NOVEL MICROPARTICULATE AND MONOLITHIC STATIONARY PHASES FOR HYDROPHILIC INTERACTION LIQUID CHROMATOGRAPHY AND AFFINITY CHROMATOGRAPHY

By

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Abstract: The overall objective of this dissertation is to develop novel polar silica-based and monolithic-based stationary phases for the separation of a wide range of polar solutes by high performance liquid chromatography (HPLC) using the so-called hydrophilic interaction liquid chromatography (HILIC) separation mode. In addition, these polar stationary phases served as ideal polar matrices to immobilize various affinity ligands for use in high performance affinity chromatography for the purpose of separation and purification of various proteins under reduced nonspecific interactions. Firstly, two different types of amine based organic polymer monolithic stationary phases were synthesized *via* direct *in situ* polymerization and *via* post polymerization functionalization approaches. This was followed by the functionalization of silica microparticles with polar neutral, cationic and anionic moieties in order to develop singly and multilayered HILIC stationary phases. In all cases, the basic silica support was coated with an epoxy active layer *via* the reaction of silica with γ-glycidoxypropyl trimethoxysilane. Thereafter, the activated epoxy silica thus obtained was covered with either tris(hydroxymethyl)aminomethane (TRIS), maltose (MALT) or sorbitol (SOR) yielding the singly layered silica stationary phases. The TRIS-silica was further coated with a layer of chondroitin sulfate A (CSA) yielding the multilayered hydrophilic silica stationary phase referred to as CSA-TRIS-silica sorbent. An extensive chromatographic characterization was conducted to assess each of the developed HILIC stationary phases. Each of these stationary phase yielded unique retention pattern and selectivity towards various polar solutes tested. In addition, the polar MALT-silica stationary phase was further functionalized by converting it first to aldehyde-activated MALT-silica and the activated surface thus obtained served as the initial and starting hydrophilic support for immobilizing various antibodies, e.g., anti-human serum albumin and anti-transferrin, and lectin affinity ligands, e.g., concanavalin A. These affinity stationary phases were successfully used to deplete high abundance proteins as well as to enrich low abundance proteins in human serum under reduced nonspecific interactions.

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LIST OF ABBREVIATIONS

2.3 CPPA	2-(3 Chlorophenoxy) propionic acid
2.4.5 T	(2,4,5-Trichlorophenoxy)-acetic acid
2.4 DPGI	2,4-Dichlorophenoxyacetic acid propylene glycol ester
2-AB	2-Aminobenzamide
3' CMP	Cytidine 3’-monophosphate
5' CMP	Cytidine 5’-monophosphate
ACN	Acetonitrile
AIBN	2-2’ Azobisisobutyronitrile
Ala	Alanine
AMP	Adenosine 5’-monophosphate
BSA	Bovine serum albumin
cAMP	Adenosine 2’:3’-cyclic monophosphate
CB	Cibacron Blue
CEC	Capillary electrochromatography
CF6	Cyclofructan-6
cGMP	Guanosine 2’:3’-cyclic monophosphate
Con A	Concanavalin A

CR	Congo red
CSA	Chondroitin sulfate A
CuAAC	Cu (I) catalyzed alkyne-azide 1, 3-dipolar cycloaddition
cUMP	Uridine 2':3'-cyclic monophosphate
DEAE-PVA	Diethylaminoethyl polyvinyl alcohol
DMF	*N*,*N*-Dimethylformamide
Dns-arg	Dansyl-L-arginine
Dns-asp	Dansyl-DL-aspartic acid
Dns-leu	Dansyl-DL-leucine
Dns-lys	*N*-Dansyl-L-lysine
Dns-met	Dansyl-DL-methionine
Dns-phe	Dansyl-DL-phenylalanine
Dns-ser	*N*-Dansyl-DL-serine
Dns-thr	Dansyl-DL-threonine
Dns-trp	*N*-α-Dnasyl-DL-tryptophan
EDC	*N*-Ethyl-*N'*-(3-dimethylaminopropyl)carbodiimide
EDMA	Ethylene glycol dimethacrylate
FO	Fullerene oxide
FTIR	Fourier transform infrared spectroscopy
Glc	Glucosyl
GlcNAc	*N*-Acetylglucosamine
GMA	Glycidyl methacrylate
GMP	Guanosine 5'-monophosphate

GPTMS	γ-Glycidoxypropyl trimethoxysilane
H	Plate height
HETP	Height equivalent to a theoretical plate
HI	Hydrophilic interaction
HILIC	Hydrophilic interaction liquid chromatography
H_{min}	Minimum plate height
HPAC	High performance affinity chromatography
HPLC	High performance liquid chromatography
HSA	Human serum albumin
id	Inner diameter
IgG	Immunoglobulin G
IL	Ion liquids
IMP	Inosine 5'-monophosphate
IPA	Isopropanol
k	Retention factor
L	Column length
LAC	Lectin affinity chromatographt
MALT-silica	Maltose silica
mecoprop	2-(4-Chloro-2-methyl phenoxy) propionic acid
Me-α-D-Man	Methyl-α-D-mannopyranoside
MPS	Mesoporous silica
N	Number of theoretical plates per column
NAPM	*N*-(3-Aminopropyl) methacrylamide hydrochloride

NHS	*N*-Hydroxylsuccinimide
NPC	Normal phase chromatography
ODS	Octadecyl silica
PAH	Poly aromatic hydrocarbons
PEG	Polyethylene glycol
PHB	*p*-Hydroxybenzoic acid
Phe	Phenylalanine
PNP	Para nitrophenyl
PVA	Polyvinyl alcohol
RPC	Reversed phase chromatography
R_s	Resolution
RSD	Relative standard deviation
SDS	Sodium dodecyl sulfate
silvex ester	(2,4,5-Trichlorophenoxy)propionic acid isooctyl ester
SOR-silica	Sorbitol silica
SWCNTs	Single walled carbon nanotubes
t_0	Dead time
TEOS	Tetraethoxysilane
TGA	Thermogravimetric analysis
t_R	Retention time
Trf	Transferrin
TRIS	Tris(hydroxymethyl) aminomethane
Tyr	Tyrosine

u	Linear flow velocity
UMP	Uridine 5’-monophosphate
u_{opt}	Optimum velocity
W	Peak width at base
W_i	Peak width at the inflection point
α	Selectivity factor
ζ-potential	Zeta potential
σ_L^2	Peak variance
σ_t^2	Peak standard deviation

CHAPTER I

BACKGROUND, RATIONALE AND SCOPE OF THE INVESTIGATION

Introduction and Some HPLC Backgrounds

At the beginning of the twentieth century, a Russian botanist Mikhail Tswett was able to separate plant pigments such as chlorophylls and xanthophyll by passing a solution of them through a glass column packed with calcium carbonate using petroleum ether as the moving liquid. He observed separated color bands on the column and named the technique chromatography, which derived from the Greek noun “chromatos” means color and the Greek verb “graphein” means to write and this was considered as the first reported chromatographic experiment. Today chromatography is a widely used technique for the separation and determination of the chemical components in complex mixtures in almost all branches of science and technology.

All chromatographic experiments use two phases namely, stationary phase, which is fixed in place mostly inside a column and a mobile phase which moves over or through the stationary phase. Generally, the components of a mixture are carried through the stationary phase by the flow of mobile phase and their separation is based on differential

migration due to differences in components distribution between the mobile phase and the stationary phase. In this regard, high performance liquid chromatography (HPLC) uses high pressure to force the mobile phase through the stationary phase in order to achieve high resolution separation in short analysis time.

Components of an HPLC System

The basic parts of an HPLC system consist of a solvent pumping system, a sample injection valve, a separation column, a detector and a recorder or any other data handling system as represented in Fig. 1. There are two types of HPLC systems namely, isocratic systems and gradient systems. Isocratic HPLC systems use a single solvent reservoir and a single pump for an equal strength elution at constant composition of the mobile phase. In contrast, an HPLC system that uses a changing composition of the mobile phase either linearly or exponentially or stepwise is called a gradient elution system.

A high pressure mobile phase pumping system is required in HPLC analysis in order to overcome the resistance to solvent flow generated by the densely packed particle based or monolithic based columns. These pumps are capable of delivering the mobile phase at different flow rates and pressures thus allowing the usage of mobile phases with wide range of compositions. In addition, the HPLC pump should deliver a steady and reproducible flow since fluctuating mobile phase flow can create detector noise leading to a poor sensitivity of the analysis. There are two major types of pumps used in LC analysis namely, reciprocating pumps and screw driven syringe type pumps [1]. Modern solvent delivery systems are often equipped with electronic or mechanical pulse dampers in order to minimize the mobile phase flow fluctuations.

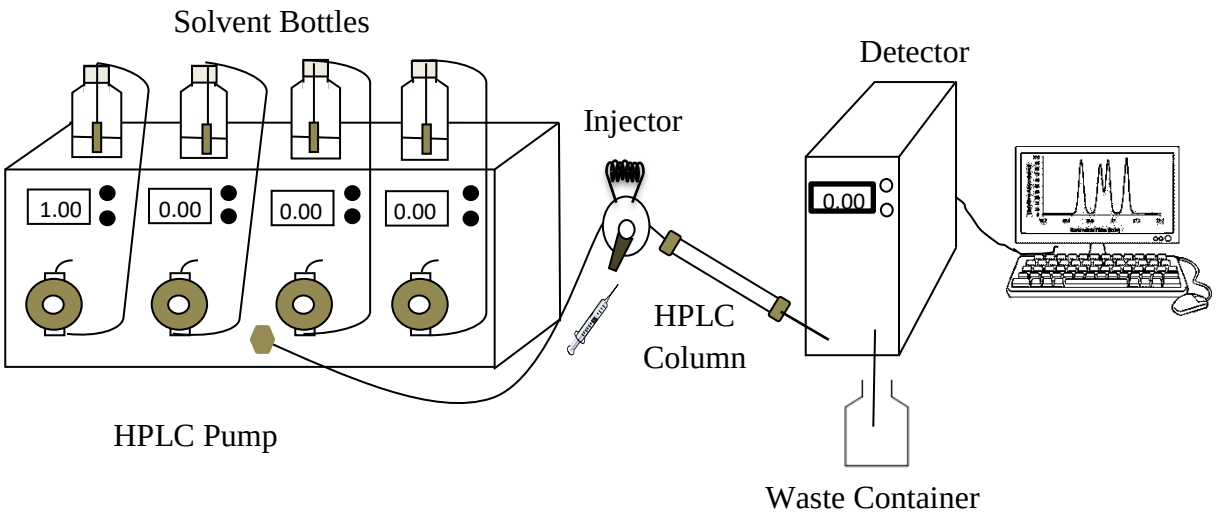

Figure 1. *A diagram showing the components of an HPLC system.*

Sampling valves are commonly used in HPLC for the introduction of the sample to the inlet of the separation column. A sampling valve is a rotary valve equipped with an interchangeable sample loop of a calibrated volume. A syringe is used to wash and load the loop with the sample solution. When the valve is rotated clockwise, the sample in the loop is injected into the column without disturbing the flow or the pressure of the system.

The analytical column where the separation of the mixture occurs is considered as the heart of the HPLC system. These HPLC columns with uniform inner diameters and very smooth inner walls are usually made of stainless steel, glass lined metal tubing or polymeric materials. The column is straight with zero dead volume inlet and outlet fittings as shown in Fig. 2. The packing is held in between two stainless steel frits inserted at both column ends. The most commonly used HPLC packing materials can be categorized into, (i) non-porous solid particles, (ii) superficially porous particles (iii) fully

porous particles and (iv) monolithic polymers. The choice of the packing materials with optimum particle and pore diameters is highly important to achieve maximum resolution in the chromatographic analysis under a moderate column backpressure.

