PREPARATION OF REACTIVE-POLYACRYLATE CAPILLARY MONOLITHS AND THEIR USE AS STATIONARY MEDIA IN HYDROPHILIC INTERACTION CHROMATOGRAPHY

REAKTİF POLİAKRİLAT KAPİLER MONOLİTLERİN SENTEZİ VE HİDROFİLİK ETKİLEŞİM KROMATOGRAFİSİNDE SABİT FAZ OLARAK KULLANIMI

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ETHICS

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16/09/2015

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ABSTRACT

THE PREPARATION OF REACTIVE-POLYACRYLATE CAPILLARY MONOLITHS AND THEIR USE AS STATIONARY MEDIA IN HYDROPHILIC INTERACTION CHROMATOGRAPHY

Sedef Damla Erkakan

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In this study, the synthesis of new polar polymer-based monoliths was performed and their chromatographic performance in hydrophilic interaction chromatography (HILIC) mode was determined. Poly(HPMA-Cl-co-EDMA) monoliths were prepared via thermally initiated block copolymerization into silanized capillary fused silica tubes having 100 µm internal diameter. In the synthesis stage, concentrations of monomer, cross-linking agent and porogen in the polymerization solution and polymerization temperature were changed. The effects of each of these changes on the structure of monolith were investigated. After obtaining appropriate pore size distribution, specific surface area and back pressure values, a chromatographic ligand, triethanolamine (TEA-OH) was covalently attached to monolith via a single step reaction. These newly produced monolithic columns with different pore structures and polarities were used successfully for the separation of nucleotides, nucleosides and benzoic acids. All chromatographic studies were performed by using acetonitrile/aqueous buffer system as the mobile phase. In the chromatographic studies, the lowest plate height was obtained as 20 µm using uracil (nucleotide), 54 µm using thymidine (nucleoside) and 25 µm for benzoic acid derivatives. According to chromatographic results, it was seen that efficient separations of benzoic acid derivatives were mainly based on hydrophilic interaction mechanisms. Under optimized separation conditions, TEA-OH attached monoliths was successfully applied for the rapid, high resolution and without obvious peak tailing separation with lower than 5 % column to column relative standard deviation values for each nucleotides. However, the first time introduced "retention-dependent column efficiency" behavior for the HILIC system

showed that the plate height raised with increasing retention factor. Its possible reasons were discussed. In this study, the first monolithic column which is capable of separating polar analytes in HILIC mode was synthesized in our country. In the light of these results, better separation efficiencies can be achieved with further studies.

ÖZET

REAKTİF POLİAKRİLAT KAPİLER MONOLİTLERİN SENTEZİ VE HİDROFİLİK ETKİLEŞİM KROMATOGRAFİSİNDE SABİT FAZ OLARAK KULLANIMI

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Bu çalışmada yeni bir polar polimer esaslı monolit sentezi yapılmış ve hidrofilik etkileşim kromatografisi (HILIC) modunda kromatografik performansları incelenmiştir. Poli(HPMA-Cl-co-EDMA) monolitler 100 μm iç çapına sahip olan silanlanmış silika kapiler tüplerde termal başlatıcılı blok kopolimerizasyonu yöntemi ile hazırlanmıştır. Sentez aşamasında, polimerizasyon çözeltisindeki monomer, çapraz bağlayıcı ve gözenek yapıcı konsantrasyonları ve polimerizasyon sıcaklığı değiştirilmiş ve bu değişimlerin monolit yapısı üzerindeki etkisi araştırılmıştır. Uygun gözenek boyutu dağılımı, özgül yüzey alanı ve geri basınç değerleri elde edildikten sonra, kromatografik ligand, trietanolamin (TEA-OH) tek basamaklı bir reaksiyon ile monolite kovalent olarak bağlanmıştır. Farklı gözenek yapıları ve polaritelere sahip olan monolitik kolonlar, nükleotid, nükleosid ve benzoik asitlerin kromatografik ayrımında başarılı bir şekilde kullanılmıştır. Bütün kromatografik çalışmalar, hareketli faz olarak asetonitril/su veya asetonitril/sulu tampon sistemi kullanılarak gerçekleştirilmiştir. Kromatografik çalışmalarda, en düşük plaka yüksekliği nükleotidler ile 20 μm, nükleosidler ile 54 μm ve benzoik asit türevleri ile 25 μm olarak elde edilmiştir. Kromatografik sonuçlar, benzoik asitlerin ayırımının hidrofilik etkileşim mekanizması ile oluştuğunu göstermiştir. TEA-OH bağlanmış monolitler % 5 den az tekrarlanabilirlik değeri ile hızlı, yüksek çözünürlüklü ve pik yayılımı olmadan analitlerin ayırımına başarılı bir şekilde uygulanmıştır. Ancak HILIC modunda ilk kez ortaya koyulmuş olan "alıkonma faktörüne bağlı kolon verimliliği" davranışı, kolon yüksekliğinin alıkonma faktörünün artması ile arttığını göstermiştir. Bunun mümkün olabilecek nedenleri tartışılmıştır. Bu çalışmanın sonunda, ülkemizde

HILIC modda polar analitleri ayırma yeteneğine sahip ilk monolitik kolonun sentezlenmiş olduğu ifade edilebilir. Bu sonuçlar ışığında, ileriki çalışmalarda daha iyi ayırma verimleri elde edilebilir.

Anahtar Kelimeler: Monolit, Hidrofilik Etkileşim Kromatografisi (HILIC), Kromatografik ayırma, HPMA-Cl, EDMA, Trietanolamin

To My Beloved Mother
and Twin Sister,

ACKNOWLEDGMENTS

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NOMENCLATURE

Abbreviations

AC	Affinity Chromatography
ACN	Acetonitrile
AIBN	2,2'-azobisizobutyronitrile
ATP	Adenosine triphosphate
BACM	4-aminocyclohexyl)methyl]cyclohexylamine
BET	Brunauer-Emmet-Teller
BSA	Bovine serum albumin
BVPE	1,2-bis(p-vinylphenyl)ethane
cAMP	Cyclic adenosine mono phosphate
CEC	Capillary electro chromatography
cLC	Capillary liquid chromatography
DAD	Diode Array Detector
Dec-OH	n-decanol
DMAA	N,N-dimethylacrylamide
DMF	N,N-Dimethylformamide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EDMA	Ethylene dimethacrylate
GC	Gas chromatography
GMA	Glycidyl methacrylate
GMM	Glyceryl monomethacrylate
h	Theoretical plate height
HILIC	Hydrophilic interaction liquid chromatography
HMMAA	N-(hydroxymethyl) methacrylamide
HPLC	High performance liquid chromatography
HPMA-Cl	3-chloro-2-hydroxypropyl methacrylate
IEC	Ion exchange Chromatography
Iso-PrOH	Isopropanol
K	Permeability
k	Retention factor
L	Column length

LC	Liquid chromatography
MAA	Methacrylic acid
MBA	Methylenebisacrylamide
MEDSA	2-(methacryloyloxy)ethyl]dimethyl-(3-sulfopropyl)ammonium hydroxide
META	[2-(methacryloyloxy) ethyl]-trimethylammonium chloride
Met-OH	Methanol
micro-HPLC	Micro-high performance liquid chromatography
MPC	2-methacryloyloxyethyl phosphorylcholine
N	Theoretical plate number
NAHAM	N-acryloyl-tris(hydroxymethyl)-aminomethane
Nano-HPLC	Nano-high performance liquid chromatography
nano-LC	Nano-liquid chromatography
NaOH	Sodium hydroxide
NPC	Normal Phase Chromatography
PEEK	Polyether ether ketone
PEG	Polyethylen glycol
PEGDA	Polyethylen glycol diacrylate
PETA	Pentaerythritoltriacrylate
R	Resolution
RNA	Ribonucleic acid
RPC	Reversed phase chromatography
RSD	Relative standard deviations
SEC	Size exclusion Chromatography
SEM	Scanning electron microscope
SPDA	N,N-dimethyl-N-acryloyloxyethyl-N-(3-sulfopropyl)ammonium betaine
SPE	N,N-dimethyl-N-methacryloxyethyl-N-3(sulfopropyl)-ammonium betaine
SPMA	3-sulfopropyl methacrylate
SPP	N,N-dimethyl-N-(3-methacryl-amidopropyl)-N-(3-sulfopropyl)ammonium betaine

SSA	Specific surface areas
TEA-OH	Triethanolamine
TEPIC	Tris(2,3-epoxypropyl) isocyanurate
TMSPM	3-(trimethoxysilyl)propyl methacrylate
t_0	Time taken during mobile phase pass through the column
t_R	Time taken until the analyte is reached to detector
t_{R_1}	Retention time of analyte 1
t_{R_2}	Retention time of analyte 2
TRIM	Trimethylolpropane trimethacrylate
u	Linear velocity
UV	Ultraviolet
VPBA	4-vinylphenylboronic acid
W_1	Peak width of analyte 1
W_2	Peak width of analyte 2
$W_{1/2}$	Peak width of half height
ZDV	Zero dead volume

Symbols

ΔP	Pressure drop across the monolithic column
γ-maps	3-(trimethoxysilyl)propyl methacrylate
η	Dynamic viscosity of mobile phase

1. INTRODUCTION

1.1. General

High performance liquid chromatography (HPLC), an important separation technique, is discovered in the mid-1970s [1]. A steady development of HPLC techniques occurs since then. In general, the trend in development of these techniques is toward faster, more efficient and sensitive systems. Nano-high performance liquid chromatography (nano-HPLC) has basically the same working principles with the conventional HPLC. Nevertheless, miniaturization of mobile phase and column diameter provide some significant advantages. The reduction of mobile phase means both economic and environmental friendly analysis [2]. The amount of sample and time needed for analysis are also reduced by reducing the column diameter. In addition, mass sensitivity is increased due to the fact that sample dilution occurs on-column is reduced [3]. Therefore, recently, nano-HPLC is frequently used.

Nano-HPLC separations are generally performed in columns having small internal diameter. These columns can be packed with particles or monolithic. Particle packed columns have some disadvantages according to monolithic columns. For example, particles are prepared outside and then packed into column while monoliths are produced into column with a single staged polymerization. Moreover, no need to perform "difficult frit production stage" for monolithic column because it is attached to column wall with silanization. In monolithic columns, higher separation efficiency is achieved with the same pressure drop since there are small-sized skeletons and no interparticular voids [4].

In liquid chromatography, according to property and polarity of analytes, different chromatographic modes are used. Although reversed phase chromatography, (RPC) is the most commonly used technique in liquid chromatography, the separation of polar analytes is difficult because of low interaction between nonpolar stationary phase and the polar analytes. A new technique called as Hydrophilic Interaction Liquid Chromatography (HILIC) is developed in order to separate polar analytes with high efficiency. A highly polar stationary phase and an aqueous less polar mobile phase are used for HILIC mode. Stationary phases used in HILIC mode are generally silica based. However, in highly acidic or basic conditions, polymeric materials are more stable than silica based materials. The development of new columns used in HILIC mode is a hot topic due to the fact that it is a new technique compared to other chromatographic modes.

1.2. Objective

The objective of this research is to synthesis of poly(3-chloro-2-hydroxypropyl methacrylate-co-ethylene dimethacrylate), poly(HPMA-Cl-co-EDMA) monoliths and to determine their capability of performing chromatographic analysis with nucleosides, nucleotides and organic acids as polary analytes in HILIC mode.

1.3. Scope

In this scope, new poly(HPMA-Cl-co-EDMA) monoliths are synthesized with block copolymerization into silica fused capillary columns. During synthesis stage, concentrations of porogenic solvents, ratio of cross-linking agent and polymerization temperature are changed until reaching the appropriate pore size distribution, specific surface area and back pressure.

After obtaining desired synthesis conditions for poly(HPMA-Cl-co-EDMA) monoliths, triethanolamine (TEA-OH), a chromatographic ligand used in HILIC mode, is covalently attached to monolith via "chloropropyl" functionality with a single step reaction. Characterization of synthesized monoliths is performed with scanning electron microscope (SEM), Brunauer-Emmet-Teller (BET) and Elemental analysis.

Ligand attached columns are used to separate polar organic compounds like nucleotides, nucleosides and organic acids. During this analysis, best conditions (ratio of mobile phase, pH and flow rate) are determined for each analysis. For each monolithic columns, Van-Deemter graphs and theoretical plate numbers are calculate via chromatograms. Finally, obtained results are compared with previous research in literature.

2. LITERATURE REVIEW

2.1. General

This chapter provides a general information about topics involved by this thesis. First, high performance liquid chromatography and its types were introduced. Then, development of monolithic columns and polymerization method were described. Finally, a brief literature review about hydrophilic interaction chromatography, used column types and general applications in this mode were given.

2.2. High Performance Liquid Chromatography (HPLC)

Chromatography is a technique that analytes in a mixture are separated from each other. For chromatographic separation, an analyte is sent to a stationary phase within the mobile phase. If this mobile phase is liquid, it is named as liquid chromatography (LC) and if mobile phase is a gas, it is termed as gas chromatography (GC) [5]. The main advantage of liquid chromatography is that the non-volatile and temperature sensitive compounds can also be analyzed.

High performance liquid chromatography (HPLC) is a type of liquid chromatography. A simplified schematically representation of HPLC system is given below in Figure 2.1. As indicated in Figure 2.1, HPLC consists of an injector, a pump, a column and a detector. HPLC can be performed at two different scales as analytical and preparative. Preparative HPLC is used for detection and purification of an analyte for further utilization, while analytical HPLC is used for qualitative and quantitative analyses of mixture of analytes. Preparative HPLC is out of scope for this work.
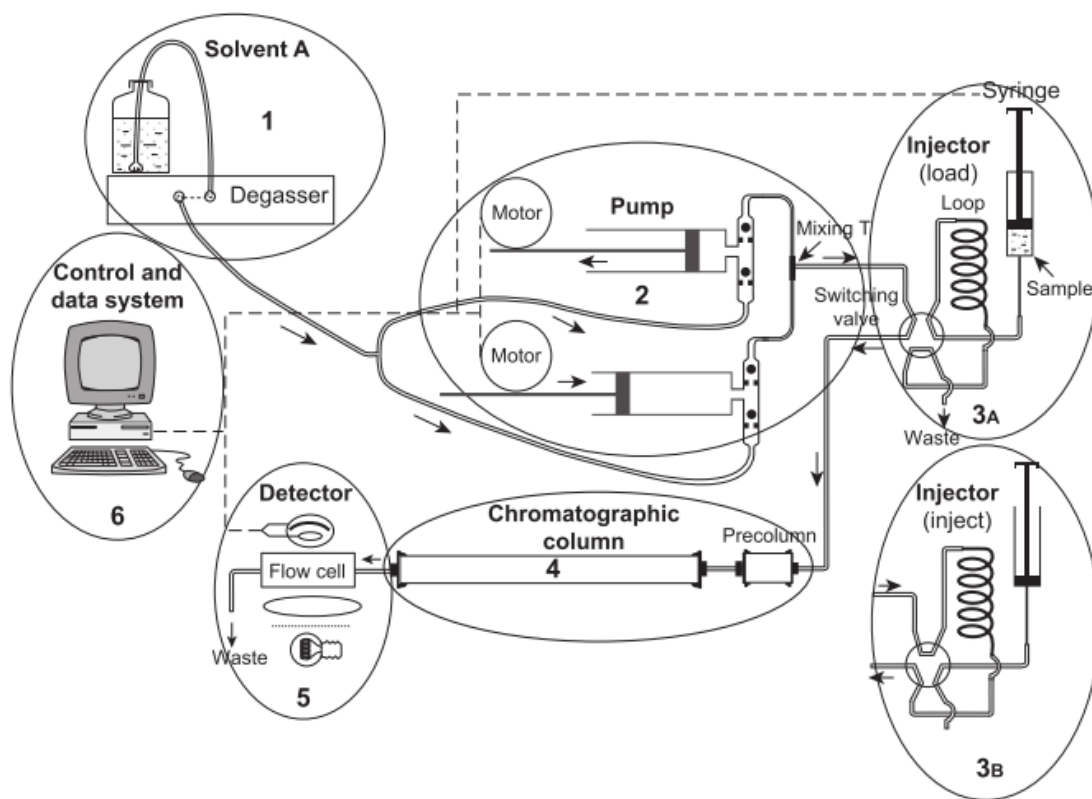
Figure 2.1. Schematic illustration of the HPLC system: 1) solvent container 2) pump 3) an injector (auto sampler) a) load loop b) inject loop 4) monolithic column 5) UV detector 6) a controller (data collecting software) [5].

Separation in HPLC based on the occurring equilibrium between the analytes in mobile phase and stationary phase. The main types of equilibrium is explained below [5];

Partition equilibrium can be explained as the retention of an analyte by the effect of interaction between the layer trapped on the surface of the stationary phase and the analyte in the bulk mobile phase. In the other words, they are separated from each other via their difference in the tendency to present in the stationary or the mobile phase.

In adsorption equilibrium, analytes are separated according to their chemical properties. It is adsorbed on the solid phase or remains in mobile phase. Separation of anionic or cationic species according to their ionic strength during stationary phase which have covalently bounded ionic groups is called as equilibria involving ions. In equilibria based on size exclusion, analytes are retained because of the difference between their molecular sizes. It takes more time when smaller molecules are passing smaller pores and longer way along the column. Larger molecules directly leave the column. Affinity interaction is used for the separation of more specific analytes like proteins and antibodies.

2.2.1. Nano-High Performance Liquid Chromatography (Nano-HPLC)

Introduction to the miniaturized liquid chromatography traced back to the years 1980-90. Some toxins and drug residues present in our bodies at very low concentrations at the beginning but accumulation of these toxins or residues with time can cause serious diseases. In quality control step of food and beverage industry, it is important to detect additives or micro-organisms even in nano levels. In such cases, it is essential to analyse very low concentrations in biological and environmental samples. Analysis which have this sensitivity become possible with nano-LC.

Nano-LC can be defined as where columns having 10-100 µm internal diameter are used and mobile phase flows in nL/min. Nano-HPLC is a modality of nano-LC that have been evolved to some extent [6].

