HYDROXY MONOLITH PRECURSOR FOR THE PREPARATION OF ORGANIC POLYMER MONOLITHIC COLUMNS BY POST-POLYMERIZATION MODIFICATION WITH VARIOUS SURFACE RETENTIVE LIGANDS FOR CAPILLARY ELECTROCHROMATOGRAPHY AND HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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Abstract: The objective of this dissertation involved furthering the development of the concept of post-polymerization modification by introducing a novel poly(HEMA-co-PETA) precursor monolithic column to which various types of ligands were chemically grafted. Three different types of monolithic stationary phases were fabricated from the above single precursor monolithic column including those with surface modified 1,2-epoxyalkane and 1-isocyanato-octadecane yielding aliphatic nonpolar surfaces, which were evaluated in the RPC mode of separation. Similarly, the immobilization of 2-biphenylylglycidylether led to the generation of aromatic nonpolar surface, which was also assessed in RPC using CEC and HPLC techniques. On the other hand, glycerol, ethylenediamine, diethylenetriamine and triethylenetetramine were immobilized on the same precursor column yielding polar monolithic stationary -phases, which were studied in hydrophilic interaction capillary electrochromatography (HI-CEC).The studies described in this dissertation have demonstrated the versatility of a hydroxy monolithic column, which can be used as a precursor or a parent monolith for the fabrication of various types of stationary phases. By using a simple and well-known epoxy ring-opening reaction, various types of ligands were immobilized on the precursor surface, a fact that demonstrated that a desired stationary phase could be tailored as required.

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LIST OF SYMBOLS AND ABBREVIATIONS

u_{eo}	interstitial electroosmotic velocity
α	selectivity factor
δ	thickness of electric double layer
ε	dielectric constant
ζ	zeta potential
η	viscosity of the electrolyte solution
μeo	electroosmotic mobility
ρ	surface charge density
σ^2_L	peak variance
υ_{eo}	electroosmotic velocity
υep	electrophoretic velocity
k'	chromatographic retention factor
t_R	migration time of retained solute
t_o	migration time of neutral solute
k*	retention factor of a charged solute in CEC
k^*_e	velocity factor of a charged solute in CEC
H	theoritical plate heigth
l	length of the separation capillary from the inlet to the detection
N	number of theoritical plates

W_b	peak width at the base
W_h	peak width at half heigth
W_i	peak width at inflexion point
R_s	resolution
cLC	capillary liquid chromatography
1,12-DoDDMA	1,12-dodecanediol
1,6-HDDMA	1,6-hexanediol dimethacrylate
1,6-HEDA	1,6-hexanediol ethoxylate
2,3,5-TCP	2,3,5-trichlorophenol
2,3,5-TMP	2,3,5-trimethylphenol
2,4-DCP	2,4-dichlorophenol
2-NAPM	2-naphthyl methacrylate
3,5-DCP	3,5-dichlorophenol
3,5-DMP	3,5-dimethylphenol
AB	alkylbenzene
ACN	acetonitrile
AF	aflatoxin
AMIM+Cl-	1-allyl-3-methylimidazolium chloride
AMPS	2-acrylamido-2-methylpropane sulfonic acid

ATRP	atom transfer radical polymerization
AZT	3'-azido-3'-deoxythymidine
BMA	butyl methacrylate
$BMIM^+Cl^-$	1-butyl-3-methylmidazolium
CAP	chloramphenicol
CD	cyclodextrin
CE	capillary electrophoresis
CEC	capillary electrochromatography
CuAAC	copper mediated azide alkyne cycloaddition
DPEPA	dipentaerythritol penta acrylate
DVB	divinyl benzene
EDA	ethylene diamine
EDMA	ethylene dimethacrylate
EOF	electroosmotic flow
FF	florfenicol
GCMA	glycerol carbonate methacrylate
GMA	glycidyl methacrylate
GNP	gold nanoparticles
HDA	hexamethylene diamine
HDDA	hexanediol diacrylate

HEMA	hydroxyethyl methacrylate
HILIC	hydrophilic interaction chromatography
HMA	hexyl methacrylate
HPLC	high performance liquid chromatography
HPMA	hydroxypropyl methacrylate
HP-β-CD	hydroxypropyl-β-cyclodextrin
IL	ionic liquid
LC	liquid chromatography
LMA	lauryl methacrylate
MAA	methacrylic acid
MOA	mercaptooctanoic acid
MOF	metal organic framework
NVC	*N*-vinylcarbazole
ODS	octadecyl silica
OHM	hydroxy monolith
OMA	octyl methacrylate
PAH	polyaromatic hydrocarbon
PEDAS	pentaerythritol diacrylate monosearate
PEGDA	polyethylene glycol
PETA	pentaerythritol tricarylate

PMA	propargyl methacrylate
RPLC	reversed phase liquid chromatography
SMA	stearyl methacrylate
TAIC	triallylisocyanurate
TAP	thiamphenicol
TEA	triethanolamine
TERP	organotellurium-mediated living radical polymerization
TFA	trifluoroacetic acid
TPTM	trimethylolpropane tris(3-mercaptopropionate)
TRIM	trimethylolpropane triacrylate
VDC	vinyl decanoate
VMNP	vinylized iron oxide magnetic nanoparticles
VPV	vinyl pivalate

CHAPTER I

BACKGROUND AND SCOPE OF THE INVESTIGATION

Introduction

Capillary electrochromatography (CEC) is an analytical technique, which has the combined advantage of chromatographic and electrophoretic separation mechanisms. It is popularly known as the hybrid of high performance liquid chromatography (HPLC) and capillary electrophoresis (CE) [1]. Thus, CEC consists of a stationary phase, which provides the separation selectivity as in HPLC and the electroosmotically driven flow (EOF) as in CE that ensures the achievement of high separation efficiency. Initially, the stationary phases in CEC were predominantly packed columns made of silica particles with surface bonded ligands [2]. However, due to disadvantages of packed columns such as retaining frits in small capillary diameter (50-100 μm), the use of continuous polymeric beds or monoliths (as they are currently used) have become popular since its introduction in 1989 by Hjertén [3]. There has been a tremendous increase in the number of different types of monolithic stationary phases for CEC in the last couple of decades. One of the main advantages of polymeric monolith stationary phases is the readily tunable surfaces,

which can be modified or tailored according to the requirement.

The scope of this dissertation is to demonstrate the potential versatility of acrylate based monoliths in tailoring various surfaces as required for use in different modes of CEC such as reversed phase, hydrophilic interaction and chiral separations, utilizing the well-known regioselective epoxy ring opening reaction in presence of boron trifluoride as a catalyst. The resulting monoliths were tested using various neutral analytes such as alkylbenzenes (ABs), polyaromatic hydrocarbons (PAHs), phenolic compounds, toluene compounds, nucleic acid bases, nucleosides and many more to explore and compare the various monolithic stationary phases in terms of their retention, separation efficiency and selectivity properties.

This chapter aims at providing an introduction to the basic concepts, background and principles along with a brief explanation of the instrumentation of CEC. In addition, this chapter will go through the recent advances and developments in the field mainly focusing on the different modes of stationary phases currently in use.

Chapter II presents the study of the effect of alkyl chain length of the monolithic surface ligands on retention, efficiency and selectivity of various solutes under reversed-phase conditions. The surface of poly (HEMA-co-PETA) monolith is modified with various epoxyalkyl ligands namely with alkyl chains C-8, C-12, C-14 and C-16 to obtain Epoxy OHM C-8, Epoxy OHM C-12, Epoxy OHM C-14 and Epoxy OHM C-16 columns. Octadecyl isocyanate was used for making an Isocyanato OHM C-18 column. Study of the effects of surface modification on the poly(HEMA-co-PETA) monoliths using

epoxyoctadecane and octadecyl isocyanate was performed by comparing Epoxy OHM C-16 and Isocyanato OHM C-18 columns.

In chapter III, non-polar aromatic surface created by using biphenyl glycidyl ether is explored in HPLC and CEC modes under reversed-phase conditions using a range of small molecules such as ABs, PAHs, phenolic compounds, aniline compounds and nitroalkanes. Phenoxy acid herbicides were also used to evaluate the columns.

Chapter IV presents the exploration of the poly(HEMA-co-PETA) monolith in HILIC mode of chromatography by modifying its surface with hydroxyl functional groups using glycerol to yield polyol column and amine functional groups using, ethylenediamine, diethylenetriamine, triethylenetetramine to yield polyamine columns. Polar solutes and nucleic acid bases were used for the evaluation of these columns.

Chapter V shows an attempt to achieve chiral stationary phases from the precursor poly(HEMA-co-PETA) monolith by the immobilization of hydroxypropyl-β-cyclodextrin (HP-β-CD). Some racemic mixtures and a positional isomer were resolved on this chiral stationary phase.

Some background of capillary electrochromatography

Historical background

In 1974, Pretorious *et al.* [4] demonstrated the use of electric field in chromatographic separation. As described by them, electroosmosis is the flow of liquid in contact with a solid surface under the influence of a tangentially applied electric field. They listed several disadvantages of pressure driven flow through particle packed column

including band broadening due, among other things, to the laminar flow profile and the detector dead volume. The authors used 75-125 μm diameter particles packed in 50 cm long glass tubes of 1 mm i.d., which gave smaller band broadening as compared to that of the flow driven by pressure. Electroosmotically driven flow has some advantages over pressure driven flow since EOF velocity is independent of the particle size and the flat velocity profile of the EOF reduces dispersion of the band of solute passing through the column which enhances the column separation efficiency [5].

However, it was not until after Jorgenson *et al.* [6] in 1980s and Knox *et al.* [7] in 1990s published their theoretical and experimental work, that electrochromatography gained popularity amongst the scientific community. Jorgensen and Lukacs used 75 μm i.d. open tubular glass capillaries and applied voltage of up to 30 kV. It was claimed by the authors that the use of small diameter capillaries allows the efficient dissipation of heat generated by the use of such high voltage [8]. Knox and Grant made several conclusions in their work including the points such as importance of miniaturization of the diameter of tubes (50-100 μm) mandatory for avoiding self-heating and the use of submicron particles for obtaining efficient Electroosmotically driven chromatography [9]. In another work, 1.5 μm particles were used and reduced plate heights and high separation efficiency were achieved [10].

Instrument overview and basic principles of capillary electrochromatography

Instrument. Similar to CE, CEC utilizes capillaries with small inner diameter (50-100 μm), which maximizes surface to volume ratio and conducts away ohmically generated heat and minimizes temperature gradient across the capillary diameter [4]. However, unlike CE, the differential partition between the solid and liquid phases is the principal behind separation

in CEC. The instrumentation of CEC is similar to that of CE in the sense that it has high voltage power supply but in addition to that, it is facilitated

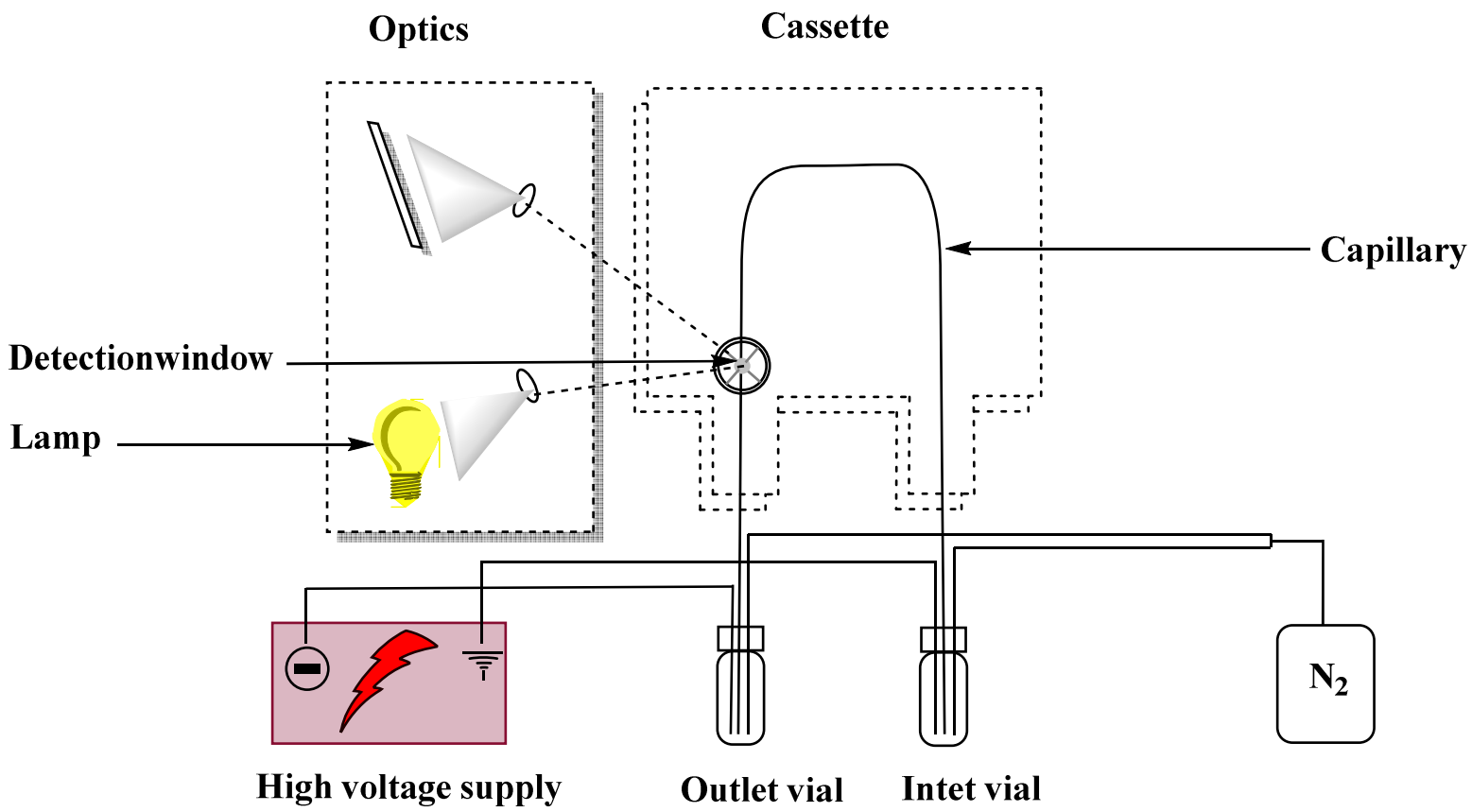

Figure 1. *Schematic diagram of a typical CEC instrumental set-up.*

with an external pressure system which can apply pressure of up to 12 bars on the inlet-outlet vials as required. A typical CEC instrument is depicted in a schematic representation as shown in Fig. 1. Typically, the instrument is equipped with two electrodes immersed in the inlet and outlet vials including the sample reservoir and a detector. Sophisticated CEC instruments are equipped with auto-sampler and column temperature control ability. The instrument has the capacity to deliver up to 30 kV through the high voltage power supply, which is applied to the platinum electrodes containing fused-silica capillary with stationary phase. The inlet electrode is connected to the high voltage supply whereas the outlet electrode is on the ground voltage.

The instrument can accommodate 25 to 100 cm long capillary with 50-100 μm i.d. contained in a capillary cartridge. Sample injection can be done either hydrodynamically by applying pressure to the inlet or electrokinetically by applying voltage. Comparatively, hydrodynamic injection is preferred over electrokinetic injection since mobility of each analyte in the electric field is different. However, hydrodynamic injection may be problematic in case of stationary phases with low porosity, which can cause high back pressure. The viscosity of sample and magnitude of applied pressure are also important factors to consider in hydrodynamic injection method. The detection is generally on-column through a detection window made by stripping the external polyimide coating of the fused silica capillary with a thermal stripper. Light from the lamp passes through it and is received by the detector. The output from detector is processed by computer software and the signal is displayed in real time on a computer screen.

Electroosmosis. Electroosmosis is the movement of liquid through a stationary phase in the presence of an applied electric field. During the process, mobile phase ions get adsorbed onto the stationary phase leading to the formation of an electric double layer at the solid-liquid interface. The double layer is made up of a layer of ions on the stationary phase, which is relatively fixed and a diffuse layer, which extends into the liquid mobile phase. In the case of columns packed with particles or in case of monoliths, the charge may arise from fixed surface charges incorporated in the stationary phase to generate enough EOF. Neutral stationary phases can acquire a charged layer by adsorption of ions from the mobile phase.

When a tangential electric field is applied, a portion of the diffuse layer moves and a shear plane is set up at some distance from the solid surface. The potential at the shear plane or the voltage drop between the stationary phase and the plane of shear is called the zeta, ζ, potential. For a negatively charged surface, the zeta potential is negative leading to a cathodal EOF. The opposite is true in the case of a positively charged surface. The velocity (υ_{eo}) of the moving liquid under the influence of an applied electric field, E, is given by:

$$\upsilon_{eo} = \frac{\varepsilon\zeta}{4\pi\eta}\mathrm{E} \qquad (1)$$

and in terms of mobility (μ_{eo}), it is expressed as:

$$\mu_{eo} = \frac{\varepsilon\zeta}{4\pi\eta}$$

(2)

where the zeta potential is expressed as:

$$\zeta = \frac{4\pi\delta\rho}{\varepsilon}$$

(3)

where ρ is the surface charge density of the capillary surface or the solid surface (e.g., a solid particle) , δ is the thickness of the electric double layer and ε is the medium dielectric constant.

The migration of cations on the negatively charged stationary phase is faster since both EOF and electrophoretic migration are in the same direction. Since neutral molecules lack electrophoretic mobility they move along with the EOF. In case of anions, their electrophoretic mobility is in the opposite direction to the EOF so they migrate slowest in comparison to cation and neutral molecules. However, they ultimately move toward the detector because the magnitude of EOF is higher than the electrophoretic mobility. The separation of ions also depends on their size and charge. The cations with double charges migrate faster than singly charged cations and the larger the size of ion the slower is its mobility. The above description is illustrated in Fig 2

Electroosmotic flow and laminar flow differ from each other in terms of flow profile. Since the flow is initiated at the walls of capillary, it is uniformly distributed along the capillary radial axis, which leads to a flat plug flow profile. Whereas, laminar flow causes parabolic velocity profile and is exhibited by a pressure-driven flow such as in HPLC.

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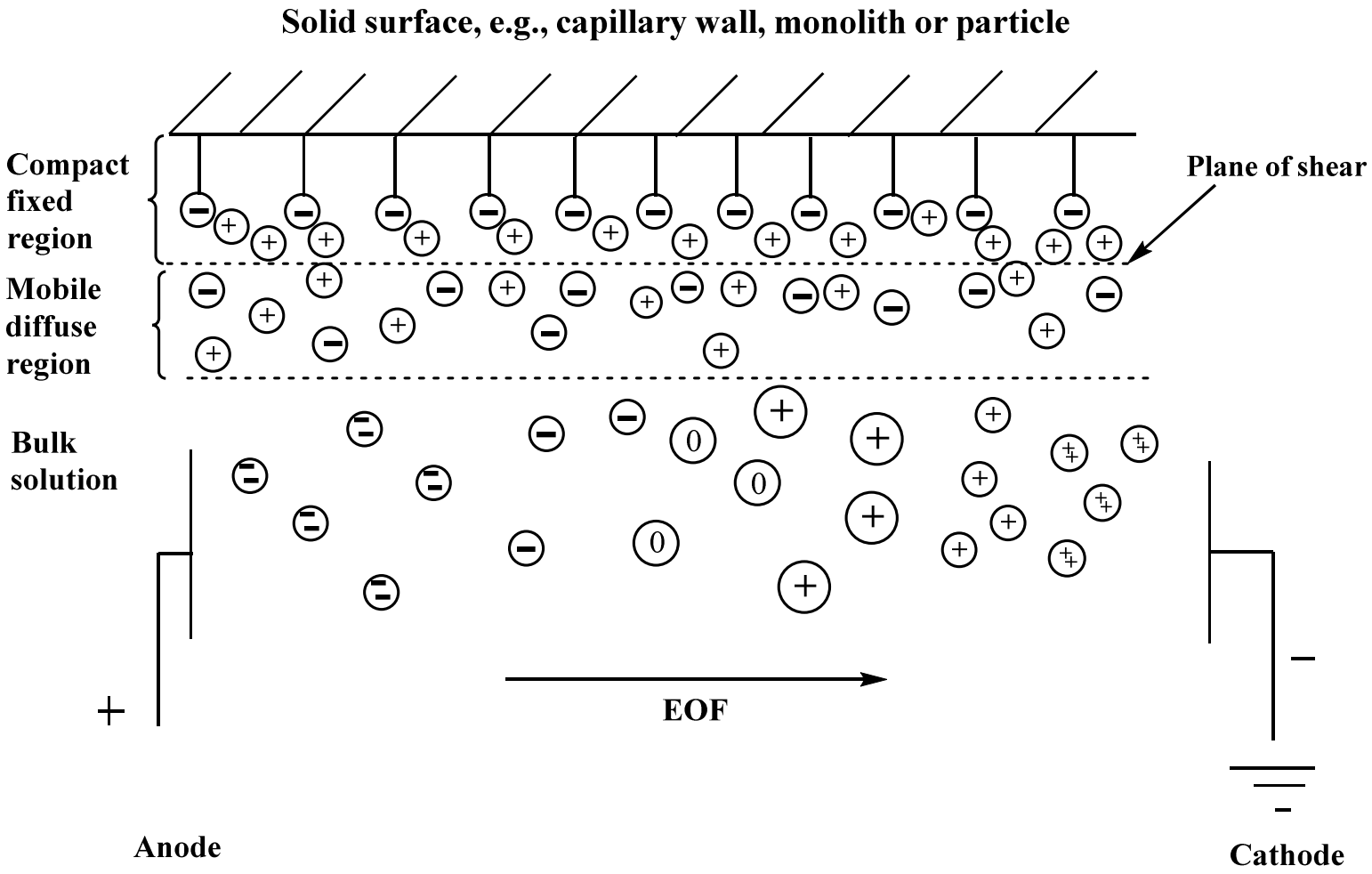

Figure 2. *Illustration of (i) the electric double layer at solid-liquid interface that is responsible for generating EOF and (ii) the migration of charged and neutral species under the influence of an electric field.*

Such parabolic flow profile increases resistance to mass transfer in the mobile phase and plate height is also increased. A flat profile of EOF reduces band broadening, increases the elution rates and yields highly efficient peaks. These phenomena are depicted in Fig 3.

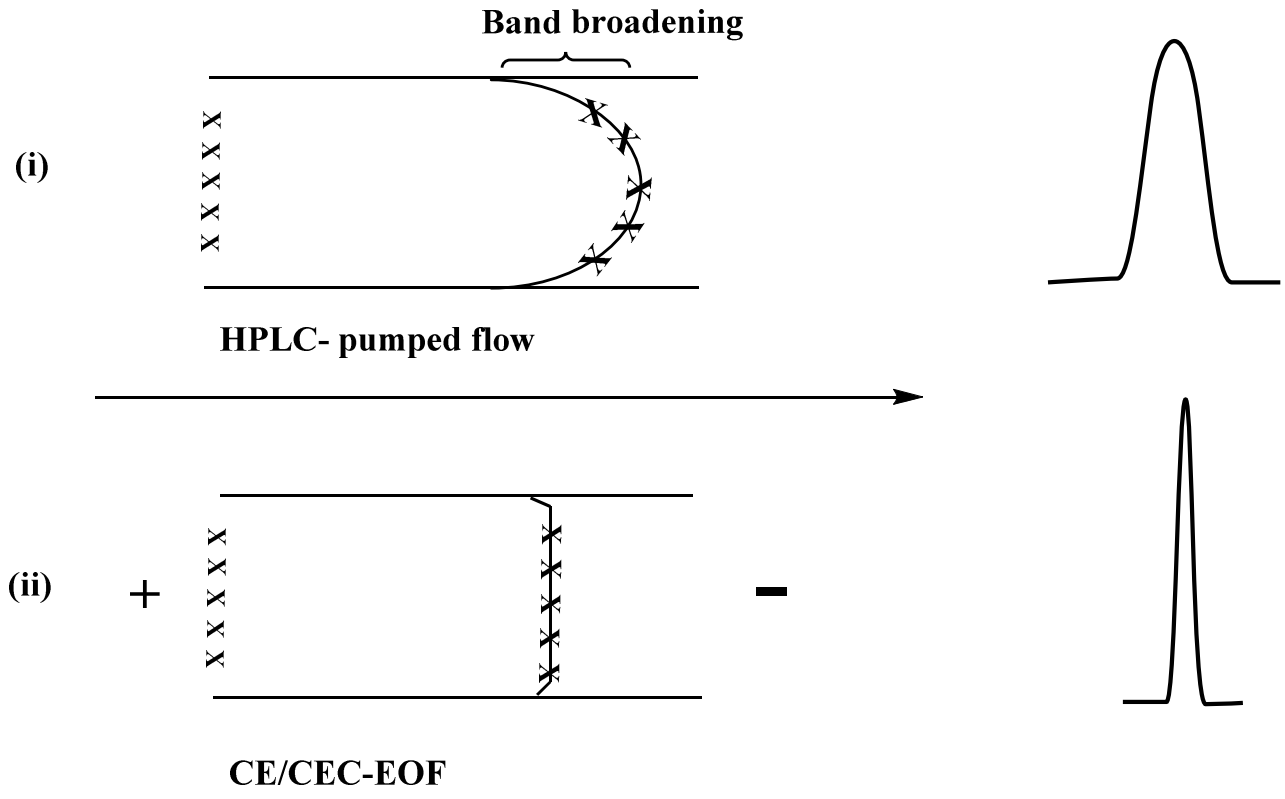

Figure 3. *HPLC and CE/CEC flow profiles and their effects on the peak shape. (i) Laminar flow observed in pumped flow system and (ii) flat/plug flow profile observed in electrically driven methods.*

Retention factor of solutes in CEC – Case of neutral solutes. The absence of charge causes neutral solutes to migrate and separate on the basis of their differential partitioning between the mobile and stationary phases in the same manner as in HPLC. This means that the retention factor, k', in both CEC and HPLC is expressed as:

$$k' = \frac{t_R - t_o}{t_o} \tag{4}$$

where t_R is the retention time of the solute and t_o is the migration time of a neutral unretained solute (i.e., EOF tracer) or dead time of the column. The EOF tracer must not interact with the stationary phase in order to obtain an accurate t_o. For instance, in RPC, neutral solutes such as thiourea, uracil, and dimethylsulfoxide are used as EOF tracer while toluene or benzene is used to trace the dead time in HILIC. The value of k' can lie anywhere between 0 to ∞. Higher values of k' indicates higher interaction of solutes with the stationary phase leading to higher retention. In other words k´ is a peak locator. The value of k is independent of column length and flow rate.

Retention factor of solutes in CEC – Case of charged solutes. Unlike neutral solutes, in the case of charged solutes, retention results from a combination of electrophoretic migration and chromatographic partitioning. Thus, the k' applicable for neutral solute does not provide a measure of chromatographic partitioning of charged solutes. Thus, another retention factor, k*, was introduced by Rathore and Horvath [11, 12] to evaluate the migration of charged solutes in CEC, and is mathematically represented as:

$$k^* = \frac{t_R(1 + k_e^*) - t_o}{t_o}$$

(5)

Where t_R is the migration time of the charged solutes, t_o is the migration time of EOF tracer and k_e^* is the velocity factor defined as:

$$k_e^* = \frac{\upsilon_{ep}}{u_{eo}}$$

(6)

Where u_{eo} is the interstitial electroosmotic velocity of the mobile phase in the CEC column and υ_{ep} is the electrophoretic velocity of the charged species. For neutral solutes, k_e^* is zero and k* reverts to k'.

Selectivity factor. Selectivity factor or relative retention, α, defines a measure of the discrimination factor between two solutes by a chromatographic system. It is mathematically represented as:

$$\alpha = \frac{t_{R1} - t_o}{t_{R2} - t_o} = \frac{k'_2}{k'_1}$$

(7)

The value of k'_2 is always greater than k'_1 so, $1 \leq \alpha < \infty$ is a measure of the separation power of the system. A value of 1.1 is indicative of a good separation.

Separation efficiency. Separation efficiency is a measure of the narrowness of bandwidth produced by a chromatographic column. The height equivalent to a theoretical plate H is used as a measure of separation efficiency and is defined as the ratio of peak variance (σ_L^2) in unit length to the column effective length (l).

$$H = \frac{\sigma_L^2}{l}$$

(8)

The number of theoretical plates per column (N) is a dimensionless unit defined as N = 1/H. By substituting H from Eq. 8 and by using migration time instead of column effective length, N can be expressed as:

$$N = \frac{l^2}{\sigma_L^2} = (\frac{t_R}{\sigma_t})^2$$

(9)

The migration time t_R and peak standard deviation σ_t are obtained from the electrochromatogram. Since chromatographic peaks are usually approximated as Gaussian distributions, peak variances can be calculated from the peak width at base (W_b), half height (W_h) or at the inflection point (W_i) and are equal to 4σ, 2.354σ, and 2σ respectively. The inflection point is located at 0.607 of peak height. Substituting σ into equation (9), N can be written as:

$$N = 4\left(\frac{t_R}{W_i}\right)^2 = 5.54\left(\frac{t_R}{W_h}\right)^2 = 16\left(\frac{t_R}{W_b}\right)^2$$

(10)

Resolution. Resolution (R_s) is defiend as the extent of separation between two adjacent peaks. It is calculated from the chromatogram by the ratio of the difference between the migration times of the two adjacent peaks (Δt_R) to the average bandwidth of both peaks at base. When the value of R_s = 1.5, baseline resolution is achieved and at R_s=1 adequate

separation is considered to be achieved. Resolution is a function of selectivity, retention factor and efficiency as shown by equation below:

$$R_s = \left(\frac{\alpha - 1}{\alpha}\right)\left(\frac{k'_2}{1 + k'_2}\right)\left(\frac{\sqrt{N}}{4}\right) \quad (11)$$

Where k'_2 is the retention factor of the more retained peak. High resolution is obtained by optimizing the three parameters, α, k′ and N. Increasing the number of theoretical plates, N, by increasing the length of column leads to an increase in retention time and causes band broadening, which is undesirable. However, the plate height can be reduced by reducing the size of the stationary phase particles. Changing the column temperature or changing the composition of the mobile phase can greatly improve the retention factor, k'. The selectivity factor, α, can also be used to influence separations. This is done by various ways like changing the nature of the stationary phase or the composition of the mobile phase or by incorporating species in the mobile phase, which can form complexes with solutes.

Types of stationary phases – A review of monolithic stationary phases

Types of stationary phases.

Columns for CEC can be broadly categorized into three classes, namely particle packed columns, continuous-bed or monolithic columns and open tubular columns. Particle packed columns are filled with fine packing materials, which are retained within the column with frits at each column end. Packed columns were the most commonly used

stationary phases for CEC however, they possess several disadvantages including difficulty of fabricating column with reproducible properties, high probability of bubble formation due to frits leading to EOF obstruction [13]. Open tubular (OT) columns have a bonded stationary phase coated on the inner surface of the open tube or bonded molecular monolayer or a porous network deposited on the capillary inner walls. The OT columns are easy to prepare, they have short conditioning time and exhibit high separation efficiencies. Unlike packed columns, OT columns do not have the disadvantages associated with fabrication difficulties and bubble generation due to frits but they have drawbacks of their own. Due to low surface area, the phase ratio is low which leads to low separation capability and limited sample loading [14, 15].

Monoliths are single continuous beds of stationary phases, which consist of highly porous interconnected network of channels. This enhances the mass transfer in monoliths compared to the particle packed columns. Since the columns are filled completely there are comparatively no voids in the monolithic stationary phases. Due to the presence of ample amount of pores, these columns have high permeability and large surface area. Moreover, they provide the flexibility to alter the porosity as required, they are easy to prepare and a wide variety of chemistries are available for fabricating the monoliths [16]. Due to the endless probability of monomers, cross linkers, porogens, post-polymerization modification, column fabrication temperature and time, monoliths with desired functionality and porosity can be prepared according to the requirement [17].

From the earliest reported monoliths made of poly(ethyleneglycol methacrylate) used for gel permeation chromatography by Kubin *et al.* in 1967 [18] and polyacrylamide compressed soft gels used for cation-exchange chromatography by Hjerten et al in 1989

[3] to current day, monoliths have increasingly become popular as effective separation media for solving important separation problems [19-21].

Nonpolar organic monoliths-Polymethacrylate/acrylate based monolithic stationary phases

Methacrylate and acrylate based monoliths are by far the most widely used have organic polymer monoliths. They are used as chromatographic stationary phases by co-polymerization along with cross linkers as well as by functionalization of the precursor or parent monolith using various methods.

Functional monomers co-polymerized with cross linkers. The most commonly practiced method of polymer monolith fabrication is by copolymerizing the monomer and cross linker together in the presence of the proper porogens and initiator. The type of monomer/cross linker and their concentration can be adjusted by varying their amount according to requirement [22].

A new porous monolith for capillary liquid chromatography (cLC) was reported recently by Alshitari *et al.* [23]. It was made by the co-polymerization of hexyl methacrylate (HMA) using 1,4 butanediol and 1-propanol as the porogens. Two different cross linkers 1,6-hexanediol ethoxylate diacrylate (1,6-HEDA), and ethylene dimethacrylate (EDMA) were used. The effects of the cross linker length on separation efficiencies of small molecules were investigated. It was reported that using 1,6-HEDA increased the separation efficiencies by tenfold. This improved efficiency was attributed to the increase in the number of non-polar methylene groups in poly(HMA-co-1,6-HEDA) monolith which resulted in increasing the number of small pores. The monolith exhibited

good selectivity for neutral non-polar molecules, weak acid molecules and basic molecules. The column showed high reproducibility for the separation of anisole and naphthalene.

A methacrylate ester based monolithic column was also made for cLC using butyl methacrylate (BMA) as the monomer and EDMA as the cross linker [24]. The influence of polymerization mixture was studied by the authors whom concluded that BMA/EDMA 60/40 % (wt/wt) and 50% (wt/wt) porogens yielded an optimum monolith. The porogens used were (36 wt% 1,4-butanediol, 54 wt% 1-propanol and 10 wt% water. The optimized monolith was used for the ultra-low determination of parabens in human urine and serum samples and it was reported that the detection was simple and fast with low sample and reagent consumptions.

The team of Zou studied the effect exhibited by the extent of cross linker on the separation efficiencies of small molecules. They made two series of monoliths using a multi-functional crosslinker the dipentaerythritol penta-/hexa-acrylate (DPEPA) and lauryl methacrylate (LMA) as the functional monomer. Hexyl alcohol and ethylene glycol were used as the porogens. In one series poly(LMA-co-DPEPA) monolith with higher content of crosslinker (63.3% w/w) was fabricated which exhibited low permeability but high column separation efficiencies (111,000-165,000 plates/m) for ABs in cLC [25]. The next series of monoliths was prepared with relatively lower content of crosslinker (52.7% w/w), which exhibited high permeability but low separation efficiencies (50,000-93,000 plates/m). According to the report, the use of multi-functional crosslinker possibly prevents the formation of gel-like micropores which in turn reduces the mass transfer resistance thereby increasing the efficiency of separation. In addition to ABs, the selectivity of the poly(LMA-co-DPEPA) monolith was demonstrated by using phenols and some basic

compounds like caffeine, carbamazepine, 2,4-dinitroaniline 2,6-dichloro-4-dinitroaniline and 2,6-dichloro-4-nitroaniline. Base line separation of these compounds on both columns was achieved. The retention as well as the separation efficiency were higher on column with higher cross linker composition with the highest plate count for 2,6 dimethyl phenol of 86,000 plates/m. The same compound exhibited a plate count of 52,000 plates/m on the column with lower cross linker composition. Faster separation with a lower separation efficiencies was obtained in the column with lower cross linker composition.

Another report from Zou and co-workers appeared recently in which the researchers developed a new approach for preparing polymeric monolith from pentaerythritol diacrylate monostearate (PEDAS) and trimethylolpropane tris(3-mercaptopropionate (TPTM) by photo induced thiol-acrylate polymerization in 10 min. The porogens n-hexanol and ethylene glycol were used. The morphology of poly(PEDAS-co-TPTM) monolith with poly(PEDAS) monolith prepared solely from PEDAS monomer by photo-induced free radical polymerization was compared and it was observed that poly(PEDAS-co-TPTM) monolith possessed better permeability than poly(PEDAS) fabricated under otherwise the same conditions [26]. It exhibited lower plate heights (15.7-17.7 μm) compared to poly(PEDAS) monolith (19.1-37.9 μm) in μLC. It was reported that the monolith was able to identify 66 unique peptides from the tryptic digest of four proteins on the poly(PEDAS-co-TPTM) monolith indicating that this monolith may have a potential in proteomics analysis.

A novel polymeric monolith was prepared by Zhong *et al.* using triallyl isocyanurate (TAIC) as the functional monomer, trimethylolpropane triacrylate (TRIM) as the cross linking monomer, polyethylene glycol 200 and 1,2-propanediol as the porogens.

The initiator for this polymerization was carbon tetrachloride in the presence of the catalyst ferrous chloride [27]. The monolith was polymerized by atom transfer radical polymerization (ATRP) technique. This poly(TAIC-co-TRIM) monolith was used for both reversed-phase HPLC and cLC to separate aromatic compounds. The column showed good stability and high separation efficiencies with theoretical plates of 125,000 plates/m and good reproducibility. Baseline separation of aromatic compounds such as phenol, naphthol, biphenyl, phenanthrene and some other compounds was obtained.

A γ-radiation induced polymethacrylate based monolithic capillary column was prepared from LMA as the functional monomer with 1,6-hexanediol dimethacrylate (HDDMA) as the cross linking monomer. This (γ –poly-(LMA-co-HDDMA)) monolith was used in the cLC mode for the separation of nine standard proteins which had a varying range of molecular weights (from 14.3 to 93.1 kDa), pI values (from 4.9 to 9.4) and hydrophobicities. The experimental conditions were optimized and the best results in terms of selectivity and peak capacity were obtained using acetonitrile with 0.1% TFA at 60 ˚C column temperature. These γ-radiated columns exhibited high permeability and mechanical stability at elevated temperatures and low pH values [28]. This allowed the use of longer columns of 50 cm and peak capacity value of more than 1000 was achieved in almost 3 h separation time window, which is a quite long analysis time. Under this time frame, one could claim that no other report of such high value of peak capacity on polymethacrylate based monolith has been published in the literature.

A robust naphthyl methacrylate monolithic column made from the co-polymerization of 2-napthyl methacrylate (NAPM) as the functional monomer with TRIM as the cross linking monomer was used in the reversed-phase mode in HPLC [29]. In this

work, Jonnada and El Rassi reported that the column showed high mechanical stability and good hydrodynamic characteristics. Compared to octadecyl silica (ODS) column, this column was shown to possess π-π interaction, due to π-electron rich naphthyl ligands, in addition to the hydrophobic interactions under reversed-phase conditions. The column was useful in the separation of aromatic compounds such as benzene, toluene, phenol and aniline derivatives. The authors carried out the separation of standard proteins under linear gradient elution at increasing acetonitrile concentration at various flow rates, characterized as shallow, steep and ultra-steep gradients. The monolithic column showed high stability even under the high mobile phase flow velocity.

A hexamethacrylate-co-ethylene dimethacrylate monolith incorporated with metal organic framework MIL-53(Al) was prepared by Yusuf *et al.* [30]. In this work, the authors showed the separation of mixtures of small aromatic compounds by cLC. 1,4-Benzenedicarboxylate moieties were present within the MIL-53(Al) structure which greatly influenced the separation of the aromatic compounds such as toluene, p-xylene, 1,2,4- trimethylbenzene due to π-π interactions [30]. The auhtors also reported the separation of ABs in less than 8 min with plate counts of 14,710 plates/m for propylbenzene. This monolith showed lower efficiency compared to hypercrosslinked mononithic columns but this kind of column has the advantage of metal organic frameworks (MOFs) manifested by the high surface area, porosity, chemical stability and designable structure as well as polymeric monoliths. Thus, it provides a challenge in fabricating a column which combines the advantages of both MOF particles and a polymeric monolith.

Single monomer/cross linker monoliths. Polymer monolithic columns synthesized from a single monomer/cross linker provide mechanical stability and high surface area due to the highly crosslinked surface [31] and the optimization of polymerization mixture is easier since only the monomer-porogens ratio need to be adjusted. Okanda and El Rassi were the first to report a polymer monolith synthesized from a pentaerythritol diacrylate monostearate (PEDAS) as the single monomer/cross linker. PEDAS provided a macroporous nonpolar monolith with C-17 surface chains and this monolith exhibited high column separation efficiencies for protein and peptides with plate number of 255,000 plates/m and 121,000 plates/m, respectively.

Recently, Aggarwal *et al*., used polyethylene glycol diacrylate (PEGDA) monolith prepared from ethylene oxide of varying molecular weights as a single cross-linking monomer. The researchers prepared an array of polymer monoliths using different combination of monomers and porogen composition. A surfactant porogen (tergitol) and typical organic liquid progens such as dodecanol, decanol, hexane, decane, isobutanol, isopropanol, ethylether and methanol were used [32]. It was reported that the monolithic reversed-phase liquid chromatography (RPLC) stationary phase showed efficiencies of > 100,000 plates/m and hydrogen bonding interactions in addition to reversed-phase behavior. Successful separations of low molecular weight polar compounds, such as hydroxyl benzoic acids, phenols, phenyl urea herbicides and non-steroidal anti-inflammatory drugs were achieved.

In another study, the same group studied the fabrication of polymer monolith based on single cross-linking monomers using three different monomers namely 1,12-dodecanediol dimethacrylate (1,12-DoDDMA), trimethylolpropane trimethacrylate

(TRIM) and pentaerythritol tetracrylate (PETA) [33]. Instead of implementing conventional free-radical polymerization, organotellurium-mediated living radical polymerization (TERP) was used and reversed-phase separation of ABs was obtained. The column gave plate counts as high as 60,200 plates/m. Upon comparison, monolithic columns made by TERP demonstrated improved morphology and separation performance compared to free radical polymerized columns.

There was yet another work demonstrating single cross-linking monomer for the fabrication of organic monolithic capillary by kucherenko *et al.*, where PETA was used for the synthesis of monolithic column. Nonanol and decanol were the porogens [34]. According to the report the best separation efficiency was obtained from the monolith with 33%-34% monomer composition and five peptides were separated.

Ionic liquid based monoliths. Ionic liquids (ILs) are defined as organic salts which are in the liquid state at or below 100 ˚C. They are composed of bulky, nonsymmetrical organic cations such as imidazolium, pyrrolidinium, pyridinium, tetraalkyl ammonium or tetralkyl phosphonium and an organic or inorganic anion such as tetrafluoroborate, hexafluorophosphate and bromide. Compared to organic solvents, ILs possess many useful characteristics including high thermal stability, negligible vapor pressure, non-flammability, varying viscosities, conductivity and miscibility in different solvents. Due to these features, ILs have been used in many analytical fields including LC, CE and CEC [35-37]. Various silica-based and organic polymer monolith based ILs stationary phases are available.