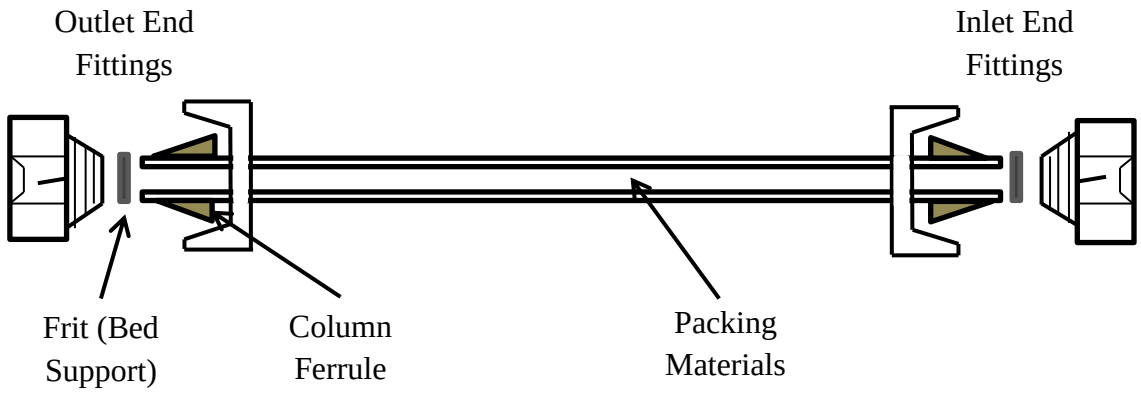

Figure 2. *Schematic diagram of a standard HPLC column.*

HPLC detectors are used to measure some property of the analytes that differ from the mobile phase and convert that measured property to a proportional electric signal. HPLC detectors are often equipped with flow cells to measure low concentration of analytes. UV-visible photometers and spectrometers are widely used as HPLC detectors. These variable wavelength detectors consist of deuterium or tungsten filament light source with grating optics to cover the UV-Vis part of the spectrum. In addition, scanning wavelength detectors or photo diode array detectors (multichannel detectors) use a computer operated devices which are capable of producing a spectrum for each separated component of the analyte in approximately 0.01 seconds. A three dimensional plot is usually obtained to help identifying peak and its purity. Other than UV-visible detectors, many other techniques including fluorescence detectors, refractive index

detectors (RI detectors), electrochemical detectors, conductivity detectors, mass spectrometric detectors can be also employed in HPLC systems.

The electric output signal of the detector is processed and converted into a chromatogram by the recorder or data handling system and a typical chromatogram of an HPLC analysis is shown in Fig. 3. Chromatogram is a plot of detector signal versus time and it is the written record of an HPLC experiment. In fact, a chromatogram can be used to obtain some important information about the solute mixture. For instance, the position of the peaks on the time axis can be used for the identification of the components in the solute mixture, whereas the area under the peak or the height of the peak can be used for the quantitative measurement of each component.

HPLC Parameters Used in this Dissertation

As it is mentioned earlier in this chapter the equilibrium distribution of substances between the two phases which in turn lead to a differential migration is responsible for the separation and retention pattern of an HPLC analysis. Moreover, the axial dispersion of the substances within the HPLC column controls the sharpness of the eluted peak or the column efficiency. In this regard, retention time (t_R) is the time taken by an analyte to reach the detector after the injection or the time that elapses between sample introduction and the appearance of the peak center at the end of the column. An unretained solute which is assumed not to interact with the stationary phase travels through the column in the minimum possible time with the mobile phase flow velocity and emerges from the column at the dead time (t_0).

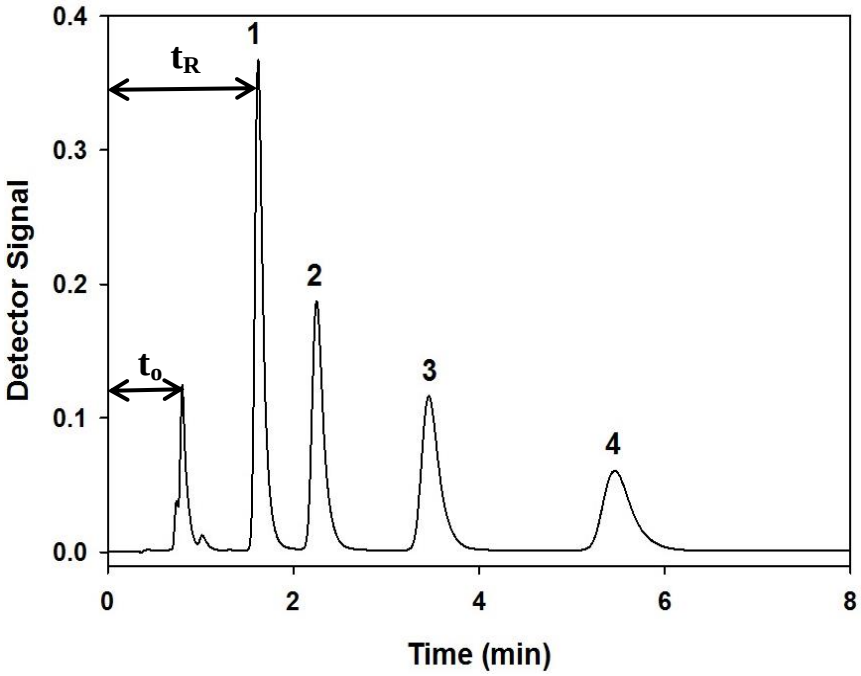

Figure 3. *Schematic of an HPLC chromatogram showing the retention times of solutes.*

The Retention Factor (k). The k value is considered as a peak locator in HPLC analysis and it is a distribution ratio for the amount of solute in the stationary phase to that in the mobile phase. The retention factor (k) is expressed as,

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

The Selectivity Factor (α). The selectivity factor α is a relative retention factor that measures the discriminative power of a given chromatographic system. It is calculated by the ratio of the k values of two adjacent peaks on the chromatogram as follows,

$$\alpha = \frac{k_2}{k_1}$$

A good separation of two species can be observed when α value is 1.1 or greater.

Separation Efficiency and Resolution. The solute, which is applied as a narrow band at the column inlet, spreads to a much longer length inside the column and elutes out at the column outlet with a Gaussian distribution shape. This process is called band broadening. The plate theory of chromatography describes the relative band broadening with respect to the solute migration through the column. In this theory, a chromatographic column is viewed as a series of thin contiguous sections or plates, each thick enough to permit a partitioning solute to equilibrate between the two phases. The movement of the solute through the column is viewed as a stepwise transfer from one plate to the next. In this regard, the separation efficiency measures the narrowness of the bandwidth and it is usually expressed in terms of the number of theoretical plates per column (N), which can be found using the following equation

$$N = \frac{t_R^2}{\sigma_t^2} = \frac{L^2}{\sigma_L^2}$$

The above equation shows the number of theoretical plates in terms of the solute retention time and the peak standard deviation (σ_t^2), and equivalently in terms of the column effective length and the peak variance (σ_L^2). The N value can be determined directly from the chromatogram using the following equation

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

In this equation W is the peak width at base ($W = 4\sigma$) and the W_i is the peak width at the inflection point ($W_i = 2\sigma$). Since the N value is dependent on the length of the column, the plate height (H) or the height equivalent to a theoretical plate (HETP) is

widely used to express the column efficiency thus permitting the comparison of different columns. The HETP can be obtained from the column length (L) and the plate number (N) as follows

$$H = \frac{L}{N}$$

The resolution (R_s) is a measure of the extent of separation between two adjacent peaks. It is a function of column efficiency (N), selectivity (α) and retention factor (k value) as shows in the following equation

$$R_s = \frac{1}{4}\left(\frac{\alpha - 1}{\alpha}\right)\left(\frac{k_2}{1 + k_2}\right)\sqrt{N}$$

where k_2 is the retention factor of the most retained peak.

Van Deemter Equation. Van Deemter equation relates column separation efficiency to the factors related to the band broadening. It describes the relationship between the HETP and the linear flow velocity (u) of the mobile phase as follows,

$$H = A + \frac{B}{u} + Cu$$

The A, B and C are constants for a given column. The multipath term A expresses the contribution of eddy diffusion to band broadening. This arises from the multiple paths in the column due to the inhomogeneity in column packing, and it is independent of the mobile phase flow velocity. The longitudinal molecular diffusion (B/u) describes the natural tendency of solute diffusion from regions of high concentrations to low

concentrations. This causes spreading of sample molecules along the column axis. Since the amount of diffusion increases with time, broadening of this type decreases with increasing flow velocity. The B/u term is increased in value when the HPLC system contains a large internal free volume than necessary due to too long or too wide tubing, incorrect fittings and usage of unions. In packed columns, the longitudinal molecular diffusion is less pronounced than in open tubes. The mass transfer resistance term (Cu) can be described as follows: As a solute moves through the column it undergoes a series of mass transfers between the two phases. The mass transfer can be viewed as diffusion from one phase to another. Broadening occurs because the two phases are not is static contact with one another (one moving and one stationary). In porous stationary phase materials, the mobile phase within the pores is slowly moving or stagnant. Solutes should diffuse through the stagnant mobile phase to reach the stationary phase ligands and then should establish the dynamic adsorption/desorption equilibrium process. The Cu term becomes more pronounced at high mobile phase flow velocity.

Van Deemter Plot. The plot of HETP verses linear flow velocity (u) is called the Van Deemter plot as shown in Fig. 4. At low flow rates, the magnitude of the band broadening is determined by the longitudinal molecular diffusion (B/u). In contrast, at high flow rates, the mass transfer resistance (Cu) controls the separation efficiency. In between there is an optimum velocity U_{OPT} at which the plot has a minimum plate height (H_{min}) giving the maximum separation efficiency for the chromatographic separation.

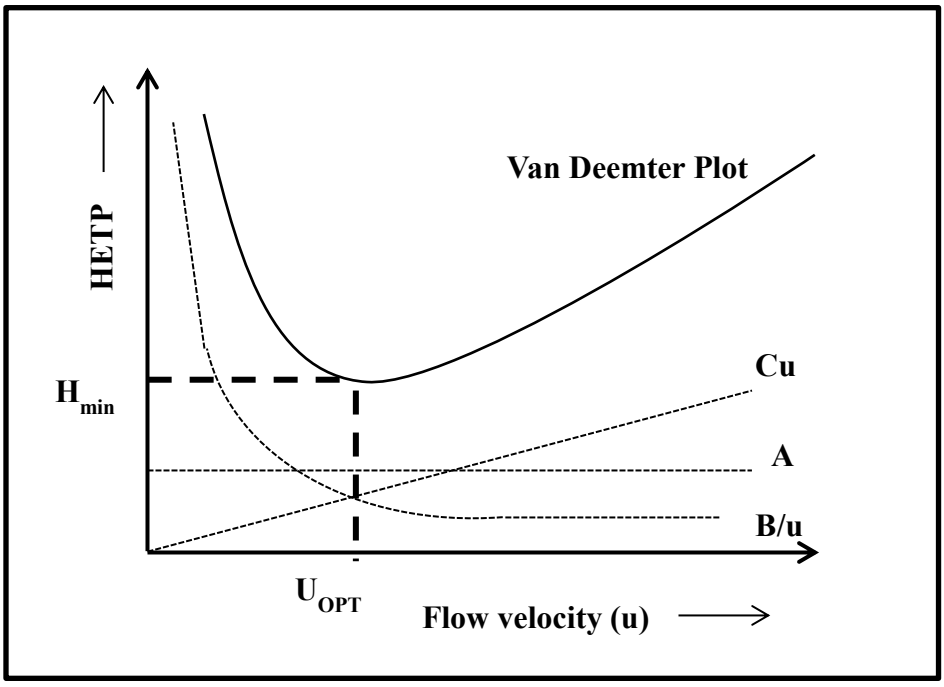

Figure 4. *Van Deemter plot.*

Hydrophilic Interaction Liquid Chromatography

Normal phase chromatography (NPC) and reversed phase chromatography (RPC) are the most commonly used chromatographic techniques in HPLC. In NPC, a polar stationary phase is used with a relatively nonpolar mobile phase such as hexane. In contrast, a nonpolar stationary phase with relatively polar mobile phase such as mixtures of water, methanol, acetonitrile or THF is used in RPC. However, both NPC and RPC separation modes most often do not provide sufficient selectivity for the separation of polar solutes. For instance, polar solutes are weakly retained on RPC stationary phases and often elute unresolved close to the column dead volume. In contrast, strong polar compounds are excessively retained in NPC systems leading to excessively long retention

times. In addition, some of the polar compounds are not sufficiently soluble in nonpolar organic solutes leading to poor reproducibility in NPC applications.

For the separation of polar solutes that may be difficult to be separated by RPC and NPC, hydrophilic interaction liquid chromatography (HILIC) was proposed as an alternative chromatographic technique by Alpert in 1990 who coined it HILIC [2]. A polar (hydrophilic) stationary phase is used with an acetonitrile (ACN) rich hydro-organic mobile phase in HILIC separation. Under these stationary and mobile phase conditions, a water rich layer is formed on the polar stationary phase surface. The separation is achieved by the differential and selective partitioning of polar solutes in between the adsorbed water layer on the stationary phase surface and the organic rich mobile phase. However, this simple retention mechanism is not sufficient to explain the separation pattern of the most polar compounds since electrostatic interactions, hydrogen bonding, dipole-dipole interactions, ion-dipole interactions are also involved in HILIC in addition to the hydrophilic partitioning [3]. The use of organic rich mobile phase in HILIC provides some additional advantages including, low column backpressure, which allows fast separation of analytes with shorter analysis time and the suitability of HILIC to direct coupling with MS detection (e.g., ESI-MS).