Miniaturization of system in nano-HPLC brings some advantages in spite of the fact that its operating principles are the same when it is compared to the conventional HPLC systems. First of all, the reduction of internal diameter of column provides shorter analysis time and lower mobile phase consumption. In this way, the pollution due to hazardous liquids is minimized and the system becomes eco-friendly [6]. Moreover, lower flow rate makes easier the connection to mass spectrometer. In addition to these, the reduction of column I.D. decreases peak width. Thereby, peaks which have high-resolution and high efficiency are obtained. When these advantages are considered, it can be said that nano-HPLC is cheaper than conventional systems [3]. It is necessary to minimize zero dead volumes in the system and the sensitivity of detectors used should be increased.

2.2.2. Chromatographic Efficiency Parameters

Chromatographic performances of columns are described via some parameters like retention factor, resolution, theoretical plate number and theoretical plate height. These parameters are calculated by using equations given below. At the end of the analyses, these parameters are calculated for each of the analyte sent to the monolithic columns via the chromatograms obtained.

2.2.2.1. Retention factor (k)

Retention factor is used to describe the migration rate of an analyte on a column [7]. It is also called as factor of capacity and calculated as given below;

$$k = \frac{t_R - t_0}{t_0} \qquad\qquad (2.1)$$

Where t_R is the time taken until the analyte is reaches the detector after injected to the column. t_0 is the time taken during mobile phase pass through the column. In order to measure t_0, a non-retained analyte (e.g. toluene) is injected to column. Since it is not retained by the stationary phase, the analyte is eluted with mobile phase. As seen from equation, retention factor is a dimensionless parameter.

2.2.2.2. Resolution (R)

Resolution is used to describe how well two analytes are separated. It is calculated as given below;

$$R = \frac{2\left(t_{R_2} - t_{R_1}\right)}{W_1 + W_2} \tag{2.2}$$

Where the time between the injection of analyte and their peaks reaching detector are termed as the retention times of analyte 1 and 2 (t_{R_1} and t_{R_2}) respectively. W_1 and W_2 are peak widths of analyte 1 and 2. Illustration for retention time is given in Figure 2.2. In case of that resolution is equal to 1.0, two peak are overlap by about 4%. If resolution value is less than 1.0, it means that two peak are overlap each other. However, if it is greater than 1.5, it means that two peaks are well separated from each other [8]. In other words, base line separation is reached [7]. Resolution can be improved. It is possible by using two methods. One of these methods is increasing column length and the other method is decreasing the particle size.
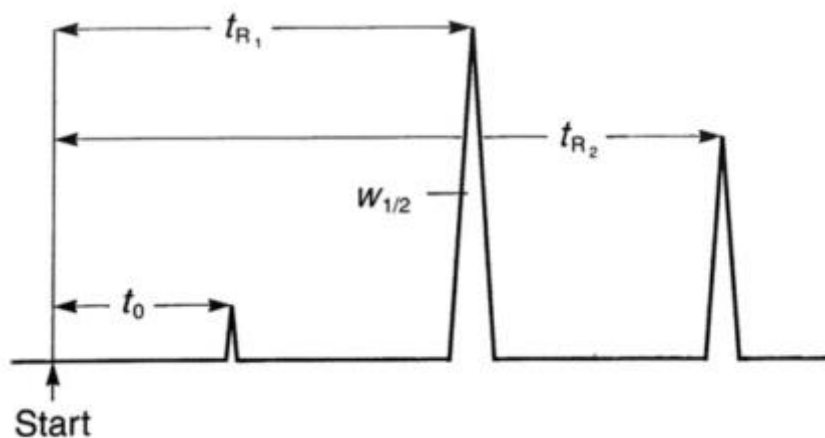
Figure 2.2: Characteristics of the chromatographic peaks [5].

2.2.2.3. Theoretical plate number (N) and Theoretical plate height (h)

Theoretical plate number is an important value to specify the efficiency of column. It is calculated as given below;

$$N = 5.54 \left(\frac{t_R}{W_{1/2}} \right)^2 \qquad (2.3)$$

Where $W_{1/2}$ is the peak width of half height. Theoretical plate number is proportional to the column length. In other words, efficiency of column increases by increasing length of column. For comparison purposes, theoretical plate numbers (N/m) are often used.

Theoretical plate height is another important value to specify the efficiency of column and denoted as h. It is calculated as given below;

$$h = \frac{L}{N} \qquad (2.4)$$

Where L is the column length. Theoretical plate height is inversely proportional to theoretical plate number.

2.3. Modes of High Performance Liquid Chromatography

There ise a number of types of liquid chromatography in literature. This classification is made by considering not only mechanism behind the separation but also stationary and mobile phase properties and various criteria. This part gives only the main classification of liquid chromatography.

2.3.1. Reversed Phase Chromatography (RPC)

Recently, it is the most commonly used HPLC mode because of its versatility and flexibility. Also, lots of compounds are able to be separated via RPC. It consists of a nonpolar stationary phase and a polar mobile phase. Stationary phase can be a silica based material nonpolar ligands are bonded on its surface (e.g. C18 bonded silica surface). The mechanism behind this separation is hydrophobic interaction with the stationary phase. Some additives (e.g. acetic acid, formic acid and trifluoroacetic acid) are used to obtain appropriate pH value for mobile phase. Retention time increases with increasing hydrophobicity of the analyte. Polar analytes are eluted first from column without being separated [8]. It can be operated in the isocratic, step gradient or continuous gradient modes.

2.3.2. Normal Phase Chromatography (NPC)

In normal phase chromatography, the polarities of the stationary and the mobile phase are reverse of that used in RPC. Mobile phase is not water soluble (e.g. hexane, tetrahydrofuran). Components are separated according to the difference in their polarity with surface adsorption mechanism. As a result of tendency of binding to hydrophilic stationary phase, the most polar component is eluted last.

2.3.3. Hydrophilic Interaction Chromatography (HILIC)

Hydrophilic interaction chromatography is used to separate polar analytes not to be separated by either NPC or RPC. It is such a technique where separation occurs by partitioning of analyte between water-enriched layer immobilized on hydrophilic stationary phase and less polar mobile phase [9]. Since all analyses are performed via HILIC mode in this study, it is described in detailed under a new title in following part.

2.3.4. Ion exchange Chromatography (IEC)

Ion exchange chromatography is frequently used for purification of proteins and other charged biomolecules. Analytes are retained according to coulombic (ionic) interactions between ionic functional groups attached onto the surface of stationary phase and analyte ions of opposite charge. This method divides into two sub-types as cation-exchange chromatography and anion-exchange chromatography.

Cation-exchange chromatography is used for the separation of cations via negatively charged ionic groups covalently bonded to a stationary support. Stronger cationic species are retained longer in column. Therefore, analytes are eluted in the order of strongest to weaker cationic strengths. Conversely, in anion exchange chromatography, anions are separated based on their different retention strengths. This time, positively charged ionic groups covalently bonded stationary phases are used. In both cases mobile phase generally consist of buffer solution [5].

2.3.5. Size exclusion Chromatography (SEC)

Separation of size exclusion chromatography is based on the difference of molecular mass or hydrodynamic volume of analytes. Stationary phase is an inert porous media which have different extents. Analytes having various molecular weights are carried by mobile phase through column. While large molecules elute earlier, small molecules penetrate every region of the stationary phase and elute late. This technique is generally used to separation and determination of molecular weight of synthetic polymers [10].

2.3.6. Affinity Chromatography (AC)

Affinity chromatography is used for separation and isolation of specific analytes from complex mixtures. It is based on biological function or individual chemical structure of molecules. Interaction between antibody and antigen, enzyme and substrate or enzyme and inhibitor can be given as an example. In this method, the target molecule should have well known and defined property to be used. Affinity ligand is attached onto the stationary phase and target molecules in the mobile phase are trapped by this ligand. These interactions are generally reversible. After undesired complex washed from media, target molecules are eluted by changing the pH, ionic strength or polarity [11].

2.4 Hydrophilic Interaction Liquid Chromatography

2.4.1. History

Hydrophilic Interaction Liquid Chromatography with the acronym HILIC was described by Alpert in 1990 for the first time [12]. It is the newest chromatographic mode among other liquid chromatography modes such as normal phase chromatography (NPC), reverse phase chromatography (RPC), size exclusion chromatography (SEC) and ion exchange chromatography (IEC). Even so, this method has been used since 1975. Linden et al. has generated this method for first time by using an amino-silica phase and aqueous acetonitrile as stationary and mobile phases for separation of carbohydrates [13]. Moreover, basics of this mode has been suggested and studied since 1952.

Hydrophilic Interaction Liquid Chromatography is referred to a variation of Normal Phase Liquid Chromatography (NPC), the oldest chromatographic mode. The reason of this, NPC is used to separate polar compounds with a polar stationary phase and a nonpolar mobile phase (e.g. hexane, tetrahydrofuran, chloroform). However, hydrophilic analytes like polar biochemical samples like carbohydrates, nucleotides, nucleotides and peptides cannot be dissolved in this non-aqueous mobile phase. In order to overcome this difficulty, mobile phase containing aqueous–organic mixture with water (e.g. aqueous acetonitrile, methanol, ethanol and isopropanol) is used and this new method is called as Hydrophilic Interaction Liquid Chromatography [14]. This replacement not only solve solubility problem but also provides higher reproducibility and convenience in interfacing with mass spectrometric detectors [15].

As indicated before, reversed phase chromatography (RPC) is a most widely used, powerful technique to separate a wide variety of compounds. However, polar analytes are first eluted from the column with less or no retention. In order to achieve adequate

retention for polar analytes in RPC highly aqueous mobile phase is needed and high water content collapses the stationary phase [16]. Aqueous mobile phase and highly hydrophilic stationary phase make polar compounds separation easier by increasing their retention times in the column.

After it has been realized that applicability on different types of polar biochemical, popularity of HILIC increases with time. The number of publications containing separation with HILIC principles from the year 1991 to 2015 is summarized in Figure 2.3 based on Web of Science. As it can be seen below, latterly the number of studies on HILIC is significantly increased.
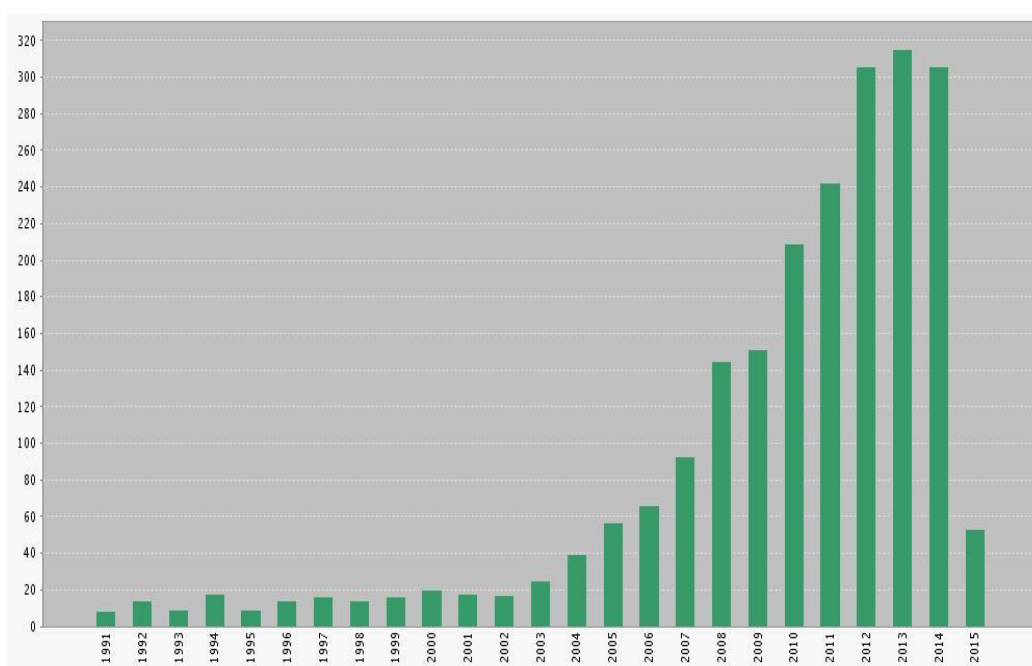
Figure 2.3: Number of publications indexed on Web of Science with term ''Hydrophilic Interaction Liquid Chromatography'' between 1991-2015 years.

2.4.2. Separation Mechanism

HILIC is such a technique, where polar groups on the stationary phase dynamically immobilize the water within the mobile phase and form a stagnant water-enriched layer. The representative illustration is given below in Figure 2.4. Analytes are separated via their tendency of existence in the bulk mobile phase or semi-immobilized aqueous layer on the stationary phase. Retention time increases with the polarity (hydrophilicity) of the analytes. The elution pattern is inverse to the RPC and analogous to NPC.
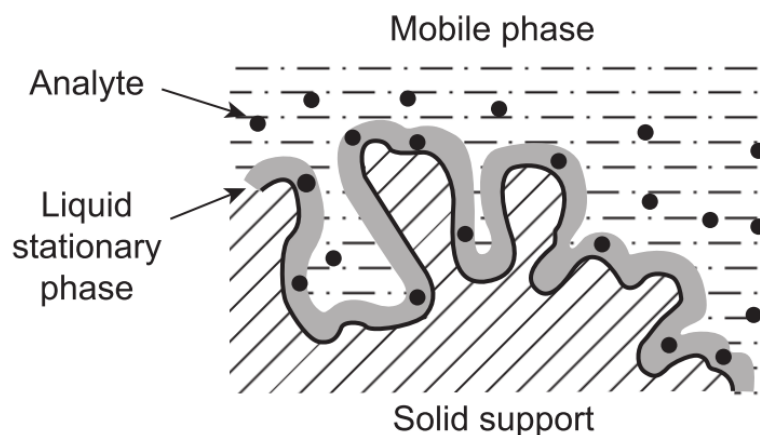
Figure 2.4. Schematic description of partition equilibrium [5].

Another distinction between the NPC and HILIC systems is their separation mechanisms. It is based on surface adsorption for conventional NPC while there is a partitioning between the bulk eluent and water-rich layer which is partially immobilized on stationary phase in HILIC [9]. Nevertheless, some multimodal separation mechanisms, involving hydrogen bonding, ion-ion, ion-dipole and dipole–dipole interactions are also suggested for HILIC [17]. Moreover, Hao et al. is stated that predominant retention mechanism can be varied with respect to the properties of analytes, mobile and stationary phases applied [18].

2.4.3. Types of Stationary Phases in HILIC

HILIC can be performed on a variety of silica-based or organic polymer-based stationary phases. Selection of suitable column type is a crucial step to achieve successful separation for HILIC. Types of stationary phases having different support materials and surface chemistries continuously increase. In HILIC, both particle-packed and monolithic columns are used. However, particle-packed columns is out of scope for this work.

Organic polymer-based monolithic column were introduced in the late 1990s and there were only a few studies for polymer based monoliths until 2007 in the HILIC mode. Limited number of polar monomers (commercially available) and need for optimization of new polymerization mixture because of limited solubility of monomer into commonly used porogens are causes of this. Most of these columns are polyacrylamide-based [16, 19, 20].

2.4.4. Applications of HILIC systems

There are many examples of HILIC applications on small polar molecules. Some examples are the separations of;

- Biomarkers
- Nucleosides

- Nucleotides
- Carbohydrates
- Amino acids
- Peptides
- Proteins
- Oligosaccharides
- Neurotransmitters
- Natural product extracts
- Polar pharmaceuticals
- Some small polar analytes that cannot be separated RPC mode

HILIC columns are used in pharmaceutical chemistry, agricultural and food chemistry, medicinal chemistry, proteomics, metabolomics, and glycomics.

In literature, it was seen that separation of nucleotide and nucleosides with HILIC was used as a model for polar compounds and not applied in real analyses. Nevertheless, it has found applications in biology, the food industry and medicine. The reason of this they are form part of RNA and DNA [21].

In biology, the determination and measurement of nucleosides in biological fluids (e.g., urine or serum) is an important subject. It is known that urinary nucleosides do serve as biomarkers of cancer and other diseases. Therefore, it has been proposed as a diagnostic and treatment-assessment method [22]. In a different case, study of receptor activity, HILIC method was used to determination of adenosine triphosphate (ATP) and cyclic adenosine mono phosphate (cAMP) in biological sample [23].

In food industry, nucleotides are identified as essential nutrients. Nucleotide monophosphates are added into cow's milk in order to make it equivalent to maternal milk. The study conducted by Inoue et al. showed that total nucleotides in infant formulas were reasonable and appropriate in comparison with referenced human-milk levels [24]. In addition to this, nucleosides and bases in *Ganoderma lucidum*, a nutritional mushroom, were investigated because of assessing the quality by Chen et al. [25].

In pharmaceutical chemistry, HILIC has been used to determine the nucleosides in flowers used as traditional Chinese medicine. For example, safflower is widely used in China for the treatment of cardiovascular and hypertensive disease. Guo et al. successfully

determined four nucleosides in Safflower (*Carthamus tinctorius L.*) [26]. After that, same research group was successfully determined twenty nucleosides in Ginko seed [27].

2.5. Monolithic Columns

A monolithic column is a single piece of a porous organic polymer or silica. It is different from particle packed columns due to its interconnected skeleton. This structure increases efficiency, owing to enhance mass transfer process by eliminating interparticle voids [19, 28]. Additionally, monoliths provide a number of advantages like no need for frits, simple preparation procedure, resistance to pH, low back pressure and high axial permeability when compared to particle packed columns [16].

Monolithic columns are used in different separation modes like RPC, IEC, HILIC, SEC, AC and molecular imprinting. Moreover, monoliths have also applicability in solid-phase extraction and sample preconcentration procedures [29].

2.5.1. History

Although the idea of single-piece separation medium was presented in late 1960s and early 1970s, first continuous media successfully used in chromatographic separation is a compressed polyacrylamide gel developed by Hjertèn in 1989. In the early 1990s, rigid macroporous polymer monoliths produced by Svec and Frèchet became the mainstream of research of monoliths [30]. Meanwhile, Nakanishi and Soga describe the preparation of porous silica monoliths via sol-gel method [31].

Since appearance of continuous media in literature, different names were suggested for this new generation of stationary phase like "continuous polymer bed", "continuous polymer rod", "porous silica rod", or "continuous column support". However, the term "monolith" was introduced by Wethen to describe a single piece of functionalized cellulose sponge used for protein separation in 1993 and rapidly adopted because of an easy-to-use single word [32].

