association of nonpolar molecules or the binding of hydrophobic moieties in aqueous solutions. When biomolecules dissolve in aqueous system, water cannot make hydrogen bonds with nonpolar moieties of the protein. Due to that reason, the neighboring water molecules get separated from each other to form a cavity for the protein [77]. This process requires energy. On the other hand,

when two or more molecules come closer, they are associated with hydrophobic interactions. As a result, their hydrophobic contact surface area is reduced and energy is released (exothermic). The amount of energy is proportional to the size of the hydrophobic contact surface area of the protein. In other words, the aggregation or (self-association) in aqueous solutions is a spontaneous process and is mainly driven by the entropy change [77-80]. The orientation of the water molecules at the cavity around the non-polar protein molecule will lead to a large entropic effect [81-82]. Sturtevant and Livingstone later demonstrated that the heat capacity change in transfer-into-water processes is driven by the change in the water-accessible non-polar surface area of the molecule [74, 83-84]. Overall, hydrophobic interactions not only involve entropic effects but are entropy driven at low temperatures, and enthalpy driven at elevated temperatures, when the heat capacity change remains constant in the range of experimental temperature [85].

1.5.3 Solvophobic theory

The solvophobic theory explains the interactions between polar solvents (aqueous mobile phase) and less or non-polar solutes (protein). In polar solvents, strong cohesive forces are present between the solvent molecules due to hydrogen bonding and other polar interactions provide a strongly structured order. Therefore, less polar solutes tend to be insoluble because these strong solvent–solvent binding interactions must be overcome to make bonds with polar solvents. According to this theory, the solute molecules adsorb to the surface of the stationary phase as a result of their rejection from the polar solvent and their attraction for the hydrophobic stationary phase [86]. This seems to be the mechanism for the molecule retention in liquid chromatography, in general. Horváth et al. used solvophobic theory to explain the basis of retention mechanisms in RPLC [87].

1.6 Principle of HIC

Hydrophobic proteins will self-associate (aggregate) or interact with each other when they dissolve in aqueous solutions. This interaction between the molecules can result in various biological interactions, such as protein folding, protein-substrate interactions, etc. HIC is used in both analytical and protein purification application to characterize biomolecules. The basic principle of HIC is hydrophobic regions in large molecules bind to hydrophobic alkyl chains of the stationary phase. These interactions occur in an environment which helps hydrophobic interactions, such as high salt solutions [68].

In general, water (a polar solvent) is a poor solvent for nonpolar molecules. Therefore, in pure water, proteins will self-associate or aggregate, in order to achieve a state of lowest thermodynamic energy. Prior to self-association, water molecules form highly ordered structures around each individual macromolecule (Figure 1.7). The self-association of nonpolar molecules (such as proteins) in the polar solvent is driven by a net increase in entropy of the environment. During the aggregation process, the overall surface area of hydrophobic sites of the protein exposed to the polar solvent is decreased, resulting in a less structured (higher entropy) condition, which is the favored thermodynamic state.

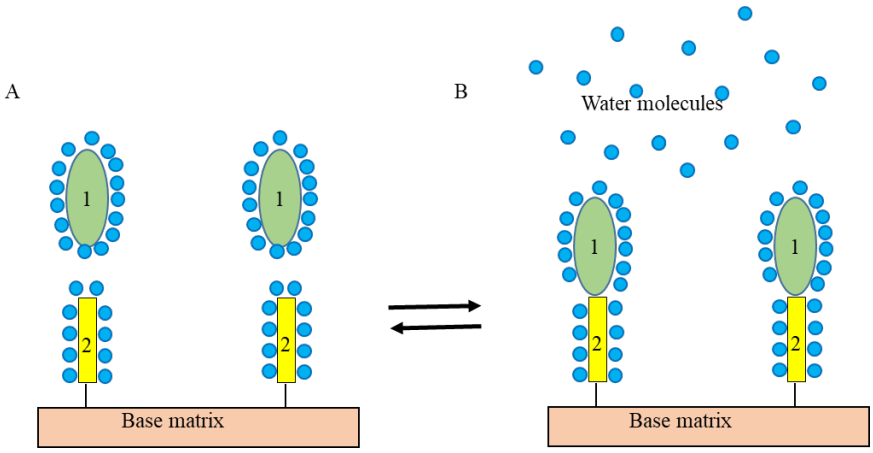

Figure 1.8. HIC separation mechanism (1) Biomolecule (2) Alkyl chain on the base matrix (A) Ordered water molecules around the hydrophobic patches of the biomolecule in low salt conditions (B) Disrupted water shell and biomolecule-stationary phase interactions in high salt conditions

HIC separations result from interactions between hydrophobic patches of proteins and low-density and moderately hydrophobic alkyl chain [88] attached to the stationary phase, which has a base matrix of silica or polymethacrylate [89-90]. In conventional HIC, the separation is performed using buffer systems by applying a linear salt gradient starting at a relatively high salt concentration [91]. In highly concentrated salt solutions (mobile phase A), proteins lose their hydrated shell and as a result hydrophobic patches will be exposed. These hydrophobic moieties will be adsorbed by the hydrophobic surface of the resin causing retention on the stationary phase. For protein elution to occur, an aqueous solvent (mobile phase B) containing no or low salt concentration is used. This low salt mobile phase will help to reassemble the water shell and enable the elution of the proteins from the column based on the surface hydrophobicity (Figure 1.8). Therefore, the elution order enables ranking of the proteins on the basis of their surface hydrophobicity, with high recovery and high sensitivity to conformational variations [63].

1.7 Theory

HIC [67, 92-94] chromatography is an established and powerful analytical tool [95] for separating biomolecules. The retention in HIC is mainly driven by the hydrophobic interactions between amino acid residues of the proteins and the alkyl chains or other non-polar functional groups located at the surface of the stationary phase [67, 76]. In HIC, retention is mainly affected by the biomolecule's surface hydrophobicity [63-64, 96-98].

A commonly used salt concentration as the starting condition in conventional HIC is in between 1 M of ammonium sulphate of ammonium phosphate and around 5.5 M ammonium acetate. The selected concentration of a salt will depend upon the lyotropic strength of the salt, the solubility of a biomolecule in the salt solution and the nature of stationary phase used in the separation [87, 99-101]. The influence of salt type plays a major role in hydrophobic interaction. The lyotropic strength of salts follow Hofmeister series (Figure 1.9) for the precipitation of biomolecules [102]. The salts that are typically used in the HIC methodology are sulfate, phosphate or citrate salts which are at a high lyotropic strength in the Hofmeister series [63]. Unfortunately, these salts are not compatible with MS analysis [47]. As an alternative, a volatile salt such as ammonium acetate can be used and is proven to reduce nonspecific sodium adducts [103-104]. According to the Hofmeister series ranking order, ammonium acetate is not a strong kosmotropic salt and acetate ions are at a low position of the series [63]. It cannot promote strong hydrophobic interactions by completely disrupting hydration shell around the biomolecule to enhance protein-surface interactions. While acetate allows for compatibility with MS [105], it was proven to provide inadequate retention when used with conventional HIC materials [106] which are mostly made with butyl alkyl chains and PolyPROPYL A [105] stationary phases. In the presence of ammonium acetate, a stationary phase which has alkyl chain length longer than butyl stationary phase may

be required to interact with amino acid residues of the mixture of biomolecules for an adequate retention. In 1986, Gooding et al. proved that HIC column with pentyl alkyl chains gave adequate retention of proteins with 4 M ammonium acetate [104].

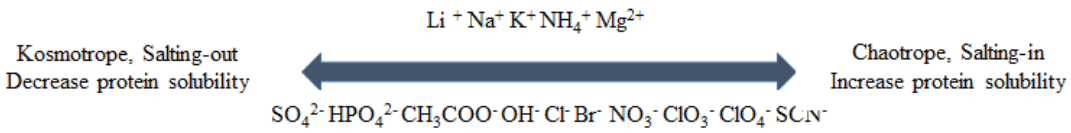

Figure 1.9. Hofmeister series with lyotropic strength

HIC columns are less retentive compared to RPLC columns. The reason for this is that the stationary phases which are used in HIC are low-density and moderately hydrophobic ligands such as butyl, phenyl or ether, and are attached to a hydrophilic under layer such as silica or polymeric material [88-90]. Because these columns have low bonding density, the strength of retentivity between biomolecules and the alkyl chains can be controlled [68]. These weakly hydrophobic ligands interact with a limited set of hydrophobic residues on the surface of the molecule's tertiary structure. With the above chromatographic conditions, usually biomolecules elute in the order of increasing hydrophobicity, generally with high recovery and high sensitivity to conformational variations [63]. In this technique, although molecule size may contribute to retention mechanism, surface hydrophobicity and bonding density of alkyl chains determine retention [64]. Since high concentrated salt solutions are used in HIC, there are very minimal secondary interactions, unlike RPLC [107]. Therefore, the elution order enables ranking of the biomolecules on the basis of their relative hydrophobicity [107].

The retention mechanism of HIC methodology can be altered by adding a small portion of organic modifiers. It has often proven as an advantageous parameter in HIC method development for decreasing the retention of highly hydrophobic compounds and also to adjust selectivity [89, 107-108]. Maintaining all of the mobile phase conditions of conventional HIC and adopting the use of an organic modifier such as acetonitrile from RPLC, hybrid mode of HIC (HHIC) (Figure 1.10) methodology enhances the ability to decrease the retention time of adsorbed solutes on HIC stationary phases. Organic solvents will help to weaken the protein-stationary phase interactions leading to a decrease in the retention time [107, 109]. Fekete et al. proved that this approach increases the recovery of biomolecules [107, 110] and also allows for the separation to be tuned while maintaining biomolecules native conformation [49, 107, 110].

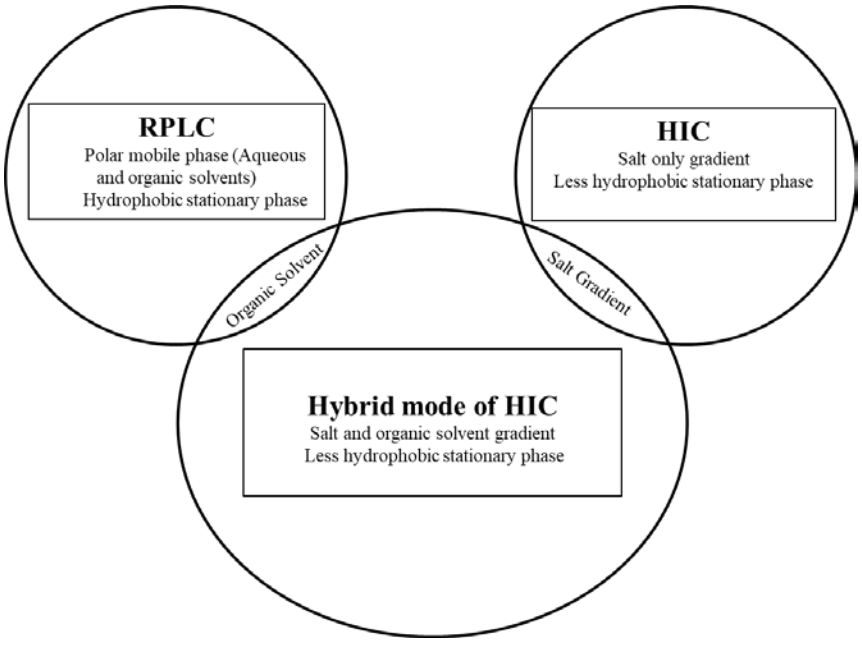

Figure 1.10. Hybrid HIC combines elements of two major chromatographic methods (RPLC and HIC)

Although conventional and Hybrid HIC compliments other chromatographic techniques, it has challenges, such as use of mobile phases with high concentrations of sulfate and phosphate salts [111]. As these are non-volatile salts [63], they prevent online coupling with mass spectrometry (MS). However, volatile salts such as ammonium acetate can be used but in higher concentrations. To overcome this limitation and to potentially provide an alternate selectivity, in 2016, Alpert used poly (alkyl aspartamide) silica HIC stationary phases that he synthesized by derivatizing short polymers such as anhydropoly(aspartic acid), or poly(succinamide) (Figure 1.11) [112 88] to develop a series of more-hydrophobic HIC materials.

1.8 Preparation of Poly (alkyl aspartamide) Silica

The following sections will describe the synthesis of poly (alkyl aspartamide) silica stationary phases which will provide an option of using lower concentrations of volatile salts with an online MS compatibility.

1.8.1 Preparation of poly (succinamide)

Various studies showed the advantages of using short polymers to prepare coatings for inorganic chromatography supports [113]. The coatings that are made with these polymers converts an inorganic support into a cation-exchange material suitable for protein chromatography. The reactive polymer is anhydropoly (aspartic acid), or poly (succinimide) which is formed in almost quantitative yield by heating aspartic acid under conditions which causes it to condense (Scheme 1) [80-84]. poly-α, β-D and L-aspartic acid [81-82] were produced from subsequent hydrolysis steps. Poly (succinimide) could be used to prepare a variety of chromatographic media in addition to the cation exchanger.

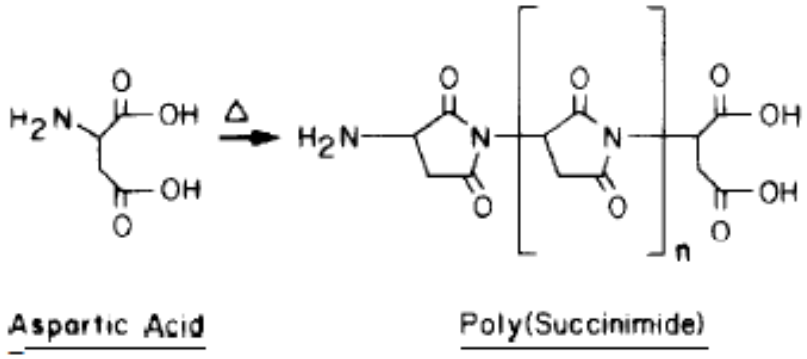

Figure 1.11. Preparation of Poly (Succinamide) from Aspartic Acid [112]
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1.8.2 Preparation of aminopropyl-silica

Aminopropyl-silica was prepared by mixing vydac silica with 3-aminopropyltriethoxysilane in toluene. After a step by step process, in a few hours the product was collected and washed well with toluene and acetone, then dried by continued suction.

1.8.3 Preparation of poly (succinimide)-silica

The dried aminopropyl-silica was swirled and degassed using poly (succinamide) in DMF. After 24 hours of swirling at room temperature the product was collected and washed well with DMF ad acetone to obtain poly (succinamide)-silica. Then above prepared poly (succinamide)-silica was swirled and degassed in a mixture of DMF and containing β-alanine and trimethylamine. After 24h of occasional swirling, washing well with HCl, water and acetone and going through the drying process, the poly (aspartic acid)-silica will be ready. The coating is simple and is easy to prepare reproducibly. The columns packed with this material showed very good performance in capacity, selectivity, recovery of enzyme activity and peak shape.

Due to its reactivity, poly (succinimide)-silica can be used to make various derivatives other than poly (aspartic acid)-silica. These derivatives can be used for steric exclusion

chromatography and various ligands could be added to the poly (succinimide) coating for use in affinity chromatography.

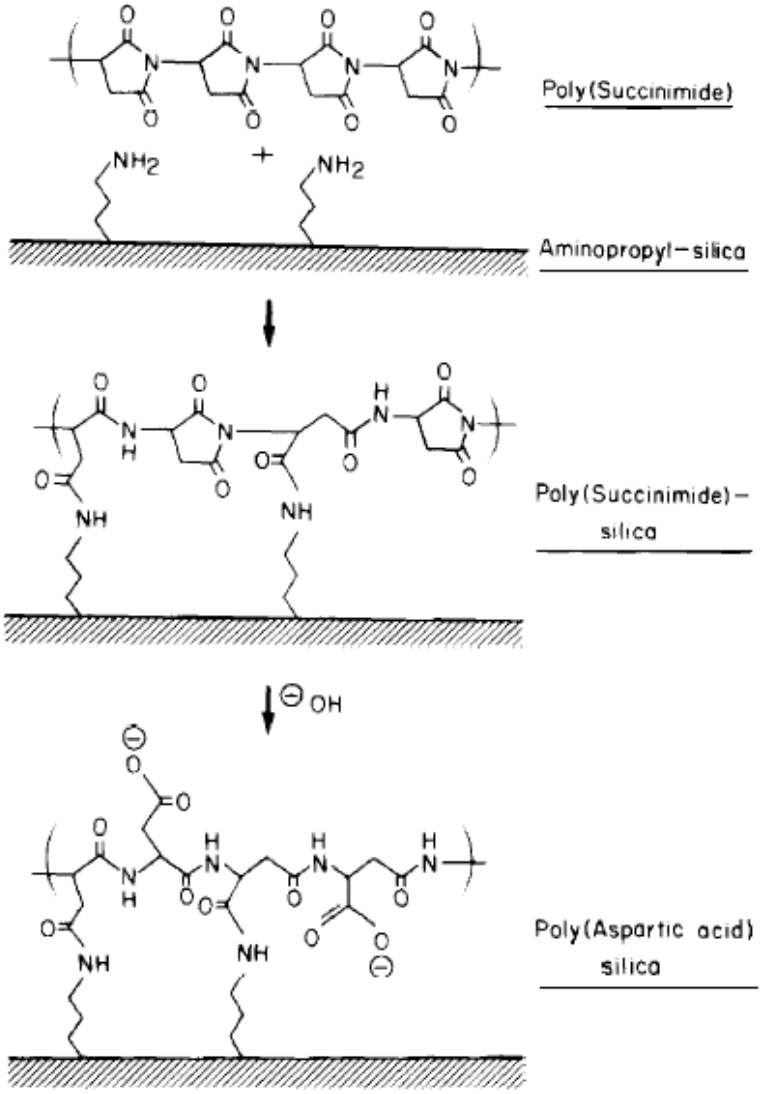

Figure 1.12. Reaction scheme for the preparation of poly (Aspartic acid) silica [112]
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1.8.4 Preparation of poly (alkyl aspartamide)-silica

Poly (succinimide)-silica was weighed into a flask and swirled in N,N-dimethylformamide (DMF). With continuous swirling alkylamine was added along with DMF. The resulting mixture was left for 24 h at room temperature with frequent swirling during the first 2 h and occasional swirling thereafter. The product was collected in a medium-porosity sintered-glass funnel and washed well with water, then hydrochloric acid, water, and acetone, and finally dried by continued suction to obtain poIy(alkyl aspartamide)-silica [88].

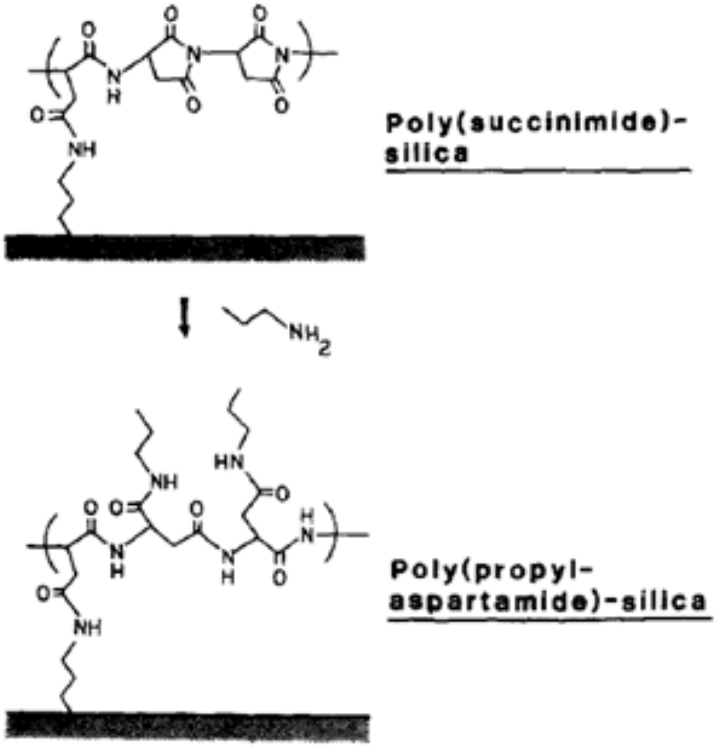

Figure 1.13. Reaction scheme for the preparation of poly (propyl aspartamide) silica [88]
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PolyPROPYL A and PolyETHYL A materials were synthesized and both cation-exchange capacity [112] and hydrophobic-interaction binding/release (HIB/R) capacity was estimated using hemoglobin [64]. The results proven the capacity of materials is very high.

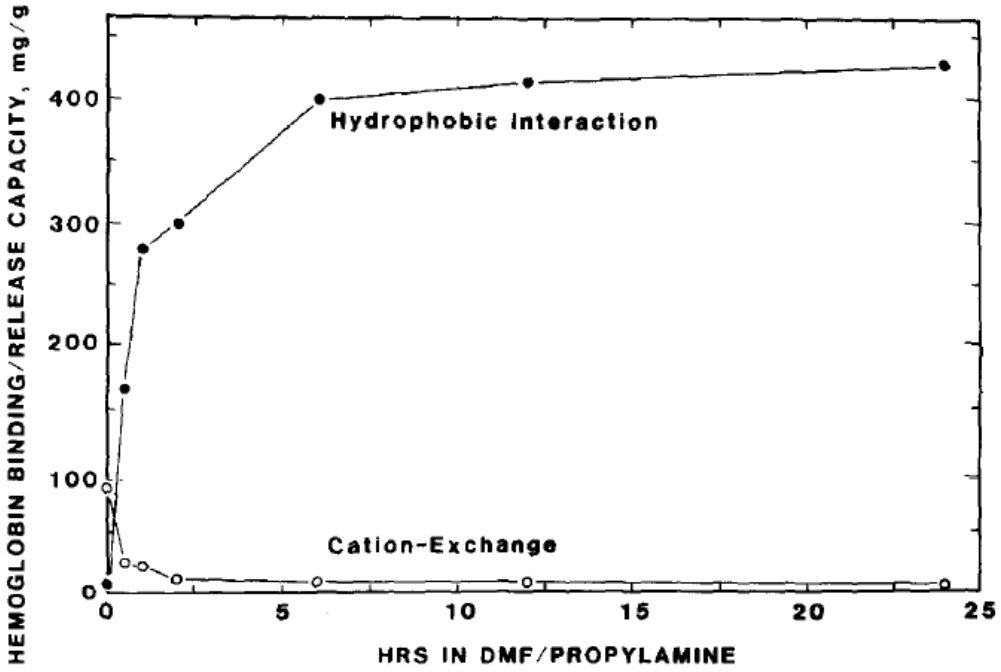

Figure 1.14. The reaction of Poly(succinimide)-silica with n-propyl amine [88]
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Experiments were carried out to study the protein recovery of PolyPROPYL A and PolyETHYL A columns and in general greater than 90% recovery was observed. The data also demonstrated that most of the molecules eluted with preservation of full activity with some exceptions. Later it was confirmed that it was due to higher ionic strength of the medium in which molecule was unstable. Research also showed that addition of detergents in the mobile phase not only may help with lower recoveries but may also increase selectivity [114]. These columns were also evaluated for estimating the retention characteristics and conformational

lability. The experimental results indicated that for some proteins there were very broad peaks or some scenarios multiple peaks were observed.

Stationary phases with these coatings demonstrated a wide range of hydrophobic properties and it was observed that proteins are generally eluted as sharp peaks with good recovery [88]. Using these columns, the native and denatured forms of biomolecules can be resolved if conformation change is slower than the migration time through the column [115-119], otherwise, a single broad peak can be expected, which is the weighted average of different conformations which are in the equilibrium [117, 119-121]. Historically it was proven that in HIC Van der Waals forces are involved [122-123] which will operate in a much shorter range than the range in electrostatic effects. This suggests that unlike ion-exchange, the access of the adsorption on the stationary phase to adsorption sites on a protein surface will be more sensitive to conformational differences in HIC. Therefore, protein with different conformations will have different binding affinity in HIC, and protein peaks in HIC usually elute as broader peaks compared to ion exchange. The same observation was made by Alpert in his research, as well [88]. Based on various experiments Alpert indicated that these poly (alkyl aspartamide) coatings of silica capacity is very high and can be used for preparative chromatography [88].

In 2016, Alpert [49] used poly (aspartamide) silica to make new stationary phases with longer alkyl chains such as Pentyl, Hexyl up to Decyl. To enhance the capabilities of these stationary phases, Chen evaluated [49] the performance of ammonium acetate with columns of the existing HIC materials PolyPROPYL A and PolyBUTYL A to estimate the concentration of ammonium acetate necessary for the retention of small proteins and to assess the effect of alkyl chain length. Through the experiments he demonstrated that these can retain the native structure of biomolecules using MS compatible concentrations of ammonium acetate [49] and elute as intact

proteins in hybrid mode using ammonium acetate concentration of 1 M or less. This salt concentration has previously been demonstrated to be compatible with mass spectrometry analysis [124]. He also confirmed the new HIC materials can function as a hybrid form of conventional HIC and RPLC [49]. In addition, he observed that with the new HIC materials some organic solvent is required in the mobile phase used in a gradient for the elution of proteins in a reasonable time frame [49, 89, 107, 110]. These columns are proven to offer high sensitivity, better speed and selectivity to simultaneously detect, identify and quantitate molecules in a complex mixture based on their mass-to-charge (*m/z*) ratio. Recent literature [49, 125-126] demonstrated that HIC technique also helps to confidently characterize unknown compounds and confirm trace components at the lowest possible levels.

1.9 Area of Interest

Commercially available HIC columns use either non-volatile mobile phases or high concentration of volatile mobile phases. Such salts are not compatible to use with online MS because they can cause a reduction in the vapor pressure and consequently a reduced signal. They can be used with off-line MS but the desalting step is required before injecting the sample onto LC/MS.

The purpose of this research was to study the chromatographic behavior of mAbs and fusion protein on poly (alkyl aspartamide) silica columns using HIC on hybrid mode and evaluate the impact of mobile phase components such as salt, pH and organic modifier. Extend the study to estimate the influence of chromatographic parameters on the separation of mAbs which helps to enhance the compatibility of online mass spectrometry. In addition, we intended to estimate the influence of different hydrophobic alkyl chain strength in HHIC separation mode.

The research also was intended to evaluate the effect of gradient time and linear flow velocity on retention and efficiency of these columns.

1.10 Scope of the Research

Protein binding to HIC adsorbents is promoted by moderately high concentrations of kosmotropic salts such as sulfate, phosphate, or citrate, which also have a stabilizing influence on protein structure. Elution is achieved by a linear or stepwise decrease in the concentration of salt in the adsorption buffer. Use of these specific salts in high concentrations is not compatible with online Mass Spectrometry (MS) analysis. To make this technique compatible with online MS, poly (alkyl-aspartamide) silica material with more hydrophobic stationary phases were synthesized and characterized [10] to obtain adequate balance between retention and denaturation with online MS compatible salts and their concentrations.

In earlier research, stationary phase alkyl chains such as butyl to decyl were synthesized to increase the ability for protein to be retained on stationary phases consisting of poly (alkyl-aspartamide) silica. However, it was clearly demonstrated in literature that the selectivity and retention on HIC material depends upon the properties of the biological molecules used in the analysis and also on several other parameters, such as stationary phase type (backbone, alkyl chain length and bonding density), salt concentration, buffer pH, temperature and mode of operation.

In this research, selectivity and retention will be evaluated by studying the effect of salt concentration, pH of the mobile phase and percentage of organic modifier. The selectivity of a mixture of mAbs will be assessed using poly (alkyl-aspartamide) silica columns with different alkyl chain lengths. Based on the experimental results, the working range of the parameters that

were studied to achieve acceptable retention while keeping the biomolecule in non-denatured form will be discussed. This knowledge will help to enhance the scope of the utilization of HIC on hybrid mode chromatography in mAbs separation.

1.11 Research Focus

This dissertation describes the factors impacting the separation of larger biomolecules including mAbs and fusion protein on poly (alkyl aspartamide) silica columns and deconvolute the impact of HHIC mobile phase components and different alkyl chain lengths of poly (alkyl aspartamide) silica stationary phases. The research also explores the impact of resident time of a biomolecule on the column in the presence of organic solvent. In addition, we evaluated the impact of linear flow velocity and gradient time on chromatographic efficiency.

Chapter 2 provides the information about the need of an organic modifier using these columns and the impact of the salt concentration on the separation. Experimental details will help to understand the chromatographic parameters that influence the retention and selectivity. This chapter also describes the impact of longer resident time of a biomolecule on the column in the presence of organic solvent. Assessment of conformational changes in the presence of moderate organic content using orthogonal methods such as intrinsic fluorescence and Mass spectrometry is also discussed. In addition, the impact of linear flow velocity and gradient time on chromatographic efficiency is presented. With these columns a small portion of organic solvent is required to elute biomolecule from the column in a reasonable timeframe. Chapter 3 describes the organic solvents that can be used for the separation and the percentage that is required to help in elution. This chapter also focuses on the effect of mobile phase pH, and the role of temperature on separation using these columns.

In the literature it was clearly discussed that to obtain adequate retention using ammonium acetate, greater than propyl alkyl chain is required. This fact indicates that alkyl chain length has a great influence on retention. Chapter 4 describes the effect of alkyl chain length on the selectivity and interdependencies of salt and the stationary phase. It will also assess mobile phase components which can influence chromatographic parameters such as retention and selectivity. Chapter 5 will summarize the research findings and provides a deep understanding on chromatographic behavior of poly (alkyl aspartamide) silica columns using hybrid HIC mobile phases.

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Chapter 2 : Hybrid Mode of Hydrophobic Interaction Chromatography of Monoclonal Antibodies and Related Biomolecules: Influence of Elution Conditions on Chromatographic Performance Using Poly (alkyl aspartamide) Silica Columns

2.1 Introduction

Operating conditions and mobile phase properties such as ionic strength, pH and organic modifier play a major role in hybrid HIC chromatographic retention [1-2]. In a recent article [2], Chen *et al.* demonstrated that adequate retention can be achieved with relatives low concentrations of NH_4OAc (~1M) with the poly (alkyl aspartamide) stationary phases using small model proteins. In addition they observed that some organic solvent such as acetonitrile is required to elute the proteins in a reasonable time frame [2-5]. The similar observations with other HIC chromatographic systems has been previously reported [6-8]. The purpose of our research was to understand the factors impacting the separation of larger biomolecules including mAbs and fusion protein on poly (alkyl aspartamide) silica columns and ascertain the impact of salt and organic modifier in HHIC mobile phases. The research was also intended to explore the impact of longer resident time of a biomolecule on the column in the presence of organic solvent. In addition, we evaluated the impact of linear flow velocity and gradient time on chromatographic efficiency.

2.2 Experimental Details

HPLC grade water and MeCN (acetonitrile) were used in all analysis. NH_4OAc (ammonium acetate) solution was purchased from Teknova, Hollister, CA. MeCN was purchased from Sigma-Aldrich and 0.1 N NH4OH solution was purchased from Ricca Chemicals. HIC columns, with various stationary phases such as PolyPROPYL A, PolyBUTYL A, PolyPENTYL

A, PolyHEXYL A, and PolyHEPTYL A with dimensions of 50 x 2.1 mm, 3µm particles with a pore size of 1000 Å were obtained from PolyLC INC, Columbia, MD. Chromatographic separations were performed using Waters Acquity H-class HPLC system with a flow rate of 1.0 mL/min at 25°C and detection by UV absorbance at 280 nm. (PolyPENTYL A column with dimensions of 50 x 2.1 mm, 3µm particles with a pore size of 1000 Å gave about 4800 psi pressure.) HPLC grade water was used to prepare mAb samples at 1 mg/mL. Molecules that were used in this experimental work were monoclonal antibodies (mAb) and a fusion protein. Biomolecule 1, 4 and 5 were IgG1s and biomolecule 2 was IgG4, biomolecule 3 was NIST (National Institute of Standards and Technology) mAb and biomolecule 6 was IgG4 fc fusion protein. Except NIST mAb, all other biomolecules were kindly provided by Bristol Myers Squibb (BMS) a Biopharmaceutical Company, New Brunswick, NJ and the names of the molecules cannot be revealed. In this dissertation, except NIST mAb, all other mAbs will be referenced as biomolecules. Unless otherwise specified chromatographic separations were performed at 25°C with initial 0.5 minutes hold and a 10 min linear gradient going 100% mobile phase A to 100% mobile phase B. 1 M NH_4OAc was used as mobile phase A and 20mM NH_4OAc (overall) with 50% MeCN as mobile phase B. pH was measured for aqueous mobile phases before combining organic solvent. For convenience NH_4OAc will be referenced as “salt” in the results and discussion sections. Intrinsic fluorescence spectra was collected from 210-400 and ratio was measured using 330 nm and 350 nm. Mass spectra were acquired using a Waters QToF Premier instrument operated in positive electrospray mode using a cone voltage of 40, desolvation temperature of either 250 or 450°C, and an ion source temperature of 150°C.

2.3 Results and Discussion

To understand the chromatographic parameters that influence the retention and selectivity of large proteins under hybrid HIC conditions, the effect of mobile phase ionic strength, pH and organic modifier content was studied on a mixture of six biomolecules. A Poly PENTYL A column was selected for this research after a brief evaluation of available stationary phases (PolyPROPYL A, PolyBUTYL A, PolyHEXYL A and PolyHEPTYL A). This column was found to be most suitable based on an evaluation of retention, peak shape and analyte recovery for the separation of selected mAbs which is in agreement with a recent report [9]. The separation was performed using gradient elution in which mobile phase A and mobile phase B with and without 50% MeCN (Figure 2.1). Under conditions 4 and 5 (which differ by a slight change in mobile phase pH) all biomolecules eluted under the influence of the dual opposing gradients of decreasing NH_4OAc concentration and increasing MeCN concentration. A very good separation was achieved for all six biomolecules using this broad linear gradient. Without any further optimization this technique demonstrated the ability to separate mixture of components some of which have a high degree of sequence homology. It should also be noted that these components were not screened for favorable chromatographic behavior prior to their inclusion in this study. Typically, under conventional HIC operating conditions, low salt concentration will promote elution. However, in this study it was found that when an NH_4OAc gradient was employed with no MeCN in mobile phase B with PolyPENTYL A column (Figure 2.1 conditions 2 and 3), the partial elution of only the two least retained (more hydrophilic) biomolecules was achieved and no elution was observed with hydrophobic biomolecules even when pure water was used as mobile phase B in place of 20 mM NH_4OAc (Refer to Section 3.4). In order to achieve adequate elution, inclusion of some MeCN in the mobile phase B was required, which was consistent with

previous findings for the separation of smaller proteins [2]. This result demonstrates even small amounts of MeCN can dramatically effect elution. For example, biomolecule 1 elutes at ~ 1.5 minutes under conditions 4 and 5 in Figure 2.1, which corresponds to MeCN concentration of ~ 2%. Nevertheless, when mobile phase B without MeCN was used retention increased and chromatographic efficiency was greatly reduced.

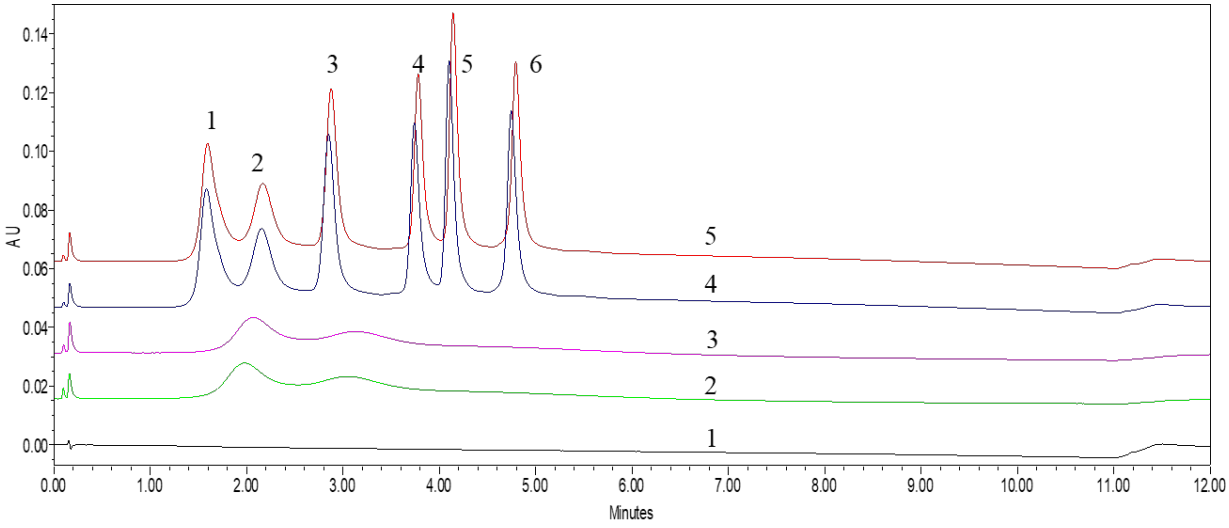

Figure 2.1. Impact of organic modifier on elution using a PolyPENTYL column at 25°C; MP A: 1M NH_4OAc and MP B: 20 mM NH_4OAc with and without organic; Sample is a mixture of 6 biomolecules; 1) MilliQ water blank using MP A and MP B: 20 mM NH_4OAc with 50% MeCN (aqueous pH@7.0); 2) Sample analysis using MP A and MP B: 20 mM NH_4OAc no pH adjustment and no MeCN; 3) Sample analysis using MP A @7.0 and MP B: 20 mM NH_4OAc pH @7.0 no MeCN; 4) Sample analysis using MP A and MP B: 20 mM NH_4OAc with 50% MeCN no pH adjustment; 5) Sample analysis using MP A @pH7.0 and MP B: 20 mM NH_4OAc with 50% MeCN (aqueous pH@7.0)

2.3.1 Effect of salt concentration on selectivity

In general, in HIC chromatography when there is an increase in salt concentration the retention of the biomolecule increases. Based on the previous set of experiments, it is apparent that the slight modifications to the mobile phase had a significant influence on the retention

behavior of the biomolecules studied. To evaluate the effect of salt, we ran a series of experiments (Figure 2.2 and 2.3) by varying NH_4OAc concentration in mobile phase A from 0.5 M to 2.5 M while all other parameters were kept constant. In these experiments, peak 1 retained longer with an increase in the salt concentration, which is an expected conventional HIC retention behavior. As the concentration increased from 1M and above, peaks 1, 2 and 3 exhibited a fairly linear increase in retention. However, an interesting behavior was observed for peak 4, 5 and 6, where the greatest retention occurred at the lowest salt concentration (0.5 M) which is a non-typical HIC retention pattern. Overall, peaks 4, 5 and 6 followed a similar pattern by eluting faster with increasing salt concentration. However, there was a slight decrease in the retention of peak 6 compared to peak 4 and 5. The retention of peak 2 showed the most complex relationship to the change in the salt concentration of mobile phase A was to widen the separation window by decreasing retention of the earlier eluting components and by increasing retention of the late eluting components. From a practical perspective it is apparent that the salt concentration of mobile phase A is a useful parameter to increase chromatographic resolution and the overall peak capacity of a separation of the large biomolecules used for this study. The improved separation of these large biomolecules at salt concentrations less than 1 M is a key difference compared to the separation of small proteins, which were reported to be poorly behaved under similar conditions which was attributed to conformational instability [2]. One measure of protein conformational stability is their thermal transition temperatures. For example, the melting temperature of α chymotrypsinogen A used in an earlier investigation of hybrid HIC is approximately 50°C at pH 7 [10], whereas, the thermal transition of a therapeutic mAb is typically in the range of 65-70°C [11] indicative of higher conformational stability. In

addition to improving peak capacity, mobile phases with lower NH_4OAc concentration generally provide an increase in analyte response by ESI mass spectrometry [12].