Recently, Wang *et al.* prepared a novel polymer monolith based on poly(PETA-co-EDMA) in the presence of 1-butyl3-methylimidazolium chloride ($BMIM^+Cl^-$) as the porogen. In this work, the effects of porogens on the properties of the monoliths were studied. 1,4-Butanediol, 1-propanol and $BMIM^+Cl^-$ in different proportions were used as porogens to make different monoliths and were evaluated using mixture of aromatic compounds such as benzene, p-xylene, naphthalene, diphenyl and anthracene. It was concluded that better chromatograms were obtained from the column with a higher total porosity which was created by $BMIM^+Cl^-$.The authors attributed this result to the high surface area of the column which would lead to quicker mass transfer. The column was further evaluated by the separation of acidic and basic compounds such as hydroquinone, phenol, p-nitrophenol, 1-naphthol, p-phenylenediamine, aniline, benzylamine, among others [38]. Some homologues of benzene were eluted in the order benzene < methylbenzene < p-xylene according to the reversed phase retention mechanism. Toluene derivatives including p-aminotoluene, p-chlorotoluene were also evaluated and the retention of these compounds increased with decreasing the concentration of acetonitrile in the mobile phase which further confirmed the monoliths reversed-phase nature. According to the authors, the use of IL was helpful in not only forming macropores but also in improving column efficiency and this method could be an alternative tool for separation of small molecules in HPLC.

Wang *et al.* published another work where the authors showed the incorporation of 1-allyl-3-methylimidazolium chloride ($AMIM^+Cl^-$) into the porous polymer monoliths to enhance the separation performance. A polymer monolith was prepared from $AMIM^+Cl^-$, TMPTA as monomers EDMA as crosslinker and 1-propanol and 1,4-butanediol as

porogens. The monolith was designated as poly(IL-co-TMPTA-EDMA) [39]. From this work, the authors were able to demonstrate the use of $AMIM^+Cl^-$ entrapped within the polymer monolith in improving the performance of the monoliths in the separation of small molecules. A series of different monoliths were prepared with and without $AMIM^+Cl^-$ to evaluate the effect of incorporating the ILs in the monolith. The poly(TMPTA-co-EDMA) monolith without $AMIM^+Cl^-$ (designated as column A) and poly(IL-co-TMPTA-co-EDMA) monoliths containing varying amounts of $AMIM^+Cl^-$ (designated B and C) were tested with aromatic hydrocarbons namely benzene, naphthalene and anthracene using ACN/H_2O (55:45, v/v) as the mobile phase. It was found that the resolution of analytes and separation efficiencies in column B and C were comparatively higher than column A. Even among the columns with ILs, higher $AMIM^+Cl^-$ containing monolith showed better resolution and higher separation efficiencies. Thus it was concluded that ILs played a significant role in the enhanced separation of small molecules in reversed-phase HPLC. Homologues of benzene (benzene, toluene and p-xylene) and isomers of phenylenediamine (*o-, m-, p-* phenylenediamine) were also tested for the evaluation of the monoliths. In comparison, the isomers m-phenylenediamine and o-phenylenediamine were overlapped on the poly(TMPTA-co-EDMA) monolith however, baseline separation of the mixture of isomers was achieved with the poly(IL-co-TMPTA-co-EDMA) monolith. Good separation of homologues was achieved on the ILs incorporated monoliths with the separation efficiencies between 24,3000 and 27,000 plates/m and the elution order was benzene < toluene < *p*-xylene. Thus, it was concluded that the addition of ILs in the polymerization solution helps to form macropores and improves the separation efficiencies and the column

exhibited good mechanical stability which makes them suitable for use in HPLC for the separation of small molecules.

Another novel IL based monolithic column was reported by Qin *et al.* wherein the monolith was fabricated by *in situ* polymerization of the IL 1-vinyl-3-butylimidazolium chloride, 1-dodecene (C-12) and TMPTA as monomers, EDMA as crosslinker and dodecanol as the porogen. Lysine from egg white and aromatic compounds were used for assessing the monoliths [40]. Aniline, p-xylene, naphthalene, diphenylamine and triphenylamine were eluted in the order of high to low polarity and the retention of these compounds increased with decreasing methanol concentration in the mobile phase showing a typical reversed-phase mode. Similarly, benzene, naphthalene, biphenyl and anthracene eluted in accordance to their polarities from high to low exhibiting separation efficiencies of between 5940-9249 plates/m. According to the authors, this monolithic column offers a potential alternative for the efficient separation of proteins and small molecules.

Precursor monolithic stationary phases with tailored surfaces. Surface modification or tailoring of polymer monolith surface to yield the desired surface functionality has attracted a great deal of attention. The main feature of this method is the possibility of obtaining a range of monolithic stationary phases from a single precursor monolith. While *in situ* co-polymerization is a well-practiced method of monolith fabrication, this approach has some disadvantages such as inaccessibility of the functional group due to its burial within the monolith structure and the need to re-adjust the polymerization conditions to achieve the desirable porosity of a given monolith can be challenging [17]. Thus, surface modification or post-polymerization modification is a good alternative since chemical modification of a

precursor monolith can generate a large number and varieties of functionalized monoliths without the need to re-adjust the polymerization conditions.

Recently, a novel glycerol carbonate methacrylate (GCMA) based monolith was reported. First, a polymerization mixture, which provided optimum permeability was identified by fabricating the monolith with different porogenic solvent mixtures. Polymerization mixture which contained 40/60% (v/v) toluene/dodecanol as porogen mixture, 39.2/60.8% (w/w) monomers/porogenic solvents and 34.4/65.6% (w/w) monomer/cross linker showed optimum permeability. After the permeability was adjusted, the glycerol carbonate functionalities available on the pore surface of the monolith were functionalized with allylamine by nucleophilic addition reaction. The alkene functionalities thus exposed on the surface of the monolith allowed a platform to generate versatile and easy subsequent thiol-ene click reaction. For this click reaction, 1-octane thiol and 4-mercaptobutyric acid were used to obtain C8-grafted monolith and carboxy functionalized monolith, respectively [41]. The carboxy-functionalized monolith was further immobilized with platinum nanoparticles and was used for flow through catalysis of p-nitrophenol. The C8 grafted monolith was evaluated with alkylbenzene solutes in the RPC mode and it was shown that the retention of ABs increased with decreasing acetonitrile concentration.

Carrasco-Corrrea *et al.* reported a poly(GMA-co-EDMA) monolithic column incorporated with vinylized iorn oxide magnetic nanoparticles (VMNPs) for CEC. VMNPs were prepared by linking vinyl group to the magnetic nanoparticles (MNPs) [42]. Varying amount of VMNPs (0.5-5 wt%) were incorporated in the polymerization mixture and the capillary monolith was obtained by UV-polymerization in 15 min. Electrochromatographic evaluation of this hybrid monolith was performed using a mixture of ABs (toluene,

ethylbenzene, propylbenzene, butylbenzene, pentylbenzene and hexylbenzene) and the effect of incorporation of VMNPs in the monolith was studied by comparing it with the poly(GMA-co-EDMA) monolith. The retention of the ABs test analytes was significantly higher in the monolith co-polymerized with VMNPs. Similarly, organophosphorous pesticides also showed increased retention on the monoliths with higher VMNP content. This behavior was attributed to the increase in the surface area of the monolith due to the presence of VMNPs.

Divinyl based monolithic stationary phases

A polymer monolithic column prepared from the low conversion polymerization or short-term polymerization of *N*-vinylcarbazole (NVC) and 1,4-divinylbenzene (DVB) was reported by Koeck *et al.* [43]. According to the authors, this NVC/DVB monolith was able to generate the lowest plate height value ever reported for small molecules on an organic monolithic column (3.9 μm corresponding to ~ 256,000 plates/m). The effect of polymerization time on the retention of a mixture of seven ABs including benzene was studied and it was shown that by increasing the polymerization time from 90 to 180 min, the elution time of hexylbenzene increased by double although there was no significant difference in resolution. A gradient separation of ABs, β-blockers, and mixture of six flavonoids (catechin, epicatechin, rutin, quercetin, hesperidin, kaempferol) and one silbenoid was performed. Baseline separation of ABs within 5 min was achieved by applying a gradient from 30-99% (v/v) ACN. Likewise, baseline separation of all six flavonoids and one silbenoid was achieved by applying a gradient from 1-40% (v/v) ACN

in 8 min. Also, four parabens and six phenones were separated by applying gradient and isocratic elution modes.

Kip *et al.* proposed new vinylester-based monoliths as the stationary phases for CEC. Vinyl pivalate (VPV) and vinyl decanoate (VDC) were used as monomers and EDMA was a cross linker. 2-Acrylamido-2-methylpropane sulfonic acid was used as the charge-bearing monomer, isoamyl alcohol and 1,4-butanediol were used as porogens [44]. Effect of porogen concentration on the morphology and ultimately on the chromatographic performance of both poly(VPV-co-EDMA) and poly(VDC-co-EDMA) monoliths was investigated. From a series of experiments it was concluded that poly(VPV-co-EDMA) monolith synthesized with isoamyl alcohol concentration of 33% (v/v) showed better resolution and exhibited 250,000 plates/m. Similarly, poly(VDC-co-EDMA) synthesized with isoamyl alcohol concentration of 66% (v/v) produced the best resolution using ABs solutes.

Hydrophobic interaction monoliths

Chen *et al.* developed a porous methacrylate-based monolithic column by co-polymerizing setaryl methacrylate, TMPTA and 2-acrylamideo-2-methyl-propanesulfonic acid in a ternary porogenic solvent including cyclohexanol, 1,4-butanediol and water. This hydrophobic poly(SMA-co-TMPTA-co-AMPS) monolithic column was used in developing a novel CEC method for the determination of nucleoside and purine compounds in human urine. The authors investigated the effect of ACN concentration on the retention of solutes namely, adenine, guanine, N^6-methyladenosine, adenosine and cytidine. The retention factors were only slightly decreased with increasing concentration of ACN [45]. The ACN concentration was varied between 0-10% (v/v). From these data it was deduced

that hydrophobic interaction was not the predominant retention mechanism exhibited by this monolithic column. According to these researchers, the presence of amino groups, hydroxyl groups, heterocyclic nitrogen groups in the above analytes could interact with the monolith surface resulting in the comprehensive interactions including hydrogen bond interactions, van der Waals interactions and Coulomb interactions. Also, separation of the analytes was brought about using a mobile phase containing 6 mM phosphate buffer, pH 5.0 without ACN within 4 min. This method was implemented successfully in the determination of the nucleosides in urine samples.

A porous functional polymer monolith was prepared from hexanediol diacrylate (HDDA) and BMA as co-monomers, EDMA as cross linker and dodecyl alcohol as porogenic solvent. This poly(HDDA-co-BMA-co-EDMA) monolith was used for the separation of small molecules in HPLC. The C-6 alkyl chain of the HDDA monomer provided hydrophobic groups on the monolithic surface. This monolith provided hydrophobic interaction at lower methanol content and the column was evaluated with dimethylbenzene, azimidobenzene, biphenyl and anthracene using methanol/water (90/10 %(v/v)) [46]. The study of the effect of methanol content in the mobile phase on the retention of the above mentioned analytes was carried out and it was found that as the methanol content went down from 95% (v/v) to 85% (v/v), solute retention increased. From the order of peaks eluted from the monolithic column, it was concluded that the column showed hydrophobic interaction mode of separation. Some other molecules such as aniline compounds and phenol compounds were also separated.

Polar organic monoliths

A poly(alkyl methacrylate-co-methacrylic acid-co-ethylene dimethacrylate) monolithic column was prepared by Lin *et al.* for the separation of polar small molecules by cCL. The methacrylic acid (MAA) was polymerized with alkyl methacrylate to increase the hydrophilicity of the monolith [47]. The effect of varying the alkyl chain length as well as the addition of MAA into the monolith composition was studied by fabricating a series of 30% (w/w) alkyl methacrylate-MAA-EDMA monolith capillaries using EDMA as the crosslinker with three different alkyl methacrylate-MAA ratios. The alkyl methacrylates used for studying the effect of chain length were butyl methacrylate (BMA), hexyl methacrylate (HMA), octyl methacrylate (OCA), lauryl methacrylate (LMA), stearyl methacrylate (SMA). The column containing 6% MAA showed very high back pressure. Also, the monolith made from BMA has the smallest pore sizes which led to high back pressure. However, larger through-pores were found in monoliths fabricated from HMA, OMA, LMA and SMA. The appearance of large pore size gave lower back-pressure resulting in higher permeability. A mixture of phenol and its derivatives namely 3,5-dimethylphenol (3,5-DMP), 2,3,5-trimethylphenol (2,3,5-TMP), 2,4-dichlorophenol (2,4-DCP), 3,5-dichlorophenol (3,5-DCP) and 2,3,5-trichlorophenol (2,3,5-TCP) were used to evaluate the monolith and six phenol derivatives were separated. Monoliths containing higher ratio of MAA (MAA:alkylmethacrylates) showed better separation which the authors attributed to the increased hydrophilic interactions between the analytes and MAA-incorporated monoliths. As far as the comparison of separation between the monoliths with the same MAA concentration was concerned, resolution among solutes decreased with the increase in alkyl methacrylate chain length due to the steric hindrance between the polar

analytes and the MAA in the alkylmethacrylates-MAA-EDMA. A steep gradient elution (30-70% ACN in 2.5 min) was also used for separation of these phenol derivatives at various flow rates (6, 12 and 18 μL min^{-1}). A fast separation of phenol derivatives at flow rate of 18 μL min^{-1} was achieved in 5 min. This monolith was applied to analyze five aflatoxins (AFs) namely AFB1, AFB2, AFG1, AFG2 and AFM1. Also, three antibiotics thiamphenicol (TAP), florfenicol (FF) and chloramphenicol (CAP) were also used to demonstrate the separation capability of selected LMA-MAA-EDMA monolith. The separation of these AFs and antibiotics were compared with the 1,6 hexanediol ethoxylate diacrylate based alkyl methacrylate monolith (LMA-HEDA) which was fabricated by the authors in their previous work which exhibited hydrophilic interaction with the analytes through two ether-linkages in the alkyl bridge of HEDA [48]. However, LMA-MAA-EDMA showed better resolution and good separations when compared to LMA-HEDA monolith for aflatoxins except (AFB2 and AFG1) and antibiotics.

Gökaltun *et al.* reported the preparation of a stationary phase for CEC using 3-chloro-2-hydroxypropyl methacrylate (HPMA-Cl) as monomer with EDMA as the cross linker. The poly(HPMA-Cl-co-EDMA) monolith was further modified with sodium bisulfite to obtain a surface with ionizable sulfonic acid group and consequently the monolith thus obtained exhibited cathodal EOF. ABs, phenol derivatives including catechol, *p*-cresol and 3-nitrophenol and benzoic acids were used for the evaluation of the monolith [49]. A comparison of separation efficiencies for some analytes between poly(HPMA-Cl-co-EDMA) monolith with that of several previously reported monoliths from the literature was done. It was concluded that the CEC column obtained from the post-polymerization modification of poly(HMPA-Cl-co-EDMA) gave comparable

separation efficiencies as that of the monoliths obtained from co-polymerization of the charge-bearing monomers.

Another monolith for HILIC was prepared from the post-polymerization modification of the above mentioned poly(HMPA-Cl-co-EDMA) by Kip *et al.* [50] using a tertiary amine, triethanolamine (TEA-OH), through the reactive chloropropyl group available on the surface of the polymer monolith. From a range of monoliths fabricated using various concentrations of monomers and porogens (isopropanol and dodecanol), monoliths made from 60% (v/v) of HPMA-Cl, porogen/monomer ratio 2.4 (v/v) and isopropanol concentration of 40% (v/v) was found to be most hydrophilic since it contained the highest amount of TEA-OH immobilized on the monolith surface. The monolith was evaluated with nucleotides, nucleosides and benzoic acid derivatives. Baseline separation of nucleotides (uracil, adenine, cytosine, and guanine) were obtained with the ACN/water ratio between 96/4 (%v/v) and 93/7 (%v/v). In the case of nucleosides (thymidine, uridine, inosine and cytidine) baseline separation was obtained using a mobile phase with ACN/water 96/4 (%v/v). The monolith is claimed to be competitive in chromatographic performance with polymer based HILIC monoliths in the recent literature. Decrease in column performance with increasing retention factor was observed which was explained to be the result of temporary gel-type micropores formed in the regions with lower crosslinking density within the polymer monoliths. Stronger interactions between analytes and stationary phase was also attributed as a reason for this behavior.

Chiral monoliths

Guo *et al.* have reported the use of a click reaction between propargyl methacrylate and mono-6-azido-β-cyclodextrin to synthesize a methacrylate derivatized β-cyclodextrin. This methacrylate derivatized β-CD was employed as a monomer for the preparation of a chiral monolithic stationary phase [51]. For the preparation of the desired monolith, first propargyl methacrylate (PMA) was reacted with mono-6-azido-β-CD and it was further modified with either methyl or sulfate group to get PMA-methylated- β-CD or PMA-sulfated-β-CD. Three β-CD monolithic columns were prepared namely poly(PMA-β-CD-co-EDMA), poly(PMA-methylated-β-CD-co-EDMA) and poly(PMA-sulfated-β-CD-co-EDMA) where EDMA was a cross linker. Poly(PMA-β-CD-co-EDMA) was the underivatized monolith and it showed dual RP and HILIC modes of separation. The enantioselectivity of the poly(PMA-methylated-β-CD-co-EDMA) monolith was evaluated using acebutolol. A good separation of this chiral compounds was obtained under RPC conditions. The separation was good with baseline resolution ($R_{s=}$ 2.692) within 30 min however, the peak efficiency was low. For further evaluating the methylated monolith, a test mixture containing thiourea, dimethylphthalate, anisole and naphthalene was analyzed in the RPC mode using isocratic elution with ACN/H_2O (40:60, v/v) as the mobile phase. A homologous series of eight alkyl ketones (acetophenone, propiophenone, butyrophenone, valerophenone, hexanophenone, heptanophenone, octanophenone and decanophenone) were also tested on the methylated monolith. The order of elution of these ketones confirmed the hydrophobicity of the monolith. Furthermore, the poly(PMA-sulfated-β-CD-co-EDMA) was evaluated using a mixture of seven analytes (nucleobases and nucleosides) including uridine, 5-methyluridine, adenosine, cytidine, cytosine,

thymine and adenine. In fact, a comparison between sulfated and underivatized monolith was made using these solutes. It was observed that under the same chromatographic conditions (using ACN/H_2O, 90/10 (%v/v)) the retention of highly hydrophilic analytes cytidine and cytosine on the sulfated monolith was significantly higher than on the underivatized monolith. This could be due to the substitution of hydroxyl groups with sulfonic acid groups. It was also reported that the sulfate derivatized monolith showed comparatively lower separation efficiencies for nucleobases and nucleosides.

In another study, Guo *et al*. reported the effect of linking spacer on the enantioseparation ability of β-CD functionalized polymer monolith. In this investigation, the authors prepared three β-CD-functionalized organic polymer monoliths having different space length using three amino-β-CDs namely mono-6-amino-6-deoxy-β-CD, mono-6-ethylenediamine-6-deoxy-β-CD and mono-6-hexamethylenediamine-6-deoxy-β-CD [52]. First, these amino-β-CDs were reacted with glycidyl methacrylate to obtain functional monomers, which were then co-polymerized with EDMA yielding monolithic columns with different amino linking spacers designated as poly(GMA-NH_2- β-CD-co-EDMA) (column A), poly(GMA-EDA-β-CD-co-EDMA) (column B) and poly(GMA-HDA-β-CD-co-EDMA) (column C) monoliths corresponding to –NH-, -NH-$(CH_2)_2$-NH-, and -NH-$(CH_2)_6$-NH- linkers, respectively. It was observed that the monoliths exhibited similar morphology, permeability, β-CD density and hydrophobicity irrespective of the spacer length. However, upon evaluation of the monoliths with 14 chiral acidic compounds the authors reported that column A provided higher R_s and α values than columns B and C for most of the chiral analytes. From the results, it was concluded that the length of spacer link could affect the enantioseparation ability of the monolithic columns to some extent.

For most of the analytes better resolution and selectivity were observed in the monolithic column with shorter linking spacer length.

A new one pot synthesis of chiral β-CD functionalized polymer monolith was reported by Ahmed *et al.* for nanoLC using 2,3,6-tris(phenylcarbamoyl)-β-cyclodextrin-6-methacrylate as the functional monomer and EDMA as the cross linker. Three different monoilths were prepared using varying concentration (wt%) of the monomer and cross linker [53]. In this work, the authors investigated the effect of monomer/cross linker concentration on the chiral separation of different classes of pharmaceuticals namely α-, β-blockers, anti-inflammatory drugs, antifuntal drugs, dopamine antagonists, norepinephrine-dopamine reuptake inhibitors, catecholamines, sedative hypnotics, diuretics, antihistaminics, anticancer drugs and antiarrhythmic drugs. The monolith with 10 (wt%) monomer/30 (wt%) cross linker (A1) as well as the monolith with 20 (wt%) monomer/20 (wt%) cross linker (A2) were permeable and good separation was obtained. However, 30 (wt%) monomer/10 (wt%) cross linker (A3) containing monolith was not permeable. Out of the tested drugs, baseline separation of propranolol, ifosfamide, alprenolol, tertalol, 1-indanol, tebucanazole, o-methoxymandelic acid, celiprolol and cizolertine was obtained under RPC conditions (methanol:water (0.1% TFA), 10:90 (%v/v)) on both A1 and A2 monoliths.

A novel chiral CD monolith based on one pot thiol-ene click reaction of allyl-β-CD with pentraerythritol tetra-(3-mercaptopropionate) was prepared for cLC by Zhang *et al*. [54]. The effects of composition of prepolymerization mixture and reaction temperature on morphology, permeability and selectivity of the chiral monolith were studied. Enantioseparation of various pharmaceutical compounds such as flurbiprofen, naproxen,

ketoprofen, synephrine and isoprenaline sulfate were achieved using ACN/0.1%Triethylammonium acetate buffer, 70/30(%v/v). The authors claimed that the chiral isomers were separated with good resolution in shorter analysis time (within 13 min) compared to that reported in the literature.

Mixed mode monoliths

Generally, a single mode of interaction occurs between stationary phase and solutes. So, these stationary phases are applied in single chromatographic mode of separation such as either RPC, HILIC or ion exchange chromatography [55, 56]. RPC is the most widely used mode of separation however, it is not always ideal especially for the retention and resolution of polar or ionic analytes such as pharmaceutical or biological solutes. Thus, mixed mode chromatography emerged as an alternative to solving the problem [57]. The resolution in mixed mode chromatography occurs from at least two different interactions between a given solute and a given stationary phase. Thus, one of the main characteristics of mixed mode stationary phases is to have a multifunctional surface [55]. This mode of separation utilizes secondary interaction to separate similar compounds that would normally co-elute. Mixed mode surfaces are generally made by modification which allows multiple retention mechanism to occur simultaneously [58].

One of such mixed mode monoliths is the mixed anion-exchange/hydrophobic interaction monolith stationary phase reported by Terborg *et al.* where the pore surface of poly(glycidylmethacrylate-co-ethylene dimethacrylate) was functionalized with thiols and coated with gold nanoparticles (GNP). Various alkanethiol and mercaptoalkanoic acid

ligands such as 11-mercaptoundecanoic acid, 1-octanethiol, 2-dimethylaminoethanethiol were attached to the poly(GMA-co-EDMA) monolith through GNP. One of these mixed mode stationary phases was prepared by attaching a mixture of 11- mercaptoundecanoic and 1-octanethiol to GNP. The mixed mode character of these stationary phases was demonstrated in the separation of proteins by using reversed phase gradient elution as well as ion exchange chromatography method [59]. The monolith functionalized with 11-mercaptoundecanoic acid was tested in reversed phase mode for the separation of a mixture of ribonuclease A, cytochrome C and myoglobin. The separation was poor with broad peaks and retention times were short. However, the monolith obtained from the surface functionalized with the mixture of 11-mercaptoundecanoic acid and 1-octanethiol yielded comparatively better separation. The pure octanethiol functionalized column showed the longest retention time. Lastly, the column with 2-diethylaminoethanethiol showed better separations than that obtained on the column with 11-mercaptoundecanoic acid, which the authors attributed to the higher surface coverage with ligands. Under ion exchange mode, the 8-mercaptooctanoic acid (8-MOA) functionalized column showed a good separation. The retention of proteins was substantially higher for the column tested under ion exchange separation mode compared to the RPC mode. This behavior confirmed the mixed mode retention mechanism of the column. However, there was no elution of proteins from any of the column except from the one functionalized with 8-MOA.

In a recent work by Aydogan, a monolithic column was shown to exhibit a dual retention mechanism of both anion exchange/hydrophobic interactions toward small organic molecules and inorganic anions in nano-LC. The stationary phase was obtained by the *in situ* polymerization of 3-chloro-2-hydroxypropylmethacrylate and EDMA. The

reactive chloro groups present on the monolithic surface thus obtained were modified by reacting these groups with *N,N*- dimethyl-*N*-dodecylamine. The resulting stationary phase showed dual anion exchange/hydrophobic mode due to the presence of ionizable as well as hydrophobic groups [60]. ABs and phenolic compounds were separated under hydrophobic conditions and inorganic anions like bromate, nitrite, iodide, and thiocyanate were separated under anion exchange mode.

Generating a multifunctional surface by post-polymerization modification allows to optimize the stationary phase porous properties as desired. However, it is generally claimed that such procedure can be time-consuming because of multiple steps of reactions required to perform on the stationary phase surface. Thus, a single step or one-pot synthesis has been practiced in order to attain such multifunctional surfaces. A trimodal monolith was prepared in a one pot procedure by polymerizing 3'-azido-3'-deoxythymidine (AZT), propargyl methacrylate (PMA) and PETA for cLC. The poly(AZT-co-PMA-co-PETA) was prepared from combination of free radical polymerization and copper mediated azide-alkyne cycloaddition (CuAAC) "click" reaction in a one pot process. The monomers were mixed with initiator in the presence of copper (I) catalyst thereby generating a polymer monolith with hydrophilic/hydrophobic and ion-exchange interaction properties. According to the report, this method of preparing a monolith was an alternative to prepare diverse polymer monoliths using a variety of azido/alkyne-organic monomers instead of going through post-polymerization modification [61]. ABs were used as test solutes to show the hydrophobic interaction of the monolith using 45.5/54.5, acetonitrile/water solution as the mobile phase. The hydrophilicity of the monolith was demonstrated by analyzing various amides like dimethylformamide, *N,N*-dimethylacetamide, formamide

and *N,N'*-methylene bisacrylamide with the maximum resolution obtained while using pure ACN as the mobile phase. The aniline compounds such as phenylamine, o-phenylenediamine, o-nitroaniline and α-naphthylamine were used for demonstrating the cation-exchange/hydrophobic interactions of the monolith. The mobile phase for this mode of separation contained 150 mM phosphate buffer with 50% (v/v) acetonitrile at various pH including pH 2.5, 4.0 and 7.0. Cation-exchange/hydrophilic interaction was also depicted by the separation of benzoic acids using 20 mM phosphate buffer containing 90.5% (v/v) ACN. In addition to the above mentioned solutes, the separations of sulfonamide antibiotics, nucleosides, nucleobases, anesthetics and proteins under various modes were shown as well.

Mixed mode stationary phases have been prepared using divinyl based monoliths as well. A poly(styrene-co-vinylbenzylchloride-co-divinylbenzene) monolith hypercrosslinked by nucleophilic substitution reaction using four different diaminoalkanes including 1,2-diaminoethane, 1,4-diaminohexane and 1,8-diaminooctane, were prepared by Janku *et al.* [62]. The effect of amine chain length on the separation efficiencies of the monoliths was investigated. Longer amine chain length on the surface of the monolith improved the monolithic column separation efficiency. The researchers reported that polymerization of the monolith at 65 ˚C for 2 h and hypercrosslinking in the presence of 3% 1,8-diaminoactane for 2h at 95 ˚C yielded an optimum column. The surface of the 1,8-diaminooctane functionalized generic poly(styrene-co-vinylbenzylchloride-co-divinylbenzene) monolith was further modified by nucleophilic substitution reaction using 2-aminoethanesulfonic acid. According to the report, the surface modified column showed a dual retention mechanism. ABs were used as test solutes and it was observed that while

using mobile phase containing 60% (v/v) ACN, the column eluted both ABs and polar compounds according to the RPC mode. However, as the concentration of ACN was increased up 95% (v/v), there was no separation of ABs and the elution order of polar compounds followed the elution order of the HILIC mode.

Rationale for the investigation

Polymer monolithic columns have proven to be strongly competitive stationary phases for chromatography in terms of the separation of a wide range of solutes. New column chemistries and polymerization methods have been developing to address the ever-increasing demands of versatile stationary phases that provide fast and efficient separation of a wide range of solutes including large and small molecules [17]. One of those ways to increase the applicability of a monolithic column is a post-polymerization modification. Post-polymerization modification opens a new door for supplying a variety of surface chemistries that can be realized on monolithic columns by taking advantage of the functional groups present on the surface of the polymer monolith. Post polymerization modification of polymers bearing various functional groups including esters, epoxides, isocyanates, aldehydes and thiols using mechanistic approaches such as substitution reaction, elimination reaction, addition reaction and recently popular thiol-ene "click" reactions are common [63, 64].

The rationale for this investigation lies in furthering the advances in post-polymerization modification of polymer monolithic columns by employing the versatile

ring opening reaction of epoxide with hydroxyl and amine functional groups. Since methacrylate based monomers are the most commonly used materials for preparing polymer monolithic columns due to their fast and easy preparation, high stability under a wide range of pH, wide selection of available monomers with a range of polarities and ease of functionalization [65], hydroxyethyl methacrylate (HEMA) was selected as the functional monomer for preparing the precursor hydroxy monolithic column. Taking advantage of the hydroxyl group of the HEMA monomer, various classes of monolithic stationary phases including non-polar, polar and chiral surfaces were fabricated and evaluated successfully. Thus, it was demonstrated that a single parent or precursor monolith can be utilized as a versatile tool in the fabrication of a wide range of useful stationary phases without having to invest a large amount of effort in optimizing various polymerization parameters. Additionally, a comparison of post-polymerization modification between a capillary column for CEC and a stainless steel column for HPLC is demonstrated in Chapter III.

Conclusions

This chapter has provided a brief introduction to the history, background and some instrumental aspects of CEC. Various technical parameters used in CEC such as retention factor, resolution and efficiency have also been described, which will be used in the subsequent chapters. An overview of history of monolithic stationary phases, including review of very recent works on different types of monolithic columns, and rationale of investigation were also presented.

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CHAPTER II

EPOXYALKANE AND OCTADECYL ISOCYANATE MODIFIED POLY (HYDROXYETHYLMETHACRYLATE-co-PENTAERYTHRITOL TRIACRYLATE) MONOLITHIC CAPILLARY COLUMNS FOR REVERSED PHASE CAPILLARY ELECTRO-CHROMATOGRAPHY

Introduction

Capillary electrochromatography (CEC) is an amalgamation of the features of HPLC and CE. The technologies have evolved through many decades from simple techniques such as low-pressure liquid chromatography and traditional slab gel electrophoresis. The introduction of continuous polymeric beds (or monoliths) and further innovation of monolithic stationary phases the in early 1990s revolutionized the area of separation science due to many advantages including, and not limited to, the ease of fabrication, good permeability, fast mass transfer, high stability and ease of modification. Likewise, the ability to optimize the porosity of the monolith due to control over the

polymerization process and consequently, the efficiency of the system is also one of the main features of monoliths [1-4]. Unlike the packed capillary columns, monolithic columns do not require retaining frits within a confinement of a capillary and packing of small diameter particles into narrow-bore tubes. These advantages have led monolithic capillary columns to flourish in the field of capillary electrochromatography (CEC). In contrast to the flow of mobile phase through the column by applied pressure, the flow of mobile phase is driven by electro-osmosis. When an electric field is applied along the length of the column, the mobile phase containing an electrolyte moves relative to the charged stationary phase thereby generating an electro-osmotic flow (EOF) [5]. Electroosmotically driven flow is the main characteristic of CEC. Thus, the inclusion of fixed charges in the monolith by polymerizing charged compounds such as vinylsulfonic acid, 2-acrylamido-2-methylpropanesulfonic acid [6] has been a norm in the preparation of monolithic stationary phases for CEC. However, the electrostatic interaction of solutes with fixed surface charges is unavoidable [7] and causes a mixed mode retention mechanism, which for some separations may be beneficial but it can also be problematic to predict the retention behavior of solutes [6]. This also leads to disadvantages like poor efficiency and asymmetric peaks among others [8]. Thus, to overcome these issues, Okanda and El Rassi introduced an acrylate based neutral monolithic stationary phase which consisted of pentaerythritol diacrylate monostearate (PEDAS) as monomer having a C-17 ligand which facilitated the adsorption of electrolyte ions from the mobile phase thus imparting the zeta potential for generating sufficient EOF [7]. Many neutral monoliths have emerged since its introduction in 2005 [6, 9-11]

Following this successful introduction, our lab has devised a series of neutral monolithic stationary phases for reversed phase CEC. Puangpila *et al.*, introduced neutral monoliths of varying *n*-alkyl chain ligands, which were made by co-polymerization of various functional monomers including C-8 methacrylate, C-12 methacrylate and C-18 methacrylate with the crosslinking monomer pentaerythritol triacrylate (PETA) to yield monoliths with surface bound C-8, C-12 and C-16 stationary phases [12]. Despite the exhilarating fact that neutral reversed phase monoliths with varying alkyl chain ligands could be designed, the process of adjusting monomer/ crosslinker composition to get the desired permeability and efficiency was arduous as is the case with all of the monoliths fabricated from co-polymerization of monomers. Additionally, the inaccessibility of functional groups due to it being "buried" during polymerization is also one of the drawbacks of co-polymerization of functional monomers.

Post-polymerization modification is one of the ways by which one can attach the desired functional group on the surface of a given precursor or parent monolith while eliminating the need for re-optimization of the monolith thereby leading to a versatile stationary phase. In addition, by the use of this approach, each time a monolith of different functionality is desired, the re-optimization of porosity can be avoided. Since the post-polymerization modification allows the incorporation of functional groups that are incompatible for co-polymerization, it increases the versatility of the parent monolith [13]. Also, several different reaction chemistries can be carried out utilizing the functional group present on the monolithic surface thereby creating an array of stationary phases from a single precursor monolith. Guerrouche and Carbonnier performed post polymerization

modification of a monolith containing *N*-acryloxysuccinamide with a series of alkylamines for reversed phased electrochromatography [14].

This chapter discusses post-polymerization modification of hydroxyl functional groups present on the surface of neutral poly (HEMA-co-PETA) precursor monolith with 1,2 -epoxyalkanes catalyzed by BF_3 ultimately providing monolithic surfaces with ligands at varying alkyl chain lengths. This type of regioselective ring-opening reaction has been utilized in the past for modification of polymeric monoliths by Ericson *et al.* [15]. However, unlike this study, a charged polymer (dextran sulfate) was included in the monolith to impart the zeta potential required for generating EOF. This study was aimed at developing a series of reversed phase monoliths having ligands at varying alkyl chain lengths for reversed capillary electrochromatography. Various parameters affecting the design and performance of these monoliths including the effects of reaction time, amount of ligand and temperature of reaction have been studied.

Experimental

Instruments

All the experiments were performed on a HP ^{3D}CE system from Hewlett-Packard (Waldbronn, Germany) equipped with a photodiode array detector. Electrochromatograms were processed with a PC running an HP ChemStation system. Heat treatment for the *in situ* polymerization initiation was performed using an Isotherm water bath (Model 105) from Fisher Scientific (Fairlawn, NJ, USA). An HPLC pump Model M-45 from Waters Associates (Milford, MA, USA) was used to condition the monolithic columns after polymerization.

Reagents and materials

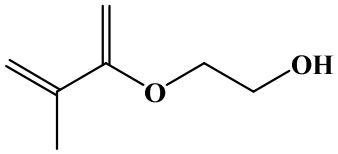
Fused silica capillaries having 100 μm internal diameter (i.d.) and 375 μm outer diameter (o.d.) were purchased from Polymicro Technologies (Phoenix, AZ, USA). Hydroxyethyl methacrylate (HEMA), pentaerythritol triacrylate (PETA), 3-(trimethoxysilyl) propylmethacrylate, cyclohexanol, 1-dodecanol, azobisisobutyronitrile (AIBN), acetone, dioxane, alkylbenzenes (ABs), 2-chlorophenol, 4-chlorophenol, 2,4-dichlorophenol, toluene, *p*-toluidine, *m*-toluidine, *p*-tolunitrile, and 2,3-dinitrotoluene were purchased from Sigma-Aldrich (Milwaukee, WI, USA). 1,2-Epoxyoctadecane was purchased from Alfa-Aesar (Ward Hill, MA, USA). 1,2-Epoxyhexadecane, 1,2-epoxytetradecane, 1,2-epoxydecane and BF_3 were purchased from TCI (Toshima, kita-ku, Tokyo, Japan). Resorcinol and phenol were from Fisher Scientific (Fairlawn, NJ, USA). Acetonitrile and methanol were obtained from Pharmco-Aaper (Brookfield, CT, USA).

Silanization of the capillary walls

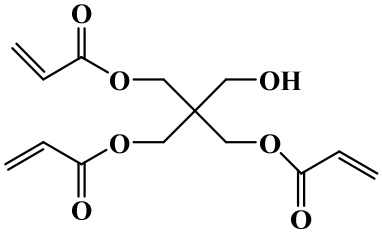
Before polymerization, the inner walls of a 40 cm long, 100 μm I.D. fused silica capillary were pre-treated by passing water for 2 min, then 1 M NaOH for 30 min followed by 0.1 M HCl for 30 min and water for 30 min. Finally, the capillary walls were silanized in the presence of a solution of 50% (v/v) of 3-(trimethoxysilyl) propylmethacrylate in acetone for 4 h at room temperature and were rinsed off with methanol for 30 min. The capillary was dried with nitrogen gas for 5 min.

In-situ polymerization

A solution of a final total weight of 0.2 g was prepared by mixing 12.8% (w/w) HEMA as the functional monomer and 12.2% (w/w) PETA as the crosslinker together with 50% (w/w) cyclohexanol and 25% (w/w) 1-dodecanol as the binary porogen (For the structures of HEMA and PETA see **(1)** and **(2)** below). Free radical initiator, AIBN, at



HEMA (1)



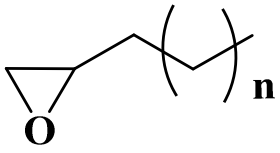
PETA (2)

1% (w/w) with respect to the monomer and the crosslinker was added to the solution and sonicated for 10 min. The polymerization solution thus prepared appeared homogenous and was free of air bubbles. To fill the silanized capillary for polymerization, one end of the 40 cm long pretreated capillary was immersed in the vial containing the solution and a negative pressure (i.e., vacuum) was applied at the capillary outlet. The solution was drawn into the capillary until it reached the 30 cm demarcation line drawn on it leaving the last 10 cm empty. Both ends of the capillary were sealed with GC septa and immersed in a water bath at 60 ˚C for 15 h. After the polymerization was complete, the capillary was washed with acetonitrile using an HPLC pump until the porogens as well as the unreacted monomer and crosslinker were completely removed. Then, a detection window was made on the column 1 mm below the monolith by using a thermal wire stripper. The resulting capillary column was trimmed to a total length of 33.5 cm with an effective length of 25

cm. The poly (HEMA-co-PETA) monolithic capillary column was then ready for post-polymerization modification.

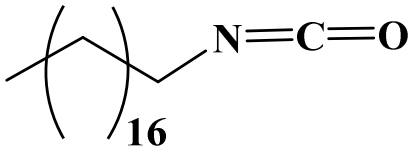
Post-polymerization modification of the precursor monolith

Immobilization of 1,2-epoxyalkanes. After *in situ* polymerization, the porogen free poly(HEMA-co-PETA) monolithic capillary column was equilibrated with dioxane for 2 h using a syringe pump. Then, it was loaded onto the HP^{3D}CE instrument for further reaction. A pressure of 8 bars was applied on the column inlet for all the reactions. The inlet vial was filled with 5% (v/v) BF_3 in dioxane and supplied to the column for 20 min. A 5% (w/v) solution of epoxyoctadecane in BF_3 was prepared and was passed onto the column for 40 min. The column was rinsed with dioxane for 20 min and the cycle was repeated six times. The epoxyoctadecane was immobilized on the surface and the resulting capillary column was designated Epoxy OHM C-16 capillary column. The reaction scheme is depicted in Figure 1. The same experimental procedure was employed for surface modification using 1,2-epoxyhexadecane, 1,2-epoxytetradecane and 1,2-epoxydecane in dioxane at 4.47 % (w/v), 3.95 % (w/v) and 2.90% (w/v), respectively. (For structures see below).