Both organic polymer-based and silica-based monoliths were also been developed for HPLC applications. Previously, silica-based monoliths were used for the separation of small molecules. Currently, organic polymer-based monoliths were used for analysis of small polar molecules in HILIC mode [33]. In the following part, the chronological progress of HILIC mode monolithic stationary phases used in micro-HPLC or capillary liquid chromatography (cLC) were summarized.

2.5.2. Organic polymer-based monolithic columns for HILIC mode

In 1997, a preliminary work was done by Xie et al. for preparation of hydrophilic monolith. Monolith was manufactured via in situ bulk polymerization of two very hydrophilic monomers acrylamide and N,N'-methylenebisacrylamide. Its pore size was controlled by changing polymerization parameters. Results shows that monoliths are feasible to be used in separation of biomolecules but their chromatographic characteristics are not given [34].

Viklund and Irgum developed two monoliths having zwitterionic character in 2000. One of them was manufactured by copolymerization of SPE with EDMA and the other one was obtained by grafting SPE onto poly(TRIM) monolith. This zwitterionic property makes them suitable for separation of biopolymers. According to the test results conducted via proteins, separation mechanism was based on Coulombic interactions because of highly charged surfaces [35].

In another study, Hoegger and Freitag produced acrylamide-based monoliths in 2003. Columns were used for the separation of positively charged amino acids and peptides under both electrochromatographic and purely chromatographic (nano-HPLC) conditions. Retention mechanism of monoliths was investigated by several variables and the results demonstrated the columns were able to support both electrostatic and hydrophilic interactions according to salt concentration and organic solvent composition in the mobile phase [36].

Freitag prepared a set of hydrophilic monolithic capillary columns based on N,N-dimethylacrylamide (DMAA) or methacrylamide (MAA). The columns were tested both nano-HPLC and the CEC mode in order to improve the understanding of the underlying separation mechanism. The retention and separation of uncharged (polar hydroxylated aromatic compounds) and charged (amino acids) analytes were investigated. In the case of uncharged analytes, separation seemed mainly based on hydrophilic interactions. In the case of charged analytes, both electrostatic and hydrophilic interactions played a role in retention as expected [37].

In 2006, Hosoya et al. introduced the first example of a high-performance, polymer-based monolithic column for small molecules. Column was manufactured by using epoxy resin-based polymer, (TEPIC-BACM column). This column had HILIC property owing to a relatively hydrophilic polymer backbone. Moreover, hydrophilic solutes afforded longer

retention time at higher acetonitrile content. Moderately efficient separation of nucleosides was achieved in 90% aqueous acetonitrile [38, 39].

Another research group, reported hydroxymethyl methacrylate-based monolithic columns which consisting of the monomer mixture consisting of N-(hydroxymethyl) methacrylamide (HMMAA) and cross-linking agent ethylene dimethacrylate (EDMA). Oligonucleotides were successfully separated by capillary liquid chromatography (cLC) in HILIC mode. Separation was achieved under gradient elution in 35 min. Columns had a very good reproducibility of preparation. The silanization was not also necessary for them [40, 41].

Jiang et al. prepared a zwitterionic poly(SPE-co-EDMA) monolithic column. This stationary phase was produced by thermally initiated copolymerization inside a 100-μm-I.D. Capillary. Separation of nucleotides (neutral), acrylamides (basic) and benzoic acid derivatives (acidic) was successfully achieved in micro-HPLC system. HILIC mechanism was observed at higher than 60% ACN content in mobile phase. Its zwitterionic property provided not only hydrophilic interaction but also weak electrostatic interaction with analytes. In addition, the pH, ionic strength, or organic solvent concentration was used to optimize the separation method owing to the electrostatic interactions [16, 41].

Later, a novel polar stationary phase was introduced by Lin et al. in 2008. Monolith was manufactured by thermally initiated copolymerizing sulfonic SPMA monomer and PETA cross-linking agent. More hydrophilic media were obtained by replacing EDMA with PETA having hydroxyl groups in its structure. Again, HILIC mechanism was observed at higher than 70% ACN content in mobile phase. Column performance was evaluated via both capillary liquid chromatography (cLC) and pressure-assisted CEC systems. In cLC system, nucleotides were separated well with 70% ACN and pH 2.5 in 20mM triethylamine phosphate as the mobile phase due to both hydrophilic interaction and electrostatic interaction. The best theoretical plate number to be reached was greater than 105.000 plates/m for cLC [42].

In 2009, the same research group reported a new monolith manufactured by copolymerizing META as monomer and PETA as cross-linking agent. It was designed for performing capillary liquid chromatography (cLC). Hydrophilicity was changed by changing META composition within the polymerization mixture. This time, HILIC mechanism was observed at higher than 20% ACN content in mobile phase. Neutral compounds (such as amides and phenols), basic compounds (such as nucleic acid bases

and nucleosides) and acidic compounds (such as benzoic acid derivatives) were well separated on poly(META-co-PETA) monolithic column without obvious peak tailing [43].

In a different investigation, Zhong and El Rassi prepared four different diol methacrylate-based polar monolithic columns used in HILIC mode for nano-LC and CEC. They used GMM or GMA as monomer with either EDMA or TRIM as cross-linking agent. Among these four columns, GMM-EDMA monolith gave the best separation efficiency, retention and linear flow velocity in nano-LC [41, 44].

Jiang et al. introduced a new zwitterionic hydrophilic porous monolithic column poly(SPV-co-MBA). It was successfully used as a stationary phase in micro-HPLC for separation of five pyrimidines & purines and nine benzoic acid derivatives. An HILIC/RPC dual separation mechanism was observed where the transition from the RP to the HILIC mode was around 70% water in ACN. Although there was slight swelling and/or shrinking, no significant influence on the reproducibility was observed. However, low solubility of MBA in a porogen caused low column-to-column reproducibility. Results of optimized column were comparable selectivity values to commercial ZIC-pHILIC phases for polar test analytes despite of the column efficiency was still relatively low [45].

In a similar approach, Jiang et al. prepared a novel phosphorylcholine monolithic column by thermal co-polymerization of MPC and EDMA within 100 µm I.D. Capillaries. This phosphorylcholine functionality provided zwitterionic character to the column. Poly(MPC-co-EDMA) was tested in micro-HPLC. Again, An HILIC/RPC dual separation mechanism was observed where the transition from the HILIC to the RPC mode was around 60% ACN in water. A good separation of small hydrophilic peptides, which was not observed on previously introduced monolithic column by same research group, was achieved on the novel column owing to biocompatibility of phosphorylcholine functionality. No swelling or shrinking was observed on monolith [46].

Urban et al. reported new polymethacrylate monolithic columns manufactured via thermally initiated radical copolymerization of [2-(methacryloyloxy)ethyl]dimethyl-(3-sulfopropyl)ammonium hydroxide (MEDSA) and ethylene dimethacrylate (EDMA) for the use in cLC. Columns showed both separation modes: reversed phase chromatography (RPC) and HILIC at different organic solvent concentrations in aqueous–organic mobile phases. The selectivity of stationary phase was also changed by changing concentration of monomer and porogen in polymerization mixture. Toluene was selected as the column hold-up time marker in the HILIC mode for separation of polar phenolic acids [47].

Another polymethacrylate monolith for micro-HPLC was introduced by Skerikova and Jandera in 2010. Applications of this column were demonstrated on the analysis of phenolic acids in a beer sample. The effects of type and the concentration of the organic solvent in the aqueous-organic mobile phase (acetonitrile and methanol), the ionic strength of the acetate buffer and temperature on separation were investigated. According to results, the best separation efficiency was achieved at high acetate buffer ionic strength and temperatures 60–80 $^{\circ}$C [48].

In 2011, Chen et al. prepared poly(SPE-co-PEGDA) monolithic columns within 75 μm-id fused-silica capillaries via photo-initiated copolymerization in isopropanol and decanol as porogens. Again its zwitterionic characterization provided electrostatic interactions addition to hydrophilic interactions. Column performance was evaluated by testing it with the separations of amides, phenols, and benzoic acids. The monolith was successfully used in HILIC mode and an efficiency of over 75 000 plates/m was obtained [17].

Another zwitterionic organic monolith was introduced by Foo et al. in 2012. It was produced by thermally initiated free radical polymerization within 200 μm capillary column at 75 $^{\circ}$C. The effect of polymerization time on monolith structure was tested for 1, 2, 4, 8, and 12 h. 2 h of polymerization time was selected since it gave the best backpressure and optimal surface area for poly(SPE-co-BVPE) column. Optimized column was successfully applied for the separation of pyrimidines and purines. Separation was rapid. Obtained peaks were sharp, symmetrical and had high-resolutions [49].

Chen et al. reported a new hydrophilic poly(NAHAM-co-PETA) monolith for capillary liquid chromatography (cLC). It was prepared by using 100 μm I.D. capillary in the presence of PEG and DMSO as porogens. The influence of the ratio of monomer to cross-linking agent, the amount and molecular weight of PEG on porosity of monolith was investigated. The optimized polymer monolith successfully separate five nucleosides, seven benzoic acids and five aniline. For acrylamide, the column efficiency was estimated to be 87.000 plates/m [50].

Same research group reported a different monolithic column for HILIC mode. The resulted poly(MAA-co-EDMA) stationary phase had "hydrophilic" property since prepared with PEG and DMSO. However, if column was prepared by toluene and dodecanol, it had "hydrophobic" property. This new polymerization mixture was optimized to obtain a

column having homogeneous and continuous bed, narrow pore size distribution and appropriate permeability. Under HILIC mode, five nucleosides were baseline separated. For acrylamide, the column efficiency was suggested to be 48,000 plates/m. Finally, monolith was used for the separation of tryptic digests of bovine serum albumin (BSA) and it was concluded that hydrophilic poly(MAA-co-EDMA) monolith is capable for the separation of complex samples [51].

Lin et al. introduced a new polymer monolith with three modes of reverse-phase, hydrophilic and cation-exchange interaction. For this purpose, group optimized new polymerization mixture consist of GMA, VPBA and EDMA. The boronic acid groups on VPBA provided cation-exchange interaction. The obtained monolith had a relatively homogeneous porous structure, good permeability and mechanical stability. In cLC, various compounds (like benzene derivatives, phenols, anilines, alkaloids, and proteins) were separated to survey column performance. For benzene derivatives, 70,000–102,000 theoretical plates/m was obtained. In addition, it had a good mechanical stability and reproducibility [52].

In 2013, poly(SPP-co-EDMA) column was manufactured via thermal-initiated copolymerization in 100 µm capillary. Liu et al. investigated the effect of cross-linking agent on column structure and separation efficiency. It was seen that the increase in proportion of EDMA caused bigger micro-globules and higher porosity. Optimized column had good permeability, stability, and column efficiency. This group also selected toluene as the column hold-up time marker. Under optimized conditions, a satisfied separation of six phenols, six benzoic acid derivatives, four nucleotides (toluene, thymine, uracil, adenine, cytosine, and thiourea) and ascorbic and dehydroascorbic acids was obtained with 90% ACN, 75% ACN, 95% ACN and 80% ACN in the mobile phase, respectively. (Liu, 2013) [53].

In 2014, Guo et al. prepared two derivatized β-cyclodextrin functionalized monolithic columns via a "one-step" strategy. This method (click chemistry) provided uniform monolithic matrices, good mechanical strength and reproducibility. One of this monolith was sulfated and the other was methylated. Sulfated monolith was useful for separation of nucleotides in HILIC mode while methylated was used for the separation of nonpolar compounds and drug enantiomers in capillary RPC [54].

Yuan et al. prepared two zwitterionic sulfobetaine monolithic columns to be used in micro-HPLC [53, 55]. They were poly(SPDA-co-EDMA) and poly(SPDA-co-MBA) copolymers. Columns were compared previously prepared poly(SPE-co-EDMA) column. Among these three columns, poly(SPDA-co-MBA) monolith yielded the highest hydrophilicity property. This property also provided the longest retention and the highest column efficiency for polar compounds. Satisfactory results for permeability, reproducibility, mechanical and chemical stability were obtained. The final optimized poly(SPDA-co-MBA) monolith successfully separated a series of polar compounds (i.e. phenols, bases, benzoic acid derivatives, small peptides, urea and allantoin). For the analysis of bases, the column efficiency of 70,000 plates/m was obtained [53, 55].

2.5.3. Production of polymer-based monolithic stationary phases

When Hjertèn prepared polyacrylamide gel, production method was a complex process requiring multiple steps also including a compression process. After that, Svec and Frèchet reported much simplifier procedure for the fabrication of monolithic columns. It was an in situ, single step polymerization of organic monomers in porogenic solvent called as bulk polymerization [19]. Bulk (Mass) polymerization is a method of free radical (addition) polymerization. Before introducing the bulk polymerization method, it is more appropriate to describe the free radical polymerization.

Thermally initiated free radical (addition) polymerization is a chain reaction initiated via radicals. Free radicals are molecules having unpaired electrons. They areproduced by decomposing initiator triggered by an external input, such as elevated temperature or UV light. This polymerization has three fundamental steps: initiation, propagation and termination.

Initiation step is where free radicals form and adds monomer units.

$$I_2 \rightarrow 2I \cdot$$

$$I \cdot + M \rightarrow IM \cdot$$
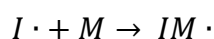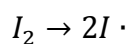
Propagation step is where length of chain increases. It is the major part of polymerization.

$$IM \cdot + M_n \rightarrow IM_{n+1} \cdot$$
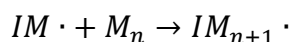
Termination step is where reaction ends.

$$IM_{n+1} \cdot + \cdot M_m I \rightarrow IM_{n+1} M_m I$$
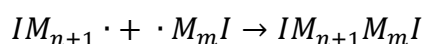
In bulk polymerization, the reaction mixture contains only initiator, monomer, cross-linking agent and porogens, no solvent. The capillary is filled with the monomer mixture. The mixture is liquid at the beginning because of the fact that all components should be dissolved each other. Then, it is heated in order to form a rigid porous polymer [19]. After initiation of reaction, the mixture become more viscous. At a later stages of polymerization reaction, nucleation and growth occurs. While molecular weight of nuclei increases, its solubility decreases in mixture. Finally, highly interconnected "cauliflower-like" polymeric material fills capillary [33]. In the synthesis of most monolithic column for HILIC, thermally initiated copolymerization is used instead of photo-initiated copolymerization [17].

3. EXPERIMENTAL PROCEDURE

3.1. General

Information about the test equipment and the materials used in polymerization, ligand attachment and analysis are given in this chapter. Column pretreatment and production procedures are also described step by step in the following headlines.

3.2. Instrumental

A nano liquid chromatography (nano-LC) system (Dionex, Ultimate 3000, A.B.D.) was used for the chromatographic experiments. A software system (Chromeleon, Version 6.8) was used to control nano-LC system and process data. A temperature-controlled water bath was used for the thermally initiated copolymerization. All chromatographic measurements were carried out at room temperature (22°C). It was required to reduce the flow rate from μLmin^{-1} to nLmin^{-1}. Therefore, a splitting device (a splitter) with a typically 100:1 split ratio was installed before the injector. There were some instrument modifications were needed. First, dead volumes in HPLC system should be minimized. For this purpose, P-704 and P-742 zero dead volume (ZDV) unions (Upchurch, PEEK, 1/16" OD tubing) were used to connect the column to the injector and detection cell. Column was installed to the system via PEEK sleeve and finger-tight fittings. Also, all units of HPLC were coupled by PEEK tubing having 65 μm ID (Upchurch). Second, detector sensitivity should be increased in order to be able to detect analyte volume in nano level. A 45-nL flow cell was applied for all runs, unless otherwise noted. Wavelength of 270 and 260 nm was used for detection.

3.3. Materials

The silanization reagents and solvent, sodium hydroxide (NaOH), methanol (Met-OH) were supplied from Riedel de Haen, Germany and 3-(trimethoxysilyl)propyl methacrylate (TMSPM) was obtained from Sigma Aldrich Co., A.B.D. In the synthesis of monolithic columns, 3-chloro-2-hydroxypropyl methacrylate (HPMA-Cl) and ethylene glycol dimethacrylate (EDMA) supplied from Aldrich Chem. Co., U.S.A. were used as the main monomer and the cross-linking agent respectively without further purification. Two alcohols used as porogens, isopropanol (Iso-PrOH) and n-decanol (Dec-OH) were purchased from Aldrich Chemical, USA and BDH Chemicals Poole, England, respectively. As an initiator, 2,2'-azobisizobutyronitrile (AIBN) was supplied by Across Organics (England) and recrystallized with methanol prior to use. After synthesis of monoliths, the solvents, absolute ethanol and HPLC-grade acetonitrile (Riedel de Haen, Germany) were

used as supplied in order to remove the polymerization media. For post-functionalization of monolith, the ligand, triethanolamine (TEA-OH) and solvent, N,N-dimethylformamide (DMF) (Aldrich Chemical, U.S.A.) were used without further purification. Ultrapure deionized water was prepared with Milli-Q water purification system (Millipore, Milford, MA) and used in all experiments. The polyimide coated fused silica capillary (100 μm i.d. and 360 μm o.d.) where polymeric monolith was synthesized, was purchased from Polymicro Technologies (Phoenix, AZ, USA). In chromatographic applications, nucleotides (uracil, adenine, cytosine and guanine), nucleosides (thymidine, uridine, inosine, cytidine) and benzoic acid derivatives (4-toluic acid, 4-aminobenzoic acid, 4-chlorobenzoic acid, 4-iodobenzoic acid) obtained from Aldrich were used. All other reagents were of analytical or special gradient.

3.4. Column pretreatment

To be able to attach the polymer onto the column wall, the inner surface of capillary should be made suitable for monolith synthesis. This procedure is called as silanization. Silanization forms bonds between an organic (polymer) and an inorganic surface (column wall) and attaches them to each other. The modification of capillary surface with γ-MAPS or TMSPM (3-(trimethoxysilyl) propyl methacrylate) was first reported by Hjertèn et al [19]. In order to provide anchoring sites for the polymer, the internal surface of fused silica capillary was treated with, TMSPM. To this end, 40 cm in length polyimide coated fused silica capillary, connected to the syringe pump, was treated with 0.2 M NaOH solution for 1.5 h. After that, capillary was flushed with deionized water and methanol for 30 min at a flow rate of 500 μL/min in this order. Then, capillary was dried with flowing nitrogen gas for 30 min. The aim is forming hydroxyl groups on the surface of silica inner wall. Then, the capillary flushed with mixture of TMSPM- methanol solution (50/50, v/v) for 30 min at a flow rate of 500 μL/min. After that, both ends of the capillary were sealed by GC septa after flow was stopped. Then the column was completely filled with the silanization solution. Capillary was kept at 35°C for 20 h by placing in a temperature-controlled water bath. In this step, alkoxy groups of TMSPM are displaced with hydroxyl groups on inner surface and forms chemically attached reactive methacrylate groups on the silica wall. In order to remove silanization mixture, capillary was washed with methanol for 10 min. Finally, the capillary was dried with nitrogen.