This observed increase in retention as the salt concentration of mobile phase A decreased suggests that there could be more than one mechanism of retention. While the observed greater retention at the reduced salt condition is consistent with ionic interactions with the stationary phase, the poly (alkyl aspartimide) stationary phase is reported to have a very low ion exchange capacity [13]. It is also important to recognize that even at the lowest salt concentration (0.5M) used in this study, there is likely more than sufficient ionic strength to suppress ionic interactions with the stationary phase. Additionally, if ion exchange was occurring it would likely be manifested by distortion of the chromatographic peaks, which was not observed.

We also investigated the possibility that the increase in the retention under low salt conditions was due to the increase in the hydrophobicity of the biomolecule resulting from conformational changes. In general, a denatured or partially unfolded molecule has longer retention compared to a native conformation due to exposure of previously buried hydrophobic residues. If there is any denaturation due to the organic solvent then a change in the retention time is expected. Examining the results from the experiment (Figure 2.5), which was conducted for the purpose of understanding the effect of flow rate on chromatographic efficiency, it is possible to gather some information on kinetics of molecular unfolding in the presence of an organic solvent. In this experiment, the gradient time and flow rate were proportionally adjusted to maintain a constant gradient volume. The result was that each component eluted under identical mobile phase conditions despite retention times varying by a factor of 10. If protein unfolding were occurring on the time scale of the chromatographic experiment, it is expected that the retention volume would increase with increasing time on column. While it is widely

assumed that conformational changes are instantaneous, as described by Sethuram's et. al., conformational changes in the presence of hydrophobic surface can be faster (less than a minute) or slower (up to 1200 minutes) [14]. Since no shifts in retention volume were observed (Figure 2.5), it can be concluded that under the conditions of 1 M NH_4OAc, if protein unfolding is occurring it is proceeding either much faster or slower than the chromatographic time scale as observed by Chen et al [2, 9].

To further evaluate conformational changes in low salt solutions, intrinsic fluorescence experiments were carried out with 0.5 M and 1M NH_4OAc mobile phases using biomolecule 6 (fusion protein) to compare the ratio of tryptophan fluorescence emission at 330 nm and 350 nm [15-16]. Biomolecule 6 was selected for this study because it is the latest eluting component and therefore exposed to greatest MeCN concentration. A mobile phase containing 0.5 M NH_4OAc was selected because it gave longest retention time and 1M was the suitable control yielding retention time similar to the higher salt concentration mobile phases. The data showed that there was no change in the ratio (Table 2.1), which indicated that there were no significant conformational changes to the biomolecule in the regions where there are tryptophan residues. To further evaluate conformational changes we also conducted electrospray mass spectrometry experiments using the same chromatographic conditions that were used for intrinsic fluorescence. It was reported that the appearance of electrospray mass spectra of a protein under hybrid HIC conditions is similar to the spectra that was obtained in folded form rather than denatured form [2]. The ESI desolvation temperature was optimized to generate mass spectra where the charge state distribution is centered at high m/z which is consistent with native or near native protein structure. For comparison, we also collected mass spectra at much higher desolvation temperature to produce a spectrum of the denatured protein where the charge state

distribution centered at significantly lower m/z. Under the non-denaturing MS conditions, a statistical significant difference in the charge state distribution was observed between the 0.5M and 1M conditions. However, the minor changes to the average change state distribution that were observed (Figure 2.4) were may be due to the differences in the solvent conditions used. This data may not be sufficient enough to conclude that it is due to conformational changes. For example, the change in charge state distribution may be due to the impact of the change in salt concentration on the electrospray ionization process rather than conformation. It has been well established that mobile phase variations can lead to minor changes in the appearance of the mass spectra. For example, in a recent article by Ding [17], it was reported that the addition of a small percentage of a basic additive to the mobile phase can act as a charge stripping agent and significantly alter the charge state distribution.

Table 2.1. Impact of Mobile Phase Salt Concentration on Biomolecule 6

Intrinsic fluorescence			
Salt Concentration	330 nm	350 nm	Ratio of 330 nm /350 nm
	Max Absorbance (eu)		
1.0 M [1]	9192.9	9182.5	1.001
0.5 M [2]	5345.6	5339.6	1.001
2.5 M [3]	10131.6	10156.2	0.998

Table 2.1. Impact of mobile phase salt concentration on mAb 6 conformation. 1) MP A: 1M NH4OAc at pH 7.0 and MP B: 20 mM NH4OAc with 50% ACN at pH 7.0, 2) MP A: 0.5 M NH4OAc at pH 7.0 and MP B: 20 mM NH4OAc with 50% ACN at pH 7.0, 3) MP A: 2.5 M NH4OAc at pH 7.0 and MP B: 20 mM NH4OAc with 50% ACN at pH 7.0). Flow rate 1 mL/min.

Even though these non-chromatographic experiments provided some basic information about conformation changes under HHIC chromatographic conditions, more techniques such as Circular Dichroism and protein NMR are required to gather details to confirm the structural changes in a biomolecule. Despite the inconclusive findings related to conformational change, the ESI experiments did provide a useful demonstration of the improved signal intensity that results from conducting analyses at lower NH_4OAc concentration. The spectral intensity of the most abundant charge state increased by approximately a factor of five with the use of 0.5 M NH_4OAc compared to 1 M (Figure 2.4).

Considered together, the results from the analyses by intrinsic fluorescence and ESI mass spectrometry suggest that extensive protein unfolding has not occurred or that it only occurs when the biomolecule is in the presence of the hydrophobic stationary phase [18-19]. If the biomolecules rapidly assume a folded conformational state after elution, the ability to detect the changes by solution analysis would be confounded. A possibly more likely explanation of the results from conformational analysis is that the changes are too subtle for detection by either fluorescence or ESI mass spectrometry but never the less significantly impact chromatographic retention. A final possible explanation for the enhanced retention under low salt conditions is hydrophobic affinity effect [18, 20]. Unfortunately, a mechanism for hydrophobic affinity has not been determined. It is possibly a distinct mechanism from HIC but it is also may be the same phenomenon of increased retention under low salt conditions resulting from protein unfolding.

While the precise mechanism for increased retention of later eluting components under low NH_4OAc conditions is not fully understood, the benefits of operating under these conditions include a widening of the elution window and the previously described enhanced response by ESI mass spectrometry. It does appear however, that the NH_4OAc concentration should be

tailored to the precise requirement of the intended separation. For example if the requirement is to separate and quantitate only components 4 and 5, a higher salt concentration would provide better resolution despite the overall narrowing of the elution window observed for the separation of this six component mixture. The behavior clearly demonstrates that the retention is not only dependent on the salt concentration but also highly dependent on the hydrophilic and hydrophobic nature of the individual biomolecule [21-24].

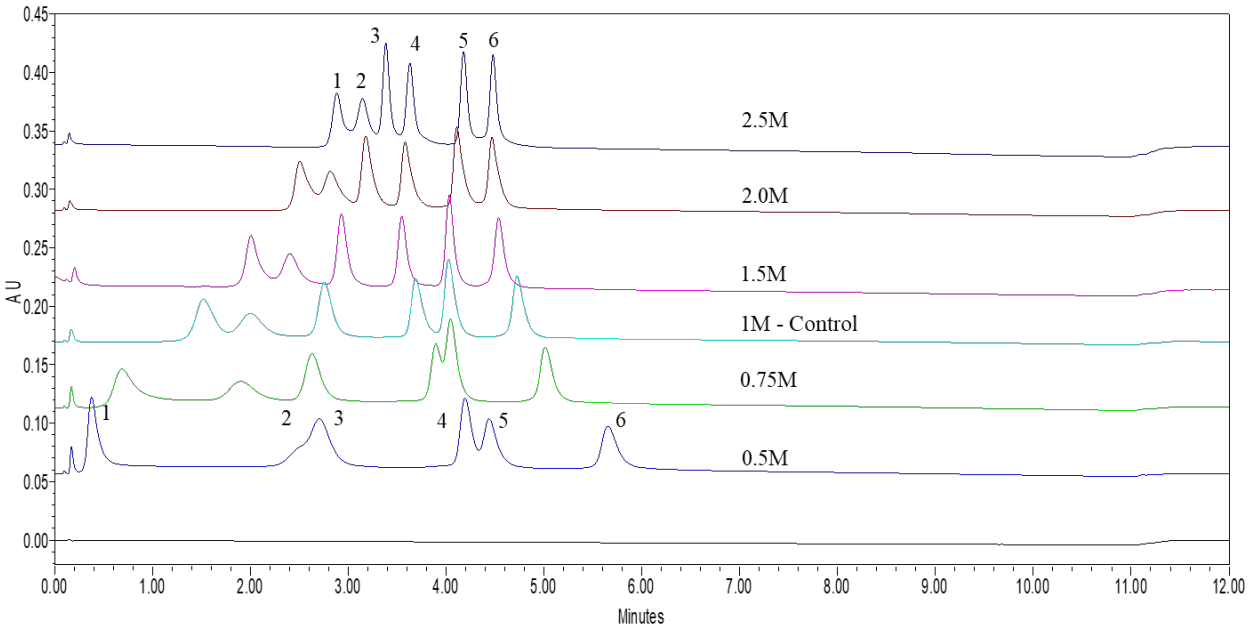

Figure 2.2. Impact of salt concentration on the selectivity (MP A: different concentrations of NH_4OAc at pH 7.0 and MP B: 20 mM NH_4OAc with 50% MeCN with an aqueous pH 7.0); Peaks 1 through 6 are biomolecules used as a sample mixture.

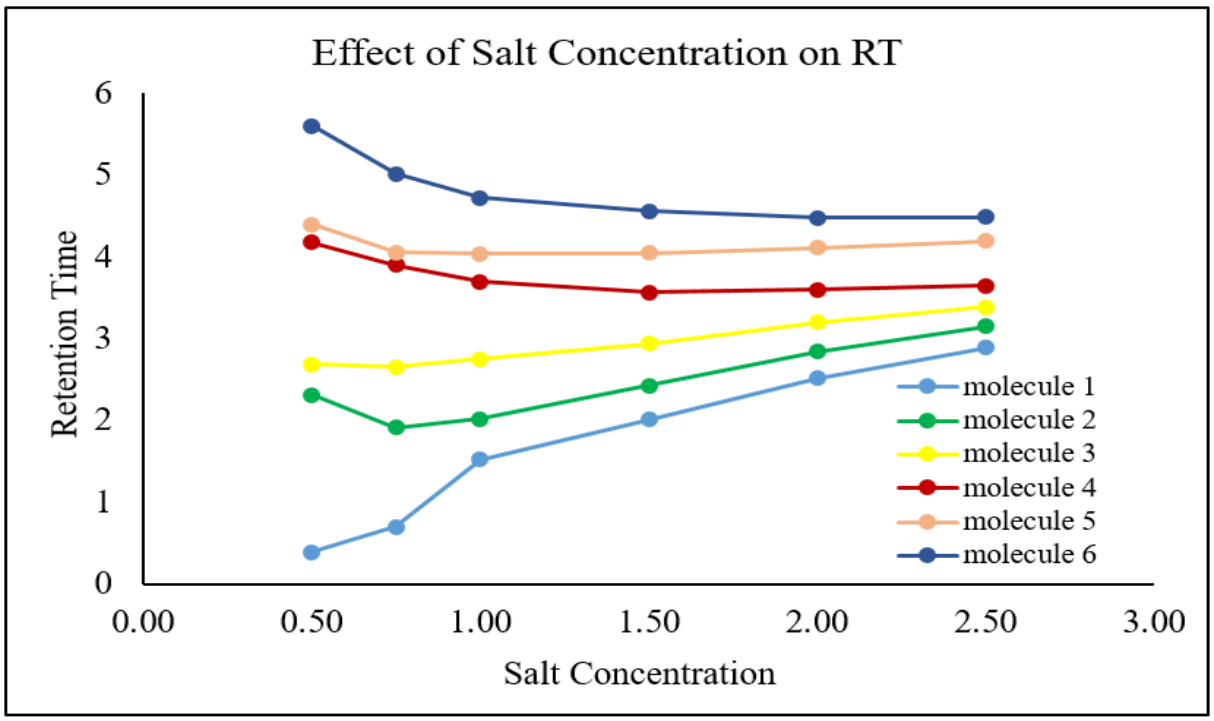

Figure 2.3. Impact of salt concentration on retention (MP A: different concentrations of NH4OAc at pH 7.0 and MP B: 20 mM NH4OAc with 50% ACN at pH 7.0)

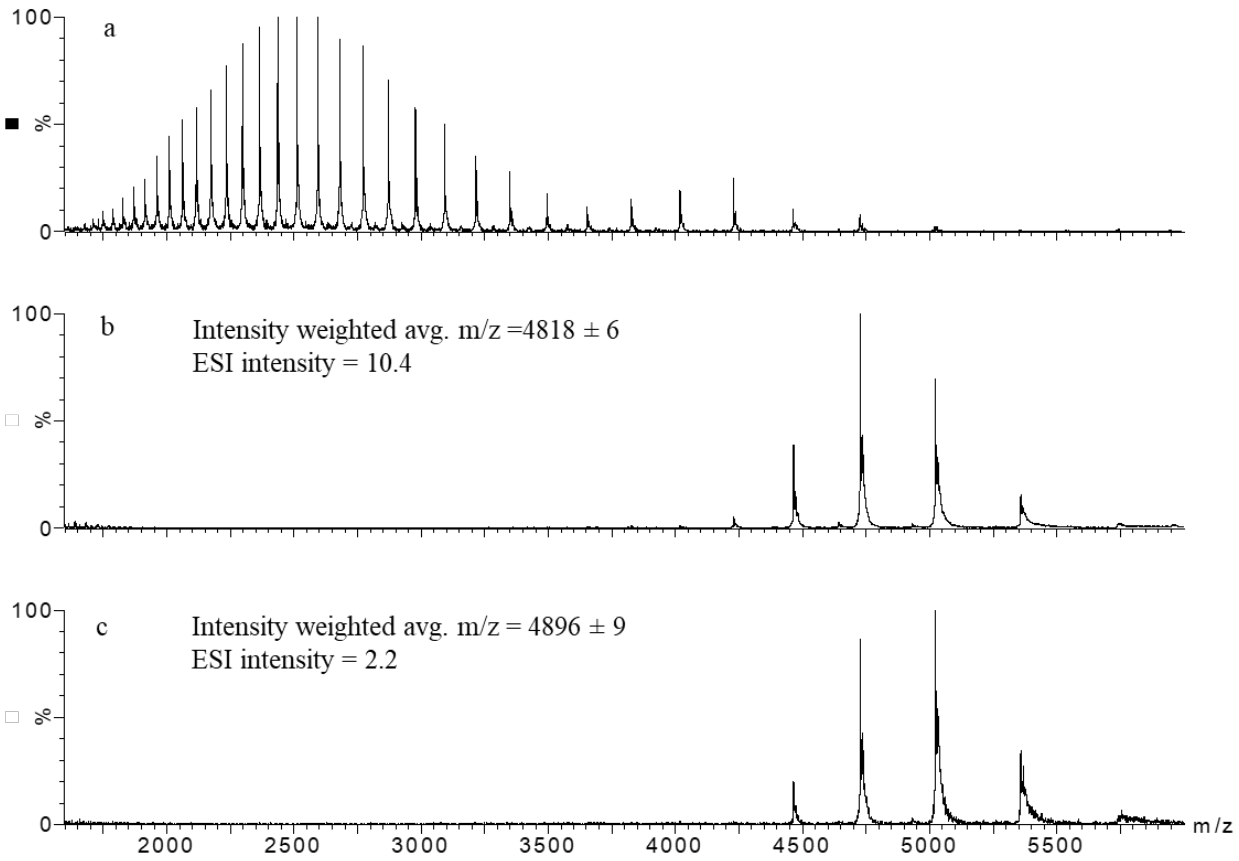

Figure 2.4. ESI-MS conducted using mobile phase A with either 1 M or 0.5 M NH4OAc and mobile phase B consisting of 20 mM NH4OAc with 50% MeCN, a) Desolvation temperature 450 °C, MP A: 1M NH4OAc, b) Desolvation temperature 250 °C, MP A: 0.5 M NH4OAc, c) Desolvation temperature 250 °C, MP A: 1.0 M NH4OAc

2.3.2 Role of linear velocity on the separation

Another parameter that plays a major role in the overall separation is mobile phase linear velocity. It not only has a significant influence on the efficiency, but also impacts the speed of the analysis. To understand the effect of linear velocity on chromatographic efficiency and resolution, mobile phase flow rates of 1, 0.8, 0.5 and 0.1 mL/min were evaluated (Figure 2.5). Gradient run time was adjusted to maintain a constant gradient volume. Based on experimental results, it was observed that when the flow rate decreased from 1 to 0.1 mL/min, there was

approximately a 30% increase in the peak capacity (Figure 2.6). In addition, there were no significant change in the selectivity and a moderate increase in the resolution at the cost of a longer run time. The modest impact of linear velocity on peak capacity is somewhat surprising since the expectation is that large molecules will exhibit poor mass transfer resulting in a more significant loss of efficiency as linear velocity increases. Even though lower flow rates may offer an increase in the peak capacity and provide enhanced resolution, a possible concern is that a longer run time with extended exposure to organic solvent may affect the conformation of the biomolecules. As described previously unfolding could lead to an increase in the hydrophobic interactions with the stationary phase and results in longer retention times, or in the case of these experiments, longer retention volumes (retention time multiplied by flow rate). However, as shown in Figure 2.5, decreasing linear velocity and increasing run time from 10 minutes to 60 minutes did not impact retention volumes suggesting no change to molecular conformation due to the longer exposure to the organic solvent. Additional experiments using biophysical characterization techniques will provide more details to confirm the structural changes.

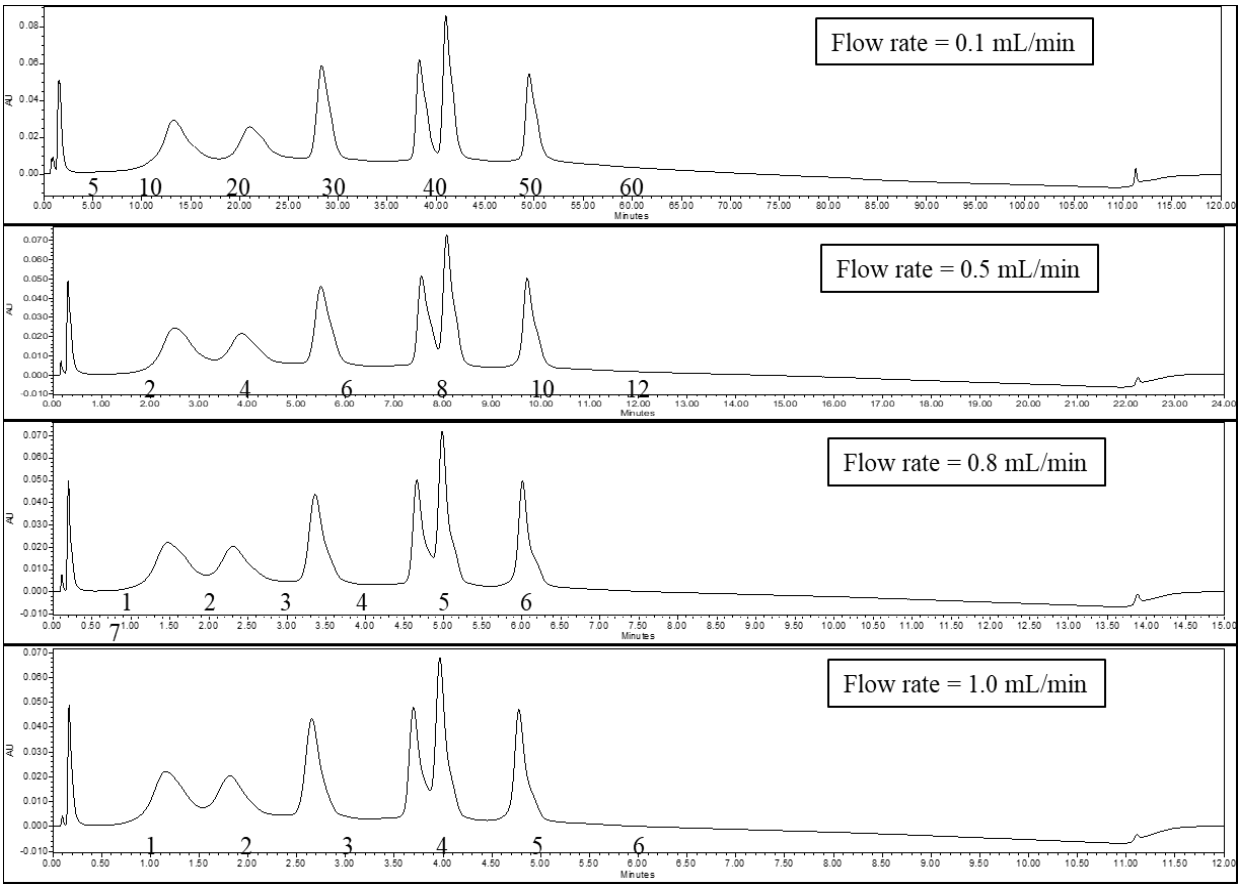

Figure 2.5. Impact of flow rate on relative retention time (MP A: 1M NH_4OAc at pH 7.0 and MP B: 20 mM NH_4OAc with 50% MeCN with an aqueous pH 7.0); Flow rates were 0.1, 0.5, 0.8 and 1 mL/min.

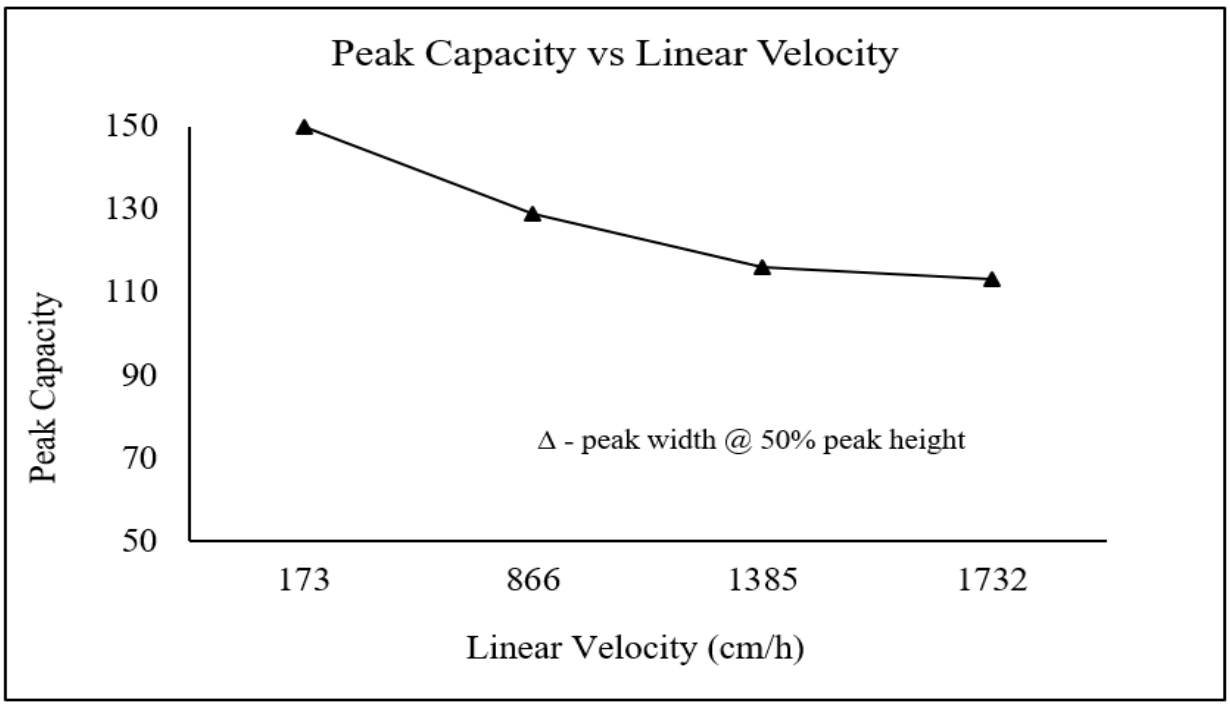

Figure 2.6. Impact of linear velocity on peak capacity (MP A: 1M NH_4OAc at pH 7.0 and MP B: 20 mM NH_4OAc with 50% MeCN with an aqueous pH 7.0). Linear velocities were 173, 866, 1385 and 1732 cm/h with flow rates of 0.1, 0.5, 0.8 and 1 mL/min respectively. Peak capacity was calculated using equation: 1+ (Gradient Time/Average peak width@50%peak height)

2.3.3 Gradient steepness

Chromatographic peak capacity is also strongly influenced by gradient steepness. The effect of gradient steepness was evaluated by conducting chromatographic separations at seven different gradient times (5, 10, 15, 20, 30, 40 and 50 minutes). Results in Figure 2.7 showed that increasing the gradient time up to 30 minutes slightly improved the peak capacity but with no significant increase with longer gradients. This observation confirms that the largest impact on peak capacity occurs from the initial increases of gradient time. For example, changing the gradient time from 5 to 10 minutes increased the peak capacity by 10% while increasing from 10 min to 30 minutes produced only a further 7% improvement.

Both linear velocity and gradient time are shown to impact peak capacity and are easily modified chromatographic parameters. With consideration to a reference point of separations conducted at 1mL/min with a gradient time of 10 minutes (added references) our results demonstrate that decreasing flow rate to 0.5 mL/min (Figure 2.6) (with a proportional increase in the gradient time to 20 min) offers a greater increase in peak capacity compared to increasing the gradient time alone (Figure 2.7). In addition, use of a lower flow rate is a more favorable condition for MS analysis due to the expected increase in sensitivity.

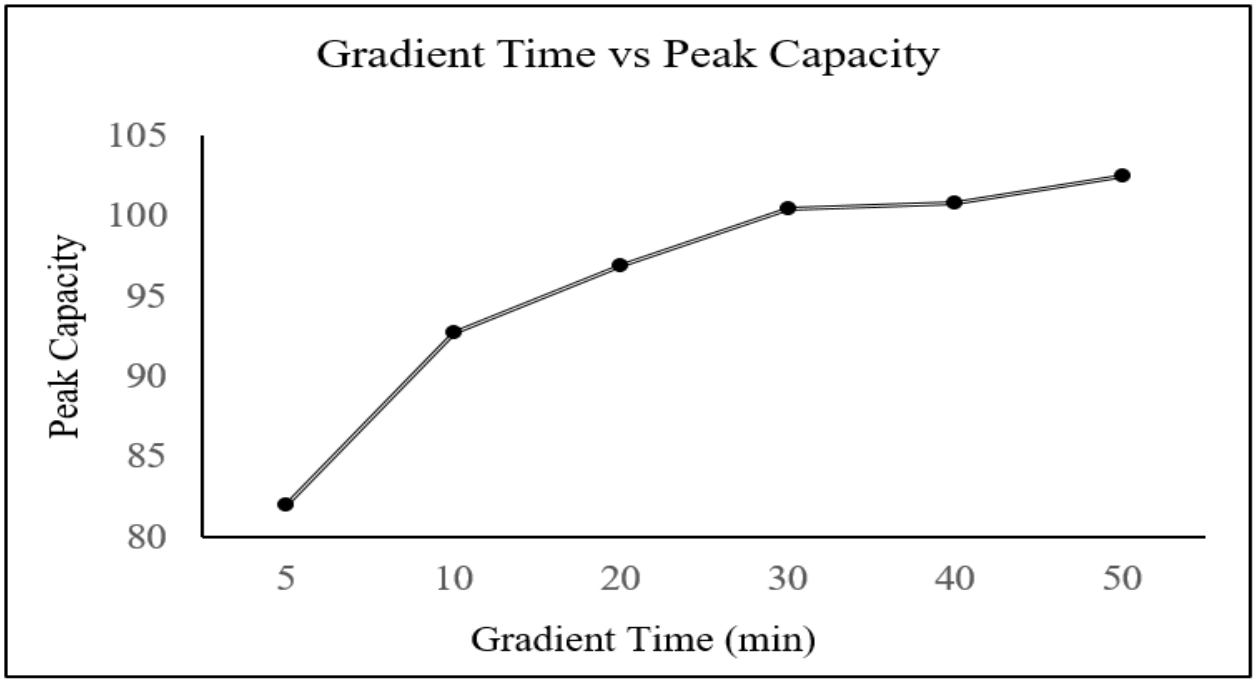

Figure 2.7. The impact of gradient time on retention. Gradient times were 5, 10, 20, 30, 40 and 50 minutes and 42, 83, 167, 250, 333 and 417 column volumes respectively.

2.4 Conclusion

In this study we evaluated the impact of elution parameters on the hybrid HIC separations of mAbs and other large therapeutic biomolecules on a previously described stationary phase that is more hydrophobic than typical used for HIC separations. This research demonstrated that an adequate separation of mAbs and related molecules is achievable using a salt concentration (0.5 M) that was lower than what was previously reported as the lowest concentration required for satisfactory chromatographic performance for a set of model proteins. Furthermore, the study showed the analytical utility of low salt conditions to widen the chromatographic elution window through the earlier elution of the hydrophilic analytes combined with the unexpected later elution of the more hydrophobic analytes. Results from intrinsic fluorescence and MS of the eluted biomolecules suggested that they were in a largely folded state despite the use of conditions that employed a low salt mobile phase containing acetonitrile (referred as either MeCN or ACN) which might be expected to cause denaturation especially in the presence of a hydrophobic stationary phase. However, there are various orthogonal and conformational analysis techniques that can provide more details to confirm the conformation changes under these chromatographic conditions. In addition to widening the elution window, another advantage of the use of a lower salt mobile phase was an approximate fivefold increase in the ESI-MS response of the analytes. Since compatibly with on-line MS analysis is one of the main drivers for the development of hybrid HIC, the increase in MS response represent a significant advance. Other elution parameters were also studied with the impact of linear velocity and gradient steepness generally followed expected trends with the relatively modest loss of efficiency at the highest linear velocity being noteworthy.

2.5 References

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Chapter 3 : Hybrid Mode of Hydrophobic Interaction Chromatography of Monoclonal Antibodies and Related Biomolecules: Influence of Mobile Phase pH, Organic Modifier and Temperature on Poly (alkyl aspartamide) Silica Columns

3.1 Introduction

Factors affecting protein adsorption in HHIC systems include the properties of mobile phase such as buffer pH, temperature, organic modifier and stationary phase in addition to salt type and concentration. Out of all these factors, salt concentration and salt type [1-7] effects have received the most attention. In HIC, Alberty *et al.* [8] derived a relationship to correlate how variations in buffer pH, temperature and salt concentration can induce Gibbs energy changes. In his work he concluded that the chromatographic affinity is driven by the change of Gibbs energy, which is related to the number of water molecules released upon protein binding. Based on this number, the effect of pH and temperature can be estimated. Xia *et al.* [9] found that when the buffer pH is close to the isoelectric point of the protein, more water is released during adsorption. Baumann *et al.* [10] conclude that pH-induced reversible structural changes and protein reorientation upon binding can increase the dynamic binding capacity affecting retention process.

An organic modifier is required to elute biomolecules (Refer to Section 2.3) in a reasonable time frame when using poly (alkyl aspartamide) silica columns with mass spectrometry compatible salts such as ammonium acetate in low concentrations. Organic solvent helps to disrupt hydrophobic interactions between the molecule and stationary phase to promote elution. Organic solvent is known to denature biomolecule conformation and the details will be discussed in Section 3.3.2. However, the impact of the organic solvent is highly dependent on the type and the percentage of organic solvent and also on the properties of the biomolecule itself. Along with mobile phase properties, column temperature also contributes to the elution. In conventional

HIC the effect of temperature on solute binding columns has been previously examined and it was found that retention is an entropically driven process at low temperature and an enthalpically driven process at high temperature. However, temperature effect on poly (alkyl aspartamide) silica columns has not been studied and is yet to be evaluated. Our research will help to demonstrate the effect of mobile pH and, organic modifier and will also provide insights on the impact of temperature on the separation.

3.2 Experimental Details

Details on the chemicals, HIC columns, mAbs, HPLC system and chromatographic conditions, refer to Section 2.2. Acetonitrile, isopropyl alcohol and methanol was purchased from Sigma Aldrich, 0.1N ammonium hydroxide was purchased from Ricca Chemicals, and glacial acetic acid was purchased from Sigma Aldrich and were used to adjust the pH of aqueous mobile phases before adding the organic solvent. Unless otherwise specified chromatographic separations were performed at 25°C with initial 0.5 minutes hold and a 10 min linear gradient going 100% mobile phase A to 100% mobile phase B. 1 M NH_4OAc was used as mobile phase A and 20mM NH_4OAc (overall) with 50% MeCN as mobile phase B. Mobile phases were prepared as pH range of 5.7 - 7.3. Isoelectric points (p*I*) of mAb 1 was 8.3, mAb2 was 7.2, mAb3 (NIST mAb) is 9.2, mAb4 is 9.5, mAb5 is 9.0 and FC protein is 7.7

3.3 Results and Discussion

In this research, mobile phase parameters such as pH, temperature, organic type and content were studied to evaluate the retention and selectivity on a mixture of six biomolecules using poly (alkyl aspartamide) silica columns. Poly PENTYL A column was selected for this

research after a brief evaluation of available stationary phases such as PolyPROPYL A, PolyBUTYL A, PolyHEXYL A and PolyHEPTYL A columns (Figure 3.1). This column was found to be most suitable for the separation of selected biomolecules and also in agreement with recent reports [7, 11].

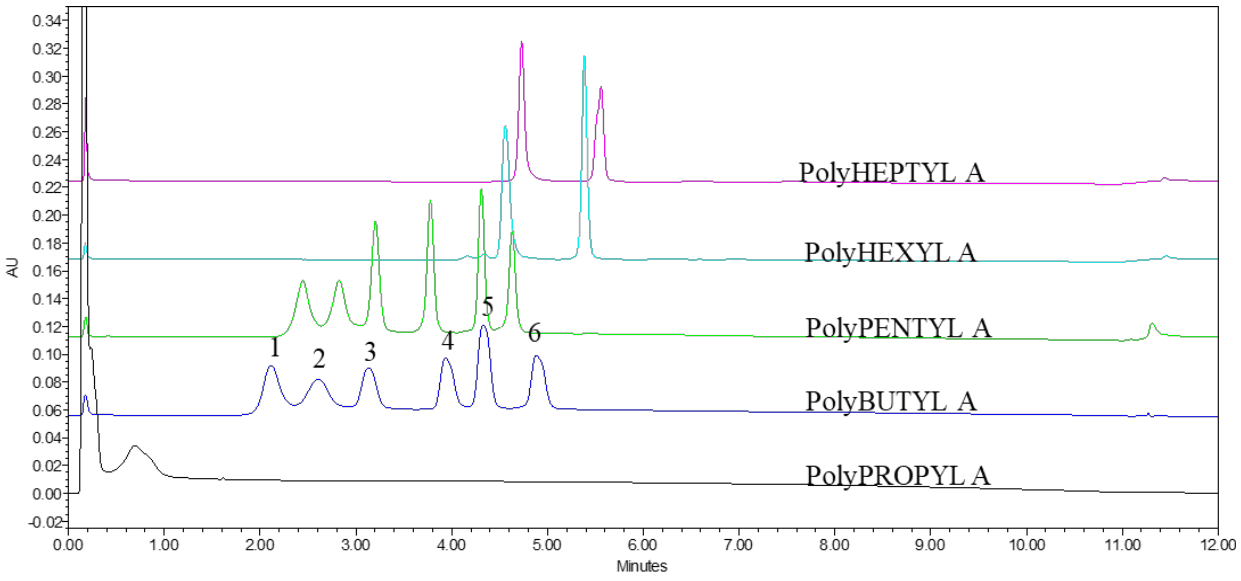

Figure 3.1. Initial evaluation of chromatographic separation using different poly (alkyl aspartamide) silica columns

3.3.1 Effect of mobile phase pH on the retention

HIC is strongly influenced by the pH of the solution, however, the effect of pH in HIC is not completely understood [12-15]. In most cases, it was observed that an increase in pH can reduce the hydrophobic interactions between molecules and the hydrophobic groups of the stationary phase, may be as a result of increased titration of charged groups, leading to the increased hydrophilicity promoted by the change (increase) in the protein charge [5, 12]. Hjerten *et al.* [16] reported that basic protein such as lysozyme displayed high binding when the buffer pH was close to its p*I* and human serum albumin capacity factor decreased as the pH increased. The net

charge on the molecule is affected by pH of its surrounding environment and can become more positively or negatively charged due to the gain or loss, respectively, of protons (H^+). Therefore, depending on the pH, the protein's net charge and its conformation can change significantly [9-10, 16-18]. It is well established in the literature, that hydrophobic interactions are stronger when solution pH is close to the isoelectric point of the protein [17]. The reason is, near its isoelectric point the net charge of the protein will become zero and the electrostatic repulsion between the protein molecules becomes small, favoring a closer packing on the adsorbent surface [17].