(3)

n= 15, 1,2-epoxyoctadecane
n= 13, 1,2-epoxyhexadecane
n= 11, 1,2-epoxytetradecane
n= 7, 1,2-epoxydecane



(4)

Octadecyl isocyanate

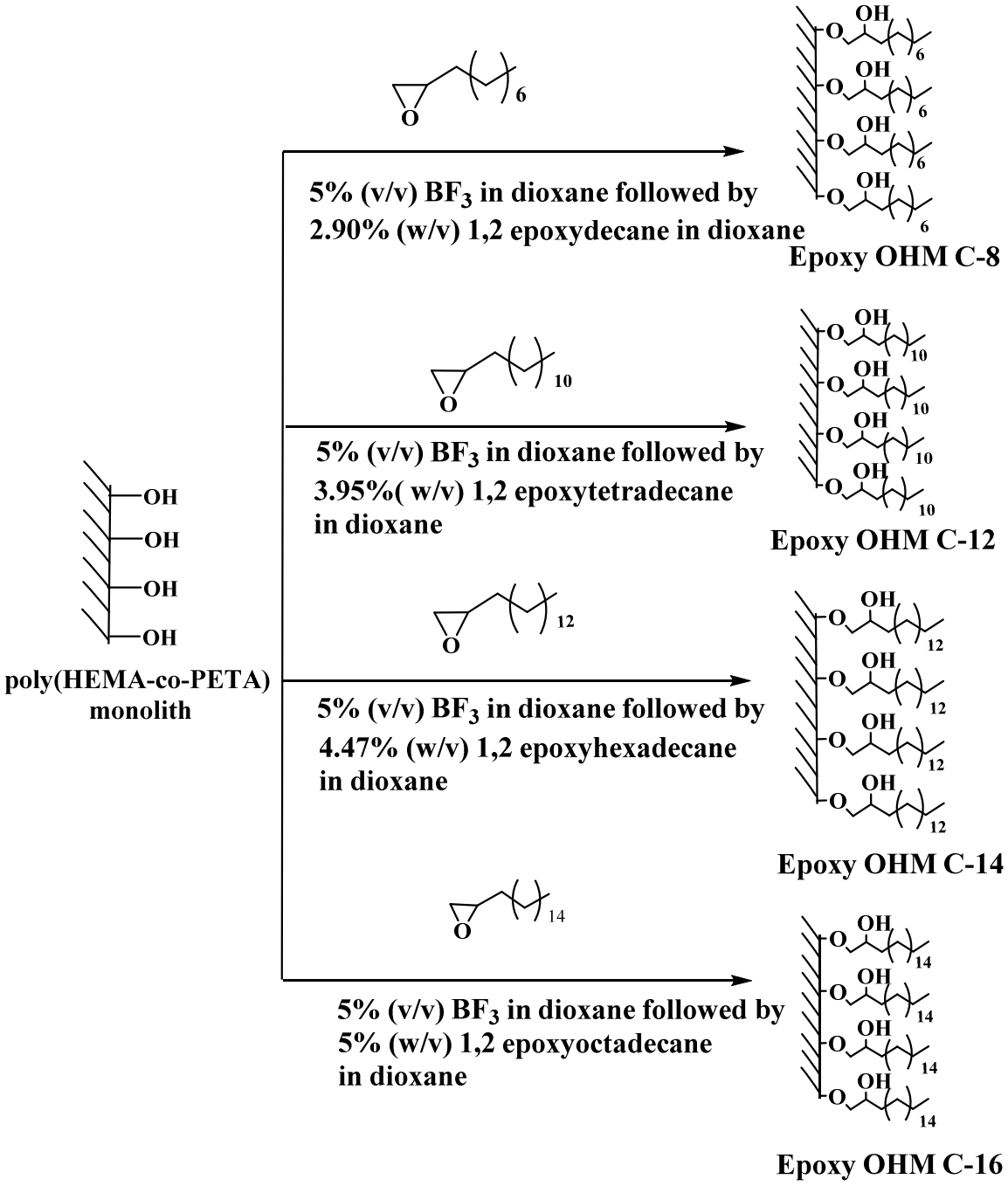


Figure 1. *Schematic of the reaction of the precursor poly(HEMA-co-PETA) monolith with 1,2-epoxyalkanes at varying alkyl chain length. The post-polymerization reaction was carried out for 6 h.*

Immobilization of octadecyl isocyanate. Octadecyl isocyanate (for structure see **(4)**, above) was immobilized on the poly(HEMA-co-PETA) precursor monolith to obtain an Isocyanato OHM capillary column.

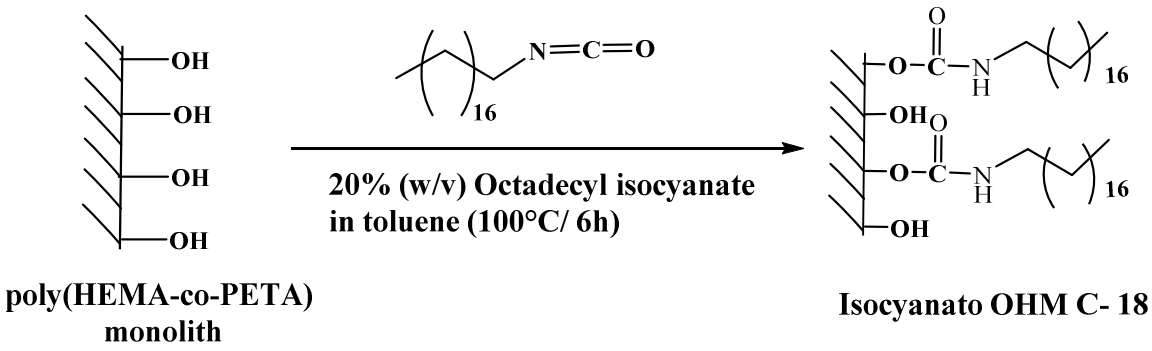


Figure 2. *Schematic for the reaction of the precursor poly(HEMA-co-PETA) monolith with octadecyl isocyanate. The post-polymerization reaction was carried out for 6 h.*

Since the reaction had to be performed at high temperature, it was not feasible to use the HP^{3D}CE instrument. Thus, an in-house experimental set up was assembled as shown in the schematic diagram in Figure 3. After removing the porogens of the precursor monolith, it was equilibrated for 2 h in dioxane using a manual syringe pump. A 200 µL 20% (w/v) solution of octadecyl isocyanate in toluene was prepared and filled in a syringe. The syringe was connected to the inlet of the capillary, which was placed inside a jacket with circulating water at 100 ˚C. The automated syringe pump was used for pushing solution into the capillary at the rate of 3.3 µL/h. The octadecyl isocyanate solution from the outlet was collected and recycled through the capillary column five more times making the total reaction time 6 h. Hot water was circulated in the jacket throughout the reaction time from the water bath connected to it thereby maintaining a constant reaction temperature. After 6 h, the capillary was flushed with toluene for 2 h using the syringe

pump, and finally it was washed with 100% ACN for 30 min followed by 80% ACN in water for another 30 min using an HPLC pump.

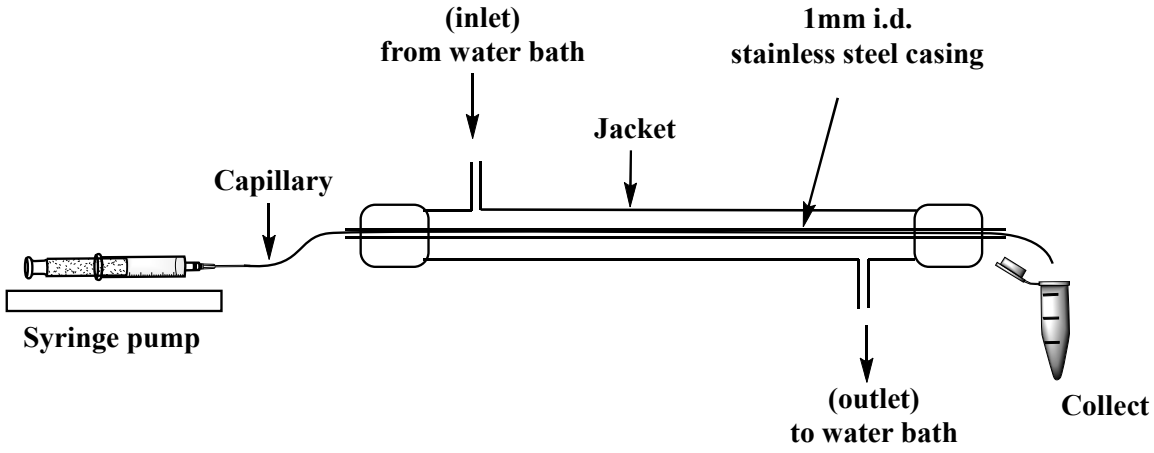


Figure 3. *Schematic of the in-house reaction set-up. The temperature of the circulating water was maintained at 100 ˚C.*

CEC conditions

All the Epoxy OHM C-n capillary columns exhibited cathodal EOF so that the experiments were performed at a running voltage of +20 kV. A pressure of 10 bars was applied to both the inlet and outlet ends of the capillary to avoid bubble formation and the injection voltage was +20 kV for 9s. At the beginning of the day, before injecting the solutes, the capillary column was equilibrated using a manual syringe pump for 1 h and then installed into the instrument for further equilibration. The voltage was ramped at a rate of 2 kV/min and equilibrated for one hour using the running mobile phase. The analysis temperature was set at 25 ˚C for all the solutes. The instrumental parameters for testing the Isocyanato C-18 OHM capillary column were the same as for the Epoxy OHM C-n capillary columns.

Results and Discussion

Part I

In our selection of the final composition for the polymerization composition (in terms of % monomers, % porogens and initiator) used for preparing the optimized poly(HEMA-co-PETA) precursor monolith, we relied on the experience gained in our laboratory from previous work on monoliths [9, 10, 16-18]. The resulting monolith was porous and free-flowing allowing further modification without any readjustments to the monomer/crosslinker composition. In order to perform post-polymerization modification of the parent monolith with alkyl ligands, the regioselective ring opening reaction of 1,2-epoxyalkanes in the presence of BF_3 as a catalyst was implemented.

Surface modification strategy

For the immobilization of 1,2-epoxyalkane ligands, two experimental conditions were performed and optimized in terms of reaction time and amount of ligands offered to the precursor monolith. The parameters were adjusted and the resulting monolithic columns were evaluated electrochromatographically using ABs as the test solutes.

Time of reaction. The effect of reaction time on the column modification was studied by offering 2% (w/v) solution of 1,2–epoxyoctadecane for varying times of 0 h (i.e., unmodified precursor), 2 h, 4 h, 6 h and 10 h, and the resulting Epoxy OHM C-16 capillary column was tested with ABs. The retention factor (k') of toluene increased from 0.142 at 0 h to 0.154 after 2 h, reached 0.795 after 6 h of modification, and almost stabilized at 0.85 after reaching 10 h of surface modification. Similarly, the k' of phenylheptane increased

from 0.221 at 0 h to 0.242 after 2 h and reached 2.05 after 6 h. However, at 10 h the k' increased further to 3.69. The increase in retention of phenylheptane as it went from 6 h to 10 h was noticeable compared to toluene. For both solutes, the observed low k' value at 0 h modification (i.e., unmodified precursor monolith) indicates that the precursor monolith possesses a relatively low hydrophobic character. Considering the constancy in the solute's retention between 6 h-10 h of post-polymerization modification (see Figure 4), and taking into account the time factor, it was more practical to consider that 6 h was enough to completely react the available hydroxyl groups providing the sites for the covalent attachment of the alkyl ligands on the surface. Thus, it was decided for the remainder of the study to keep the time of post-polymerization reactions at 6 h.

Amount of epoxy alkyl ligand offered to the column. The amount of alkyl ligands required to obtain maximum functionalization of the surface hydroxyl groups was optimized by employing various amounts of 1,2-epoxyoctadecane for a duration of 6 h. Taking the effect of reaction time on retention of ABs test solutes from the above study into consideration, it was obvious that a 2% (w/v) solution was not enough for reaching an effective surface coverage (i.e., the maximum possible surface density). Thus, it was planned to perform a series of reactions using 5% (w/v), 6.5% (w/v), 8% (w/v) and 10% (w/v) of 1,2-epoxyoctadecane solution, thus resulting in four different Epoxy OHM capillary columns. All reactions were carried out at 25 ˚C. The moles of ligand that reacted on the column was calculated by measuring the volume of the solution collected at the column outlet after the standard reaction time, i.e., 6 h.

TABLE 1

EFFECT OF TIME OF REACTION ON THE RETENTION FACTORS (k′) OF AB HOMOLOGOUS SERIES

Time of reaction (h)	k′ values of AB homologous series						
	C1	C2	C3	C4	C5	C6	C7
0	0.14	0.15	0.18	0.18	0.19	0.21	0.22
2	0.15	0.16	0.21	0.23	0.24	0.25	0.27
4	0.36	0.65	0.98	1.32	1.80	2.40	3.20
6	0.80	0.99	1.27	1.62	2.05	2.57	3.24
10	0.85	1.07	1.38	1.79	2.28	2.90	3.69

Conditions: Epoxy OHM C-16 capillary column, 33.5 cm (effective length 25 cm) × 100 μm i.d.; running voltage, 20 kV; column temperature, 25 ˚C; hydro-organic mobile phase, 2 mM Na_2HPO_4 (pH 7.0) at 30% (v/v) ACN; EOF tracer, thiourea.

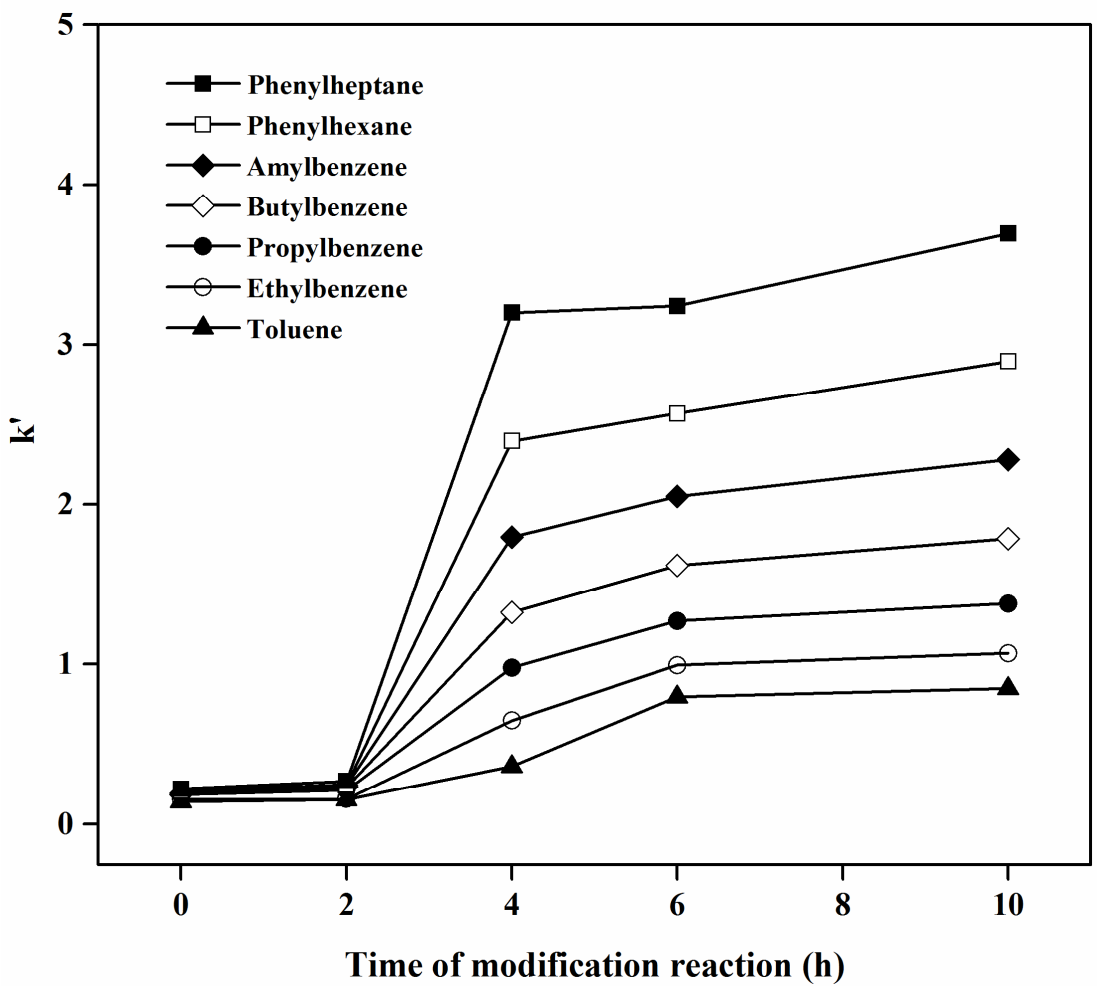


Figure 4. *Plots of k' of ABs vs. hours of modification of the poly(HEMA-co-PETA) precursor monolith with 1,2 epoxyoctadecane. Conditions: Epoxy OHM C-16 capillary column, 33.5 cm (effective length 25 cm) × 100 µm i.d.; running voltage, 20 kV; column temperature 25.*

The moles of ligands added to the column was derived from the concentration and volume of the solution offered as shown in Table 2. Each of the Epoxy OHM C-16 columns was evaluated electrochromatographically using ABs as the test solutes under reversed phase conditions and the observed k' values were plotted against the µmoles of Epoxy C16 offered to the OH monolith (see Fig. 5). The effect of increasing the moles of epoxy offered

to the column was as expected more pronounced on phenylheptane than on toluene. The k' of toluene for 1.19 x 10^{-6} moles was 1.43 which increased to 2.47 for 3.27 x 10^{-6} moles. There was almost a two fold increase in the retention of toluene for around three fold increase in the mole of ligands offered to the parent monolithic column. In the case of phenylheptane, and for the same moles of epoxyalkane offered to the column, the k' went up from 10.83 to 32.6 which is an appropriate three-fold increase around 3 folds. The more significant increase in retention of phenylheptane (three fold) with respect to that of toluene (two fold) reflects the more nonpolar character of the former solute than the later. Although the 10% (w/v) solution resulted in the saturation of k' for ABs, the preparation of the column was tedious. This was due to the increased viscosity and needed more effort to remove the unreacted 1,2-epoxyoctadecane at the end of reaction, which noticeably increased the time of column fabrication. For the column prepared from a 5% (w/v) solution, which corresponds to offering 2.23 x 10^{-6} moles of 1, 2-epoxyoctadecane, the k' values were comparable to that of the column made from 10 % (w/v) solution. Thus, for the fabrication of Epoxy OHM C-14, Epoxy OHM C-12 and Epoxy OHM C-8, the initial concentration of the 1,2-epoxyalkane was adjusted such that 2.23 x 10^{-6} moles of ligand would be offered to the column.

TABLE 2*

AMOUNT OF 1, 2-EPOXYOCTADECANE OFFERED TO THE PRECURSOR MONOLITH

1,2 epoxyoctadecane (%)	Volume of epoxy collected in the outlet (μL)	moles of epoxy
2.0	16.0	1.19×10^{-6}
5.0	12.0	2.23×10^{-6}
6.5	6.0	1.45×10^{-6}
10.0	8.8	3.27×10^{-6}

*****_The listed amount of 1,2-epoxyoctadecane were offered to the poly(HEMA-co-PETA) precursor monolith for optimizing the amount of ligand required for the post-polymerization modification reaction. Conditions: OHM capillary column, 33.5 cm (effective length 25 cm) × 100 μm i.d.; pressure, 10 bars; column temperature, 25 ˚C; total run time, 6 h._

CEC parameters - Retention behavior of solutes

Percent ACN in the mobile phase. The non-polarity of Epoxy OHM C-16 capillary column was evaluated using an ABs homologous series of test solutes under reversed phase conditions using hydro-organic mobile phase containing 2 mM Na_2HPO4, pH 7, and ACN at varying percentage (v/v).

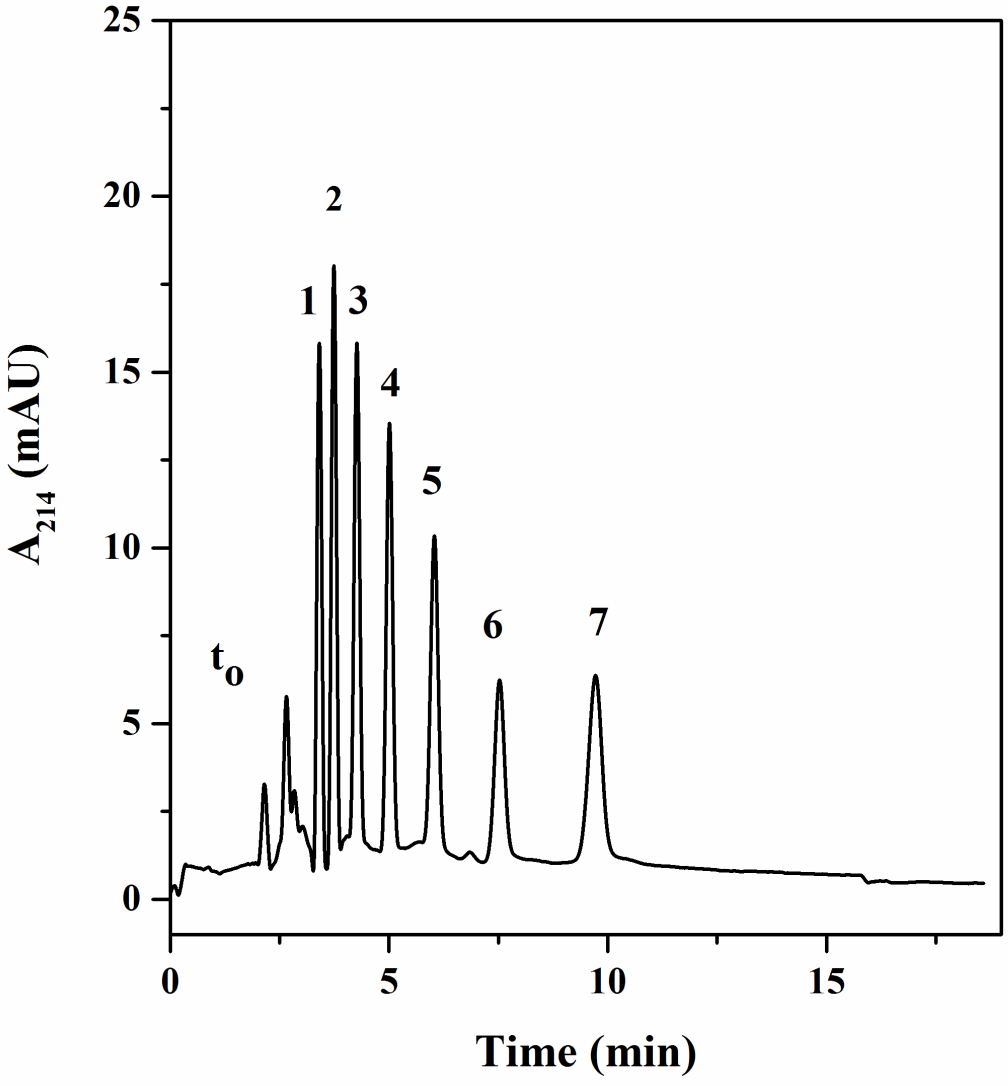


Figure **6** shows the separation of ABs at 55% (v/v) ACN which allowed the complete separation of the seven ABs in around 8 min with baseline resolution. The sharp Gaussian

peaks (see

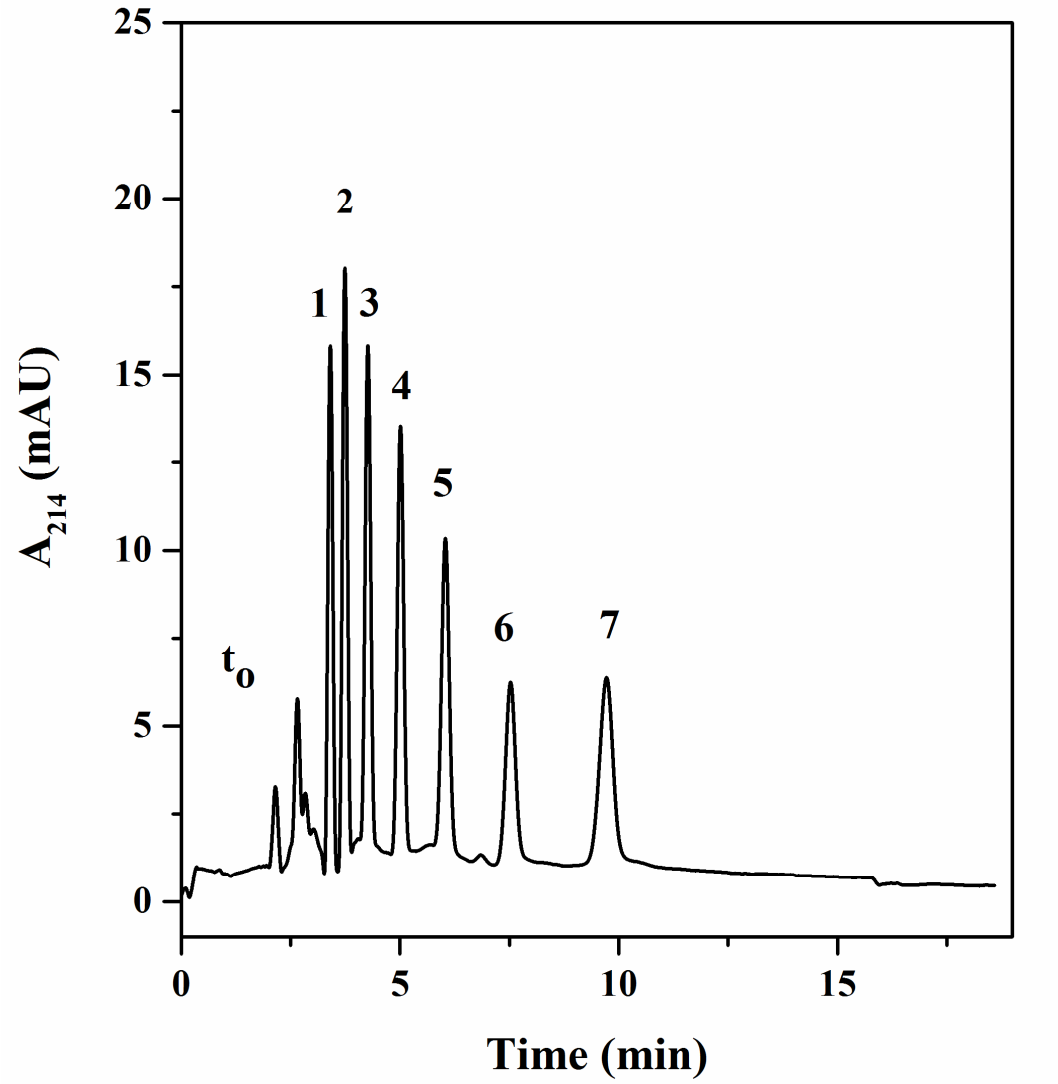


Figure **6**) indicate that there is an efficient mass transfer between the stationary phase and the mobile phase. The Epoxy OHM C-16 capillary column showed a good separation

efficiency with an average plate number of 33,000 plates/m under the conditions of

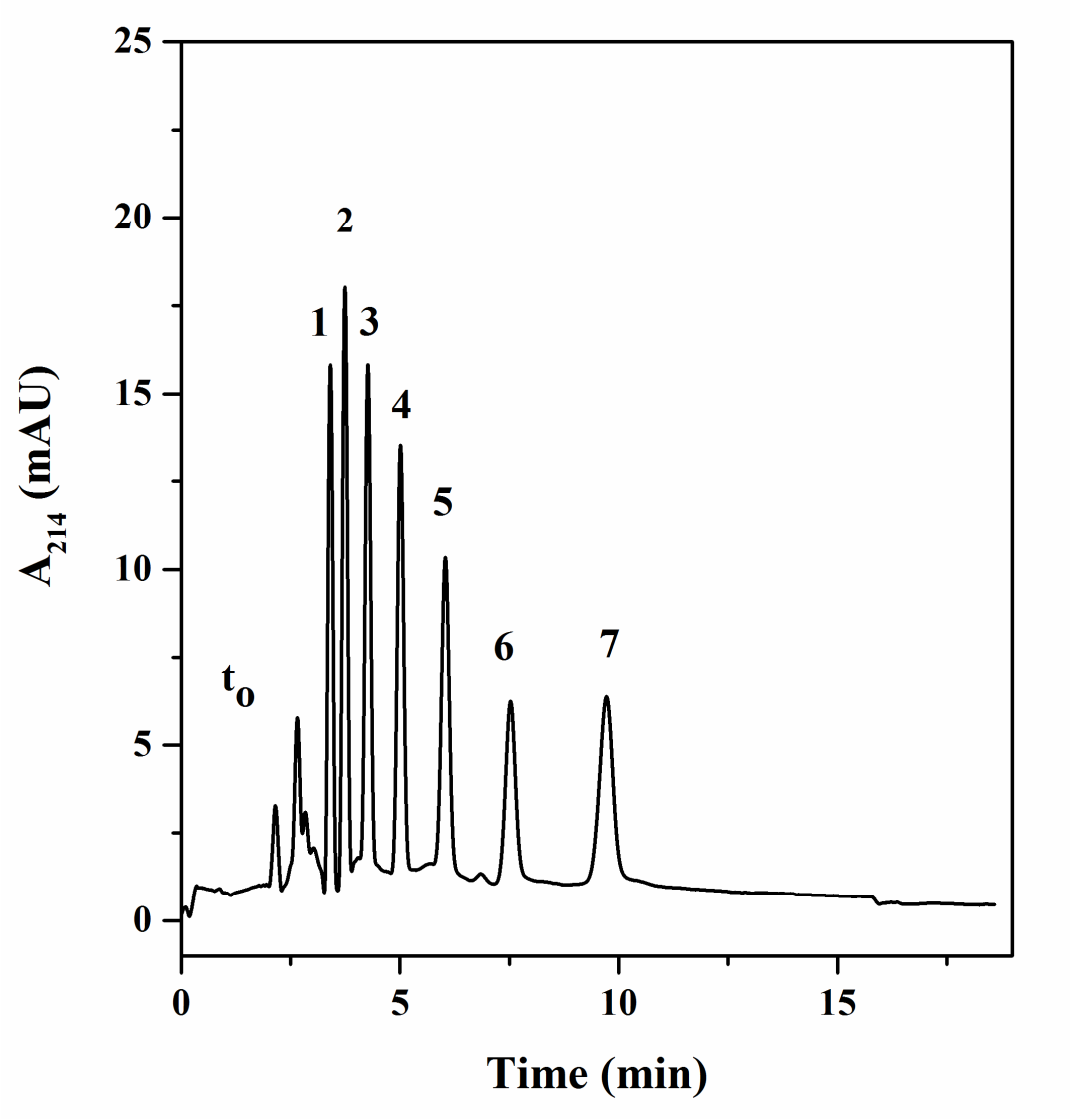


Figure **6**.

To further investigate the effect of % ACN in the mobile phase on the retention of an AB homologous series, several experiments were performed. Figure 5 shows the plots of log k' *vs.* the % ACN (v/v) in the hydro-organic mobile phase. As can be seen in this figure, log k' decreases quasi-linearly with increasing % ACN in the mobile phase. The slope of these lines increased with the size of the solute. That is, the effect of decrease in

ACN concentration was more profound for phenylheptane, which is due to the increased interaction of the solute with the Epoxy OHM C-16 stationary phase. This behavior further revealed the hydrophobicity of the Epoxy OHM C-16 column. This indicates that the separation of ABs on the Epoxy OHM C-16 capillary column exhibits a typical reversed phase behavior.

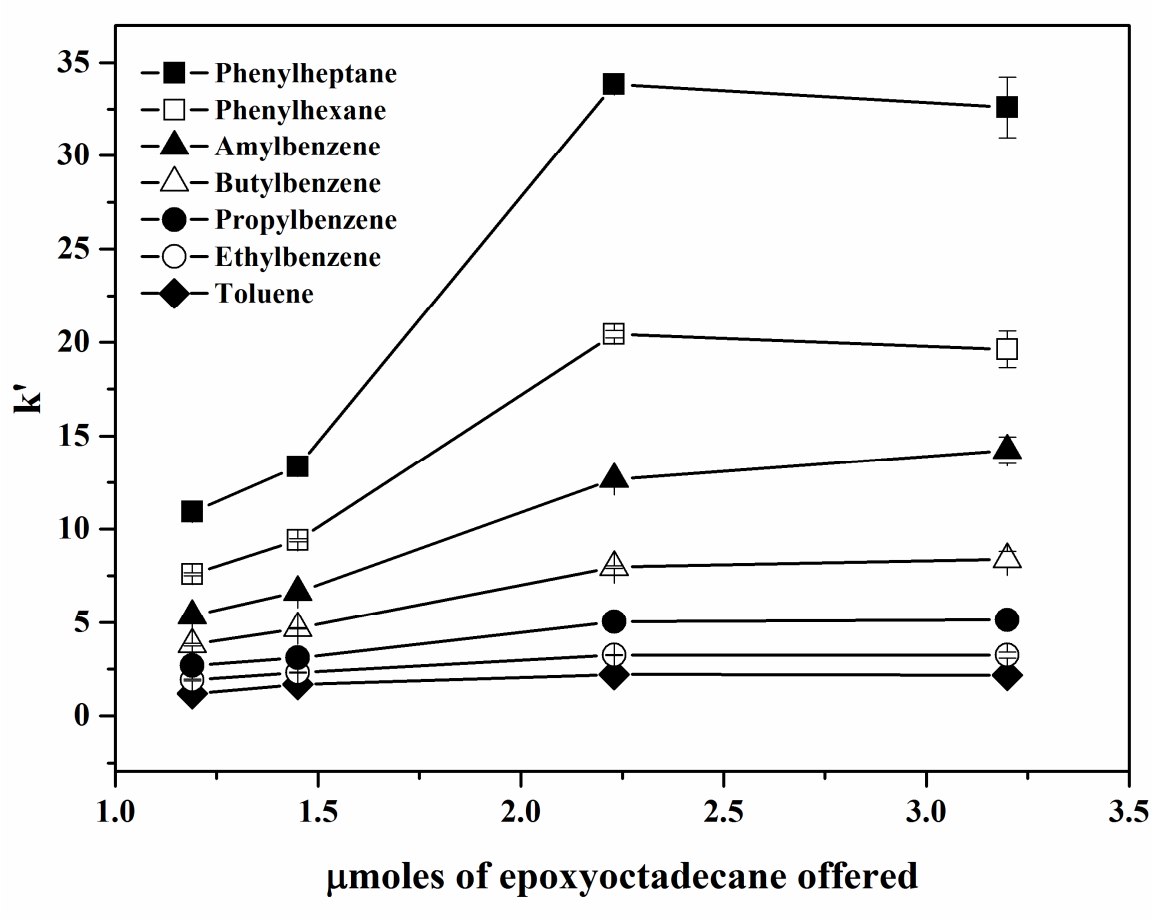


Figure 5. *Plots of k' of ABs vs. µmoles of Epoxy C-16 ligand offered to the precursor OHM capillary column. Conditions: Epoxy C16 monolithic capillary column, 33.5 cm (effective length 25 cm) × 100 µm i.d.; running voltage, 20 kV; column temperature, 25 ˚C; hydro-organic -mobile phase, 2 mM Na_2HPO_4 (pH 7.0) at 40% (v/v) ACN; EOF tracer, thiourea.*

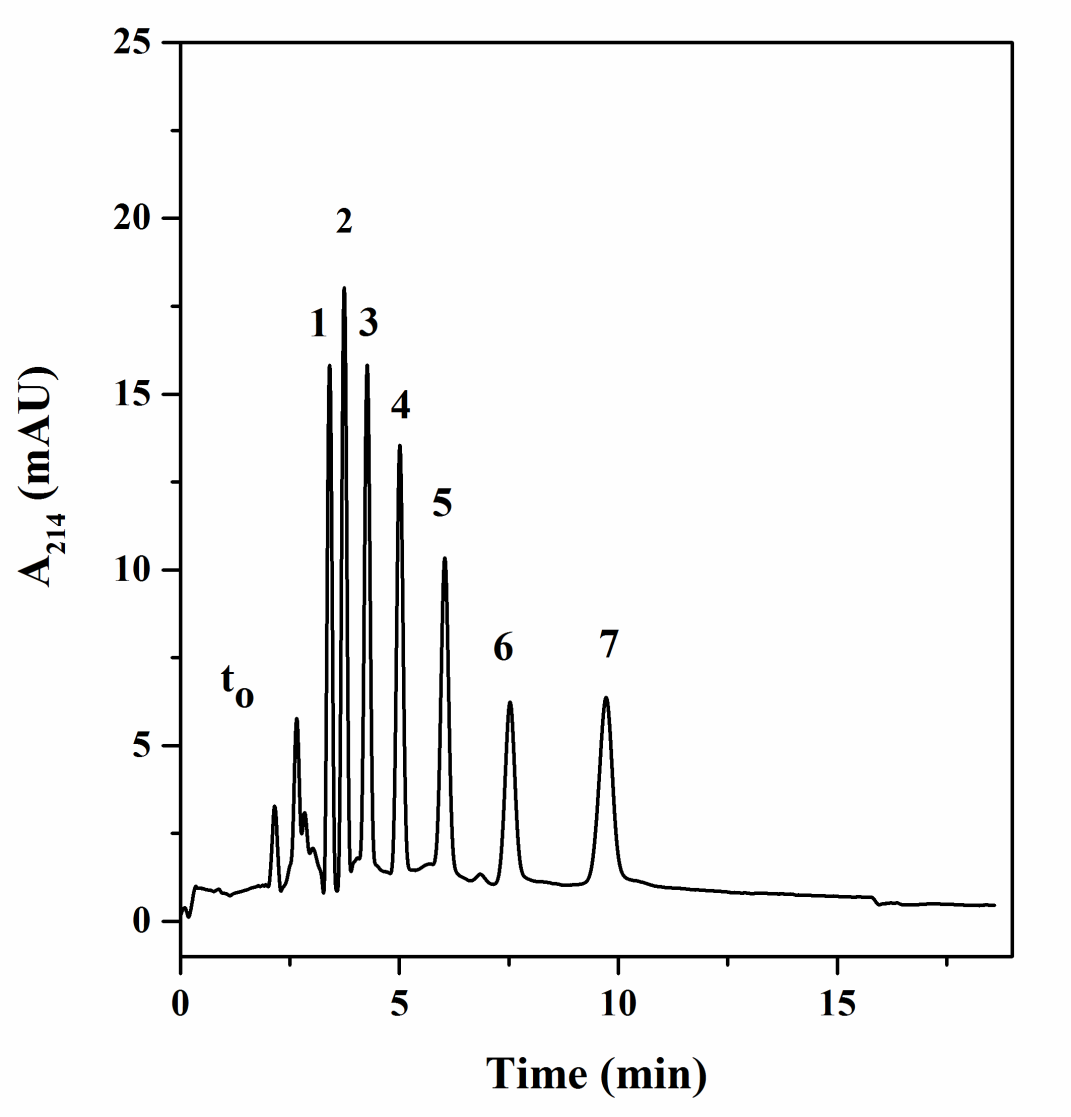


Figure 6 *Electrochromatogram of ABs obtained on the Epoxy OHM C-16 capillary column 33.5 cm (effective length 25 cm) × 100 µm i.d. Conditions: running voltage, 20 kV; column temperature, 25 ˚C; hydro-organic mobile phase, 2 mM Na_2HPO_4 (pH 7.0) at 55 % (v/v) ACN. Solutes: 1) toluene, 2) ethylbenzene, 3) propylbenzene, 4) butylbenzene, 5) amylbenzene, 6) phenylhexane, 7) phenylheptane.*

Methylene group selectivity. To gain further insight into the retention behavior of ABs, the log k' values of ABs obtained at each % ACN were plotted against the number of carbons of the AB homologous series to evaluate the methylene group selectivity, α_{CH_2} (see Figure 8). A slope in the range of ~0.13 to ~0.20 was obtained for the lines, and this slope increased with decreasing the % ACN in the mobile phase, see Figure 8. The values of α_{CH2}, which are the antilog of the slopes (~0.13 to ~0.20) ranged from ~1.35 to ~1.58, reflecting the effect of % ACN on the retention selectivity in RPC. Higher selectivity is achieved as the percent of ACN in the mobile phase is decreased. This behavior confirms the nonpolar character of the Epoxy OHM C-16 column.

Ligand chain length. After the hydrophobicity of the Epoxy OHM C-16 column was evaluated, the effect of the length of the alkyl chain ligand of the modified surface on the non-polarity of the columns was also investigated using the homologous ABs under reversed-phase mobile phase conditions. As shown above, the hydrophobicity of the reversed phase column is generally characterized by its methylene group selectivity (α_{CH2}) which can be calculated from the slope of the plots of log k' against the carbon number of the AB solutes. For each Epoxy OHM C-ns, the logarithmic methylene group selectivity increased from 0.1257 to 0.1444, 0.1642 and then to 0.1982 when going from Epoxy OHM C-8 to Epoxy OHM C-12, Epoxy OHM C-14 and then to Epoxy OHM C-16 (see Figure 9). That is, the α_{CH2} values (antilog of each slope) are 1.34, 1.39, 1.46 and 1.58 for Epoxy OHM C-8, Epoxy OHM C-12, Epoxy OHM C-14 and Epoxy OHM C-16, respectively. As shown in Figure 10, these values of α_{CH2} are plotted against the number of carbons in the alkyl chain of the chromatographic ligand. As can be seen in this figure, the increase in the carbon chain length of the surface alkyl ligand leads to an increase in the overall

methylene selectivity of the non-polar solutes as shown by the steep increase in selectivity as the surface ligand goes from C-8 to C-16.