3.5. Monolith synthesis

The chemical structures of monomer and cross-linking agent used for the synthesis of capillary monoliths via block copolymerization are shown in Figure 3.1.
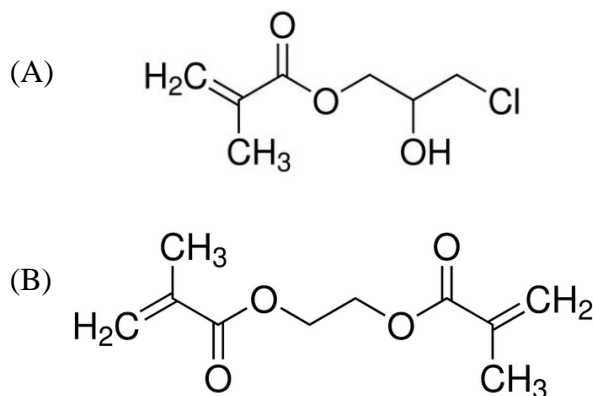
Figure 3.1. Monomer and cross-linking agent (A) HPMA-Cl (B) EDMA

When choosing the monomer, it was considered that monomer can be post-functionalized with different functional ligands after polymerization. HPMA-Cl containing a reactive chloropropyl group satisfying this demand. Typically, the monomer phase was prepared by mixing an appropriate volumes of HPMA-Cl and EDMA in a pyrex glass tube with 7.0 mm i.d.. The volume of monomer phase was kept constant at 1 mL. The concentration of monomer in polymerization mixture is important because of its effect on density and efficiency of column [33]. Therefore, the volumetric ratio of monomer and cross-linking agent was adjusted as 60/40 (v/v) in order to obtain a suitable structure. Iso-PrOH (short-chain alcohol) and Dec-OH (long-chain alcohol) were selected as microporogen and macroporogen respectively for the binary porogen solvent system. The porogen mixture was added into glass tube and mixed with monomer phase. Finally, the thermal initiator, AIBN (20 mg) was dissolved in this mixture. Polymerization mixture was sonicated in ultrasonic bath at 200 W for 5 min to obtain a homogenous solution. After that, capillary electro chromatography system (CEC, Prince, Model 760, Holland) was used to fill the (40 cm length) pre-treated fused silica capillary. Input vial of CEC was filled with the prepared mixture and the mixture was passed through the capillary tubing by applying 1 bar pressure, for 5 min. After both ends were sealed with GC septa, the capillary was placed in a temperature-controlled water bath to keep at the polymerization temperature overnight. The synthesis was performed at two different temperatures, 50 and 60°C. The rest part of the mixture in a glass tube sealed by GC septa, was also placed in a temperature-controlled water bath to obtain the bulk monolith. Then, it was used for the specific surface area

measurement and elemental analysis. The properties of poly(HPMA-Cl-co-EDMA) monoliths synthesized at 50 and 60°C are given in Table 3.1 and Table 3.2.

Table 3.1. Polymerization mixture of poly(HPMA-Cl-co-EDMA synthesized at 50°C.

Monolith Code	HMPA-Cl (µl)	EGDMA (µl)	Iso-PrOH (µl)	Dec-OH (µl)	M/P Ratio	AIBN (mg)
TH-1	600	400	1300	1700	3.0	20
TH-2	600	400	1350	1650	3.0	20
TH-3	600	400	1400	1600	3.0	20
TH-4	600	400	1080	1620	2.7	20
TH-5	600	400	1170	1530	2.7	20
TH-6	600	400	1400	1300	2.7	20

Table 3.2. Polymerization mixture of poly(HPMA-Cl-co-EDMA) synthesized at 60°C.

Monolith Code	HMPA-Cl (µl)	EGDMA (µl)	Iso-PrOH (µl)	Dec-OH (µl)	M/P Ratio	AIBN (mg)
TH-7	600	400	1300	1700	3.0	20
TH-8	600	400	1350	1650	3.0	20
TH-9	600	400	1400	1600	3.0	20
TH-10	600	400	1080	1620	2.7	20
TH-11	600	400	1170	1530	2.7	20
TH-12	600	400	1220	1480	2.7	20
TH-13	600	400	1270	1430	2.7	20
TH-14	600	400	1704	696	2.4	20
TH-15	600	400	1296	1104	2.4	20
TH-16	600	400	960	1440	2.4	20
TH-17	600	400	1040	1360	2.4	20
TH-18	600	400	1090	1310	2.4	20
TH-19	600	400	1140	1260	2.4	20

As seen from these tables, the variety in the structure of monolith was caused by difference between compositions of the compounds of binary porogen system since the monomer to cross-linking agent ratio was kept constant. After the synthesis was completed, the capillary monolith was washed with absolute ethanol to remove the porogenic solvent for 1 hour at maximum flow rate to be reached within workable pressure limits in the nano-LC system (Dionex, Ultimate 3000, A.B.D.). Finally, the column was washed with water at previous conditions and prepared for derivatization step.

3.6. Derivatization of monolith

In this step, poly(HPMA-Cl-co-EDMA) monoliths were reacted with chromatographic ligand, TEA-OH carrying cationic hydrophilic groups. The purpose was producing a stationary phase, suitable for hydrophilic interaction chromatography. The procedure of attachment was described below:

3.6.1. Triethanolamine (TEA-OH) attachment

The monolithic capillary column was washed with DMF for 1 hour. Meanwhile, the ligand solution was prepared by dissolving 15 mL of TEA-OH in 15 mL of DMF. Then, column was placed in a temperature controlled water bath at 80°C, and flushed with this solution for 16 h at a flow rate of 0.05 µl/min approximately. The ligand attached poly(HPMA-Cl-co-EDMA) columns were washed with ultrapure water for 2 h to remove the excess reagent and solvent used in the derivatization. After this stage, column became ready for chromatographic applications.

For elemental analysis and specific surface area measurements, the bulk monolith, polymerized in pyrex glass tube, was ground into the powder form and extensively washed with ethanol. It was dried at 60°C in oven for overnight. 0.5 g of powder was dispersed in 10 mL of reaction medium, identical to those used in the derivatization of capillary monoliths, by ultra-sonication and reacted at given conditions specified to selected ligand.

3.7. Monolith characterization

For the characterization of monolithic columns, the following methods were used. For the purpose of observation of morphology of monolithic columns, a piece of monolith approximately 4 mm in length was cut and attached onto pin horizontally to be able to observe the cross section. Then, all pieces were coated with a 10 nm layer of gold by a sputter-coater system. The pins were placed into Scanning electron microscopy (SEM, FEI

Quanta 200 FEG, USA) and the images of the cross section of capillary monolith were taken with the magnifications of 2000x and 8000x.

To characterize the mechanical performance and permeability of the monoliths, the back pressures of the monoliths at different flow rates were recorded in a nano liquid chromatography (LC) system (Dionex, Ultimate 3000, A.B.D.) in an isocratic mode. The mobile phase is (90/10 mL/mL) acetonitrile/water solution containing 0.05 % v/v acetic acid.

While measuring the specific surface area of monolith, the nitrogen adsorption-desorption method was used via pore size analyzer apparatus (Quantachrome, Nova 2200E, U.K.). Calculations were performed according to BET equation. After derivatization of monoliths with TEA-OH ligand, the attachment proportion was determined by elemental analysis (i.e. N or S determination).

3.8. Hydrophilic Interaction Liquid Chromatography (HILIC) runs

All experiments were performed on a nano-LC system (Dionex, Ultimate 3000, A.B.D.) at room temperature ($22^{o}C$). During the chromatographic runs, columns TH-16 and TH-17 were tested in HILIC mode. The columns were connected to the system and washed by using mobile phase used during test at appropriate flow rate until base-line became stable. This step was repeated before each test having different separation condition. By the way, accurate retention time determination was ensured. Experiments were performed in isocratic conditions. Acetonitrile-ammonium acetate buffer mixture and acetonitrile-water mixture prepared at various volume ratios were used as the mobile phase. The pH of mobile phase was adjusted to 4.5 via acetic acid (approximately 0.05% v/v). Performance tests carried out with three sets of sample mixtures containing four different polar analytes and one marker (Set 1: toluene, adenine, uracil, cytosine, guanine, Set 2: toluene, cytidine, thymidine, uridine, inosine and Set 3: toluene, p-toluic acid, p-aminobenzoic acid, 4-chlorobenzoic acid, 4-iodobenzoic acid.) The structures of used analytes structures were given in Figure 4.5. The injection volume was 100 nL. As indicated before, the peak detection was performed via diode array detector (DAD), a type of UV detector, operated at 270 and 260 nm and a 45-nL flow cell. A software system (Chromeleon, Version 6.8) was used to control nano-LC system and process data.

4. RESULTS AND DISCUSSION

4.1. General

The properties of monolithic columns (i.e. surface area, permeability, morphology etc.) were given in this chapter. In addition, the results of chromatographic applications like separation of nucleotides, nucleosides and benzoic acid derivatives were given and discussed in the following headlines.

4.2. Characterization of poly(HPMA-Cl-co-EDMA) monoliths

It is essential to control the morphology of monolith in order to control the chromatographic performance [56, 57]. The structure of monolith is influenced by polymerization mixture composition. In the preparation of poly(HPMA-Cl-co-EDMA) monolithic columns, the ratio of porogen to monomer, the composition of binary porogenic solvent system and the temperature were changed. In order to investigate the physical and chemical properties of the monoliths prepared in this study, SEM, nano-HPLC, a surface area and pore size analyzer apparatus based on the nitrogen adsorption-desorption method, were used.

Poly(HPMA-Cl-co-EDMA) monoliths having different porous properties were synthesized as an alternative reactive starting material. For this purpose, three sets of monolithic columns, where the volume ratio of porogen to monomer was altered between 2.4 to 3.0 v/v, were prepared keeping the volume ratio of monomer to cross-linking agent (i.e. HPMA-Cl/EDMA) was adjusted to an appropriate value (i.e. 60/40 v/v) with respect to the preliminary syntheses as it can be seen from Tables 4.1 and 4.2.

Porogen composition is a remarkable variable affecting on the monolith porosity due to its minor variation greatly changes inner structure of the monolith. In addition to this, for each porogen/monomer volume ratio, microporogen concentration in the binary porogen mixture was changed between 40-51 % v/v. In this study, the mixture of two alcohols was selected as the binary porogen mixture. This mixture contains a short chain alcohol, Iso-PrOH and a long chain alcohol, Dec-OH as the microporogen and macroporogen.

Temperature is also an important parameter affecting on the porosity of monolith. It is known that at a higher temperatures, the initiator decomposes faster. It causes small nuclei formation in larger quantities. So, smaller globules resulted smaller pores. Hereby, higher temperatures provides larger surface area but smaller permeability values [33]. Temperature for the preparation of polyacrylate based monoliths, commonly used is $60^{o}C$

in the literature [47, 50, 51]. Therefore, all TEA-OH attachment experiments in each set were performed at these temperatures.

The porous structure of monoliths was determined by using SEM photographs given below (Figure 4.1-2). Images of the cross section of capillary monolith were taken with the magnification of 2000x. From images, it can be seen that poly(HPMA-Cl-co-EDMA) matrix was fully formed and attached to the inner wall of silica capillary column. Moreover, the monoliths possessed different porous structures. The SEM photographs of columns TH-1, TH-5, TH-7 and TH-11 are given in Figure 4.1. For all columns, the microporogen concentration, Iso-PrOH in the binary porogen mixture was fixed to 43.3 % v/v. Monolithic columns TH-1 and TH-7, exemplified with SEM photographs in left side of Figure 4.1, have 3.0 v/v of porogen/monomer ratio. Monolithic columns TH-5 and TH-11, exemplified with SEM photographs in right side of Figure 4.1, have 2.7 v/v porogen/monomer ratio. Moreover, the images in the top row of Figure 4.1 were obtained from the columns synthesized at 50°C while the images in the bottom row were obtained from columns synthesized at 60°C.
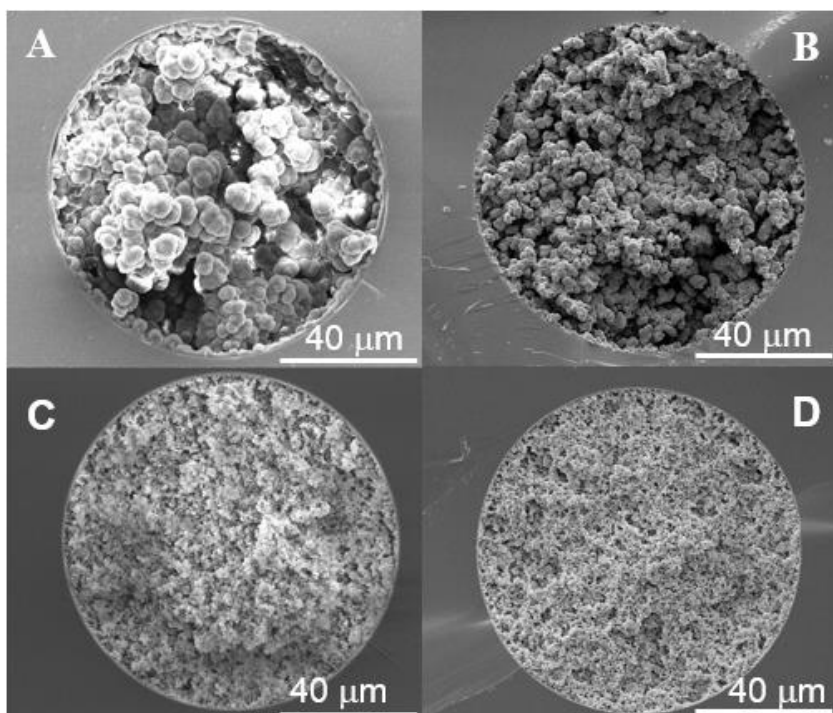
Figure 4.1. SEM photographs of poly(HPMA-Cl-co-EDMA) monoliths synthesized at different temperatures with Iso-PrOH concentration (v/v %): 43.3 and different porogen/monomer ratios. Temperature (°C) and porogen/monomer ratio (v/v): (A) 50 and 3.0, (B) 50 and 2.7.

As seen here, a macroporous structure containing crater-like voids was observed between agglomerates having approximately 1 μm size in the top row. However, as it can be seen from the bottom row, both agglomerates and average pore size significantly became smaller when the temperature was increased from 50 to 60°C at both porogen/monomer ratio. In the case where the temperature is constant, decreasing porogen/monomer ratio caused also a decrease in the aggregate size (i.e. pore size). As a result of these, it can be expected that the smallest agglomerates and pore size were observed in poly(HPMA-Cl-co-EDMA) monoliths (Columns TH-17, TH-18 and TH-19) synthesized with the lowest porogen/monomer ratio (i.e. 2.4 v/v) at 60°C. The SEM photographs of columns are given below in Figure 4.2.
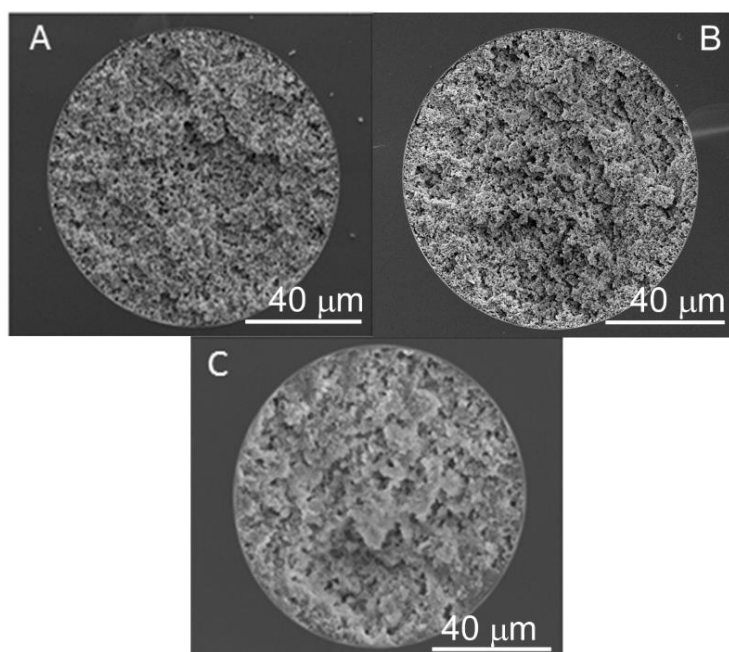
Figure 4.2. SEM photographs of poly(HPMA-Cl-co-EDMA) monoliths synthesized at 60°C with the porogen/monomer ratio of 2.4 v/v and with different Iso-PrOH concentrations. Iso-PrOH concentration (v/v %): (A) 40.0, (B) 43.3, (C) 45.4, Magnification: 2000x.