In general, the commonly used pH conditions for larger biomolecules are in the range of physiological pH which is in between 6.4 and 7 [6]. However, most of the biomolecules stored in the range pH 5.7 - 7.3, because they exhibit high chemical and physical stability. Keeping this in mind, in this research, pH range was evaluated between 5.7 and 7.3, while all other chromatographic parameters were kept constant (Figure 3.1). The results demonstrated that peaks 1 and 2, where the p*I*s are close to 8, retained longer and exhibited low resolution at low pH (5.7) and eluted faster with increased resolution as the pH increased to 7.3. In general, when the pH is less than p*I*, a molecule contains positive charge. The increased protein retention at low pH could be due to partial denaturation [19] or disruption in both ionic and hydrophobic interactions. This scenario will result in stronger adsorption onto stationary phase. Based on earlier research [18], it was explained that the ionization state of amino acids in the contact surface area influences the strength of the hydrophobic interaction. Hence, not only hydrophobicity, but hydrophilicity also affects HIC retention. In conclusion, there is a strong influence of the buffer pH on the adsorption strength resulting from both configurational changes and electrostatic effects [20]. As the pH increases and gets close to p*I* of the molecule, the net charge of the molecule gets closer to 0, causing a decrease in electrostatic interactions. As a result, only

hydrophobic interactions of the molecule play a role and it is easy for organic to disrupt these interactions by causing earlier elution.

In this research, for peaks 1 and 2, as the pH increased from 5.7 to 7.3, there was a decrease in peak retention and increase in peak resolution. The reason for this observation may be similar to what was described [9] as the change in pH impacts the total number of released water molecules upon protein binding. This number increases as the buffer pH approaches the molecule's p*I* and decreases when the pH was away from its p*I*. This influence impacts the selectivity of the biomolecule on HIC systems [9]. A very similar trend was observed for peak 3 (NIST mAb), where the p*I* is 9.2. A decrease in the retention time occurred when the pH increased to pH 7.3. Peaks 4 and 5 have similar p*I* values as peak 3 but there was no major shift in the retention observed. However, there is a slight decrease in the resolution between these two peaks. There was an interesting observation made with peak 6, where the p*I* of the molecule is similar to peak 2. The change in the retention was completely negligible. Hjertén *et al.* observed that the retention of various test proteins changed more drastically at pH values above 8.5 and/or below 5 than in the range pH 5 – 8.5 [16]. The shift in retention caused by the pH is highly dependent on the biomolecule. The p*I* and the number of charged amino acid residues in the biomolecule have an impact [5]. Therefore, although the p*I* of the mAbs is typically higher than biological pH, the effect of buffer pH may vary depending on the molecule properties such as the available amino acids in the contact surface area and the number of the water molecules released. Although there has been an explanation for the impact of pH on the retention mechanism, it is very complex to predict the retention process as there are multiple parameters simultaneously changing during the gradient run. As described in previous findings and the outcome of this

research, pH may not be a critical parameter [6] but it has an impact on selectivity (Figure 3.2) and peak capacity (Figure 3.3). Hence, it can be used to optimize the separation of biomolecules.

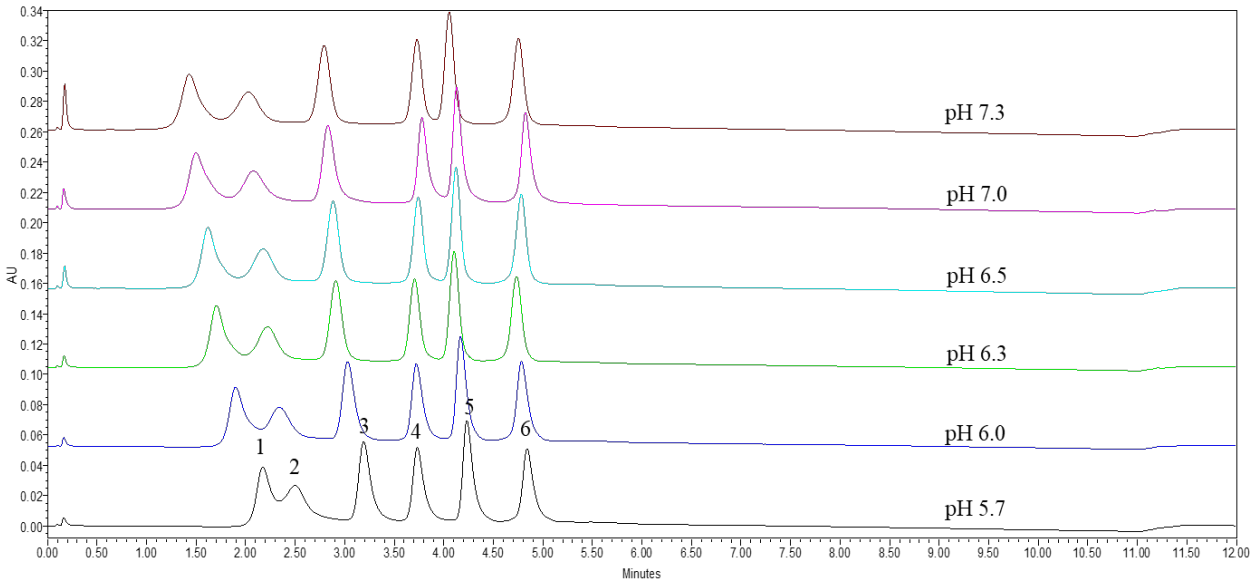

Figure 3.2. Effect of mobile phase pH on retention and selectivity

Table 3.1. Changes in the retention factor in different mobile phase pH

	Mobile phase pH					
Peaks	5.7	6.0	6.3	6.5	7.0	7.3
mAb1	20.7	18.0	16.0	15.2	14.0	13.3
mAb2	24.1	22.5	21.2	20.8	19.8	19.3
NIST	30.9	29.3	28.1	27.8	27.3	26.9
mAb4	36.3	36.2	36.1	36.4	36.8	36.3
mAb5	41.3	40.7	40.1	40.2	40.3	39.6
mAb6	47.4	46.8	46.4	46.8	47.2	46.5

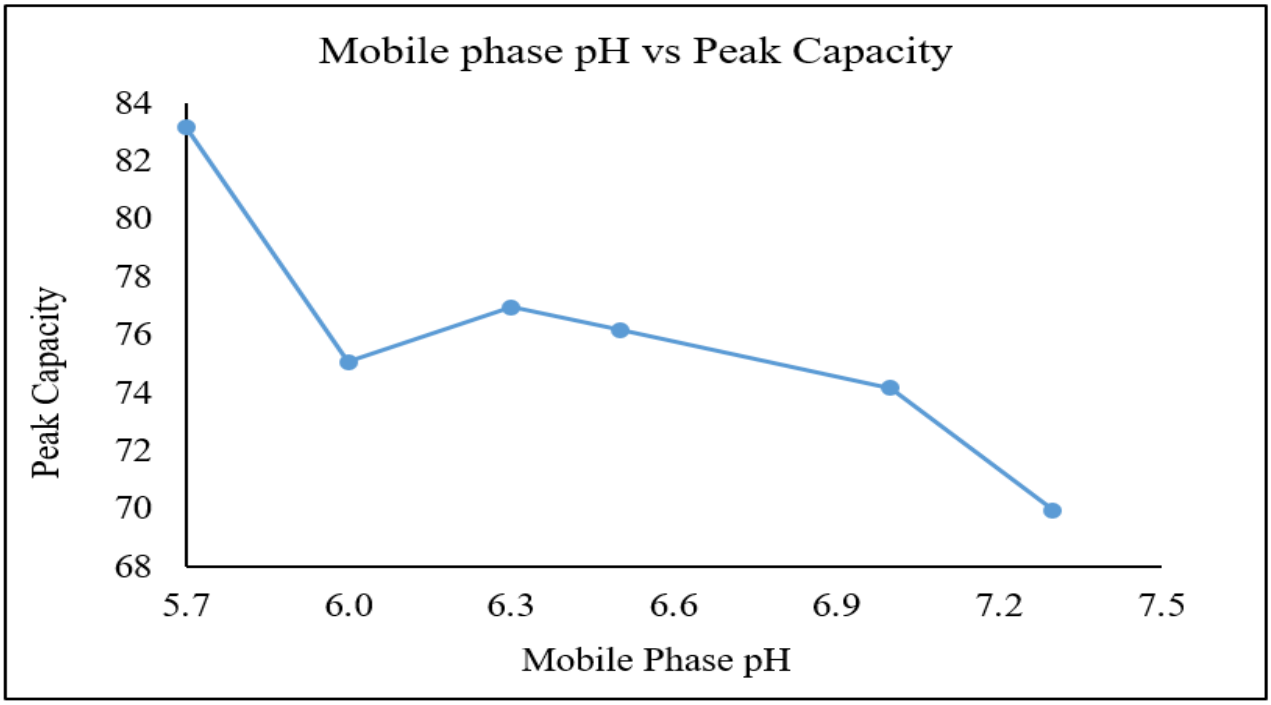

Figure 3.3. Effect of mobile phase pH on the peak capacity

3.3.2 Effect of organic modifier concentration on the retention

Adding a small portion of organic solvent will contribute to disruption of hydrophobic interactions in HIC, resulting in elution of bound molecules. However, the impact of the organic solvent depends on the properties of the organic solvent and the molecule as well. In general, molecules with α-helix seems to be more stable compared to molecules with β-sheets. The reason for this is that β-sheets have more solvent accessibility compared to α-helix, therefore, molecules with β-sheets tend to denature faster [21]. In the absence of water, proteins in hydrophobic solvents were thought to retain their native structure as a result of kinetic trapping [22], which is due to stronger hydrogen bonding between the protein atoms and a more rigid structure. In hydrophobic water-immiscible solvents, the available water will tend to stay at the protein surface as a result of the solvophobic and hydrophilic nature of the protein surface [23]. In 1999, Klibanov indicated that a small amount of water (1% v/v) will have significant impact on increase of catalytic activity because water plays an important role in the structure and dynamics of the protein [24]. On the other hand, polar solvents such as dimethyl sulfoxide [DMSO], dimethylformamide [DMF] and, formamide can easily strip water from protein surface and compete strongly for hydrogen bonds between protein atoms by denaturing to a significantly unfolded state of the molecule [25]. However, due to the presence of a hydrophilic component, alcohols moderately compete for amide hydrogen bonds and will disrupt tertiary structure leaving secondary structure interactions intact [26].

Methanol is a commonly used organic solvent for chromatographic separations. The assumption is, due to its polar protic nature it works as a denaturant and increases the concentration of possible folding intermediates [26], hence, it may not be a good choice for the separation. Previous experimental results and theoretical studies have shown that the addition of

methanol to aqueous protein solutions stabilizes (or even induces) the α-helical structure. However, it denatures other protein structures, caused by the accumulation of methanol near the protein surfaces [27-29]. This behavior induces the expansion of protein structure (Figure 3.4) possibly by the reduction of hydrophobic effects. Simultaneously, the replacement of water molecules from the protein surface decreases the hydrogen bonding between water and the protein and increases the protein-protein hydrogen bonds [30]. Although disruption of water shell in the presence of methanol increases the exposure of the protein to solvent. Combination of these effects locally decrease the polar interactions between the solvent and the protein causing an increase in the possibilities for secondary structure formation [30]. However, the effect of methanol on protein structure depends on the sequence and the position of the amino acids. Therefore, it is a combination of interdependent direct (preferred binding of methanol) and indirect (e.g., reduced protein-water hydrogen bonding) effects [30]. Based on the reference, the influence of methanol depends on multiple factors such as the amino acid sequence, the environment of the biomolecule and also the balance between the interactions. Combination of all these effects will determine whether methanol tightens or loosens the protein structure [30], which may act as a protein denaturant. Various studies were conducted over a wide range of water/organic solvent mixtures and it was observed that proteins in solvents containing different proportions of water and organic solvent showed very different behavior than that observed in either water alone or neat organic solvent [22].

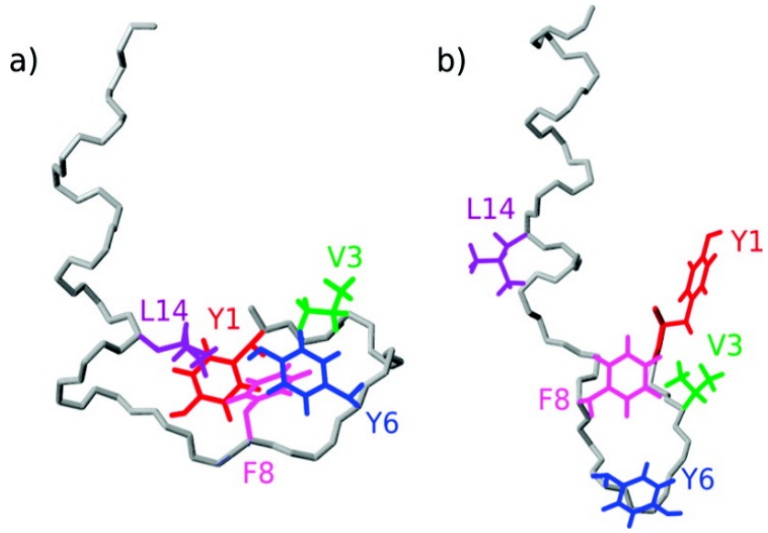

Figure 3.4. NMR structure of BBA5 in a) water, and b) MeOH/water solution. The side-chains of hydrophobic residues participating in the hydrophobic cluster in water are indicated [30].
(Reprinted with the permission of ACS)

The effect of MeCN-water mixtures on the solubility of amino acids in lysozyme indicated very similar observations as with other organic solvents. They tend to weaken the hydrophobic interactions by enhancing the peptide-peptide hydrogen bonding leading to the denaturation of proteins. Circular dichroism confirmed that the confirmation of lysozyme remained native up to 40% of MeCN [31]. 2016 Bobaly *et al.* [32] performed experiments to evaluate the effect of MeCN using commercial HIC columns. MAbs with low or moderate hydrophobicity demonstrated longer retention with lower percentage of MeCN and lower retention was observed as the percentage of MeCN increased, whereas for most hydrophobic mAbs, the retention decreased continuously as the concentration of MeCN increased. Longer retention indicated some possible structural changes depending on the proportion of the MeCN, which impacts the confirmation and contact area of the proteins with the stationary phase. Larger proteins exhibit deviations from the linear solvent strength (LSS) retention mechanism in RPLC mode indicating

potential conformational changes in the molecule [33-34]. In HIC mode the effect of organic depends on the aprotic nature because some mAbs showed decreasing retention in previous studies (at ambient temperature), when the amount of IPA (protic solvent) in the mobile phase increased [6]. Bobaly *et al.* [32] mentioned that protic solvent (IPA) can be better solvent than aprotic solvent (ACN) to minimize the denaturation when used up to 10-15%. However, the effect of organic will depend on the nature of the molecules. Small percentage of organic solvent may have very minor effect on conformation changes but will have significant effects on folding thermodynamics [35].

Organic modifier in the mobile phase plays a significant role in HIC in selectivity and resolution. As per earlier research findings [3-4, 6-7, 11, 32, 36-37] small amount of organic in mobile phase B is useful and will dramatically improve the separation without impacting the conformation of the biomolecule. HIC separation using poly (alkyl aspartamide) silica columns require some percentage of organic solvent to elute molecules using low concentration of ammonium acetate [4]. In earlier research it was mentioned [4-6, 32] that in HIC separation, alcohols (specifically isopropanol) improved selectivity and it also been reported that in many cases isopropanol was a less denaturing solvent than MeCN when temperature kept below 40°C [32].

In this research a sequence of experiments were conducted separately to evaluate the impact of different percentages of methanol, isopropanol and MeCN to compare the selectivity and separation. Of the molecules that were used in this research, MeCN provided better separation and selectivity as compared to IPA and MeOH when ammonium acetate gradient was used (manuscript). MeOH and isopropanol percentages were adjusted to match MeCN solvent strength and the impact of MeOH was evaluated using 40%, 62.5% and 75% concentrations in mobile phase B. There was no elution observed with 40% and partial elution was observed with

62.5% and 75% MeOH (Figure 3.5). This may be due to the conformation changes and result in interactions with the stationary phase. Similarly, 15% and 25% IPA (Figure 3.6) in mobile phase did not provide an adequate separation. However, 40% IPA enables the separation of variants which was not observed with MeCN. Use of IPA can be further evaluated to gain knowledge on separating variants. This important observation can lead to new opportunities of quantifying variants of a mAb.

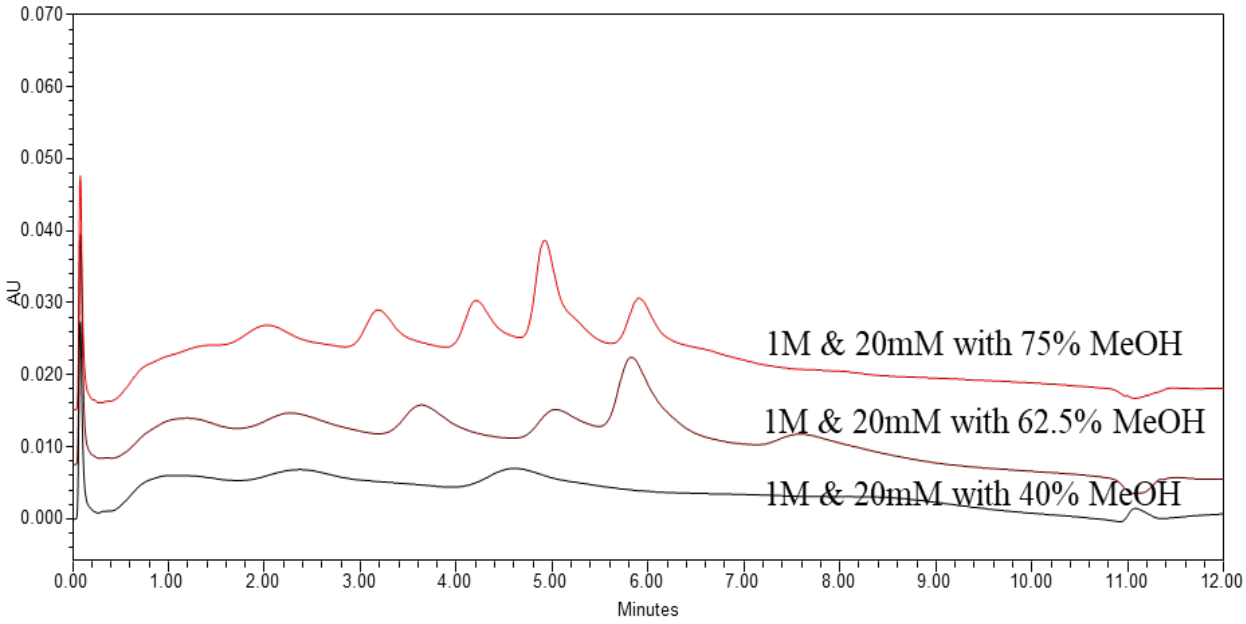

Figure 3.5. The effect of methanol on the separation of six mAbs

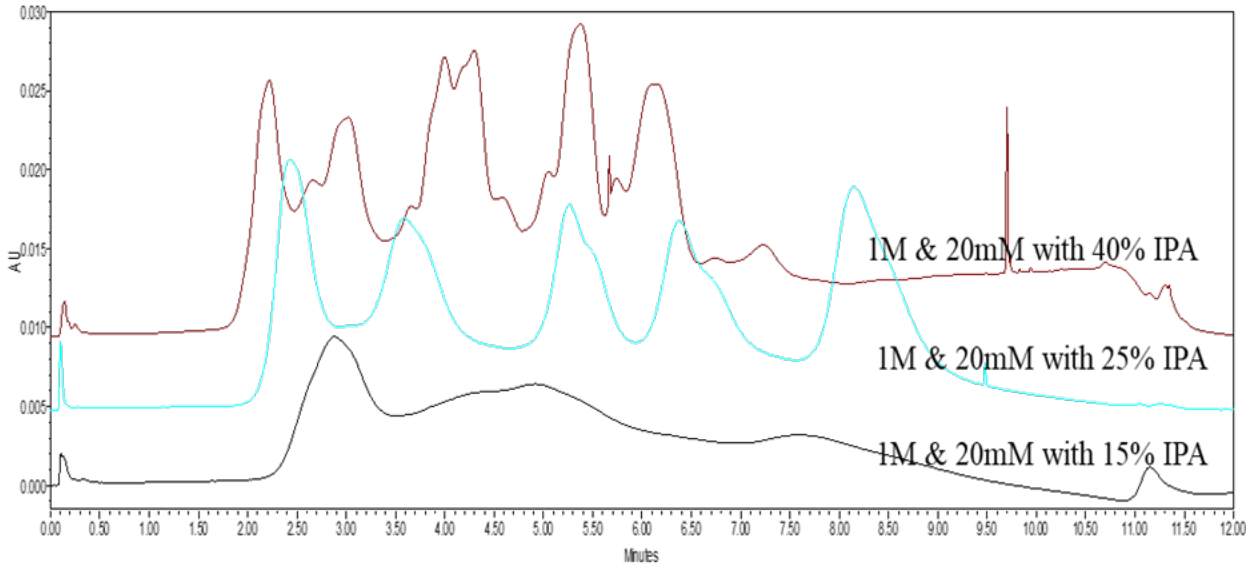

Figure 3.6. The effect of IPA on the separation of six mAbs

To study the workable range of MeCN, a series of experiments were carried out (Figure 3.7) by varying the concentration (25% to 75%) in mobile phase B while all other parameters were kept constant. Results of mobile phase B with 25% MeCN showed that more hydrophilic molecules retained longer compare to higher concentrations of MeCN and no elution was observed for more hydrophobic molecules indicating that the MeCN concentration is not enough to disrupt the hydrophobic interactions to promote elution. Mobile phase B with 35% MeCN contributed to elute the peaks, but a wider elution window was observed. This condition can be effectively utilized to increase the peak capacity. In mobile phase B with 50% MeCN, the separation was not only adequate but also earlier elution was observed. As MeCN percentage increased from 50% to 75% the trend continued with good selectivity (Figure 3.7) and increase in peak capacity (Figure 3.8). In addition, peaks appeared sharper than mobile phase B with 50% MeCN demonstrating similar peak recovery. As there was neither co-elution nor peak splitting observed, it was assumed that molecules retained their mostly folded state or native-like

conformation even with 75% MeCN in mobile phase B. However, to confirm the conformational changes more experiments are required. In these experiments, it was evident that peaks retained longer with an increase in the salt concentration (higher mobile phase A percentage), which is an expected conventional HIC retention behavior.

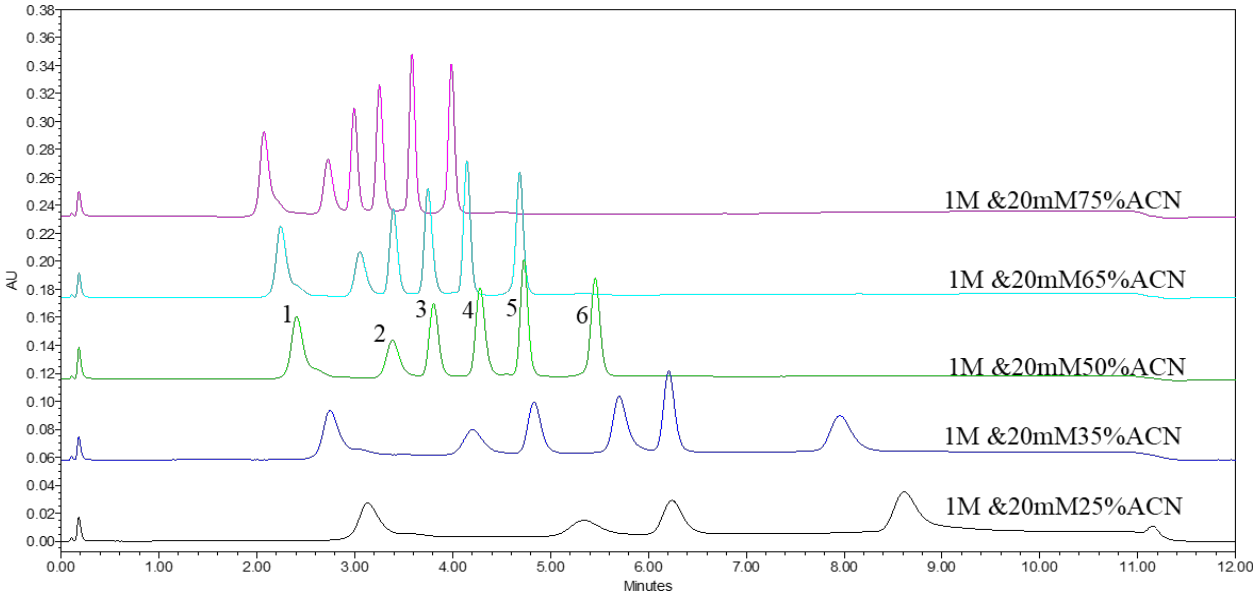

Figure 3.7. Effect of MeCN on the separation of six mAbs

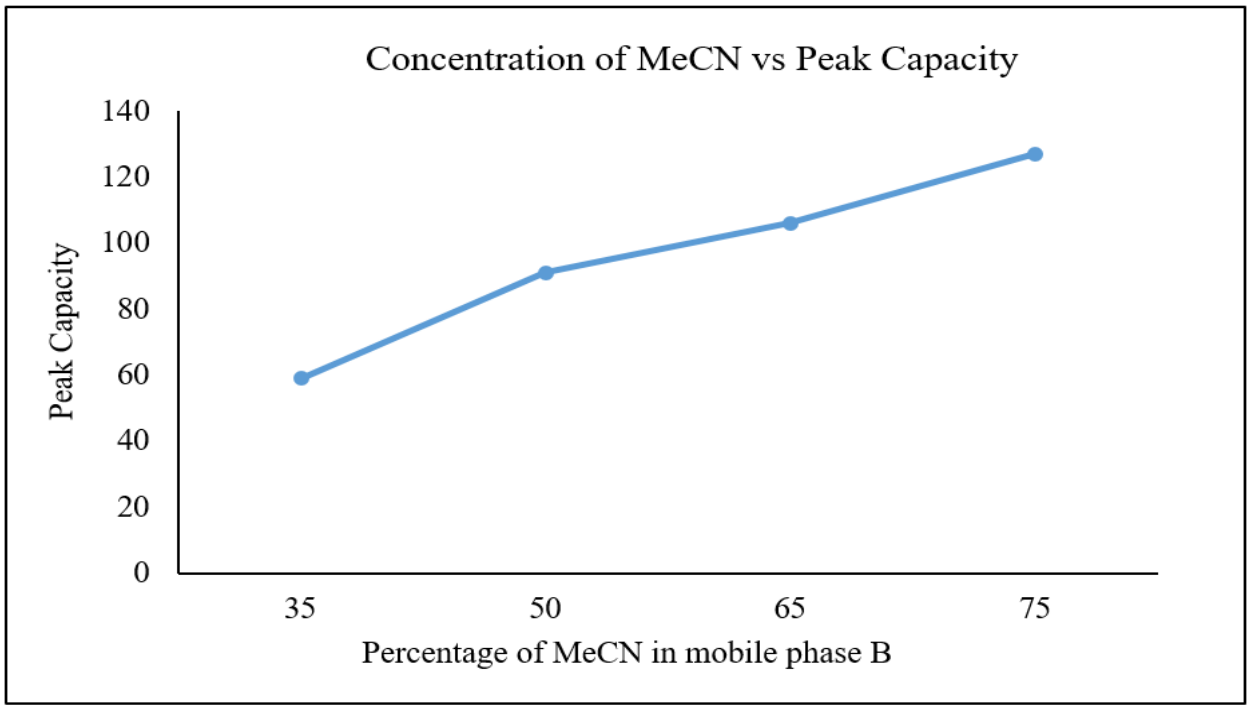

Figure 3.8. Increase in peak capacity with an increase in percentage MeCN in mobile phase B in the presence of 1M salt in mobile phase A

3.3.3 Effect of column temperature on the retention

The temperature effect on HIC performance has been very well studied [9, 16, 38-41]. In general, in HIC, the retention factor, *k*, increase with an increase in temperature [38, 42] and lowering the temperature enhances the protein elution [38]. It is well known that in the folded state protein retention is less in HIC compare to an unfolded state [43]. This is because in an unfolded state, the increase in the retention is due to the increase of hydrophobic interactions as a result of the temperature-induced conformational changes of biomolecules and/or related to the increase in the hydrophobic contact area upon binding to the chromatographic surface [43-45]. Hence, retention is nonlinear with temperature due to protein conformational changes, which leads to an increase in the conformational entropy at higher temperature [42-43]. Wei *et.al* [42] proved that the total moles of solvent released at the contact region between the stationary phase and the solute

interface when 1 mole of solvated solute is absorbed is not same for all the molecules. This number highly depends on the conformational stability of the molecule. The conformational change of proteins will contribute to the increase in hydrophobic contact area between the molecule and the stationary phase. With that, it was confirmed that the increase in the solvent depends on two factors, one is the changes in the number of water molecules surrounding the molecule due to conformational changes of molecules, and the other is a result of an increase in hydrophobic contact area between the protein and the stationary phase. On the other hand, it was clear that the adsorption is accompanied by the release of a large number of water molecules. This supports the expectation of an entropically driven process in which the release of a large number of ordered water molecules provides the driving force for adsorption. This confirmation supports Hjertén *et. al* findings [38].

At low temperatures large and positive enthalpy and entropy changes were observed. In the literature [41, 46-48], HIC is an entropy-driven process and the Gibbs free energy is given according to the Eq. (1).

$$\Delta G = \Delta H - T\Delta S \quad (1)$$

Since ΔH may be a small positive or negative value, ΔG is controlled by a positive entropy change and hence increases with an increase in temperature. According to El Rassi, in HIC the retention factor k' increases with increasing temperature according to the below equation [40]:

$$\ln k' = \ln \varphi - \Delta G/RT \quad (2)$$

Where R is the gas constant, φ is the phase ratio and T is the absolute temperature. However, based on the impact of temperature on the conformational state of different biomolecules and on

their solubility in aqueous solutions, an opposite behavior can be observed in protein retention. In HIC system, protein unfolding is affected by hydrophobic and static electronic force between protein and stationary phase beside the force of the interior of the molecule as temperature increased [42]. Enthalpy–entropy compensation relationship can be used to explain HIC retention process [47, 49]. Even though the effect of temperature in HIC is not straightforward, this parameter can be used to promote elution by weakening the interactions and separating proteins under mild conditions without denaturing the molecule [40].

To demonstrate the effect of temperature using poly(alkyl aspartamide) silica columns and assess the retention in HHIC, an experimental study was designed by varying temperatures in the range of 20°C and 30°C, with 25°C being a control for this research. The temperature limitation for these columns is 35°C. The columns used in the study were PolyBUTYL A, PolyPENTYL A, PolyHEXYL A and PolyHEPTYL A. No significant difference was observed on the retention going from 20°C to 25°C using PolyPENTYL A columns, therefore 20°C was not evaluated for PolyBUTYL A, PolyHEXYL A and PolyHEPTYL A columns. However, as the temperature increased from 25°C to 30°C (Figure 3.9) unlike conventional HIC, an earlier elution was observed for PolyPENTYL A column (Figure 3.10) and the same trend was observed in all other columns. This is an atypical behavior compared to what was described for conventional HIC methodology. The hypothesis for this behavior is that increase in temperature may be effecting in the diffusion of the molecule through the stationary phase or in lower temperatures hydrophobic effect becomes weaker, resulting in early elution [42, 50]. This experiment demonstrated that overall there is no significant impact of separation of these mAbs at the range of temperatures 20°C - 30°C (Figure 3.10).

The aim of this research is to enhance the fundamental knowledge about the retention behavior of hybrid HIC using poly (alkyl aspartamide) silica columns by studying chromatographic parameters such as concentration of salt, pH, and organic solvent and demonstrate the effect of temperature with no intention of evaluating thermodynamic model. The results generated in this research based on chromatographic parameters, such as concentration of salt, pH, and organic solvent are not suitable to assess thermodynamic properties such as entropy and enthalpy. Therefore, a thermodynamic model was out of the scope of this research.

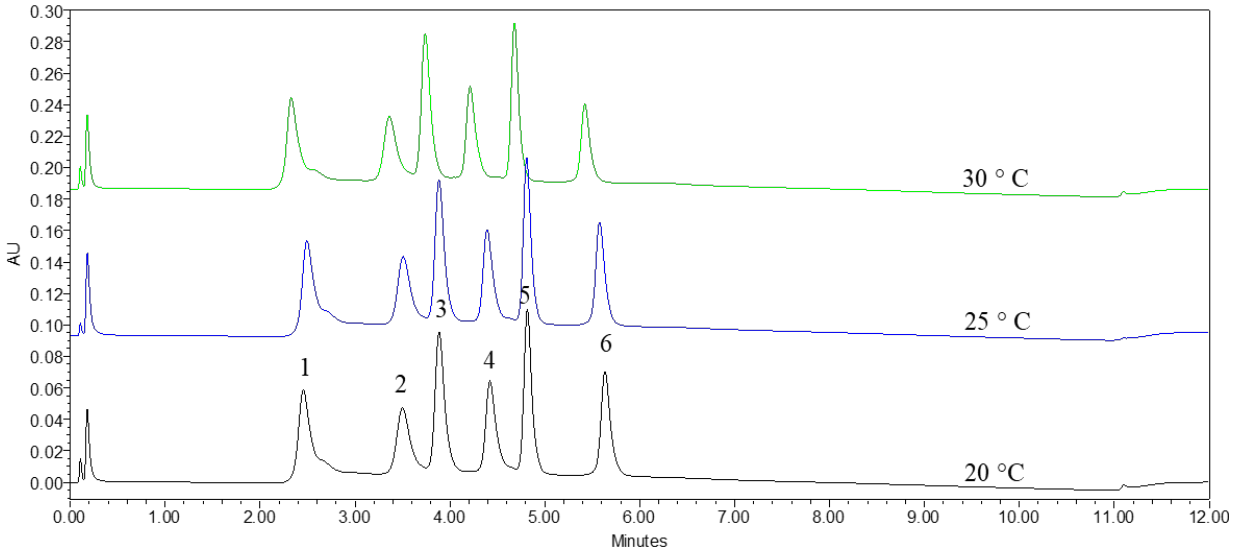

Figure 3.9. Effect of temperature on PolyPENTYL A column

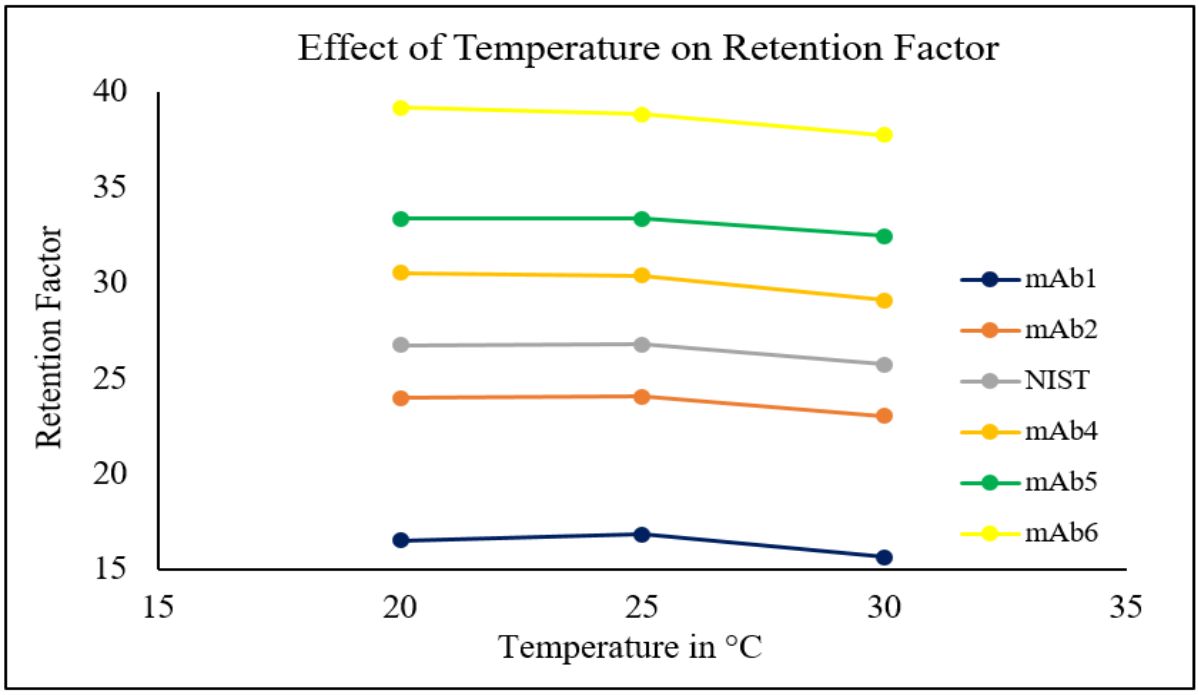

Figure 3.10. Changes in the retention factor with the column temperature using PolyPENTYL A column.

3.3.4 Effect of Salt in Mobile Phase B (20mM ammonium acetate with 50% MeCN)

Salt concentration in mobile phase A plays a significant role in molecule retention on HIC methodology. In this research the separation was based on a decreasing salt gradient with 20mM salt in mobile phase B which is a very low concentration. With a closer look it is obvious that all six peaks eluted before 5 minutes in the presence of half the amount of mobile phase A where the concentration is about 500 mM. The assumption is, in the presence of high salt (500 mM) in mobile phase A and low salt (~10 mM) in mobile phase B, there may not be a great impact on the elution and separation. To study the importance and measure the role of salt

concentration on mobile phase B, we performed experiments (Figure. 3.11) using mobile phase A with 1 M and mobile phase B with pure water and 50% MeCN in water (no salt).