Stationary Phases for HILIC

The main requirement for a HILIC stationary phase is to have a good polarity. Due to the possibility of the multiple modes of interaction in between the stationary phase and the mobile phase, the surface chemistry variation of HILIC stationary phases is wider than that of RPC columns. Different types of HILIC stationary phases provide diverse

retention behavior, selectivity and efficiencies for addressing a wide range of separation challenges. The polarity and the charge of the stationary phase can be used to classify HILIC stationary phases into (i) neutral (ii) anionic (iii) cationic and (iv) zwitterionic types

Monolithic HILIC Stationary Phases

Since a brief attempt was made to synthesize polar monolithic stationary phases in this dissertation research, and also the advances in polar and nonpolar monoliths for HPLC and CEC over the past five years have been reviewed by Jonnada et al. and Rathnasekara et al. and published in the Journal of Electrophoresis recently [3, 4], only a very short description of polymer monolithic columns is given here. Briefly, polymer based monolithic stationary phases have become an attractive choice for the preparation of novel polar HILIC stationary phases due to their distinct advantages including ease of preparation, high permeability, stability under wide pH conditions and possibility of miniaturization in nano-LC and CEC. Organic polymer monoliths are generally prepared by free radical polymerization of functional monomers with cross-linking agents in the presence of pore forming solvents (i.e., porogens). Most often post polymerization functionalization is also conducted to introduce the desired ligands into the polymer surface.

Although monolithic stationary phases showed excellent pH stability and ease of preparation, they undergo swelling in organic solvents leading to poor mechanical stabilities. Also, the pore structure of most polymer monoliths is not suitable for separation of small molecules. Due to these shortcomings, silica based stationary phases

have been proposed as alternatives to monolithic stationary phases. The major advances made in silica based stationary phases are summarized in the following sections.

Silica Based Stationary Phases for HILIC

Compared to monolithic stationary phases, silica based stationary phases offer high mechanical stabilities and high resolution in HILIC separation of polar solutes. In this regard, un-derivatized or bare silica is widely used as HILIC stationary phases. However, bare silica stationary phases are not stable under high pH conditions. Moreover, acidic silanol groups on the surface of un-derivatized silica stationary phases undergo electrostatic interactions with the basic solutes leading to peak tailing. Therefore, a wide range of polar functionalities has been attached onto the silica surface in order to increase the hydrophilicity of the silica as well as to get different selectivity and higher resolution for the HILIC separation of polar analytes.

Neutral Polar Silica Bonded Stationary Phases for HILIC. Among various available stationary phases for HILIC separations, neutral polar stationary phases undergo little or no electrostatic interactions with charged solutes leading to higher separation efficiencies and different separation selectivity compared to those of charged HILIC surfaces. In this regard, a diterpene glycoside compound namely, rebaudioside A which is a low calorie sugar substitute was immobilized onto mercapto-silica particles *via* “thiol-ene” click reaction [5]. The separation of charged solutes was dominated by the hydrophilic partitioning mechanism at lower pH condition (pH 3) revealing the neutral polar character of the stationary phase. However, the unreacted residual silanol groups get ionized at higher pH (pH 6.6) giving a cation exchange contribution to the retention

process. The resulting stationary phase displayed excellent selectivity and efficient retention for various polar compounds including oligosaccharides, nucleic acid bases and nucleosides. The structure of the rebaudioside A consists of hydrophobic aglycone and hydrophilic sugar units. Therefore, separation of saponins was performed in both HILIC and RPC modes by just changing the mobile phase composition.

New HILIC stationary phases for HILIC were reported by covalently attaching native cyclofructan-6 (CF6) to fully porous silica particles and superficially porous silica particles [6, 7]. Cyclofructans consist of six or more β-(2→1) linked D-fructofuranose units and each unit contains hydroxyl groups which are responsible for the HILIC behavior of the stationary phase. Native CF6 was chemically bonded to isocyanate modified silica particles *via* carbamate linkages produced by the reaction between isocyanate groups on the sialylated silica and the hydroxyl groups on the CF6. These stationary phases were successfully used to separate a large number of polar solute mixtures including nucleic acids bases and nucleosides, xanthines, β-blockers, salicylic acid and derivatives and carbohydrates under HILIC separation conditions. Furthermore, HILIC stationary phases based on native CF6 bonded to superficially porous particles, fully porous particles with 5 μm and 3 μm particle diameters were evaluated in terms of separation efficiency, analysis time and resolution in HILIC separations. These results indicated that bonded HILIC phases of superficially porous particles show clear advantages in both efficiency and analysis time without loss in selectivity when compared to those of fully porous particles.

Fullerene oxide (FO) was successfully immobilized onto aminopropyl-silica microparticles to form FO modified silica stationary phases for HILIC separations [8]. The epoxy groups and the carboxyl groups of the FO reacted with the amine groups of the aminopropyl-silica particles yielding the HILIC surface. According to the authors' the study of the effect of buffer concentration on the retention of charged solutes revealed that the electrostatic interactions and ion-exchange effects were not significant towards retention of charged solutes on the FO-modified silica stationary phase. The retention factors (k values) of tested polar solutes increased with increasing ACN content of the mobile phase indicating typical HILIC behavior of the FO-silica stationary phase. Since particle size of the FO is on the same order of magnitude with the pore path size of the silica surface, FO would inevitably damage the original pore structure of silica causing a decrease in separation efficiency on the FO modified silica stationary phase.

A new hydrophilic and nonionic poly(2-vinyloxazoline)-grafted silica stationary phase was synthesized and applied in HILIC separation of polar solutes [9]. Oxazolines are five membered cyclic compounds having an endo-imino ether (-N=C-O-) group. The poly(2-vinyloxazoline) was attached to the silica surface *via* 3-mercaptopropyltrimethoxysilane. The resulting material exhibited high hydrophilicity even though it is neutral. As a result, good separation behavior was observed during the separation of nucleobases and nucleosides. Furthermore, the k values of all selected nucleosides and nucleobases decreased with increasing the water content of the mobile phase revealing the typical HILIC behavior of the oxazoline based column.

Cucurbit(n)urils (n=5-8, 10) which are macrocyclic host molecules composed of glycoluril monomers joined by pairs of methylene bridges have been immobilized onto silica and evaluated under HILIC/RPC mixed mode chromatography. Cucurbit(n)urils have a symmetrical structure with a hydrophobic inner cavity and two identical hydrophilic carbonyl fringe portals, providing selective interaction sites with a variety of molecules *via* hydrophobic, hydrogen bonding and ion-dipole or dipole-dipole interactions [10, 11]. In this regard, cucurbit(7)uril was immobilized onto silica surface by a sol-gel approach in the presence of formic acid [12]. Ethanol was used during the preparation to facilitate the dissolution of tetraethoxysilane (TEOS) and formation of the sol coating on silica. Since cucurbit(7)uril has a cavity and polar fringe portals, it may provide hydrophobic, dipole-dipole, H-bonding and electrostatic interactions for the separation of charged solutes. The retention behavior of alkaloids was studied with different % methanol content in the mobile phase. The "U shaped" curves were observed for all examined alkaloids indicating RPC/HILIC mixed mode retention behavior. At methanol rich region, the retention was based on HILIC interactions caused by the carbonyl groups of the cucurbit(7)uril, while in water rich region, solute retention was based on hydrophobic interactions caused by the solute inclusion in the cavity. Cucurbit(7)uril would be protonated to some degree at low pH giving cationic characteristics to the column. Therefore, analyzed cationic solutes showed weaker retention at low pH (2.7) than that of higher pH.

In another report, cucurbit(6)uril was immobilized onto the silica surface by a sol-gel approach to synthesize a novel HPLC stationary phase [13]. The cucurbit(6)uril contain 6 glycoluril units linked with each other by a pair of methylene groups.

Furthermore, six carbonyl groups on the cucurbit(6)uril molecule provide sufficient hydrophilicity for the HILIC applications [14]. According to the authors the retention mechanism of the cucurbit(6)uril silica was dependent on the nature of the analytes. For less polar solutes, both hydrophobic and hydrophilic interaction could contribute to the solute retention, while for polar analytes, hydrophilic interaction may be predominant. The novel stationary phase was successfully used to separate mixtures of polar solutes including nucleobases.

Cationic Silica Bonded Stationary Phases for HILIC. The HILIC stationary phases in this category are positively charged over the pH range used in HILIC applications. These stationary phases have the ability to form electrostatic interactions with charged solutes in addition to hydrophilic partitioning under HILIC conditions. Therefore, the retention, selectivity and the separation efficiency of the charged solutes separated on cationic HILIC stationary phases are largely affected by the mobile phase pH and the ionic strength.

There are several reported recent studies on the functionalization of silica microparticles with neutral polar groups *via* amine based coupling agents such as (3-aminopropyl)trimethoxysilane and ethylenediamine. Although the attached functional groups are uncharged over the typical pH range used in HILIC separations, the residual amine functionalities in the coupling agents get deprotonated at lower pH giving weak-anion exchange characteristics to the prepared stationary phases. In this regard, a novel stationary phase containing β-cyclodextrin and poly(*N*-isopropylacrylamide) polar functionalities was reported recently [15]. Firstly, the bare silica was functionalized with

3-(methacryloyloxy)-propyl-trimethoxysilane to provide the proper anchoring for the polymer coating. Then, a poly(*N*-isopropylacrylamide-co-glycidyl methacrylate) polymer layer was formed on the silica particles by free radical polymerization. Finally, ethylenediamine functionalized β-cyclodextrin was attached onto the epoxy polymer layer by the nucleotide ring opening reaction. The prepared stationary phase has showed HILIC/RPC mixed mode retention behavior. According to the author the retention time of the test solute benzoic acid was almost unchanged in the pH range from 6.2 to 4.5 showing the typical neutral polar stationary phase properties. However, the stationary phase has undergone electrostatic interactions with charged solutes at lower pH possibly due to the protonation of ethylenediamine groups present on the stationary phase surface.

Recently, maltose and dextran were covalently bonded to aminopropyl silica using carbonyl di-imidazole as the crosslinker and the stationary phases thus obtained were used as HILIC columns for the separation of polar compounds [16, 17]. According to the authors this method is a simple way to bond various carbohydrates onto the silica gel. The ζ- potential analysis revealed a positive surface charge within the pH 2 to 7 on sugar bonded silica due to the unreacted amino groups on the silica surface. Both stationary phases showed typical HILIC behavior, whereby the retention factor of polar solutes decreased with increasing water content in the mobile phase. Moreover, the effect of mobile phase pH and salt concentration on the retention factor of anionic orotic acid revealed that the electrostatic interactions between the charged solutes and the cationic stationary phases are also playing an important role towards the HILIC separations on these sugar modified silica. Both maltose and dextran modified silica stationary phases

were successfully used for the HILIC separations of nucleobases, nucleosides, carbohydrates, amino acids and peptides.

Thiolated-β-cyclodextrin was covalently attached onto gold nanoparticles, which were immobilized on aminopropyl silica [18]. The resulting stationary phase was evaluated in chiral liquid chromatography and HILIC. Five nucleobases and nucleosides were baseline separated on the new stationary phase using a mobile phase containing 92% ACN. Moreover, the k values of polar solutes showed a small change with increasing temperature. According to the authors, the existence of gold nanoparticles on the new stationary phase surface led to the rapid mass transfer rate and it is responsible for the slight decrease of k values with increasing column temperature.

Recently, chitosan derivatized with calix[4]arene, which is a cavity shaped cyclic molecule consisting of four phenol units linked *via* methylene bridges was covalently bonded to the glutaraldehyde modified 3-aminopropyl silica [19]. The resulting stationary phase was evaluated under RPC-HILIC mixed mode chromatography. While phenol groups of the calix[4]arene provides the hydrophobic and π-π interactions for RPC separation of nonpolar solutes, the polar NH and OH groups on calixarene and chitosan provides the hydrophilic and hydrogen bonding interactions for the HILIC separations. The RPC-HILIC mixed mode chromatographic retention behavior of mono-substituted benzenes, phenols and nucleosides on claix[4]arene derivatized chitosan silica were investigated and compared with an octadecyl silica (ODS) stationary phase.

Multiple embedded polar groups containing C_{18} silica based stationary phase for the separation of versatile analytes including shape-constrained isomers, nonpolar, polar

and basic compounds with good selectivity in both RPC and HILIC modes was reported [20]. This material contains four amide groups providing hydrophilicity, wettability and hydrogen bonding interactions for HILIC separations and rigid C_{18} groups providing the hydrophobicity for the RPC separations. In HILIC, hydrophilic interactions and weak anion exchange multiple interactions were observed and the residual aminopropyl functionalities of the base silica support are supposed to influence these ion exchange interactions.