To characterize the mechanical performance and permeability of the monoliths, the back pressures of the monoliths at different flow rates were measured in nano-liquid chromatography system (Dionex, Ultimate 3000, A.B.D.) in the isocratic mode by using a typical mobile phase for hydrophilic interaction chromatography (90/10 mL/mL: ACN/Water solution containing 0.05% AAc). In these runs, the flow rate was increased until reaching the upper pressure limit (35 MPa) of system. Measured back pressure versus

the flow rate values for the monolithic columns is plotted in Figure 4.3. It could be possible to reach 100 µL/min flow rates with the columns produced at 50°C (Fig. 4.3A). But the maximum flow rate was only 1 µL/min for the columns produced at 60°C (Fig. 4.3B). The reason is lower permeability of the monoliths synthesized at 60°C resulted in reasonably higher back-pressure.
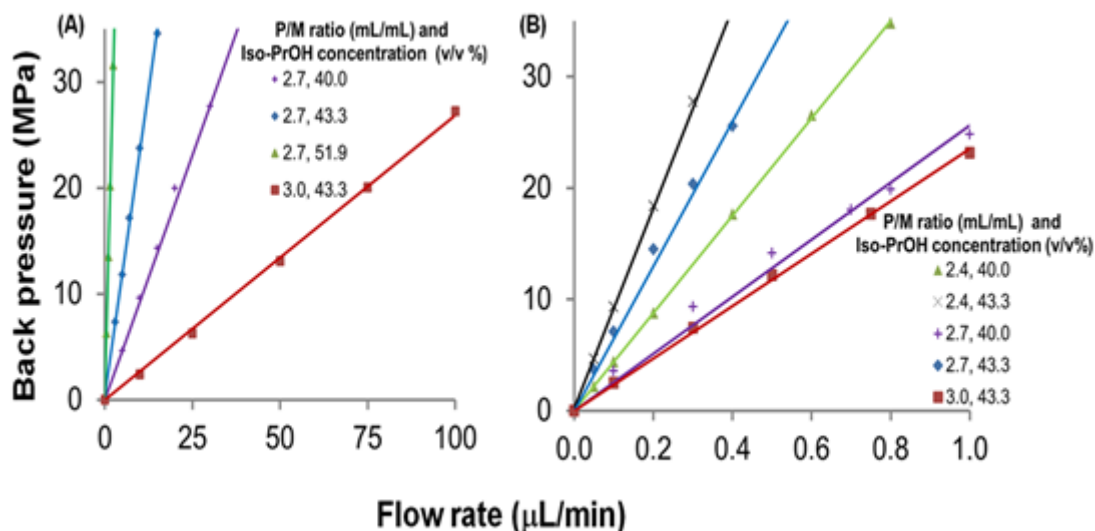
Figure 4.3. The variation of back pressure with the flow rate for poly(HPMA-Cl-co-EDMA) monoliths obtained at different temperatures, with different porogen/monomer ratios. Polymerization temperature (°C): (A) 50, (B) 60. The mobile phase was 90/10 v/v ACN/water solution containing 0.05 % v/v acetic acid. Column: 360 mm x100 µm i.d.

As shown in Figure 4.3A and B, back-pressure behaviors can be changed with both microporogen concentration and porogen/monomer ratio at constant temperature. While porogen/monomer ratio was kept constant, an increase in the microporogen concentration cause an increase in the back-pressure (i.e. the straight lines sketched for the porogen/monomer ratio of 2.7 v/v in Figure 4.3A). In contrast, decreasing porogen/monomer ratio cause an increase in the back-pressure (i.e. the straight lines sketched for the microporogen concentration of 43.3 % v/v in Figure 4.3B). Appearing of linear plots (back-pressure vs. flow rate) showed that porous structure of poly(HPMA-Cl-co-EDMA) monoliths were mechanically stable to withstand the pressure and no appreciable deformation occurred.

The specific surface areas (SSAs) and permeability values of poly(HPMA-Cl-co-EDMA) monoliths are listed in Table 4.1 and 4.2. The specific surface area plays important role in column efficiency [40]. As indicated before, it was measured by the nitrogen adsorption-

desorption method and the calculations were performed according to BET equation. The permeability values were calculated as described in following pages.

Permeability can be described as the inverse of resistance to mobile phase flowing through the monolithic column. Its measurement was performed by micro-HPLC system under constant flow of mobile phase and it is calculated by Darcy's Law according to following equation (Equation 4.1).

$$K = \eta \frac{uL}{\Delta P} \qquad (4.1)$$

Where u (m/s) is the linear velocity and η is the dynamic viscosity of mobile phase (90/10 v/v: ACN/Water at 25°C). L is the column length and ΔP is the pressure drop across the monolithic column (Pa) [16, 51].

Table 4.1. Synthesis conditions and properties of poly(HPMA-Cl-co-EDMA) monoliths synthesized at 60°C with different HPMA-Cl feed concentrations and porogen/monomer ratios.

Monolith Code	Temperature (°C)	Porogen/Monomer Ratio (v/v)	Iso-PrOH concentration (v/v %)	Specific Surface Area (m²/g)	Permeability (m²) x 10¹³
TH-7	60	3.0	43.3	9.3	0.15
TH-8	60	3.0	45.0	10.1	0.10
TH-9	60	3.0	46.7	16.8	0.07
TH-10	60	2.7	40.0	21.5	0.14
TH-11	60	2.7	43.3	25.0	0.05
TH-12	60	2.7	45.2	30.8	Impermeable
TH-13	60	2.7	46.7	39.3	Impermeable
TH-14	60	2.4	71.0	22.3	0.210
TH-15	60	2.4	54.0	28.3	0.119
TH-16	60	2.4	40.0	29.8	0.099a
TH-17	60	2.4	43.3	36.3	0.049a
TH-18	60	2.4	45.4	43.6	Impermeable
TH-19	60	2.4	47.5	52.2	Impermeable

Table 4.2. Synthesis conditions and properties of poly(HPMA-Cl-co-EDMA) monoliths synthesized at 50°C with different HPMA-Cl feed concentrations and porogen/monomer ratios.

Monolith Code	Temperature (°C)	Porogen/Monomer Ratio (v/v)	Iso-PrOH concentration (v/v %)	Specific Surface Area (m²/g)	Permeability (m²) x 10¹³
TH-1	50	3.0	43.3	6.2	12.29
TH-2	50	3.0	45.0	9.5	10.04
TH-3	50	3.0	46.7	15.7	8.94
TH-4	50	2.7	40.0	4.4	3.68
TH-5	50	2.7	43.3	7.7	1.49
TH-6	50	2.7	51.9	22.9	0.27

As shown in Table 4.1, the use of relatively higher porogen/monomer ratio at 50°C generated monoliths having too high porosity (or permeability) and low specific surface area. Therefore, these monolithic columns were not suitable for pressure-driven liquid chromatographic applications. It can be expected that higher specific surface areas and lower permeability values for the monoliths synthesized at 60°C since an increase in the synthesis temperature causes a decrease in the average pore size, ergo the specific surface area increases as explained before.

Nevertheless, the monoliths synthesized by using the porogen/monomer ratio of 2.4 v/v were in the same order of magnitude with those used for HILIC and reversed phase separations in nano and micro-liquid chromatography systems [40, 52, 58-63]. For this reason, Column TH-16 and TH-17 were used as the base material for the new stationary phases for nano-HILIC. According to these tables, it can be concluded that monolithic columns having between 45% and 47% (v/v) microporogen concentration were impermeable.

In addition to these, a new set poly(HPMA-Cl-co-EDMA) monoliths were synthesized with different specifications for the attachment of hydrophilic chromatographic ligand (TEA-OH). Ligand contents, SSAs and permeability values of new set are given in Table 4.3. The reactive character of "chloropropyl" group on monomer allowed the covalent

attachment of TEA-OH by simple and single-stage reaction. The attachment of ligand is described in Figure 4.4.
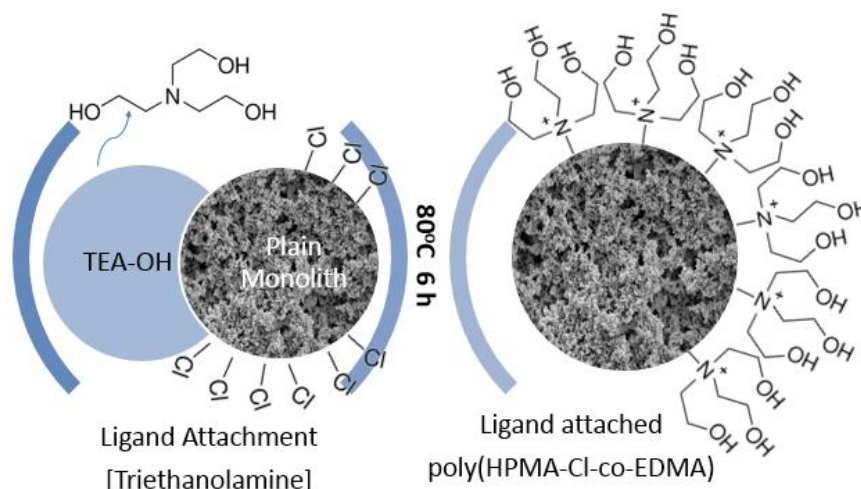
Figure 4.4. The chemical route used for the derivatization of poly(HPMA-Cl-co-EDMA) monolith using TEA-OH.

In the new set, the porogen/monomer ratio in the reaction mixture was fixed to 2.4 v/v for all columns. On the other hand, the monomer/cross-linking agent and the microporogen/macroporogen ratios were changed separately. Basically, HPMA-Cl concentration was changed between 40-60 % v/v. According to this change, an appropriate microporogen concentration was selected by aiming to obtain a satisfactory permeability.

Table 4.3. Ligand contents, specific surface areas and permeability values of derivatized monoliths.

Monolith Code	HPMA-Cl (v/v %)	P/M Ratio (v/v)	Iso-PrOH concentration (v/v %)	Ligand Content (mg/g)	Specific Surface Area (m²/g)	Permeability (m²) x 10¹³
TH-20	40	2.4	71.0	118.3	22.3/21.3	0.210/0.112
TH-21	50	2.4	54.0	129.1	28.3/22.9	0.119/0.097
TH-16	60	2.4	40.0	142.8	29.8/26.2	0.099/0.082
TH-17	60	2.4	43.3	137.2	36.3/30.3	0.049/0.046

From Table 4.3, it can be seen a remarkable decrease in the permeability with increasing HPMA-Cl concentration for the first three syntheses. This finding can also be explained as the reduction of cross-linking agent concentration into the monomer mixture (i.e. HPMA-Cl and EDMA mixture) cause to decrease of porosity of monolithic structure. On the other hand, the reduction of cross-linking agent concentration into the monomer mixture causes

an increase in the ligand content of monolith. The reason is the stationary phase containing more chloropropyl moiety to which TEA-OH can be covalently attached.

Based on these results, for the purpose of obtaining poly(HPMA-Cl-co-EDMA) monoliths having higher specific surface area and ligand content, the fourth column (Column TH-17) was synthesized with the highest HPMA-Cl concentration (i.e. 60 % v/v) and higher microporogen concentration (i.e. 43.3 % v/v) with respect to that used in the previous set. Naturally, permeability was decreased by using a higher microporogen concentration even if the SSA was further increased. The morphology of the monolith after the ligand attachment was also investigated by SEM but no significant difference was observed.

4.3. Chromatographic applications of poly(HPMA-Cl-co-EDMA) monoliths in HILIC mode

Besides the SEM images, chromatographic behaviors of the columns were also examined in HPLC. In this part, the chromatographic performances of monolithic columns were investigated and the optimum separation conditions for each analyte groups were determined. All columns were equilibrated with the mobile phases prior to use. Poly(HPMA-Cl-co-EDMA) surface can provide a hydrophilic environment due to its hydroxyl functionality. Among the optimized and derivatized monoliths, Column TH-16 was the most hydrophilic one. Because it had the highest functional monomer (HPMA-Cl) concentration (i.e. 60 % v/v) in polymerization mixture where hydroxyl content coming from. Its hydrophilic character makes easier to use for HILIC mode as a base material. In the derivatized form, Column TH-16 also had the highest TEA-OH content that covalently attached "chloropropyl" group on monomer as it is shown in Table 4.3.

To demonstrate the chromatographic performance of the poly(HPMA-Cl-co-EDMA) monolith, a group of polar compounds including some nucleotides, nucleosides and benzoic acids were selected as the analytes. The separation of these analytes is very difficult on the reversed-phase liquid chromatography mode. The chemical structures of analytes used for the evaluation of chromatographic performance of monoliths are shown in Figure 4.5.
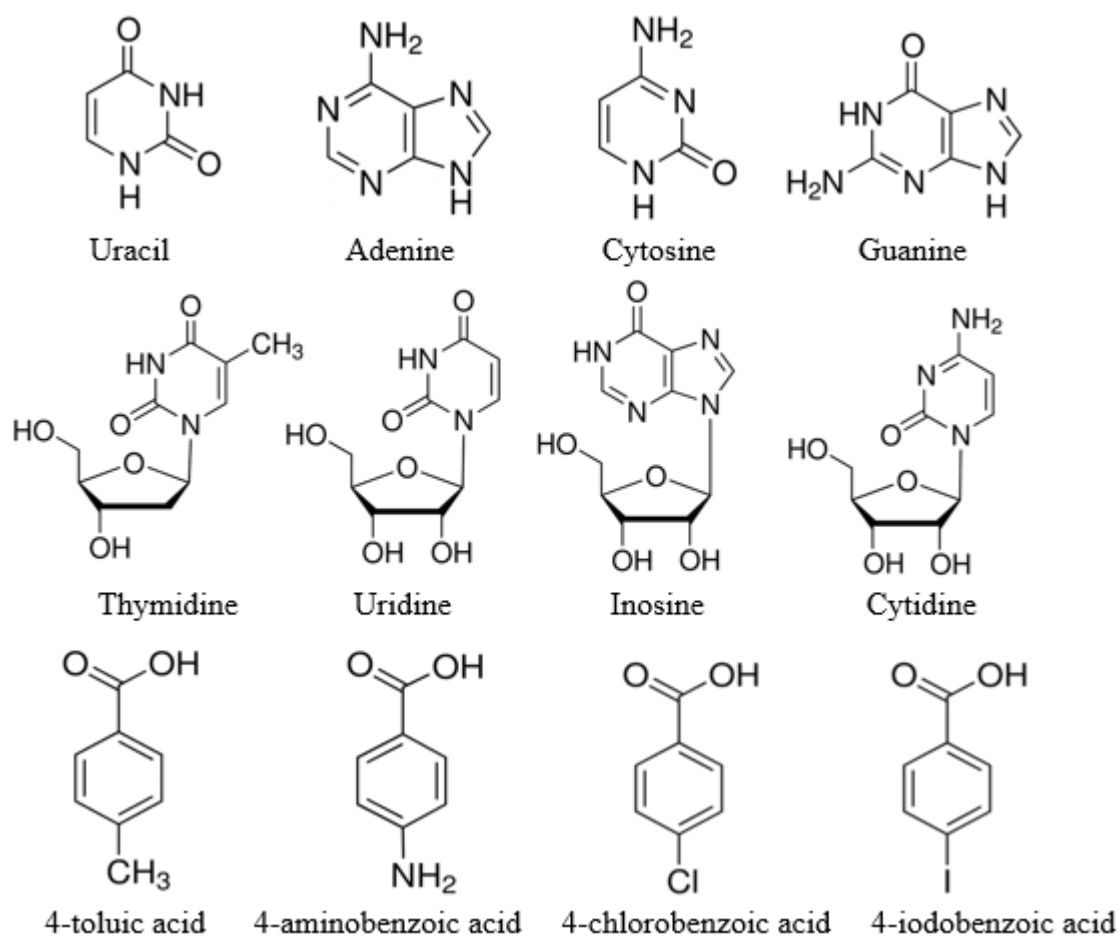
Figure 4.5. Chemical structures of investigated nucleotides, nucleosides and benzoic acid derivatives

4.3.1. Separation of Nucleotides

In HILIC mode, the proportion of organic solvent in the mobile phase greatly influence the retention time of analytes [20]. Figure 4.6 demonstrate that the Column TH-16 could separate nucleotides by hydrophilic interaction mechanism at different ACN concentrations between 90/10 to 96/4 (v/v) in the mobile phase.
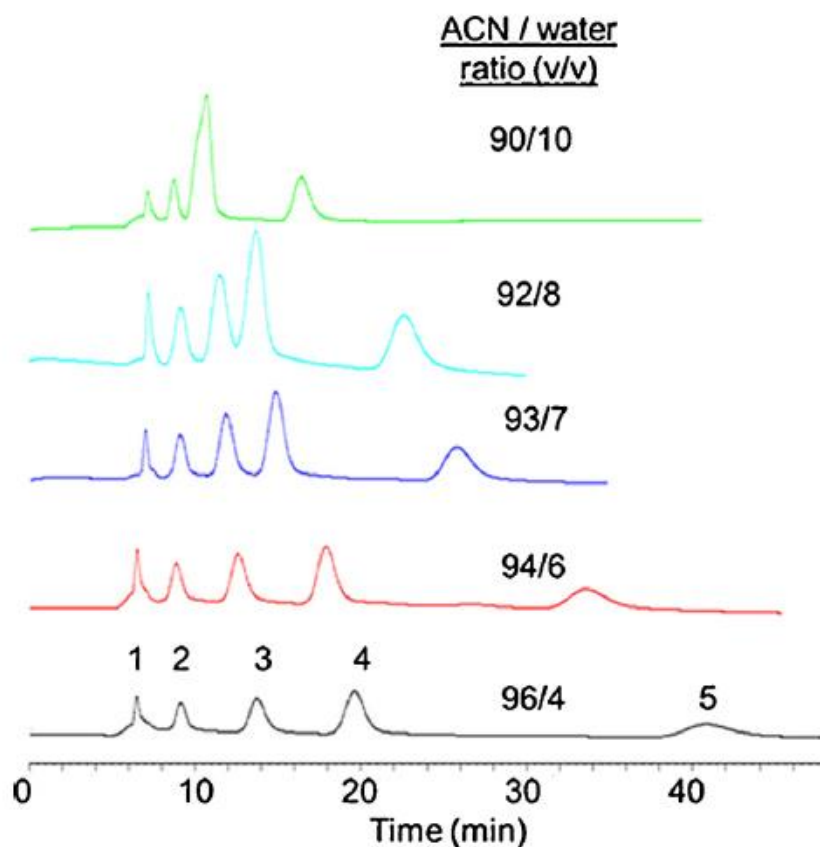
Figure 4.6. The chromatograms obtained in the HILIC separation of nucleotides with different ACN/water ratios using TEA-OH attached poly(HPMA-Cl-co-EDMA) monolith. Synthesis conditions: HPMA-Cl feed concentration of 60% v/v, Porogen/monomer ratio: 2.4 v/v. Iso-PrOH concentration: 40% v/v, Temperature: 60°C. Chromatographic conditions: Flow rate: 0.8 µL/min, Detection: DAD at 270 nm, Column: 300 mm × 100 µm i.d. Injection volume: 100 nL. Mobile phase: ACN/water solution containing 0.05% v/v acetic acid (pH 4.5). Order of elution: 1. Toluene, 2. Uracil, 3.Adenine, 4. Cytosine, 5. Guanine.