The results confirmed that as described in section (2.3) organic solvent is required for the elution of the peaks using PolyPENTYL A column when used with low concentrations of ammonium acetate as mobile phase A. It was evident that the separation was very similar (Table. 3.2) with and without 20 mM salt present in mobile phase B. Therefore, it was clear that the low salt that achieved through the gradient steepness may be low enough to rebuild the hydration shell around the biomolecule and organic solvent will help to break hydrophobic interactions by promoting the elution. This experiment confirmed that an adequate chromatographic separation can be achieved using 1M ammonium acetate as mobile phase A and mobile phase B with or without 20mM ammonium acetate (overall) with 50% MeCN using PolyPENTYL A column.

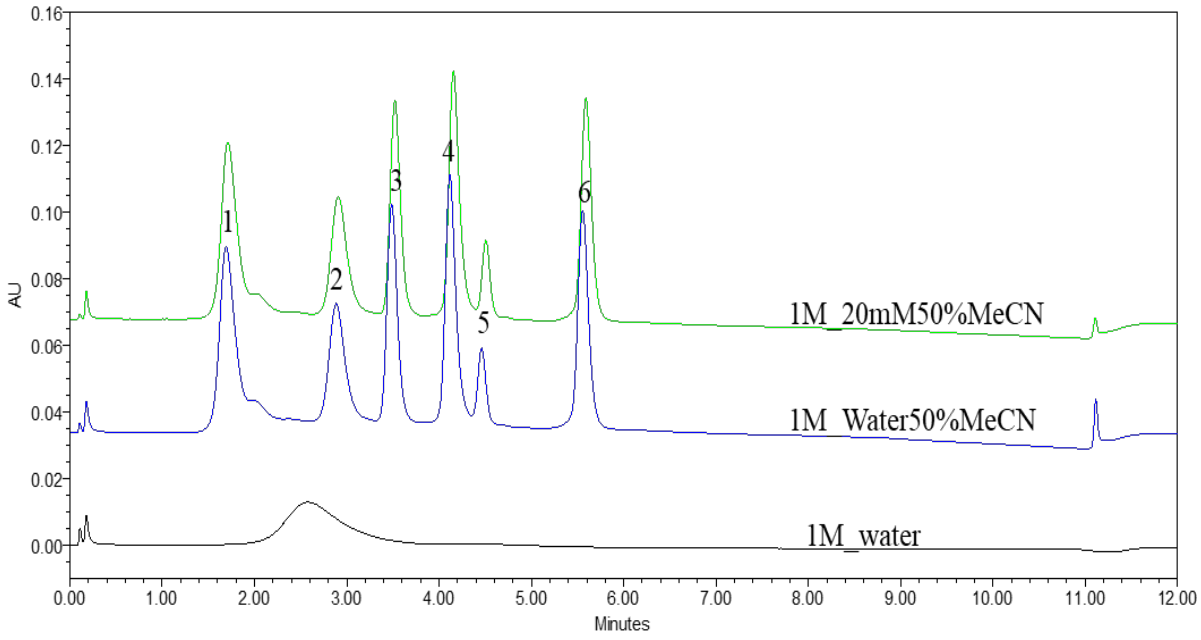

Figure 3.11. PolyPENTYL A column with 1000 Å and 3 um: Evaluation of chromatographic separation (MP A: 1 M NH_4OAc at pH 7.0 and MP B: water, water with 50% MeCN and 20 mM with 50% MeCN @ an aqueous pH 7.0)

Table 3.2. Comparison of mobile phase B using PolyPENTYL A column with 1000 Å and 3 um particle size: 1 M ammonium acetate as mobile phase A and 50% of MeCN with and without 20 mM ammonium acetate as mobile phase B

Mobile phase composition	Peak name	Tailing Factor	Resolution	Retention Factor	Selectivity	Peak Capacity
1M_20mM50%MeCN	mAb1	1.05	NA	16.1	NA	76
	mAb2	1.17	3.93	28.1	1.75	
	NIST	1.17	2.37	34.2	1.22	
	mAb4	1.24	3.08	40.6	1.19	
	mAb5	1.11	1.96	44.1	1.09	
	mAb6	1.12	5.99	54.9	1.25	
1M_water50%MeCN	mAb1	1.05	NA	15.9	NA	71
	mAb2	1.15	3.94	27.9	1.76	
	NIST	1.19	2.31	33.9	1.22	
	mAb4	1.23	3.06	40.2	1.19	
	mAb5	1.14	1.91	43.6	1.09	
	mAb6	1.10	5.88	54.6	1.25	

3.4 Conclusion

The purpose of this study was to demonstrate the influence of chromatographic parameters that impact selectivity and retention. Parameters such as mobile phase pH, organic type and percentage and temperature are shown to effect chromatographic performance. The research finding suggests that mobile phase pH variation in the range of 5.7 - 7.3 may not be a critical parameter to improve the separation for some mAbs, but for other mAbs the impact is significant. The reason for this behavior is, the changes in the pH will change the charge on the molecule due to the ionization of acidic groups. Hence, it is worth to evaluate mobile pH to tune the selectivity using poly (alkyl aspartamide) silica columns.

It was demonstrated that an organic solvent has a significant impact on the elution using HHIC technique due to the use of longer alkyl chains in stationary phase in the presence of low concentrations of NH_4OAc. In this research, different organic solvents and their percentages in mobile phase B showed a significant difference in selectivity. The data proved that MeOH cannot provide adequate elution for the selected mixture of biomolecules and to provide adequate separation, at least 35% MeCN is required for acceptable separation. 40% IPA demonstrated complex chromatogram with multiple peaks for each component, which appears it has the capabilities to separate variants. This observation can be further evaluated. It was well established that in conventional HIC along with mobile phase parameters, temperature plays a major role. Unlike conventional HIC, in HHIC with poly (alkyl aspartamide) silica columns increasing temperature from 25°C to 30°C showed an early elution which is an atypical HIC behavior. Based on this observation, temperature may not be a critical parameter but may contribute to increase the peak capacity.

The experiment to assess the importance of salt in mobile phase B confirmed that using pure water as mobile phase B was unable to elute biomolecules from the column. However, small percentage of MeCN in mobile phase B with or without 20 mM ammonium acetate provided adequate separation with little or no distinguishable differences. The results confirmed that salt in mobile phase B does not play a major role in biomolecule separation.

3.5 References

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Chapter 4 : Hybrid Mode of Hydrophobic Interaction Chromatography of Monoclonal Antibodies and Related Biomolecules: Impact of Stationary Phase and Its Interplay with Mobile Phase Parameters

4.1 Introduction

Protein conformation plays a significant role on chromatographic behavior in protein chromatography [1]. Structural changes in the secondary, tertiary or quaternary structure can have a major impact on retention, peak width and peak shape [1-2]. In addition to mobile phase conditions, stationary phase ligands play a significant role and result in structural modifications which can lead to changes in the retention behavior. Strong protein-surface interactions can force partial exposure of the molecule hydrophobic interior [3]. The reorientation of the molecule is required to achieve optimal interaction between the hydrophobic groups and the alkyl chains on stationary phase to adsorb on the chromatographic surface. These interactions will determine the nature of adsorption-induced structural changes [3]. Protein adsorption at stationary phase surface is driven by the various interactions which exist between the protein and the groups that are attached to the stationary surface. The interactions that play a role in protein adsorption are hydrophobic interactions, electrostatic interactions, van der Waals interactions, hydrogen bonding, coordination bonding, and conformational entropy [4-6]. Based on the knowledge acquired from the literature Yu *et al.* [7] illustrated protein behavior in a diagram (Figure. 4.1). Yano and Rabe *et al*. [6, 8] mentioned that when molecules exists as an individual entity and as an ensemble, the protein adsorption phenomena at solid surfaces is mainly driven by their attraction towards solid surfaces.

The amino acids nature and diversity such as hydrophobicity/hydrophilicity or charged/neutral properties, leads to structural and functional complexity of the molecule. A

protein consists of both positive and negatively charged groups and exists in a secondary or tertiary structure. Protein folding patterns lead to a heterogeneous surface exhibiting specific properties such as hydrophobic/hydrophilic as demonstrated in Figure 4.2. Protein behavior at chromatographic surface becomes extremely complex due to this diversified surface properties and as a result, adsorption often results in an interplay of attraction and repulsion interactions. These interactions will drive to the protein preferred binding orientation, which can further effect mass transport and protein conformational transitions such as unfolding and refolding. Unfolding commonly occurs at the surface of the molecule due to complicated molecular interactions with surfaces, especially for unstable proteins. Hence, separation of unstable biomolecules may require more attention. Literature suggests either avoid using HIC technique [9-12] or to carefully select mobile phase solvents and its composition. Other alternative is to add protein stabilizers [13-15] as additives [16-18] in the mobile phase before performing the analysis [7]. Not only stationary phase surface properties but protein properties, and operating parameters such as mobile phase conditions, can drive all of these effects as well [7].

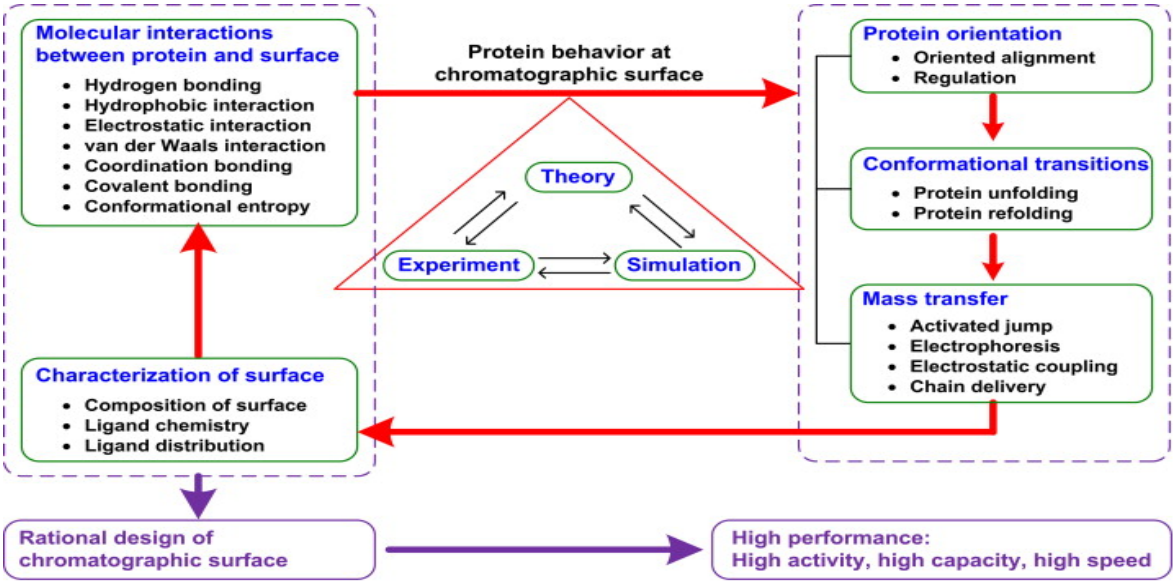

Figure 4.1. Protein behavior on chromatographic surface [7]
(Adopted with permission from Elsevier)

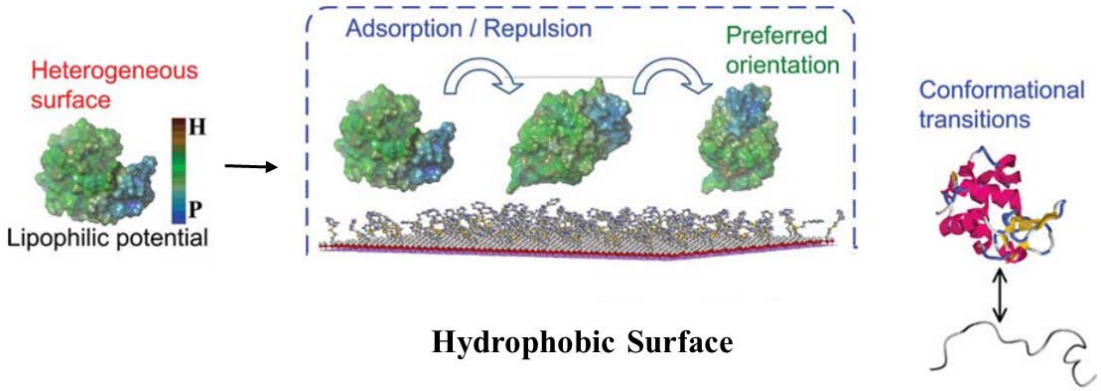

Figure 4.2. Biomolecule with heterogeneous surface composed of hydrophobic/hydrophilic patches. The re-orientation and favored binding orientation of the molecule at hydrophobic surface [7] *(Adopted with permission from Elsevier)*

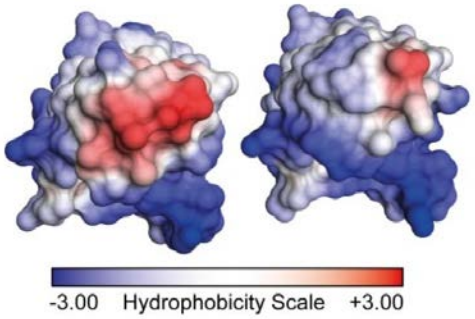

Figure 4.3. Biomolecule representing hydrophobic (Red) and hydrophilic (blue) areas [19] (Adopted with permission from creativecommons.org)

HIC stationary phases typically consist silica or a polymer bonded with alkyl or aryl groups that have relatively limited hydrophobicity, for example butyl, phenyl, ether, amide, and propyl (Figure 4.4). Straight chain alkyl groups exhibit hydrophobic character, whereas aryl groups show mixed-mode behavior where both aromatic and hydrophobic interactions can play a role in the adsorption process. Commercially available HIC stationary phases are non-porous and made with either silica (MAbPac HIC-Butyl) or polystyrenedivinylbenzene (Proteomix HIC Butyl) as a base material; however, there are also some porous and nonporous polymethacrylate-based (TSKgel Butyl-NPR) particles available. These columns will offer separation using non-volatile salts such as phosphates and sulfates, which are incompatible with online MS. Therefore, to extend MS compatibility Andrew Alpert synthesized new stationary phases (Section 1.8) which can provide the separation using volatile salts such as acetate and tartrate.

Stationary phase properties such as base matrix, hydrophobicity, particle size and pore size have a major impact on the selectivity. Base matrix plays a significant role in the separation due to non-specific interactions such as electrostatic interactions, which can contribute to the selectivity. Even though there is one alkyl group difference from propyl to butyl to pentyl, propyl has access only when high salt has disrupted the hydration layer around the molecule.

However, longer alkyl chains such butyl and pentyl can reach the hydrophobic patches even when the water molecules are intact with the biomolecule, showing an adequate selectivity. It is a well-established fact that smaller particles provide higher surface area, shorter diffusion paths and increased mass transfer kinetics which will contribute to the reduction in plate height and increase the efficiency. Similarly, the results from the columns with higher pore size demonstrate shorter retention times, higher resolution and better efficiency. Even though larger pore size provides low surface area, molecules can freely enter into these larger pores and can able to interact with the stationary phase alkyl chains. In addition to column properties, mobile phase properties such as buffer conditions can modify the apparent size of the biomolecule effecting the resolution. As a whole, not only stationary phase properties by itself have a profound impact on the selectivity but the interplay of both mobile phase and stationary phase can highly influence the separation of biomolecules. In general, for biomolecules, large pore size with shorter alkyl chain lengths provide better resolution.

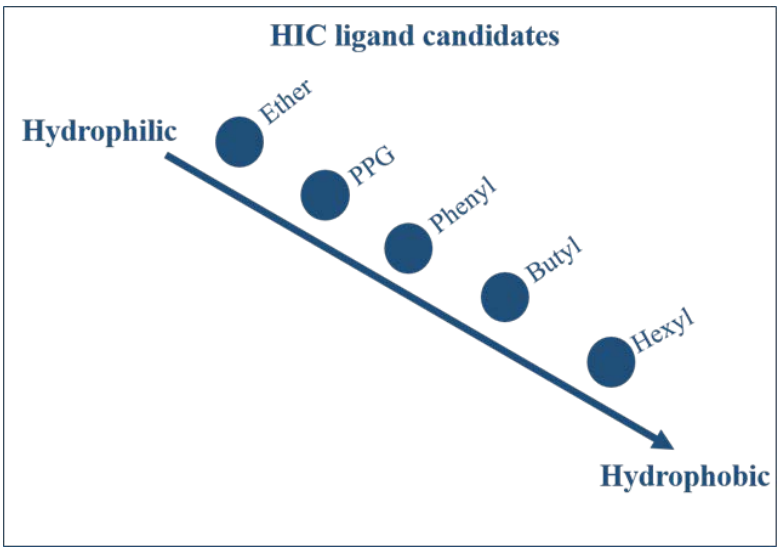

Figure 4.4. Increase in the hydrophobicity of HIC stationary phases.

HIC stationary phases are less hydrophobic compared to RPLC phases as the functional groups are sparsely distributed, resulting in mild interactions [20]. Retention in HIC technique is very sensitive and highly dependent on the alkyl type, alkyl chain length and alkyl density along with mobile phase parameters demonstrated that salt type can have different effects on retention depending on the hydrophobicity of the protein to be separated and the hydrophobicity of the stationary phase itself. They also emphasized the importance of a well selected stationary phase and the salt (phase system) [20-21] on the retention and selectivity. In addition, it was demonstrated that the hydrophobicity of the stationary phase plays a significant role in maintaining native state conditions upon elution from the chromatographic column [1].

HIC stationary phases made with poly (alkyl aspartimide) are more hydrophobic than typical HIC stationary phases. The impact of mobile phase parameters on these columns play a very similar role to conventional HIC columns. Due to their more hydrophobic nature and highly retentive behavior the non-typical HIC columns can provide adequate retention using low

concentrations of MS compatible salts. There are several stationary phases available with different alkyl lengths such as propyl, butyl, pentyl hexyl, heptyl etc. with different pore size such as 1000 Å and 1500 Å and particle sizes of 2 µm, 3 µm, 5 µm and 12 µm. The selection of phase system and chromatographic conditions for therapeutic biomolecules are mostly subjective and chosen based on the trial-and-error approach [22]. The separation and selectivity is highly dependent on the stationary phase and mobile phase parameters and as well as, the interplay of both parameters. As multiple parameters act simultaneously, the chromatographic behavior is considered to be complex due to the interdependency of multiple gradients such as dynamic changes in pH, salt and organic solvent. To demonstrate the capabilities and differences of these stationary phases, we studied the selectivity of different columns with different pore sizes and different particle sizes using the biomolecules which are used in this research.

4.2 Experimental Details

Details on the chemicals, HIC columns, mAbs, HPLC system and chromatographic conditions, refer to Section 2.2. Unless otherwise specified chromatographic separations were performed at 25°C with initial 0.5 minutes hold and a 10 min linear gradient going 100% mobile phase A to 100% mobile phase B. 1 M NH_4OAc was used as mobile phase A and 20mM NH_4OAc with 50% MeCN as mobile phase B. pH of all the mobile phases were measured for aqueous solutions. Experiments were performed using columns with different column parameters and also using mobile phases with different salt and organic concentrations. Please refer to the details in Tables 4.1 and 4.2.

Table 4.1. PolyBUTYL A and PolyPENTYL A column dimensions used in this research.

Columns (50 x 2.1 mm)	1000 Å		1500 Å	
PolyPENTYL A	3 µm	2 µm	3 µm	2 µm
PolyBUTYL A	3 µm	-	-	-

Table 4.2. List of mobile phase A and mobile phase B compositions.

Mobile phase A @ pH 7.0	Mobile phase B: % of MeCN in 20mM ammonium acetate @ pH 7.0				
1M	25%	35%	50%	65%	75%
0.5 M	X	X	X	X	X
0.35 M	X	X	X	X	X
0.25 M	X	X	X	X	X

4.3 Evaluation and Comparison of Retention in Different Poly (alkyl aspartamide) Stationary Phases

The properties of these stationary phases result in different retention and selectivity. The effect of salt and organic concentration on the chromatographic separation of the molecules can vary considerably on different stationary phases with same hydrophobicity but different pore size and particle size. The selected biomolecules were screened using PolyPROPYL A, PolyBUTYL A, PolyPENTYL A, PolyHEXYL A and PolyHEPTYL A columns that were evaluated by Chen *et al.* [23-25] and the initial conditions for the separation were adopted from their research findings [23]. After an initial evaluation (Figure 4.1) PolyBUTYL A and PolyPENTYL A columns demonstrated very similar selectivity with an adequate separation. PolyHEXYL A column showed poor recovery of early eluters and co-elution of late eluters. PolyHEPTYL A column demonstrated similar chromatographic performance as PolyHEXYL A for late eluters but showed no elution for early eluters. This observation of both stronger retention and co-elution of the peaks may be due to the more hydrophobic nature of the longer alkyl chains stationary phases. Compare to PolyBUTYL A, PolyPENTYL A column has more surface area leading to higher efficiency. Therefore, it is found to be most suitable based on the tailing factor, resolution, efficiency, peak capacity (Table 4.3) for the selected molecules which is in agreement with previous report [23, 26].

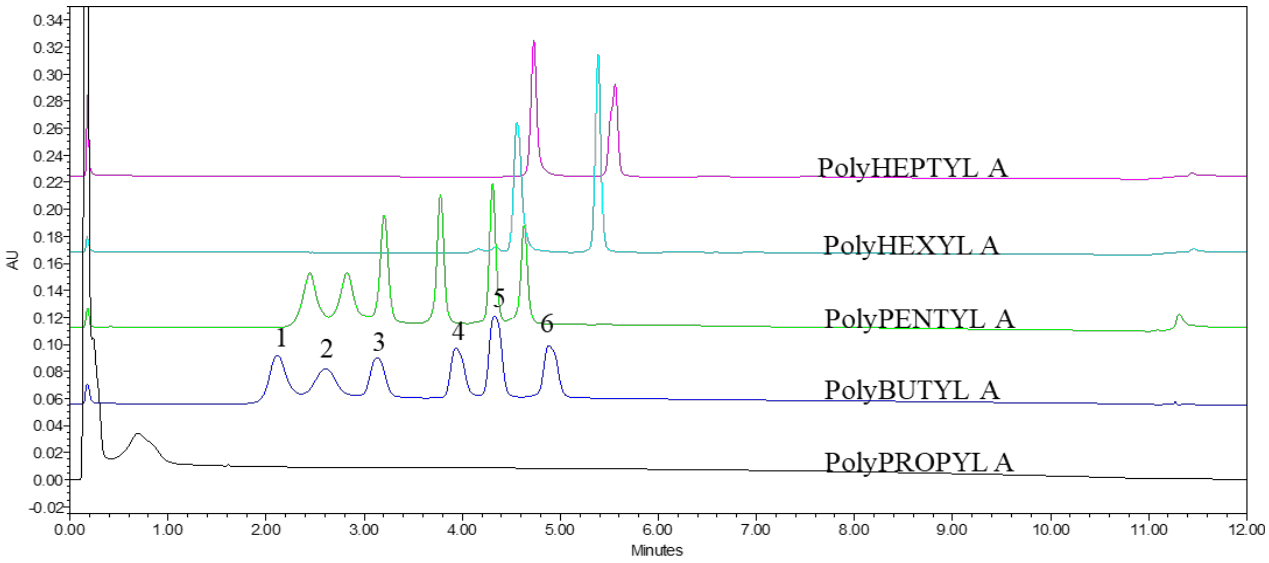

Figure 4.5. Comparison of the selectivity using different poly (alkyl aspartamide) columns (MP A: 1 M NH_4OAc and MP B: 20 mM NH_4OAc with 50% MeCN @ pH 7.0)

Table 4.3. Comparison of PolyBUTYL A and PolyPENTYL A columns chromatographic performance.

Column Type	Peak name	Resolution	Retention Factor	Selectivity	Peak Capacity
PolyBUTYL A	mAb1	NA	10.7	NA	62
	mAb2	1.52	13.5	1.25	
	NIST	1.68	16.4	1.22	
	mAb4	3.26	20.9	1.27	
	mAb5	1.77	23.0	1.10	
	mAb6	2.52	26.1	1.13	
PolyPENTYL A	mAb1	NA	12.6	NA	105
	mAb2	1.61	14.7	1.17	
	NIST	2.01	16.8	1.14	
	mAb4	4.03	20.0	1.19	
	mAb5	4.17	22.9	1.15	
	mAb6	2.62	24.7	1.08	

Note: Poor recovery was observed using PolyPROPYL A, PolyHEXYL A and PolyHEPTYL A columns. Hence, data was not included. Peak capacity was calculated using peak width@50% height.

In sight of initial evaluation, both PolyBUTYL A and PolyPENTYL A columns were chosen to extend the research to study the effect of different particle sizes and pore sizes on chromatographic separation. The main purpose of the study was to evaluate the separation using different phase systems (stationary phase and salt) in combination with different concentrations of MeCN in mobile phase B. This study was performed to check if a lower salt concentration (>0.5 M) can provide an adequate separation using columns packed with different pore sizes and particle sizes for the mixture of biomolecules that are used in this research. To evaluate the separation capabilities, an experiment was designed to use PolyPENTYL A columns with both 1000 Å and 1500 Å pore sizes with 3 um and 2 um particle sizes and PolyBUTYL A column with 1000 Å pore size with 3 µm particle size. Different concentrations of ammonium acetate as mobile phase A and 20 mM ammonium acetate with different percentages of MeCN as mobile phase B was used to perform sample analysis. All these experiments were conducted using 1 mL/min flow rate at a column temperature 25°C unless otherwise specified.

4.3.1 PolyPENTYL A column with 1000 Å pore size with 3 µm particle size

PolyPENTYL A column with 1000 Å with 3 µm particle size was used for all previous research experiments, hence, this column was considered as a control (Figure 4.6) for this study. Peaks 1 through 6 are biomolecules used as a sample mixture. The difference in selectivity was observed with 1M ammonium acetate as mobile phase A and 20mM ammonium acetate with different concentrations of MeCN as mobile phase B (Table 4.4). Refer to Section 3.3 for the details of the retention hypothesis. 25% MeCN was not strong enough to break the hydrophobic interactions between the molecule and the stationary phase alkyl chains. The increase in MeCN concentration showed good selectivity and a decrease in the resolution. Peaks appeared sharper

showing a decrease in the retention time with a similar recovery. There was at least a two fold increase in the peak capacity observed (Figure 4.7).

A significant difference in the selectivity (Figure 4.8) was observed with 0.5 M ammonium acetate as mobile phase A and mobile phase B changing from 25% MeCN to 75% MeCN. 25% and 35% MeCN was not enough to elute all the peaks and co-elution was observed with 75% MeCN with a decrease in the resolution. Mobile phase A containing 0.5 M with 50% and 65% MeCN with 20mM ammonium acetate gave adequate separation and the impact of the salt concentration on the selectivity was explained in section 2.3. An important observation was made that the chromatogram obtained in this study showed wider elution window compared to the one obtained for Figure 2.2 using similar PolyPENTYL A columns. The reason may be due to column to column variability, please refer to Section 4.10 for more details.

Mobile phase A with 0.35 M (Figure 4.9) and 0.25 M (Figure 4.11) in combination with 25%, 50%, 65% and 75% MeCN in mobile phase B resulted in co-elution or no elution of peaks. To confirm the peaks order of the elution, individual molecules were injected using mobile phase A with 0.35 M and 75% MeCN in mobile phase B and found that mAb4 and 6 retained on the column showing no elution Figure 4.11. To enhance the elution of molecules in the mixture with 0.25 M, temperature was increased from 25°C to 30°C and coeluted peaks showed some separation but not a significant difference from what was observed with 25°C. In summary, PolyPENTYL A 1000 Å with 3 µm column give good separation with 1M and 0.5 M as mobile phase A and 20 mM ammonium acetate with 50% and 65% MeCN as mobile phase B but lower salt concentrations did not promote elution of the molecules. Peak capacity was calculated only for the conditions where chromatographic separation was achieved.

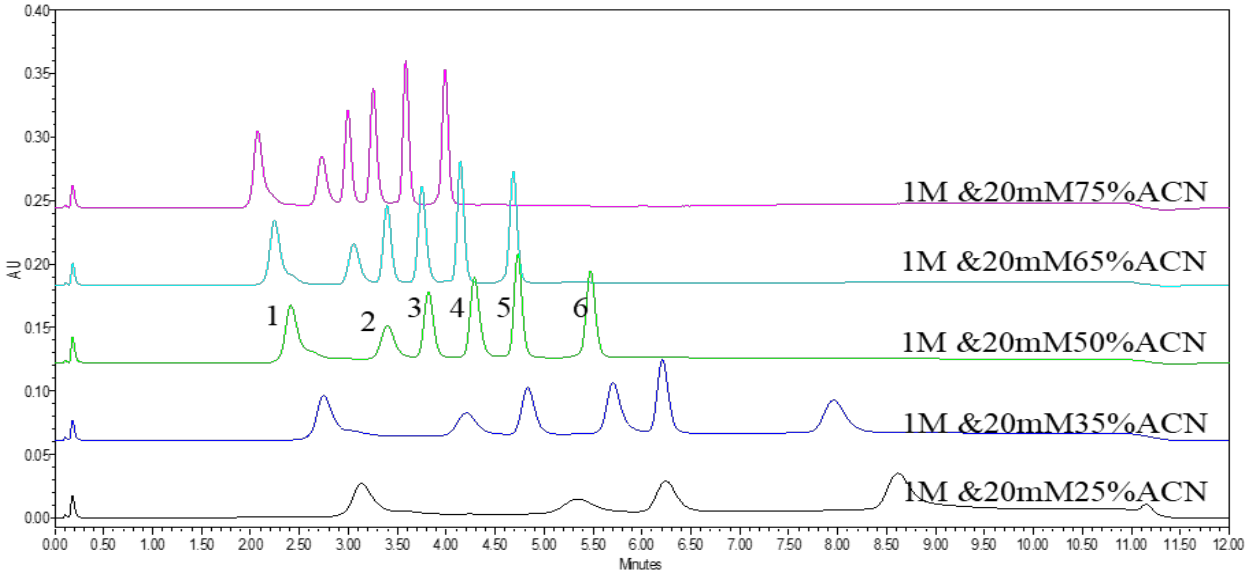

Figure 4.6. PolyPENTYL A column with 1000 Å and 3 µm particle size: Impact of MeCN percentage on the selectivity (MP A: 1M NH_4OAc at pH 7.0 and MP B: 20 mM NH_4OAc with different percentages of MeCN with an aqueous pH 7.0).

Table 4.4. Comparison of chromatograms with 1 M ammonium acetate as mobile phase A and different percentages of MeCN with 20 mM ammonium acetate as Mobile phase B using PolyPENTYL A column with 3 µm, 1000 Å pore size.

Mobile phase composition	Peak name	Resolution	Retention Factor	Selectivity	Retention Factor
1M and 20mM with 35%MeCN	mAb1	NA	24.0	1.56	59
	mAb2	4.31	37.2	1.56	
	NIST	2.04	42.9	1.16	
	mAb4	3.38	50.8	1.19	
	mAb5	2.13	55.4	1.09	
	mAb6	5.65	71.3	1.29	
1M and 20mM with50%MeCN	mAb1	NA	20.9	NA	91
	mAb2	4.14	29.8	1.43	
	NIST	1.99	33.6	1.13	
	mAb4	2.66	37.9	1.13	
	mAb5	2.74	42.0	1.11	
	mAb6	4.47	48.6	1.16	
1M and 20mM with 65%MeCN	mAb1	NA	19.5	NA	106
	mAb2	3.98	26.8	1.38	
	NIST	1.90	29.9	1.12	
	mAb4	2.35	33.1	1.11	
	mAb5	2.82	36.7	1.11	
	mAb6	3.94	41.6	1.13	
1M and 20mM with 75%MeCN	mAb1	NA	17.8	NA	127
	mAb2	3.79	23.8	1.38	
	NIST	1.80	26.2	1.12	
	mAb4	2.06	28.5	1.11	
	mAb5	2.79	31.6	1.11	
	mAb6	3.47	35.3	1.13	

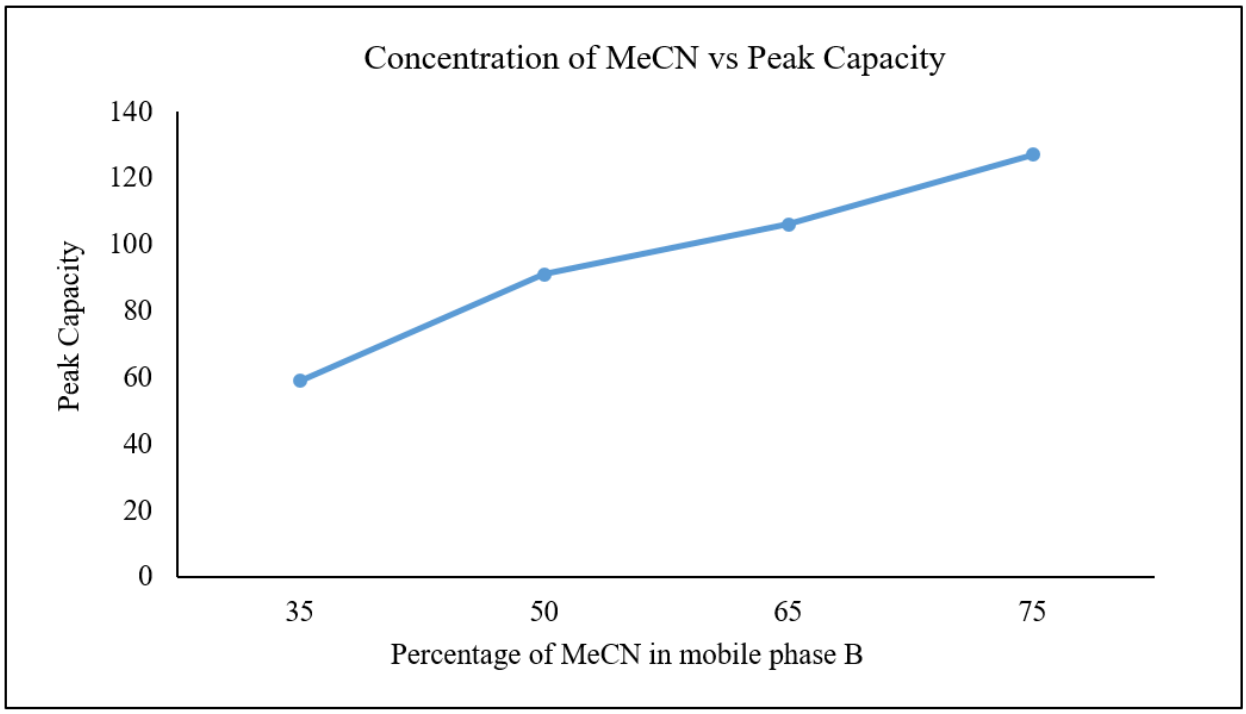

Figure 4.7. PolyPENTYL A column with 1000 Å and 3 µm particle size: Impact of MeCN percentage on the peak capacity (MP A: 1M NH_4OAc at pH 7.0 and MP B: 20 mM NH_4OAc with different percentages of MeCN with an aqueous pH 7.0).

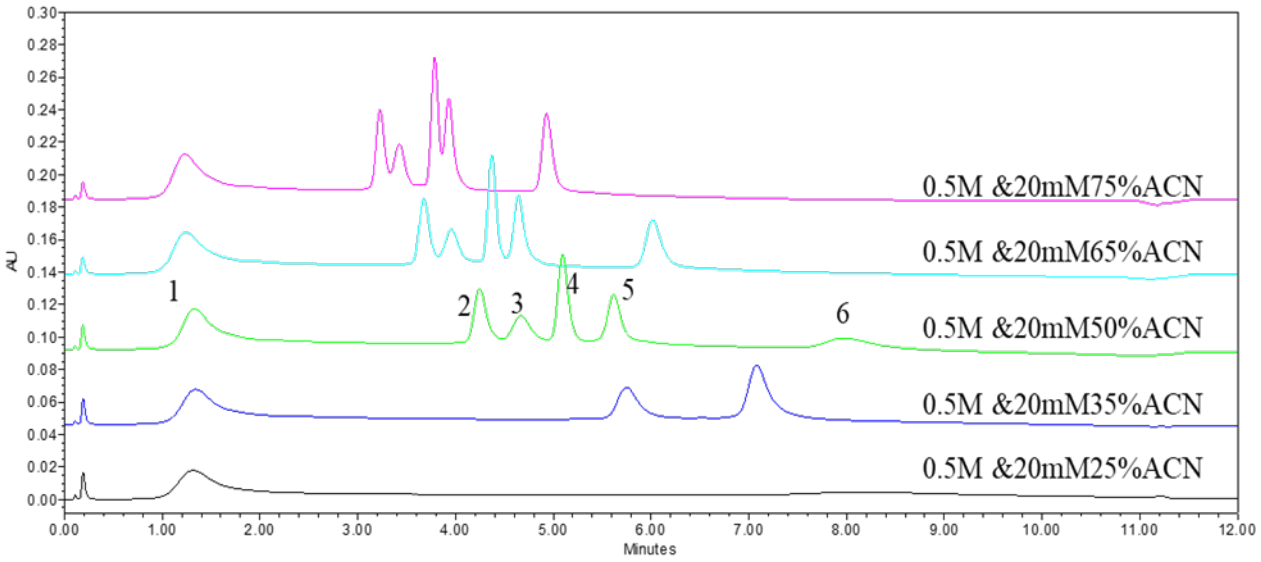

Figure 4.8. PolyPENTYL A column with 1000 Å and 3 µm particle size: Impact of MeCN percentage on the selectivity (MP A: 0.5 M NH_4OAc at pH 7.0 and MP B: 20 mM NH_4OAc with different percentages of MeCN with an aqueous pH 7.0).

Table 4.5. Comparison of chromatograms with 0.5 M ammonium acetate as mobile phase A and different percentages of MeCN with 20 mM ammonium acetate as Mobile phase B using PolyPENTYL A column with 1000 Å and 3 µm column.