A novel poly(L-lactic acid)-modified silica stationary phase was prepared by amide bond reaction between amino groups on aminopropyl silica and carboxylic acid groups at the end of the poly(L-lactic acid) chain [21]. The retention of test compounds exhibited "U-shaped" curves with the %ACN content in the mobile phase indicating the RPC/HILIC mixed mode retention behavior. Mixture of nucleobases and nucleosides were successfully separated on the novel stationary phase under HILIC separation conditions. In addition, authors have claimed that the carbonyl groups included into the poly(L-lactic acid) backbone provide $\pi-\pi$ interactions for the separation of polycyclic aromatic hydrocarbon in the RPC mode.

Similarly, a surfactin modified silica stationary phase was prepared by amide bond formation between amino groups on aminopropyl silica and the carboxylic acid groups of L-Glu and L-Asp residues in surfactin [22]. Surfactin is an efficient bio surfactant consisting of seven amino acids and a β-hydroxyl fatty acid. The retention of polar solutes showed "U-shaped" curves depending on the acetonitrile content in the

mobile phase indicating HILIC/RPC mixed mode separation behavior due to the presence of polar and nonpolar groups on the same surfactin modified stationary phase.

Amino acids are naturally polar zwitterionic molecules. Moreover, some of the amino acids contain polar or charged side chains attached to their structures thus increasing their hydrophilicity. Therefore, many attempts have been made recently to immobilize amino acids or oligopeptides onto the silica surface in the purpose of synthesizing HILIC stationary phases.

A novel amide based HILIC stationary phase was synthesized by transferring the amino groups of natural amino acids asparagine to the corresponding azido group *via* imidazole-1-sulfonyl azide hydrochloride reagent and then clicked with terminal alkyne-silica gel [23]. The performance of the click asparagine stationary phase under HILIC mode was evaluated using mixtures of nucleobases and nucleosides and organic bases. The novel stationary phase showed high running stability relative to the amino-based HILIC stationary phases owing to the less reactive amide groups. The existence of 1, 2, 3-triazole group introduced during the click reaction make the stationary phase possible to behave as an anion exchanger. This was demonstrated by separating five inorganic anions in less than 4 min on this novel stationary phase.

An L-lysine based, urea containing organogelator-modified silica stationary phase was synthesized for the separation of chiral analytes and polar analytes [24]. The lysine based gelator-silica stationary phase was successfully used for the separation of nucleobases and sulfonamide antibiotics under HILIC separation conditions. Moreover, the retention times of the sulfur drugs separated on the lysine-silica stationary phase

decreased with increasing salt concentration in the mobile phase indicating the influence of ionic interactions on the HILIC mode retention mechanism.

A novel glutamine containing silica based stationary phase was reported for HILIC/RPC mixed mode chromatography [25]. First, an amide ligand was synthesized by reacting glutamine with aniline and then it was covalently bonded to the epoxy activated silica. The new stationary phase bears multiple amide groups and a secondary amine group giving hydrophilic characteristics and phenyl and alkyl groups giving non-polar characteristics to the separation surface. The novel stationary phase was used to separate mixtures of nucleobases and nucleosides under HILIC separation conditions and to separate phytohormones and phenolic compounds under RPC conditions.

A tripeptide functionalized silica based stationary phase has been developed for HILIC enabling the efficient separation of bioactive polar molecules [26]. One short peptide molecule having the sequence of Boc-Tyr-Ala-Tyr-OH was synthesized for this purpose. The phenolic OH group of tyrosine and carbonyl groups give the hydrophilic nature to the stationary phase while terminal tertiary butyl group (Boc-) and alanine residue impart a hydrophobic character. The tripeptide molecule was immobilized onto the aminopropyl grafted silica surface by forming covalent linkages using diethyl phosphorocyanidate as the coupling reagent. The retention factor of sulfur compounds separated on this stationary phase showed a sharp decrease at high salt concentrations indicating the influence of ionic interactions in the HILIC-mode separation mechanism.

Another novel tri-peptide based monomeric silica stationary phase for use in RPC-HILIC mix mode chromatography was reported [27]. The N-terminally protected

short tripeptide Boc-Phe-Aib-Phe-OH that contains four hydrophilic amide moieties and two hydrophobic phenyl groups is responsible for the mixed mode behavior. Elemental analysis, FTIR, thermogravimetric analysis and solid-state ^{13}C NMR were used to identify the chemical modifications of the silica phase. These results confirmed the successful grafting of the Boc-Phe-Aib-Phe-OH onto the silica surface. Mixtures of alkylbenzenes, PAHs and steroids were well separated on the tripeptide functionalized column under RPC conditions. This stationary phase showed good retention and selectivity for nonpolar solutes in the RPC mode when compared to conventional ODS and phenyl stationary phases due to the presence of multiple phenyl and carbonyl groups for establishing $\pi-\pi$ interactions with the solutes. Due to the presence of chiral centers in the tripeptide groups, the novel stationary phase was used for enantiomeric separations as well. Moreover, peptide based stationary phase showed excellent performance in HILIC separations of sulfur based polar compounds, nucleobases and nucleosides with high retention factors and separation efficiencies.

Ion liquid (IL) functionalized silica has evolved as a new class of HILIC stationary phases and many works in this area have been reported in last few years. ILs contain organic cation and organic or inorganic anion. The versatile properties of ILs such as high hydrophilicity, electrostatic interactions involved in the cation/anion pairs, ability to interact with other molecules through ion exchange interactions, possibility of establishing hydrogen bonding interactions make them attractive as HILIC ligands for the functionalization of silica [28]. When IL is bonded to silica, it loses its liquid state, but not other unique properties [29]. Among various commercially available ILs, those with imidazolium cations have been widely used for the preparation of HILIC stationary

phases. Imidazole is a highly polar five membered cyclic planer molecule with a protonated nitrogen atom making it cationic. In this regard, a novel poly(ionic liquid)-grafted silica stationary phase was prepared by polymerizing an IL monomer 1-(2-acryloyloxyundecyl)-3-methyl-imidazolium bromide onto the mercaptopropyl modified silica [30]. The polar imidazolium, carbonyl and alkyl chain groups in this stationary phase give the possibility of multiple interaction mechanisms, including anion exchange, hydrophobic, π–π, and ion-dipole interactions. Mixture of inorganic anions was successfully separated on this novel IL modified silica stationary phase under anion-exchange conditions due to the greater cationic character of the column. In addition, mixtures of nucleobases and nucleosides were also successfully separated on this IL modified silica column under HILIC separation conditions. Furthermore, this multi-mode IL stationary phase was effectively used for the separation of PAHs under RPC conditions.

In another study, several germinal dicationic ILs based on 1,4-bis(3-allylimidazolium)butane and 1,8-bis(3-allylimidazolium)octane were bonded to a mercaptopropyl modified silica *via* "thiol-ene" click reaction yielding stationary phases for HILIC [31]. The studies of retention changes with the chromatographic conditions demonstrated HILIC/anion exchange mixed-mode retention mechanism for the separation of charged solutes on these dicationic IL based stationary phases. The obtained dicationic stationary phases showed higher efficiency, retention and selectivity towards typical hydrophilic compounds in HILIC mode compared to those on analogue monocationic IL columns under identical conditions. However, longer alkyl chain spacer linking two imidazolium cations would decrease the hydrophilicity of the stationary phase due to the

increase of hydrophobicity and dispersion of ionic nature. The same research group has evaluated tricationic IL modified silica under both HILIC and RPC chromatographic conditions [32]. According to the authors, the tricationic IL columns showed higher separation, enhanced retention and better selectivity toward flavonoid standards than reported HILIC columns, and show great prospect for application in the analysis of complex samples.

A novel imidazolium embedded *N*,*N*-dimethylaminopropyl functionalized silica was prepared *via* "thiol-ene" click reaction between mercaptopropyl silica and vinyl imidazole followed by dimethylaminopropyl group attachment for use as a stationary phase in HILIC/RPC mixed-mode chromatography [33]. Simultaneous separation of polar and nonpolar compounds was achieved in a single column with high resolution. Recently, a similar approach was followed with a surface radical chain-transfer polymerization step instead of the dimethylaminopropyl attachment for the preparation of the imidazolium embedded iodoacetamide functionalized silica stationary phase [34]. Fast and efficient separation of polar analytes, such as nucleosides and nucleobases, water soluble vitamins and saponins were achieved on the prepared column in HILIC mode while hydrophobic compounds and positional isomers were separated under HILIC/RPC mixed mode.

Moreover, a novel imidazole functionalized stationary phase for HILIC was prepared *via* surface initiated atom transfer radical polymerization [35]. In this regard, α bromoisobutyryl bromide was bonded to the aminopropyl functionalized silica to obtain Sil-Br as a macro-initiator. Then, 1-vinyl imidazole was polymerized on Sil-Br *via*

surface initiated atom transfer radical polymerization. Three types of poly(1-vinylimidazole)-grafted silica particles of different polymer chain lengths were prepared by controlling the monomer concentration in the polymerization system. These stationary phases showed an increase in retention factors of polar solutes with increasing the grafted imidazole groups in the stationary phase under HILIC separation conditions. The effect of buffer pH on retention of polar analytes indicated the involvement of electrostatic interactions in the HILIC separation mechanism.

A novel HILIC/anion exchange mixed-mode stationary phase was recently synthesized by immobilizing 2-methylimidazole onto the chloropropyl functionalized silica [36]. The separation performance of the prepared column was investigated by HILIC separation of nucleobases and nucleosides, water-soluble vitamins, sulfonamides and saccharides and ion chromatographic separation of inorganic anions.

The 1,3-butyldibromo *p*-tert-butyl calix[4]arene–allylimidazole IL was grafted on mercaptopropyl modified silica gel in order to prepare mixed-mode chromatographic stationary phase [37]. Due to the presence of polar, nonpolar and charged functionalities on the same surface, the prepared IL based stationary phase was evaluated in HILIC/RPC/anion exchange mixed mode chromatographic conditions and satisfactory results were achieved. In this regard, the imidazolium gives the polar characteristics to the column and mixtures of nucleosides and ginsenosides were successfully separated under HILIC separation conditions.

Glucaminium-based IL is a new class of ILs, which is prepared by functionalization of the amine groups of *N*-methyl-D-glucamine. Due to the presence of

glucose units and the amine functionalities, glucamine based ILs can be effectively used in HILIC stationary phases. In this regard, N,N-diallyl-N-methyl-D-glucaminium bromide was bonded to the 3-mercaptopropyl modified silica surface through "thiol-ene" click chemistry [38]. Neutral polar nucleosides and flavonoids were well separated on this novel stationary phase with high separation efficiencies in the HILIC mode. Moreover, mixtures of anionic nucleotides were separated under HILIC/anion exchange mixed mode chromatographic conditions. In another study, glucaminium based IL was bonded to the glycidoxypropyl functionalized silica *via* facile epoxy-amine reaction in a three step one-pot synthesis [39]. Elemental analysis and infrared spectroscopy were used to validate the successful immobilization of the IL. The study of the effect of mobile phase ionic strength and pH on retention factor of charged solutes revealed a hydrophilic partitioning/adsorption/ion-exchange mixed mode type retention mechanism.

A series of amine based cationic HILIC stationary phases have been also reported. In this regard, a novel stationary phase containing primary and tertiary amine groups was synthesized by first bonding methyl acrylate onto the aminopropyl functionalized silica to obtain ester groups modified silica. Then, ethylenediamine was reacted with the ester-modified silica to afford an amine terminated silica material. Finally, the amine silica thus obtained was further reacted with acrylonitrile followed by ammonium thiocyanate to obtain the HILC stationary phase containing primary ad tertiary amine functionalities [40]. The ζ potential analysis on this novel stationary phase revealed that the material is positively charged and the ζ potential decreased with increasing pH value due to the deprotonation of the amine groups. A series of quinolone compounds were used to probe

the effect of ACN content in the mobile phase on retention. The corresponding curves were “U shaped” indicting HILIC/RPC mixed-mode retention behavior.

Moreover, a silica based amino stationary phase was prepared by immobilizing propargylamine on azide-silica *via* click chemistry [41]. Cu (I) catalyzed alkyne-azide 1, 3-dipolar cycloaddition (CuAAC) has been employed in this synthetic procedure. The regioselective 1, 2, 3-triazole ring formed in this reaction plays a major role in HILIC separation mechanism of the stationary phase. According to the authors, the stationary phase has a very simple structure and easy to prepare. Inorganic anions were well separated under ion chromatography mode and polar compounds including nucleosides, organic acids and bases were also separated under HILIC mode. In another study, a new type of silica based HILIC-stationary phase was synthesized by covalently bonding organic azide bearing 2-(*N*,*N*-dimethylamino)-1,3-propanediol moiety onto alkyne-silica surface *via* CuAAC click reaction [42]. This new stationary phase showed good HILIC characteristics and high column separation efficiencies in the separation of polar compounds such as nucleosides and bases, organic acids, cephalosporins and carbapenems.