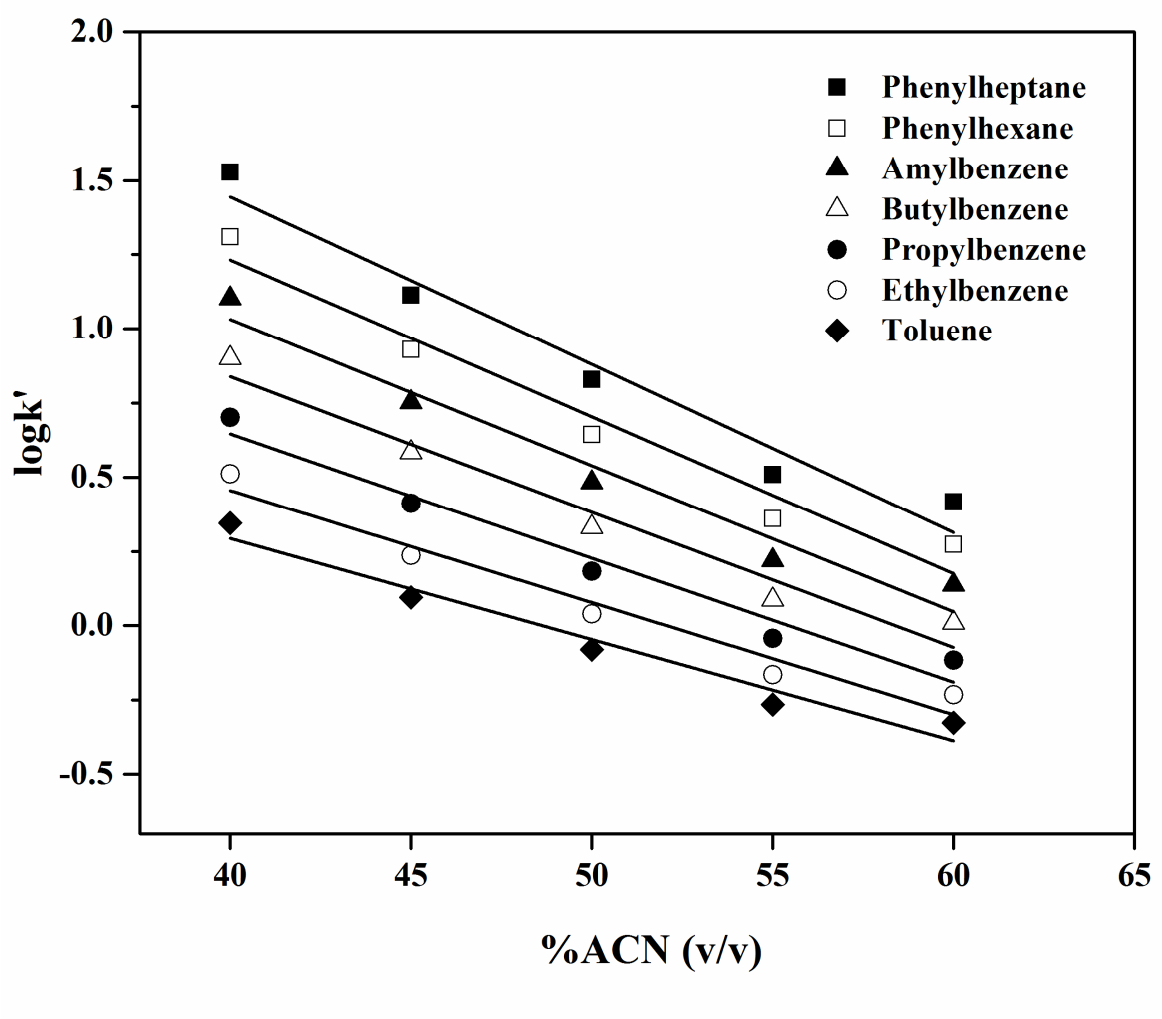


Figure 7. *Plots of log k' of ABs vs. % ACN (v/v). Conditions: Epoxy OHM C-16 capillary column, 33.5 cm (effective length 25 cm) × 100 µm i.d.; running voltage, 20 kV; column temperature, 25 ˚C; hydro-organic mobile phase, 2 mM Na2HPO4 (pH 7.0); EOF tracer, thiourea.*

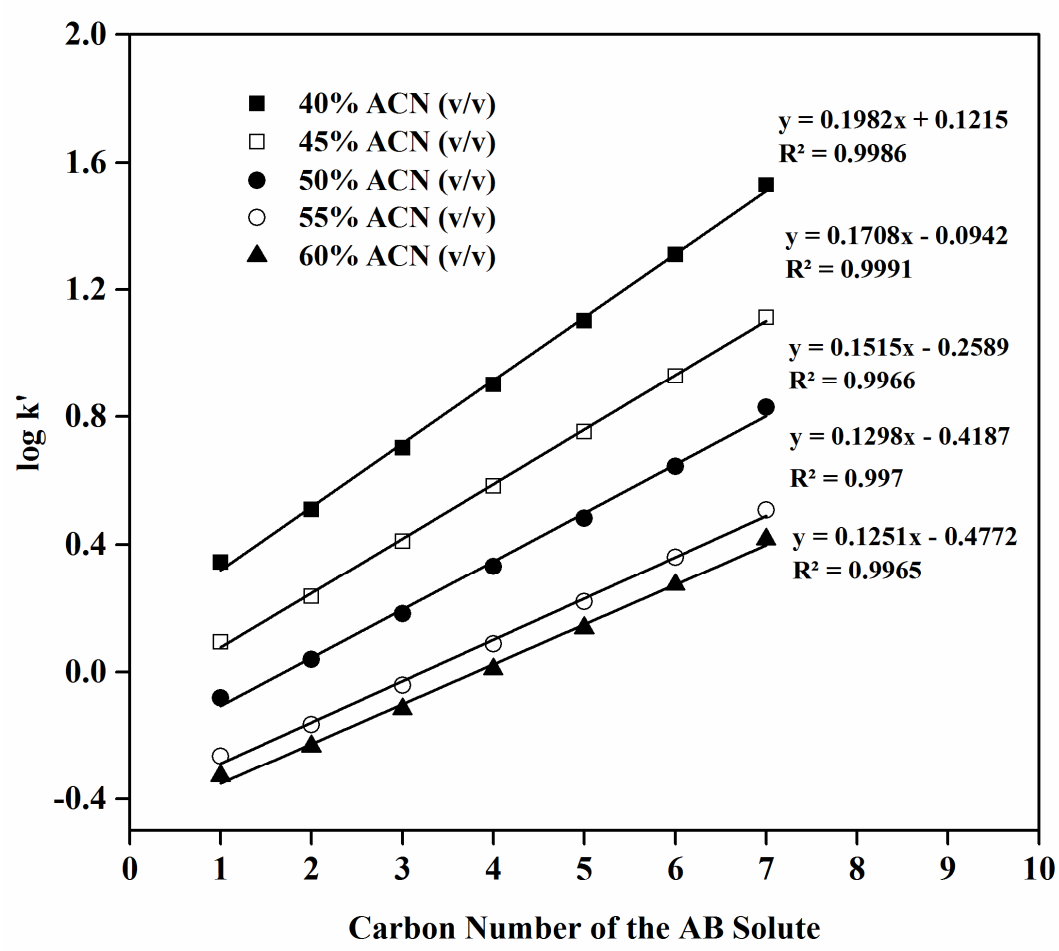


Figure 8. *Plot of log k' of ABs at various ACN : H_2O composition vs. number of carbon in the homologous series of ABs. Conditions: Epoxy OHM C-16 capillary column, 33.5 cm (effective length 25 cm) × 100 µm i.d.; running voltage, 20 kV; column temperature, 25 ˚C; hydro-organic mobile phase, 2 mM Na_2HPO_4 (pH 7.0), EOF tracer; thiourea.*

TABLE 3

k' OF AB HOMOLOGOUS SERIES OBTAINED ON THE Epoxy OHM CAPILLARY COLUMNS

	k'			
Alkylbenzenes	Epoxy OHM C8	Epoxy OHM 12	Epoxy OHM 14	Epoxy OHM 16
Toluene	1.17	1.35	1.95	2.21
Ethylbenzene	1.54	1.83	2.71	3.24
Propylbenzene	2.07	2.57	3.65	5.03
Butylbenzene	2.79	3.62	5.78	7.96
Amylbenzene	3.73	5.10	8.49	12.64
Phenylhexane	4.98	7.17	12.48	20.42
Phenylheptane	6.58	10.09	18.59	33.80

Conditions: Epoxy OHM C-n capillary columns, 33.5 cm (effective length 25 cm) × 100 µm i.d.; running voltage, 20 kV; column temperature, 25 ˚C; hydro-organic mobile phase, 2 mM Na_2HPO_4 (pH 7.0) at 40% (v/v) ACN; EOF tracer, thiourea.

Furthermore, the effect of surface alkyl chain length on the retention of individual solutes of the ABs homologous series was also evaluated by plotting either k´ values vs. the number of carbon in the alkyl chain of the monolithic surface or by log k´ vs. log C as shown in Figure 11 A and B. Since k', the phase ratio ϕ and the equilibrium constant K are related by the equation $k' = \phi K$, where the phase ratio is directly proportional to C, both plots should have yielded straight lines if one of the parameters (ϕ or K) stayed constant while varying the other. The plots in Figure 11 A and B were nonlinear thus indicating that the size or the length of the alkyl chain seem to affect both ϕ and K.

For illustration, the electrochromatograms of ABs obtained on the various Epoxy OHM C-n columns under otherwise the same elution conditions are shown in Figure 12. They provide evidence of the successful covalent attachment of the various epoxyalkanes to the OHM capillary.

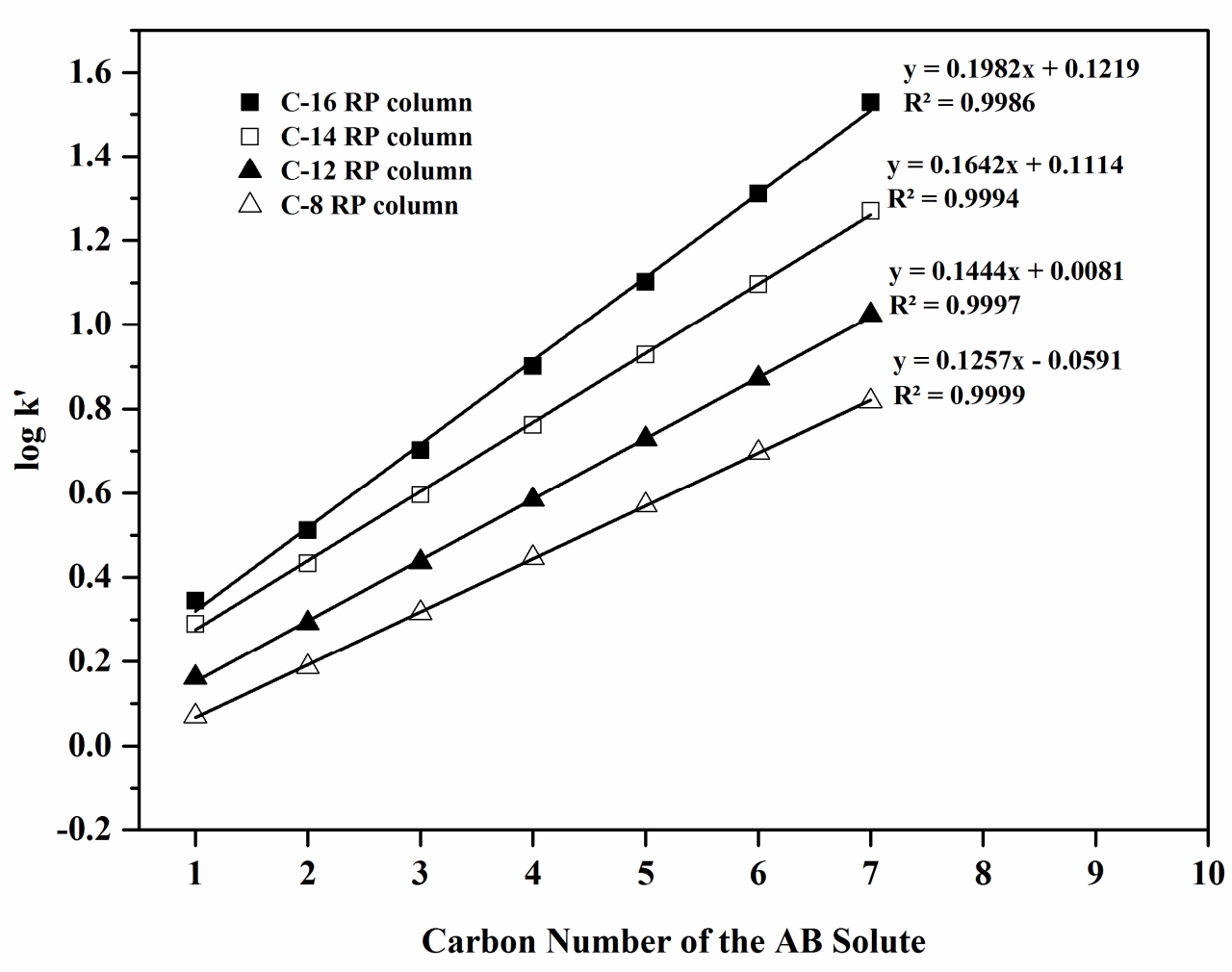


Figure 9. *Plots of log k' of ABs obtained on the Epoxy OHM C-n capillary columns vs. carbon number in the alkyl chain of ABs. Conditions: Epoxy alkyl OH capillary column, 33.5 cm (effective length 25 cm) × 100 µm i.d.; running voltage, 20 kV; column temperature, 25 ˚C; hydro-organic mobile phase, 2 mM Na_2HPO_4 (pH 7.0) at 40% (v/v) ACN; EOF tracer, thiourea.*

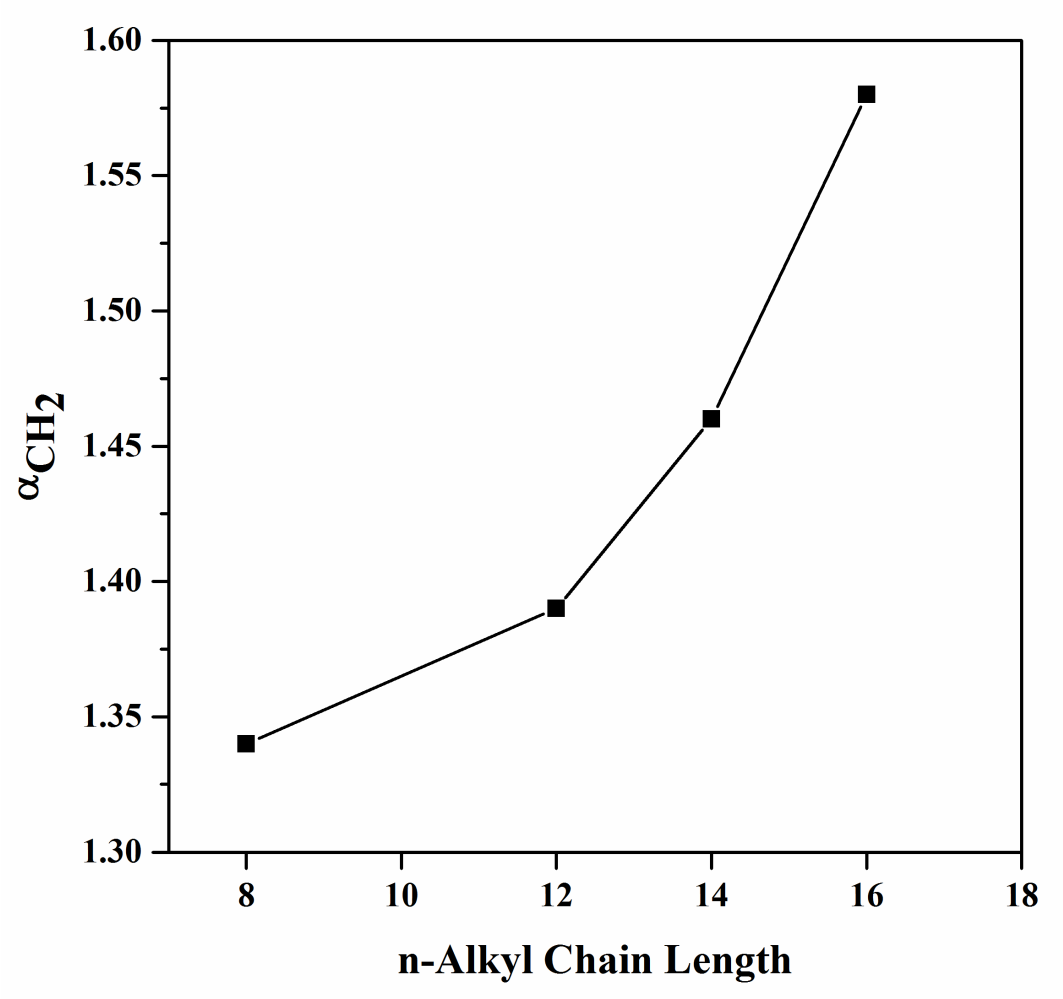


Figure 10. *Plots of methylene group selectivity vs. the number of carbon in the surface alkyl chain of the monolithic column. Conditions: Epoxy OHM C-n capillary columns, 33.5 cm (effective length 25 cm) × 100 μm i.d.; running voltage, 20 kV; column temperature, 25 ˚C; hydro-organic mobile phase, 2 mM Na_2HPO_4 (pH 7.0) at 30% (v/v) ACN; EOF tracer, thiourea.*

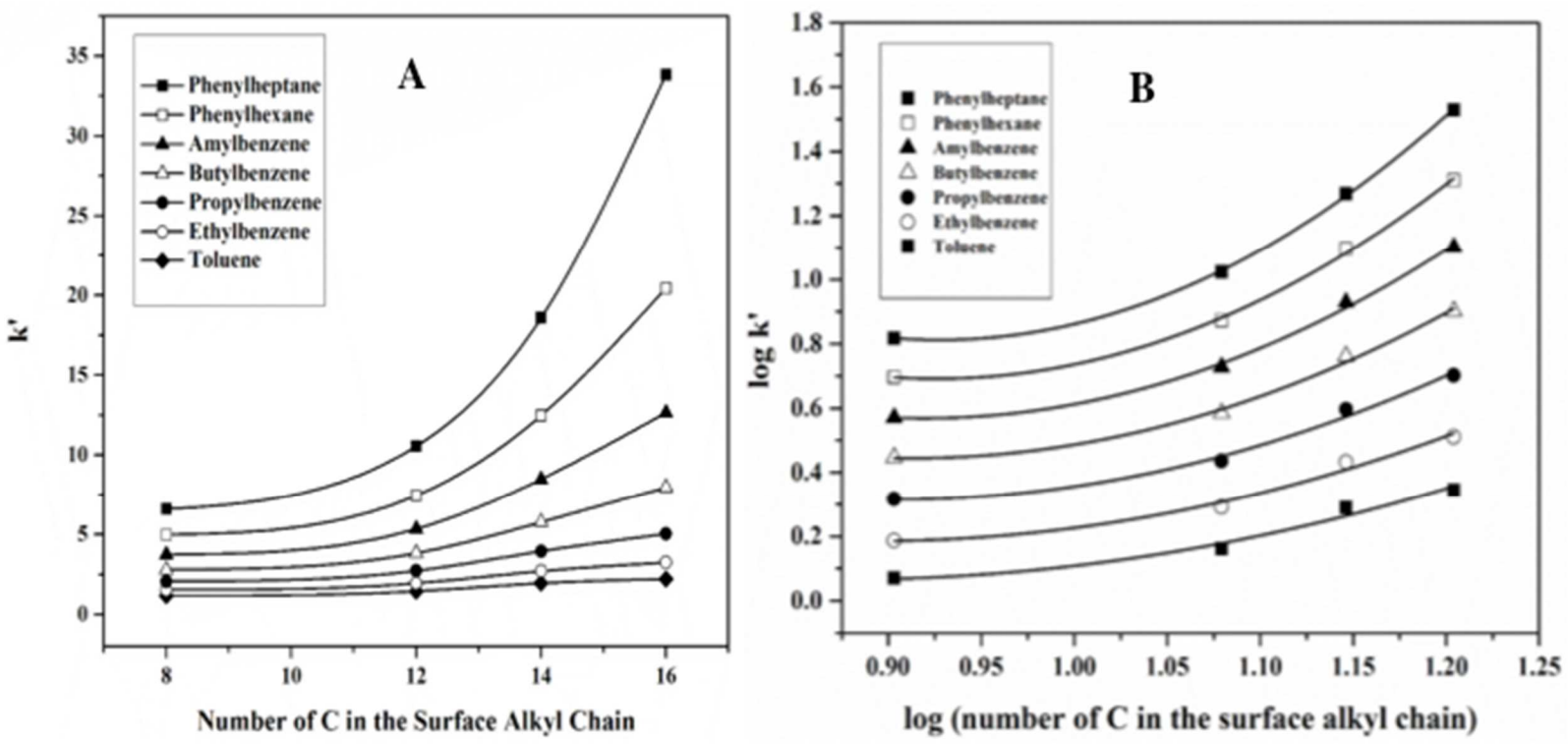


Figure 11. *Plots of k' values of ABs vs. the number of C in the surface alkyl chains in (A) and of log k' vs. log C in (B). Conditions: Epoxy OHM C-n capillary columns, 33.5 cm (effective length 25 cm) × 100 μm i.d.; running voltage, 20 kV; column temperature, 25 ˚C; hydro-organic mobile phase, 2 mM Na_2HPO_4 (pH 7.0) at 40% (v/v) ACN; EOF tracer, thiourea*

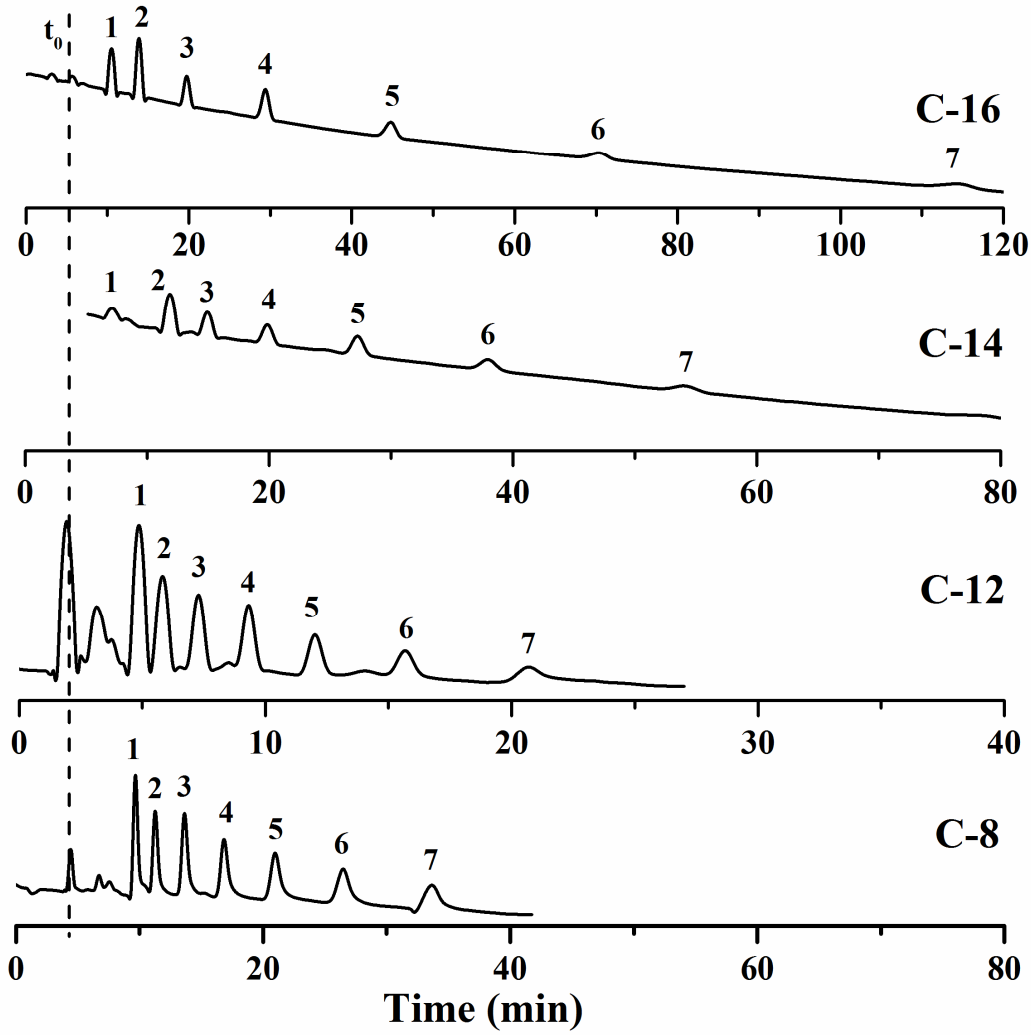


Figure 12.*Electrochromatograms of ABs obtained on (from bottom to top) Epoxy OHM C-8, Epoxy OHM C-12, Epoxy OHM C-14 and Epoxy OHM C-16 capillary columns. Conditions: Epoxy OHM C-n capillary columns, 33.5 cm (effective length 25 cm) × 100 µm i.d.; running voltage, 20 kV; column temperature, 25 ˚C; hydro-organic mobile phase, 2 mM Na_2HPO4 (pH 7.0) at 40% (v/v) ACN; solutes, 1, toluene; 2, ethylbenzene; 3, propylbenzene; 4, butylbenzene; 5, amylbenzene; 6, phenylhexane; 7, phenylheptane, EOF tracer, thiourea.*

Retention behaviors of slightly polar and nonpolar solutes

Phenolic compounds. Few phenol compounds including positional isomers of chlorophenols were electrochromatographed on all of the Epoxy OHM capillary columns under investigation (see Figure 13). Resorcinol being more polar than phenol, due to the presence of two hydroxyl groups on the benzene ring, its interaction with the hydrophobic surface was less compared to that of the phenol resulting in lesser retention of the prior. These surfaces were also able to resolve the positional isomers 2-chlorophenol and 4-chlorophenol. The higher retention of the 2,4-dichlorophenol compared to that of the monosubstituted chlorophenols was justified because of the decrease in the polarity of 2,4-dichlorophenol due to the presence of two chlorine atoms. The effect of increased hydrophobicity of the stationary phase also had an influence on the magnitude of retention of the phenolic compounds. As the chain length of the stationary phase was doubled from C-8 to C-16, the k' of resorcinol also increased by almost double from 0.32 to 0.68 (see Table 4). But in the case of phenol, k´ increased by 2.6 times. Similarly, as the non-polarity of the solute increased from 2-chlorophenol and 4-chlorophenol to 2,4-dichlorophenol, k´ increased by 3.2, 3.4 and 4.2 times, respectively. Although a noticeable effect of surface hydrophobicity on the retention of the monosubstituted chlorophenols was observed, this effect is more pronounced in the case of the disubstituted chlorophenols.

TABLE 4

k' OF PHENOLIC COMPOUNDS OBTAINED ON Epoxy OHM CAPILLARY COLUMNS

Phenolic compounds	k'			
	Epoxy OHM C- 8	Epoxy OHM C- 12	Epoxy OHM C- 14	Epoxy OHM C-16
Resorcinol	0.32	0.58	0.63	0.68
Phenol	0.53	1.01	1.11	1.37
2-Chlorophenol	0.94	1.76	2.37	3.03
4-Chlorophenol	1.07	2.02	2.92	3.72
2,4-Dichlorophenol	1.61	2.97	5.31	6.83

Conditions: Epoxy OHM C-n capillary columns, 33.5 cm (effective length 25 cm) ×100 µm i.d.; running voltage, 20 kV; column temperature, 25 ˚C; hydro-organic mobile phase, 2 mM Na_2HPO_4 (pH 7.0) at 30% (v/v) ACN; EOF tracer, thiourea.

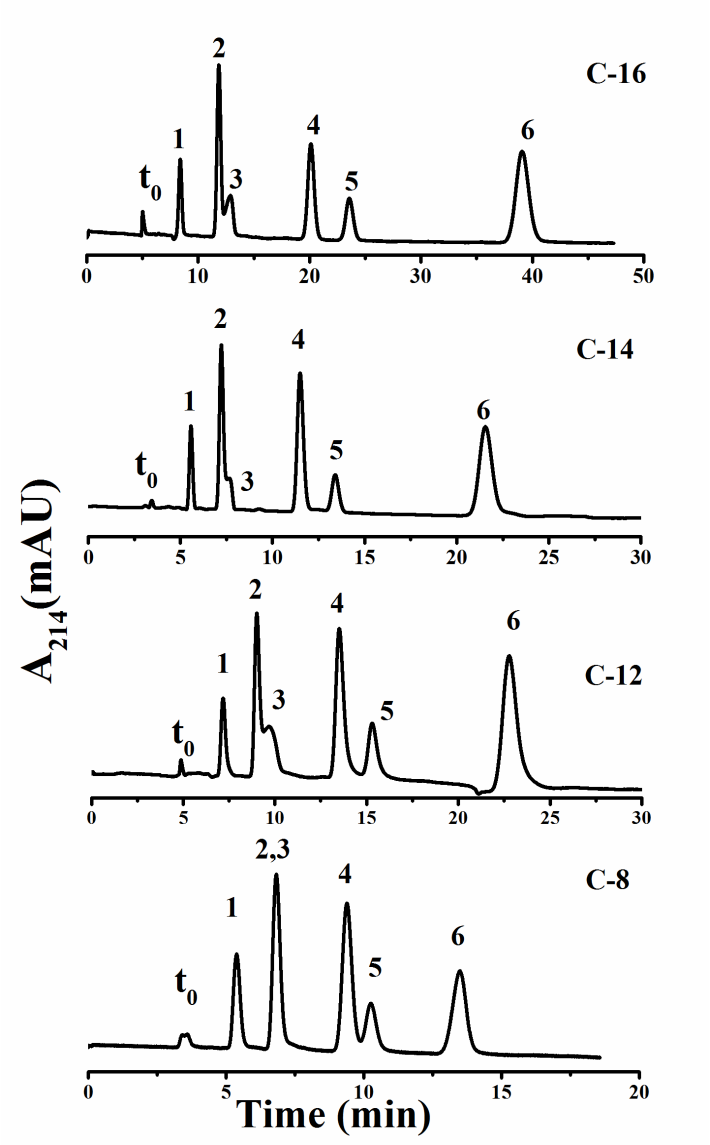


Figure 13. *Electrochromatograms of phenolic compounds obtained on (from bottom to top) on Epoxy OHM C-8, Epoxy OHM C-12, Epoxy OHM C-14 and Epoxy OHM C-16 capillary columns. Conditions: Epoxy OHM C-n capillary columns, 33.5 cm (effective length 25 cm) × 100 µm i.d.; running voltage, 20 kV; column temperature, 25 ˚C; hydro-organic mobile phase, 2 mM Na_2HPO_4 (pH 7.0) at 30% (v/v) ACN; EOF tracer, thiourea; solutes, 1, resorcinol; 2, phenol; 3, 4-nitrophenol; 4, 2-chlorophenol; 5, 4-chlorophenol; 6, 2,4-dichlorophenol.*

Toluene compounds. The behavior of toluene compounds on the Epoxy OHM C-n capillary columns was studied and it showed that the presence of an electron withdrawing group increased the retention of a given solute when compared to the retention of another solute with electron donating groups. The k´ values of all toluene solutes increased by almost 1.8 as the surface alkychain of the stationary phase increased form C-8 to C-16 as can be seen in Table 5. There were no anomalies in the retention behavior of the solutes and predictable results were obtained on each of the columns.

Polyaromatic hydrocarbons. Polyaromatic hydrocarbons are carcinogenic compounds and considered as pollutants, which can cause adverse effects on health. A range of polyaromatic hydrocarbons were injected onto the Epoxy OHM C-n capillary columns. The retention of these PAHs increased with increasing the number of aromatic rings and also their retention increased with increased hydrophobicity of the stationary phase. The k' of benzene increased from 0.87 to 1.27 while going from Epoxy OHM C-8 to Epoxy OHM C-16 whereas k' of pyrene increased from 6.76 to 15.65 as shown in Table 5.

TABLE 5

k' OF TOLUENE DERIVATIVES OBTAINED ON Epoxy OHM C-n CAPILLARY COLUMNS

Toluene Compounds	k’			
	Epoxy OHM C-8	Epoxy OHM C-12	Epoxy OHM C-14	Epoxy OHM C-16
p-Toluidine	1.16	1.50	2.14	2.00
m-Toluidine	1.27	1.63	2.33	2.22
p-Tolunitrile	1.60	2.07	2.95	2.90
2,3-Dinitrotoluene	3.12	4.07	5.82	5.82

Conditions: Epoxy OHM capillary columns, 33.5 cm (effective length 25 cm) × 100 µm i.d.; running voltage, 20 kV; column temperature, 25 ˚C; hydro-organic mobile phase, 2 mM Na_2HPO_4 (pH 7.0) at 30% (v/v) ACN; EOF tracer, thiourea.

TABLE 6

k' OF PAHs OBTAINED ON Epoxy OHM C-n CAPILLARY COLUMNS

Polyaromatic hydrocarbons	k’			
	Epoxy OHM C-8	Epoxy OHM C-12	Epoxy OHM C-14	Epoxy OHM C-16
Benzene	0.87	1.00	1.19	1.27
1-Cyanonapthene	1.51	1.76	2.01	2.48
1-Naphthol	1.68	1.98	2.20	3.56
Fluorene	3.34	3.95	5.88	6.56
Phenanthrene	4.67	5.58	8.28	9.39
Fluoranthrene	6.23	7.70	12.59	14.06
Pyrene	6.76	8.51	14.02	15.65

Conditions: Epoxy OHM C-n capillary columns, 33.5 cm (effective length 25 cm) × 100 μm i.d.; running voltage, 20 kV; column temperature, 25 ˚C; hydro-organic mobile phase, 2 mM Na_2HPO_4 (pH 7.0) at 30% (v/v) ACN; EOF tracer, thiourea.

Part II

Isocyanato OHM C-18 capillary column

Selection of solvent. The reaction of hydroxyl group with isocyanato forming a carbamate linkage requires a high temperature [19]. So, the solvent had to be selected such that, it would be stable at high temperature. Since the boiling point of dioxane is 101.1^{o}C, it was concluded that dioxane is not suitable for the reaction. Toluene on the other hand has a boiling point of 110.6^{o}C and 20% (w/v) of octadecyl isocyanato is also freely soluble in toluene. Thus, it was decided to use toluene as the solvent for this reaction.

Optimization of the reaction temperature. The reaction was performed at 75 ˚C and 100 ˚C and the Isocyanato OHM C-18 columns thus obtained from the modification at these two temperatures were electro-chromatographically evaluated using ABs as the test solutes. The retention of ABs as shown in Table 7 is higher for the column prepared at 100 ˚C compared to that prepared at 75 ˚C. However, plot of log k' vs. carbon number of ABs shows that the slope of the line obtained on the column prepared at 75 ˚C is slightly higher than that obtained on the column prepared at 100 ˚C indicating that the methylene group selectivity was roughly about the same on both columns (see Figure 14).

TABLE 7

k' OF ABs OBTAINED ON Isocyanato OHM C-18 CAPILLARY COLUMNS AT DIFFERENT TEMPERATURES

Solutes	Temperature of reaction	
	75ºC	100º C
	k’	
Toluene	0.82	1.04
Ethyl benzene	1.15	1.35
Propyl benzene	1.61	1.82
Butyl benzene	2.22	2.44
Amyl benzene	3.03	3.27
Phenyl hexane	4.06	4.39
Phenyl heptane	5.48	5.92

Conditions: Isocyanato OHM capillary columns, 33.5 cm (effective length 25 cm) × 100 µm i.d.; running voltage, 20 kV; column temperature, 25 ºC; hydro-organic mobile phase, 2 mM Na_2HPO_4 (pH 7.0) at 40% (v/v) ACN; EOF tracer, thiourea.

Effects of CEC parameters

Retention of ABs test solutes. For the evaluation of the hydrophobicity of the stationary phase under investigation, the ABs were used as model test solutes. As it can be seen from Table 7, toluene showed the least retention (k'=1.04) followed by ethylbenzene (k'=1.35), propylbenzene (k' = 1.82), butylbenzene (k' = 2.44), amylbenzene (k' = 3.27), phenylhexane (k' = 4.39) and phenylheptane (k' = 5.92). This retention behavior is typical for reversed phase sorbents.

Even though the Isocyanato OHM C-18 has longer alkyl chain on its surface compared to Epoxy OHM C-16, we can see from the plot of log k' vs. carbon number (see Figure 15) that the log of methylene group selectivity for Isocyanato OHM C-18 is ~ 0.13 (α_{CH2} = 1.35) compared to that of Epoxy OHM C-16, which is ~0.2 (α_{CH2} = 1.58). This could be attributed to the fact the Isocyanato OHM C-18 possesses carbamate linkage, which could provide more polarity to the surface than in the case of the Epoxy OHM C-16 which possesses a hydroxy-ether group near to the stationary phase surface.

Phenols. The phenolic compounds were separated in the order of their polarity (see Table 8). Resorcinol being the most polar of the tested phenols was eluted first followed by phenol, 4-nitrophenol, 2-chlorophenol, 4-chlorophenol and 2,4-dichlorophenol. The selectivity for 4-nitrophenol was better on Isocyanato OHM C-18 (α=1.40) compared to Epoxy OHM C-16 (α = 1.19) (see Table 8). This could be due to the presence of a carbamate linkage on the Isocyanato OHM C-18 stationary phase, which would have interacted with the nitro group.

TABLE 8

k' OF PHENOLIC COMPOUNDS OBTAINED ON Epoxy OHM C-16 AND Isocyanato OHM C-18 CAPILLARY COLUMNS

Solutes Phenolic compounds	k' Epoxy C-16 OHM		k' Isocyanato C-18 OHM	
Resorcinol	0.68		0.62	
Phenol	1.37		1.04	
		α = 1.19		α=1.40
4- Nitrophenol	1.58		1.46	
2-Chlorophenol	3.03		2.30	
4-Chlorophenol	3.72		2.50	
2,4-Dichlorophenol	6.83		4.23	

Conditions: Epoxy OHM C-n capillary columns, 33.5 cm (effective length 25 cm) × 100 µm i.d.; running voltage, 20 kV; column temperature, 25 ˚C; hydro-organic mobile phase, 2 mM Na_2HPO_4 (pH 7.0) at 30% (v/v) ACN; EOF tracer, thiourea; Isocyanato OHM capillary columns, 33.5 cm (effective length 25 cm) × 100 µm i.d.; running voltage, 20 kV; column temperature, 25 ºC; hydro-organic mobile phase, 2 mM Na_2HPO_4 (pH 7.0) at 30% (v/v) CAN; EOF tracer, thiourea.

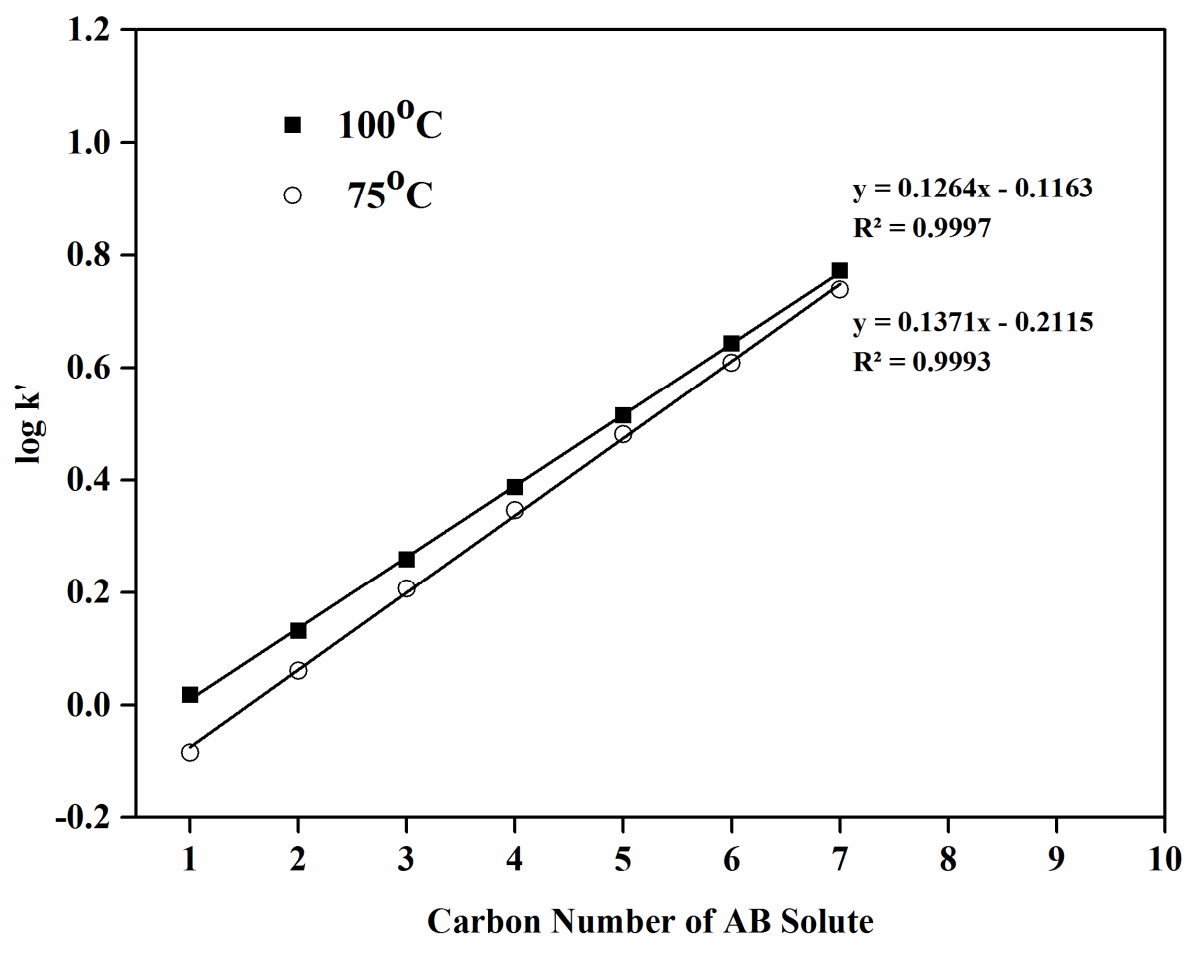


Figure 14. *Plots of log k' of ABs vs. carbon number of the ABs solutes. Conditions: Isocyanato OHM C-18 capillary column, 33.5 cm (effective length 25 cm) × 100 µm i.d.; running voltage, 20 kV; column temperature, 25 ˚C; hydro-organic mobile phase, 2 mM Na_2HPO4 (pH 7.0) at 30% (v/v) ACN; EOF tracer, thiourea.*

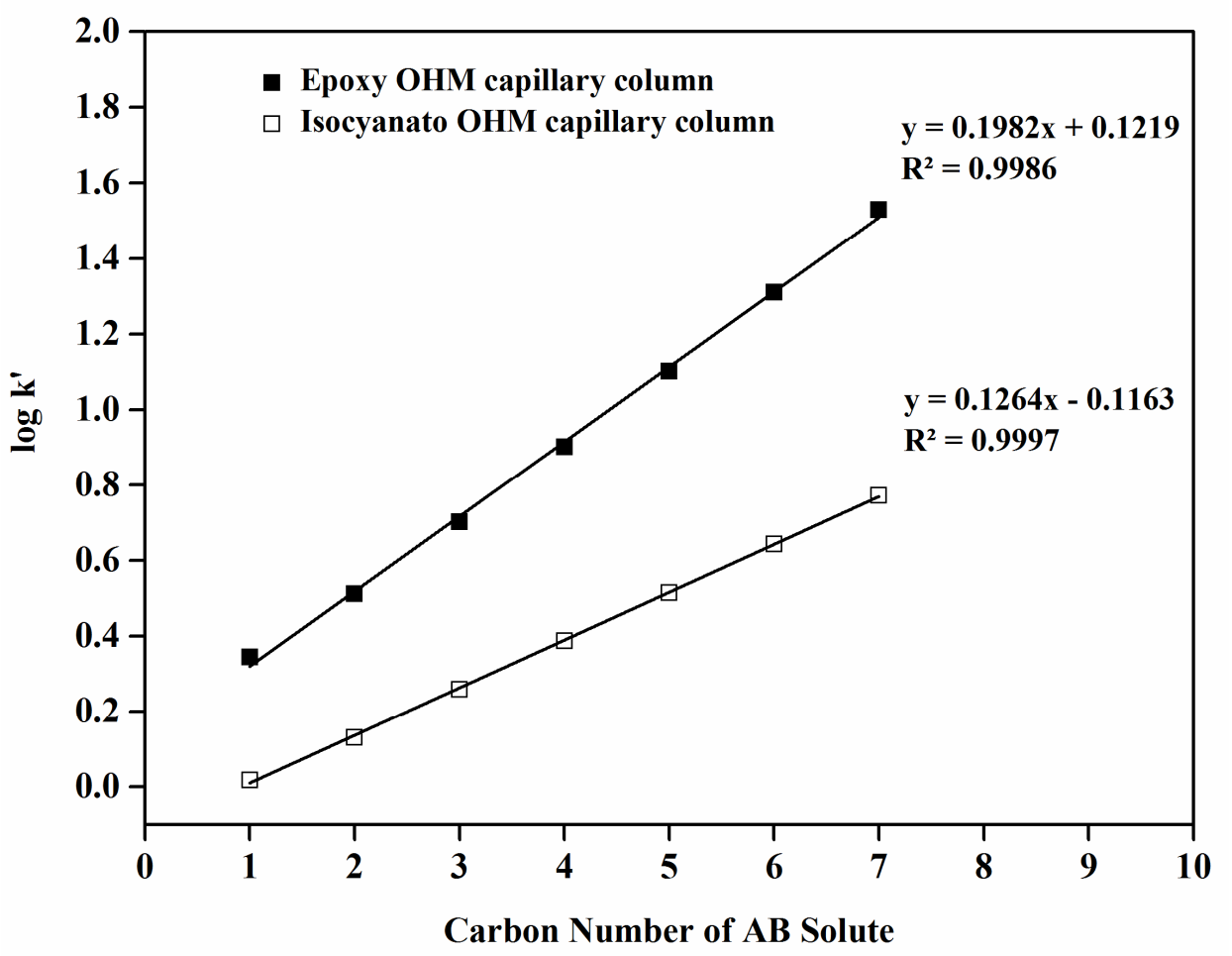


Figure 15. *Plots of log k' of ABs vs. carbon number of ABs solutes. Conditions: Epoxy OHM C-16 and Isocyanato OHM C-18 capillary column, 33.5 cm (effective length 25 cm) × 100 μm i.d.; running voltage,20 kV; column temperature, 25 ˚C; hydro-organic mobile phase, 2 mM Na2HPO4 (pH 7.0) at 30% (v/v) ACN; EOF tracer, thiourea.*

Toluene compounds. The effect of hydrophobicity of the stationary phases including the Epoxy OHM C-n and Isocyanato OHM C-18 columns on the toluene compounds is compared in Table 9 below.