Mobile phase composition	Peak name	Resolution	Retention Factor	Selectivity	Retention Factor
0.5M and 20mM with 50%MeCN	mAb1	NA	11.0	NA	48
	mAb2	7.93	37.6	3.47	
	NIST	1.60	41.4	1.10	
	mAb4	1.73	45.3	1.09	
	mAb5	2.39	50.1	1.11	
	mAb6	4.82	71.5	1.43	
0.5M and 20mM with 65%MeCN	mAb1	NA	10.2	NA	66
	mAb2	6.50	32.4	3.23	
	NIST	1.39	35.0	1.08	
	mAb4	2.14	38.7	1.11	
	mAb5	1.57	41.2	1.06	
	mAb6	5.71	53.7	1.30	
0.5M and 20mM with 75%MeCN	mAb1	NA	10.1	NA	77
	mAb2	5.89	28.3	2.85	
	NIST	1.05	30.1	1.06	
	mAb4	2.07	33.4	1.11	
	mAb5	0.93	34.7	1.04	
	mAb6	5.52	43.8	1.26	

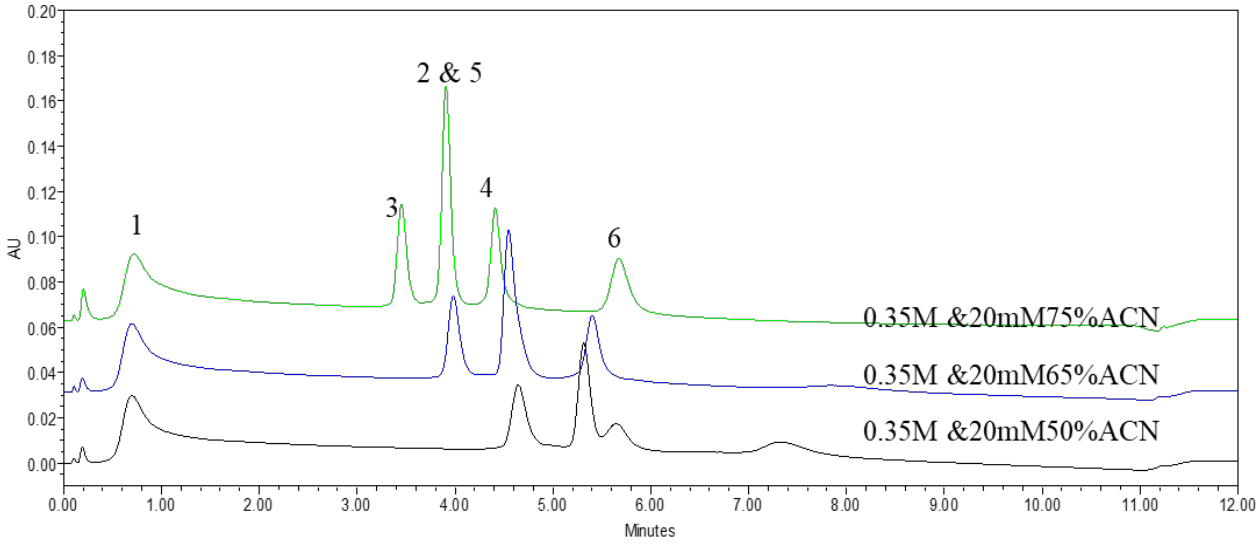

Figure 4.9. PolyPENTYL A column with 1000 Å and 3 µm particle size: Impact of MeCN percentage on the selectivity (MP A: 0.35 M NH_4OAc at pH 7.0 and MP B: 20 mM NH_4OAc with different percentages of MeCN with an aqueous pH 7.0).

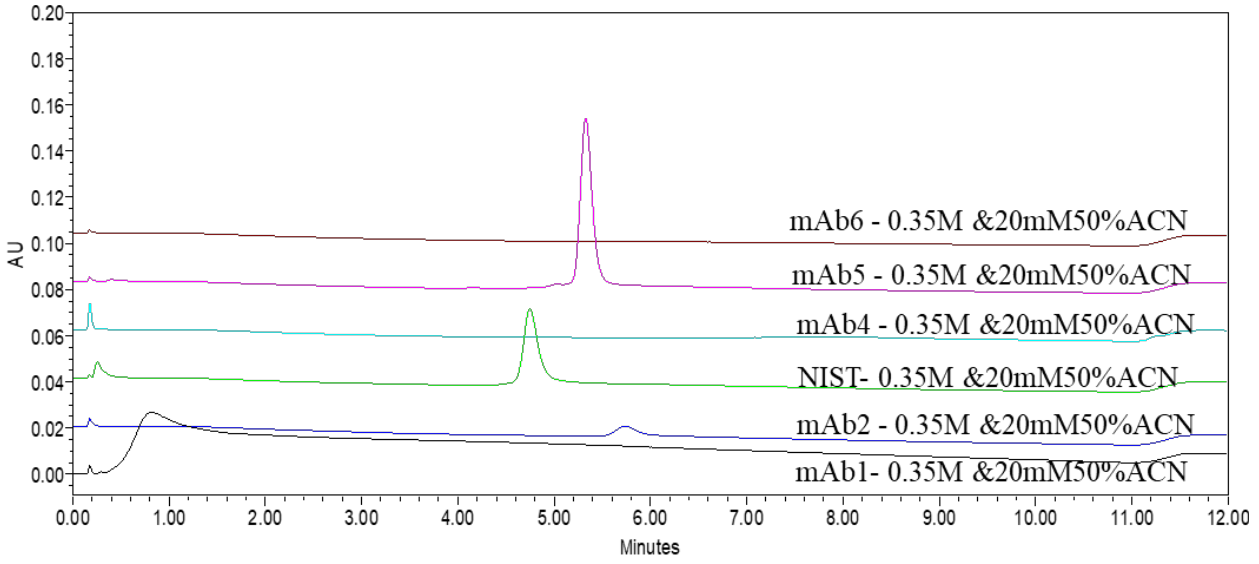

Figure 4.10. PolyPENTYL A column with 1000 Å and 3 µm particle size: Impact of MeCN percentage on the selectivity of individual mAbs (MP A: 0.35 M NH_4OAc at pH 7.0 and MP B: 20 mM NH_4OAc with 75% of MeCN with an aqueous pH 7.0).

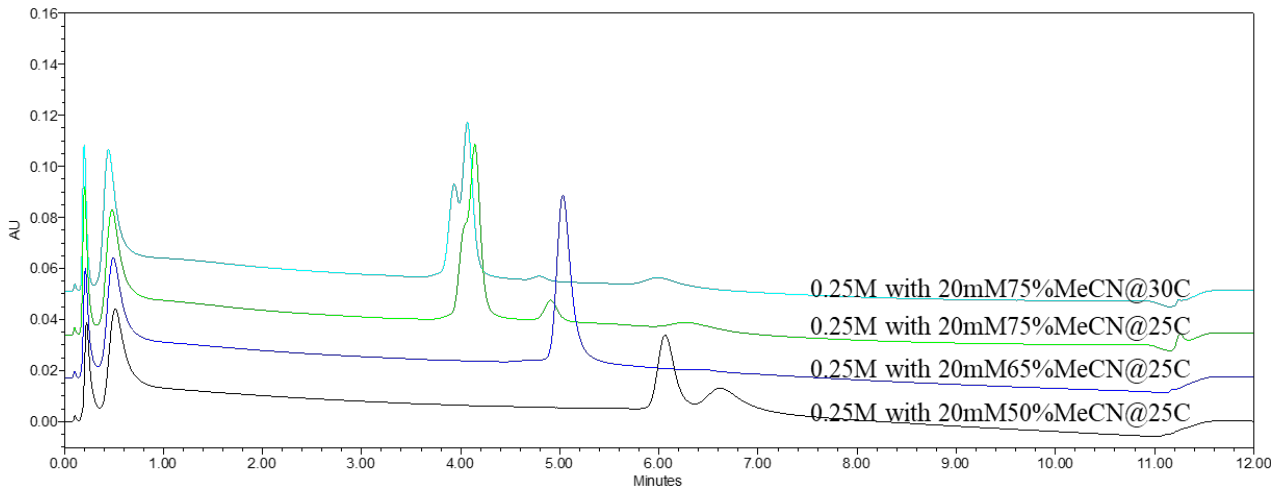

Figure 4.11. PolyPENTYL A column with 1000 Å and 3 µm particle size: Impact of MeCN percentage on the selectivity (MP A: 0.25 M NH_4OAc at pH 7.0 and MP B: 20 mM NH_4OAc with different percentages of MeCN with an aqueous pH 7.0).

4.3.2 PolyPENTYL A column with 1000 Å pore size with 2 µm particle size

PolyPENTYL A column with 1000 Å with 2 µm particle size in combination of 1M ammonium acetate as mobile phase A and 20mM ammonium acetate with different concentrations of MeCN as mobile phase B (Figure 4.12) demonstrated very similar selectivity to PolyPENTYL A column with 1000 Å with 3 µm particle size column. 1 M with 25% MeCN did not elute all the peaks but as the organic concentration increased to 35% and above, all six molecules eluted with improved resolution. Mobile phase A with lower salt concentrations such as 0.5 M, 0.35 M and 0.25 M (Figures 4.14, 4.15 and 4.16) with different percentages of MeCN as mobile phase B, either did not elute or co-eluted peaks showed lack of selectivity under these conditions. This experiment confirmed that PolyPENTYL A column with 1000 Å with 2 µm particle size can be used with only 1 M with 35%, 50%, 65% and 75% MeCN in 20 mM ammonium acetate. Peak capacity was calculated only for the conditions where chromatographic separation was achieved.

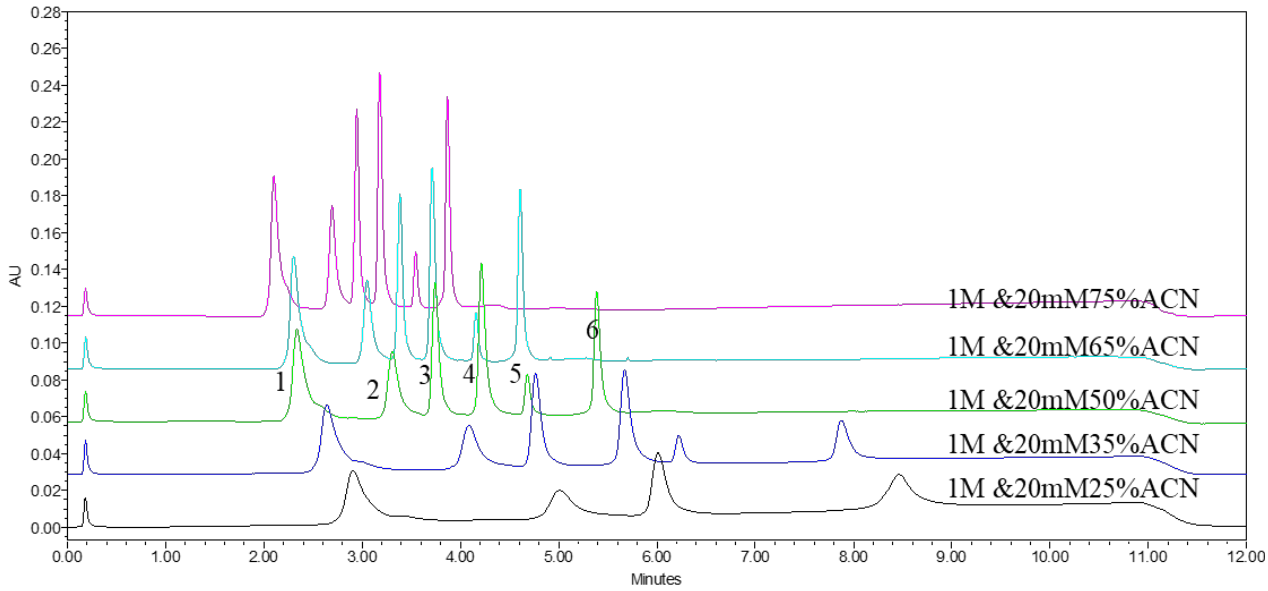

Figure 4.12. PolyPENTYL A column with 1000 Å and 2 µm particle size: Impact of MeCN percentage on the selectivity (MP A: 1 M NH_4OAc at pH 7.0 and MP B: 20 mM NH_4OAc with different percentages of MeCN with an aqueous pH 7.0).

Table 4.6. Comparison of chromatograms with 1 M ammonium acetate as mobile phase A and different percentages of MeCN with 20 mM ammonium acetate as Mobile phase B using PolyPENTYL A column with 1000 Å and 2 µm column.

Mobile phase composition	Peak name	Resolution	Retention Factor	Selectivity	Retention Factor
1M and 20mM with 35%MeCN	mAb1	NA	12.9	NA	82
	mAb2	5.21	20.5	1.58	
	NIST	2.98	24.1	1.17	
	mAb4	4.96	28.8	1.20	
	mAb5	3.41	31.7	1.10	
	mAb6	9.03	40.4	1.27	
1M and 20mM with50%MeCN	mAb1	NA	11.3	NA	121
	mAb2	4.82	16.4	1.44	
	NIST	2.77	18.7	1.14	
	mAb4	3.81	21.2	1.13	
	mAb5	4.13	23.6	1.11	
	mAb6	6.5	27.3	1.16	
1M and 20mM with 65%MeCN	mAb1	NA	11.1	NA	154
	mAb2	4.72	15.1	1.35	
	NIST	2.69	16.8	1.11	
	mAb4	3.33	18.5	1.10	
	mAb5	4.94	20.9	1.12	
	mAb6	5.22	23.3	1.11	
1M and 20mM with 75%MeCN	mAb1	NA	10.1	NA	184
	mAb2	4.57	13.2	1.30	
	NIST	2.45	14.5	1.10	
	mAb4	2.83	15.7	1.08	
	mAb5	4.72	17.7	1.12	
	mAb6	4.36	19.4	1.10	

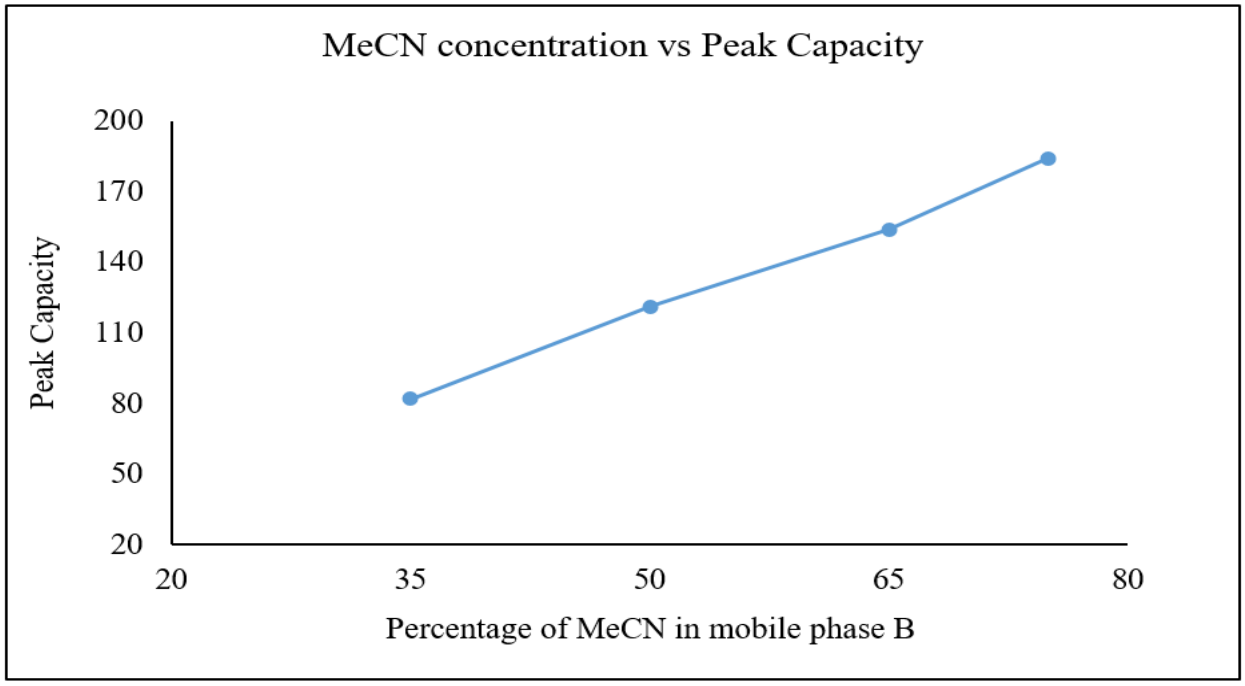

Figure 4.13. PolyPENTYL A column with 1000 Å and 2 µm particle size: Impact of MeCN percentage on peak capacity (MP A: 1 M NH_4OAc at pH 7.0 and MP B: 20 mM NH_4OAc with different percentages of MeCN with an aqueous pH 7.0).

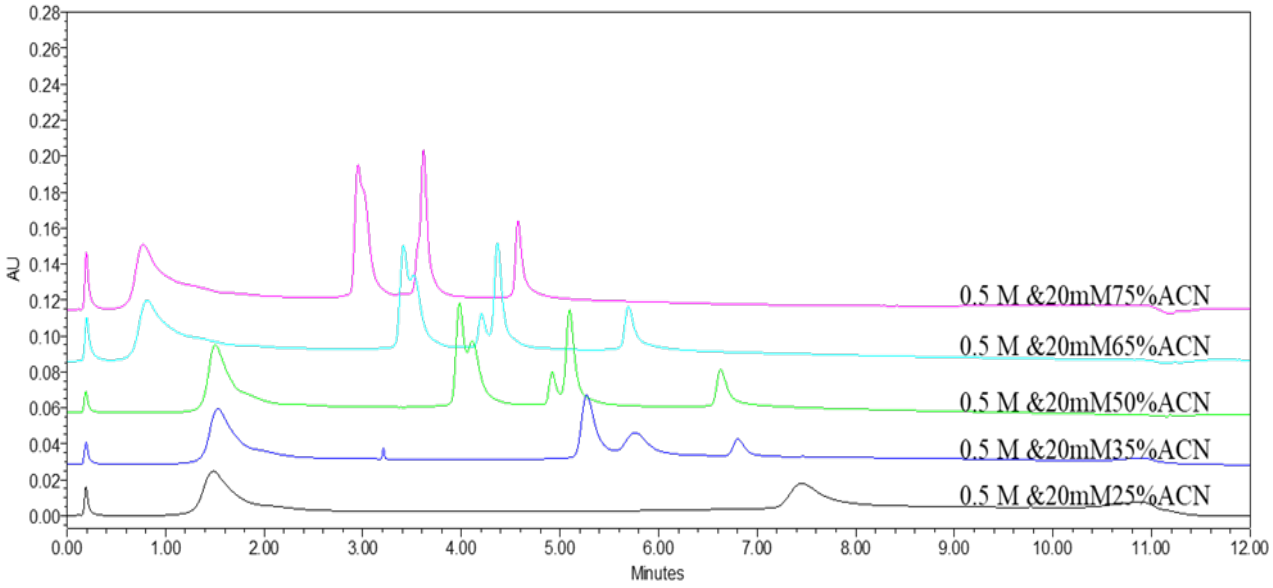

Figure 4.14. PolyPENTYL A column with 1000 Å and 2 µm particle size: Impact of MeCN percentage on the selectivity (MP A: 0.5 M NH_4OAc at pH 7.0 and MP B: 20 mM NH_4OAc with different percentages of MeCN with an aqueous pH 7.0)

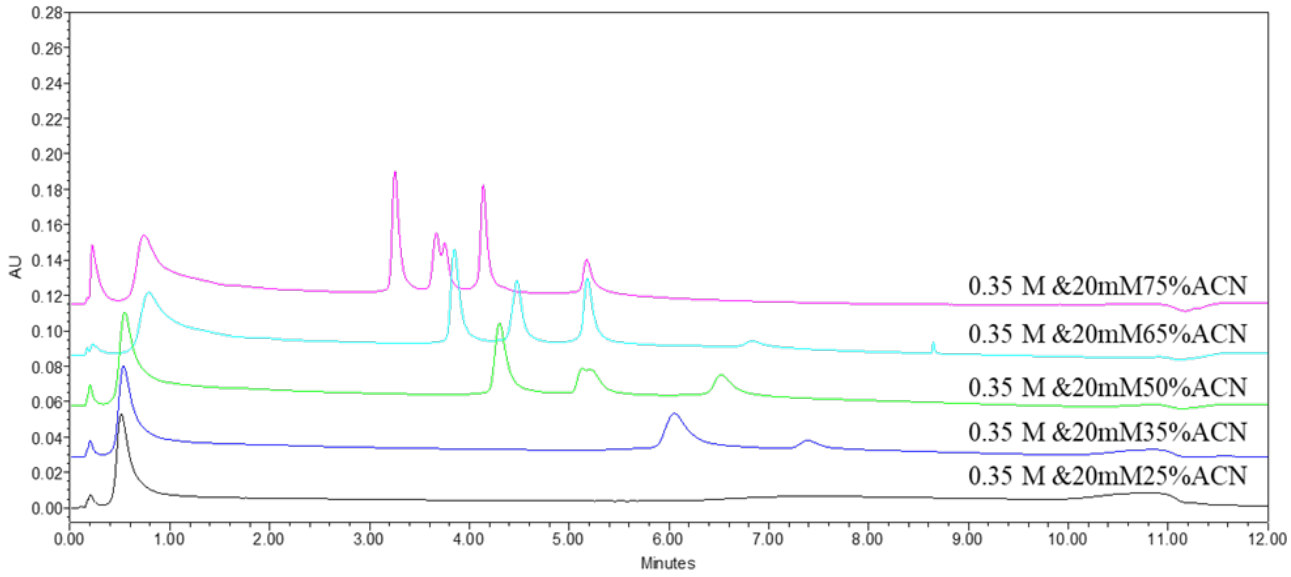

Figure 4.15. PolyPENTYL A column with 1000 Å and 2 µm particle size: Impact of MeCN percentage on the selectivity using (MP A: 0.35 M NH_4OAc at pH 7.0 and MP B: 20 mM NH_4OAc with different percentages of MeCN with an aqueous pH 7.0).

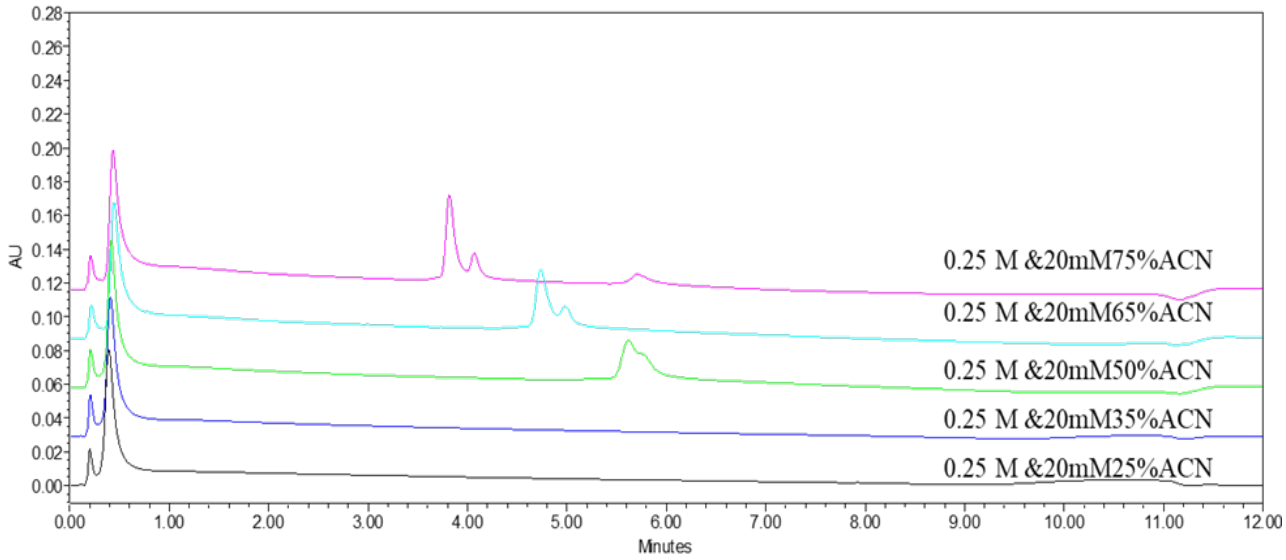

Figure 4.16. PolyPENTYL A column with 1000 Å and 2 µm particle size: Impact of MeCN percentage on the selectivity (MP A: 0.25 M NH_4OAc at pH 7.0 and MP B: 20 mM NH_4OAc with different percentages of MeCN with an aqueous pH 7.0).

4.3.3 PolyPENTYL A column with 1500 Å pore size with 3 µm particle size

The selectivity of PolyPENTYL A column with 1500 Å with 3 µm particle size was not adequate in 1 M with 25% MeCN in 20 mM ammonium acetate, however, as the organic concentration increased to 35% and above, resolution of the peaks increased (Figure 4.17) as did peak capacity (Figure 4.18). 0.5 M salt concentration did not provide acceptable separation (Figure 4.19) with an increase of MeCN that was used in this research. However, 0.35 M as mobile phase A with 50%, 65% and 75% MeCN as mobile phase B showed early elution of peak 1 but provided good chromatographic performance demonstrating these conditions can be used for chromatographic separation (Figure 4.20). Resolution decreased as the organic content in mobile phase B increased due to improved peak capacity (Figure 4.21). Surprisingly, 0.25 M in combination with 65% and 75% MeCN displayed adequate elution (Figure 4.22) proving that this column can be one of the choices to achieve chromatographic separation at low salt concentrations. Peak capacity was calculated only for the conditions where chromatographic separation was achieved.

In this study it appeared that there was a change in the selectivity of mAbs at comparatively low starting salt concentrations such as 0.35 M and 0.25 M. The hypothesis for this finding was that the concentration of salt plays a significant role in disrupting hydration shell around the biomolecule and it is highly dependent on the properties of the biomolecule. The thickness of the hydration shell can contribute to the changes in the strength of hydrophobic interactions between the molecule and the stationary phase due to the exposure of more hydrophobic patches resulting in an increase in the adsorption for some mAbs. Pore size is a well-established column parameter and increase in pore size decreases the surface area. The pore size of these columns are large enough to accommodate biomolecule diffusion and the results

from the experiments do not provide details to predict the impact of the pore size on the chromatographic separation. The influence and the interdependency of low salt concentration on increased pore size is not completely understood and yet to be determined.

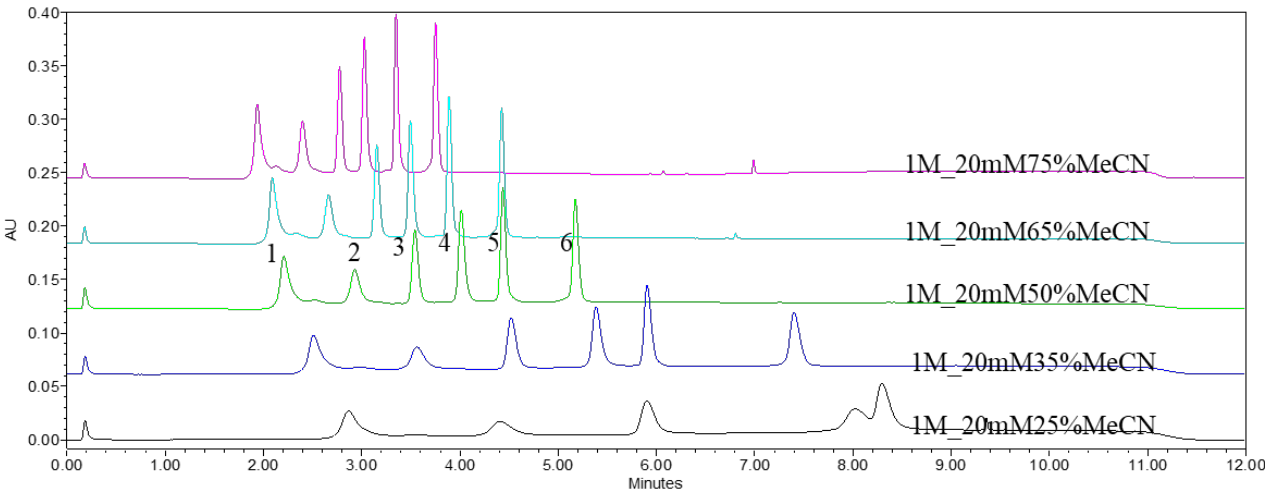

Figure 4.17. PolyPENTYL A column with 1500 Å and 3 µm particle size: Impact of MeCN percentage on the selectivity (MP A: 1 M NH_4OAc at pH 7.0 and MP B: 20 mM NH_4OAc with different percentages of MeCN with an aqueous pH 7.0).

Table 4.7. Comparison of chromatograms with 1 M ammonium acetate as mobile phase A and different percentages of MeCN with 20 mM ammonium acetate as Mobile phase B using PolyPENTYL A column with 3 µm, 1500 Å pore size.

Mobile phase composition	Peak name	Resolution	Retention Factor	Selectivity	Retention Factor
1M and 20mM with 35%MeCN	mAb1	NA	12.9	NA	94
	mAb2	4.48	18.8	1.44	
	NIST	4.66	24.1	1.28	
	mAb4	5.24	28.9	1.20	
	mAb5	3.47	31.8	1.10	
	mAb6	9.17	40.1	1.26	
1M and 20mM with50%MeCN	mAb1	NA	11.3	NA	134
	mAb2	4.25	15.3	1.35	
	NIST	4.24	18.7	1.22	
	mAb4	4.05	21.3	1.14	
	mAb5	3.97	23.7	1.11	
	mAb6	7.13	27.8	1.17	
1M and 20mM with 65%MeCN	mAb1	NA	10.6	NA	164
	mAb2	4.17	13.8	1.29	
	NIST	4.12	16.5	1.19	
	mAb4	3.57	18.4	1.11	
	mAb5	4.40	20.6	1.12	
	mAb6	6.15	23.6	1.14	
1M and 20mM with 75%MeCN	mAb1	NA	9.78	NA	189
	mAb2	3.88	12.3	1.26	
	NIST	3.70	14.4	1.17	
	mAb4	3.03	15.8	1.10	
	mAb5	4.18	17.6	1.11	
	mAb6	5.31	19.8	1.13	

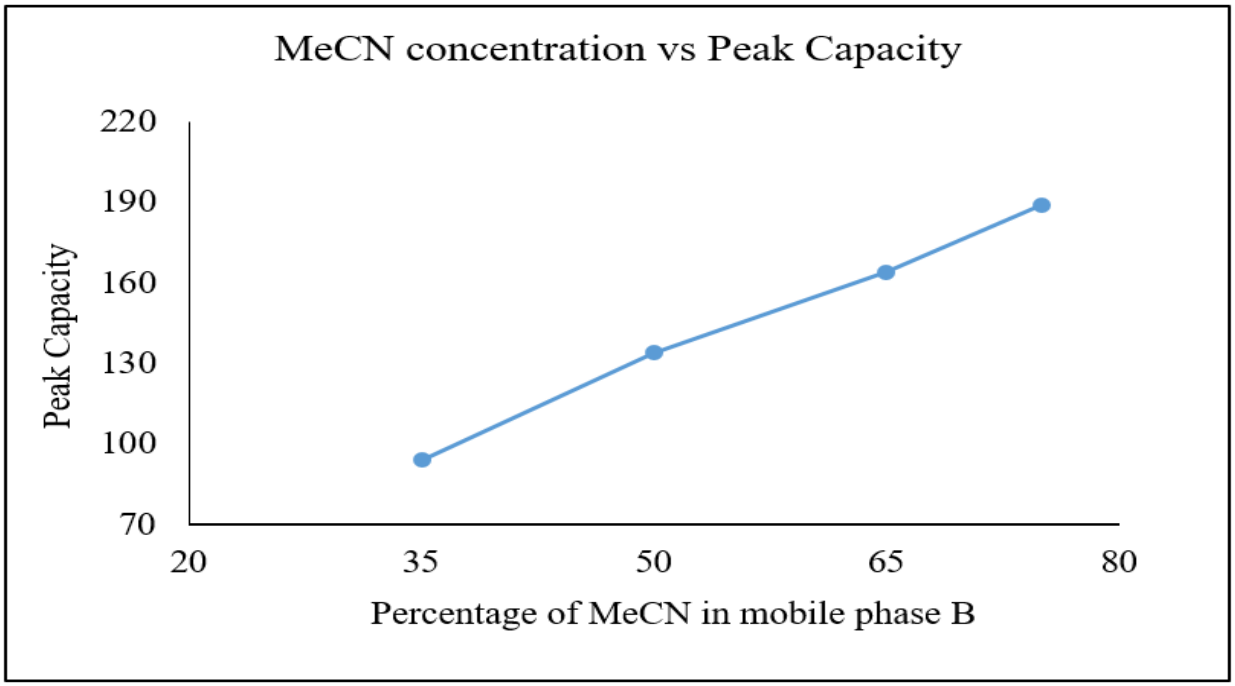

Figure 4.18. PolyPENTYL A column with 1500 Å and 3 µm particle size: Impact of MeCN percentage on peak capacity (MP A: 1 M NH_4OAc at pH 7.0 and MP B: 20 mM NH_4OAc with different percentages of MeCN with an aqueous pH 7.0).

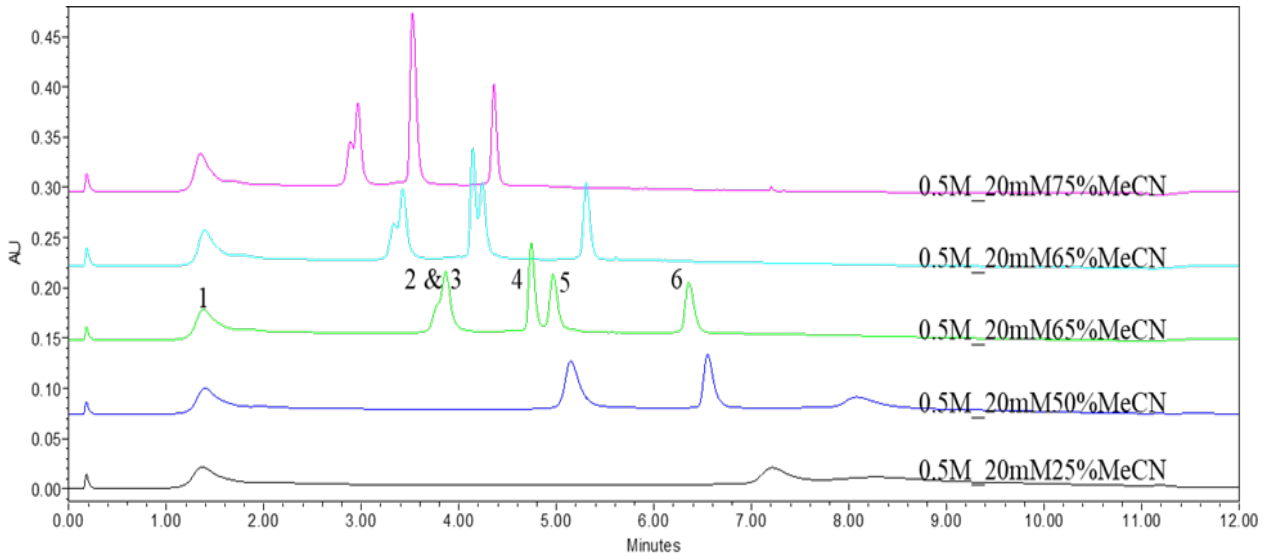

Figure 4.19. PolyPENTYL A column with 1500 Å and 3 µm particle size: Impact of MeCN percentage on the selectivity (MP A: 0.5 M NH_4OAc at pH 7.0 and MP B: 20 mM NH_4OAc with different percentages of MeCN with an aqueous pH 7.0).

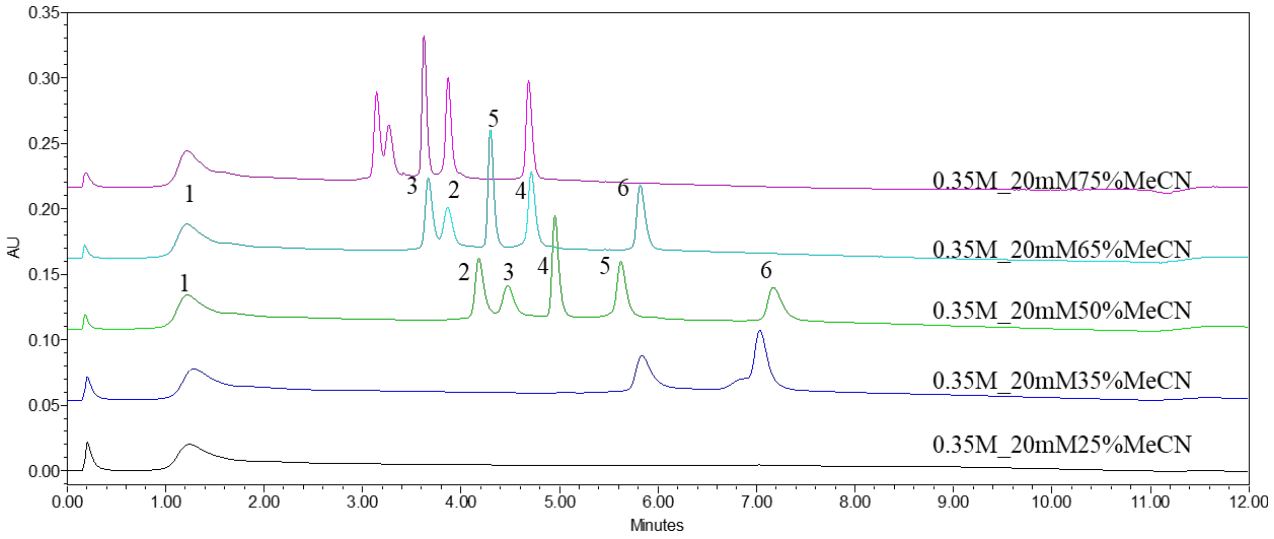

Figure 4.20. PolyPENTYL A column with 1500 Å and 3 µm particle size: Impact of MeCN percentage on the selectivity (MP A: 0.35 M NH_4OAc at pH 7.0 and MP B: 20 mM NH_4OAc with different percentages of MeCN with an aqueous pH 7.0.