Another amine based HILIC stationary phase was prepared by coupling neomycin onto the aminopropyl functionalized silica using cyanuric chloride as the coupling agent [43]. Neomycin is an aminoglycoside antibiotic containing strong hydrophilic six amino groups, seven hydroxyl groups and six glycosidic oxygen functionalities. The amine groups of neomycin could offer anion exchange interactions in addition to the hydrophilic interactions during HILIC separations. Application of this novel stationary phase in

HILIC applications indicated that the retention mechanism of nucleosides were mainly based on hydrophilic-interactions while for organic acids retention mechanism was involved with a mixed-mode hydrophilic/ion-exchange interactions.

Another HILIC stationary phase containing two bidentate urea-type functionalities was synthesized *via* facile one-pot, two step procedure by first reacting 1,2-ethylene diamine with (3-isocyanatopropyl)trimethoxysilane to give an intermediate bis-urea, followed by anchoring onto the silica surface [44]. Moreover, a variant of the bidentate urea-type stationary phase was realized in which free amino groups were intentionally left on the silica surface. In order to get a higher stability of the stationary phase, silanization with 1, 2-bis(trichlorosilyl)ethane was used in the final stages of the synthesis. According to the authors, the urea-type phase bearing free amino groups was specifically designed for application to sugar analysis. Free amino groups on the silica surface have a potential catalytic effect on the α/β anomer interconversion on reducing sugars, and this effect in turn translates in α/β anomer peak averaging thus giving a single sharp peak. The new HILIC stationary phase are chemically and thermally stable and showed good selectivity for a broad set of chemically diverse polar compounds.

Recently, an amino derivatized silica gel was non-covalently coated with carboxylated single walled carbon nanotubes (SWCNTs) to preserve the structure of the nanotubes and their physio-chemical properties [45]. Six nucleobases were baseline separated on this novel stationary phase under HILIC separation conditions. The retention times of all nucleobases decreased with increasing the water content in the mobile phase indicating typical HILIC behavior of the column.

Anionic Silica Bonded Stationary Phases for HILIC. Several polar stationary phases containing bonded anionic functionalities were reported in recent literature and these types of stationary phases were often evaluated in HILIC/cation exchange mixed-mode chromatographic conditions. These anionic stationary phases provide different selectivity for the separation of charged solutes compared to that of cationic or neutral polar HILIC stationary phases. In this regard, Armstrong's research group has synthesized stationary phases composed of native cyclofructan 6 (CF6) and benzoic acid modified CF6 and evaluated them under HILIC separation conditions [46]. Benzoic acid moieties were covalently attached to impart ionizable character to the CF6. The modified CF6 was bonded to isocyanate-functionalized silica by reacting the hydroxyl groups of the sugar CF6 with the isocyanate groups. According to the authors, the large bonded bulky CF6 molecule protects the silica surface from hydrolytic attack while maintaining the high efficiency of the bonded phases. The relative retention factor of benzyltrimethylammonium chloride/cytosine (BTMA/cytosine) revealed that the benzoic acid CF6 had stronger cation exchange property than the native CF6 since the attached carboxyl groups were negatively charged and therefore strongly retained cationic BTMA. The benzoic acid CF6 appears to be a good HILIC stationary phase for the separation of polar pharmaceutical drugs, salicylic acids analogues, β-blockers as well as nucleic acids and their bases.

Another carboxyl bonded HILIC stationary phase was prepared by coupling thioglycolic acid to vinyl-bonded silica *via* "thiol-ene" click reaction [47]. The carboxyl groups in the novel stationary phase are responsible for the HILIC/weak cation exchange mixed mode chromatographic behavior of this column. The negatively charged surface of

the carboxylic stationary phase was proven by the ζ-potential measurements. The study of the effect of the water content in the mobile phase on the retention of some nucleosides on the novel stationary phase demonstrated the contribution of hydrophilic partitioning, hydrogen bonding and ion-exchange interactions towards the retention of polar solutes.

A novel sulfonic-azobenzene functionalized silica based stationary phase was synthesized through the preparation of a new sulfonic-azobenzene monomer and its grafting on mercaptopropyl modified silica by a surface-initiated radical chain transfer reaction [48]. Due to the presence of multiple nonpolar groups within its structure, this novel stationary phase was successfully used for the separation of aromatic isomers, PAHs and steroids in the RPC mode. Moreover, the sulfonic, amide and other polar groups of the sulfonic-azobenzene functionalized silica make it appropriate to be used as a HILIC stationary phase. According to the authors, this stationary phase can also be used in strong cation-exchange mode owing to the presence of the negatively charged sulfonic acid groups.

A novel cellulose based stationary phase was prepared by coating cationic cellulose onto a sulfonated silica through ionic interactions [49]. In this regard, silica was first functionalized with γ-mercaptopropyl triethoxysilane followed by the oxidization of the thiol groups by reacting them with H_2O_2 and H_2SO_4 to obtain sulfonated silica. The cationic cellulose was then coated onto anionic silica surface *via* ionic interactions. However, the coated cationic groups could not cover all the surface sulfonate anions and the stationary phase showed negative ζ-potential from pH 2 to pH 7 indicating the net negative surface charges. After coating with cellulose the silica surface had large

amounts of hydroxyl groups giving excellent hydrophilicity for HILIC applications. The column was successfully used for the HILIC separation of carbohydrates and for the selective enrichment of glycopeptides.

Zwitterionic Silica Bonded Stationary Phases for HILIC. Zwitterionic molecules contain both anionic and cationic functionalities in the same molecules, thus providing hydrophilic interaction, weak cation-exchange interactions and weak anion exchange interactions, and in turn multi-mode retention possibilities. In this regard, sulfoalkylbetaine molecules possessing both positively charged quaternary ammonium and negatively charged sulfonic acid groups are a popular choice for the functionalization of silica in order to achieve zwitterionic HILIC stationary phases. An acrylamide type sulfobetaine monomer, 3-[2-(acrylamide)-ethyldimethylammonio] propane sulfonate was coated on mercaptopropyl modified silica *via* controlled thiol-initiated surface polymerization strategy in order to synthesize a sulfobetaine zwitterionic polymeric layer type stationary phase [50]. The resulting polymer coating has hydrophilic polyacrylamide backbone and zwitterionic sulfobetaine side chains giving the hydrophilic character to the prepared column. According to the authors, electrostatic interaction between the stationary phase and the tested analytes was weak while hydrophilic interaction played a dominant role in the retention of polar compounds.

A zwitterionic stationary phase with controllable ratio of positively charged tertiary amine groups and negatively charged carboxyl groups was synthesized by bonding amino functional silane, (N,N-diethyl-3-aminopropyl)trimethoxysilane and carboxyl functional silane, 2-((2-(trimethoxysilyl)ethyl)thio)acetic acid onto the silica

surface [51]. The net charge of the zwitterionic stationary phase was controlled by changing the ratio between attached amino functional silane and the carboxyl functional silane onto the silica surface. Effect of salt concentration of the mobile phase on retention factor of charged solutes were used to determine the net surface charged on several prepared zwitterionic stationary phases. According to the authors the prepared zwitterionic stationary phases demonstrated a mixed-mode retention mechanism composed of surface adsorption, partitioning and electrostatic interactions.

A novel type of tridentate zwitterionic HILIC stationary phase was prepared by covalently bonding *N*-4-azidobenzyl-iminodiacetic acid (IDA) on alkyne-silica gel *via* copper (I) catalyzed azide-alkyne 1, 3-dipolar cycloaddition reaction (CuAAC) [52]. The chromatographic mechanism showed that the prepared stationary phase possessed multi retention mechanisms, such as partition mechanism, hydrogen bonding interaction, chelate and electrostatic interaction mechanisms. The column was successfully used to separate a wide range of moderate to highly polar compounds under HILIC isocratic elution conditions.

An arginine functionalized zwitterionic HILIC stationary phase was prepared by clicking arginine onto silica gel [53]. In this study, the amine group of the arginine was transferred to an azide, which was then clicked onto an alkyne-functionalized silica *via* CuAAC click reaction. The ζ-potential studies demonstrated a positive value up to pH 5.5 and a negative value above pH 5.5 on this stationary phase. The polar functionalities of arginine, triazole ring and the carbonyl functionalities on the prepared stationary phase provided the hydrophilicity for the column. In addition to the hydrophilic partitioning,

hydrogen bonding and electrostatic interaction were also involved in the separation of charged solutes. In addition to charged solutes, six sugars were baseline separated with a very high separation efficiency on this column and these sugars were eluted in the order of increasing polarity indicating the typical HILIC retention behavior of the column. According to the authors, the click-arginine stationary phase demonstrated excellent selectivity and high capacity toward glycopeptides and thus could become a promising material in glycomics and glycoproteomics.

Another zwitterionic stationary phase containing both negatively charged phosphate groups and positively charged amino groups was synthesized by the modification of *N*-β-aminoethyl-γ-aminopropyl-functionalized silica particles with trichlorophosphine oxide ($POCl_3$) for use in HILIC [54]. The effect of ionic strength of the mobile phase on the retention of acidic and basic solutes revealed that the separation mechanism of the novel stationary phase might be based on partitioning, surface adsorption and electrostatic interactions. The effective separation of several smaller polar compounds including water-soluble vitamins, nucleosides and nucleobases and organic acids was achieved on the novel zwitterionic stationary phase under HILIC separation conditions.

A 4-propylaminomethyl benzoic acid bonded silica was synthesized by reacting an aminopropyl modified silica with 4-carboxybenzaldehyde and reducing the resulting Schiff base with sodium cyanoborohydride *in situ* in order to achieve a zwitterionic stationary phase [55]. The novel zwitterionic stationary phase can be operated in anion and cation exchange modes as well as HILIC mode and is capable of separating a range

of polar compounds, including aromatic sulfonates, amines, amino acids and carboxylic acids.

A novel Congo red (CR) derivatized silica stationary phase was prepared through the bonding of CR to glycidoxypropyl modified silica gel [56]. Congo red is a sulfonic-azobenzene dye, which is commonly used as an acid-base indicator. The two azo groups are protonated in the pH range 3.0-5.2 causing a color change in the dye from blue to red. Therefore, CR is considered as a zwitterion. Due to the presence of multi-functional groups, the novel stationary phase was able to separate both hydrophilic and hydrophobic solutes mixture under both HILIC and RPC separation conditions showing mixed-mode chromatographic behavior.

Based on the brief overview provided above, silica-based polar stationary phases are still evolving toward improved selectivity and enhanced separation efficiencies to meet the major goal of HPLC analysis, which high performance separations and rapid analysis. More concerted efforts are still needed, and this thesis is attempting to make a solid contribution in this area.

Overview of Affinity Chromatography

Affinity chromatography is a variant of adsorption chromatography where the target molecule to be separated is specifically and reversibly adsorbed onto a biologically related complementary ligand usually immobilized on a chromatographic support material. The principle of affinity chromatography is shown in Fig. 5. First, one pair of interacting molecules (affinity ligand) is immobilized onto a rigid, insoluble solid support and packed into a column. Then the complex sample containing the target molecule is

introduced into the column. Inside the column, the target molecule is specifically captured by the affinity ligand and other sample components will be washed away with the mobile phase. In a later step, elution of the adsorbed target molecule is achieved by disturbing the interactions between the affinity pair.