Thus, the effect of organic eluent concentration on nucleotide separation in HILIC mode was studied. For all runs, in order to obtain good separations, the maximum flow rate to be reached for the system was selected (0.8 µL/min). In Figure 4.6, as expected, the total analysis time became shorter with increasing water content of the mobile phase. Moreover, the retention times of nucleotides decreased with a slight drop in the ACN content from 94 to 93 % (v/v). This situation can be explained with that the main retention mechanism is partitioning. Resolution values were calculated via Equation 2.2 given in literature review part are listed below in Table 4.4.

Table 4.4. The effect ACN/water ratio on the resolutions obtained in the separation of nucleotides using the TEA-OH attached-poly(HPMA-Cl-co-EDMA) monolith.

ACN/water ratio (v/v)	Peak resolution			
	R(2/1)	R(3/2)	R(4/3)	R(5/4)
98/2	3.0	3.9	4.9	9.1
96/4	2.8	3.7	4.2	8.3
94/6	2.5	3.2	3.0	7.5
93/7	2.4	2.9	2.2	6.5
92/8	2.2	2.7	1.9	6.2
90/10	2.0	2.4	NR	5.5

NR: The related successive peaks are not resolved. The conditions for monolith synthesis conditions and the chromatographic conditions are given in Figure 4.6.

Retention factor values were calculated via Equation 2.1 given in literature review part. Toluene was used as the unretained analyte while retention factors were calculated. Similarly, toluene was also selected as unretained analyte in studies conducted by Liu et al. and Urban et al. for HILIC mode [47, 64]. Figure 4.7 showed that the retention factors of four nucleotides increased with the increase of ACN content in the mobile phase from 90% to 96% (v/v), validating a typical HILIC retention mechanism for poly(HPMA-Cl-co-EDMA) monoliths.
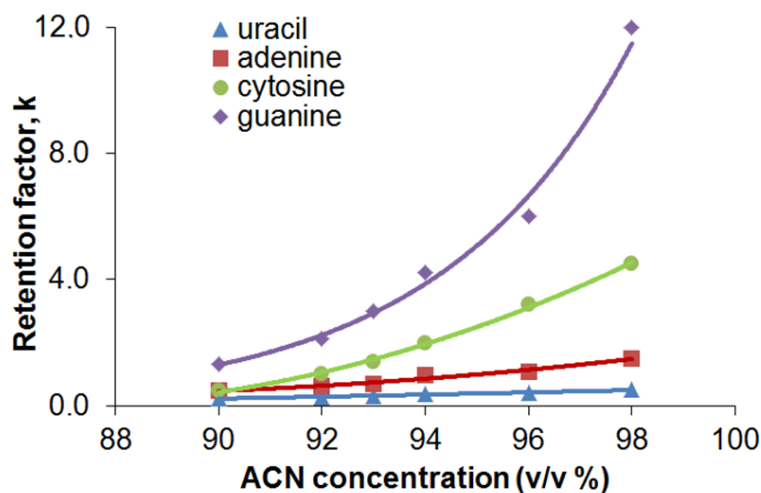
Figure 4.7. The variation of retention factor for nucleotides with the ACN concentration in the mobile phase using TEA-OH attached poly(HPMA-Cl-co-EDMA) monolith. The conditions for monolith synthesis and the chromatographic conditions are given in Figure 4.6.

When the water content was 6% (v/v), the chromatographic peaks on the column exhibited much serious tailing and band broadening. In addition to this, the separation time was consumed. When water content was 8% (v/v), the baseline separation could not be obtained. This case also supported by Jiang et. al., baseline separation for four nucleotide (thymine, adenine, uracil, and cytosine) was obtained at low water concentration in mobile phase (<10%) [16]. It can be said that the best result was obtained with the ACN/water ratio of 93/7 v/v mobile phase since the separations completed with both the shortest time (i.e. 28 min) and satisfactory resolutions.
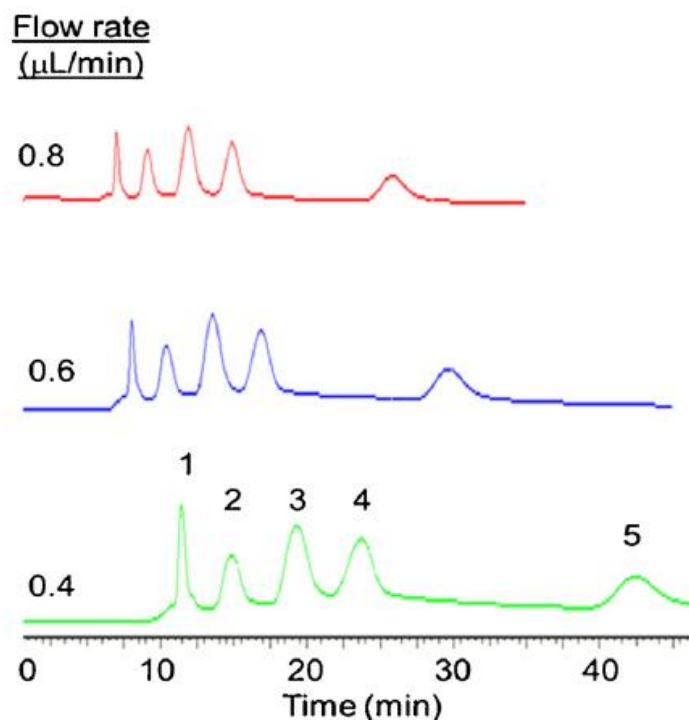
Figure 4.8. The chromatograms obtained from the nucleotide separation with different flow rates via TEA-OH attached poly(HPMA-Cl-co-EDMA) monolith. Synthesis conditions of poly(HPMA-Cl-co-EDMA) monolith are given in Fig. 4.6. Chromatographic conditions: ACN concentration: 93% v/v. Detection DAD at 270 nm, Columns: (A) 300 mm × 100 μm i.d. Injection volume: 100 nL. Mobile phase: ACN/water solution containing 0.05% v/v acetic acid (pH 4.5). Order of elution: 1. Toluene, 2. Uracil, 3. Adenine, 4. Cytosine, 5. Guanine. The back-pressures are 17, 26 and 34 MPa for the flow rates of 0.4, 0.6, and 0.8 μL/min, respectively.

New runs were performed with the different flow rates between 0.4 and 0.8 μL/min using the best conditions obtained from the previous set (i.e. the ACN/water ratio of 93/7 v/v mobile phase). In this way, the effect of flow rate on separation could be examined. The chromatograms are plotted above in Figure 4.8 and calculated resolution values are given in Table 4.5. For each flow rate, the base-line separation was achieved because of high ACN concentration. As expected, the higher resolution values were obtained at lower flow rates since the retention time of each analyte in column increased with lower flow rates so that each analyte was separated better from each other. The back-pressures are 17, 26 and 34 MPa for the flow rates of 0.4, 0.6, and 0.8 μL/min, respectively.

Table 4.5. The effect of flow rate on the resolutions obtained in the separation of nucleotides using the TEA-OH attached-poly(HPMA-Cl-co-EDMA) monolith.

Flow rate (cm/s)	Peak resolution			
	R(2/1)	R(3/2)	R(4/3)	R(5/4)
0.011	3.5	4.2	4.0	8.8
0.021	3.2	3.8	3.7	8.4
0.042	2.9	3.6	3.4	8.0
0.085	2.8	3.4	2.4	7.6
0.127	2.6	3.2	2.3	7.2
0.170	2.4	2.9	2.2	6.5

The conditions for monolith synthesis are given in Figure 4.6. The chromatographic conditions are given in Figure 4.8.

The Van Deemter plot shows the dependence of the theoretical plate height (HETP) on the mobile phase linear velocity. It has a hyperbolic function that predicts an optimum velocity where the column efficiency is maximum. The Van Deemter curve obtained from nucleotide separation is given in Figure 4.9.
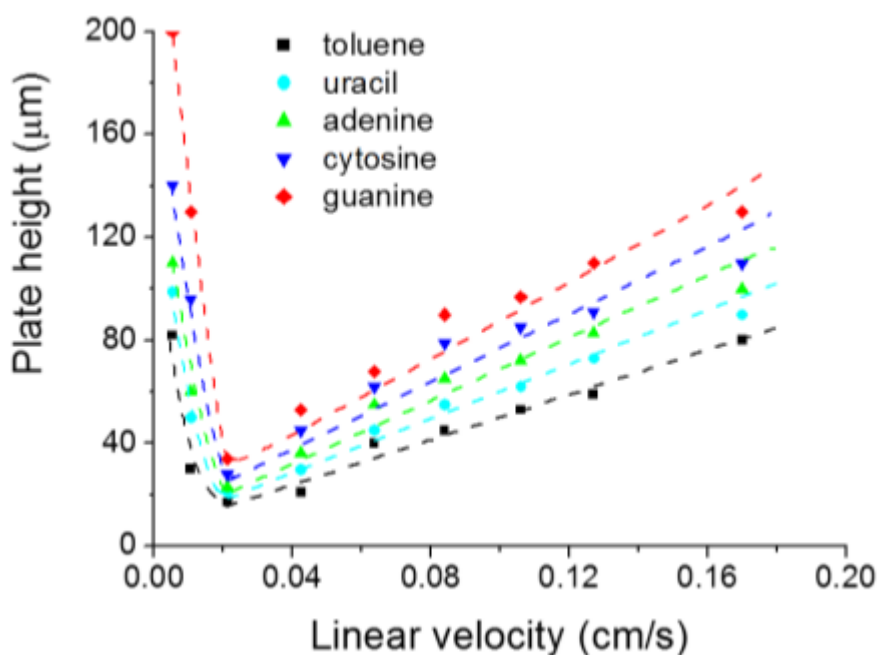
Figure 4.9. The variation of plate height with the linear flow rate in the separation of nucleotides by using TEA-OH attached-poly(HPMA-Cl-co-EDMA) monolith. Synthesis

conditions of poly(HPMA-Cl-co-EDMA) monolith are given in Figure 4.6. Chromatographic conditions are given in Figure 4.8.

From Figure 4.8, it can be seen that the plate height first decreased till the linear velocity became 0.02 cm/s. However, after that point, almost linear increase of plate height was observed from 0.02 to 0.18 cm/s. Figure 4.9 showed the lowest plate height was approximated 20 μm for uracil where flow velocity was 0.02 cm/s. The plate heights obtained for TEA-OH attached polymethacrylate monolith synthesized with the HPMA-Cl feed concentration of 60 % v/v are in the range of 20-128 μm.

Nucleotide separation on polymer based monoliths was studied by different research groups as indicated in the literature review part. Hosoya et al. separated three nucleosides (thymine, uracil and adenine) via (TEPIC-BACM) column. Their plate heights were approximately 37, 68 and 56 μm respectively [38]. Foo et al. studied effect of polymerization time on the column efficiency with poly(SPE-co-BVPE) monolith. The best results was 210 μm for uracil, 314 μm for adenine and 251 μm for cytosine with the monolithic column produced with a 2 h polymerization time [49]. Liu et al. produced poly(SPP-co-EDMA) column used to separate same four nucleotides in 2013. Their approximate plate height values were calculated as 88 μm for thymine, 76 μm for adenine and 36 μm for cytosine [64]. In 2014, Guo et al. prepared β-cyclodextrin modified methacrylate based monolith and it was successfully applied to the hydrophilic interaction liquid chromatography separation of nucleotides. The plate heights were calculated as 122 and 27 μm for thymine and cytidine respectively [54].

According to referred studies, the plate heights in the range of 27-314 μm were calculated for the polymer based HILIC columns except for one study. The efficiency of TEA-OH attached poly(HPMA-Cl-co-EDMA) monolith is suitable among polymer based HILIC monoliths in the recent literature. The variation of the plate height with the retention factor of nucleotides, plotted by using the chromatographic conditions, are given in Figures 4.6 and 4.8 and given below in Figure 4.10.
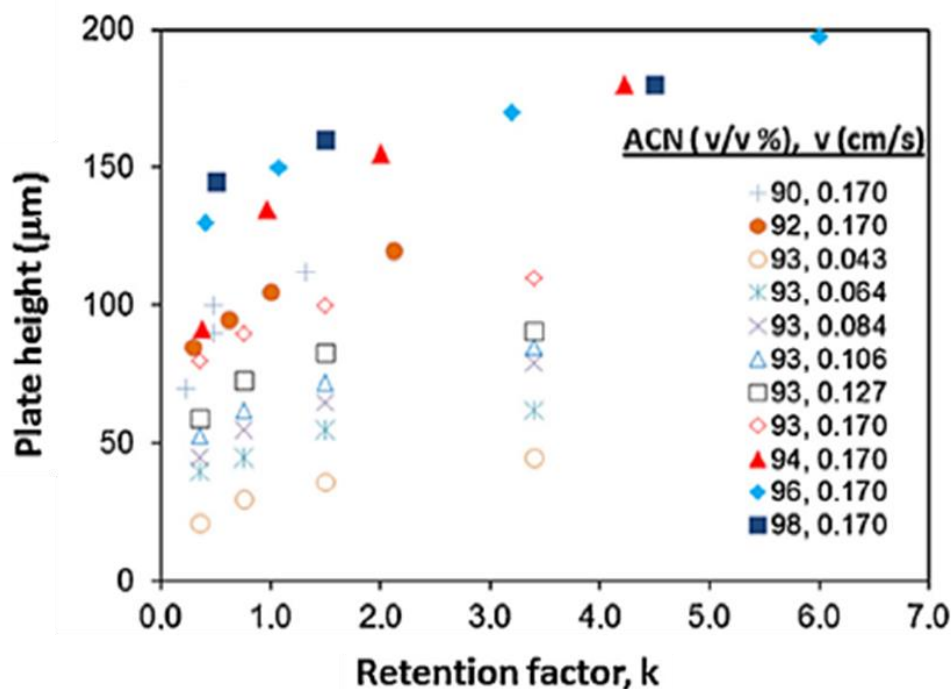
Figure 4.10. The variation of plate height with retention factor for nucleotides under HILIC conditions.

As seen from figure above, the plate height significantly increased with the increasing retention factor. A relationship between the plate height and the retention (i.e. retention dependent plate height) was first obtained in a study conducted by Nischang and Bruggemann in the reversed phase mode [65]. The graph was plotted using the data obtained in the separation of alkylbenzenes with poly(butyl methacrylate-co-ethylene dimethacrylate), poly(BMA-co-EDMA) column in the reversed phase mode [65]. However, the retention-dependent plate height was observed for HILIC mode first in this study. The plot obtained in HILIC mode was very similar to that obtained for the reversed phase mode. This similarity proves that there is an analogy between the reversed phase chromatography and the hydrophilic interaction liquid chromatography.

The increase in plate height (i.e. decrease in column performance) was explained by Nischang and Bruggemann as formation of gel porosity on the surface of globules [65]. The formation of gel porosity was related to conventional free radical polymerization reaction. As described in literature part, polymerization process has different steps. In early steps of reaction, highly cross-linked nucleation forms and phase separation occurs. In the growth step in the reaction, these nuclei become globules. By the way, the degree of cross-linking decreases considerably. As a result, globules formed with slightly cross-linked chains swells in hydro-organic solvents and causes a non-uniform gel porosity [65].

Poor performance in separation of small molecules with polymer based monoliths could be explained with not only the lack of mesopores or low surface area but also gel porosity formation. Gel porosity creates stagnant mass transfer zones to be also expressed as mass transfer resistance for the small analytes. Thus, these analytes shows undesirable surface diffusion, retained more than expected and eluted with boarder bands [65].

The possible partitioning equilibrium occurs within TEA-OH attached poly(HPMA-Cl-co-EDMA) monolith can be demonstrated with the equilibrium adsorption of each analyte onto the stationary phase. For this purpose, the monolith was produced in batch fashion and derivatized with TEA-OH as described in the experimental part and dried in the vacuum. The dried monolith was grinded. In the adsorption runs, the initial concentration of each nucleotide was 0.17 mg/mL in the adsorption medium since this value was very close to the value used in sample mixture. 0.1 g polymer powder was used within the 3.0 mL ACN/water mixture at pH 4.5 at room temperature with magnetic stirring at 300 rpm. Batch adsorption runs performed at seven different ACN/water ratios. The equilibrium period was selected as 6 h. Results obtained from experiments is plotted in Figure 4.11. The magnitude of equilibrium adsorption onto the monolithic phase was in the order of Guanine > Cytosine > Adenine > Uracil according to the polarity of these compounds from high to low. Same order was also observed from previous nucleotide separations in HILIC mode (Figure 4.7) suggesting a typical hydrophilic interaction liquid chromatography (HILIC) retention mechanism.
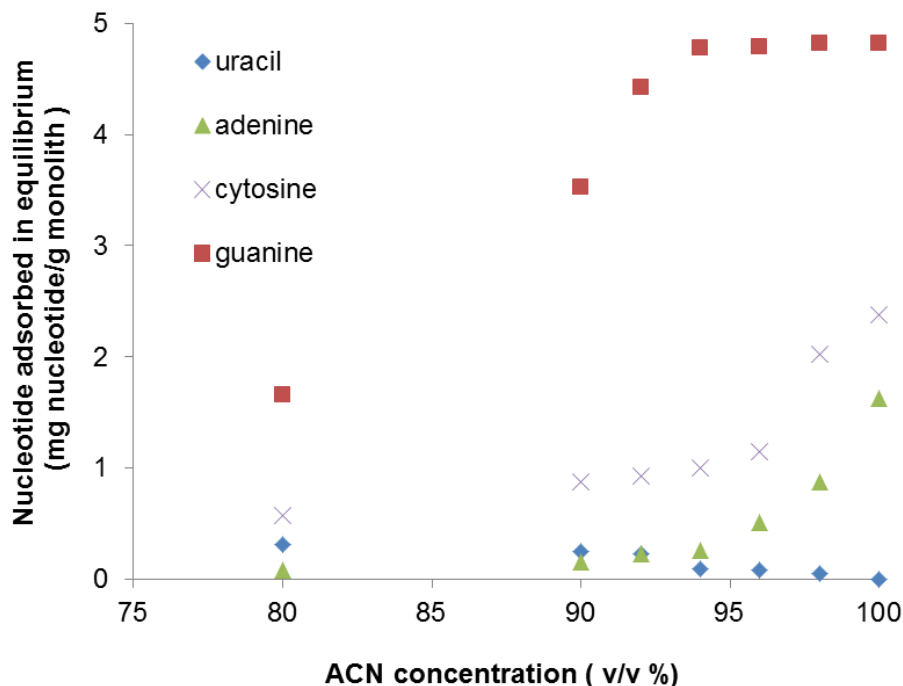
Figure 4.11. The variation of nucleotide adsorbed in equilibrium onto TEA-OH attached-poly(HPMA-Cl-co-EDMA) monolith (grinded form) with the ACN concentration in the adsorption medium.