Table 4.8. Comparison of chromatograms with 0.35 M ammonium acetate as mobile phase A and different percentages of MeCN with 20 mM ammonium acetate as Mobile phase B using PolyPENTYL A column with 3 µm , 1500 Å pore size.

Mobile phase composition	Peak name	Resolution	Retention Factor	Selectivity	Retention Factor
0.35M and 20mM with 50%MeCN	mAb1	NA	4.81	NA	75
	mAb2	8.68	18.9	3.74	
	NIST	1.68	20.3	1.07	
	mAb4	2.99	22.6	1.11	
	mAb5	4.5	25.8	1.14	
	mAb6	7.05	33.1	1.28	
0.35M and 20mM with 65%MeCN	mAb1	NA	4.81	NA	91
	mAb2	4.25	16.5	3.28	
	NIST	4.24	17.4	1.06	
	mAb4	4.05	19.5	1.12	
	mAb5	3.97	21.4	1.10	
	mAb6	7.13	26.7	1.24	
0.35M and 20mM with 75%MeCN	mAb1	NA	4.81	NA	108
	mAb2	7.00	14.0	2.79	
	NIST	0.97	14.6	1.04	
	mAb4	3.09	16.3	1.11	
	mAb5	2.53	17.4	1.07	
	mAb6	7.57	21.3	1.21	

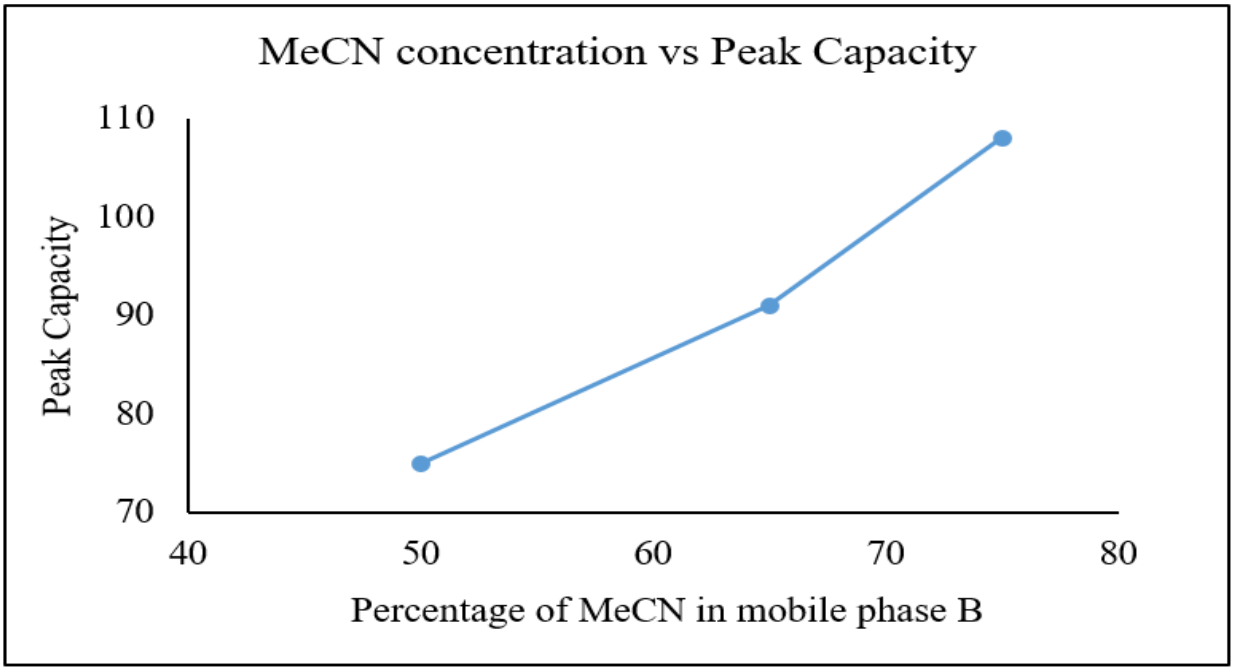

Figure 4.21. PolyPENTYL A column with 1500 Å and 3 µm particle size: Impact of MeCN percentage on peak capacity (MP A: 0.35 M NH_4OAc at pH 7.0 and MP B: 20 mM NH_4OAc with different percentages of MeCN with an aqueous pH 7.0).

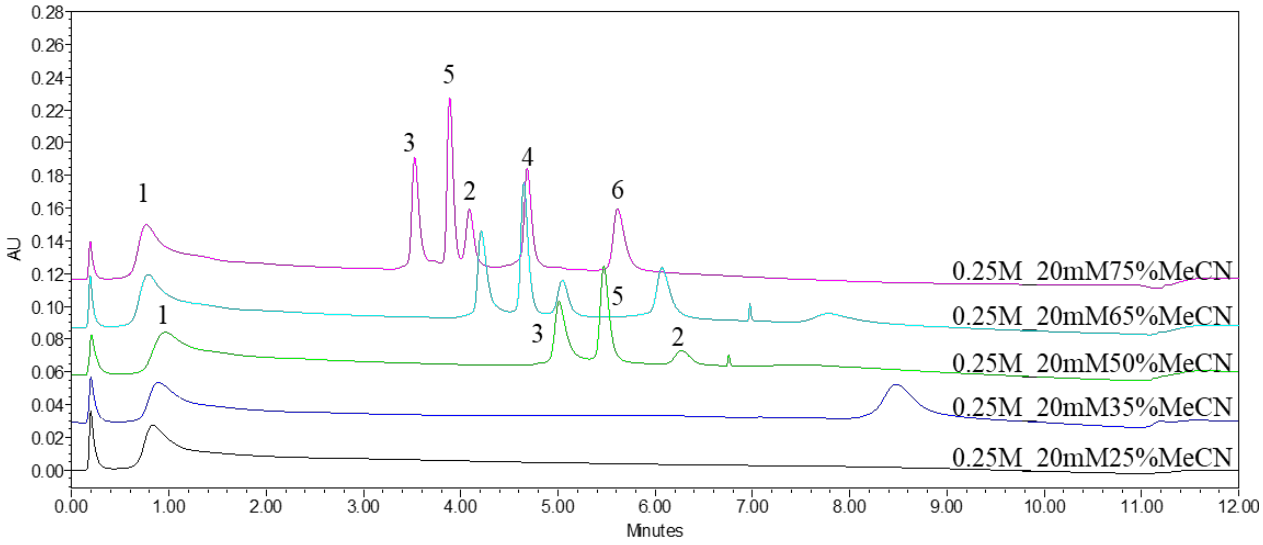

Figure 4.22. PolyPENTYL A column with 1500 Å and 3 µm particle size: Impact of MeCN percentage on the selectivity (MP A: 0.25 M NH_4OAc at pH 7.0 and MP B: 20 mM NH_4OAc with different percentages of MeCN with an aqueous pH 7.0).

Table 4.9. Comparison of chromatograms with 0.25 M ammonium acetate as mobile phase A and 65% and 75% of MeCN with 20 mM ammonium acetate as Mobile phase B using PolyPENTYL A column with 3 µm, 1500 Å pore size.

Mobile phase composition	Peak name	Resolution	Retention Factor	Selectivity	Retention Factor
0.25M and 20mM with 65%MeCN	mAb1	NA	2.91	NA	60
	mAb2	12.06	20.1	6.35	
	NIST	2.82	22.3	1.11	
	mAb4	2.38	24.3	1.09	
	mAb5	4.71	29.4	1.21	
	mAb6	3.86	37.9	1.29	
0.25M and 20mM with 75%MeCN	mAb1	NA	2.80	NA	95
	mAb2	10.51	16.7	5.43	
	NIST	2.95	18.5	1.11	
	mAb4	1.59	19.5	1.05	
	mAb5	4.23	22.4	1.15	
	mAb6	5.25	27.1	1.20	

4.3.4 PolyPENTYL A column with 1500 Å pore size with 2 µm particle size

PolyPENTYL A column with 1500 Å with 2 µm demonstrated adequate selectivity using 1M ammonium acetate as mobile phase A in combination with all five concentrations of MeCN in 20mM ammonium acetate as mobile phase B that were evaluated in this study (Figure 4.23). Every mobile phase condition demonstrated sharper peaks, high resolution, and selectivity with high peak capacity (Figure 4.24) compared to other columns that were evaluated in this study. Separation in 0.5 M with different percentages MeCN resulted in co-elution of peaks as the organic concentration increased from 25% to 75% (Figure 4.25). 0.35 M as mobile phase A with 50% MeCN and higher concentration of organic in mobile phase B showed very good selectivity demonstrating an option to achieve adequate separation under low salt conditions (Figure 4.26) with an enhanced peak capacity (Figure 4.27). An interesting observation was made in the presence of mobile phase A containing 0.25 M with mobile phase B with 75% MeCN. This condition demonstrated the separation of variants for mAb1 and mAb2. If conditions are optimized, this combination can be exploited to separate minor variants of a mAb (Figure 4.28). Peak capacity was calculated only for the conditions where chromatographic separation was achieved.

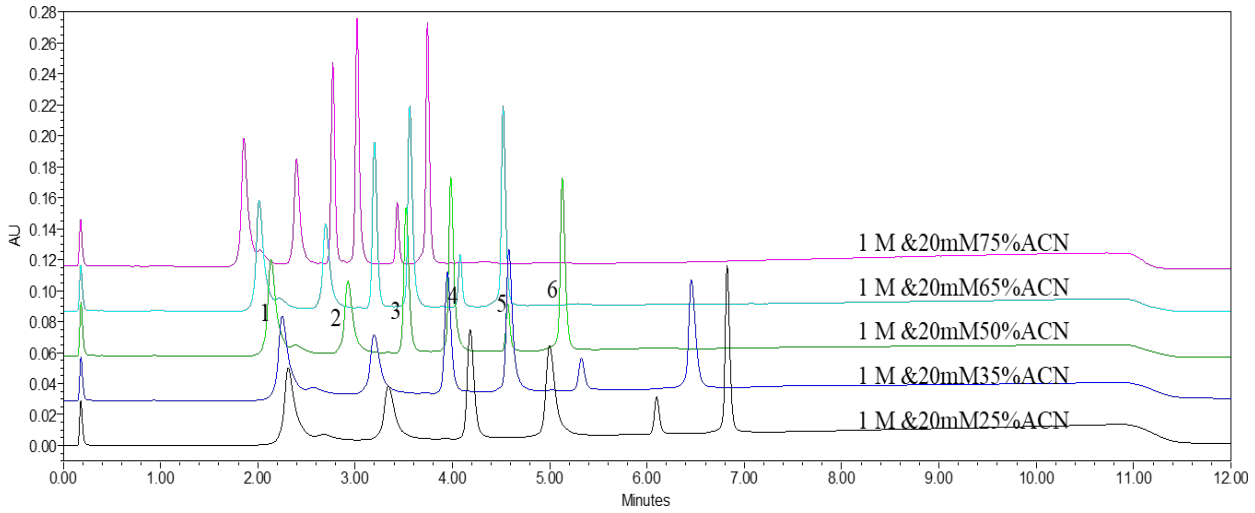

Figure 4.23. PolyPENTYL A column with 1500 Å and 2 µm particle size: Impact of MeCN percentage on the selectivity (MP A: 1 M NH_4OAc at pH 7.0 and MP B: 20 mM NH_4OAc with different percentages of MeCN with an aqueous pH 7.0).

Table 4.10. Comparison of chromatograms with 1 M ammonium acetate as mobile phase A and 65% and 75% of MeCN with 20 mM ammonium acetate as Mobile phase B using PolyPENTYL A column with 2 µm, 1500 Å pore size.

Mobile phase composition	Peak name	Resolution	Retention Factor	Selectivity	Retention Factor
1M and 20mM with 25%MeCN	mAb1	NA	11.8	NA	123
	mAb2	5.37	17.6	1.48	
	NIST	5.14	22.2	1.26	
	mAb4	5.41	24.0	1.20	
	mAb5	8.30	32.9	1.23	
	mAb6	7.97	36.9	1.12	
1M and 20mM with35%MeCN	mAb1	NA	11.5	NA	128
	mAb2	5.36	16.8	1.45	
	NIST	5.07	20.9	1.25	
	mAb4	5.41	24.4	1.17	
	mAb5	6.64	28.6	1.17	
	mAb6	9.43	34.9	1.22	
1M and 20mM with 50%MeCN	mAb1	NA	10.9	NA	159
	mAb2	5.32	15.3	1.40	
	NIST	4.80	18.6	1.22	
	mAb4	4.67	21.1	1.14	
	mAb5	6.75	24.4	1.15	
	mAb6	6.69	27.5	1.13	
1M and 20mM with 65%MeCN	mAb1	NA	10.2	NA	161
	mAb2	5.34	13.9	1.37	
	NIST	4.66	16.8	1.20	
	mAb4	4.20	18.8	1.12	
	mAb5	6.60	21.7	1.15	
	mAb6	5.81	24.1	1.11	
1M and 20mM with 75%MeCN	mAb1	NA	9.3	NA	215
	mAb2	5.00	12.3	1.32	
	NIST	4.16	14.4	1.17	
	mAb4	3.45	15.8	1.10	
	mAb5	6.25	18.1	1.14	
	mAb6	4.82	19.8	1.09	

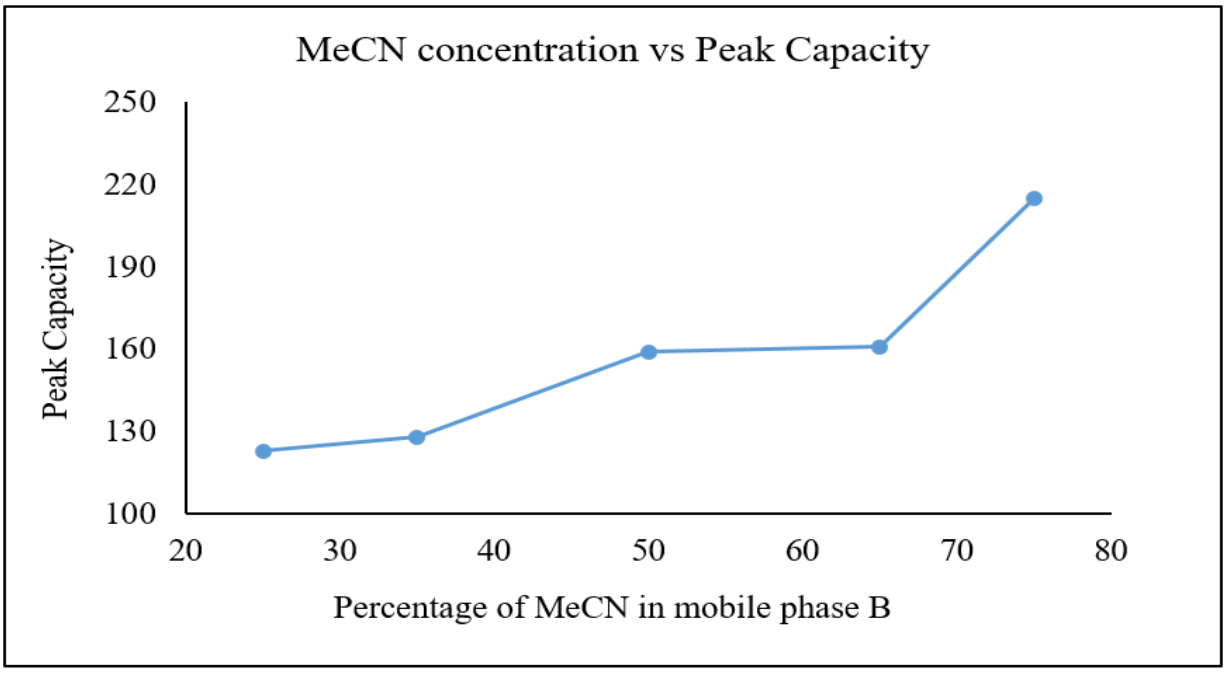

Figure 4.24. PolyPENTYL A column with 1500 Å and 2 µm particle size: Impact of MeCN percentage on peak capacity (MP A: 1 M NH_4OAc at pH 7.0 and MP B: 20 mM NH_4OAc with different percentages of MeCN with an aqueous pH 7.0.

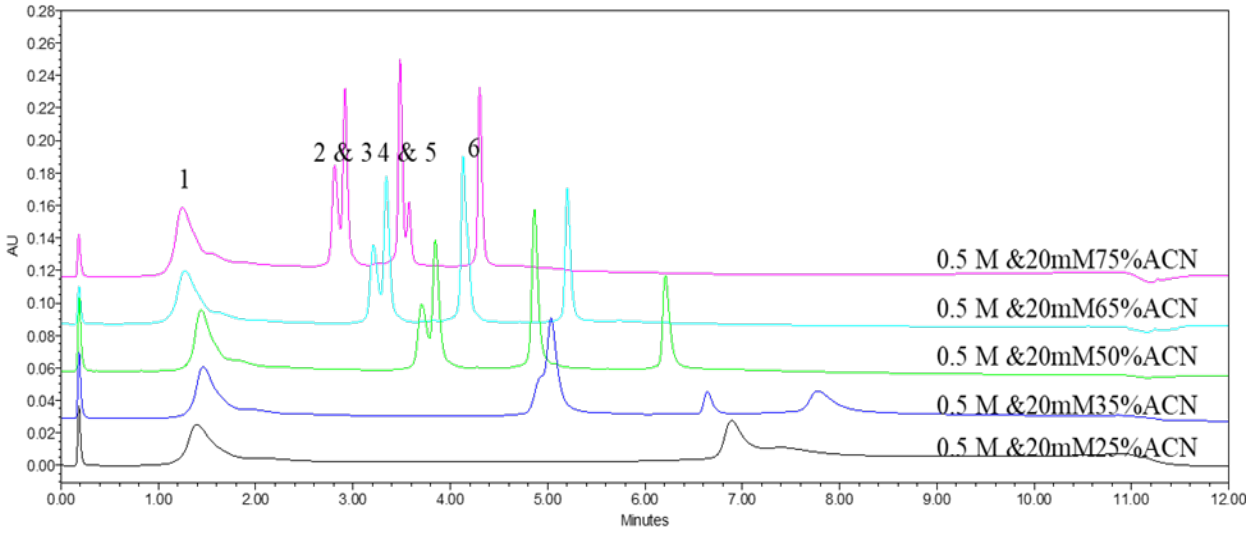

Figure 4.25. PolyPENTYL A column with 1500 Å and 2 µm particle size: Impact of MeCN percentage on the selectivity (MP A: 0. 5 M NH_4OAc at pH 7.0 and MP B: 20 mM NH_4OAc with different percentages of MeCN with an aqueous pH 7.0.

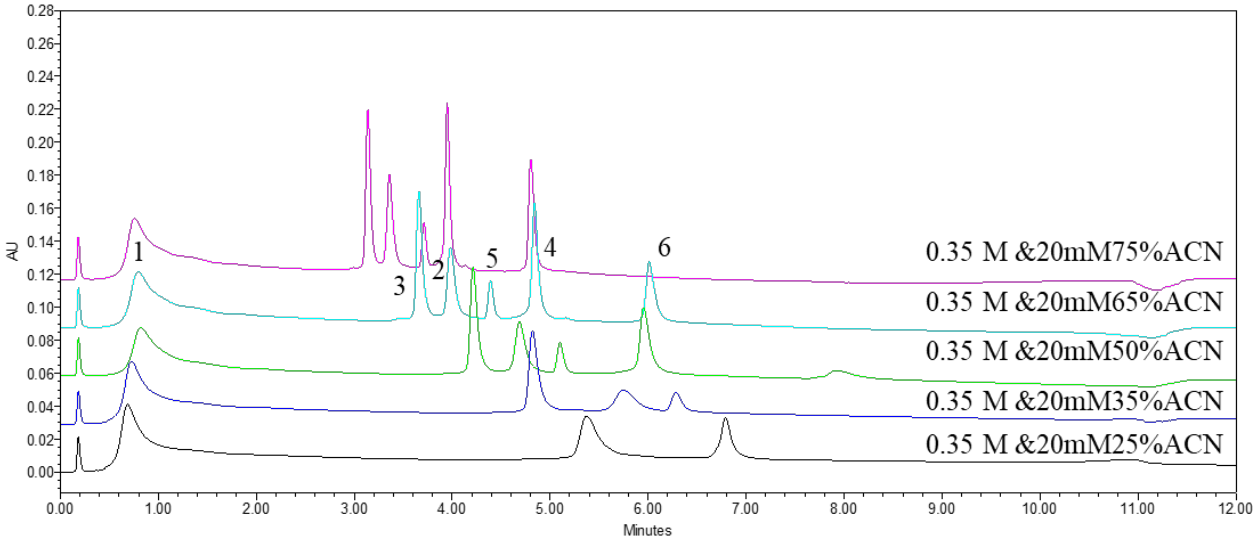

Figure 4.26. PolyPENTYL A column with 1500 Å and 2 µm particle size: Impact of MeCN percentage on the selectivity (MP A: 0. 35 M NH_4OAc at pH 7.0 and MP B: 20 mM NH_4OAc with different percentages of MeCN with an aqueous pH 7.0).

Table 4.11. Comparison of chromatograms with 0.35 M ammonium acetate as mobile phase A and 50%, 65% and 75% of MeCN with 20 mM ammonium acetate as Mobile phase B using PolyPENTYL A column with 2 µm, 1500 Å pore size.

Mobile phase composition	Peak name	Resolution	Retention Factor	Selectivity	Retention Factor
0.35M and 20mM with 50%MeCN	mAb1	NA	3.32	NA	74
	mAb2	12.73	21.2	6.08	
	NIST	2.97	23.7	1.12	
	mAb4	2.70	25.8	1.09	
	mAb5	5.68	30.3	1.17	
	mAb6	6.36	40.7	1.34	
0.35M and 20mM with 65%MeCN	mAb1	NA	3.16	NA	105
	mAb2	11.29	18.3	5.47	
	NIST	2.58	19.9	1.09	
	mAb4	3.41	22.1	1.11	
	mAb5	4.16	24.5	1.11	
	mAb6	8.48	30.6	1.25	
0.35M and 20mM with 75%MeCN	mAb1	NA	3.00	NA	128
	mAb2	10.46	15.5	4.92	
	NIST	2.21	16.7	1.07	
	mAb4	3.69	18.5	1.11	
	mAb5	2.85	19.8	1.07	
	mAb6	8.89	24.3	1.22	

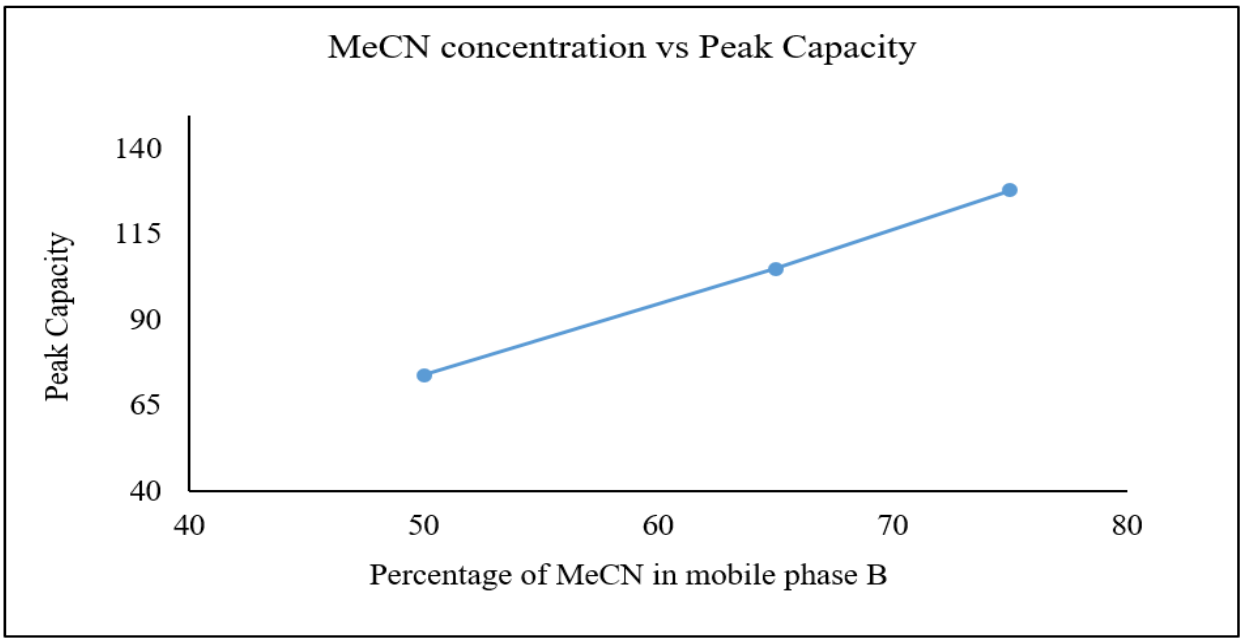

Figure 4.27. PolyPENTYL A column with 1500 Å and 2 µm particle size: Impact of MeCN percentage on peak capacity (MP A: 0.35 M NH_4OAc at pH 7.0 and MP B: 20 mM NH_4OAc with different percentages of MeCN with an aqueous pH 7.0).

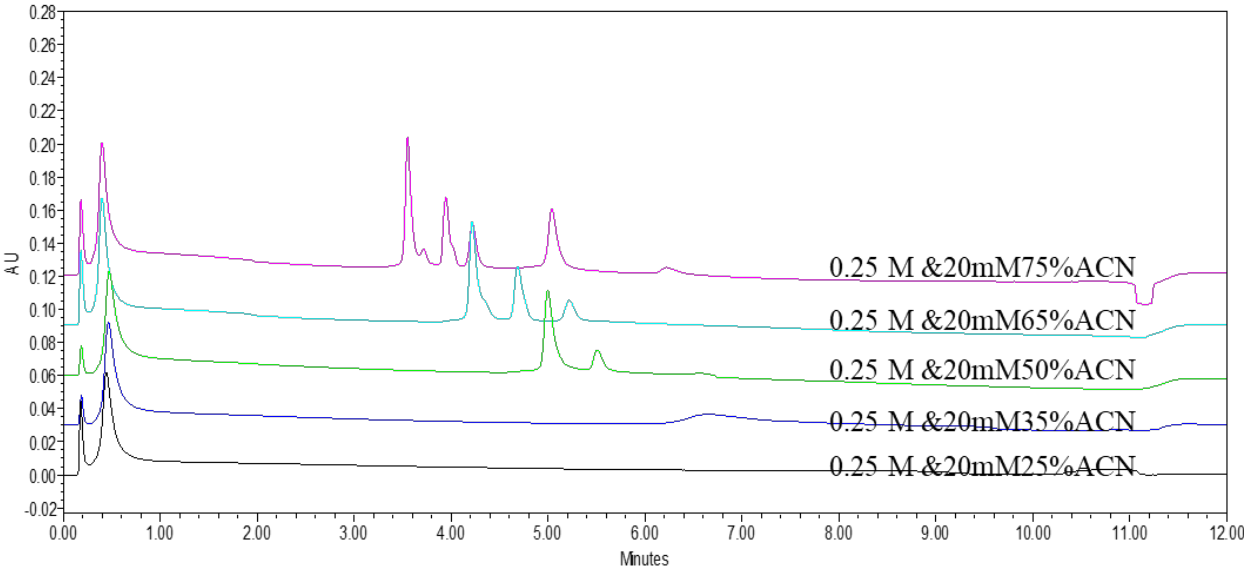

Figure 4.28. PolyPENTYL A column with 1500 Å and 2 µm particle size: Impact of MeCN percentage on the selectivity (MP A: 0. 25 M NH_4OAc at pH 7.0 and MP B: 20 mM NH_4OAc with different percentages of MeCN with an aqueous pH 7.0).

4.3.5 PolyBUTYL A column with 1000 Å pore size with 3 µm particle size

PolyBUTYL A column with 1000 Å with 3 µm particle size showed very similar selectivity to PolyPENTYL A column (control) with the same column parameters using 1M ammonium acetate as mobile phase A and 20mM ammonium acetate with different concentrations of MeCN as mobile phase B. Mobile phase B with 25% MeCN did not elute all the peaks but selectivity improved significantly with 35% MeCN. With 1M salt concentration, adequate chromatographic separation was achieved in a combination with a MeCN concentration of 50% and above (Figure 4.29) showing an increase in the peak capacity (Figure 4.30). Results of the chromatographic parameters are listed in Table 4.12. Mobile phase A with 0.5 M and mobile phase B with different percentages MeCN demonstrated no elution or co-elution of peaks (Figure 4.31). However, and the unlike PolyPENTYL A column with same pore and particle size, butyl column with 1000 Å and 3 µm showed good selectivity and acceptable resolution using 0.35 M with 65% and 75% MeCN (Figure 4.32). This behavior is a good example to explain the importance of stationary phase alkyl chain length which plays a significant role it plays in obtaining good selectivity in HHIC separation. Similar to 1500 Å with 2 µm column, this column also showed the separation of variants for mAb4 in the presence of mobile phase A containing 0.25 M with mobile phase B with 75% MeCN. This combination of stationary and mobile phase conditions can be helpful to separate minor variants of a mAb (Figure 4.33). Peak capacity was calculated only for the conditions where chromatographic separation was achieved.

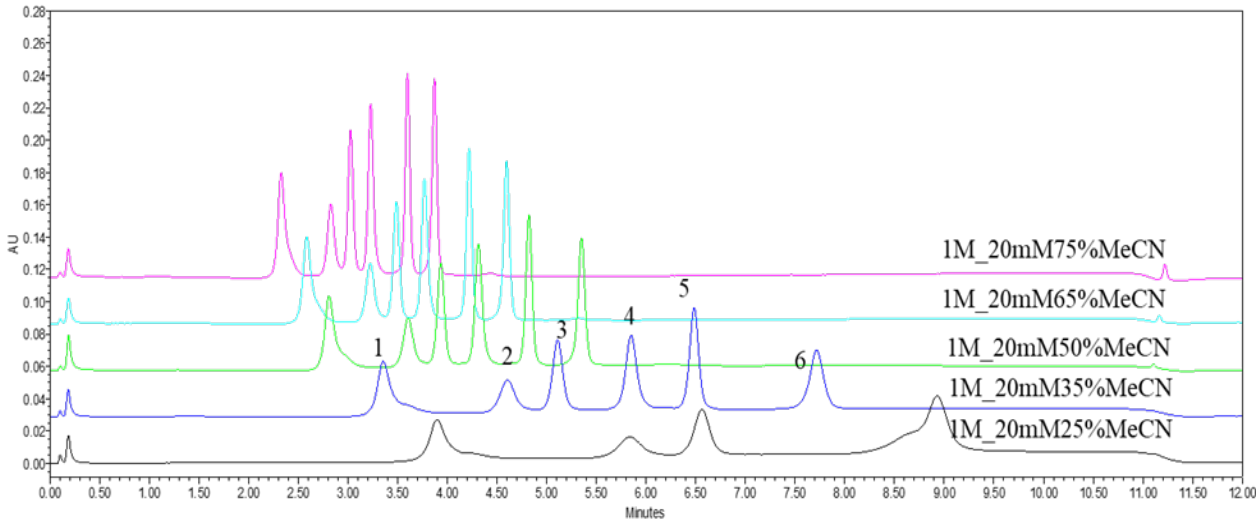

Figure 4.29. PolyBUTYL A column with 1000 Å and 3 µm particle size: Impact of MeCN percentage on the selectivity (MP A: 1 M NH_4OAc at pH 7.0 and MP B: 20 mM NH_4OAc with different percentages of MeCN with an aqueous pH 7.0).

Table 4.12. Comparison of chromatograms with 1 M ammonium acetate as mobile phase A and 35%, 50%, 65% and 75% of MeCN with 20 mM ammonium acetate as Mobile phase B using PolyBUTYL A column with 3 µm, 1000 Å pore size.

Mobile phase composition	Peak name	Resolution	Retention Factor	Selectivity	Retention Factor
1M and 20mM with 35%MeCN	mAb1	NA	29.5	NA	79
	mAb2	4.88	40.8	1.39	
	NIST	2.17	45.5	1.11	
	mAb4	3.65	52.2	1.15	
	mAb5	3.30	57.9	1.11	
	mAb6	5.69	69.2	1.19	
1M and 20mM with50%MeCN	mAb1	NA	24.5	NA	118
	mAb2	4.34	31.7	1.30	
	NIST	2.06	34.7	1.10	
	mAb4	2.77	38.2	1.10	
	mAb5	3.94	42.8	1.12	
	mAb6	4.20	47.6	1.11	
1M and 20mM with 65%MeCN	mAb1	NA	22.5	NA	131
	mAb2	4.06	28.3	1.26	
	NIST	1.94	30.7	1.09	
	mAb4	2.40	33.3	1.08	
	mAb5	4.02	37.4	1.12	
	mAb6	3.48	40.8	1.09	
1M and 20mM with 75%MeCN	mAb1	NA	20.2	NA	164
	mAb2	3.79	24.7	1.23	
	NIST	1.77	26.5	1.07	
	mAb4	2.06	28.4	1.07	
	mAb5	3.94	31.7	1.12	
	mAb6	2.98	34.4	1.08	

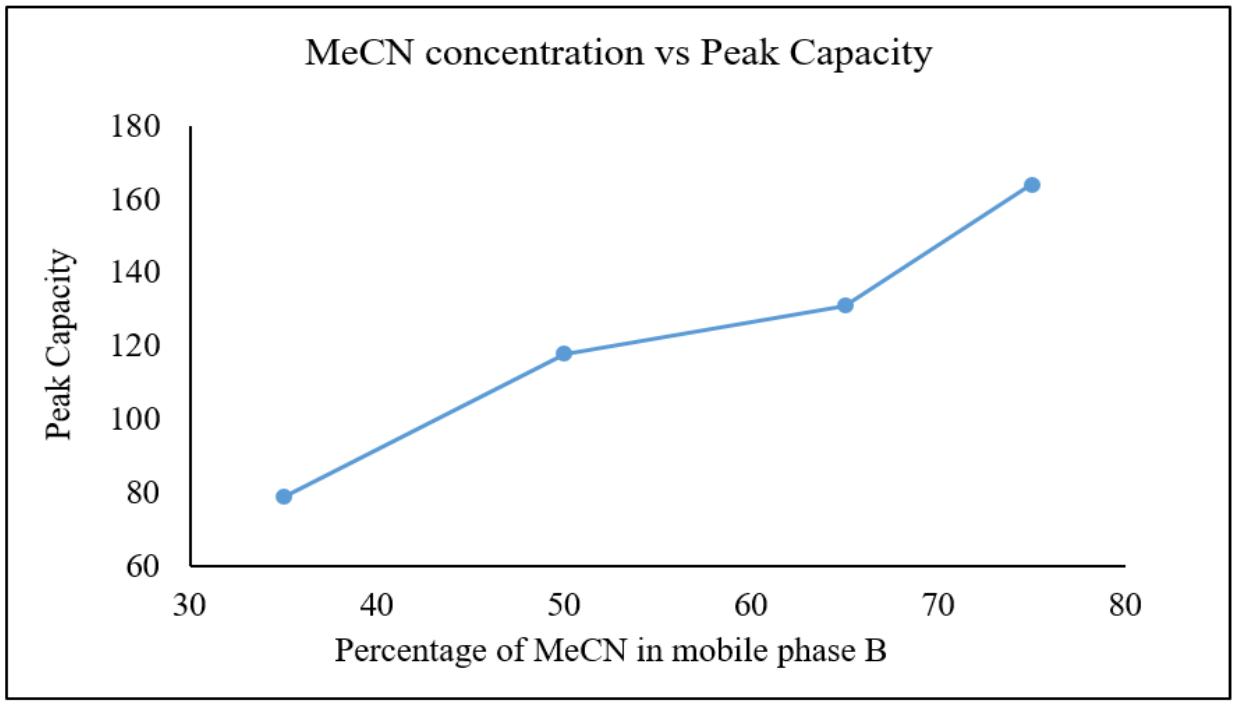

Figure 4.30. PolyBUTYL A column with 1000 Å and 3 µm particle size: Impact of MeCN percentage on peak capacity (MP A: 1 M NH_4OAc at pH 7.0 and MP B: 20 mM NH_4OAc with different percentages of MeCN with an aqueous pH 7.0).

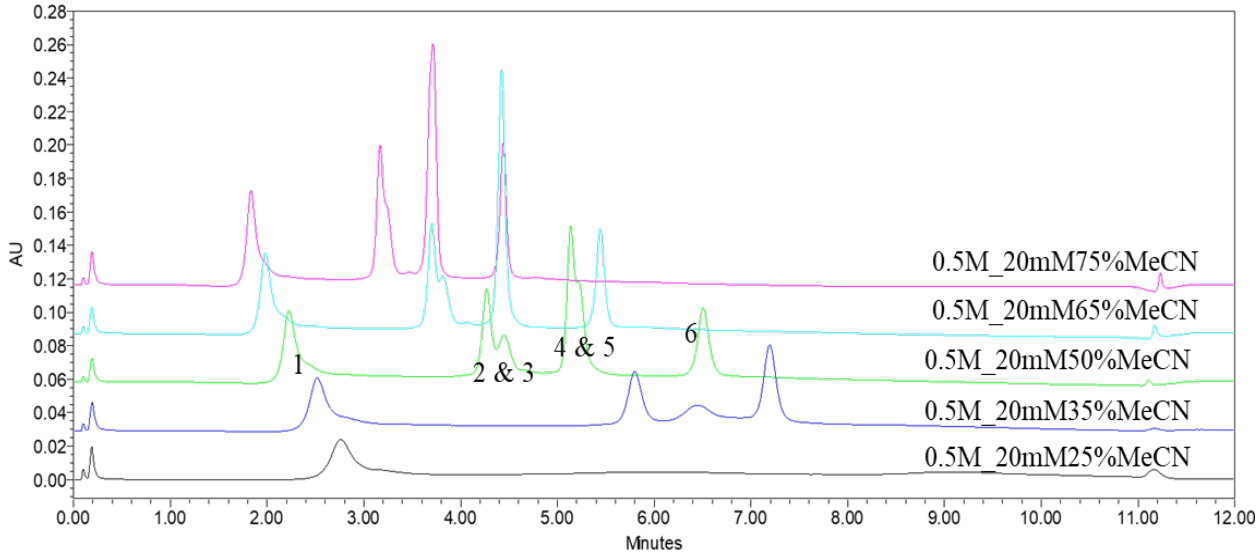

Figure 4.31. PolyBUTYL A column with 1000 Å and 3 µm particle size: Impact of MeCN percentage on the selectivity (MP A: 0.5 M NH_4OAc at pH 7.0 and MP B: 20 mM NH_4OAc with different percentages of MeCN with an aqueous pH 7.0).