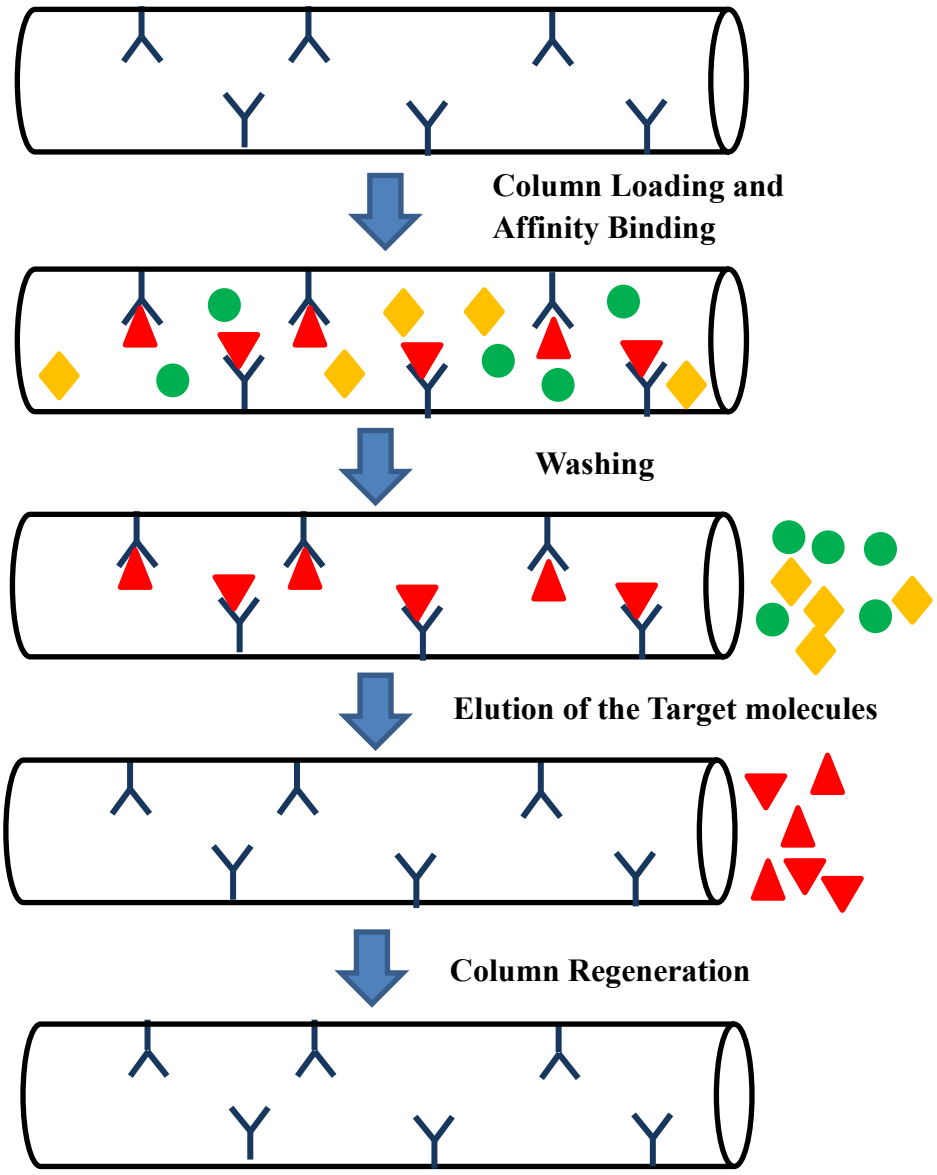

Figure 5. *Schematic representation of the principle of affinity chromatography.*

The binding in between the affinity ligands and the target molecule is due to several non-covalent type of interactions such as hydrogen bonding, vander waals interactions, hydrophobic and electrostatic interactions. The affinity ligands used for the immobilization can be divided into two categories, namely specific and general [57]. In this regard, specific ligands such as antibodies bind only with one particular target molecule (antigen). Antibodies are Y shaped glycoproteins (MW ~ 150, 000 kDa) related to the immune system containing two heavy chains and two light chains, which are connected by disulfide bonds. Antibodies have high affinity towards the target antigen and they are divided into two categories based on the way of production namely monoclonal and polyclonal antibodies. The chromatographic technique, which uses antibodies as the affinity ligands is called immunoaffinity chromatography. In contrast, general ligands such as lectins, bind with certain groups of molecules such as glycoproteins. These affinity ligands, mostly proteins, are usually immobilized onto the chromatographic supports by using covalent bonding in between the chromatographic support and the free amino groups in the ligands. Other functional groups in the proteins such as carboxylic acid, phenol and thiol groups are also occasionally used.

The elution of captured target molecules can be done using either bio-specific methods or nonspecific methods [57]. In bio-specific elution methods, a modifier (inhibitor), which is similar in chemical nature to the affinity ligand or the target molecule is added into the elution mobile phase. These modifier molecules compete with the affinity pair to bind with them and elute out the target molecule. In nonspecific elution methods, the interactions between the affinity pair are weakened by means of changing pH or ionic strength, using chaotropic agents or organic solvents.

The ideal chromatographic support in affinity chromatography should be mechanically strong, hydrophilic, biocompatible, provides low levels of nonspecific interactions and provides ease of ligand immobilization [58]. Although the matrices based on carbohydrates can be used for this purpose owing to their distinct advantages including low levels of nonspecific interactions and high density of available functionalities for the ligand immobilization, they undergo severe shrinkage and compressibility in HPLC applications due to their low mechanical stabilities. Therefore, silica supports with excellent mechanical stabilities are widely used in affinity chromatography applications. However, acidic silanol groups on the silica surface can undergo severe nonspecific interaction with the sample thus lowering the specificity of the affinity chromatographic technique. Therefore, various attempts have been made to synthesize silica-based affinity chromatographic stationary phases for the purification of a wide range of molecules under reduced nonspecific interactions. In this regard, recent advancements of the silica based affinity chromatographic stationary phase development are discussed below.

Recent Advances in Silica Based Lectin Affinity Chromatography

Lectin affinity chromatography (LAC) is an important tool for the selective enrichment and fractionation of proteins in glycoproteomics analysis. A silica-based concanavalin A (Con A) material using oxidized dextran as the spacer was synthesized for the enrichment of glycoproteins/glycopeptides [59]. Oxidized dextran not only served as the molecular spacer to minimize non-specific adsorption, but also provided a large number of aldehyde groups to maximize the conjugation capacity. In this study, oxidized

dextran was immobilized onto a aminopropyl functionalized silica in order to immobilize the lectin Con A *via* a Schiff base method. For comparison, another silica based Con A material was prepared *via* conventional 3-glycidoxypropyl trilmethoxysilane as the initial spacer arm. Higher binding capacity of ovalbumin was observed on the dextran modified silica compared to the epoxy activated silica possibly due to the higher volumetric ligand density and minimum steric hindrance of the dextran modified stationary phase. Furthermore, ζ–potential measurements demonstrated that the residual silanol groups of silica were effectively shielded on dextran-modified silica leading to lower level of nonspecific interactions. Moreover, the evaluation of both Con A columns with model glycoproteins/glycopeptides revealed the higher specificity and higher trapping efficiency of dextran modified silica Con A stationary phase towards the target high mannose type and complex type glycoproteins.

Another lectin affinity chromatography material was synthesized by covalent bonding of Con A onto silica gel *via* simple, rapid and robust method [60]. In this regard, silica gel was first functionalized with a spacer arm containing an amide bond and a carboxyl residue obtained *via* reaction between 3-aminopropyl trimethoxysilane and glutaric anhydride. Then, the modified silica particles were activated by *N*-ethyl-*N'*-(3-dimethylaminopropyl)carbodiimide (EDC) and *N*-hydroxylsuccinimide (NHS). Subsequently, Con A was immobilized onto EDC/NHS activated silica followed by blocking the unreacted NHS groups with ethanolamine. The prepared silica bonded Con A material was successfully utilized for the selective and efficient enrichment of *N*-linked glycoproteins and glycopeptides with high binding capacity, excellent affinity selectivity and low sample loss.

A novel mechanically stable spherical silica particle of less than 2 μm, containing an interconnected network of macropores (100 nm pore diameter) has been developed [61]. As a result of their unique mesh like pore architecture, these particles offer high specific surface area of 200 m^2/g. To demonstrate their applicability, they have been functionalized with two different lectins namely Con A and *Aleuria aurantia* lectin (AAL) and their binding properties were tested with different standard glycoproteins. According to the authors, the high surface area of the novel material allows a high density of ligands to be immobilized for the sake of affinity chromatography. The apparent increase in binding affinity was observed due to the high density of immobilized lectins. The separation of a series of standard glycoproteins and microliter volume of blood serum samples revealed the applicability of these prepared lectin micro columns for high sensitive glycomics and quantitative glycoproteomic profiling.

Recently, a macroporous silica coated with polyvinyl alcohol (PVA) or its cationic derivative diethylaminoethyl PVA (DEAE-PVA) and cellulose were functionalized with Con A to compare the effectiveness of polymer coated silica and cellulose based adsorbents for lectin affinity chromatography [62]. The results showed that the coated silica possessed higher non-specific interaction with glucose oxidase compared to that of the cellulose based stationary phase. Moreover, the sorption capacity for cellulose based Con A stationary phase was higher than that of the silica based stationary phases showing significant advantages of natural polymers as chromatographic supports despite their poor mechanical stabilities.

Recent Advances in Silica Based Immunoaffinity Chromatography

Immunoaffinity chromatography, which is a sub group of affinity chromatography utilizes the high specificity in antibody-antigen interactions for the purification and pre-concentration of target molecules (antigen). In this regard, Nakanishi et al. have immobilized staphylococal protein A onto mesoporous silica (MPS) for the specific capturing of the immunoglobulin G (IgG) [63]. It is well known that protein A has high affinity for the Fc fragment of IgG. In addition, protein A is capable of binding to human IgM, IgA and IgE [64]. Protein A was immobilized onto MPS *via* three different methods, namely, covalent attachment, hydrophobic bonding and disulfide bonding. These results suggested that the covalent bonding between the Protein A and MPS functionalized with 3-glycidoxypropyl trimethoxysilane enhanced the stability of the immobilized protein A and also the IgG binding specificity. According to the authors, the optimization of the pore size of MPS can notably enhance the specific activity and stability of the protein A by correctly fit them into the pore and allowing easy access of the IgG.

The same research group reported the functionalization of MPS with anti-IgG monoclonal antibodies for use in immunoaffinity chromatography as an extension to the their previous work [65]. Antibodies were immobilized onto four types of MPSs with different pore structures and different particle morphologies. The MPS with 2-D hexagonal pore structure and polyhedral particle morphology had the highest antigen binding capacity of all of the MPS prepared. Furthermore, the antibody/MPS composites retain their stability in chaotropic and low-pH eluting solvents with no decrease in the

binding capacity showing the reusability of the prepared immunoaffinity stationary phases.

Furthermore, an immunoaffinity stationary phase for the selective extraction and determination of aflatoxins in agriproducts have been synthesized by covalently coupling monoclonal antibody 1C11 against aflatoxins to amino functionalized silica gel microparticles [66]. In this regard, phosphate buffer and methanol were used as the binding mobile phase and eluting mobile phase, respectively. A calibration curve was constructed using the chromatographic peaks of the aflatoxins at different concentration levels for the determination of aflatoxins in agriproducts. Under the optimized conditions, the method was validated for linearity, precision, recovery and detection limits.

In view of the above overview of the most recent literature, the field of affinity stationary phases at reduced nonspecific interactions that are mechanical rigid and chemically stable to operate in the field of HPLC is still a major theme of research. This dissertation is aiming at making a contribution in the same direction.

Rationale and the Scope of the Investigation

As discussed earlier in this chapter, HILIC stationary phases with various support materials and different surface chemistries is being continuously developed, to address complicated separation challenges. In fact, there is no common stationary phase for HILIC applications such as octadecyl stationary phase in RPC. In addition due to the complex nature of interactions between polar solutes and the polar HILIC stationary phase, the same solute mixture might give different selectivity and resolution on different HILIC columns. Despite the fact that numerous HILIC stationary phases have been

developed so far, there is always the need of novel stationary phases with higher hydrophilicity, different surface chemistries and good stability for the fast separation of a wide range of polar solutes with high resolution to reach towards an ideal HILIC separation material. In addition, the existing chromatographic supports for affinity chromatographic separations are not capable to fulfill the requirement of an ideal separation matrix including the mechanical and chemical stability, low level of non-specific interactions and ease of functionalization with affinity ligands. For instance, although carbohydrate based chromatographic supports are biocompatible and provide low levels of nonspecific interactions they undergo severe shrinkage and compressibility during HPLC analysis due to their poor mechanical stabilities. On the other hand, silica supports with excellent mechanical stabilities exhibit high levels of nonspecific interactions with analytes reducing the selectivity of the analysis. Therefore, the development of mechanically stable chromatographic support with reduced nonspecific interactions for affinity chromatographic applications remains a challenge until today. Further rationales are provided in the introduction of each chapter concerning each particular investigation.

Therefore the primary objective of this dissertation is to develop novel robust polar silica-based and monolithic-based stationary phases for the separation of a wide range of polar solutes under HILIC separation conditions and to utilize them in affinity chromatography as the initial and hydrophilic support to immobilize various affinity ligands in the purpose of selective depletion and enrichment of proteins in human serum under reduced nonspecific interactions.

In the work described in Chapter II, two different types of amine based monolayer and multilayer monolithic based HILIC stationary phases were developed. In Chapter III, tris(hydroxymethyl) aminomethane (TRIS) was bonded to epoxy functionalized silica microparticles to developed a cationic HILIC stationary phase for the separation of a wide range of polar solutes. The TRIS-silica stationary phase thus obtained was further coated with an addition layer of anionic sulfated polysaccharide chondroitin sulfate A (CSA) in order to get multilayer HILIC separation matrix.

In Chapter IV two different sugar functionalized silica-based stationary phases, namely sorbitol silica (SOR-silica) and maltose silica (MALT-silica) were developed and characterized under HILIC separation conditions. The developed neutral but polar sugar based stationary phases are believed to have expanded the selectivity of HILIC applications by separating charged solutes under little or no electrostatic interactions.