It is evident that the k' values obtained on Isocyanato OHM C-18 are lower compared to Epoxy OHM C-16 and moreover, it is almost similar to that of the Epoxy OHM C-8 values which could be also due of the underlying surface polarity of the Isocyanato OHM C-18. However, the retention order on all the columns is almost the same, indicating that hydrophobic interactions of the solutes with the various stationary phases are the predominant interactions.

Polyaromatic hydrocarbons. The order of elution of polyaromatic hydrocarbons on the Isocyanato OHM C-18 and Epoxy OHM C-16 was the same (see Figure 16). However, the retention values in the case of the former were significantly lower compared to that of the later. This retention behavior could also be due to the carbamate linkage present in the backbone of the stationary phase. However, the selectivity of the solutes on the Isocyanato OHM C-18 and all of the Epoxy OHMs were comparable.

TABLE 9

k’ OF TOLUENE COMPOUNDS OBTAINED ON Isocyanato OHM C-18 CAPILLARY COLUMN-COMAPRISON WITH Epoxy OHM C-n COLUMNS

	k'				
Toluene Compounds	Epoxy OHM C-8	Epoxy OHM C-12	Epoxy OHM C-14	Epoxy OHM C-16	Isocyanato OHM C-18
p-Toluidine	1.16	1.50	2.14	2.00	1.16
m-Toluidine	1.27	1.63	2.33	2.22	1.27
p-Tolunitrile	1.60	2.07	2.95	2.90	1.59
2,3-Dinitrotoluene	3.12	4.07	5.82	5.82	3.33

Conditions: For both Epoxy OHM and Isocyanato OHM capillary columns, 33.5 cm (effective length 25 cm) × 100 µm i.d.; running voltage, 20 kV; column temperature, 25 ˚C; hydro-organic mobile phase, 2 mM Na_2HPO_4 (pH 7.0) at 30% (v/v) ACN; EOF tracer, thiourea.

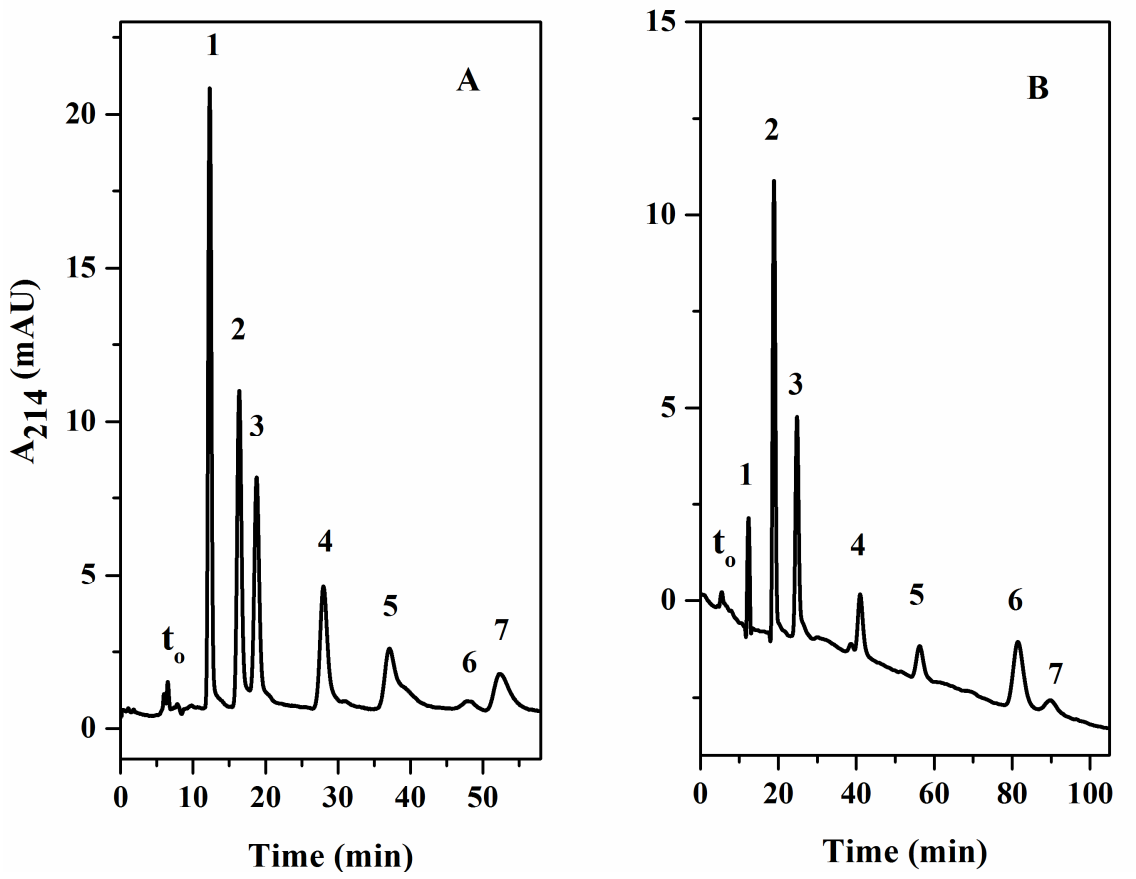


Figure 16 *Electrochromatograms of PAHs obtained on Isocyanato OHM C-18 column in (A) and on Epoxy OHM C-16 capillary column in (B), Conditions: 33.5 cm (effective length 25 cm) × 100 µm i.d.; running voltage, 20 kV; column temperature, 25 ˚C; hydro-organic mobile phase, 2 mM Na_2HPO_4 (pH 7.0) at 30% (v/v) ACN. Solutes: 1) benzene, 2) 1-cyanonaphthalene, 3) 1-naphthol, 4) fluorene, 5) phenanthrene, 6) fluoranthrene, 7) pyrene; EOF tracer, thiourea.*

Conclusions

The poly(HEMA-co-PETA) precursor monolith was applied for the functionalization of various 1,2-epoxyalkanes and octadecyl isocyanate to obtain Epoxy OHM alkyl and Isocyanato OHM C-18 stationary phases. Two different experimental methods were utilized in the fabrication of these columns. The preparation of Isocyanato OHM C-18 column was easier compared to that of the Epoxy OHM C-n due to the absence of catalyst and lesser steps. The presence of carbamate linkage provided some hydrophilicity to the surface of Isocyanato OHM C-18 rendering it less non-polar than even the lower alkyl chain Epoxy OHM C-n. However, both stationary phases showed relatable solute elution order.

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CHAPTER III

NONPOLAR AROMATIC MONOLITHS WITH SURFACE BOUND BIPHENYL LIGANDS FOR REVERSED PHASE CAPILLARY ELECTROCHROMATOGRAPHY AND HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Introduction

Owing to the limitation of particulate sorbents used traditionally for packing chromatographic columns, arising particularly from high mass transfer resistance (e.g., slow diffusion of solutes in and out of the porous structure), stationary phases have evolved through various stages of innovation and development. The introduction of small particles with short pores, and non-porous and superficially porous particles were some of the early remedies to the high mass transfer resistance [1, 2]. The Early 1990s saw the dawning of the revolutionary continuous macroporous polymeric beds [3] which would later be coined as monoliths. The ability of mobile phase to flow through the large pores of monoliths [4]

and the lack of void volume are some of the attractive features which led to high-resolution separations [3] in short flow paths thus making separations faster and better.

Earlier, monoliths were prepared from polymerizing the monomers such as monovinyl and divinyl methacrylate and were used as thin disks for chromatographic separations. However, with time, monolithic columns made from styrene, divinylbenzene and their derivatives were introduced [5]. These initial monoliths involved in addition to the main constituent monomers the co-polymerization of small amounts of charged monomers like 2-acrylamido-2-methylpropanesulfonic acid with the aim of providing fixed charges on the monolithic surface, which were thought necessary to generate EOF [6, 7]. Through the course of development, aromatic acrylate monolithic capillary columns emerged [8], and more recently, other promising aromatic monolithic stationary phases appeared [9-11]. This is not surprising since there has been a noticeable rise in the commercial availability of phenyl type reversed-phase (RP) columns for HPLC separations due to their potential orthogonal retention mechanism. The ability of the phenyl stationary phases to undergo π-π interactions with aromatic solutes in addition to nonpolar interactions contributed to making them complimentary to their counterparts, the alkyl bonded stationary phases [12]. As far as the area of RP-CEC is concerned, it also witnessed the introduction of naphthyl methacrylate based monoliths [10] by a strategy that got away from introducing fixed charges to the monolithic surface following the onset of neutral stationary phases that started in our laboratories [13]. The novel naphthyl methacrylate based monolith for RP-CEC introduced by Karenga and El Rassi [9] was adapted by Jonnada and El Rassi to the fact that this stationary phase was capable of possessing retention mechanism based on both hydrophobic and π-π interactions [11].

Availing to the various benefits of the post-polymerization modification of a given precursor monolith including avoidance of re-optimization of the polymeric mixture and ability to design specific surfaces as desired, there have been some contributions in this area. For instance Guerroauche *et al.* have used the post-polymerization modification approach in functionalizing the surface of a polymer precursor monolith with aromatic ligands. In this work, they functionalized the surface of poly(NAS-co-EDMA) monolith [poly(*N*-acryloxysuccinimide-co-ethylene glycol dimethacrylate)] with phenylbutylamine where butyl and phenyl functional groups would provide the monolithic stationary phase with both hydrophobic and π-π interaction properties [14].

The scope of this chapter is to investigate the post-polymerization modification to obtain a non-polar aromatic monolithic column and to evaluate the benefits of such a stationary phase chromatographically as well as electrochromatographically. Comparison of the retention behavior and efficiency of these non-polar aromatic monoliths have been done both in HPLC and CEC modes. The HPLC results seem to match that of the CEC especially in terms of efficiency.

Experimental

Instruments

All CEC experiments were performed on an HP ^{3D}CE system from Hewlett-Packard (Waldbronn, Germany) equipped with a photodiode array detector (PDA). Electrochromatograms were processed with a PC running an HP ChemStation software. Heat initiation for the polymerization reaction was performed using an Isotherm water bath

(Model 105) from Fisher Scientific (Fairlawn, NJ, USA). An HPLC pump Model M-45 from Waters Associates (Milford, MA, USA) was used to condition the monolithic columns after polymerization. HPLC experiments were performed on a Waters Alliance (Model w2690/5) system from Waters Associates (Milford, MA, USA) equipped with a PDA detector (Model w2996) using Empower2 (Build 2154) software. Another HPLC solvent delivery system Model M-45 was also obtained from Waters Associates. It was used to wash away the porogens and the unreacted monomers after the *in situ* polymerization of the monoliths. A constant pressure pump obtained from Shandon Southern Products Ltd. (Cheshire, UK) was used to transfer the monolithic mold at high pressure to an HPLC column of the desired length.

Reagents and materials

Fused silica capillaries having 100 μm internal diameter (i.d.) and 375 μm outer diameter (o.d.) were purchased from Polymicro Technologies (Phoenix, AZ, USA). Stainless steel tubing of 4.6 mm i.d., end column fittings and all other HPLC fittings were obtained from Alltech Associates (Deerfield, IL, USA). Hydroxyethyl methacrylate (HEMA), pentaerythritol triacrylate (PETA), 3-(trimethoxysilyl)propylmethacrylate, cyclohexanol, 1-dodecanol, azobis(isobutyronitrile) (AIBN), acetone, dioxane, alkylbenzenes (ABs), nitroalkanes, 2-chlorophenol, 4-chlorophenol, 2,4-dichlorophenol, 4-chloroaniline, 3-chloroaniline, 4-bromoaniline and 3,4-dibromoaniline were purchased from Sigma-Aldrich (Milwaukee, WI, USA). BF_3 was purchased from TCI (Toshima, kita-ku, Tokyo, Japan). 2-Biphenylyl glycidyl ether was purchased from Sigma-Aldrich (St. Louis, MO). Phenol and resorcinol were from Fisher Scientific (Fairlawn, NJ, USA). Acetonitrile and methanol were obtained from Pharmco-Aaper (Brookfield, CT, USA).

Phenoxy acid herbicides including cloprop, dichloroprop, and 2,4-dichloroprop were from Chem Service (Westchester, PA, USA).

Silanization of the capillary walls

Before polymerization, the inner wall of a 40 cm long, 100 μm i.d. fused silica capillary column was pre-treated by passing water for 2 min, then 1M NaOH for 30 min followed by 0.1M HCl for 30 min and with water for 30 min. Finally, the capillary walls were silanized in the presence of 50% (v/v) solution of 3-(triethoxysilyl) propylmethacrylate in acetone for 4 h at room temperature and were rinsed off with methanol for 30 min. The capillary was dried with nitrogen gas for 5 min.

In-situ polymerization in capillary

A solution containing 12.8% (w/w) HEMA as the functional monomer, 12.2% (w/w) PETA as the crosslinker together with 50% (w/w) cyclohexanol and 25% (w/w) 1-dodecanol as the binary porogen were mixed to yield a final total weight of 0.2 g. A 1% (w/w) of free radical initiator AIBN with respect to the monomer and crosslinker was added to the solution and sonicated for 10 min. The polymerization solution thus prepared appeared homogenous and was free of air bubbles. To fill the silanized capillary for polymerization, one end of the 40 cm long pretreated capillary was immersed in the vial containing the solution and negative pressure was applied to the capillary outlet. The solution was drawn into the capillary until it reached the 30 cm demarcation line drawn on it leaving the rest of the 10 cm void. Both ends of the capillary were sealed with GC septa and immersed in the water bath for 15 h at 60 ˚C. After the polymerization was complete,

the capillary was washed with acetonitrile using an HPLC pump until the porogens as well as unreacted monomer and crosslinker were completely removed. Then, a detection window was made on the column at 1mm below the monolith by using a thermal wire stripper. The resulting capillary column was trimmed into a total length of 33.5 cm with an effective length of 25 cm. The poly (HEMA-co-PETA) monolithic capillary column was then ready for post-polymerization modification.

Post-polymerization modification in capillary

After the *in situ* polymerization, the washed poly (HEMA-co-PETA) monolithic capillary column was equilibrated with dioxane for 2 h using a micro-syringe pump 0.1 mL/h.Then, it was loaded onto the HP ^{3D}CE instrument for further modification. A pressure of 8 bars was applied on the capillary inlet during all post-polymerization modification reactions. The inlet vial was filled with 5% (v/v) BF_3 in dioxane and supplied to the column for 20 min.

Preparation of biphenyl capillary monolithic column

A solution of 5% (w/v) 2-biphenylyl glycidyl ether in BF_3 solution was prepared and was passed through the column for 40 min. The column was rinsed with dioxane for 20 min and the cycle was repeated five more times. Finally, the capillary was rinsed with dioxane for 1 h followed by acetonitrile for another 1 h. The resulting capillary column was designated Biphenyl OHM capillary column (see Figure 1 for the reaction scheme).

In-situ polymerization in stainless steel column

A solution weighing 3.5 g was prepared using the exact same composition used for preparing the capillary monolith. It was drawn into stainless steel tubing made up of a 10

cm x 4.6 mm i.d. stainless steel column connected to a 5 cm x 4.6 mm i.d. stainless steel column *via* a ¼" union. The column assembly was provided with frits at both ends and sealed with end plugs. It was polymerized under the same conditions as described above for the capillary. After the polymerization was completed, it was taken out and rinsed by an HPLC pump using acetonitrile until all the porogens and unreacted monomer and crosslinker were washed out. The column assembly was equilibrated with isopropyl alcohol, and then 8000 psi pressure was used to push down the monolith into a firmer position in the 10 cm column using a constant pressure pump obtained from Shandon Southern Products Ltd. (Cheshire, UK). This process ensured that there was no void in the final 10 cm column. This column was then ready for post-polymerization modification.

Preparation of Biphenyl OHM HPLC column

The porogen free precursor monolith in the stainless steel column was equilibrated with dioxane using an HPLC pump at a flow rate of 1 mL/min for 10 min. The reactants were introduced into the column inlet using an injector. A 5% (v/v) solution of BF_3 in dioxane was injected into the column at a flow rate of 0.05 mL/min for 20 min, which was immediately followed by 10% (w/v) 2-biphenylyl glycidyl ether at a flow rate of 0.025 mL/min for 40 min. The process was repeated five more times. During each run, the 2-biphenylyl glycidyl ether was collected from the outlet and recycled for the subsequent injections (see Figure 1 for the reaction scheme).

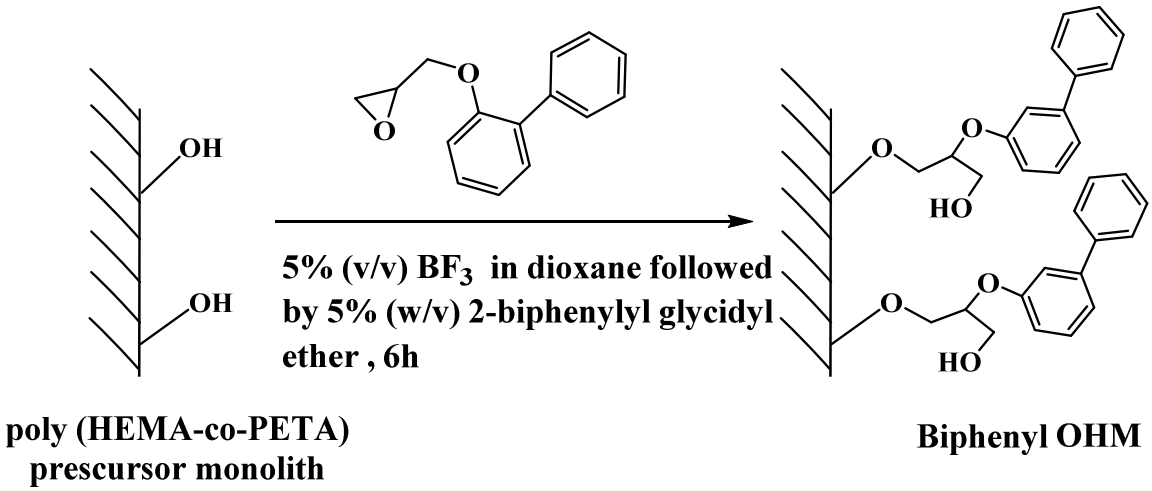


Figure 1. *Post-polymerization modification of the poly(HEMA-co-PETA) precursor monolith for the preparation of the Biphenyl OHM HPLC column and the Biphenyl OHM capillary column. The reaction was performed at 20 ˚C for 6 h.*

Results and discussion

Evaluation of the Biphenyl OHM columns in both HPLC and CEC modes

Alkylbenzenes. The strategy of reacting the surface hydroxyl groups of the OHM precursor monolith to tailor it into a desired surface was utilized and the biphenyl ligands were immobilized on the monolith in a capillary format as well as in a stainless steel column format to generate an aromatic non-polar stationary phase. The resulting monoliths were evaluated for their reversed phase behavior using ABs as the test solutes. Figure 2 and Figure 3 show plots of log k' of ABs against various % ACN (v/v) in the mobile phase for both the monolithic capillary and the HPLC columns. As can be seen in these figures, the plots linearly decrease with increasing the % ACN (v/v) indicating that the separation of

ABs on the two columns in HPLC and CEC follow the typical reversed phase chromatography (RPC) behavior. We can see that the slope of the line goes from -0.04 for toluene to ~ -0.08 for phenylheptane for the capillary column (see Figure 2) and

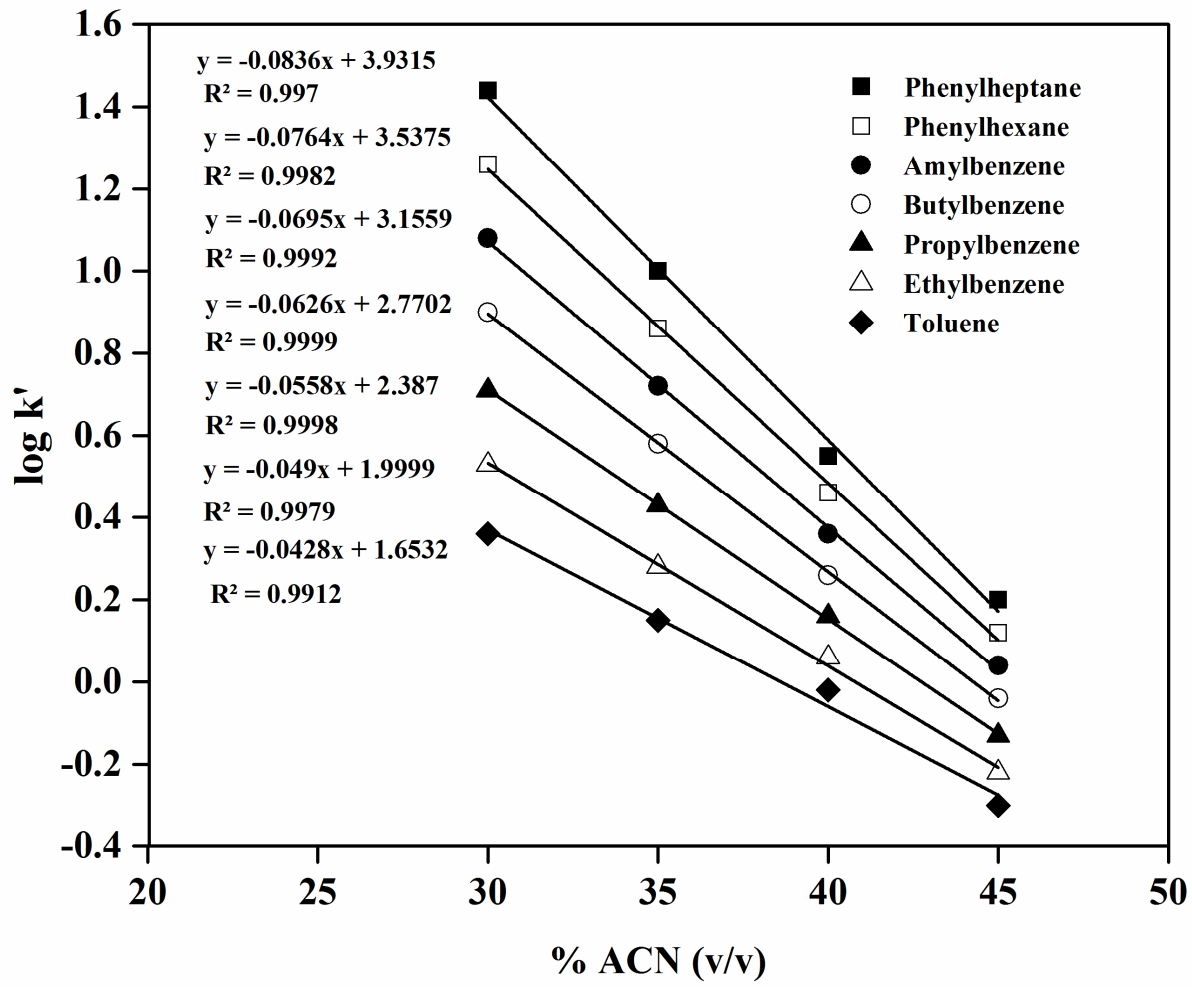


Figure 2. *Plots of log k' vs. % ACN (v/v) in the mobile phase obtained on the Biphenyl OHM capillary column. Conditions: 33.5 cm (effective length 25 cm) × 100 µm i.d.; running voltage, 20 kV; column temperature, 25 ˚C; hydro-organic mobile phase, 2 mM Na_2HPO_4 (pH 7.0) at various % (v/v) ACN.*

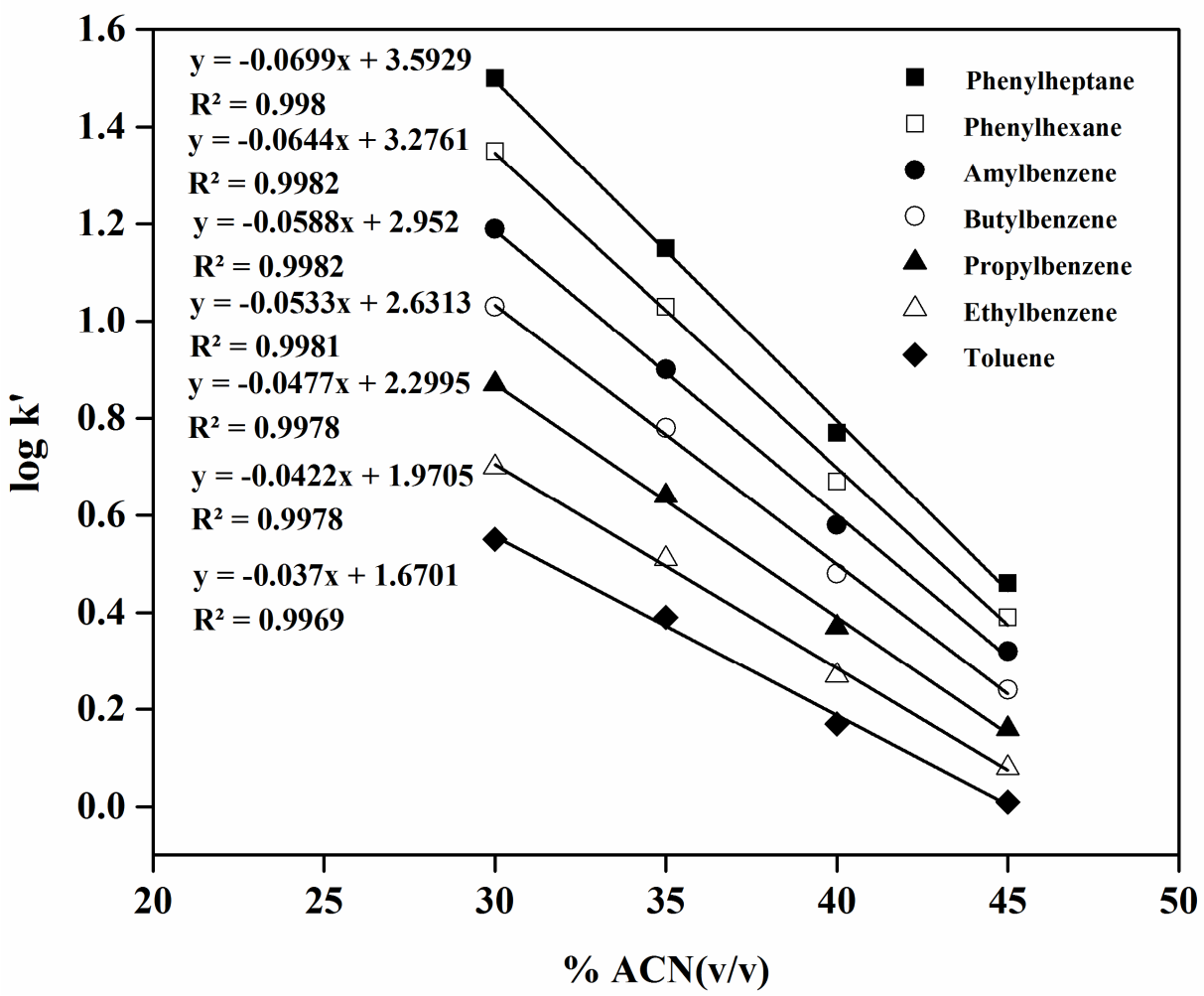


Figure 3. *Plots of log k' vs. % ACN (v/v) in mobile phase obtained on the Biphenyl OHM HPLC column (10 cm × 4.6 mm i.d.). Conditions: mobile phase, H2O at various % (v/v) ACN; flow rate, 1 mL/min.*

goes from ~ -0.037 for toluene to ~ -0.070 for phenylheptane on the HPLC column (see Figure 3). The slope of the line increased with the size of the solute, which suggests increased interaction between the solute and the biphenyl stationary phase, which is indicative of an RPC behavior.

The nonpolar selectivity of a given reversed phase column is determined by its methylene group selectivity (α_{CH_2}), which is the antilog of the slope of the linear plot of log k′ vs. the number of recurring methylene groups (or carbon number) in a homologous series, e.g., ABs. This is represented in Figure 4 for the Biphenyl OHM capillary column tested in CEC where it can be seen that the slope of the line increases from ~0.084 (α_{CH_2} = 1.2) for % 45 (v/v) ACN to 0.17 (α_{CH_2} = 1.45) for % 30 (v/v) ACN. Similar methylene group selectivity result is obtained on the Biphenyl OHM HPLC as shown in Figure 5. Indeed, the slope increased from 0.076 (α_{CH_2} ~ 1.2) to 0.16 (α_{CH_2} = 1.45) when going from 45% (v/v) to 30% (v/v) ACN in the mobile phase.

The AB homologous series showed baseline separation in both HPLC and CEC modes baseline separation with excellent resolution (see Figure 6). The average plate number of AB peaks in HPLC was 20,000 plates/m, which was comparable to that of the CEC mode with average plate number of 18,000 plates/m.

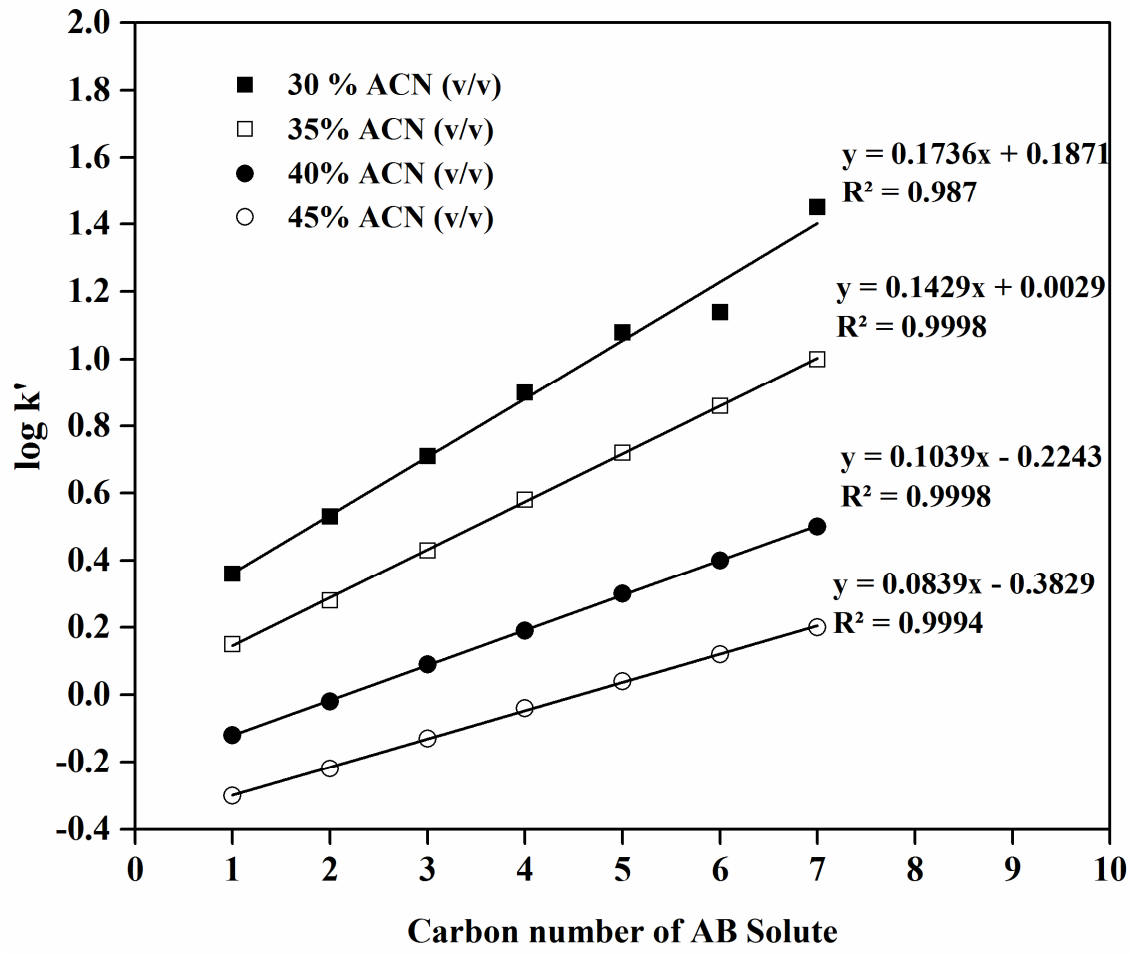


Figure 4. *Plots of log k' vs. number of carbon in AB homologous series obtained on the Biphenyl OHM capillary column at various % (v/v) ACN. Conditions: 33.5 cm (effective length 25 cm) × 100 µm i.d.; running voltage, 20 kV; column temperature, 25 ˚C; hydro-organic mobile phase, 2 mM Na_2HPO_4 (pH 7.0).*

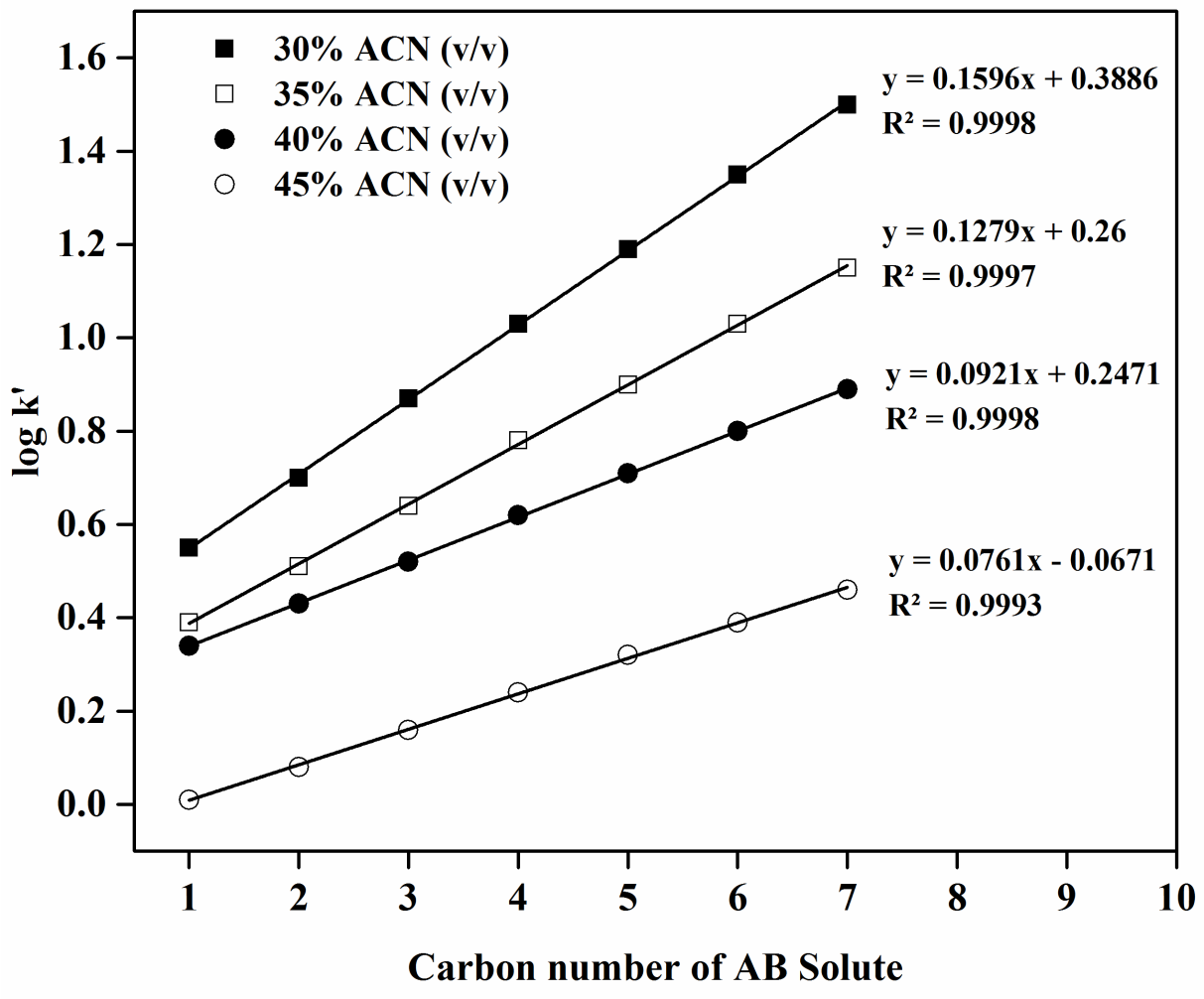


Figure 5. *Plots of log k' vs. the number carbon of AB homologous series obtained on the Biphenyl OHM HPLC column (10 cm × 4.6 mm i.d.) at various % (v/v) ACN. Conditions: mobile phase, H2O at various % (v/v) ACN; flow rate, 1 mL/min.*

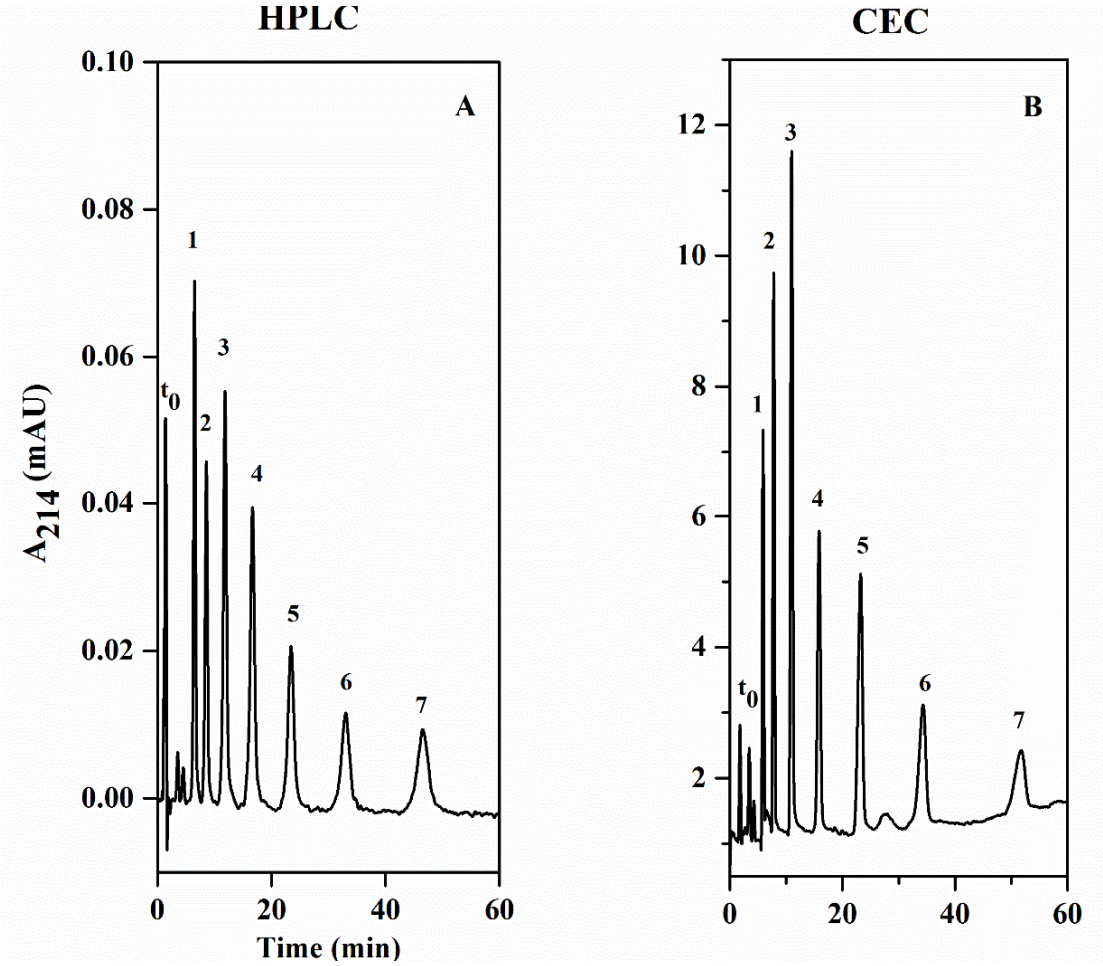


Figure 6. *Comparison of the retention of ABs obtained on the Biphenyl OHM HPLC column (10 cm × 4.6 mm i.d. in (A) to that obtained on the Biphenyl OHM capillary column 33.5 cm (effective length 25 cm) × 100 µm i.d. in (B).Conditions: (A) mobile phase, ACN: H_2O, (35:65, v/v); flow rate, 1 mL/min. (B) running voltage, 20 kV; column temperature, 25 ˚C; hydro-organic mobile phase, 2 mM Na_2HPO_4 (pH 7.0) at 35% (v/v) ACN. Solutes 1) toluene 2) ethylbenzene 3) propylbenzene 4) butylbenzene 5) amylbenzene 6) phenylhexane 7) phenylheptane.*

Polyaromatic hydrocarbons. Several polyaromatic hydrocarbons were tested in both the CEC and HPLC modes using the same % ACN (v/v) in the mobile phase and the chromatograms are shown in Figure 7. The retention factors obtained in HPLC and CEC for the polyaromatic hydrocarbons under investigations are listed in Table 1. Despite the use of the same % ACN in the mobile phase, the k´ values are higher (almost 2 fold higher), in HPLC than in CEC (see Table 1). This may reflect a higher surface coverage of biphenyl ligands of the monolith prepared in the stainless steel column than that prepared in the fused silica capillary column. The higher surface coverage in the biphenyl ligands may be explained by the ease with which the reactants for the post-polymerization modification of the precursor monolith could be supplied to the column in HPLC than in CEC. Indeed, in the former case an accurate HPLC pump was used versus a micro-syringe pump was used in the latter case. Higher surface coverage with ligands leads to higher retention and in turn higher separation efficiency. For equivalent surface coverage, i.e., same stationary phases, a CEC column usually yields a 3 fold higher plate count than an HPLC column. The lower surface coverage of the CEC column in biphenyl ligands, would explain the reason for the fact that the average plate numbers obtained in HPLC and CEC were about the same: 15,400 plates/m and 16,600 plates/m, respectively. Although the separation efficiency is slightly higher in CEC, the band broadening was dependent on the nature of the solute. In fact, the peak of fluorene looks sharper in CEC when compared to that in HPLC but the peaks of fluoranthrene and pyrene look narrower in HPLC. In both modes, the elution order of the solutes was the same. The selectivity values were also comparable in both modes (see Table 1)

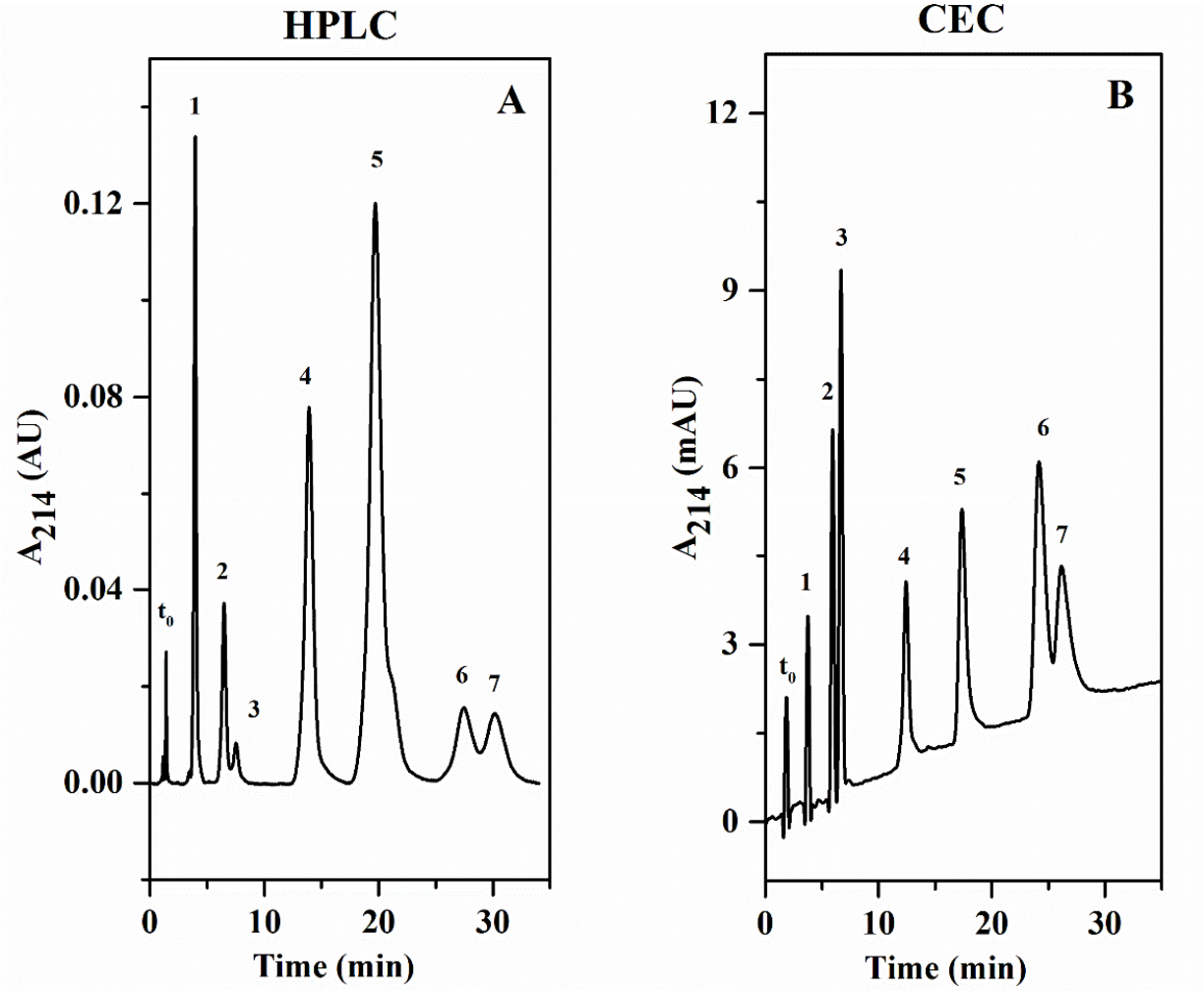


Figure 7. *Comparison of the separation of PAHs obtained on the Biphenyl OHM HPLC column (10 cm × 4.6 mm i.d. in (A) to that obtained on the Biphenyl OHM capillary column 33.5 cm (effective length 25 cm) × 100 µm i.d. in (B).Conditions: (A) mobile phase, ACN: H_2O, (35:65, v/v); flow rate, 1mL/min. (B) running voltage, 20 kV; column temperature, 25 ˚C; hydro-organic mobile phase, 2 mM Na_2HPO_4 (pH 7.0) at 35% (v/v) ACN, Solutes 1) benzene 2) 1-cyanonapthene 3) 1-naphthol 4) fluorene 5) phenanthrene 6) fluoranthrene 7) pyrene.*

TABLE 1

k' AND SELECTIVITY α VALUES OF PAHs OBTAINED ON BIPHENYL OHM COLUMNS IN HPLC AND CEC MODES

	HPLC		CEC	
Polyaromatic Hydrocarbons	k'	α	k'	α
Benzene	1.80		0.99	
		1.99		2.08
1-Cyanonapthene	3.59		2.07	
		1.21		1.16
1-Naphthol	4.33		2.40	
		2.04		2.07
Fluorene	8.85		4.97	
		1.46		1.43
Phenanthrene	12.95		7.09	
		1.42		1.41
Fluoranthrene	18.42		10.01	
		1.11		1.13
Pyrene	20.40		11.33	

The experiments were performed on the Biphenyl OHM HPLC column (10 cm x 4.6 mm i.d.) using the following conditions: mobile phase, ACN: H_2O, (35:65, v/v); flow rate, 1mL/min; UV detection at 214 nm and on the Biphenyl OHM capillary column, 33.5 cm (effective length 25 cm) × 100 μm i.d. using the following conditions: running voltage, 20 kV; column temperature, 25 ˚C; hydro-organic mobile phase, 2 mM Na_2HPO_4 (pH 7.0) at 35% (v/v) ACN.