The results obtained with ACN concentration range of 90-100% v/v were more appreciable. According to Figure 4.11, higher equilibrium adsorption obtained with higher ACN concentrations. This case can be explained with high ACN/water ratio increases physical interaction between the analyte and the stationary phase. In other words, equilibrium adsorption changes depending upon nucleotide type and ACN concentration. Moreover, an increase in hydro-organic solvents ratio causes greater amount of gel porosity formation of slightly cross-linked polymer and provides larger surface area for parking of analytes.

In addition, the change of retention factor of nucleotide with the equilibrium adsorption for all ACN/water ratios is plotted and given below in Figure 4.12. The plot was sketched in logarithmic scale to show clearly the tendency by including the lower values of equilibrium adsorption and retention factor.
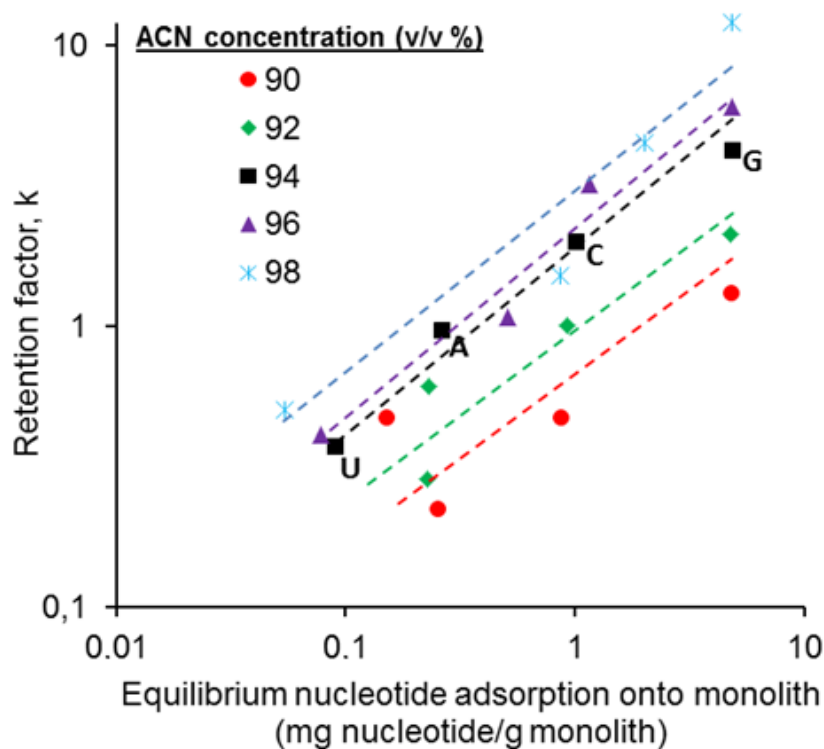
Figure 4.12. The variation of retention factor in HILIC separation with the equilibrium adsorption in batch fashion. Analytes: uracil, adenine, cytosine and guanine. Mobile phase flow rate in HILIC separations: 0.8 µL/min. Other HILIC conditions are given in Figure 4.6. The equilibrium adsorption conditions are given in Figure 4.11.

As seen from the Figure above, the retention factor was directly proportional to the equilibrium nucleotide adsorption for all ACN concentrations. The points obtained with the ACN concentration of 94 % v/v were marked with uracil (U), adenine (A), cytosine (C) and guanine (G) as an example. By starting from left to right each point represent nucleotides with same order for all ACN concentrations (v/v %). This can be concluded as retention factor is a function of both equilibrium nucleotide adsorption and ACN concentrations.

Finally, the cases described previous pages resulted that either gel porosity or high equilibrium adsorption make analyte retention increase (i.e. more difficult to elute) leading to a decrease in the column efficiency.

4.3.2. Separation of Nucleosides

In the second part of the chromatographic applications, nucleosides were well retained and separated completely in HILIC by using same Column TH-16. Likewise, the effect of acetonitrile content on the retention was investigated by varying the percentage of acetonitrile between 90/10 to 96/4 (v/v) in the mobile phase. The chromatograms are given in Figure 4.13. For all runs, the flow rate was selected as 0.8 μL/min where the back-pressure was 32 MPa. Because of this, higher flow rates were not tried since upper pressure limit of the system was 35 MPa.
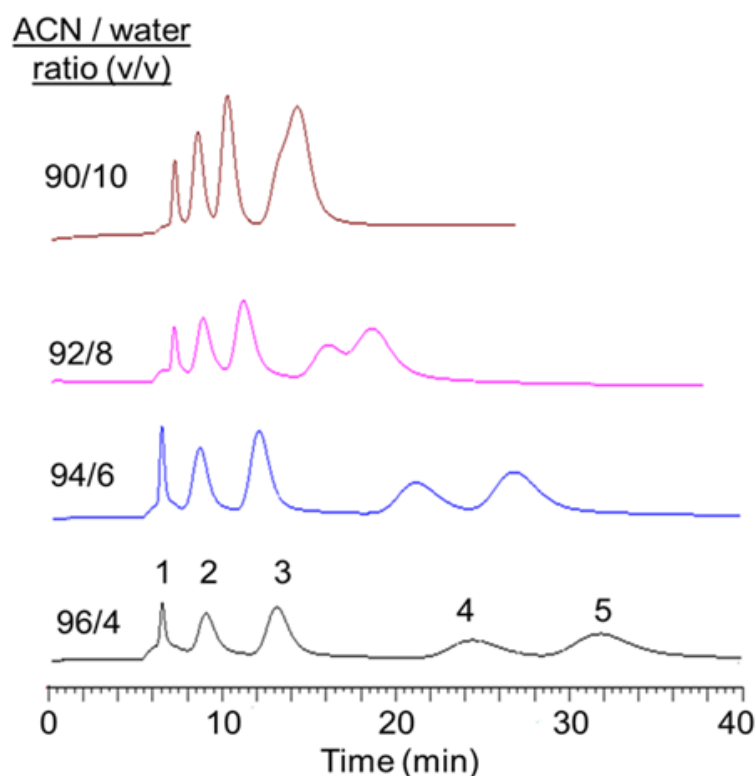
Figure 4.13. The chromatograms obtained in the HILIC separation of nucleosides with different ACN/water ratios using TEA-OH attached poly(HPMA-Cl-co-EDMA) monolith. Synthesis conditions: HPMA-Cl feed concentration of 60% v/v, Porogen/monomer ratio: 2.4 v/v. Iso-PrOH concentration: 40% v/v, Temperature: 60°C. Chromatographic conditions: Flow rate: 0.8 μL/min, Detection: DAD at 270 nm, Column: 300 mm × 100 μm i.d. Injection volume: 100 nL. Mobile phase: ACN/water solution containing 0.05% v/v acetic acid (pH 4.5). Order of elution: 1. Toluene, 2.Thymidine, 3.Uridine, 4.Inosine, 5.Cytidine.

The performance of a stationary phase in which TEA-OH is bonded to poly(HPMA-Cl-co-EDMA) was tested in HILIC of nucleosides and found that it is effective in resolving mixtures of thymidine, uridine, inosine and cytidine with aqueous acetonitrile mobile phases (i.e. 90-96% v/v). As shown from Figure 4.13, the baseline separation of nucleosides were obtained with both the ACN/water ratios of 94/6 and 96/4 v/v. From results, it can be said that a better separation was achieved when ACN content was 94% (v/v) since separations completed with both the shortest time (i.e. 30 min) and satisfactory resolutions. Therefore, it is appropriate to choose this condition for studying the effect of flow rate. Calculated resolution values are given in Table 4.6. As seen from this table, the resolution values of nucleotides decrease with a drop in the ACN content from 98 to 90 % (v/v) in mobile phase as expected. However, the resolution between the more polar compounds cytidine and Inosine decreased remarkably while the resolution between the relatively less polar compounds thymidine and uridine decreased slightly. Moreover, peaks of cytidine and inosine were not resolved in 90% (v/v) ACN content.

Table 4.6. The effect of ACN/water ratio on the resolutions obtained in the separation of nucleosides using TEA-OH attached poly(HPMA-Cl-co-EDMA) monolith.

ACN/water ratio (v/v)	Peak resolution			
	R(2/1)	R(3/2)	R(4/3)	R(5/4)
98/2	2.8	2.9	3.3	2.0
96/4	2.4	2.5	3.0	1.7
94/6	2.2	2.3	2.9	1.5
92/8	2.0	2.2	2.5	0.9
90/10	1.8	1.9	2.3	NR

NR: The related successive peaks are not resolved. The conditions for monolith synthesis are given in Figure 4.6. The chromatographic conditions are given in Figure 4.13.

Under the chromatographic conditions determined above, the chromatograms obtained with different flow rates are given in Figure 4.14. The back-pressures are 16, 25 and 32 MPa for the flow rates of 0.4, 0.6, and 0.8 μL/min, respectively. Resolution values were calculated via Equation 2.2 using the chromatograms in Figure 4.14 are listed in Table 4.7.

Again as expected, the higher resolution values were obtained at lower flow rates. Each analyte can interact longer time with the hydrophilic surface of stationary phase with lower flow rates (i.e. higher retention time) in the column so that the analytes are separated better from each other. It is also noted that the increase in resolution between uridine and inosine depending on the decrease of flow rate is much greater when it is compared with the others. Hence, the polarity difference between these analytes (Inosine/Uridine) is greater than those between Uridine/Thymidine and Cytidine/Inosine.
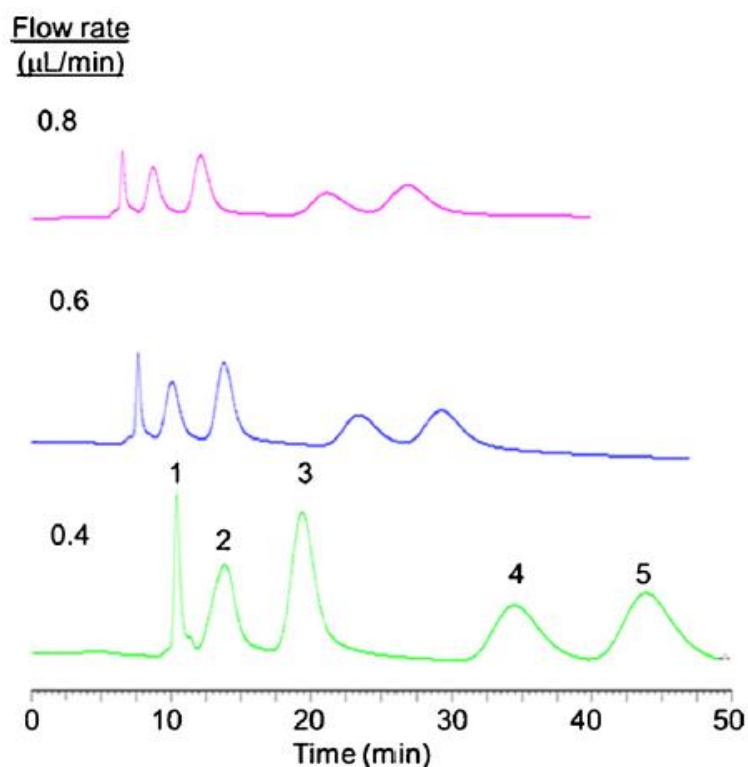
Figure 4.14. The chromatograms obtained with different flow rates in separation of nucleosides using TEA-OH attached-poly(HPMA-Cl-co-EDMA) monolith. Synthesis conditions of poly(HPMA-Cl-co-EDMA) monolith are given in Figure 4.6. Chromatographic conditions: ACN/water ratio: 94/6 v/v, detection DAD at 260 nm, Column: 300 mm x 100 µm i.d. Mobile phase: ACN/water solution containing 0.05 % v/v acetic acid (pH 4.5). Injection volume: 100 nL. Order of elution: 1.Toluene, 2.Thymidine, 3.Uridine, 4.Inosine, 5.Cytidine.

Table 4.7. The effect of mobile phase flow rate on the resolutions obtained in the separation of nucleosides using TEA-OH attached poly(HPMA-Cl-co-EDMA) monolith the HPMA-Cl feed concentration of 60 % v/v.

Flow rate (cm/s)	Peak resolution			
	R(2/1)	R(3/2)	R(4/3)	R(5/4)
0.011	3.3	3.8	6.7	2.1
0.021	2.9	3.6	5.3	2
0.042	2.7	3.2	4.3	1.9
0.085	2.5	2.8	3.5	1.7
0.127	2.4	2.6	3.1	1.6
0.170	2.2	2.3	2.9	1.5

The conditions for monolith synthesis are given in Figure 4.6. The chromatographic conditions are given in Figure 4.14.

Theoretical plate height (HETP) values were calculated for each nucleosides via Equation 2.4 and used to construct the Van Deemter plot. Figure 4.15 shows the Van Deemter plots for toluene, thymidine, uridine, inosine and cytidine on TEA-OH attached poly (HPMA-Cl-co-EDMA) monolith.
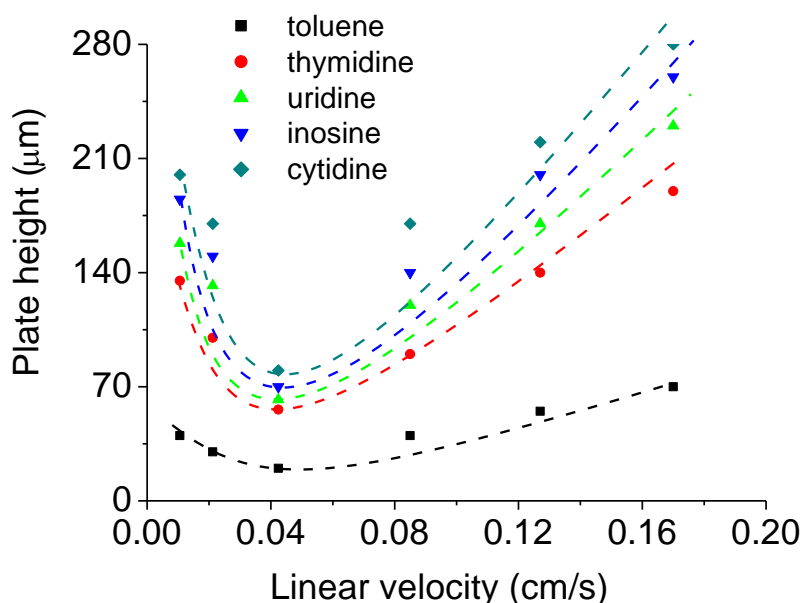
Figure 4.15. The variation of plate height with the linear flow rate using for nucleosides using TEA-OH attached poly(HPMA-Cl-co-EDMA) monolith. Synthesis conditions of

poly(HPMA-Cl-co-EDMA) monolith are given in Figure 4.6. Chromatographic conditions are given in Figure 4.6. Chromatographic conditions are given in Figure 4.11.

From figure 4.15, it can be seen that the plate height first decreased till the linear velocity became 0.04 cm/s and almost linearly increased from 0.04 to 0.18 cm/s. Figure 4.15 showed the lowest plate height was approximated 54 μm for thymidine where flow velocity was 0.04 cm/s. The plate heights obtained in the HILIC separation of nucleosides is in the range of 54-270 μm.

As previously indicated in literature review part, the separation of nucleosides was also studied by different research groups. In 2006, (TEPIC-BACM) column produced by Hosoya et al. was successfully separated three nucleosides (adenosine, guanosine and uridine). Their plate heights were found as approximately 110, 309 and 183 μm respectively [38]. Chen et al. produced poly(NAHAM-co-PETA) column used to separate nucleosides in 2012. The plate height of thymidine was calculated as 50 μm [50]. Poly(MAA-co-EDMA) column was prepared by Chen et al. and used to separate nucleosides. Their approximate plate height values were calculated as 44 μm for cytidine and 135 μm for guanosine [51]. Same research group, Guo et al. also used β-cyclodextrin modified methacrylate based monolith to separate of nucleosides. The plate height of uridine was calculated as 50 μm [54].

According to referred studies, the plate heights in the range of 50-309 μm were calculated for the polymer based HILIC columns. The separation efficiency of TEA-OH attached poly(HPMA-Cl-co-EDMA) monolith is close to the polymer based HILIC monoliths in the recent literature.

The variation of the plate height with the retention factor of nucleosides was obtained by using the chromatograms recorded by different flow rates of mobile phases having different ACN/water ratios (i.e. Figures 4.13 and 4.14) and is given below in Figure 4.16.
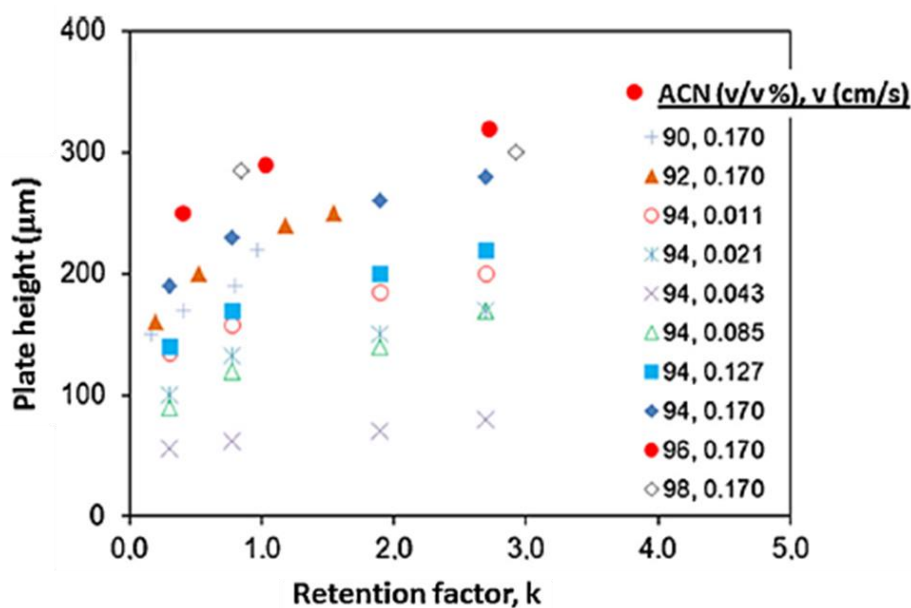
Figure 4.16. The variation of plate height with retention factor for nucleosides under HILIC conditions.