Finally, in Chapter V, MALT-silica stationary phase which was developed Chapter IV by combining the excellent mechanical stability of silica with the chemical inertness and the biocompatibility of sugars was used as the chromatographic support for the immobilization of various affinity ligands (e.g., antibodies and lectins) in order to utilize them in high performance affinity chromatographic applications under reduced nonspecific interactions. These affinities MALT-silica based stationary phases were successfully used for the depletion of high abundance proteins as well as for the fractionation and enrichment of low abundant proteins in human serum.

Concluding Remarks

This chapter provides an overview of the fundamental aspects of HPLC with its basics and some of the chromatographic parameters as well as an overview of various separation techniques including HILIC and affinity chromatography. Furthermore, the current chapter has summarized and discussed the advances made in silica-based stationary phases in the areas of HILIC and affinity chromatography over past 3-4 years. Also, the overall rationale and the scope of the current studies have been summarized.

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CHAPTER II

PREPARATION AND CHARACTERIZATION OF SINGLY AND MULTILAYERED MONOLITHIC STATIONARY PHASES FOR HYDROPHILIC INTERACTION LIQUID CHROMATOGRAPHY

Introduction

Hydrophilic interaction chromatography (HILIC), as coined by Alpert who introduced this mode of chromatography in 1990 has gained an increasing popularity in the last couple of decades for the separation of polar solutes in complex mixtures [1-3]. As it already mentioned in the Chapter I, HILIC analyses are carried out with a high organic, low aqueous mobile phase on a polar stationary phase and therefore it is considered as a variant of the normal phase chromatography (NPC) technique. Polar solutes are separated by differential and selective partitioning between an adsorbed water layer on the polar stationary phase and the hydro-organic mobile phase. HILIC has been applied to separate various analytes including, carbohydrates, peptides, proteins, nucleotides and numerous other polar compounds.

Organic polymer monolithic columns for chromatography have been developed as continuous, single cylindrical blocks of highly porous materials that contain both large channels for unrestricted rapid flow through of mobile phase and a number of small pores that provide the retentive surface for the separation [4]. Porous organic polymer monolithic columns with amine [5-7], diol [8, 9], cyano [10], amide [6, 11], polyhydroxyl [6] and sulfoalkylbetaine [12] functionalities have been developed as HILIC stationary phases during the last two decades. These monolithic stationary phases have some unique chromatographic characteristics compared to particle packed stationary phases, including (i) high permeability, which leads to lower backpressure, (ii) low resistance to mass transfer, (iii) simple preparation procedure and ease of surface functionalization, (iv) stability under extreme pH conditions and (v) ease of miniaturization [13]. Among various HILIC monolithic columns reported, those containing amine functionalities are more attractive due to their ability to provide a multimode retention mechanism including hydrophilic partition and anion exchange. However, HILIC applications of amine based monoliths are limited by the possibility of forming Schiff bases between the amine groups on the monolith and some aldehyde containing sample components, thus reducing the life time of the column.

HILIC polymer monoliths are generally prepared by free radical polymerization of functional monomers with cross-linking agents in the presence of pore-forming solvents (i.e., porogens). This direct synthesis is convenient in the sense that the functionalities needed for solute retention are already present on the monolithic surface and there is no need for performing a post functionalization step [8]. Direct synthesis of HILIC monoliths with desired functionalities is not always possible due to (i) lack of

commercially available polar monomers, (ii) limited solubility of highly polar monomers in commonly used porogenic solvents, and (iii) tedious re-optimization of the polymerization conditions from monolith to monolith [14]. Therefore, most often post polymerization functionalization is also conducted to introduce the desired ligands to the polymer monolithic surface. One of the major advantages of this strategy is that starting from one template monolith matrix with optimized mechanical and flow through porous properties, one can prepare other monoliths with the bonded surface ligands of choice [13]. Although poly(glycidyl methacrylate-co-ethylene dimethacrylate) (poly(GMA-co-EDMA)) is a popular precursor monolith for post polymerization functionalization, relatively few reports have been published regarding the use of functionalized poly(GMA-co-EDMA) monoliths in the separation of low molecular weight solutes under HILIC mode [7, 15-18].

Two different types of directly synthesized and post polymer functionalized amine based HILIC monolithic stationary phases are reported in this chapter. In this regard, a cationic amine based polymer monolith (AP monolith) was prepared from the direct *in situ* co-polymerization of the functional monomer *N*-(3-aminopropyl) methacrylamide hydrochloride (NAPM) and the cross linker ethylene glycol dimethacrylate (EDMA) and then modified with maltose sugars to obtain a multilayer HILIC stationary phase as a continuation of the work done by Gunasena and El Rassi [6] in CEC using 100 µm id. capillaries. Furthermore, a reactive poly(GMA-co-EDMA) monolith was synthesized according to the procedure reported by Jmeian and El Rassi [19], which was then functionalized with various polar groups to obtain single and multilayered HILIC stationary phases with amine functionalities. All synthesized polar

stationary phases were extensively tested with polar solute mixtures to evaluate their HILIC separation performances.

Experimental

Instrumentation

All HPLC separations were performed using an HPLC system consisting of a quaternary solvent delivery system Q-grad pump from Lab Alliance (State College, PA, USA), a Model 3100 UV–Vis variable wavelength detector from Milton Roy, LDC division (Riviera Beach, FL, USA) and a Rheodyne injector Model 7010 injector from IDEX Health & Science LLC (Rohnert Park, CA, USA) equipped with a 20 µL loop. Chromatograms were recorded using the clarity version, 3.0.06.589 advanced chromatographic software from Data Apex (Prague, Czech Republic) running on a Dell PC computer. Some of the chromatograms were occasionally recorded with a C-R501 CHROMATOPAC integrator from Shimadzu (Kyoto, Japan). Analyses were carried out at 214 nm and 254 nm wavelengths at 1 mL/min flow rate at room temperature. The retention factors (k) were determined from the retention time of toluene as an unretained marker (t_0) and solute retention time (t_R).

A Model 590 programmable HPLC pump from Waters Corporation (Milford, MA, USA) was used to wash the monolithic column from porogen and unreacted monomers as well as to pass the derivatizing solutions through the columns. A constant pressure pump from Shandon Southern Products Ltd. (Cheshire, UK) was used to transfer the monolith from mold column to the HPLC column. The *in situ* polymerization of the monolithic columns was carried out in an Isotemp Model 2100 water bath from Fisher

Scientific (Fairlawn, NJ, USA). A Sigma 2100 GC oven from Perkin Elmer (Norwalk, Connecticut, USA) or Dupont 860 column compartment oven from E.I. du. Pont de Nemours and Co. (Wilmington, Delware, USA) were used for heating the columns during derivatization.

Reagents and Materials

N-(3-Aminopropyl) methacrylamide hydrochloride (NAPM) was purchased from polyscience Inc. (Warrington, PA, USA). Ethylene glycol dimethacrylate (EDMA), glycidyl methacrylate (GMA), cyclohexanol, dodecanol, glucose, *N*-acetylglucosamine (GlcNAc), adenine, adenosine, cytosine, uracil, uridine, cytidine, vanillic acid, thiourea and ninhydrin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). 2, 2' Azobisisobutyronitrile (AIBN), sinapic acid and caffeic acid were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). Phenol, resorcinol, maltose, sodium acetate and toluene were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Maltooligosaccharide was obtained from Pfanstiehl laboratories (Waukegan, IL, USA). ACS grade acetonitrile (ACN), isopropanol (IPA), acetone and methanol were obtained from pharmco-AAPER (Brookfield, CT, USA). Ammonium acetate and ammonium hydroxide were obtained from Spectrum Quality Products (New Brunswick, NJ, USA). Sodium cyanoborohydride was purchased from Acros Organics (New Jersey, USA). *N*,*N*-Dimethylformamide and formamide were purchased from EM Science (Gibbstown, NJ, USA). Pyrogallol was obtained from J.T Baker Chemical Co. (Phillipsburg, NJ, USA). Thymine was obtained from Nutritional Biochemical Corporation (Cleveland, OH, USA).

Preparation of Sugar Modified AP Monolith

The polymerization solutions used for the preparation of AP monolith have a final weight of 5 g of monomer and porogens. The monomers (NAPM and EDMA), porogens (cyclohexanol, dodecanol and methanol) and the polymerization initiator (AIBN ~ 1% wt% with respect to the monomers weight) were vigorously mixed on a vortex mixer in various ratios, as detailed in Table 1. Each polymerization solution was degassed by sonication for 15 min and purging with N_2 for 5 min. Thereafter, the polymerization solution was introduced into a stainless steel column (25 cm × 4.6 mm id) with fittings at both column ends, which were sealed with end plugs. The polymerization was allowed to proceed at 50 °C for 24 h in a water bath. The end pugs were removed and the column was washed with acetonitrile to remove porogens and unreacted monomers. The column was then washed with DI water followed by 50 mM sodium acetate solution, pH 5.0 for 1 h. After that, a 10% sugar solution consisting of maltose prepared in the acetate solution and containing 50 mM of sodium cyanoborohydride was passed through the column followed by heating at 60 °C in a GC oven overnight. Thereafter, the column was flushed with water and equilibrated with IPA. The modified monolith thus obtained was transferred from the mold column into a shorter HPLC column (10 cm × 4.6 mm id) with packing pressure of 6000 psi- 7000 psi, using IPA as the packing solvent as shown in Fig. 1. Thereafter the column was equilibrated for 1 h with the running mobile phase prior to use in chromatographic analysis.

Preparation of an Amine Modified poly(GMA-co-EDMA) Monolith

The polymerization solutions used for the preparation of poly(GMA-co-EDMA) monolith have a final weight of 5 g of monomers and porogens. The monomers (GMA and EDMA), porogens (cyclohexanol and dodecanol) and the polymerization initiator (AIBN ~ 1% wt% with respect to the monomers weight) were vigorously mixed on a vortex mixer in various ratios, as detailed in Table 2. Each polymerization solution was degassed by sonication for 15 min and purging with N_2 for 5 min. Thereafter, the polymerization solution was introduced into a stainless steel column (25 cm × 4.6 mm id) with fittings at both column ends, which were sealed with end plugs. The polymerization was allowed to proceed at 50 °C for 24 h in a water bath. The end pugs were removed and the column was washed with acetonitrile (ACN) to remove porogens and unreacted monomers. The column was then equilibrated with water followed by continuous passing of 30% (v/v) NH_4OH aqueous solution at 80 °C at 0.5 mL/min flow rate for 3 h. The modified monolith was thoroughly washed with water for about 4 h until the pH of the eluent became neutral. Thereafter, the monolith was transferred from the 25 cm long mold column into another shorter stainless steel column (10 cm × 4.6 mm id, occasionally 5 cm × 4.6 mm id) at 6000 – 7000 psi pressure for 20 min using water as the packing solvent as shown in Fig. 1. Finally, the column was equilibrated for 1 h with running mobile phase prior to use in chromatographic analysis.

TABLE 1

COMPOSITION OF MONOMERS AND POROGENS USED IN THE PREPARATION OF SMAP MONOLITH

Column	wt% Monomers		wt% Porogens			wt% Monomers	wt% Porogens
	NAPM	EDMA	Cyclohexanol	Dodecanol	Methanol		
SMAP 1	5.50	11.73	53.25	9.55	19.97	17.23	82.77
SMAP 2	13.35	11.15	48.50	8.70	18.20	24.50	75.40
SMAP 3	21.00	9.00	37.80	13.30	18.90	30.00	70.00
SMAP 4	17.10	12.90	37.80	13.30	18.90	30.00	70.00

TABLE 2

COMPOSITION OF POLYMERIZATION MIXTURES AND POLYMERIZATION CONDITIONS USED FOR THE PREPARATION OF POLY(GMA-CO-EDMA) MONOLITH

Column	% wt of monomers and porogens				Temperature (˚C)	Polymerization Time (h)
	EDMA	GMA	Cyclohexanol	Dodecanol		
EGAN 1	12.0	18.0	59.5	10.5	50	24
EGAN 2	15.0	15.0	59.5	10.5	50	24
EGAN 3	18.0	12.0	59.5	10.5	50	24
EGAN 4	12.0	18.0	35.0	35.0	50	24
EGAN 5	15.0	15.0	58.0	12.0	50	24
EGAN 6	15.0	15.0	54.0	16.0	50	24
EGAN 7	12.0	18.0	59.5	10.5	70	15

Functionalization of Amine Modified poly(GMA-co-EDMA) Monolith with Sugars

The amine modified poly(GMA-co-EDMA) monolith prepared according to the above procedure was washed with DI water followed by 50 mM sodium acetate solution, pH 5.0 for 1 h. After that, a 10% sugar solution consisting of glucose, maltose, maltooligosaccharide or *N*-acetylglucosamine (GlcNAc) prepared in the acetate solution and containing 50 mM sodium cyanoborohydride was passed through the column followed by heating at 60 °C in a GC oven overnight. Thereafter, the column was flushed with DI water and equilibrated with the running mobile phase for 1 h before using it in a chromatographic analysis.