Phenol derivatives. Similar to the above solutes, the phenol derivatives tested were retained almost twice in HPLC than in CEC. The resolution between resorcinol and phenol was better in HPLC than in CEC, see Figure 8. The k' values are listed in Table 2 and it is evident that the solutes are retained more in HPLC when compared to CEC. Also, the average plate number in HPLC (19,800 plates/m) is higher than that in CEC (18,300 plates/m). The selectivity between hydroxybenzoic acid and resorcinol was comparatively higher in HPLC (see Table 2). However, the selectivity between phenol and hydroxybenzoic acid was higher in CEC. This can be attributed to the electromigration of the acidic compound hydroxybenzoic acid in CEC.

Aniline compounds. The aniline compounds showed a similar elution order in both HPLC and CEC modes, see Figure 9. The k' and α values are listed in Table 3. Again, the retention of these solutes is greater in the HPLC mode than in the CEC mode. However, the selectivity between 4-bromoaniline and 3,4-dichloroaniline in HPLC is 1.66 vs. 1.14 in CEC. In addition to the hydrophobic interactions the presence of two chlorine groups makes the compound more electron deficient thus contributing to the π-π interactions. Also, the biphenyl HPLC column exhibited a higher average plate number (20,370 plates/m) than the biphenyl CEC column (14,200 plates/m). The significantly higher average plate

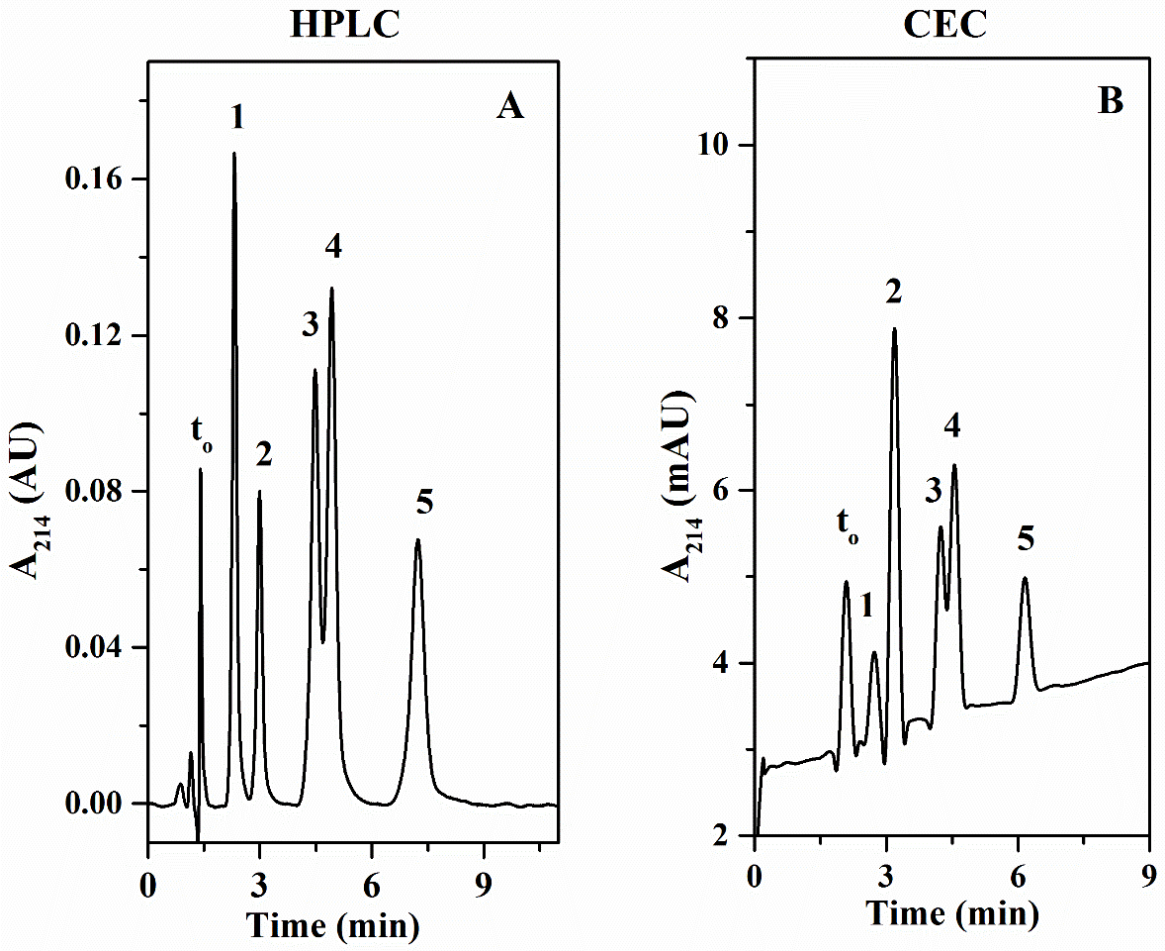


Figure 8. *Comparison of the separation of phenols obtained on the Biphenyl OHM HPLC column (10 cm × 4.6 mm i.d. in (A) to that obtained on the Biphenyl OHM capillary column, 33.5 cm (effective length 25 cm) × 100 µm i.d. in (B).Conditions: (A) mobile phase, ACN: H_2O, (35:65, v/v); flow rate, 1mL/min. (B) running voltage, 20 kV; column temperature, 25 ˚C; hydro-organic mobile phase, 2 mM Na_2HPO_4 (pH 7.0) at 35% (v/v) ACN. Solutes 1) resorcinol 2) phenol 3) 2-chlorophenol 4) 4-chlorophenol 5) 2,4-dichlorophenol.*

TABLE 2

K' AND α VALUES OF PHENOLS OBTAINED ON BIPHENYL OHM COLUMNS IN HPLC AND CEC MODES

Phenolic compounds	HPLC		CEC	
	k'	α	k'	α
Resorcinol	0.64		0.36	
		2.13		1.30
Hydroxybenzoic acid	1.35		0.47	
		0.82		1.36
Phenol	1.11		0.64	
		1.27		1.57
Cresol	1.40		1.01	
		1.53		1.24
2-Chlorophenol	2.14		1.25	
		1.13		1.14
4-Chlorophenol	2.43		1.42	
		1.67		1.64
2,4-Dichlorophenol	4.06		2.33	

The experiments were performed on Biphenyl OHM HPLC column (10 cm x 4.6 mm i.d.). Condition: mobile phase, ACN: H_2O, (35:65, v/v); flow rate, 1mL/min; UV detection at 214 nm and on Biphenyl OHM capillary column, 33.5 cm (effective length 25 cm) × 100 µm i.d.; running voltage, 20 kV; column temperature, 25 ˚C; hydro-organic mobile phase, 2 mM Na_2HPO_4 (pH 7.0) at 35% (v/v) ACN.

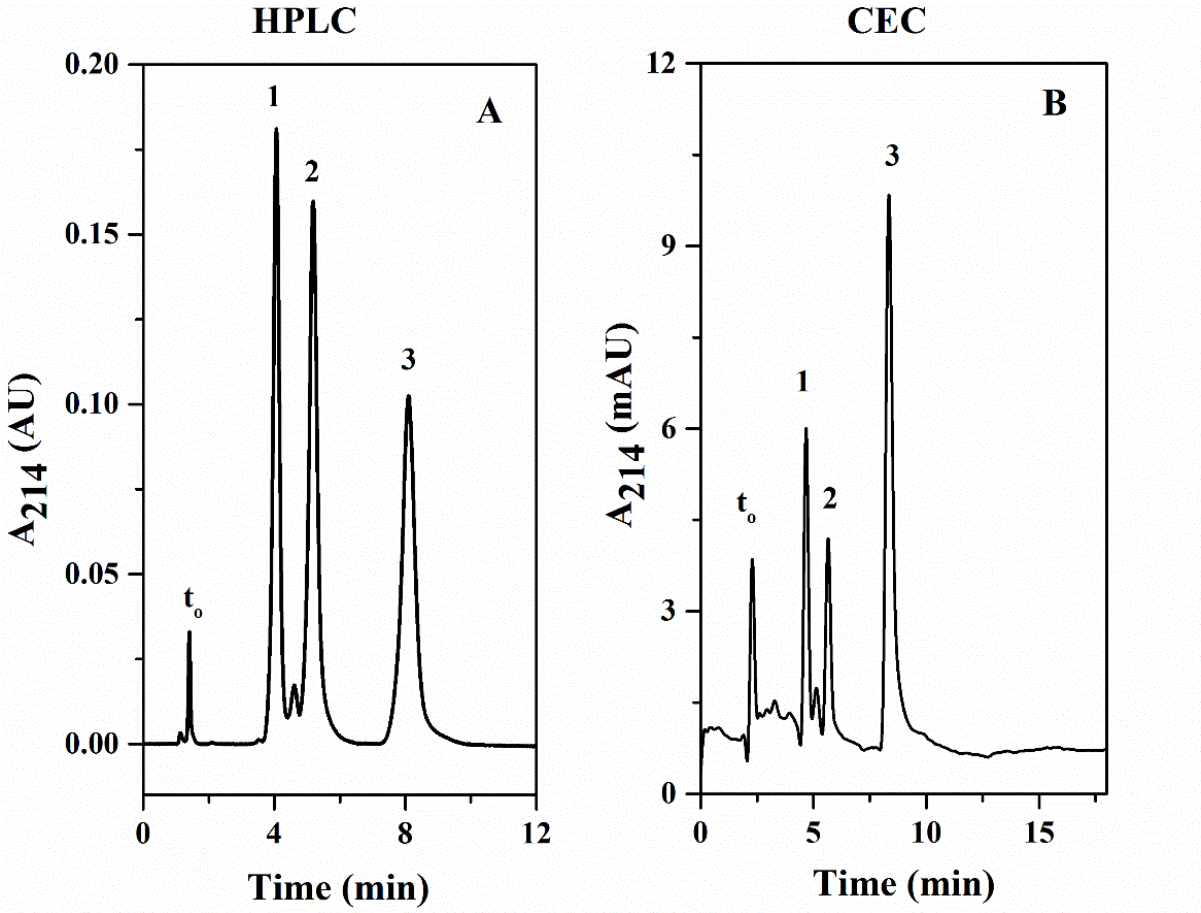


Figure 9. *Comparison of the separation of aniline derivatives obtained on the Biphenyl OHM HPLC column (10 cm × 4.6 mm i.d.) in (A) to that obtained on the Biphenyl OHM capillary column 33.5 cm (effective length 25 cm) × 100 µm i.d. in (B).Conditions: (A) mobile phase, ACN: H_2O, (35:65, v/v); flow rate, 1mL/min; (B) running voltage, 20 kV; column temperature, 25 ˚C; hydro-organic mobile phase, 2 mM Na_2HPO_4 (pH 7.0) at 35% (v/v) ACN. Solutes 1) 4-chloroaniline 2) 3-chloroaniline 3) 3,4-dichloroaniline.*

TABLE 3

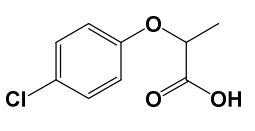
k' AND α VALUES OF ANILINE DERIVATIVES OBTAINED ON BIPHENYL OHM COLUMNS IN HPLC AND CEC MODES

Aniline Compounds	HPLC		CEC	
	k'	α	k'	α
4-Chloroaniline	1.90		0.65	
		1.42		1.43
3-chloroaniline	2.70		0.93	
		1.05		1.32
4-Bromoaniline	2.84		1.23	
		1.66		1.22
3,4-Dichloroaniline	4.71		1.50	

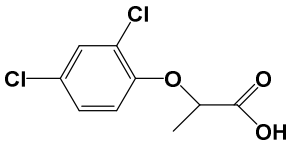
The experiments were performed on the Biphenyl OHM HPLC column (10 cm × 4.6 mm i.d.) using the following conditions: mobile phase, ACN: H_2O, (35:65, v/v); flow rate, 1 mL/min; UV detection at 214 nm and on the Biphenyl OHM capillary column, 33.5 cm (effective length 25 cm) × 100 µm i.d. using the following conditions: running voltage, 20 kV; column temperature, 25 ˚C; hydro-organic mobile phase, 2 mM Na_2HPO_4 (pH 7.0) at 35% (v/v) ACN.

number for HPLC when compared to that of CEC can be attributed to the higher retentivity of the Biphenyl OHM HPLC column than the Biphenyl OHM CEC column.

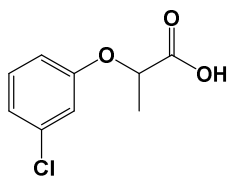
Phenoxy acid herbicides. The biphenyl column was able to retain and separate some phenoxy acid herbicides (see solute structures shown below) as shown in Table 4, and Figure 10. All of the solutes were more retained in HPLC than in CEC. Also, the elution order of these herbicides differed between HPLC and CEC. For HPLC, the solutes eluted in the order cloprop < mecoprop < dichloroprop < 2,4-D while in CEC the elution order varied in between the last two solutes and eluted cloprop < mecoprop < 2,4-D < dichloroprop. The difference in retention order is due to the fact that the phenoxy acid are negatively charged at pH 7, and therefore would migrate electrophoretically in CEC, which would explain the better separation obtained in CEC. Furthermore, CEC was more efficient than HPLC in the case of the negatively charged phenoxy acid herbicides yielding an average plate number of 13,350 plates/m in CEC versus an average plate number of 4,700 plates/m in HPLC. Three out of the four listed phenoxy acid herbicides were resolved in CEC compared to just two in HPLC. Three solutes namely cloprop, mecoprop and dichloroprop co-eluted in the HPLC mode.



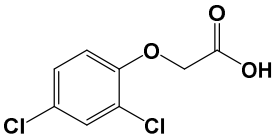
Mecoprop



Dichloroprop



Cloprop



2,4-D

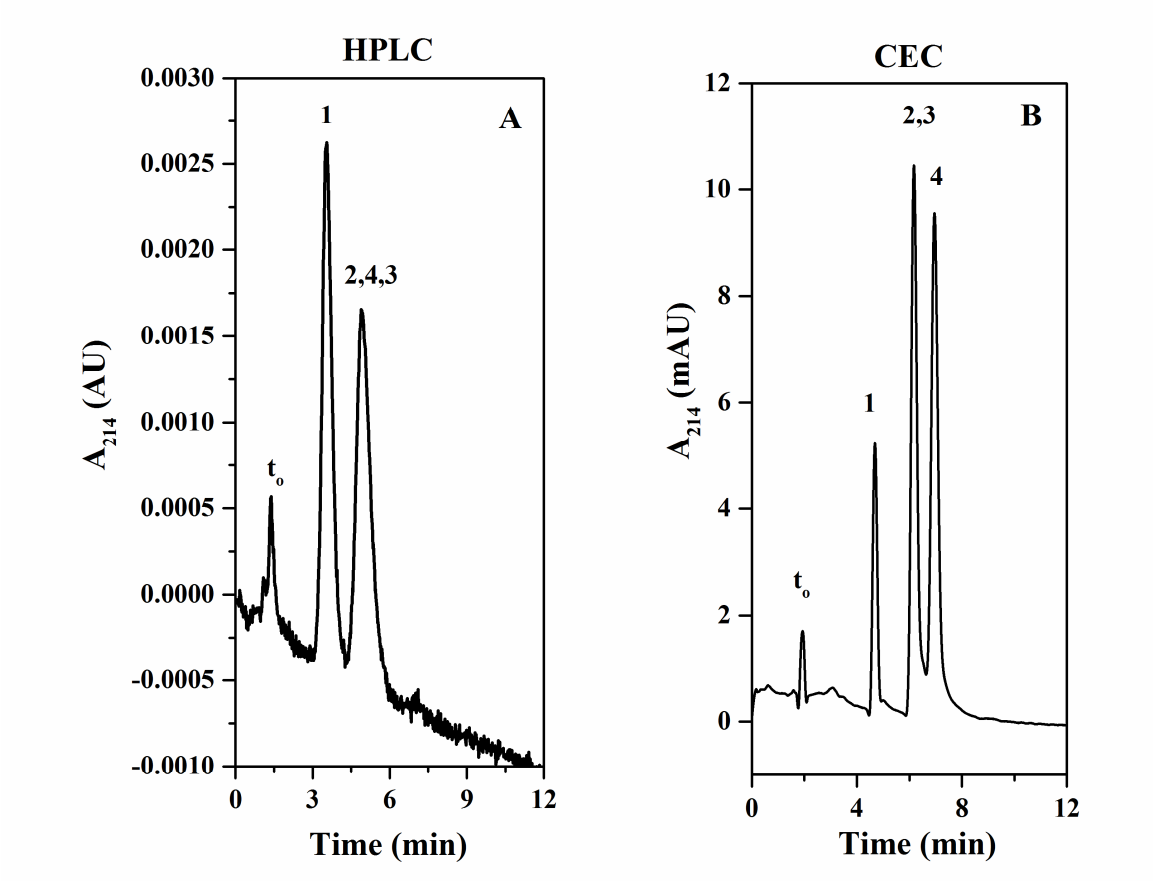


Figure 10. *Comparison of the separation of the phenoxy acid herbicides obtained on the Biphenyl OHM HPLC column (10 cm × 4.6 mm i.d.) in (A) to that obtained on the Biphenyl OHM capillary column 33.5 cm (effective length 25 cm) × 100 µm i.d. in (B).Conditions: (A) mobile phase, ACN: H_2O, (35:65, v/v); flow rate, 1 mL/min. (B) running voltage, 20 kV; column temperature, 25 ˚C; hydro-organic mobile phase, 2 mM Na_2HPO_4 (pH 7.0) at 35% (v/v) ACN; solutes 1, cloprop; 2, mecoprop; 3,2,4-D; 4, dichloroprop.*

TABLE 4

k' VALUES OF PHENOXYHERBICIDES OBTAINED ON BIPHENYL OHM COLUMNS IN HPLC AND CEC MODES

	k'	
Phenoxy acid herbicides	HPLC	CEC
cloprop	1.50	1.35
Mecoprop	2.48	1.94
Dichloroprop	2.72	2.31
2,4-D	2.83	2.06

The experiments were performed on the Biphenyl OHM HPLC column (10 cm x 4.6 mm i.d.) using the following conditions: mobile phase, ACN: H_2O, (35:65, v/v); flow rate, 1mL/min; UV detection at 214 nm and on the Biphenyl OHM capillary column, 33.5 cm (effective length 25 cm) × 100 µm i.d.; using the following conditions: running voltage, 20 kV; column temperature, 25 ˚C; hydro-organic mobile phase, 2 mM Na_2HPO4 (pH 7.0) at 35%(v/v) ACN.

Nitroalkanes homologous series. Some of the homologous series of nitroalkanes (NAs) were used as test solutes on the Biphenyl OHM columns in both the HPLC and CEC modes. The retention of nitroalkanes in HPLC and CEC is depicted by the chromatograms in Fig. 11 and the values of k' are given in Table 5. As can be seen in this table, the NAs solutes are more retained in HPLC than in CEC. However, we can say that the selectivity is almost the same for all the solutes in both modes. To further characterize the biphenyl column, log k' of NAs obtained in HPLC and CEC were plotted against the carbon number of the NAs solutes in Figure 12 andFigure **13**, respectively, and these plots were compared to those obtained for ABs. Linear plots were obtained in all cases, and the slopes of these lines are the log methylene group selectivity (log α_{CH_2}).

The slope of the line for NAs was greater than that of ABs in HPLC (compare ~ 0.19 to ~ 0.13) yielding an $\alpha_{CH_2} = 1.55$ for NAs versus an $\alpha_{CH_2} = 1.35$ for ABs. Similar results were obtained in CEC in the sense that the slope of the line for NAs is ~ 0.18, which was higher than the slope of the line obtained for ABs (~0.14) yielding an $\alpha_{CH_2} = 1.5$ for NAs versus an $\alpha_{CH_2} = 1.38$ for ABs. In both cases, the higher value of α_{CH_2} for NAs indicates the presence of π-π interactions in addition to hydrophobic interactions. The nitro groups in NAs are rich in π electrons and can associate with the biphenyl ligands of the monolithic columns. The peaks were narrower in HPLC than in CEC with an average plate number of 18,234 plates/m and 10,172 plates/m, respectively. This is not surprising given the fact that the NAs were more retained on the HPLC column than on the CEC column. This is shown in the plots of log k' of NAs vs. number of carbons in the solutes shown in Figure 14.

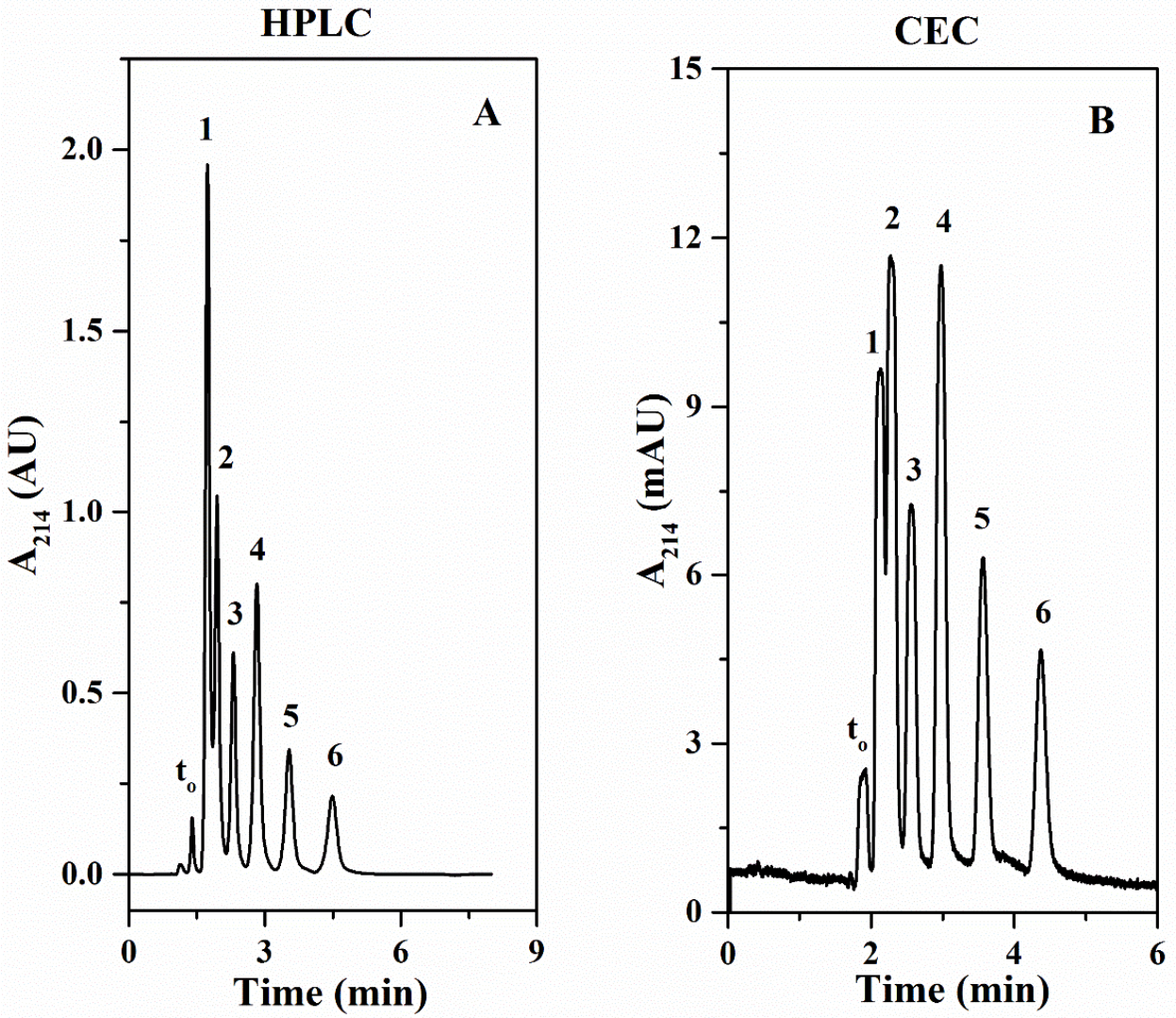


Figure 11. *Comparison of the separation of NAs obtained on the Biphenyl OHM HPLC column (10 cm × 4.6 mm i.d.) in (A) to that obtained on the Biphenyl OHM capillary column 33.5 cm (effective length 25 cm) × 100 µm i.d. in (B). Conditions: (A) mobile phase, ACN: H_2O, (35:65); flow rate 1mL/min. (B) running voltage, 20 kV; column temperature, 25 ˚C; hydro-organic mobile phase, 2 mM Na_2HPO4 (pH 7.0) at 35% (v/v) ACN; solutes 1, nitromethane; 2, nitroethane; 3, nitropropane; 4, nitrobutane; 5, nitropentane; 6, nitrohexane.*

TABLE 5

k' AND α VALUES OF NITROALKANES OBTAINED ON Biphenyl OHM COLUMNS IN HPLC AND CEC MODES

Nitroalkanes	HPLC		CEC	
	k'	α	k'	α
Nitromethane	0.24	1.61	0.12	1.58
Nitroethane	0.39	1.65	0.19	1.79
Nitropropane	0.65	1.57	0.34	1.65
Nitrobutane	1.02	1.50	0.56	1.55
Nitropentane	1.53	1.44	0.87	1.48
Nitrohexane	2.21		1.29	

The experiments were performed on the Biphenyl OHM HPLC column (10 cm × 4.6 mm i.d.) using the following conditions: mobile phase, ACN: H_2O, (35:65, v/v); flow rate, 1 mL/min; UV detection at 214 nm and on the Biphenyl OHM capillary column, 33.5 cm (effective length 25 cm) × 100 µm i.d. using the following conditions: running voltage, 20 kV; column temperature, 25 ˚C; hydro-organic mobile phase, 2 mM Na_2HPO_4 (pH 7.0) at 35% (v/v) ACN.

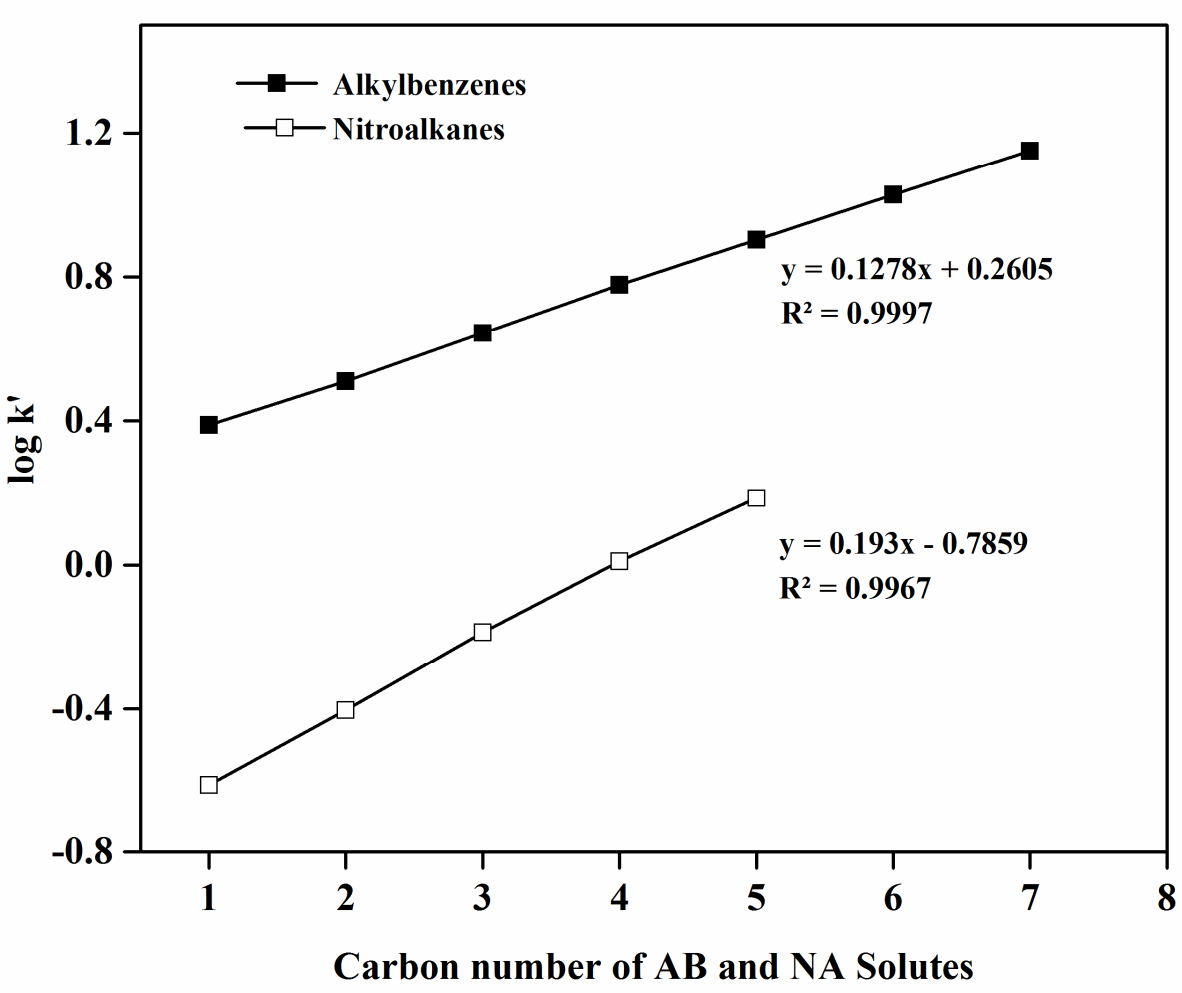


Figure 12. *Plot of log k' vs number of carbons in AB and NA homologous series obtained on the Biphenyl OHM HPLC column (10 cm × 4.6 mm i.d.). Conditions: mobile phase, ACN: H2O, (35:65, v/v); flow rate, 1 mL/min.*

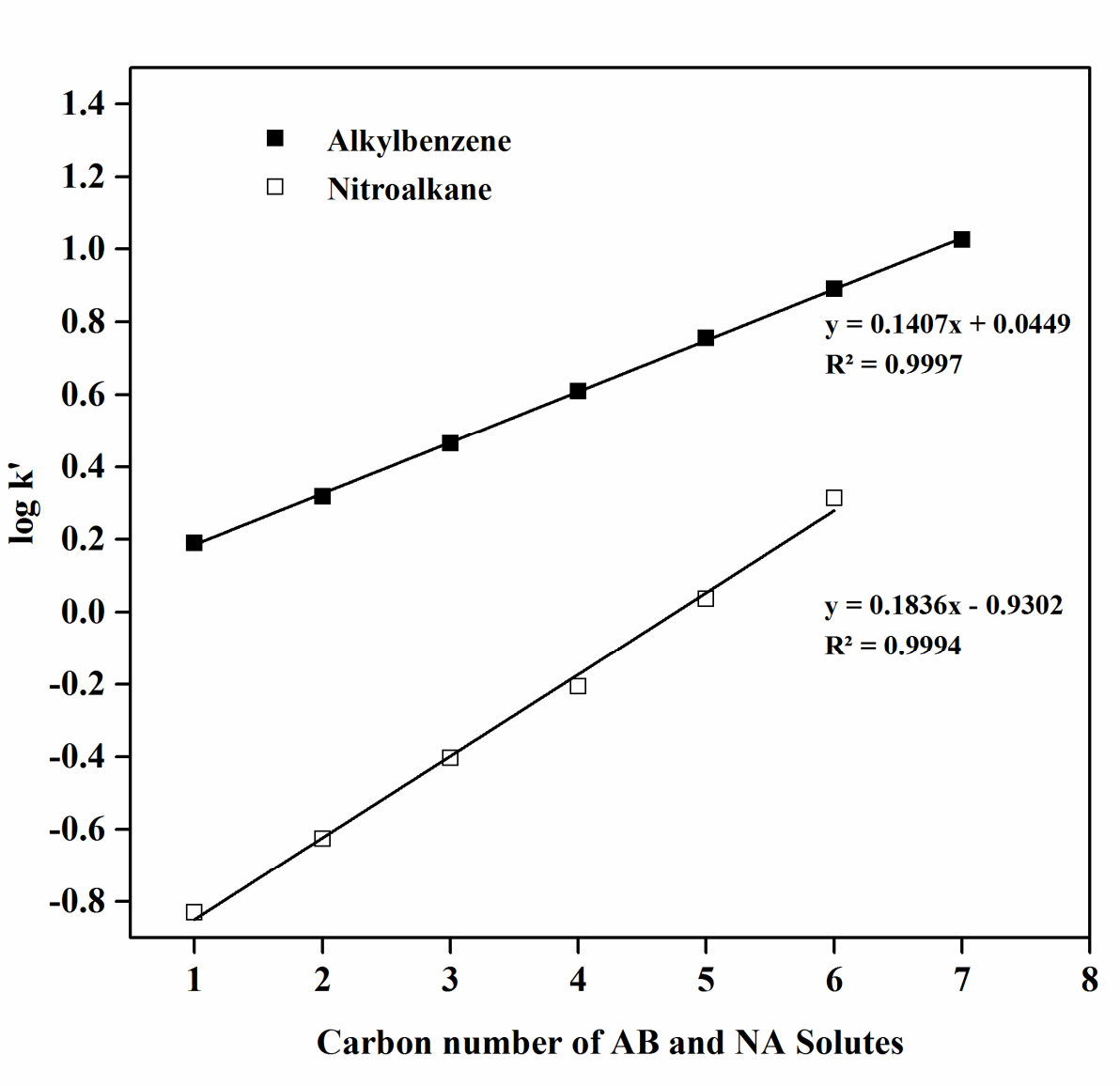


Figure 13. *Plots of log k' vs. the number of carbons in AB and NA homologous series obtained on Biphenyl OHM CEC column, 33.5 cm (effective length 25 cm) × 100 µm i.d. Conditions; running voltage, 20 kV column temperature, 25 ˚C; hydro-organic mobile phase, 2mM Na_2HPO_4 (pH 7.0) at 35% ACN.*

The slope of the line for NAs in HPLC is ~0.19 a slightly higher value than ~0.18 obtained in CEC. This means that the methylene group selectivity of NAs is about the same in both HPLC (α_{CH_2} = 1.55) and CEC (α_{CH_2} = 1.52).

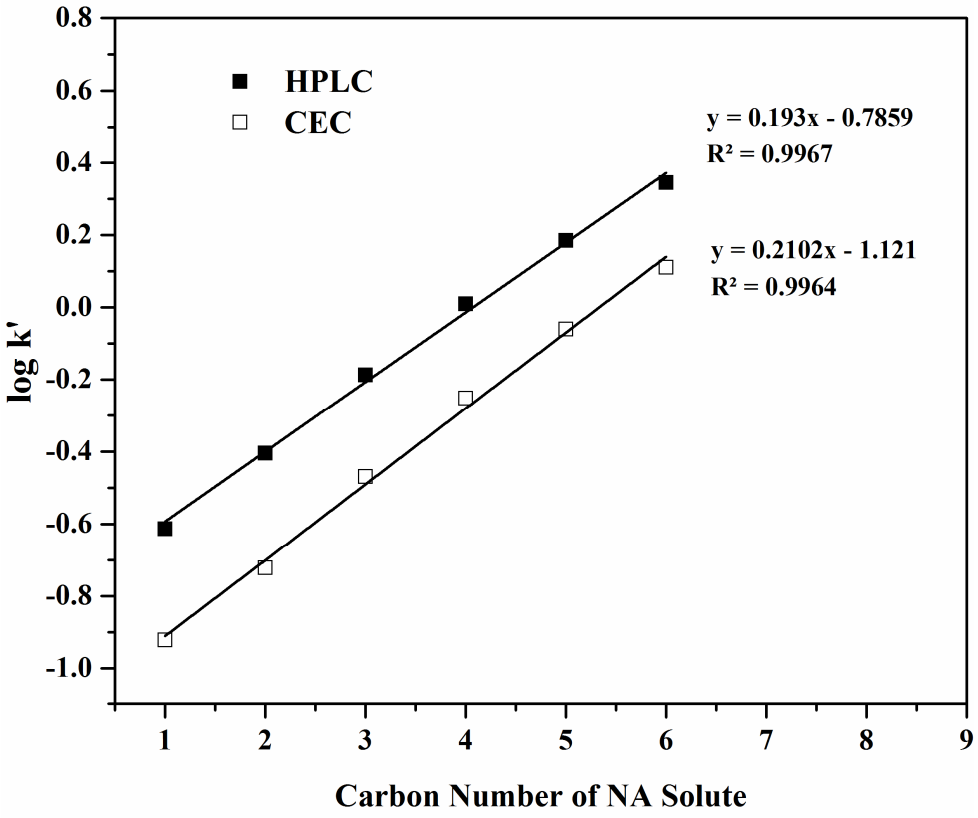


Figure 14. *Plot of log k' vs. number of carbons of NAs obtained on the Biphenyl OHM HPLC column (10 cm × 4.6 mm i.d.). Conditions: mobile phase, ACN: H2O, (35:65, v/v); flow rate, 1 mL/min and on the Biphenyl CEC capillary column;33.5 cm (effective length 25 cm) × 100 µm i.d. conditions; running voltage, 20 kV column temperature, 25 ˚C; hydro-organic mobile phase, 2 mM Na_2HPO_4 (pH 7.0) at 35% (v/v) ACN.*

Conclusions

The poly (HEMA-co-PETA) monolith was tailored into an aromatic non-polar stationary phase and was investigated in the HPLC and CEC modes. Most of the solutes including the AB homologous series, the NA homologous series, and the phenol derivatives showed the same selectivity in both separation modes but HPLC was little bit more efficient than CEC. This may be due to the higher surface area and surface coverage of the HPLC monolith with the biphenyl ligands. Phenoxy acid herbicides showed peculiar behavior for the two modes of separations. Three out of four chosen phenoxy acid herbicides resolved in CEC compared to only two in HPLC and the observed separation efficiency was also higher in CEC. This was attributed to the fact that the negatively charged phenoxy acid herbicides underwent electromigration in CEC under the influence of the applied field strength. In terms of the ease of fabrication of monoliths, HPLC was more favorable mainly while carrying out the post-polymerization modification reaction. Unlike the HPLC monoliths, the capillary monoliths in CEC were more readily blocked by the unreacted reagents.