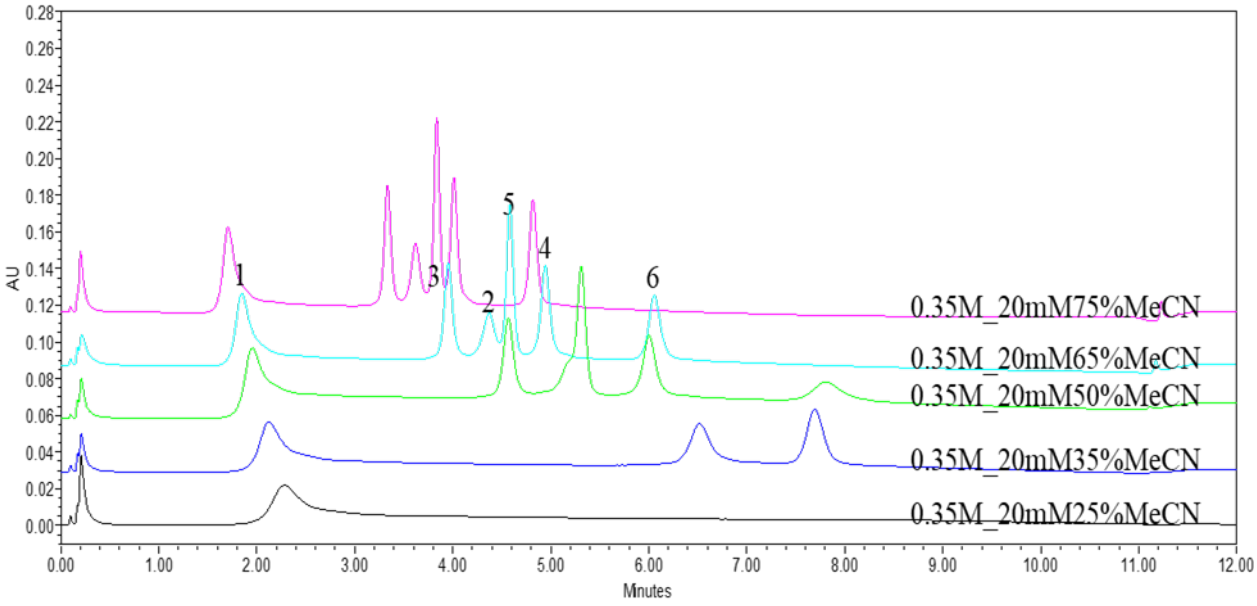

Figure 4.32. PolyBUTYL A column with 1000 Å and 3 µm particle size: Impact of MeCN percentage on the selectivity (MP A: 0.35 M NH_4OAc at pH 7.0 and MP B: 20 mM NH_4OAc with different percentages of MeCN with an aqueous pH 7.0).

Table 4.13. Comparison of chromatograms with 0.35 M ammonium acetate as mobile phase A and 65% and 75% of MeCN with 20 mM ammonium acetate as Mobile phase B using PolyBUTYL A column with 3 µm, 1000 Å pore size.

Mobile phase composition	Peak name	Resolution	Retention Factor	Selectivity	Retention Factor
0.25M and 20mM with 65%MeCN	mAb1	NA	17.5	NA	37
	mAb2	9.68	39.0	2.23	
	NIST	2.08	42.7	1.11	
	mAb4	1.13	44.8	1.05	
	mAb5	2.33	48.5	1.08	
	mAb6	5.58	59.6	1.23	
0.25M and 20mM with 75%MeCN	mAb1	NA	16.1	NA	52
	mAb2	9.22	32.4	2.04	
	NIST	1.97	35.2	1.09	
	mAb4	1.58	37.4	1.06	
	mAb5	1.46	39.1	1.05	
	mAb6	5.86	47.2	1.21	

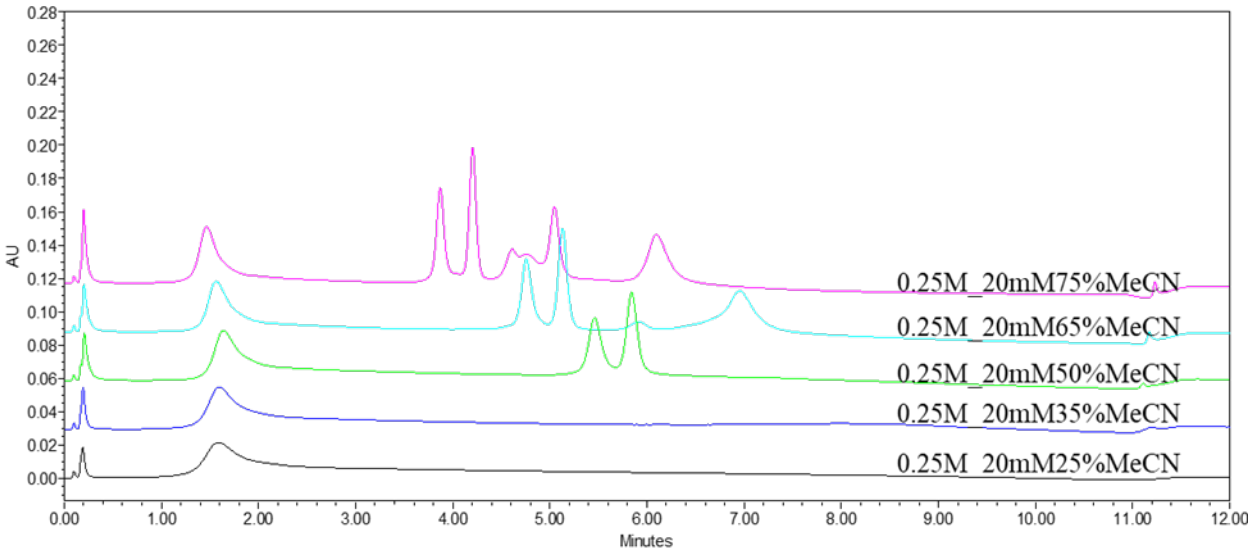

Figure 4.33. PolyBUTYL A column with 1000 Å and 3 µm particle size: Impact of MeCN percentage on the selectivity (MP A: 0.25 M NH_4OAc at pH 7.0 and MP B: 20 mM NH_4OAc with different percentages of MeCN with an aqueous pH 7.0).

Upon reviewing the above five columns, considerable selectivity differences were observed with respect to alkyl chain lengths, pore and particle sizes. In addition, there are some very important observations in the chromatographic separations which can open the doors to explore separate variants of biomolecules in HHIC, which has been a challenges thus far.

PolyPENTYL A column with 1000 Å with 2 and 3 µm particle sizes gave very similar selectivity with 1 M mobile phase A, but 3 µm showed selectivity with 0.5M which was not obtained using 2 µm column. When compared columns with same pore sizes but different particle sizes, columns with smaller particle size retained molecules much stronger resulting in poor recovery. Comparing both PolyPENTYL A column with 1500 Å pore size with 2 µm and 3 µm particle sizes, unlike 3 µm column, 2 µm particle size column was able to elute peaks with both 1 M in combination with 25% MeCN and 0.35 M with 50% to 75% MeCN. However, between 2 µm and 3 µm, 3 µm column provided adequate elution in 0.25 M in the presence of

65% and 75% MeCN. The reason for stronger retention in 2 µm for both 1000 Å and 1500 Å is the availability of a larger surface area for adsorption.

Experimental results demonstrated that PolyPENTYL A and PolyBUTYL A columns gave adequate separation using 1 M in combination with 35% and higher MeCN concentration in 20mM ammonium acetate. However, column with 1000 Å, 3 µm separated molecules using 0.5 M in the presence of 50% and 65% MeCN in mobile phase B. PolyPENTYL A 1500 Å and PolyBUTYL A 1000 Å columns with 3 um pore size were able to provide separation using 0.35 M with 50% and higher MeCN content. PolyPENTYL A column with 1500 Å with 2 µm pore size can separate molecules with 0.25 M with 75% MeCN in mobile phase B. Unlike any other conditions in this study, 0.25 M salt as the starting concentration, the 2 µm, 1500 Å PolyPENTYL A column and 3 µm, 1000 Å PolyBUTYL A column demonstrated two minor variant peaks. With these results it was clear that the variations in column parameters such as alkyl chain with different particle and pore sizes have a significant impact on the chromatographic separation. In addition, it was proven that salt concentration played a critical role and impacted the hydrophobic interactions by altering the selectivity of mAbs. Hence, it is highly beneficial to screen the stationary phase parameters to achieve chromatographic performance based on the separation requirements. It has been well established that increase in pore size will decrease the surface area, but in these columns, the difference in the surface area due to the changes in pore and particle size may not be significant. However, the difference in the pressure may contribute to subtle changes in the molecule conformation. If any there are any minor structural alterations in molecule's tertiary structure are prevented by intramolecular crosslinks, then the access to the hydrophobic patches can be restricted and can result in faster elution. As HIC is very sensitive to these minor changes it may affect the adsorption. However,

the interplay of salt concentration and pore size between 1000 Å and 1500 Å is inconclusive and need further investigation.

The columns and the conditions that showed adequate separation are listed in the below table.

Table 4.14. Summary of different phase systems that gave adequate separation.

Column	Pore and particle size	Mobile phase A	% of MeCN in mobile phase B
PolyPENTYL	1000 Å, 3 µm	1 M	50 (Control)
		1 M	35, 65 and 75
		0.5 M	50 and 65
	1000 Å, 2 µm	1 M	35, 50, 65 and 75
	1500 Å, 3 µm	1 M	35, 50, 65 and 75
		0.35 M	50 and 65
		0.25 M	75%
	1500 Å, 2 µm	1 M	25, 35, 50, 65 and 75
		0.35 M	50, 65 and 75
PolyBUTYL	1000 Å, 3 µm	1 M	35, 50, 65 and 75
		0.35 M	65 and 75

Note: Mobile phase A is ammonium acetate concentration, mobile phase B is 20 mM ammonium acetate (overall) with different percentages of MeCN.

4.3.6 Comparison of peak capacity in different phase systems

To provide a head to head comparison all five columns were compared using the selectivity that was obtained at the control mobile phase conditions of 1M in combination with 50% MeCN in 20 mM salt concentration. In these conditions all five columns gave adequate separation (Figure 4.34). The above experimental data demonstrated that column pore size and particle size contributed to the modification of molecule retention due to the differences in the available surface area. Columns with low surface area gave better peak shape and resolution compared to other columns, in addition, low surface area promoted early elution for some mAbs but not all. These stationary phases are newly developed and not completely optimized. The chromatographic performance changes might be due to the differences in the other parameters not limited to only pore size and particle size. Drawing a conclusion to predict the differences in selectivity using the low salt conditions with different column parameters needs further evaluation.

The novelty of this research was to demonstrate the utility of HHIC for the first time by studying a mixture of mAbs under these salt conditions using poly (alkyl aspartamide) silica columns. The goal was to achieve adequate separation at lower salt concentrations with an organic percentage which can still maintain the folded confirmation of the molecule and enhance the MS signal. This data provided very useful insights indicating that these mobile phase and stationary phase conditions can be further evaluated to achieve HHIC separation with online MS capability.

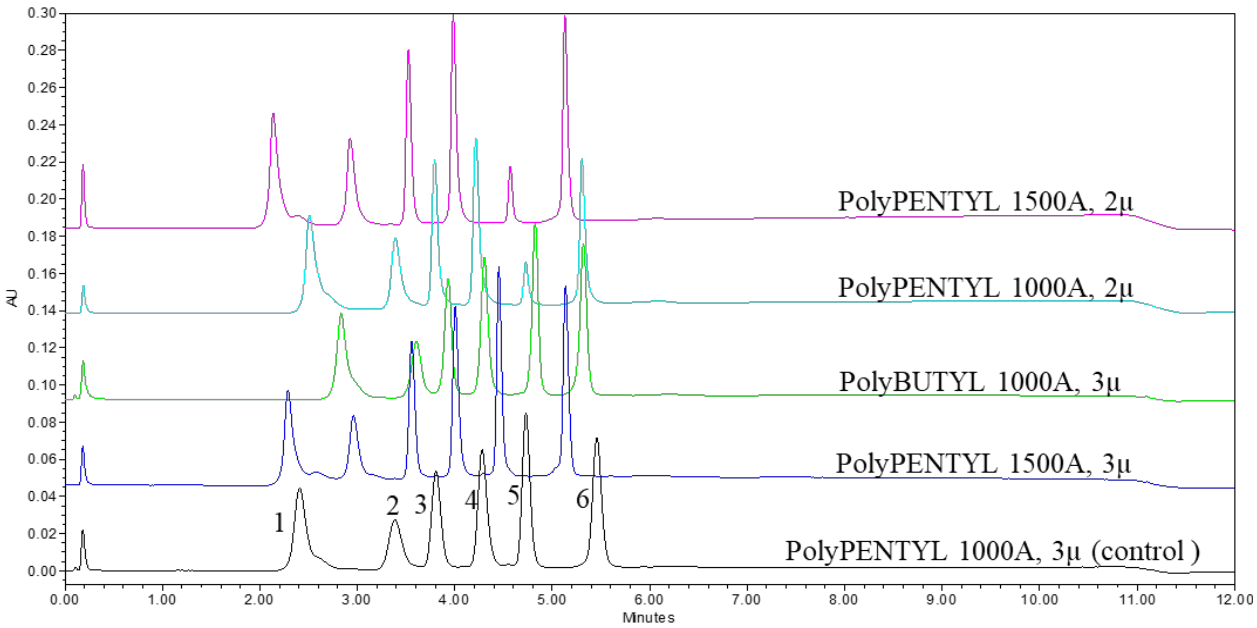

Figure 4.34. Comparison of PolyPENTYL A and PolyBUTYL A columns with different pore and particle sizes: (MP A: 1 M NH_4OAc and MP B: 20 mM NH_4OAc with 50% MeCN with an aqueous pH 7.0). Note: Sample mixture used in 2 µm was different from the sample mixture used with 3 um columns.

4.3.7 Impact of high organic content in mobile phase B

Biomolecules retention on HIC columns is very sensitive to the percentage of organic solvent, specifically MeCN, in this case. This study demonstrated that some columns gave adequate separation and increased peak capacity with more than 50% MeCN in mobile phase B. Under conditions with more than 50% MeCN in combination with low salt concentrations, neither spilt peaks nor additional peaks were observed indicating conformational changes. However, it is a well-known fact that an increase in organic content can induce conformational changes, and it is important to make sure mobile phase with higher organic content will not disturb the conformational stability of a molecule. To gain some preliminary knowledge about the structural changes of a biomolecule in the presence of acetonitrile, experiments using

orthogonal techniques such as intrinsic fluorescence were performed and results were presented in Chapter 5.

4.3.8 Column to column variability: Comparison of PolyPENTYL A columns

In our research it was observed that chromatographic retention under HHIC stationary phases is highly sensitive to mobile phase and stationary phase parameters. Chromatographic variations from batch to batch were observed (Figure 4.35). This result can be due to minor differences in the columns such as pore diameter, pore volume, surface area in the stationary phase and packing of the material. In addition, the combination of stationary phase properties with minor differences in mobile phase composition can impact the retention. As these stationary phases are newly developed and additional optimization may enhance chromatographic performance. For qualitative assessment, the shift in the peaks may not have an impact, however, it is important to keep in mind during method development experiments and method transfer.

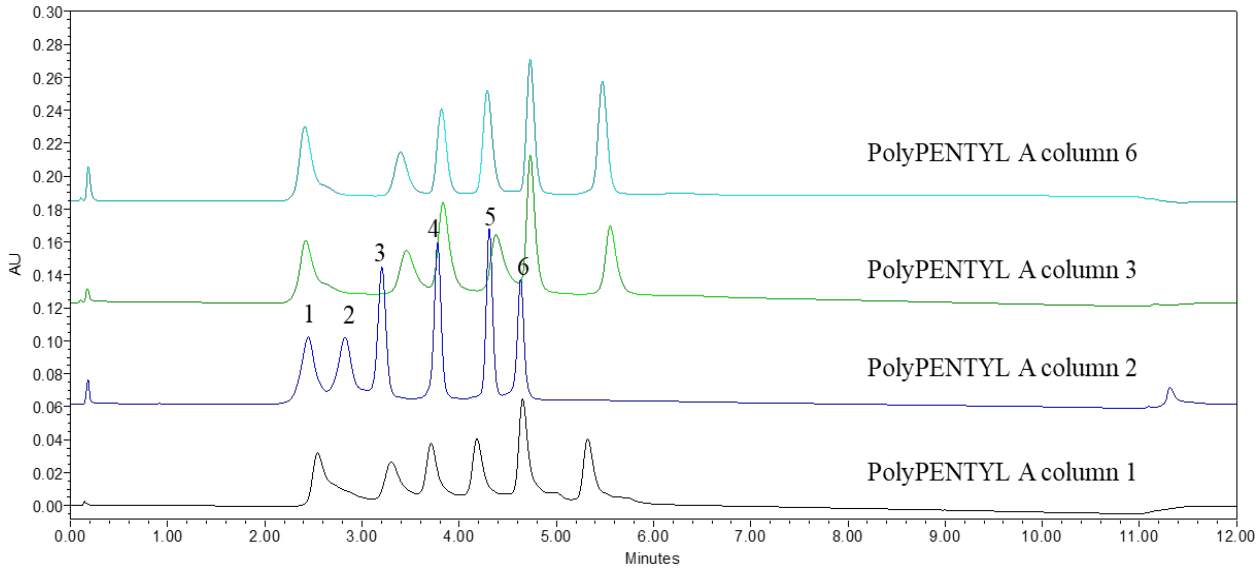

Figure 4.35. Comparison of PolyPENTYL A columns using the same lot of bulk stationary phase (MP A: 1 M NH_4OAc and MP B: 20 mM NH_4OAc with 50% MeCN with an aqueous pH 7.0).

4.3.9 Comparison of TSKGel Butyl-NPR and PolyPENTYL A columns

To compare the selectivity of the selected mAbs on commercially available columns, a sample mixture and individual mAbs were analyzed using Tosoh TSKGel Butyl-NPR column with phosphate and sulfate as mobile phases, which are commonly used for HIC separations. Peaks co-eluted on the Butyl-NPR column showing lack of selectivity (Figure 4.36 and Figure 4.37). This experiment demonstrates that either Butyl-NPR column may not be suitable to separate this mixture or the method needs to be optimized to evaluate suitable mobile phase conditions. The same sample mixture gave an adequate separation with PolyPENTYL A column using a combination of ammonium acetate and 50% MeCN showing the utility of the column to separate molecules with very low surface hydrophobicity differences.

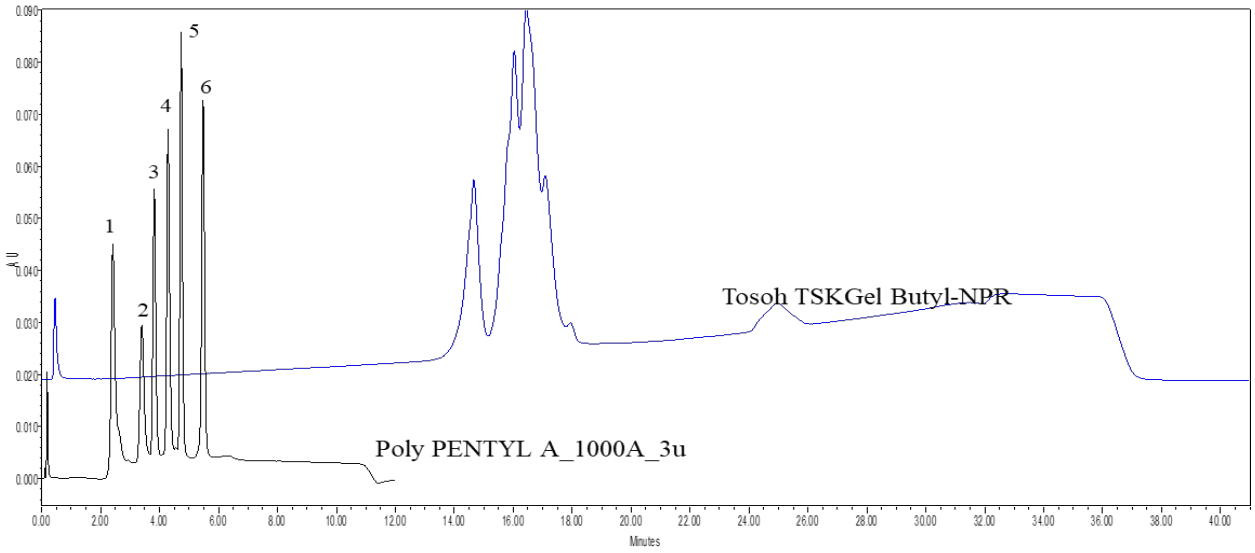

Figure 4.36. Comparison of PolyPENTYL A and Tosoh TSKGel Butyl-NPR columns with (MP A: 1 M NH_4OAc and MP B: 20 mM NH_4OAc with 50% MeCN with an aqueous pH 7.0 and MP A: 20 mM sodium phosphate, 1.5 M ammonium sulfate, 500 mM Arg and MP B: 20 mM sodium phosphate, 500 mM Arg, pH 7.5).

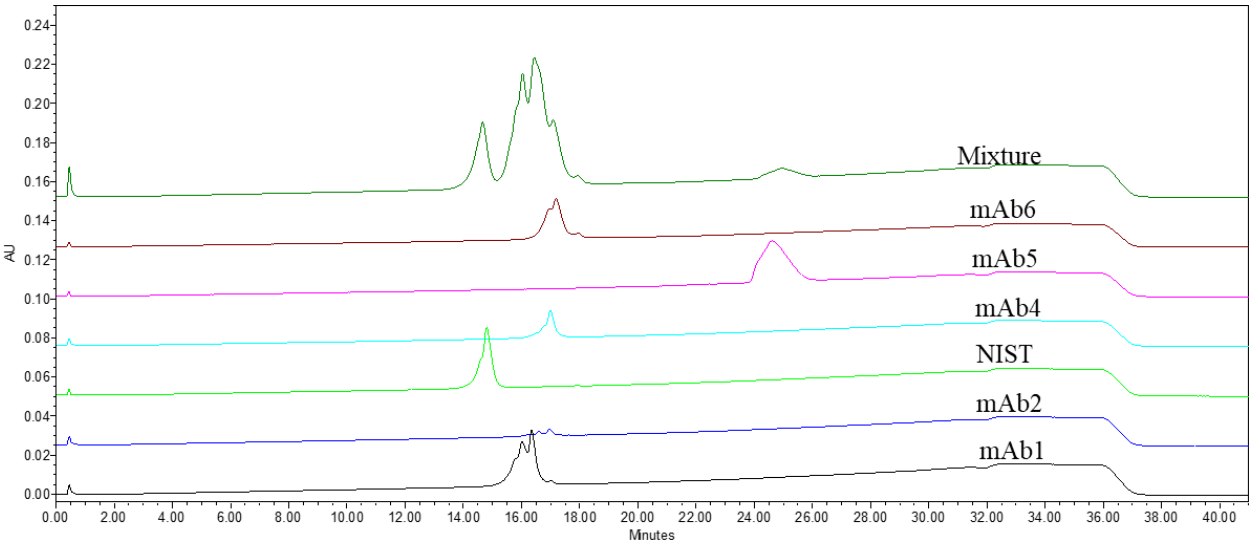

Figure 4.37. Selectivity of individual mAbs on Tosoh TSKGel Butyl-NPR columns with MP A: 20 mM sodium phosphate, 1.5 M ammonium sulfate, 500 mM Arg and MP B: 20 mM sodium phosphate, 500 mM Arg, pH 7.5.

4.4 Conclusion

The purpose of this study was to evaluate the impact of chromatographic parameters on the chromatographic performance using poly (alkyl aspartamide) silica columns. It was evident that pore size, particle size, alkyl length has a significant impact on the selectivity due to the available surface area. Together, these parameters effected diffusion and retention of the molecule. Along with column parameters, mobile phase parameters such as concentration of salt at the starting condition and percentage of MeCN used to disrupt the hydrophobic interactions contributed to adequate elution. In addition, concentration of salt at the starting condition altered the order of mAbs elution by modifying the selectivity.

Assessment of chromatographic performance on multiple PolyPENTYL A columns demonstrated that even though pore size and particles sizes appear to be similar, there may have been some variations in column parameters that needs further investigation to achieve robustness and overcome column to column variability.

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Chapter 5 : Evaluation of mAb Conformational Changes Using Intrinsic Fluorescence: Influence of Chromatographic Parameters

5.1 Introduction

HHIC mobile phase conditions can contribute to significant changes in biomolecule conformation. Salts can either promote solubility or induce self-association or aggregation. In addition, structural differences such as secondary and tertiary structure can be highly impacted by the organic content including the type, amount and contact time with the biomolecule[1-6]

The evaluation of poly (alkyl aspartamide) stationary phases with butyl and pentyl alkyl chains with 1000 Å and 1500 Å pore sizes and 2 µm and 3 µm particle sizes showed adequate separation with different mobile phase conditions described in Table 4.2.4. With respect to chromatography (Refer to Chapter 4), the peaks in most of these experiments appeared homogeneous with very low or no observation of variants. To study conformational changes and structure evaluation of a biomolecule, there are various techniques available, such as Circular Dichroism (CD) [7-9], protein NMR [10-13] and intrinsic Fluorescence resonance energy transfer (iFRET) [14-15]. To conduct a preliminary screening of conformational changes, experiments were performed using intrinsic fluorescence, which is an indicator for conformational changes in biomolecules with respect to changing tryptophans environment. Even though this technique does not provide conformation details of a biomolecule as NMR [16-17] does, it can be very useful tool for screening because of its sensitivity to changes in the conformational and dynamic properties due to solvent changes [18], high throughput and faster analysis time.

Intrinsic fluorescence, also known as Tryptophan fluorescence, is widely used as a tool to monitor changes in proteins and to make inferences regarding local structure and dynamics [19]. Out of the three fluorescent amino acids that are present in biomolecules, tryptophan is the most abundant [20]. The indole group of tryptophan is the source of UV absorbance at ~280 nm and emission is at a of range 330 - 350 nm [21] depending on the polarity of its local environment [20, 22-27]. In addition, tryptophan fluorescence efficiency is strongly impacted by external and internal quenchers [14]. Tryptophan is a relatively rare amino acid; many biomolecules contain only one or a few tryptophan residues. However, intrinsic protein fluorescence is very weak and will not occur in biomolecules if tryptophans are absent [18]. Compared to extrinsic probes, this technique has an advantage of keeping the molecule unchanged. This technique is very sensitive, fast and can be automated. Specifically, observing conformational changes in conjunction with thermal changes, due to solvent differences can be very helpful. Identifying conformational changes under temperature variations will help to characterize the thermal conformational stability of molecules in solution as a function of the solution properties [18].

The basic principle of this technique is, when a biomolecule is in a folded state, the spectral shift is towards lower wavelengths (i.e. higher energy and higher frequency), which is a blue shift [28], indicating tryptophans are in a hydrophobic environment [29]. Unfolded molecule shifts towards higher wavelengths (i.e. lower energy and lower frequency) indicating a red shift [18] and the exposed tryptophans are in a hydrophilic environment. The spectral shift from blue to red represents an unfolding of a biomolecule [30]. Similarly a folded molecule 330/350 ratio is high and as the molecule unfolds the ratio decreases [31]. The magnitude of the wavelength shift depends on the extent to which the protein is buried in the native conformation and is exposed to polar environment in the unfolded state [18].

The aim of the study was to get an initial screening of the conformational changes under the chromatographic conditions which provided satisfactory chromatographic separation. The experiments were designed to evaluate the conformational changes separately in the presence of different concentrations of salt (mobile phase A), different percentages of MeCN in 20 mM salt (mobile phase B), and also in combination of both mobile phase A and mobile phase B. All six mAbs were screened to select a most sensitive probe to observe conformation changes. This screening experiment was carried out using an offline batch mode intrinsic fluorescence.

The results from this preliminary research provided useful information about mAb5 conformation in the presence of each mobile phase condition using offline batch mode intrinsic fluorescence in addition to sample analysis using HPLC with a fluorescence detector. All of these results will help to hypothesize the insights on structural modifications during the chromatographic separation. However, this information may not be sufficient enough to draw conclusions about conformational changes and additional techniques are required to evaluate the changes in the conformation.

5.2 Experimental Details

HPLC grade water and MeCN were used in all analysis. NH_4OAc solution was purchased from Teknova, Hollister, CA. MeCN, was purchased from Sigma-Aldrich, 0.1N ammonium hydroxide was purchased from Ricca Chemicals. HIC column PolyPENTYL A, with dimensions of 50 x 2.1 mm, 3 µm particles with a pore size of 1000 Å was obtained from PolyLC INC, Columbia, MD. Chromatographic separations were performed using Waters Acquity H-class HPLC system with FLR detector with a flow rate of 1.0 mL/min and spectra was collected from 210-400 nm. Details about the molecules and chromatographic conditions

were listed in Section 2.2. pH of aqueous solutions is 7. Offline batch mode intrinsic experiments were performed using Prometheus NT.Plex nanoDSF. Intrinsic fluorescence spectra was collected and the ratio was measured using 330 nm and 350 nm. Control (ctrl) sample was prepared using HPLC grade water at 1 mg/mL to get a baseline of the native conformation in addition to the samples that were prepared in mobile phase A and B according to Tables 5.1 and 5.2.

5.3 Structural Evaluation Using Intrinsic Fluorescence

To perform an initial assessment using intrinsic fluorescence, samples were prepared at 1 mg/mL concentration using HPLC grade water as the diluent to get a base line of the conformation and also to select a best suitable mAb to perform experiments for further conformational evaluation. The experiment was conducted by using thermal stress to unfold the mAbs. MAb which showed the highest difference in F330/350 ratio was an indication of highest unfolding. With a preliminary scan, mAb2 showed very low signal at 30% intensity compared to other mAbs. Therefore, mAb2 was prepared at 5 mg/mL and rest of the mAb5 were kept at 1 mg/mL concentration. Experiments were carried out at a temperature range of 25°C to 90°C using 30% excitation power to study deep conformational changes as compared to subtle changes. The study was designed to observe conformational changes at 25°C, and also monitor maximum shift in F330/350 ratio with respect to temperature variations to assess conformational changes which can help to identify the better probe out of all six mAbs that were used. In addition, thermal range was considered as a variable to observe conformational changes and to have as an option to use higher temperatures as needed if the column is stable under high temperatures. The results of F330/F350 ratio (Figure 5.1) showed that at 25°C, mAb1, mAb3 and

mAb6 exhibited mostly exposed tryptophans which were exposed to outside hydrophilic environment and mAb2 and mAb4 had some exposed. Conversely, the data showed that mAb5 existed with least number of exposed tryptophans, which were in hydrophobic environment. Based on these observations, mAb5 was considered as the best choice to study the conformational changes using different mobile phase conditions as described in Tables 5.1 and 5.2.

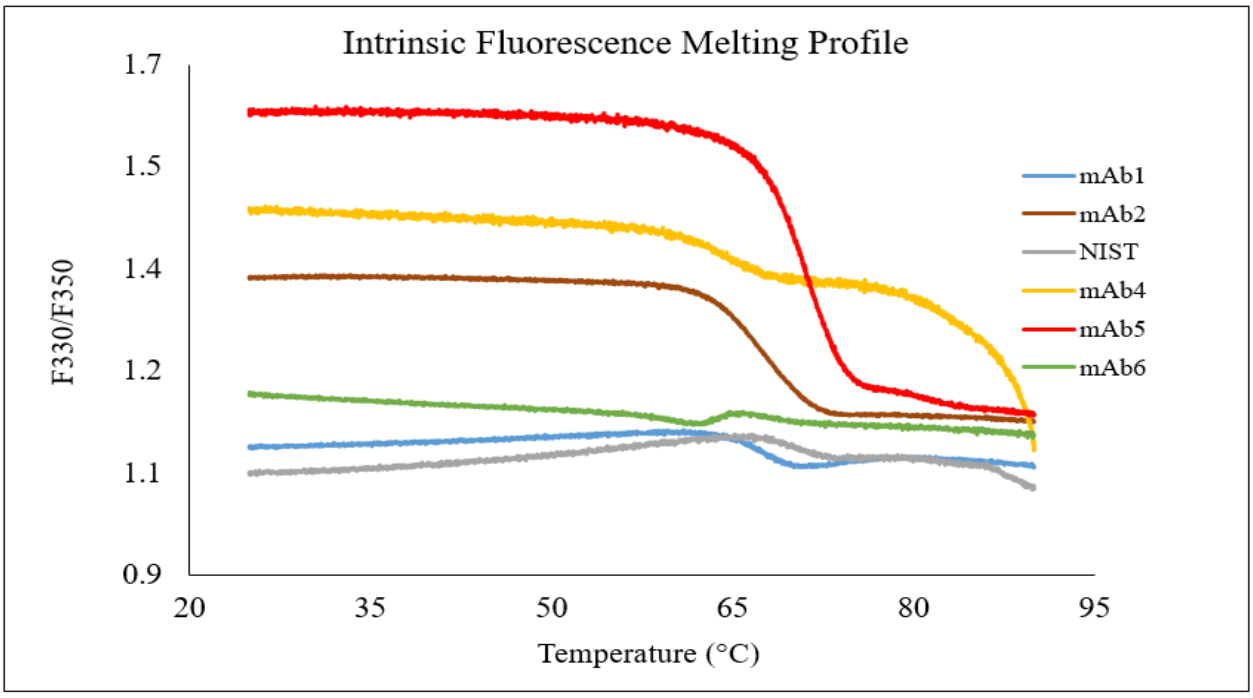

Figure 5.1. The measure of unfolding in water at 25°C to 90°C using F330/F350 ratio.

To study the conformational changes, mAb5 samples were prepared separately using different concentrations of mobile phase A, 20 mM salt with different percentages of MeCN as mobile phase B and also mixing both mobile phases to perform experiments as described in the following tables 5.1 and 5.2.

Table 5.1. Samples in different concentrations of mobile phase A and mobile phase B.

Sample	Mobile Phase A: Concentration of ammonium acetate (M) @ pH 7.0				
mAb5	1	0.5	0.35	0.25	
	Mobile Phase B: % of MeCN in 20 mM ammonium acetate @ pH 7.0				
	25	35	50	65	75

Table 5.2. Samples prepared using mobile phase A with different percentages of mobile phase B at 70:30 ratio.

	70% of Mobile Phase A	30% of Mobile Phase B				
mAb 5	1M	25	35	50	65	75

5.3.1 Impact of salt concentration (mobile phase A)

To study the impact of salt concentration, mAb5 samples were prepared using water as control (Ctrl), 1 M, 0.5 M, 0.35 M and 0.25 M concentrations of ammonium acetate. The biomolecule demonstrated no significant difference in F330/F350 ratio in these salt conditions at 25°C. In addition, at 25°C, the conformation of mAb5 was very similar to the conformation that was observed in water. High F330/350 ratio indicated that the tryptophans were folded and reside in protein's interior hydrophobic environment. As the temperature increased from 25°C to 90°C, the molecule produced a red shift representing that the molecule had undergone unfolding due to the thermal stress (Figure 5.2) which was also confirmed by the decrease in F330/350 ratio. This observation confirmed that tryptophans were exposed from a protein interior hydrophobic environment to an external hydrophilic polar environment. At 25°C, the same behavior was observed in all four salt concentrations, indicating that the changes in the molecule

conformation among this concentration range were insignificant. Based on the results, it was confirmed that at 25°C mAb5 existed in a folded state and also proved that salt with a concentration range of 1 M - 0.25 M did not show any impact on mAb 5 conformation. Again the salt impact may be different on different biomolecules.

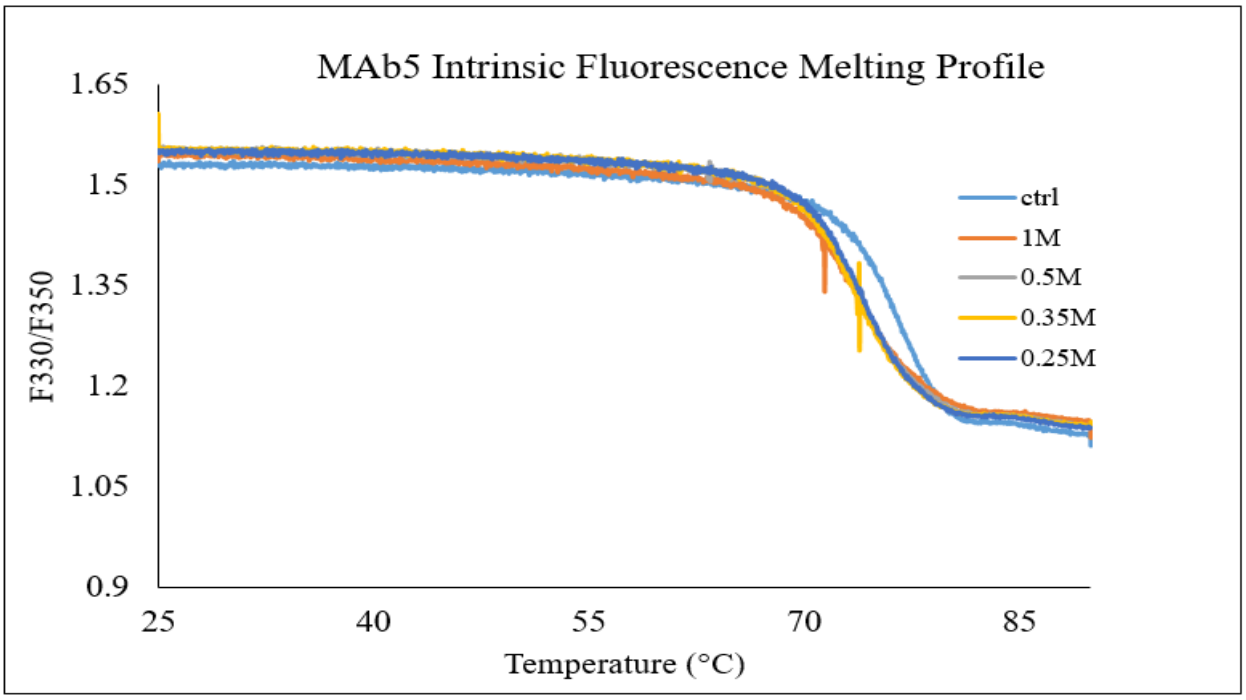

Figure 5.2. MAb5 molecule conformation measured using F330/350 ratio in water and different salt concentrations as mobile phase A in a temperature range of 25°C to 90°C.