As seen from the figure above, the column performance significantly decreased with increasing retention factor. As mentioned before, the same dependency of column performance based on retention was found in Nischang's study of separation of alkylbenzenes with polymeric monolith [65]. Curves obtained for both nucleotides and nucleosides have almost the same shape. This also supports the analogy between HILIC and RPC modes. Again, this decrease in column performance was expressed as formation of gel porosity [65]. The formation of temporary gel porosity should be the main reason decreasing the diffusion rate of analyte between the stationary and mobile phases [66].

4.3.3. Separation of Benzoic acid derivatives

Then, same column (Column TH-16) was also tried for the isocratic separation of benzoic acids by HILIC. But it was found that benzoic acid separation was not possible via Column TH-16. Hence, a new monolith was synthesized with higher surface area and lower permeability by increasing only microporogen concentration into polymerization mixture (Table 4.1). The results demonstrated that changing the ratio of microporogen affected both the formation of monolithic skeleton and its chromatographic behaviors. So, Column TH-17 was adopted for further experiments. Nevertheless, satisfactory separation of benzoic acids could be done not only synthesizing a new column but also using ACN/buffer volume ratio of 99/1 v/v. The effect of ACN concentration on benzoic acid separation was described with retention factor vs. ACN concentration plot which is given in Figure 4.17.
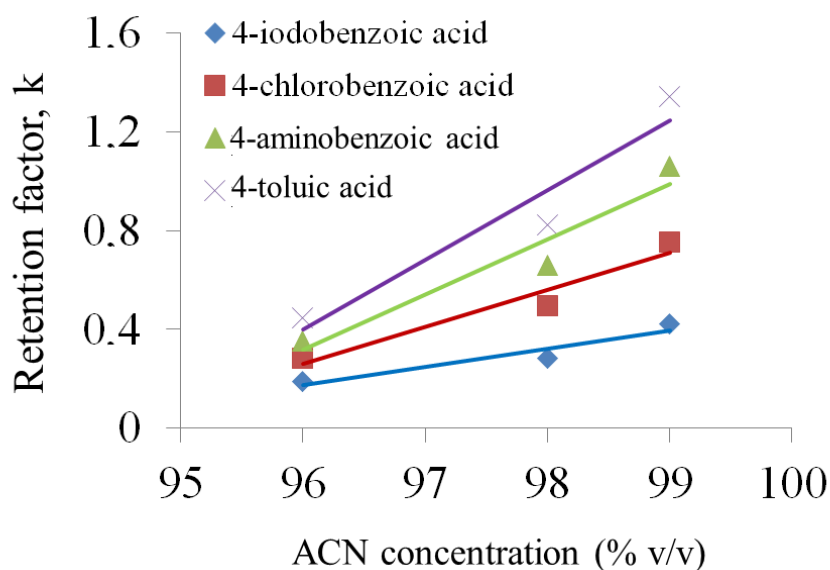
Figure 4.17. The variation of retention factor with the ACN concentration in the mobile phase for the separation of benzoic acids using TEA-OH attached poly(HPMA-Cl-co-EDMA) monolith. Synthesis conditions: HPMA-Cl feed concentration: 60 % v/v, Porogen/monomer ratio: 2.4 v/v, Iso-PrOH concentration: 43.3 v/v %, Temperature: 60°C. Chromatographic conditions. Column: 240 mm x 100 μm i.d. Flow rate: 0.3μL/min. Detection: DAD at 270 nm, Injection volume: 100 nL. Mobile phase: ACN-ammonium acetate buffer (pH 4.5).

From the plot, it was seen that desired separation was able to be achieved at 99/1 v/v. Lower than that value benzoic acid derivatives could not be resolved with. Thus, this condition was chosen for studying the effect of flow rate. Chromatograms obtained with different flow rates are represented in Figure 4.18 and resolutions are given in Table 4.8. The back-pressures are 12, 18 and 24.5 MPa for the flow rates of 0.2, 0.3 and 0.4 μL/min, respectively.
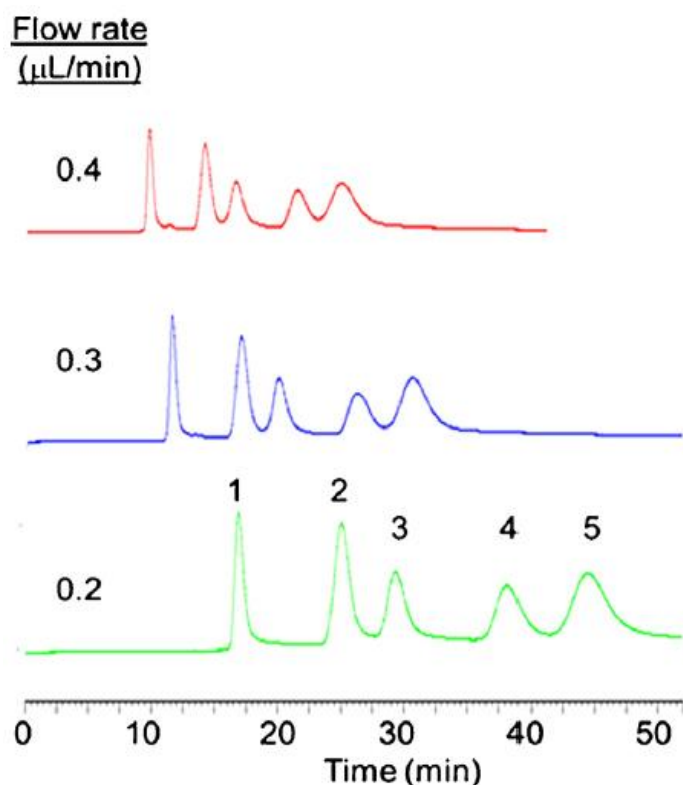
Figure 4.18. The chromatograms obtained with different flow rates for the separation of benzoic acids using TEA-OH attached poly(HPMA-Cl-co-EDMA) monolith. Synthesis conditions: HPMA-Cl feed concentration of 60 % v/v, Porogen/monomer ratio: 2.4 v/v. Iso-PrOH concentration: 43.3 % v/v, Temperature: 60°C. Chromatographic conditions. Detection DAD at 270 nm, Injection volume: 100 nL. Column: 240 mm x 100 μm i.d. Mobile phase: ACN-ammonium acetate buffer ratio (pH 4.5): 99/1 v/v. Injection volume: 100 nL. Order of elution: 1.toluen, 2.p-toluic acid, 3.p-aminobenzoic acid, 4. 4-chlorobenzoic acid, 5. 4-iodobenzoic acid.

From Figure 4.14, benzoic acids including p-toluic acid (pKa: 4.37), p-aminobenzoic acid (pKa: 4.65), 4-chlorobenzoic acid (pKa: 3.98) and 4-iodobenzoic acid (pKa: 4.02) were well separated [65, 67, 68]. There is no significant ionic interaction between analytes and mobile phase or stationary phase containing protonated ethanolamine moiety since there was no correlation between the pKa values and the elution order of benzoic acids. In other words, the retention factors of benzoic acids are not dependent on the pH of the mobile phase. It can be explained with that ionization degree of analytes is too low. Consequently, hydrophilic interaction is more dominant with respect to the ionic interaction.

Table 4.8. The effect of mobile phase flow rate on the resolutions obtained in the separation of benzoic acids using the TEA-OH attached poly(HPMA-Cl-co-EDMA) monolith.

Flow rate (cm/s)	Peak resolution			
	R(2/1)	R(3/2)	R(4/3)	R(5/4)
0.021	7.5	2.8	4.6	2.4
0.042	6.8	2.5	4.2	2.1
0.064	6.1	2.6	3.7	1.6
0.085	5.4	2	3.1	1.6

The conditions for monolith synthesis are given in Figure 4.17. The chromatographic conditions are given in Figure 4.18.

For the separation of benzoic acids, the variation of plate height with the linear velocity is given in Figure 4.19.
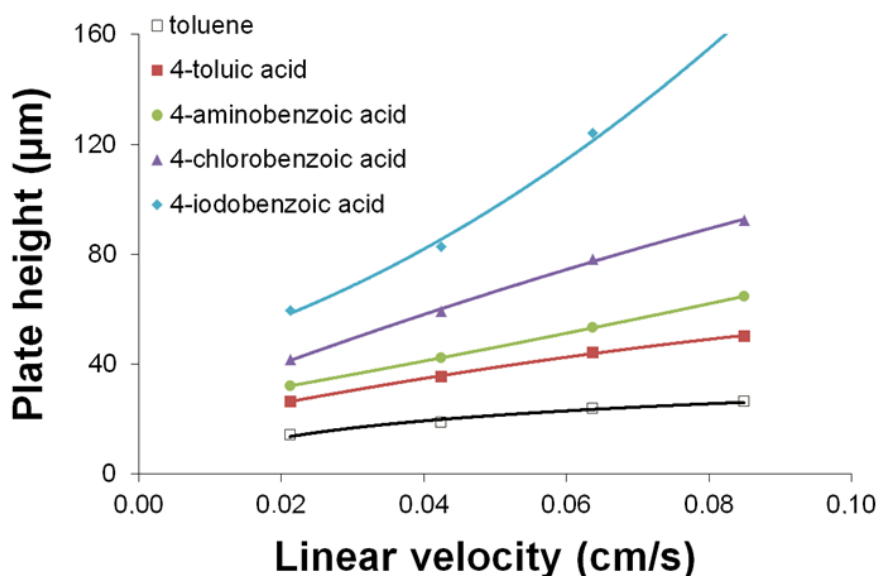
Figure 4.19. The variation of plate height with the linear velocity for different benzoic acid derivatives using TEA-OH attached poly(HPMA-Cl-co-EDMA) monolith. The chromatographic conditions are given in Figure 4.18.

This Van Deemter curve showed the lowest plate height was approximated 25 μm for 4-toluic acid with a flow velocity of 0.02 cm/s. The plate heights obtained in the HILIC separation of benzoic acid derivatives is in the range of 25-165 μm. Previously indicated research groups were also studied separation of benzoic acid derivatives. Chen et al. produced poly(MAA-co-EDMA) column used to separate benzoic acids in 2012.

The plate height of benzoic acid and o-aminobenzoic acid was achieved as 46 and 240 µm, respectively [51]. Poly(NAHAM-co-PETA) column was prepared by Chen et al. and used to separate benzoic acids. Their approximate plate height values were calculated as 270 µm for benzoic acid and 202 µm for p-hydroxybenzoic acid [50]. Guo et al. also used β-cyclodextrin modified methacrylate based monolith to separate of benzoic acid derivatives. The plate heights were calculated as 47 µm for 2,6 dihydroxybenzoic acid and 67 µm for 2,6 dihydroxybenzoic acid [54]. According to referred studies, the plate heights in the range of 46-270 µm were calculated for the polymer based HILIC columns. The separation efficiency of TEA-OH attached poly(HPMA-Cl-co-EDMA) monolith is in the similar value with polymer based HILIC monoliths in the recent literature.

The variation of the plate height with the retention factor of benzoic acid derivatives were plotted by using the chromatographic conditions are given in Figures 4.17 and 4.18 and given below in Figure 4.20.
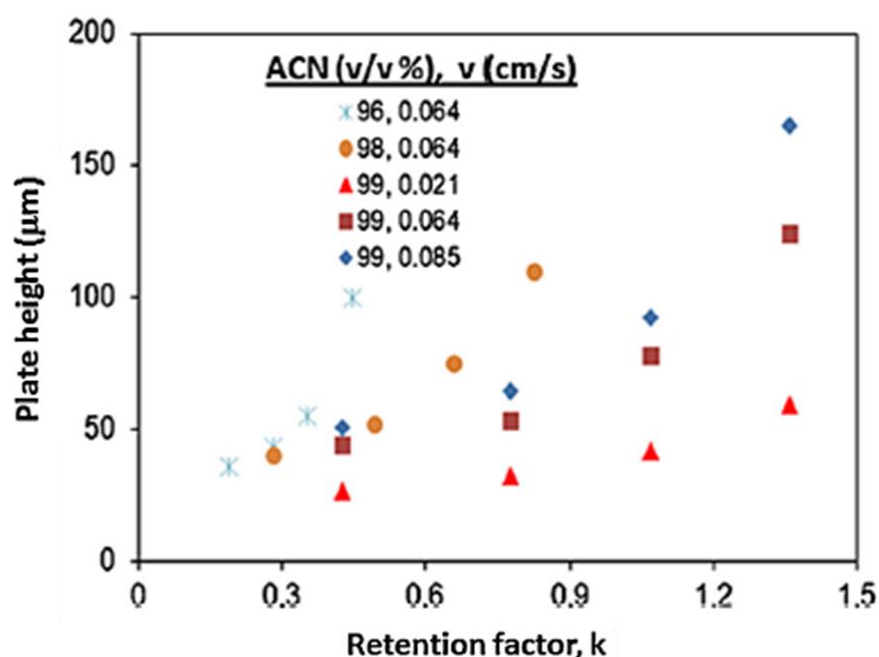
Figure 4.20. The variation of plate height with retention factor for benzoic acids under HILIC conditions.

The plate height markedly increased with increasing retention factor for benzoic acids. Nevertheless, the curve shape is different than that observed in Figure 4.10 and Figure 4.16.

4.4. Column Stability

Reproducibility is a parameter playing an important role in quality characterization of the monolithic column. Therefore, the relative standard deviations (RSDs) values for "day-to-day" "column-to-column" and "run-to-run" reproducibilities were assessed. The calculated reproducibility parameters are summarized in Table 4.9.

Table 4.9. "Run to run", "Day to day" and "Column to column" reproducibility values for TEA-OH attached-poly(HPMA-Cl-co-EDMA) capillary monolith.

Analyte	Run to run (RSD %)	Day to day (RSD %)	Column to column (RSD %)
Toluene	0.45	2.69	3.82
Uracil	0.34	2.76	4.10
Adenine	0.48	1.75	3.40
Cytosine	0.20	1.25	3.10
Guanine	0.12	0.75	2.90

Synthesis conditions: Porogen/monomer ratio: 2.4 v/v, IsoPrOH concentration: 40 % v/v , HPMA-Cl feed concentration: 60 % v/v. Temperature: 60°C. Chromatographic conditions: ACN/water ratio: 93/7 v/v, Flow rate: 0.6 µL/min, Injection volume: 100 nL, Column: 300 mmx100 µm i.d., Detection: DAD at 270 nm.

To study day-to-day repeatability of the column, each day same injection of test compounds, consisting of nucleotides, were analyzed for five days. As shown Table 4.9, the day-to-day repeatability was less than 3% in terms of relative standard deviation (RSD) of the retention factor. In the fifth day of runs performed, a series of injections of test compounds were analyzed in order to study run-to-run repeatability. The run-to-run repeatabilities were lower than 1 %.

On the other hand, "column to column" RSD values obtained by analyzing test compounds with three TEA-OH attached-poly(HPMA-Cl-co-EDMA) monoliths produced by three parallel syntheses. RSD values for each nucleotides were less than 5 %. This can be explained with the high solubility of polymer contents. Hereby, homogeneous polymerization mixture resulted in high column-to-column reproducibility.

These results indicated that reproducibility and repeatability of TEA-OH attached-poly(HPMA-Cl-co-EDMA) monolith were good and satisfactory in nano-HLIC mode.

5. CONCLUSION

A highly hydrophilic porous poly(HPMA-Cl-co-EDMA) monolithic column prepared by thermally initiated free radical copolymerization of HPMA-Cl and EDMA in the presence of porogens, was proposed as a new reactive stationary phase with preparation and derivatization flexibility in nano-HILIC. Its reactive group, "chloropyl moiety" permits the attachment of different hydrophilic ligands. Using this facility a hydroxylamine type chromatographic ligand, TEA-OH was covalently attached via the direct reaction onto the reactive starting material in this study.

Variation of the polymerization parameters, described in the previous chapter, has a considerable impact on the properties of monoliths prepared. The increase in temperature, monomer ratio and microporogen concentration resulted in stationary phases having lower permeability and higher surface area. Accordingly, the presence of a sufficiently high amount of micropores provides the higher ligand content and contact area needed to efficiently separate low-molecular weight compounds.

The effect of ACN content, salt concentration and flow rate on the separation of analytes was used to manipulate analysis conditions when optimizing separation methods. Under the optimized separation conditions, TEA-OH attached monoliths were successfully applied for the rapid, high resolution and without obvious peak tailing separation of nucleotides, nucleosides and benzoic acid derivatives.

However, it was observed that plate height increased when the retention factor also increased. It should be noted that, for HILIC mode, "retention-dependent plate height" value was introduced first time with this study. The possible reasons of this were formation of gel porosity and strong physical interaction between stationary phase and analyte because of high ACN concentration. This problem can be solved by increasing cross-linking ratio of globules during growth step of polymerization reaction.

Typical HILIC retention was observed when the content of ACN was relatively high. However, it was not expected ionic interaction between analytes and mobile phase or stationary phase since ionization degree of analytes is too low. In other words, efficient separations of benzoic acid derivatives were mainly based on hydrophilic interaction mechanisms.

Consequently, poly(HPMA-Cl-co-EDMA) monolith exhibited satisfactory chromatographic performance based on the chromatographic results obtained in HILIC

mode. Evaluating this monolith as a new polymer-based stationary phase for HILIC mode is appropriate. Further investigations and experiments are underway in order to extend the applicability and variety of poly(HPMA-Cl-co-EDMA) monolith.

6. REFERENCES

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Publications

Ç. Kip, D. Erkakan, A. Gökaltun, B. Çelebi, and A. Tuncel, "Synthesis of a reactive polymethacrylate capillary monolith and its use as a starting material for the preparation of a stationary phase for hydrophilic interaction chromatography," *Journal of Chromatography A,* vol. 1396, pp. 86-97, **2015.**

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