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CHAPTER IV

GLYCEROL AND AMINE MODIFIED HYDROXYL MONOLITH FOR HYDROPHILIC INTERACTION CAPILLARY ELECTROCHROMATOGRAPHY

Introduction

The main rationale behind the introduction of hydrophilic interaction chromatography (HILIC) was to provide an alternative to reversed-phase chromatography (RPC) [1] mainly for the separation of small polar compounds [2] and biologically active compounds like proteins, peptides, nucleotides, nucleosides, pharmaceutical drugs etc. [3]. This chromatography is a branch of normal phase chromatography (NPC) in the sense that the retention increases with decreasing the polarity of the mobile phase but it is comparatively more complex in terms of separation mechanism [2].

The first generation of HILIC sorbents used amino silica bonded phases for the separation of carbohydrates. The use of diol and amide silica bonded phases proceeded

into the next generation of HILIC stationary phases. silica bonded phases, alkylamide silica bonded phases, and mixed mode phases [4-6] just to name a few. As HILIC has proceeded into the second and third generation, a wide variety of stationary phases became available nowadays and contained either neutral polar or ionic surfaces [2]. Organic polymer monoliths have always been a center of attraction in chromatography due to their well-known features such as high permeability, low mass transfer resistances, simple preparation and availability of a plethora of functional monomers [7]. Thus, it is not a staggering realization that the area of HILIC separation is also attracted to the rewards of organic polymer monoliths.

The separation of polar solutes by capillary electrochromatography (CEC), which mostly uses reversed phase sorbents, is troublesome mainly because of the necessity to use highly aqueous electrolyte solutions as the mobile phases. As it is well known, CEC already suffers from the problem of bubble formation which is further aggravated by the presence of highly aqueous mobile phases required to retain polar solutes in RP-CEC [8]. Thus, hydrophilic interaction capillary electrochromatography (HI-CEC) is a perfect match for the need of separation of very polar and moderately polar solutes. Many organic polymer cationic, anionic and zwitterionic monoliths for HI-CEC have been fabricated along with some neutral monoliths [9, 10].

Methacrylate based polymer monoliths are predominantly used for reversed phase chromatography due to reasons like presence of hydrophobic backbone and the commercial unavailability of polar monomers. However, Zhong and El Rassi reported neutral polar methacrylate-based monoliths and demonstrated the success of this class of stationary phases for use in HI-CEC mode. Glyceryl monomethacrylate (GMM) and glycidyl

methacrylate (GMA) were used as monomers for monoliths fabrication. While GMA-based monoliths were subsequently surface modified to obtain diol methacrylate monoliths, GMM-based monoliths were already in a diol form. Although naturally neutral, EOF was generated on these monoliths due to the adsorption of electrolyte ions from the mobile phase [11].

Wang *et al*. have also detailed the hydrophilicity of methacrylate-based monoliths namely using 2-hydroxyethylmethacrylate (HEMA), ethylenediamine methacrylate and methacrylic acid (MAA) co-polymerized monoliths, where MAA was included to provide fixed charges on the surface for imparting EOF [8]. The same authors reported the preparation of a neutral hydrophilic monolith made of HEMA co-polymerized with pentaerythritol triacrylate (PETA), and showed the effect of varying the composition of HEMA and PETA on the hydrophilicity of the surface [12]. Gunasena and El Rassi demonstrated the usefulness in HI-CEC of a diol monolith consisting of the co-polymerization of GMM with PETA [13], which was initially used with immobilized antibodies on its surface for subsequent use in immunoaffinity chromatography at reduced non-specific interactions [14].

Furthermore, polar monolithic columns based on polymerized glycidyl methacrylate were modified with diethylamine *via* the functionalization of their surface epoxy groups in order to obtain amino-functionalized monoliths. These monoliths were successfully used for the chromatographic separation of proteins [15].

The research described in this chapter is directed towards further exploring the potential of methacrylate-based monoliths in HI-CEC. HEMA and PETA were co-polymerized and evaluated for the hydrophilicity of the resulting monolith due to the

presence of surface hydroxyl groups. The surface was then functionalized with various polar ligands such as glycerol, ethylenediamine, diethylenetriamine and triethylenetetramine to provide the surface with more polar sites for bringing about more effective hydrophilic properties.

Experimental

Instruments

All the experiments were performed on an HP ^{3D}CE system from Hewlett-Packard (Waldbronn, Germany) equipped with a photodiode array detector. Electrochromatograms were processed with a PC running an HP ChemStation software package. Heat treatment for the polymerization initiation was performed using an Isotherm water bath (Model 105) from Fisher Scientific (Fairlawn, NJ, USA). An HPLC pump Model M-45 from Waters Associates (Milford, MA, USA) was used to condition the monolithic columns after polymerization.

Reagents and materials

Fused silica capillaries having 100 μm internal diameter (i.d.) and 375 μm outer diameter (o.d.) were purchased from Polymicro Technologies (Phoenix, AZ, USA). 2-Hydroxyethyl methacrylate (HEMA), pentaerythritol triacrylate (PETA), 3-(trimethoxysilyl) propylmethacrylate, cyclohexanol, 1-dodecanol, AIBN, acetone, dioxane, *n*-alkylbenzenes (ABs), glycerol diglycidyl ether, ethylenediamine,

diethylenetriamine and triethylenetetramine were purchased from Sigma-Aldrich (Milwaukee, WI, USA). BF_3 was purchased from TCI (Toshima, kita-ku, Tokyo, Japan). Phenol, resorcinol and pyrogallol were from Fisher Scientific (Fairlawn, NJ, USA). Acetonitrile and methanol were obtained from Pharmco-Aaper (Brookfield, CT, USA). DMF and formamide were from EM-Science (Gibbstown, NJ, USA). Uracil, cytosine, adenine, thymine and thiourea were from Sigma (St. Louis, MO, USA).

Silanization of the capillary walls

Before performing a polymerization inside the fused silica capillary column, the inner walls of this capillary (40 cm x 100 μm i.d.) were pre-treated by passing water for 2 min, then 1 M NaOH for 30 min followed by 0.1 M HCl for 30 min and rinsed with water for 30 min. Finally, the capillary walls were silanized in the presence of 50% (v/v) solution of 3-(triethoxysilyl) propylmethacrylate in acetone for 4 h at room temperature and were rinsed off with methanol for 3 min. The capillary was dried with nitrogen gas for 5 min.

In-situ polymerization

A solution of a final total weight of 0.2 g was prepared from 12.8% (w/w) HEMA as the functional monomer, 12.2% (w/w) PETA as the crosslinker together with 50% (w/w) cyclohexanol and 25% (w/w) 1-dodecanol as binary porogens. A 1% (w/w) of free radical initiator AIBN with respect to the monomer and crosslinker was added to the solution and sonicated for 10 min. The polymerization solution thus prepared appeared homogenous and was free of air bubbles. To fill the silanized fused silica capillary with the polymerization solution, one end of the 40 cm long pretreated capillary was immersed in the vial containing the solution and a vacuum was applied at the outlet end. The solution was drawn into the capillary until it reached the 30 cm demarcation line drawn on it leaving

the rest of the 10 cm void. Both ends of the capillary were sealed with GC septa and heated in a water bath at 60 ˚C for 15 h. After the polymerization was complete, the capillary was washed with acetonitrile using an HPLC pump until the porogens as well as the unreacted monomer and crosslinker were completely removed. Then, a detection window was made on the column at 1 mm below the monolith by using a thermal wire stripper. The resulting capillary column was trimmed to a total length of 33.5 cm with an effective length of 25 cm. The poly(HEMA-co-PETA) monolithic capillary column was then ready for post-polymerization modification.

Post-polymerization modification

After the *in situ* polymerization, the washed poly(HEMA-co-PETA) monolithic capillary column was equilibrated with dioxane for 2 h using a syringe pump. It was then loaded onto the HP^{3D}CE instrument for further modification. A pressure of 8 bars was applied at the capillary inlet for all the reactions. The inlet vial was filled with 5% (v/v) BF_3 in dioxane and supplied to the column for 20 min. A 5% (v/v) solution of glycerol diglycidyl ether was subsequently passed for another 20 min. This step immobilizes the glycerol diglycidyl ether on the surface via one of the epoxy groups leaving the other epoxy group available for further reaction (see reaction scheme, Figure 1).

Immobilization of glycerol.

After the glycerol diglycidyl ether was immobilized onto the monolithic surface, 5% (v/v) BF_3 in dioxane was again passed for 20 min immediately followed by a saturated solution of glycerol in dioxane for 20 min, and the cycle was repeated three more times. Between each cycle the column was rinsed with dioxane for 20 min. The glycerol-

immobilized column thus obtained was designated as a polyol column due to the presence of multiple hydroxyl groups on the surface of the monolith.

Immobilization of ethylenediamine, diethylenetriamine and triethylenetetramine

For the immobilization of the polyamines, a similar approach as that of immobilizing glycerol was implemented. The glycerol diglycidyl ether was immobilized on the monolithic surface as described above. Then, 5% (v/v) BF_3 in dioxane was supplied to the column for 20 min immediately followed by 3.08% (v/v) solution of ethylenediamine in dioxane for 20 min. The amine group of ethylenediamine forms a complex with BF_3 which then reacts with the epoxy group on the surface of the monolith provided by the immobilization of glycerol diglycidyl ether [16]. The diamine monolith was then obtained (see reaction scheme,2). This cycle was repeated three more times with 20 min of rinsing with dioxane between each cycle. Similar experimental strategy was employed for the immobilization of diethylenetriamine and triethylenetetramine using 5% (v/v) and 6.08% (v/v) solutions in dioxane, respectively (see reaction scheme, Fig. 2). The columns with surface immobilized groups were washed with acetonitrile for 1 h using an HPLC pump and stored at 2-8 ˚C until use.

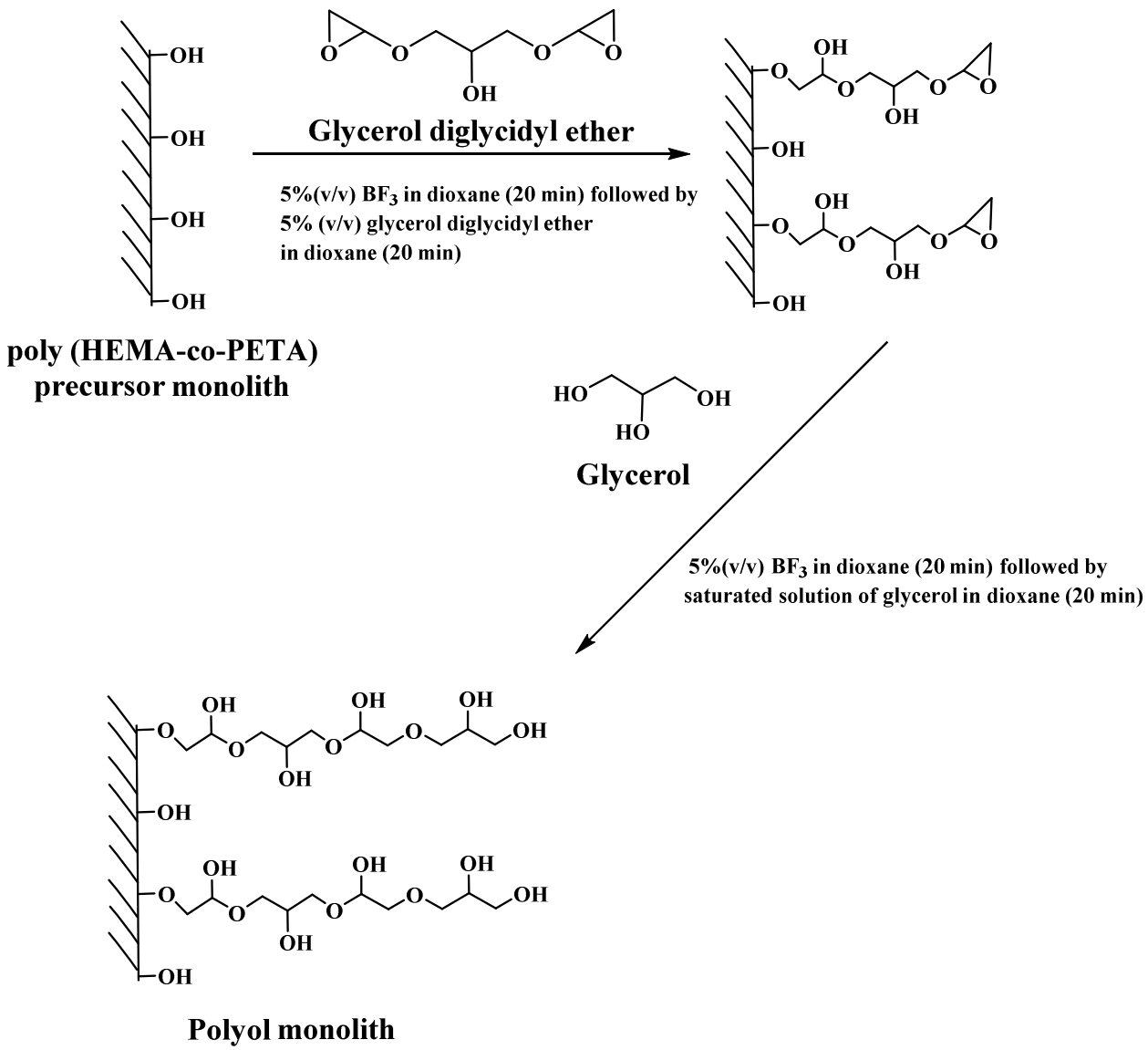

Figure 1. *Reaction scheme of post-polymerization modification of poly(HEMA-co-PETA) precursor monolith for making a polyol monolith.*

Column conditioning

Before each run, the glycerol functionalized monolithic capillary column (i.e., polyol monolithic column) and polyamine functionalized monolithic capillary column were first equilibrated with the running mobile phase using a manual micro-syringe pump. The running mobile phase consisted of 5% (v/v) of 3.5 mM ammonium acetate, pH 7.0, and 95 % (v/v) ACN. The columns were then ramped at the rate of 2 kV/min in the instrument until it reached 20 kV and equilibrated for 1 h at 25 ˚C. The solutes were injected at a pressure of 10 bars for 9 s.

Results and discussion

The poly(HEMA-co-PETA) precursor monolith possesses hydroxyl groups exposed on the surface, which impart hydrophilic properties to the OHM capillary column. To test this characteristic, some polar compounds were studied using mobile phases that promote hydrophilic interactions. The precursor monolith is neutral with hydroxyl groups on the surface but it generated a cathodal EOF due to the adsorption of mobile phase ions, which provided the surface with the negative zeta potential [17-19].

Effect of solvent on post-polymerization modification of the precursor monolith

For the post-polymerization modification of poly(HEMA-co-PETA) monolith with glycerol, a suitable solvent needed to be selected first. Dioxane and DMF were chosen since these two solvents were aprotic and would not interfere with the post-polymerization

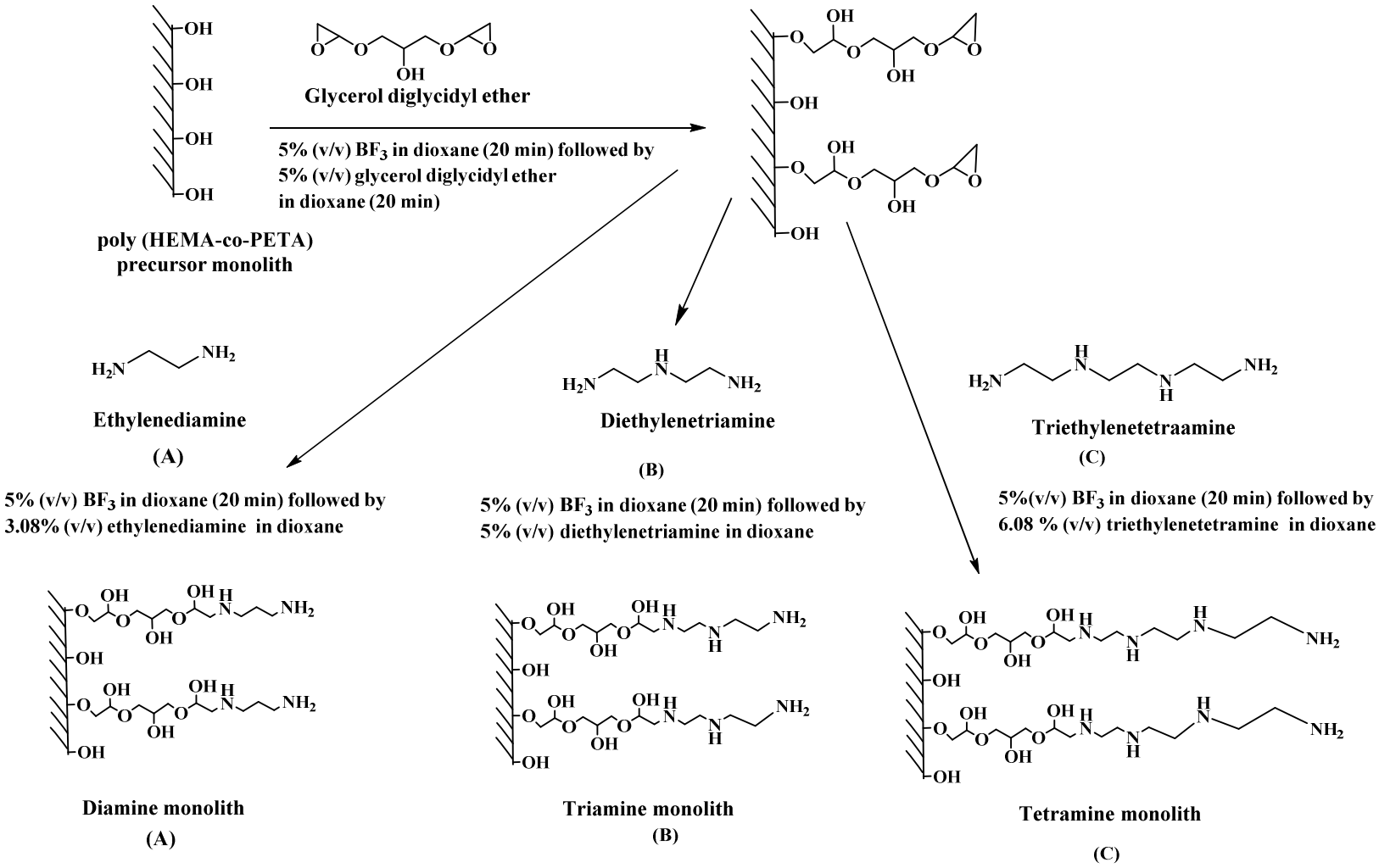

Figure 2. *Reaction scheme of post-polymerization modification of poly(HEMA-co-PETA) precursor monolith for making polyamine bonded monoliths.*

modification reaction. The same experimental conditions were employed for the reaction using these two different solvents. The polyol monolith obtained from the modification in the presence of DMF as the solvent was compared to the precursor monolith using some polar compounds, namely DMF, formamide and thiourea to evaluate the extent of success for using DMF, and the results are shown in Figure 3. As can be seen in the figure, the elution order of the three polar test solutes on the precursor monolithic column was predictable in the sense that DMF being the least polar exhibited less interaction with the polar surface and eluted first followed by formamide and thiourea according to the solute polarity order. When DMF was used as the modification solvent, the retention time of the three polar solutes decreased when compared to the retention time on the precursor column, compare Figure 3B to Figure 3A.

The experiment performed with dioxane as the modification solvent was carried out in the same manner, and the resulting polyol column was compared to the precursor monolithic column using the same polar test solutes, see Figure 4. As can be seen in this figure, the retention time of the 3 test solutes increased significantly when compared to the retention time obtained on the precursor monolithic column (compare Figure 4B to Figure 4A). It should be noted that the magnitude of the retention time also reflects the velocity of the mobile phase, which is controlled by the EOF. In other words, the difference in retention times may not reflect commensurate difference in k´ values as shown in Table 1. The k´ values of the above 3 polar solutes (i.e., DMF, formamide and thiourea) obtained on the polyol columns using DMF and dioxane as the modification solvents in comparison to the k´ values obtained on the precursor monolithic column, under otherwise the same elution conditions is listed in Table 1.

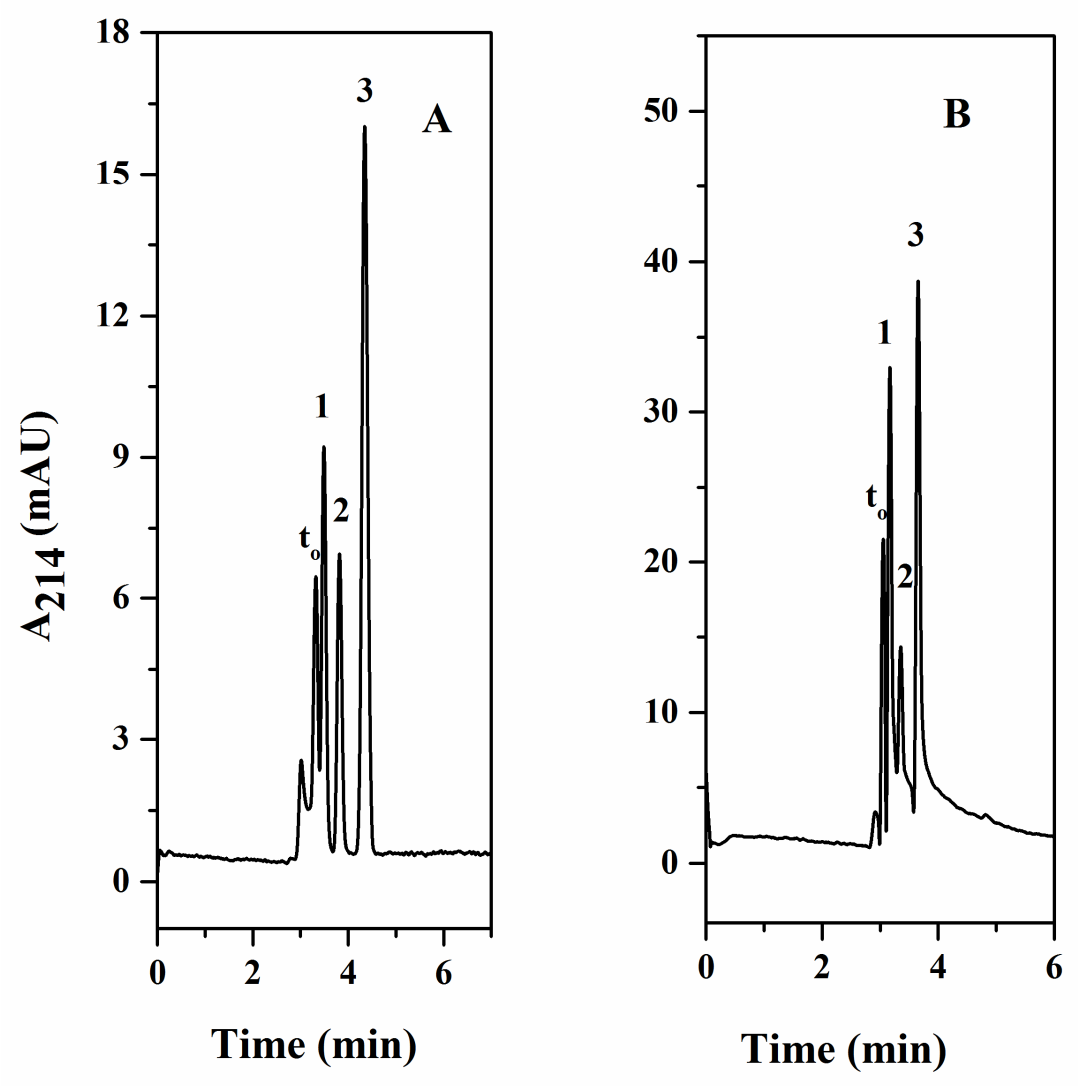

Figure 3. Electrochromatograms of some polar solutes obtained on the precursor OHM capillary column in (A) and on the polyol monolith capillary column modified in the presence of DMF (B).Conditions: 33.5 cm (effective length 25 cm) × 100 µm i.d.; running voltage, 20 kV; column temperature, 25 ˚C; hydro-organic mobile phase, 5% (v/v) of 3.5 mM NH_4Ac, pH 7.0, 95% (v/v) ACN; solutes: 1, DMF; 2, formamide; 3, thiourea; EOF tracer, toluene.

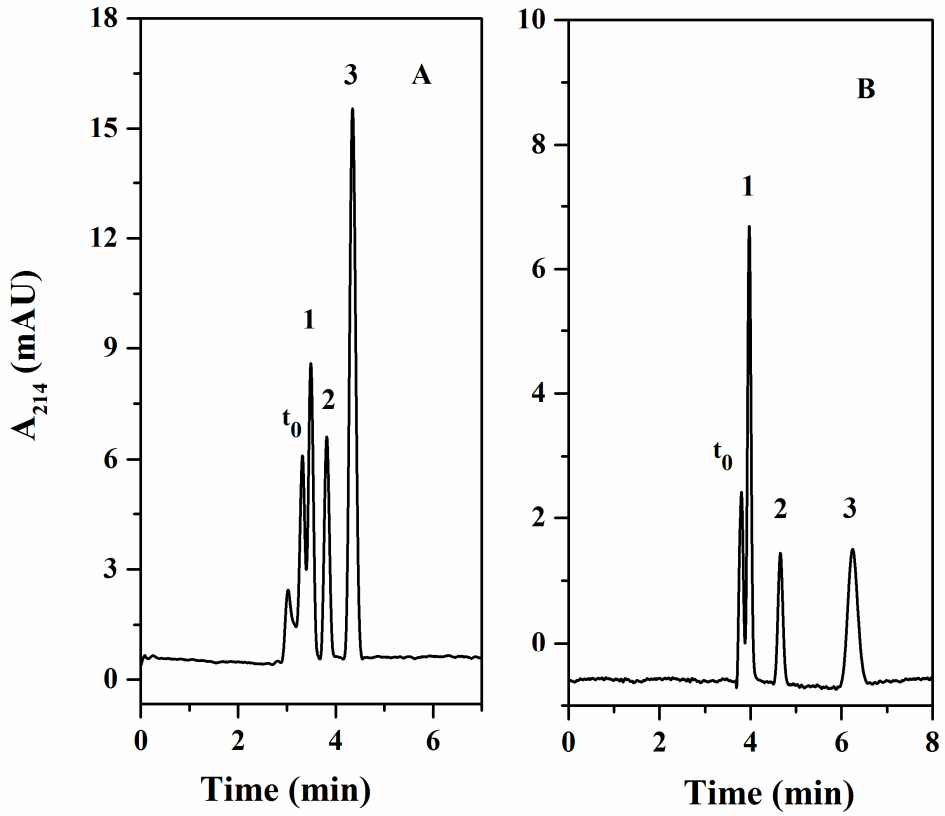

Figure 4. *Electrochromatograms of some polar solutes obtained on the precursor OHM capillary column in (A) and on the polyol OHM capillary column modified in the presence of dioxane in (B).Conditions: 33.5 cm (effective length 25 cm) × 100 µm i.d.; running voltage, 20 kV; column temperature, 25 ˚C; hydro-organic mobile phase, 5% (v/v)of 3.5 mM NH_4Ac, pH 7.0, 95% (v/v) ACN; solutes: 1, DMF; 2, formamide; 3, thiourea; EOF tracer, toluene.*

TABLE 1

EFFECT OF THE NATURE OF MODIFICAITON SOLVENT ON THE k' AND α VALUES OF SOME POLAR SOLUTES

Polar solutes	Polyol monolith prepared in Precursor OHM		Polyol monolith prepared in DMF		Polyol monolith prepared in dioxane	
	k'	α	k'	α	k'	α
DMF	0.052		0.035		0.053	
		2.88		2.80		4.55
Formamide	0.150		0.098		0.241	
		2.07		2.02		2.79
Thiourea	0.310		0.198		0.672	

Conditions:33.5 cm (effective length 25 cm) × 100 μm i.d.; running voltage, 20 kV; column temperature, 25 ˚C; hydro-organic mobile phase, 5% (v/v) of 3.5 mM NH_4Ac, pH 7.0 , 95% (v/v) ACN; solutes: 1, DMF; 2, formamide; 3, thiourea; EOF tracer, toluene.

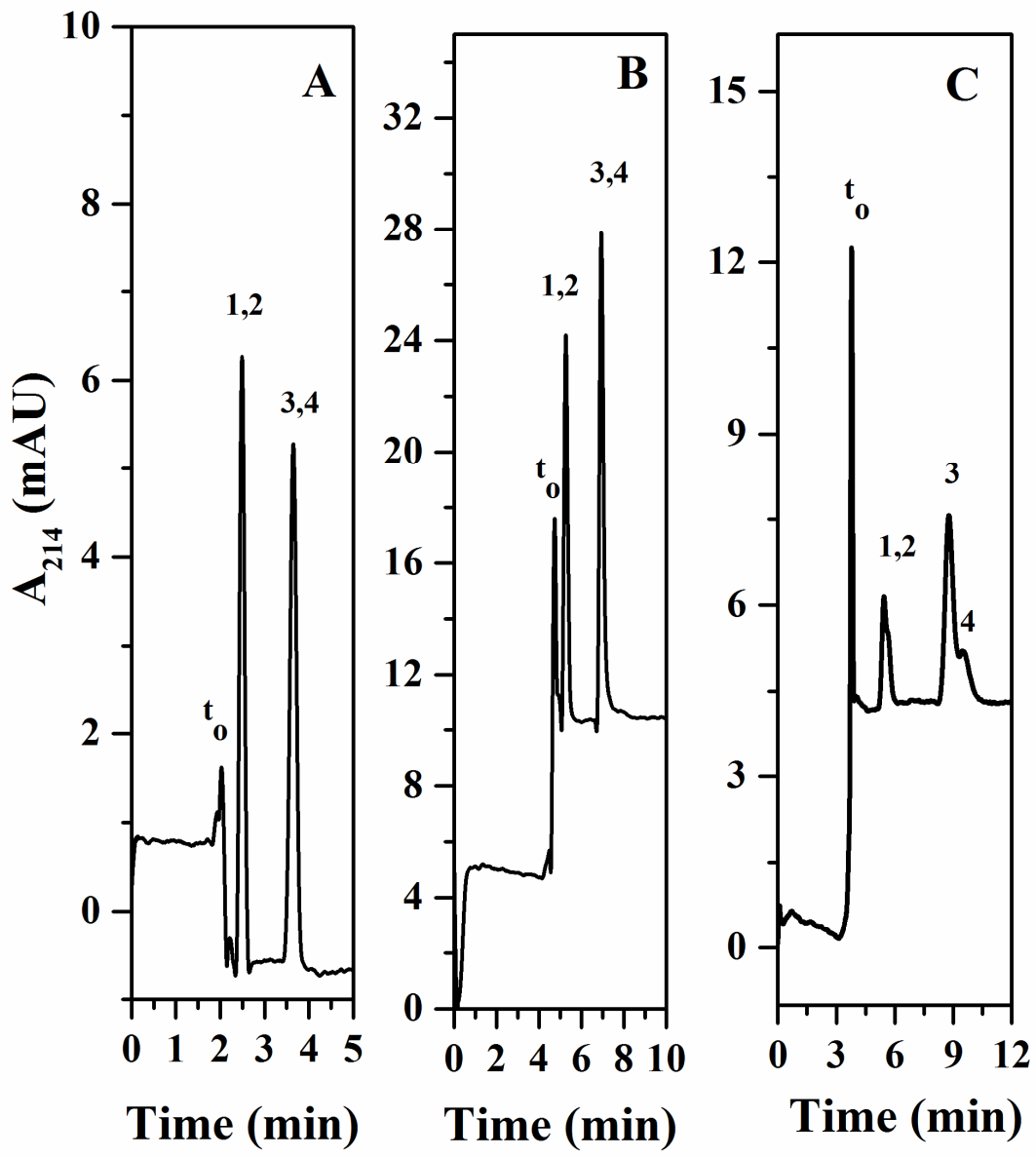

Figure 5. *Electrochromatograms of nucleic acid bases obtained on the precursor OHM capillary column in (A) and on the polyol monolith capillary column modified in the presence of DMF in (B) and on the polyol monolith capillary column modified in the presence of dioxane in (C). Conditions: 33.5 cm (effective length 25 cm) × 100 µm i.d.; running voltage, 20 kV; column temperature, 25 ˚C; hydro-organic mobile phase, 5% (v/v) of 3.5 mM NH_4Ac, pH 7.0, 95% (v/v) ACN; solutes 1, thymine; 2,uracil; 3,cytosine; 4, adenine; EOF tracer, toluene.*

On the polyol monolithic column produced in the presence of dioxane, the k' values for DMF, formamide and thiourea went up to 0.053, 0.241 and 0.672 respectively. In contrast, the k´ values decreased and went down to 0.035, 0.098 and 0.198 for DMF, formamide and thiourea, respectively, on the polyol monolith obtained using DMF as the modification solvent. Furthermore, not only the retention times and k´ decreased on the polyol column prepared in the presence of DMF as solvent, but also the values of the selectivity factors, see Table 1. On the other hand, the column prepared in dioxane yielded almost the same selectivity factors as those obtained on the precursor column

Retention of nucleic acid bases

In addition to the above three polar solutes, several nucleic acid bases were also used to evaluate the effect of different solvents on the post-polymerization modification of the precursor monolithic column. Nucleic acid bases namely thymine, uracil, adenine and cytosine were tested under hydrophilic interaction conditions similar to those used with the above three polar solutes. Thymine and uracil co-eluted and so did adenine and cytosine with a k' of 0.206 and 0.626, respectively. Then, the precursor modified in the presence of DMF solvent was evaluated and it was observed that thymine/uracil and adenine/cytosine still co-eluted but their k' values decreased to 0.128 and 0.471, respectively (see Table 2). Further, the precursor monolith modified in the presence of dioxane was evaluated with these nucleic acid bases. There was some improvement in the polarity of the resulting polyol column as can be seen in Fig. 5. The k´ values of thymine/uracil increased to 0.433 and that of adenine increased to 1.323 with cytosine shouldering at k´ = 1.530.

Currently, there are not enough data to provide a clear explanation on why the polarity of the “polyol” column decreased when using DMF as the modification solvent,

as manifested by the decrease in solute retention. From the above results, dioxane was selected as the best solvent for the post-polymerization modification of the precursor column with glycerol to yield a polyol monolith.

Retention of phenolic compounds

Some phenolic compounds including phenol, resorcinol and pyrogallol were tested under hydrophilic interaction conditions on the precursor monolithic column and on the polyol monolithic column prepared in dioxane. The results are illustrated in the electrochromatograms in Figure 6. The hydrophilicity of the precursor monolith was further confirmed with the phenolic compounds being retained and eluted in the order of increasing number of hydroxyl groups of the solutes. The k' values listed in Table 3 show that phenol is retained the least, which is logical since it has one hydroxyl group as compared to 2 and 3 hydroxyl groups in resorcinol and pyrogallol molecules. The k' value for phenol on the precursor monolith was 0.044 and increased to 0.145 on the polyol monolith, resorcinol increased from 0.099 to 0.401, and similarly pyrogallol increased from 0.154 to 0.626. However, the selectivity values obtained on all the columns were comparable.

Scavenging the unreacted epoxy groups

Glycerol diglycidyl ether is used as a bi-functional anchor in the post-polymerization modification of the precursor monolith. There may be some unreacted epoxy groups on the surface of the monolith, which could possibly reduce the hydrophilicity of the resulting polyol monolith. So, the polyol monolith was further

TABLE 2

COMPARISON OF k′ AND α VALUES OF NUCLEIC ACID BASES OBTAINED IN PRECURSOR OHM MONOLITH POLYOL MONOLITHS MODIFIED IN DIFFERENT SOLVENTS

Polar solutes	Precursor OHM		Polyol monolith prepared in DMF		Polyol monolith prepared in dioxane	
	k'	α	k'	α	k'	α
Thymine	0.206		0.128		0.433	
		1.00		1.00		1.11
Uracil	0.206		0.128		0.481	
		3.04		3.68		2.75
Cytosine	0.626		0.471		1.323	
		1.00		1.00		1.16
Adenine	0.626		0.471		1.530	

Conditions:33.5 cm (effective length 25 cm) × 100 μm i.d.; running voltage, 20 kV; column temperature, 25 ˚C; hydro-organic mobile phase, 5%(v/v) 3.5 mM NH_4Ac, pH 7.0 , 95% (v/v) ACN; solutes:1 , thymine; 2,uracil; 3,cytosine; 4, adenine.

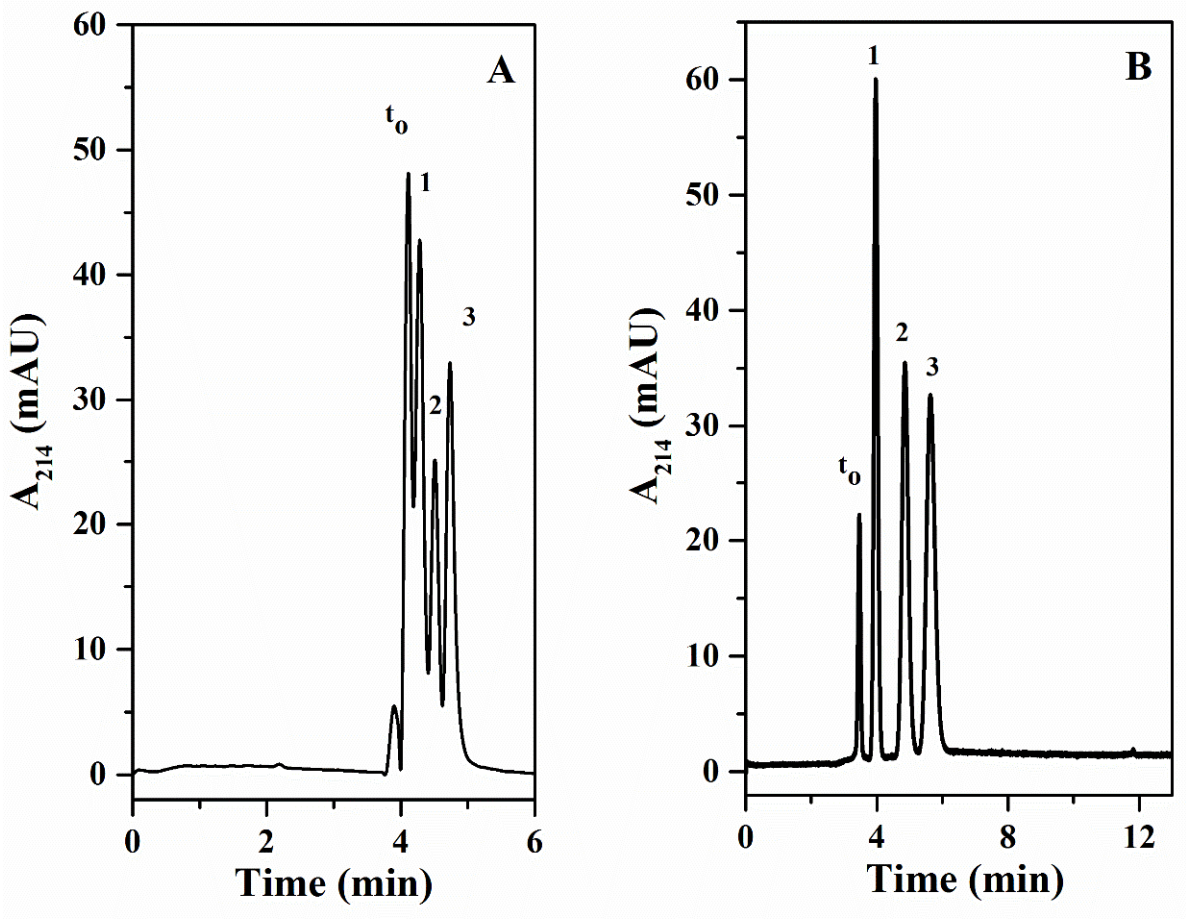

Figure 6. *Electrochromatograms of some phenols obtained on the precursor OHM capillary column in (A) and on the polyol monolith capillary column modified in dioxane (B).Conditions: 33.5 cm (effective length 25 cm) × 100 µm i.d.; running voltage, 20 kV; column temperature, 25 ˚C; hydro-organic mobile phase, 5%(v/v) of 3.5 mM NH_4Ac, pH 7.0, 95% (v/v) ACN; solutes: 1, phenol; 2, resorcinol; 3, pyrogallol; EOF tracer, toluene.*

TABLE 3

k' AND α VALUES OF SOME PHENOLIC COMPOUNDS OBTAINED ON THE PRECURSOR OHM AND THE POLYOL MONOLITHS

Polar solutes	Precursor OHM		Polyol monolith modified in DMF		Polyol monolith modified in dioxane	
	k'	α	k'	α	k'	α
Phenol	0.044	2.25	0.040	2.30	0.145	2.77
Resorcinol	0.099	1.56	0.092	1.57	0.401	1.56
Pyrogallol	0.154		0.144		0.626	

Conditions: 33.5 cm (effective length 25 cm) × 100 μm i.d.; running voltage, 20 kV; column temperature, 25 ˚C; hydro-organic mobile phase, 5% (v/v) of 3.5 mM NH_4Ac, pH 7.0, 95% (v/v) ACN.

reacted with sulfuric acid to open the epoxy rings of the unreacted glycerol glycidyl ether on the surface of the monolith thereby providing more hydroxyl groups and consequently increasing the polarity of the polyol monolith. The scavenging was carried out by circulating 0.1 M H_2SO_4 for 2 h using the $HP^{3D}CE$ instrument under similar parameters of temperature as the post-polymerization modification reaction. For comparison purposes, the scavenged monoliths were evaluated with the same polar solutes as those used in evaluating the polyol monolith.