5.3.2 Impact of different percentages of MeCN (mobile phase B)

The unfolding nature of the molecule was illustrated using mobile phase B, 20 mM ammonium acetate (overall) with different percentages of MeCN. Experimental results demonstrated that at 25°C, there was a significant difference in the molecule conformation with an increase of MeCN content from 25% to 75% in mobile phase B with 20 mM ammonium acetate. MAb5 showed a blue shift in the presence of 25% MeCN which confirmed the

molecule's folded conformation. This observation was very similar to the conformation in water and also in different salt concentrations (Figure 5.2 and 5.3). As MeCN concentration in mobile phase B increased from 25% to 50%, the molecule showed unfolding which was represented by the decrease in F330/350 ratio and a red shift by moving to a higher wavelength. The concentration further increased to 65% and to 75% MeCN, the F330/350 ratio slowly increased showing indications of aggregation resulting in a blue shift (Figure 5.4). Using this technique, the presence of 20 mM with 25% MeCN demonstrated no major changes in the conformation. The assumption was, 25% MeCN in mobile phase may be not strong enough to disrupt non-covalent interactions to unfold the molecule, and however, as the percentage increased to 35% and above, the conformation was impacted due to disrupted interactions and lead to an unfolding which eventually resulted in aggregation. A non-monotonic change of shifting to red and then back to blue generally indicates that the tryptophans were turning into a more hydrophobic environment which is associated with an aggregation. The hypothesis for the aggregation at higher concentrations of salt solutions can be supported by the physical observation of cloudiness during the sample preparations.

The impact of organic content varies the way a biomolecule is exposed to the organic content. Samples were prepared using premixed mobile phase B, 20 mM with 25% to 75% of MeCN. The effect may vary if water added first and MeCN follows or vice versa. Earlier research [32] proved that proteins denature in aqueous-organic mixture but not pure organic content. Organic solvents expected to disrupt hydrophobic and other non-covalent interactions of a protein (Refer to Section 3.2). As demonstrated by Meng, water and MeCN mixture tend to weakens electrostatic interactions [33].

The observations from this experiment supported the hypothesis of possible conformational changes in the presence of 20 mM with different concentrations of MeCN due to low salt environment. However, to gain more knowledge on structural changes under the above solvent conditions other conformational evaluation experiments are required.

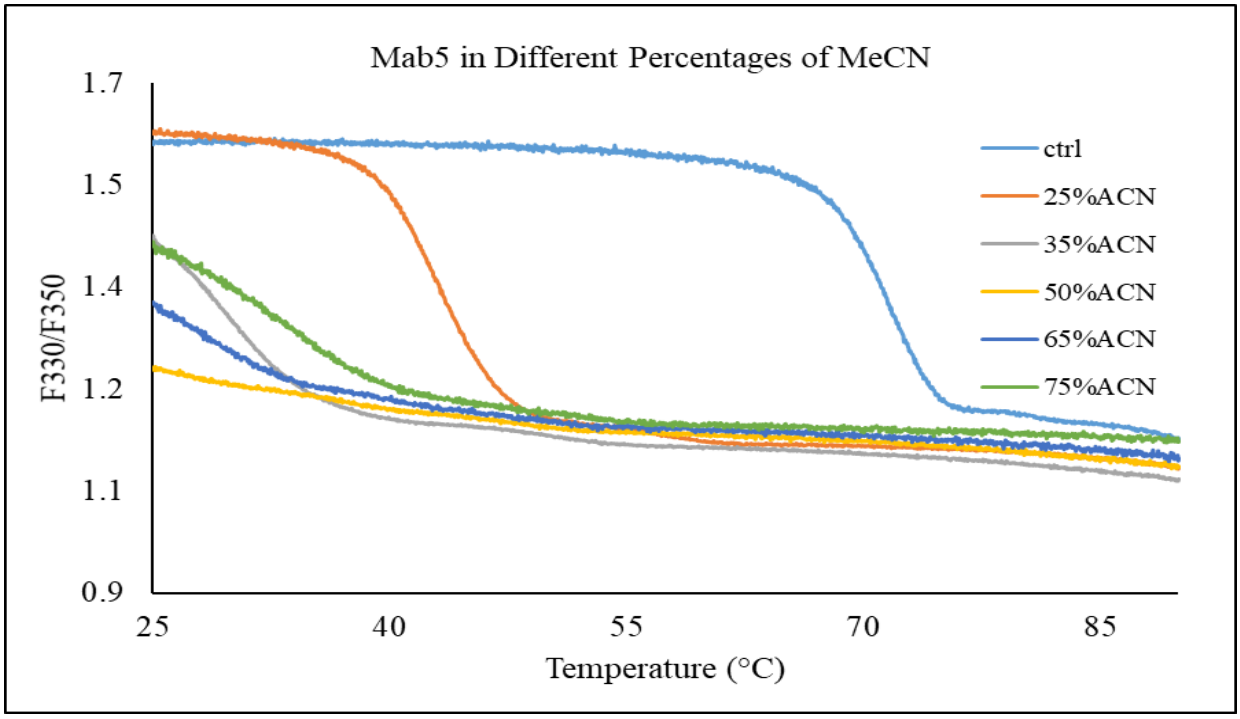

Figure 5.3. MAb5 confirmation measured in different concentrations of MeCN in mobile phase B using F330/F350 ratio from 25°C to 90°C.

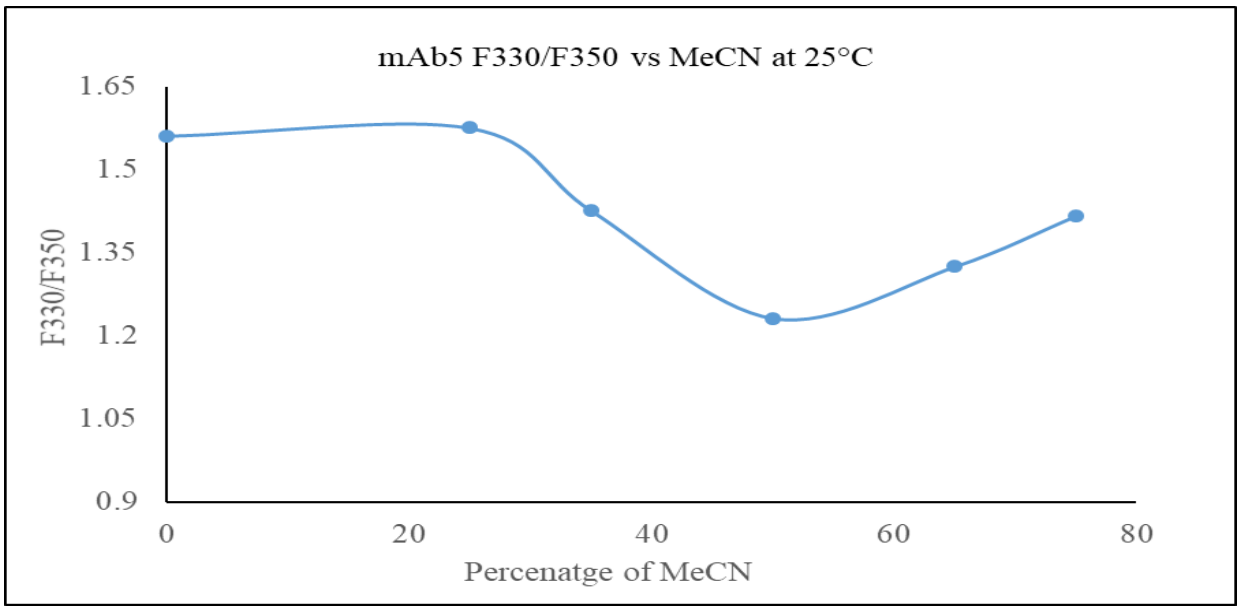

Figure 5.4. MAb5 confirmation measured in 20 mM with different concentrations of MeCN as mobile phase B using F330/F350 ratio at 25°C.

5.3.3 Impact of mobile phase composition

HHIC experiments with various concentrations of mobile phase A and mobile phase B showed that mAb5 eluted within a range of 50% - 62% mobile phase A in combination with 54% - 27% of mobile phase B containing 20 mM ammonium acetate. Samples were prepared only in those conditions where the separation was adequate. In an effort to mimic the chromatographic elution conditions, the sample was first mixed with mobile phase A then mobile phase B was added to bring the sample concentration to 1 mg/mL. Experimental design was as indicated in the Table 5.3.

The purpose of this experiment was to assess the tolerability of the molecule conformation in the mobile phase composition. Based on the observation, there was a significant variation in the molecule conformation with an increase from 25% to 75% of mobile phase B

with 20 mM ammonium acetate in combination with mobile phase A salt concentration that was used in this study.

Table 5.3. Samples prepared using mobile phase A and mobile phase B.

MAb5	Mobile Phase A	20 mM ammonium acetate containing different % of MeCN as mobile phase B			
	Ammonium acetate concentration	35	50	65	75
	1 M	x	x	x	x
	0.5 M	-	x	x	x
	0.35 M	-	x	x	x
	0.25 M	-	-	x	x

Note: "x" - Conditions used to prepare samples

Intrinsic fluorescence results from 1 M and 0.35 M as mobile phase A with different percentages of organic in mobile phase B (Figure5.5, 5.6 and 5.7) at 25°C supported the assumption of biomolecule conformational stability under high salt concentrations. No difference in fluorescence ratio demonstrated that there was no change in tryptophan environment under these conditions and molecule existed in folded state. As the temperature increased from 25°C to 90°C, a red shift indicated that tryptophans were exposed to hydrophilic environment due to an unfolding of the molecule. Unlike other higher salt conditions, 0.25M as mobile phase A and 20 mM with 65% and 75% MeCN as mobile phase B showed considerable change in fluorescence ratio, which was an indication of change in molecule confirmation due to low salt condition (Figure 5.9). However, a non-monotonic increase in F330/350 ratio under 0.25M and 20 mM with 75% MeCN can be a sign of possible aggregation. By evaluating the

effect of mixture of mobile phase (A and B) with mobile phase A (section 5.5) and mobile phase B (section 5.6) separately, it was clear and supported the hypothesis of the role of MeCN is highly dependent upon the salt concentration. 0.25 M as mobile phase A, the salt concentration was helping to reduce the unfolding by holding the tryptophan hydrophobic environment. Where as in mobile phase B containing 20 mM with 65% and 75% MeCN, the absence of salt was promoting unfolding by weakening the non-specific interactions.

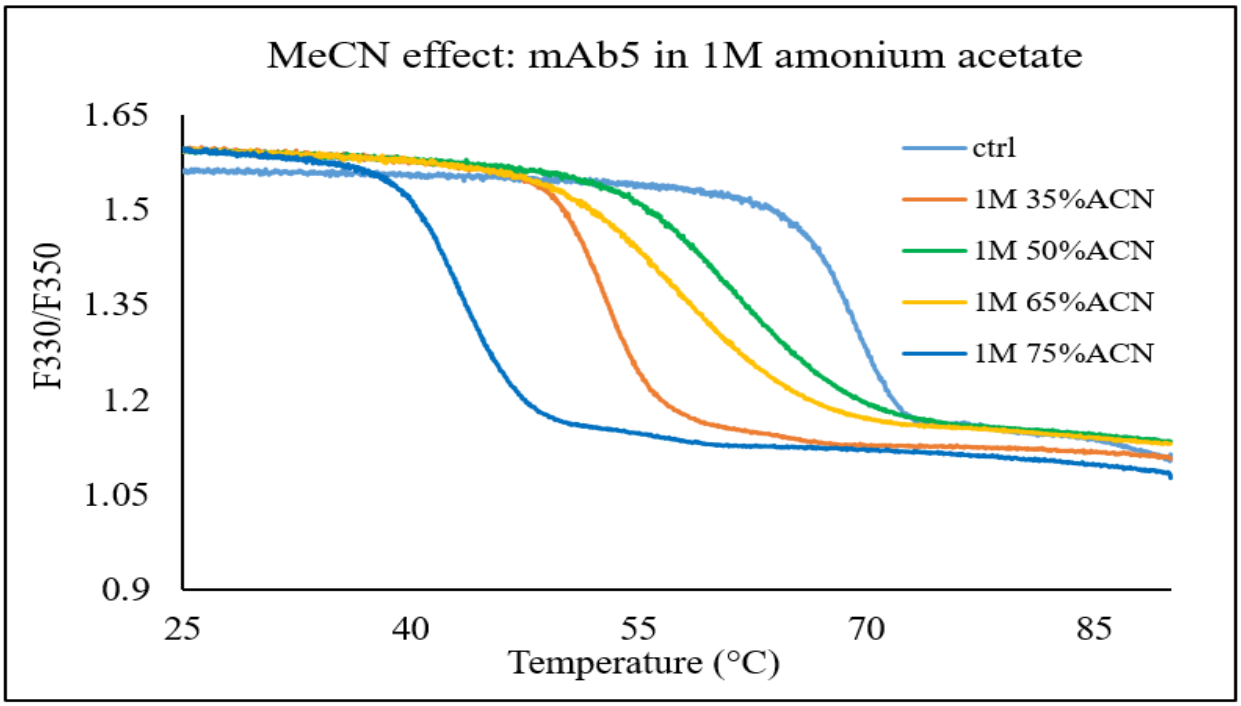

Figure 5.5. MAb5 confirmation measured in 1M with different concentrations of MeCN in mobile phase B using F330/F350 ratio from 25°C to 90°C

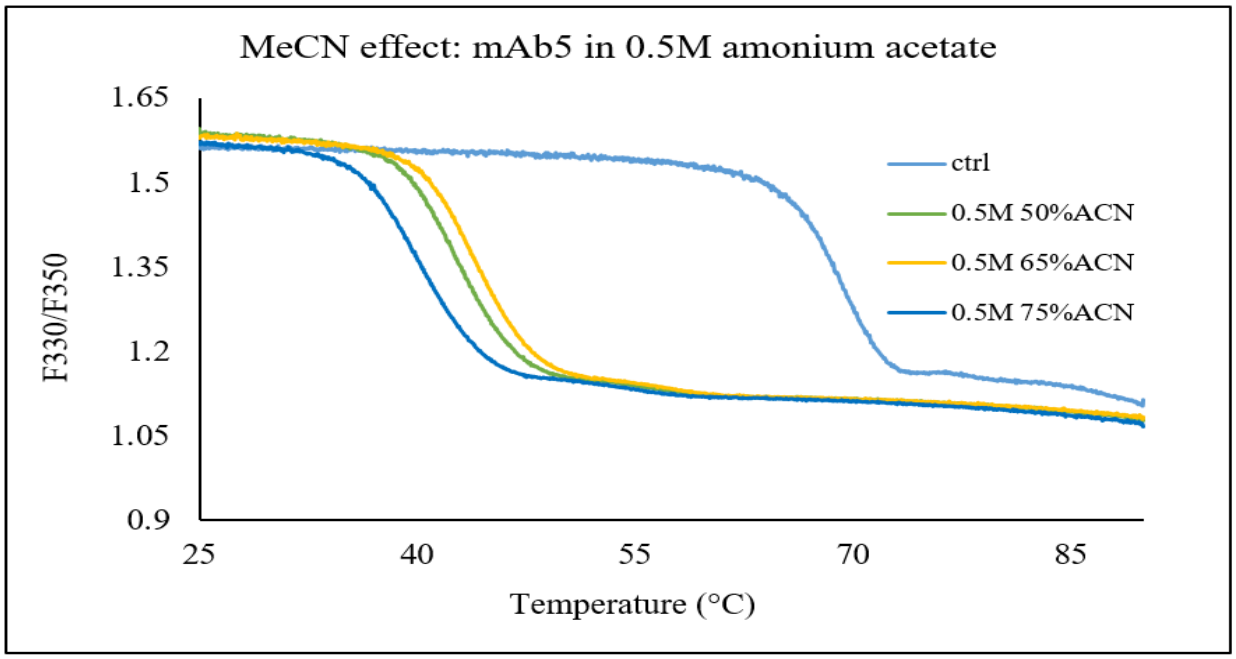

Figure 5.6. MAb5 confirmation measured in 0.5M as mobile phase A with different concentrations of MeCN in mobile phase B using F330/F350 ratio from 25°C to 90°C

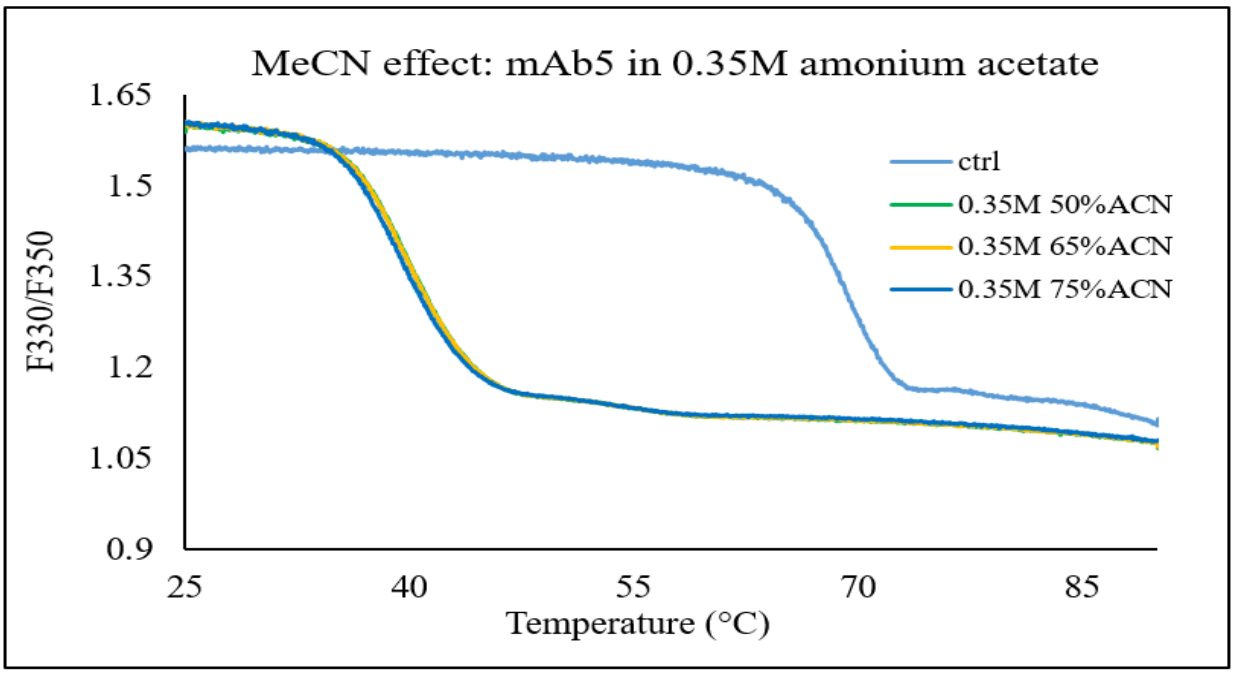

Figure 5.7. MAb5 confirmation measured in 0.35M as mobile phase A with different concentrations of MeCN in mobile phase B using F330/F350 ratio from 25°C to 90°C

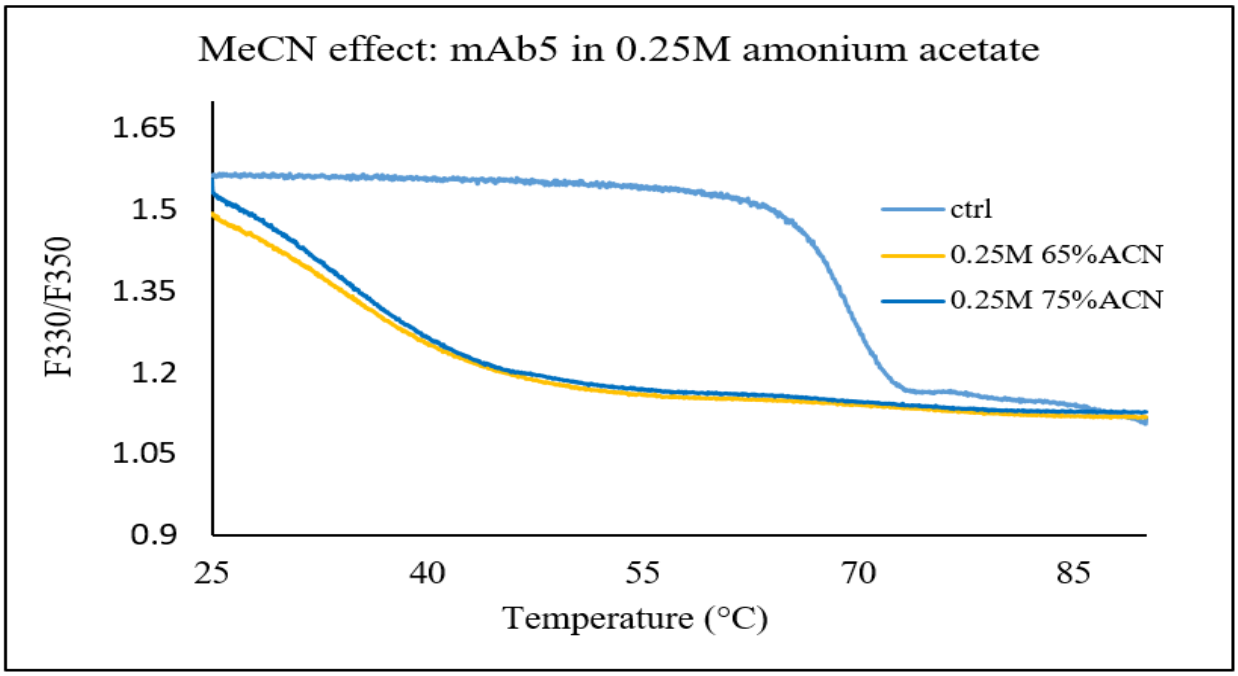

Figure 5.8. MAb5 confirmation measured in 0.25M as mobile phase A with different concentrations of MeCN in mobile phase B using F330/F350 ratio from 25°C to 90°C

Intrinsic fluorescence data demonstrated that mAb5 showed conformation changes in the presence of mobile phase A, mobile phase B and in the mixture of both mobile phases. Out of all these conditions, mAb5 retained its folded conformation in a mixture of mobile phase A with a range of 1 M to 0.35 M and mobile phase B as 20 mM with 75% MeCN. However, mAb5 aggregated in the presence of mobile phase B containing 20 mM with 75% MeCN alone. Upon reviewing the above two experiments, it was clear that when mobile phase A (1 M to 0.35 M) was added to mobile phase B with 75% MeCN, the tryptophan environment was protected by keeping the molecule in its folded state. This result demonstrated that the impact of 75% MeCN was insignificant on intrinsic florescence changes in the presence of higher salt concentration which is above 0.35 M ammonium acetate. The assumption was, that higher salt may have protected mAb5 conformation. Under high salt conditions, the biomolecule existed in the folded

state resulting in a blue shift with high F330/350 ratio. However, the data indicated that mAb5 may have unfolded in the presence of mobile phase A with 0.25 M and mobile phase B containing 20 mM with 75% MeCN showing a decrease in F330/350 ratio (Figure 5.8 and Figure 5.9). This intrinsic experimental data indicating possible unfolding in low salt with high organic content environment supports the hypothesis of high organic content can have a huge impact on molecule conformation, however, in the presence of high salt environment the impact is low due to the conformational stability. In contrary, in low salt environment high organic content can influence the molecule confirmation by causing partial unfolding or unfolding. The observation of red shift in 0.25 M and 20 mM with 75% MeCN was an indication of change in tryptophan environment which was caused as a result of the molecule partial unfolding or unfolding.

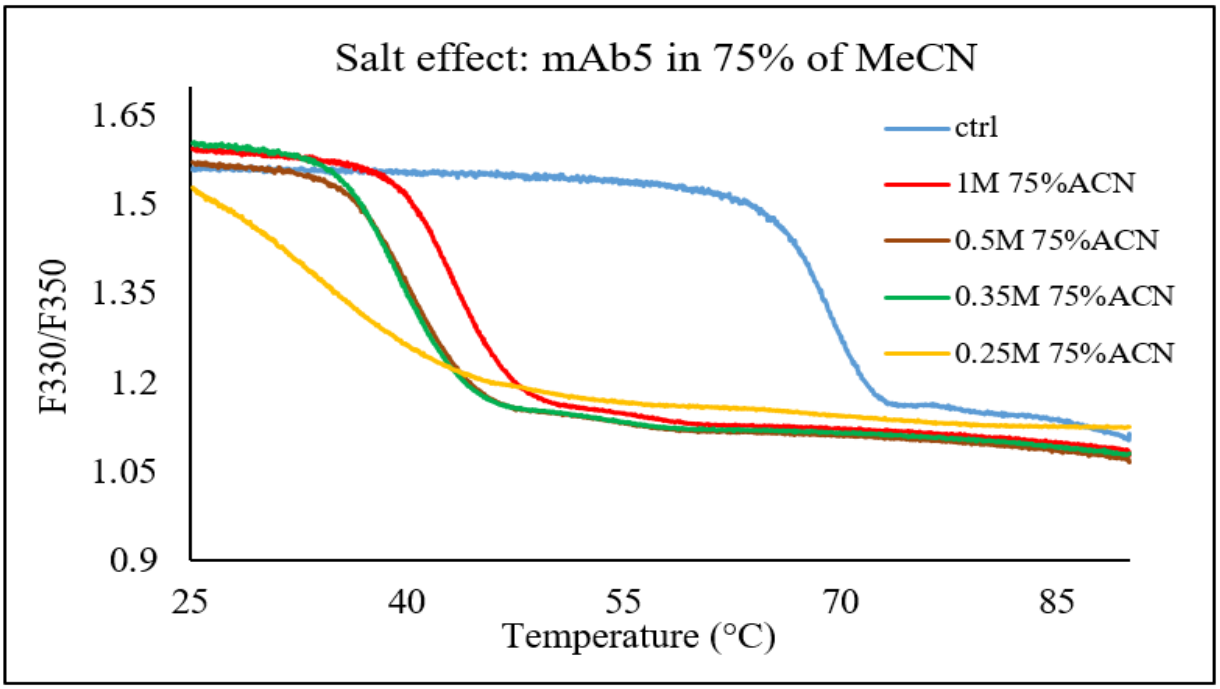

Figure 5.9. MAb5 confirmation measured in different concentrations of salt as mobile phase A and 75% of MeCN in mobile phase B using F330/F350 ratio from 25°C to 90°C

5.3.4 Impact of mobile phase and stationary phase interplay

Stationary phase evaluation (Chapter 4) demonstrated PolyPENTYL A 1500 Å with 2 um and 3 um particle size and PolyBUTYL A 1000 Å with 2 um and 3 um particle size columns exhibited adequate separation using 0.25 M and 0.35 M as mobile phase A in combination with 20 mM with 65% and 75% MeCN as mobile phase B. To investigate the biomolecule conformation in these relatively low salt concentrations with high MeCN, the same HHIC experiments were repeated using a HPLC system with intrinsic fluorescence detector. The data was calculated using F330/350 ratio. The primary aim of this experiment was to study the role of hydrophobic stationary phase on the biomolecule conformation in the presence of mobile phase gradient. The F330/350 ratio from chromatographic data and the standalone intrinsic experimental results were evaluated to build a hypothesis to explain the conformational changes in mAb5 when exposed to chromatographic conditions.

Results from the experiments indicated that along with mobile phase A with 1 M and mobile phase B containing 20 mM with 50% MeCN, mobile phase A with 0.25 M and 0.35 M in a combination with 20 mM with 65% and 75% MeCN in mobile phase B, showed very similar F330/350 ratios. Correlating chromatographic data with batch mode intrinsic data, mAb5 behavior in different salt concentrations in combination with different percentages of organic solvent at 25°C (Figure 5.10) showed that the molecule existed mostly in aggregated state. However, as there were not enough data points to observe the trend of conformational changes, the outcome is inconclusive. To perform a complete assessment on unfolding and aggregation of mAb5 molecule, a future experimental study should be planned to gather information with multiple data points using different percentages of MeCN in combination with different salt concentrations.

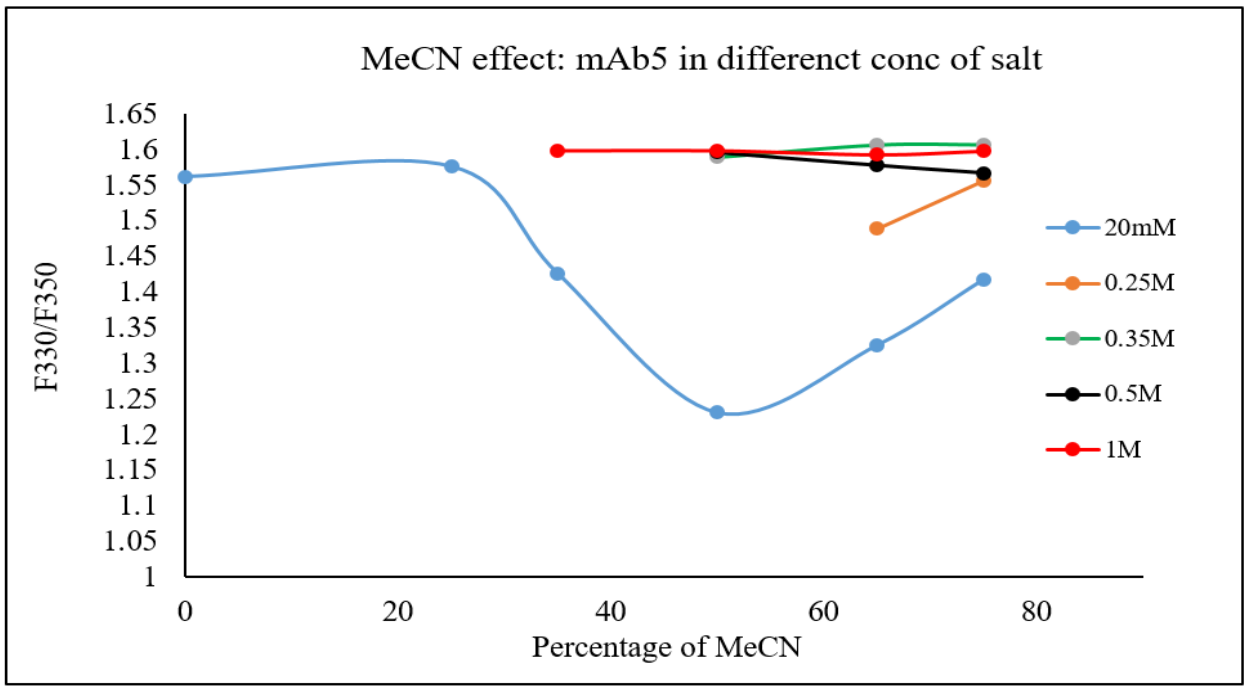

Figure 5.10: MAb5 confirmation measured in mobile phase A and different concentrations of MeCN in the presence of mobile phase B using F330/F350 ratio at 25°C.

5.4 Conclusion

The results from batch mode intrinsic fluorescence and HHIC fluorescence detector experiments indicated that the salt in mobile phase A (1 M - 0.35 M) helped to retain mAb5 conformation from unfolding in the presence of 20 mM containing 75% MeCN by supporting the hypothesis. According to these preliminary experiments using mAb5 and intrinsic fluorescence technique, the results from both mobile phases A and B and the mixture of mobile phases (A and B) suggested that the low salt conditions mentioned in Table 4.14 (such as 0.35M and 0.25M with 20 mM containing 65% and 75% MeCN in mobile phase B) can be used to achieve adequate separation by keeping the molecule in non-denatured form. To obtain more details on the trend of unfolding/aggregation, additional experiments need to be performed by changing the MeCN concentration from 25% to 75% MeCN in mobile phase B.

The conformational evaluation using intrinsic fluorescence was done for the first time to gain knowledge on a mAb's existence under the influence of HHIC chromatographic parameters. Results obtained from this small set of experiments using mAb5 provided fundamental knowledge about possible conformational changes based on intrinsic fluorescence data and also brought out some important questions to extend the research and further evaluate to obtain deeper understanding on the conformation of biomolecules under HHIC chromatographic solvent conditions. All of these experiments were performed with one mAb using one technique to assess the conformational changes. To expand this knowledge, the research can be continued to evaluate the changes in multiple mAbs and related biomolecules using techniques such as intrinsic fluorescence Förster resonance energy transfer (FRET), Circular Dichroism (CD), Static Light Scattering (SLS) and Dynamic Light Scattering (DLS) and also protein NMR can provide details about the structure, weight and size along with aggregation and unfolding of a molecule.

Collectively, these techniques can help to provide more insights to confirm the existence of biomolecule conformation (unfolded state, native-like or aggregated state).

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Chapter 6 : Research Conclusions

Co-formulated monoclonal antibodies (mAbs) and other related biomolecules are very important and fastest growing therapeutic modalities for various diseases. To develop, characterize and accurately quantitate multiple biomolecules simultaneously is extremely important and highly challenging. There is a need to develop innovative technologies to address limitations and resolve the challenges. This research is to study chromatographic retention and enhance knowledge about HHIC and also provide possibilities to resolve some of the limitations to assess critical parameters of chromatographic separation.

This dissertation provides new insights about HHIC capabilities to evaluate mixture of mAbs using poly (alkyl aspartimide) columns, which can also facilitate MS compatibility. This research provides useful information on working range of mobile phase parameters such as salt concentration, pH, organic modifier and temperature to achieve adequate separation. The study design also expands the knowledge of stationary phase properties such as hydrophobicity, particle size and pore sizes that contribute towards achieving adequate separation under low salt conditions to enhance ESI/MS signal, while keeping the molecule in folded or native-like confirmation. The highlights are:

- This novel research demonstrated the utility of HHIC for the first time using a mixture of mAbs and separate them simultaneously.
- In this research the utility of salt concentration was illustrated by demonstrating that adequate separation is achievable using low salt concentration despite earlier reports to the contrary. In addition, low salt (0.5M) provided superior chromatographic separation and significant improvement in ESI/MS response. The improvement in separation is a

result of reduced retention of early eluting components combined with an unexpected greater retention of later eluting components which results in a widening the chromatographic window (increased peak capacity). The new finding is use of low salt as a starting condition to obtain greater retention which is atypical HIC behavior. The improvement in ESI/MS response, which is a direct result of the use of lower salt concentration, also represents a significant enhancement since the main motivating factor of hybrid HIC is to enable online MS compatibility to HIC separation.

- With 0.25 M salt as the starting concentration, the 2-µm, 1500-Å PolyPENTYL A column exhibited two minor variant peaks that were not observed in any other chromatogram in this research. This research demonstrated that low salt not only improves ESI/MS signal but can be further evaluated to separate minor variants of a mAb.
- Conformational analysis confirmed mAb5 existed in folded or native-like conformation under low salt conditions (0.25 M and 0.35M) in combination with high MeCN (65% and 75%).
- The results of gradient steepness and linearity velocity indicate that the impact of these parameters followed expected tendencies with a relatively modest loss of efficiency at the highest linear velocity.
- A pH range of 5.7 - 7.3 was evaluated as most of the biomolecules exhibit high chemical and physical stability. The results indicated that when pH was close to the *pI* of the molecule a decrease in retention was observed for some molecules and no impact on others. This behavior was a result of a decrease in the charge of a molecule which was a result of pH of the mobile phase. The study also demonstrated the impact of pH is

complex because it is mainly driven by the properties of the molecule with respect to the charged moieties. Even though pH may not a critical parameter, it can be used to widen the chromatographic window and optimize the separation.

- It has been well established that in HIC methodology organic modifier decreases the retention. These experimental results confirmed that to perform HIC on poly(alkyl aspartimide) columns, organic solvent is required to disrupt hydrophobic interactions and elute peaks when low concentrations of ammonium acetate was used for the separation. Evaluation of different organic solvents illustrated that adequate separation was achieved using a non-polar solvent, such as MeCN but a polar solvent such as IPA produced more complex chromatogram with multiple peaks for each component. It appears IPA enables the separation of variants which was not observed with MeCN. Use of IPA can be further evaluated to gain knowledge on separating variants. This is a valuable finding opens up an opportunity to use IPA for separating variants and use MeCN to quantitate as an ensemble of biomolecule depending upon the type of analysis.
- In conventional HIC columns, increase in the temperature increase the retention. For the first time the effect of temperature (20°C - 30°C) on these columns was evaluated and the results indicated that with an increase in temperature, retention decreased. This is atypical behavior of HIC columns. This result may be due to the changes in the diffusion of the molecule through the stationary phase or in lower temperatures hydrophobic effect becomes weaker.
- The study to evaluate the effect of stationary phase hydrophobicity indicated that column parameters significantly impact selectivity. PolyPROPYL column was unable to retain molecules, however, as the alkyl chain length increased to HEPTYL early eluters

demonstrated failed to elute and later eluters coeluted showing the impact of stationary phase hydrophobicity.

- Column pore size and particle size contributed to the modification of molecule retention due to the differences in available surface area. Columns with low surface area gave better peak shape and resolution compare other columns. The low surface area promoted early elution for some mAbs but not all. These stationary phases are newly developed and yet to be optimized, therefore, some of the chromatographic performance changes may be due to the differences in other column parameters than just pore size and particle size. Drawing a conclusion to predict the difference in selectivity using the low salt conditions with different column parameters needs further evaluation.