Table 4 shows the effect of scavenging on the k' and α values of some polar test solutes. The effects of scavenging varied among the various solutes. The k' values of DMF and formamide slightly increased but decreased for thiourea. But, the selectivity values decreased slightly after scavenging. Similarly, in the case of phenolic compounds, the k´ values of the first two eluting compounds phenol and resorcinol increased slightly and then decreased for pyrogallol. In this case also, the selectivity decreased slightly after scavenging. On the contrary, the k' values for the nucleic acid bases decreased after scavenging. The selectivity value between thymine and uracil increased slightly but decreased for the rest.

From the above results, it can be concluded that hydrolyzing the unreacted epoxy rings by sulfuric acid treatment may have resulted in removing some the polyol functions on the surface of the polyol monolithic column as reflected by decreasing the polar character of the column. Thus, the removal of the presumably unreacted epoxy groups is not worth attempting because it has an adverse effect on the polar property of the polyol monolithic column.

TABLE 4

EFFECT OF SCAVENGING ON k' AND α VALUES OF VARIOUS SOLUTES OBTAINED ON THE POLYOL MONOLITH COLUMN

Solutes	unscavanged polyol monolith		scavanged polyol monolith	
	k'	α	k'	α
DMF	0.053		0.062	
		4.55		3.98
Formamide	0.241		0.247	
		2.79		2.55
Thiourea	0.672		0.631	
Phenol	0.145		0.161	
		2.77		2.51
Resorcinol	0.401		0.404	
		1.56		1.51
Pyrogallol	0.626		0.611	
Thymine	0.433		0.233	
		1.11		1.80
Uracil	0.481		0.420	
		2.75		2.41
Cytosine	1.32		1.01	
		1.16		1.22
Adenine	1.53		1.24	

Conditions:33.5 cm (effective length 25 cm) × 100 µm i.d.; running voltage, 20 kV; column temperature 25 ˚C; hydro-organic mobile phase,5% (v/v) of 3.5 mM NH_4Ac, pH 7.0 , 95% (v/v) ACN.

Polyamine monolithic columns

Utilizing the hydroxyl groups on the surface of the precursor monolith as the reactive sites was further exploited, and a series of polyamine modified monoliths was prepared. Ethylenediamine, diethylenetriamine and triethylenetetramine were covalently attached to the surface of the precursor column to generate polyamine monolithic columns with two, three and four amine groups on the surface of the monolith. In this post-polymerization modification process, the surface hydroxyl groups of the precursor monolith were first reacted with the bi-functional glycerol diglycidyl ether to provide an epoxy group available for linking with the amine group of the ethylenediamine, diethylenetriamine and triethylenetetramine.

CEC parameters

The diamine OHM and triamine OHM columns exhibited cathodal EOF and these columns were tested under HILIC conditions using mobile phase with 5% (v/v) of 3.5 mM NH_4Ac (pH 7.0) and 95% (v/v) ACN. Other instrumental parameters included 25 ˚C capillary column temperature, 20 kV as the running voltage under 5 bars pressure applied on both column ends and an injection voltage of 20 kV for 9 s. In contrary, the tetramine OHM exhibited anodal EOF and was tested under HILIC conditions using a mobile phase consisting of 5% (v/v) of 3.5 mM NH_4Ac (pH 7.0) and 95% (v/v) ACN. The running voltage was set at -20 kV, 5 bars pressure was applied on both column ends and the injection voltage was effected at -20 kV for 9s.

Retention of polar solutes

Similar to the polyol columns, DMF, formamide, thiourea, phenol, resorcinol and pyrogallol were first used to test the polarity of the polyamine columns. From Table 5, it can be seen that on the diamine OHM column, DMF and formamide co-eluted with a k' of 0.290 and thiourea was retained longer with a k' of 0.936. Similarly, on the triamine OHM column, DMF and formamide co-eluted with a k' of 0.279 and thiourea with a k' of 1.12. Phenol, resorcinol and pyrogallol were retained with a k' of 0.221, 0.615 and 0.980, respectively, on the diamine column and with k´ value of 0.235, 0.823 and 2.17, respectively, on the triamine column. On the tetramine OHM column, the k' values for phenol, resorcinol and pyrogallol decreased to 0.155, 0.432 and 0.833, respectively. The selectivity values of these solutes are higher on the triamine OHM column as compared to the diamine OHM column. But, the selectivity values decreased in the case of the tetramine OHM column for all the solutes under investigation.

Retention of nucleic acid bases

The retention of the nucleic acid bases were also evaluated on the diamine OHM column in order to assess the extent of solute-stationary phase association. It was observed that the retention was in the order of thymine < uracil < cytosine < adenine. It can be said that the solutes possessing primary amine groups (i.e., cytosine and adenine) eluted after the solutes with secondary amines (i.e., thymine and uracil). Among the solutes with secondary amines, thymine eluted before uracil which shows that the presence of the methyl group in thymine may have made it elute first due to its relative non-polarity.

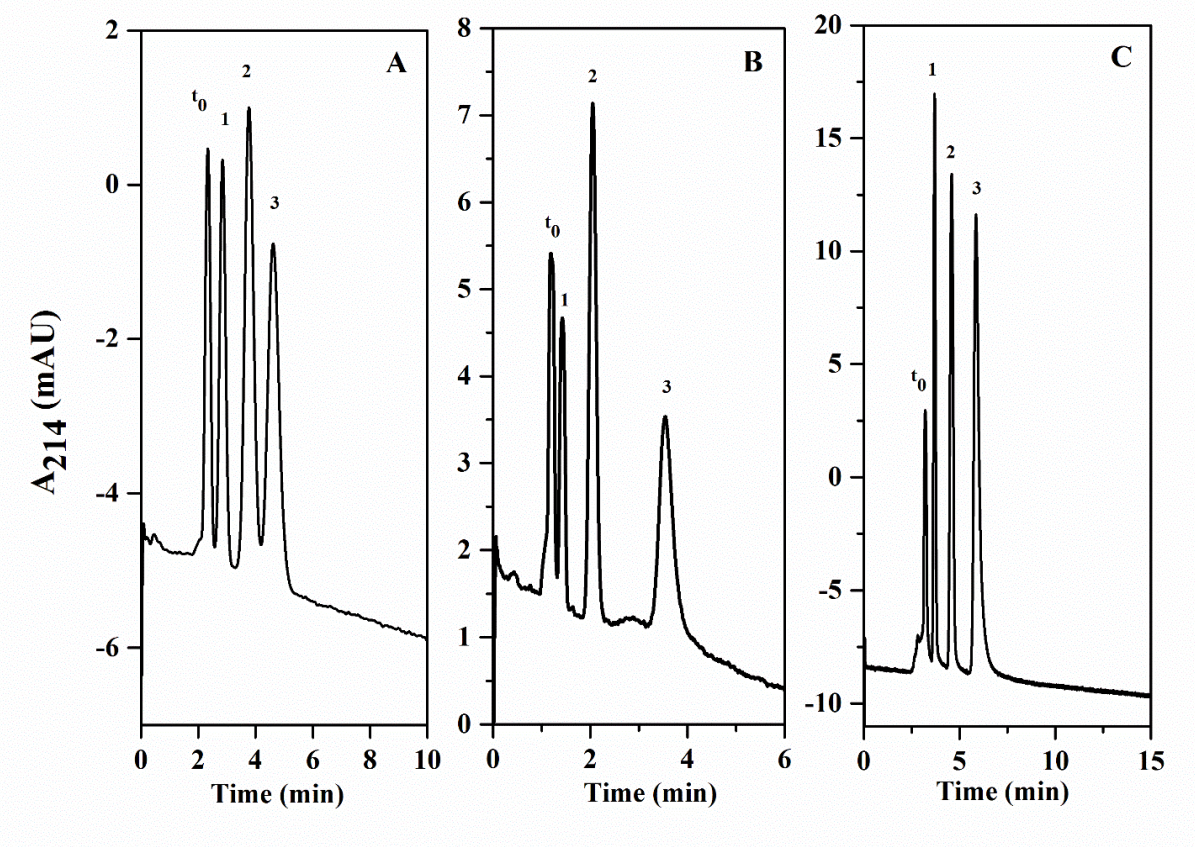

Figure 7. *Electrochromatograms of phenols obtained on the diamine monolithic capillary column in (A); triamine monolithic capillary column in (B) and tetramine monolithic capillary column in (C).Conditions: 33.5 cm (effective length 25 cm) × 100 μm i.d.; running voltage, 20 kV; column temperature, 25 ˚C; hydro-organic mobile phase, 5% (v/v) of 3.5 mM NH_4Ac, pH 7.0, 95% (v/v) ACN; running voltage, -20 kV; solutes: 1, phenol; 2, resorcinol; 3, pyrogallol; EOF tracer: toluene.*

TABLE 5

k' AND α VALUES OF SOME POLAR SOLUTES OBTAINED ON VARIOUS AMINE MONOLITH COLUMNS

Polar solutes	Diamine OHM		Triamine OHM		Tetramine OHM	
	k'	α	k'	α	k'	α
Phenol	0.221		0.195		0.155	
		2.78		3.69		2.79
Resorcinol	0.615		0.720		0.432	
		1.59		2.74		1.93
Pyrogallol	0.98		1.97		0.833	
DMF	0.29		0.279		0.189	
		1.00		1.00		1.00
Formamide	0.29		0.279		0.189	
		3.23		4.01		3.12
Thiourea	0.936		1.12		0.590	

Conditions: 33.5 cm (effective length 25 cm) × 100 μm i.d.; for diamine OHM and triamine OHM, running voltage, 20 kV; for tetramine OHM column, running voltage, -20 kV; temperature, 25 °C; hydro-organic mobile phase, 5% (v/v) of 3.5 mM NH_4Ac, pH 7.0, 95% (v/v) ACN.

When comparing the diamine and triamine columns, the diamine column was not able to resolve thymine and uracil. These two solutes shouldered on the triamine column. However, on both the diamine and triamine columns, some resolution was obtained between cytosine and adenine. Overall, the tetramine column yielded higher resolution for all the nucleic acid bases under investigation. These results corroborate well with the prediction that increasing the number of functional amine groups on the polyamine column should provide an increased selectivity for polar solutes.

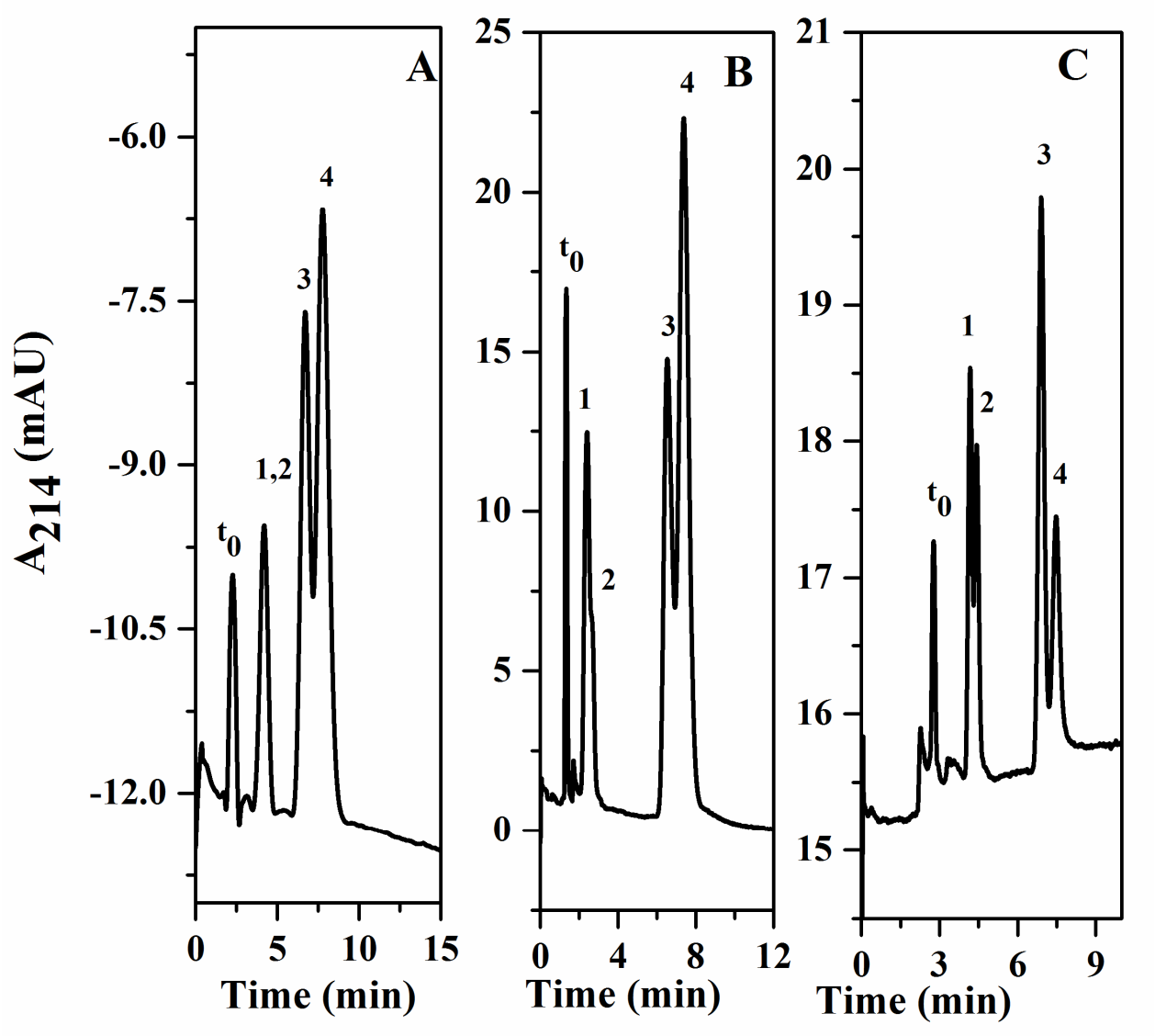

Figure 8. *Electrochromatograms of nucleic acid bases obtained on diamine monolithic capillary column in (A); triamine monolithic capillary column in (B); tetramine monolithic capillary column in (C). Conditions: 33.5 cm (effective length 25 cm) × 100 µm i.d.;running voltage, 20 kV; column temperature 25 ˚C; hydro-organic mobile phase, 5% (v/v) of 3.5mM NH_4Ac, pH 7.0, 95% (v/v) ACN; C) running voltage, -20 kV; solutes: 1, thymine; 2,uracil, 3; cytosine; 4, adenine; EOF tracer: toluene.*

TABLE 6

k' AND α VALUES OF NUCLEIC ACID BASES OBTAINED ON VARIOUS AMINE MONOLITH COLUMNS

Polar solutes	Diamine OHM		Triamine OHM		Tetramine OHM	
	k'	α	k'	α	k'	α
Thymine	0.851		0.827		0.454	
		1.00		1.00		1.22
Uracil	0.851		0.827		0.552	
		2.30		4.80		2.56
Cytosine	1.96		3.97		1.412	
		1.23		1.16		1.15
Adenine	2.42		4.62		1.62	

Conditions: 33.5 cm (effective length 25 cm) × 100 μm i.d.; for diamine OHM and triamine OHM, running voltage, 20 kV; for tetramine OHM column, running voltage, -20 kV; temperature, 25 °C; hydro-organic mobile phase, 5% (v/v) of 3.5 mM NH_4Ac, pH 7.0, 95% (v/v) ACN.

Conclusions

Similar reaction strategies were used for making the polyol and polyamine columns. The polyol column was compared to the precursor OHM monolith and it was confirmed that it exhibited increased polar properties, which was due to the addition of more hydroxyl groups to the surface of the OHM monolith *via* post-polymerization modification reaction with glycerol. However, the polyol column was not able to adequately resolve the nucleic acid bases. The polyamine columns were also made using a modification process similar to that used for the polyol columns and their polarity was assessed with the same polar solutes. Overall, the polyamine columns yielded better resolution and separation for nucleic acid bases than the polyol and the tetramine column was even better than the diamine and triamine columns. Diamine and triamine columns possessed cathodal EOF while the tetramine column exhibited an anodal EOF.

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CHAPTER V

HYDROXYPROPYL-β-CYCLODEXTRIN POST-MODIFIED CAPILLARY MONOLITHIC COLUMN FOR CHIRAL CAPILLARY ELECTROCHROMATOGRAPHY

Introduction

It has been known for a long time that stereochemistry governs the chemical or biological activity of a given chiral substance. In chromatographic terms, chiral recognition refers to the interaction of an enantiomer of a substance preferably with an enantiomer of another substance. When such an interaction occurs on the surface of a chiral stationary phase (CSP) a diastereomeric complex between enantiomer of an analyte forms with a chiral selector on the surface of CSP [1]. Structurally, there are various classes of CSPs, few examples being macrocyclic, polymeric, π-π association and ligand exchange. Cyclodextrin (CD) falls under the macrocyclic class of CSP and is the most used in enantiomeric separations [2]. CDs are naturally occurring cyclic oligosaccharides and are composed of several glucose units linked together by an α-1,4 linkage to form a truncated cone like shape ranging between 5-8 Å in diameter.

This forms inclusion complex with smaller molecules that would fit inside this cavity [5, 6]. Generally, they are of 6, 7 and 8 glucosyl units and are designated as α-CD, β-CD and γ-CD, respectively [3, 4]. These CDs have a hydrophilic surface and hydrophobic cavity Several CD derivatives have been immobilized on the solid support through covalent amine, urethane, amide and ether linkages, which are some of the early examples of CSPs [7-9]. Due to the shortcomings of these CSPs such as structural instability, structurally well-defined CD-CSPs were developed by the immobilization of permethylated, peracetylated and perphenylcarbamated azido-CDs onto aminated silica [6].

Although, these CD-CSPs were mostly packed bed columns, the use of silica monoliths and organic polymer monoliths have been increased to avoid problems like difficulty in column packing, high back pressure and low flow rates [10]. It is also notable that the functionalization of particles with CDs can be a tedious process, which can be avoided by the use of a functional monomer in fabricating the monoliths. CD and their derivatives have been utilized as chiral selectors in various chromatographic techniques like gas chromatography, high performance liquid chromatography (HPLC), capillary electrophoresis (CE), capillary electrochromatography (CEC) and supercritical fluid chromatography [11].

Chiral separation in capillary mode is attractive for reasons like use of small amounts of expensive chiral selectors and solvents and use of small sample volumes. CEC in particular has an added advantage of high efficiency and selectivity due to its technological overlap between CE and HPLC [12]. CEC also uses a variety of supporting materials as CSPs including polyarcylamides, polymethacrylates, silica-based monoliths and particle fixed materials [13]. The fabrication of silica-based monoliths is tedious due

to the tremendous amount of time and effort required to generate chiral capillary columns compared to organic polymer monoliths. Various silica based monoliths have been reported, which were post-modified using various chiral selectors [14].

In one example, Zhang and El Rassi reported the *in situ* conversion of a commercially available diol-silica microparticles packed in fused silica capillary by dynamically coating the microparticles with hydroxypropyl-β-cyclodextrin (HP-β-CD) and proved useful in the separation of organochlorine pesticides and dansyl amino acids by CEC [15]. The separation of these neutral and anionic enantiomers was possible due to the inclusion of HP-β-CD in the mobile phase, which allowed the rapid transport of the anionic solutes across the capillary at relatively low EOF. In another work, they described silica microparticles with a surface bound sulfonated sublayer, which was covalently bonded with HP-β-CD and packed in fused silica capillary columns. The chiral capillary column thus obtained was also able to separate the anionic dansyl amino acids and phenoxy acid herbicides by CEC [16]. Epoxy activated polymethacrylate monoliths, e.g., poly(glycidyl methacrylate-co-ethylene dimethacrylate) monoliths, are more versatile stationary phases as they provide an opportunity to carry out a single-step polymerization using functional monomers and subsequently an easy functionalization step of the epoxy activated monolith can be performed using chiral selectors like CD or CD derivatives [17].

In addition to chiral separation, CDs are versatile tools in separation and have been shown to discriminate between positional isomers and functional groups as well by a variety of separation techniques including capillary gel electrophoresis, capillary isotachophoresis, capillary isoelectric focusing as well as by preparative scale electrophoretic techniques and thin layer chromatography just to name a few [18]. There

are reports of CDs used successfully in the separation of nucleobases and nucleosides [19], dichlorobenzene, dinitrotoluene and positional isomers of cresol [20, 21].

There are numerous examples of CD-CSP fabrication by post modification of the surface functional groups. Li *et al.* have reported the use of hydroxyl functional groups present on the surface of poly(glycidyl methacrylate-co-ethylene dimethacrylate) as reactive sites for chemically grafting them with β-CD and its various derivatives including HP β-CD. They were able to achieve the separation of several racemic drugs and amino acids by CEC [17].

In this research, the versatility of polymethacrylate monoliths was further investigated. 2-Hydroxyethyl methacrylate was co-polymerized with pentaerythritol triacrylate to obtain poly(HEMA-co-PETA) monolith and the versatility of this monolith was further explored by functionalizing the surface with HP-β-CD through the hydroxyl group present on the surface of the precursor monolith. The resulting HP-β-CD OHM column was tested with chiral compounds like DL-phenylalanine, warfarin and hexobarbital and some positional isomers.

Experimental

Instruments

All the experiments were performed on an HP ^{3D}CE system from Hewlett-Packard (Waldbronn, Germany) equipped with a photodiode array detector. Electrochromatograms were processed with a PC running an HP ChemStation software package. Heat treatment

for the polymerization initiation was performed using an Isotherm water bath (Model 105) from Fisher Scientific (Fairlawn, NJ, USA). An HPLC pump Model M-45 from Waters Associates (Milford, MA, USA) was used to condition the monolithic columns after polymerization.

Reagents and materials

Fused silica capillaries having 100 μm internal diameter (i.d.) and 375 μm outer diameter (o.d.) were purchased from Polymicro Technologies (Phoenix, AZ, USA). 2-Hydroxyethyl acrylate (HEMA), pentaerythritol triacrylate (PETA), 3-(trimethoxysilyl) propylmethacrylate, cyclohexanol, 1-dodecanol, azobis(isobutyronitrile) (AIBN), acetone, dioxane, glycerol diglycidyl ether, DL-phenylanaline, warfarin, hexobarbital, 2-chloroaniline and 4-chloroaniline were from Sigma-Aldrich (Milwaukee, WI, USA). BF_3 was purchased from TCI (Toshima, kita-ku, Tokyo, Japan). Acetonitrile (ACN) and methanol were obtained from Pharmco- Aaper (Brookfield, CT, USA).

Silanization of the capillary inner walls

Before polymerization, the inner wall of a 40 cm long, 100 μm i.d. fused silica capillary was pre-treated by passing water for 2 min, then 1 M NaOH for 30 min followed by 0.1 M HCl for 30 min and with water for 30 min. Finally, the capillary inner walls were silanized in the presence of 50% (v/v) solution of 3-(triethoxysilyl) propylmethacrylate in acetone for 4 h at room temperature and were rinsed with methanol for 30 min. The capillary was dried with nitrogen gas for 5 min.

In-situ polymerization

A solution of a final total weight of 0.2 g was prepared from the following: 12.8% (w/w) HEMA as the monomer, 12.2% (w/w) PETA as the crosslinker together with 50% (w/w) cyclohexanol and 25% (w/w) 1-dodecanol as the binary porogens. A 1% (w/w) of free radical initiator AIBN with respect to the monomer and crosslinker was added to the solution and the mixture was sonicated for 10 min. The polymerization solution thus prepared appeared homogenous and free of air bubbles. To fill the silanized capillary with the polymerization solution, one end of the 40 cm long pretreated capillary was immersed in the vial containing the solution and a vacuum was applied to the outlet. The solution was drawn into the capillary until it reached the 30 cm demarcation drawn on it leaving the rest of the 10 cm void. Both ends of the capillary were sealed with GC septa and immersed in a water bath at 60^{o}C for 15 h. After the polymerization was complete, the capillary was washed with acetonitrile using an HPLC pump until the porogens as well as the unreacted monomer and crosslinker were completely removed. A detection window was then made on the column 1 mm below the monolith by using a thermal wire stripper. The resulting capillary column was trimmed to a total length of 33.5 cm with an effective length of 25 cm. The poly (HEMA-co-PETA) monolithic capillary column was then ready for post-polymerization modification.

Post-polymerization modification

After the *in situ* polymerization, the washed poly (HEMA-co-PETA) monolithic capillary column was equilibrated with dioxane for 2 h using a syringe pump. Then, it was loaded onto the HP^{3D}CE instrument for further modification. A pressure of 8 bars was

applied on the inlet for all the reactions. The inlet vial was filled with 5% (v/v) solution of BF_3 in dioxane and supplied to the column for 20 min. Then, a 5% (v/v) solution of glycerol diglycidylether was passed for another 20 min. This step immobilizes the glycerol diglycidylether on the monolithic surface through an epoxy group, leaving the other epoxy group available for further reaction, see **Error! Reference source not found.**.

Immobilization of HP-β-CD

After the glycerol diglycidylether was immobilized on the monolithic surface (see Fig. 1), 5% (v/v) BF_3 in dioxane was again passed for 20 min immediately followed by a saturated solution of HP-β-CD in dioxane for 20 min. and the cycle was repeated four times. Between each cycle the column was rinsed with dioxane for 20 min. The HP-β-CD immobilized column was thus ready for evaluation. The column was sealed and stored at 2-8°C when not in use.

Results and discussion

In this study, the chiral recognition ability of the HP-β-CD modified poly(HEMA - co-PETA) precursor monolith was examined. The experiments were performed with a mobile phase containing 30% of 10 mM NH_4Ac (pH 7.0) and 70% (v/v) ACN. Three stereoisomers and one positional isomer were successfully separated using CEC, see Figure 2 through Figure **5**.

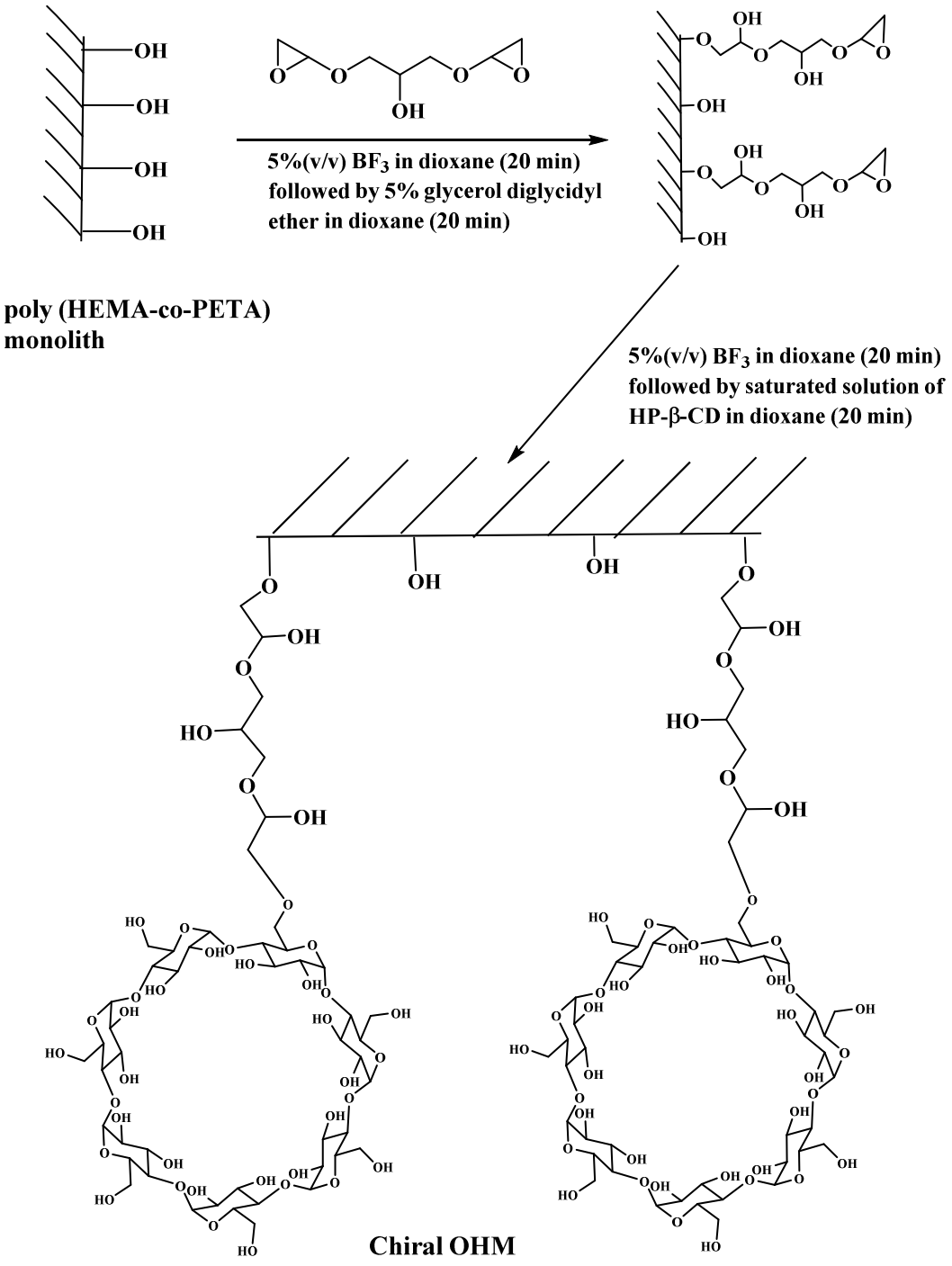

Figure 1.*Reaction scheme for the post-polymerization modification of poly(HEMA-co-PETA) for making HP-β-CD OHM column.*

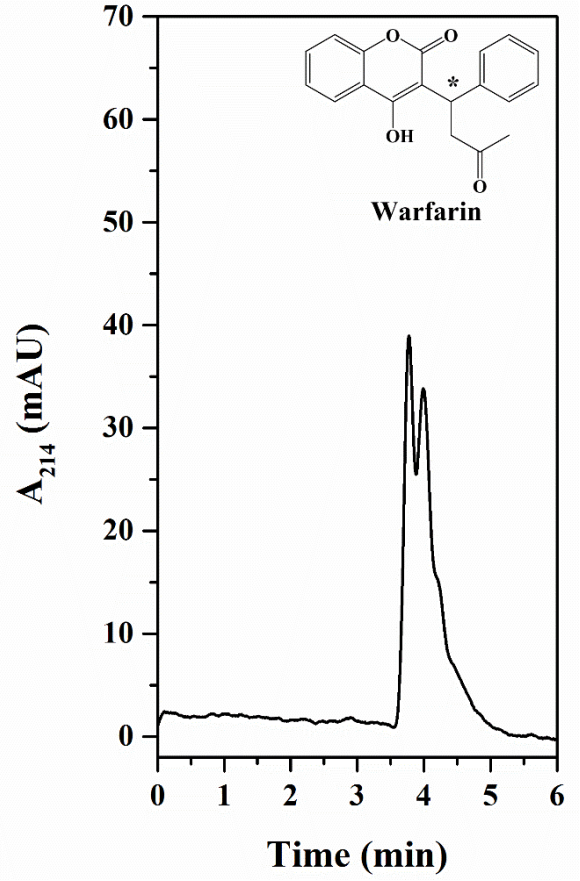

Figure 2. *Electrochromatogram of warfarin obtained on the HP-β-CD OHM capillary column, 33.5 cm (effective length 25 cm) × 100 µm i.d. Conditions: running voltage, 20 kV; sample injection time, 9 s; column temperature, 25 ˚C; hydro-organic mobile phase, 30% (v/v) of 10 mM NH_4AC (pH 7.0) and 70% (v/v) ACN.*

To meet the basic requirements of an ideal chiral selector in chromatography, especially in CEC, it must exhibit the ability to retain chiral recognition in the background electrolyte and also the ability to generate, or at least not restrict, the EOF [22]. The post-polymerization modification of poly(HEMA-co-PETA) monolith with various ligands was studied as shown in previous chapters and a similar reaction scheme was employed here (see Figure 1) to anchor the HP-β-CD onto the monolithic surface. The anchoring of the

chiral selector HP-β-CD was made possible by functionalizing the precursor monolith with the bi-functional glycerol diglycidyl ether, which leaves one of the epoxy groups available for further reaction. This exposed epoxy group was then reacted with the hydroxyl group of the chiral selector, which led to the chiral monolithic column with surface bound HP-β-CD.

The enantioseparation of pharmaceutical drugs and biological substances is important due to the different biological effects exhibited by the enantiomers of the same compound. So, to avoid complications, it is important for the pharmaceutical and biological products to be enantiomerically pure. Some representatives of the biological and pharmaceutical compounds were evaluated on the HP-β-CD OHM capillary column under investigation. Figure 2 shows the separation attempt of warfarin on the HP-β-CD OHM column. As can be seen in this figure, the enantiomers were not fully resolved under the given experimental conditions. Similarly, a racemic mixture of hexobarbital was injected. Here also, the resolution was not better but the peaks are less broadened compared to warfarin. The retention time was about the same for both types of chiral compounds (see Figure 3).

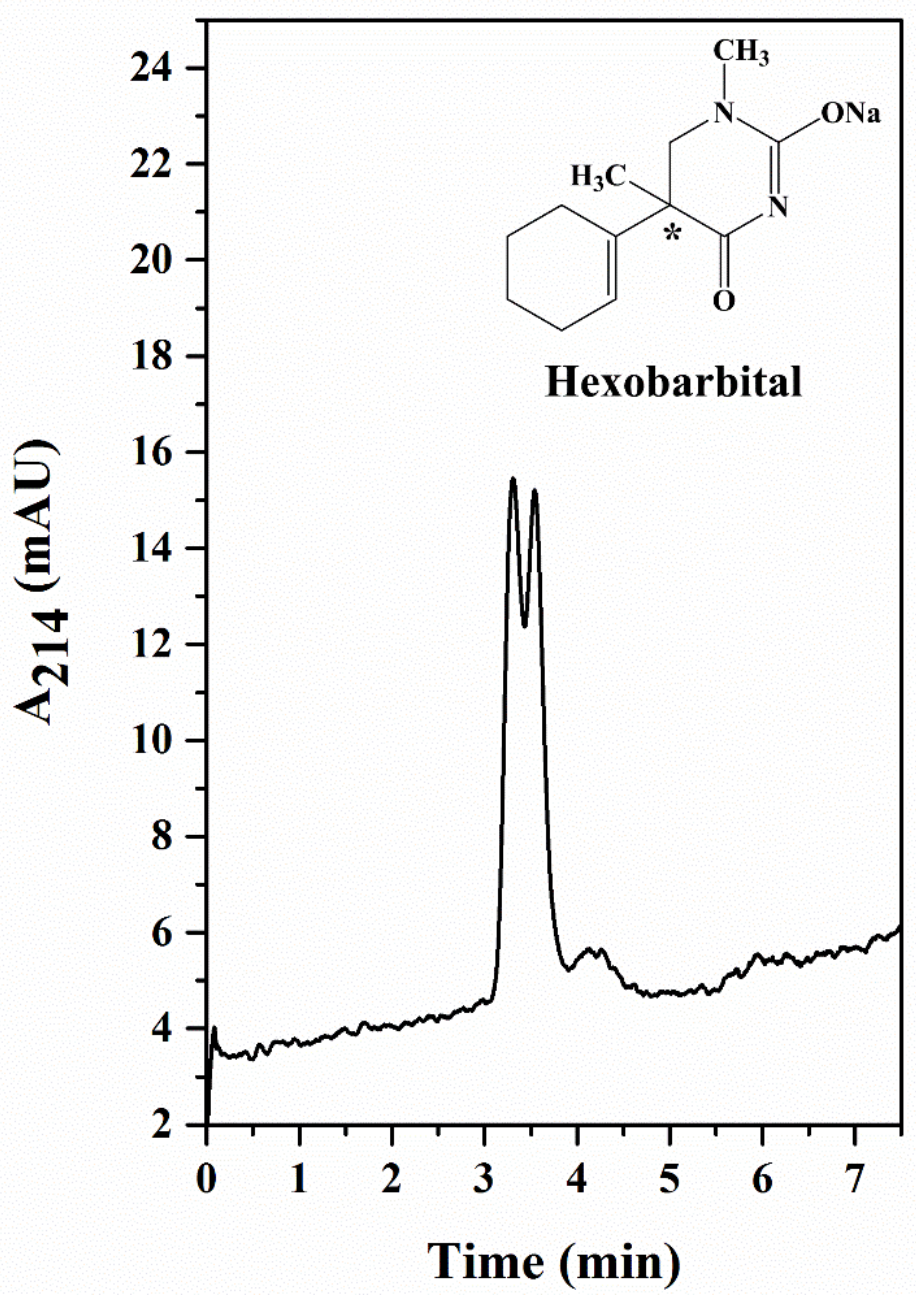

Figure 3. *Electrochromatogram of hexobarbital obtained on the HP-β-CD OHM capillary column, 33.5 cm (effective length 25 cm) × 100 µm i.d. Conditions: running voltage, 20 kV; sample injection time, 9s; column temperature, 25 ˚C; hydro-organic mobile phase, 30% (v/v) of 10 mM NH_4Ac (pH 7.0) and 70% (v/v) ACN.*

In the next attempt, DL-phenylalanine was tested (see Fig. 4). As can be seen in this figure, a better enantioresolution was obtained as compared to both warfarin and hexobarbital. However, peak broadening and tailing were observed. The retention times of all the three racemic mixtures were around the same (compare Figs 2, 3 and 4)

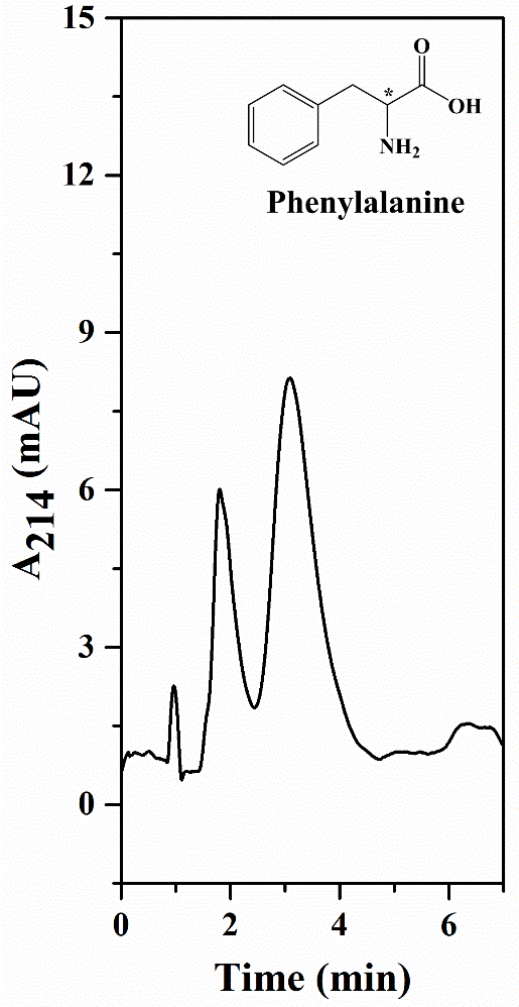

Figure 4. *Electrochromatogram of hexobarbital obtained on the HP-β-CD OHM capillary column, 33.5 cm (effective length 25 cm) × 100 µm i.d. Conditions: running voltage, 20 kV; sample injection time, 9s;column temperature, 25 ˚C; hydro-organic mobile phase, 30% (v/v) of 10 mM NH_4Ac (pH 7.0) and 70% (v/v) ACN.*

Positional isomers of chloroaniline were also tested (see Fig. 5). Among all the compounds tested, 3-chloroaniline and 4-chloroaniline showed better resolution and sharper peaks were obtained. The retention time was also comparatively longer. 3-chloroaniline eluted later than 4-chloroaniline.

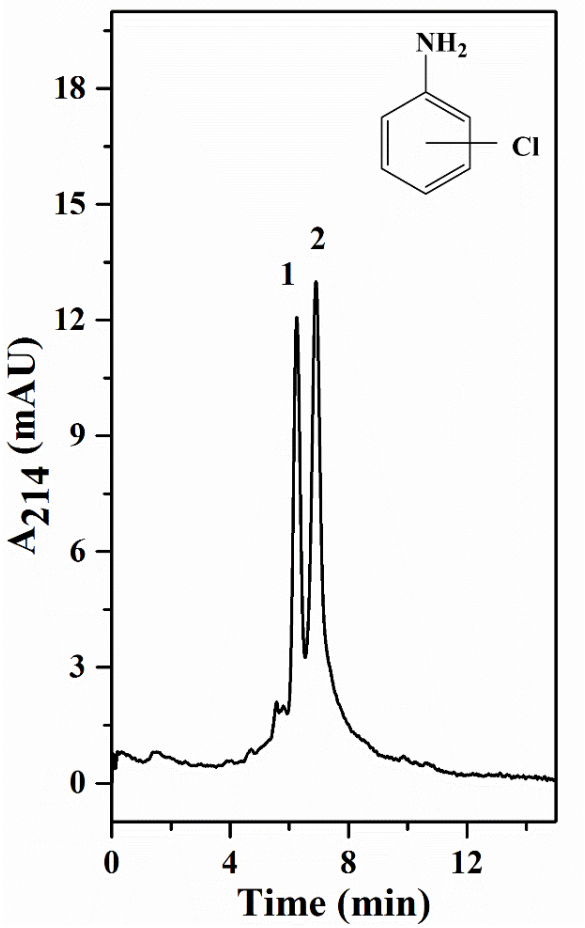

Figure 5. *Separation of chloroaniline positional isomers obtained on the HP-β-CD OHM capillary column, 33.5 cm (effective length 25 cm) × 100 µm i.d. Conditions: running voltage, 20 kV; sample injection time, 9s;column temperature, 25 ˚C; hydro-organic mobile phase, 5% (v/v) of 10 mM NH_4AC (pH 7.0) and 95% (v/v) ACN. Solutes: 1) 4-chloroaniline, 2) 3-chloroaniline.*

Conclusions

The HP-β-CD OHM column obtained by post-polymerization modification was evaluated with some pharmaceutical and biological products and it was observed that there was not sufficient separation obtained for any of the racemic mixtures. However, it can be said that the HP-β-CD chiral selector was successfully functionalized on the surface of the precursor poly(HEMA-co-PETA) monolith due to the racemic peaks obtained as depicted by the electrochromatograms shown in the chapter. However, there is room for further optimization of reaction conditions in terms of the surface coverage of the monolith by the chiral selector. Experimental conditions also need to be improved to get better separation for a wider range of solutes.

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