Chromatographic Conditions for HILIC Separations

Analyses were carried out at 214 nm and 254 nm wavelengths at 1 mL/min flow rate at room temperature. Solution mixtures containing acetonitrile and ammonium acetate buffer were used as the mobile phases unless otherwise specified. The molarity and the pH of the mobile phases are pertinent to the aqueous portion. The pH of the aqueous ammonium acetate buffer was adjusted using formic acid or ammonium hydroxide, before mixing it with the acetonitrile. All the mobile phases were freshly prepared, filtered through 0.1 Whatman filter paper and sonicated for 30 min before use. The retention factors, k, were determined from the retention time of toluene as the unretained marker (t_0) and solute retention time (t_R).

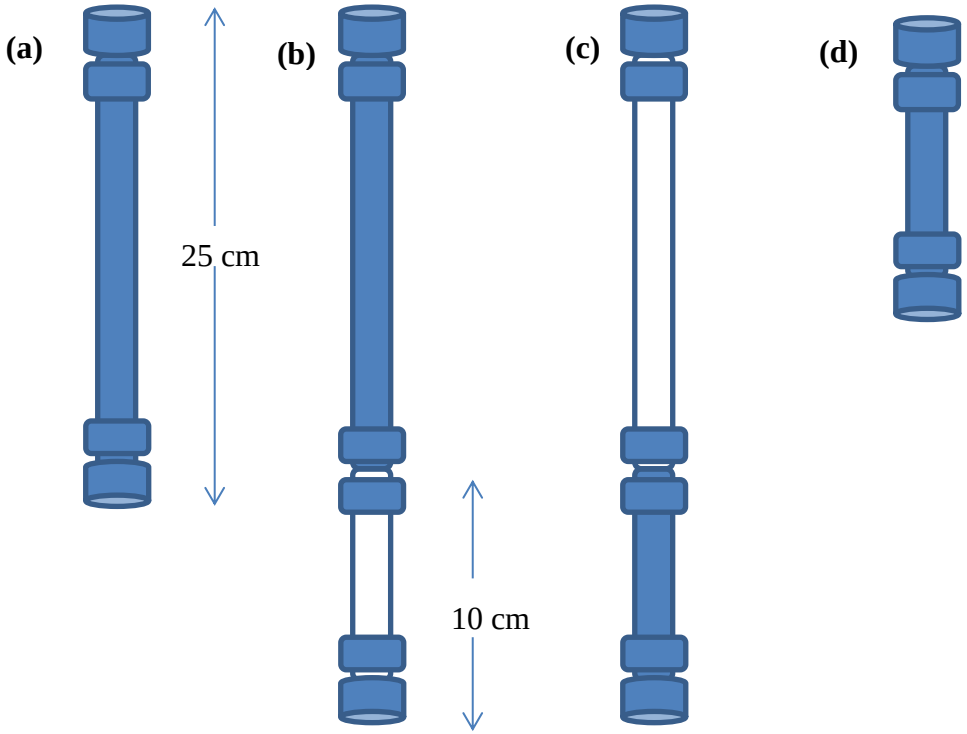


Figure 1. *Monolithic column preparation process. In situ polymerization of monomers along with the porogens was carried out in 25 cm × 4.6 mm id mold column (a). The mold column was connected to an empty 10 cm × 4.6 mm id column by means of ¼ inch union (b). The monolith was pushed from the mold column into the 10 cm long HPLC column using a constant high pressure (6000 – 7000 psi) (c). The 10 cm × 4.6 id column was used for the further study (d).*

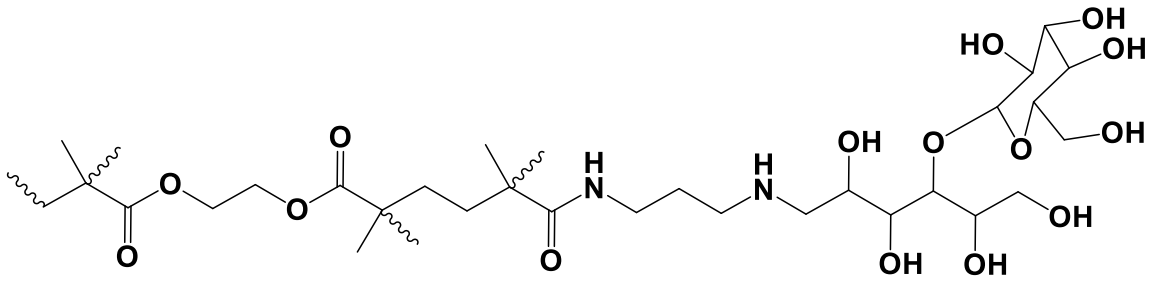
Ninhydrin Test

About 1 cm piece of amine modified poly(GMA-co-EDMA) monolith was forced out from the stainless steel column after removing the column end fittings into a 10 cm test tube. Four milliliters of DI water and 1 mL of 8% (w/v) ninhydrin in acetone were added to that test tube. The test tube was covered with an aluminum foil and heated in boiling water bath for 15 min. Finally, 1 mL of ethanol was added to the test tube after cooling to the room temperature. A control test was done in a similar way using a 1 cm long unmodified poly(GMA-co-EDMA) monolith.

Results and Discussion

Sugar Modified poly(NAPM-co-EDMA) Monolith (SMAP Monolith)

Firstly, monolithic poly(NAPM-co-EDMA) stationary phase containing both amide and primary amine functionalities was prepared by direct copolymerization of NAPM functional monomer and EDMA crosslinker (AP monolith). The composition of the polymerization mixture was systematically optimized using the recipe given by Gunasena and El Rassi as a guide, in order to upscale the CEC monolith in a 100 µm id capillary to an HPLC monolith in a 4.6 mm id stainless steel column [6]. Thereafter, the primary amine groups on this AP monolith were further functionalized with maltose sugars using a reductive amination reaction in order to get the SMAP monolith with amine/amide and polyhydroxy functionalities as shown in scheme 1.



Scheme 1. *Schematic diagram showing maltose modified poly(NAPM-co-EDMA) stationary phase (SMAP monolith)*

Although the effects of the composition of the polymerization mixtures and the polymerization conditions on the surface area, pore properties, retention, and resolution of monolithic columns have been studied extensively in the literature, the choice of porogens, polymerization temperature and other conditions to make a desired monolith still remains semi-empirical. In this regard, the selection of suitable porogenic solvent/solvents which provides sufficient solubility to both relatively nonpolar crosslinker and relatively polar functional monomer is even difficult in HILIC monolithic preparations. Therefore, a ternary porogenic mixture containing cyclohexanol, dodecanol and methanol was chosen to prepare poly(NAPM-co-EDMA) monolith. Methanol in the polymerization solution dissolves both polar solid NAPM and relatively nonpolar EDMA completely leading to a homogeneous polymerization solution.

It is well known that the structure and porosity of monolithic stationary phases can be altered by minor changes in the polymerization mixture. Therefore, a series of

monolithic columns was prepared by varying the monomer and porogen composition in the polymerization mixture, for the purpose of preparing a robust monolithic column which can withstand the high pressure during HPLC analysis. In order to examine the effect of monomer to porogen ratio towards separation performance, three monolithic columns were prepared by increasing the %wt of monomers from 17.23% to 30% while decreasing %wt of porogens from 82.77% to 70%. As can be seen from the results in Table 3, the retention factor (k value) of all analyzed polar solutes increased with increasing weight percentage of monomer in the polymerization mixture due to the increase of the surface functionalities on the monolithic structure. When the porogen content was kept constant at 70.0 % and the weight content of EDMA increased from 9.00% to 12.9%, the k values of all analyzed polar compounds were also increased. This behavior indicates that even with a higher proportion of functional monomers in the polymerization solution, sufficient crosslinker content is also needed in order to incorporate a high percentage of those functional monomers into the monolithic structure. Moreover, increasing the crosslinker content in the polymerization solution might lead to a monolith with smaller pores and greater surface area, which in turn could improve the retention properties as well.

HILIC analyses are usually done using a mobile phase containing relatively high organic content. Therefore, a mobile phase containing 95% acetonitrile and 5% water was used for all HILIC analyses on SMAP columns. First, a neutral, polar, low molecular mass amide mixture containing DMF, formamide and thiourea was baseline separated on

TABLE 3

RETENTION FACTORS (k VALUES) OF POLAR SOLUTES OBTAINED ON FOUR DIFFERENT SMAP COLUMNS

Column	k value								
	DMF	**Formamide**	**Thiourea**	**Phenol**	**Resorcinol**	**Pyrogallol**	**Thymine**	**Uracil**	**Adenine**
SMAP 1	0.150	0.490	0.880	0.0500	0.230	0.650	0.780	1.90	1.69
SMAP 2	0.220	1.11	2.36	0.100	0.820	2.66	1.73	3.06	4.70
SMAP 3	0.320	1.79	3.95	0.190	0.970	3.61	2.56	4.62	7.53
SMAP 4	0.320	1.68	4.48	0.320	1.88	4.41	3.18	5.92	9.38

the SMAP 3 column as can be seen in Fig. 2A. These solutes were separated in the order of increasing polarity, showing a typical HILIC behavior where the least polar DMF eluted first while the highest polar thiourea (which is used as the unretained marker in RPC) eluted last.

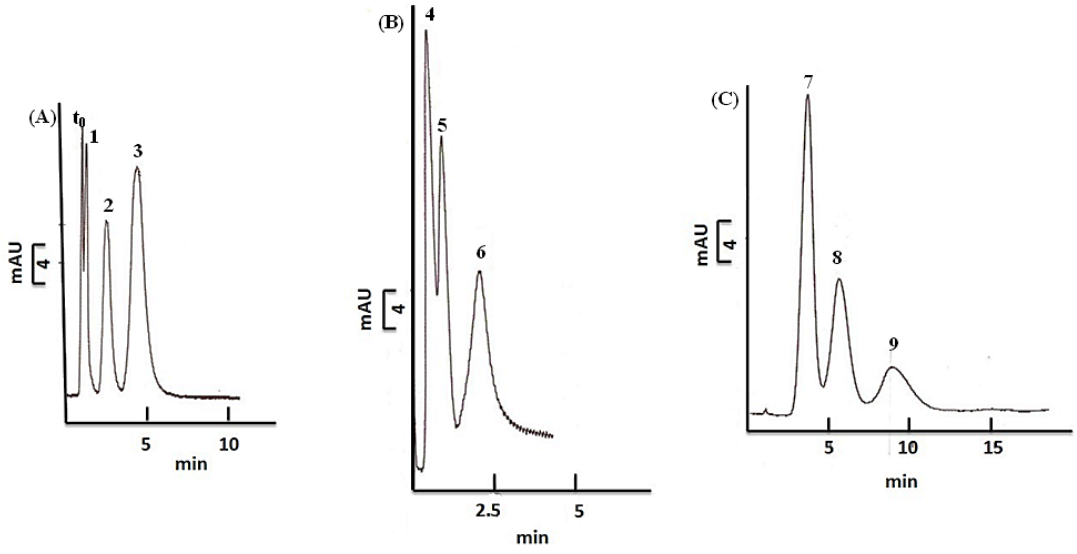


Figure 2. *Chromatograms of three neutral polar compounds (A), three phenols (B) and three nucleobases (C) obtained on SMAP columns. Separation conditions; columns, SMAP 3 column (10 cm × 4.6 mm id) in (A) and (C); SMAP 2 column (5 cm × 4.6 mm id) in (B); mobile phase, 95% ACN (v/v), 5% water (v/v); detection, UV at 254 nm; flow rate, 1 mL/min; injection volume, 20 µL; temperature, room temperature. Solutes; t_0, toluene; 1, N,N-dimethylformamide (DMF); 2, formamide; 3, thiourea; 4, phenol; 5, resorcinol; 6, pyrogallol; 7, thymine; 8, uracil; 9, adenine.*

Thereafter, a mixture of phenolic compounds was separated on the SMAP 2 column as shown in Fig. 2B. As expected, the retention time increased from phenol to pyrogallol in the order of increasing number of hydroxyl groups within the molecule.

The SMAP 3 column was again used to separate polar charged nucleic acid bases as shown in Fig. 2C. As before, all the solutes were eluted in the order of increasing polarity showing the suitability of the column for bio separations in HILIC mode.

Finally, two SMAP 2 columns were prepared with and without functionalization with maltose. The column functionalized with sugars showed higher retention towards all analyzed polar solutes as shown in Fig. 3 due to the increased hydrophilicity given by polyhydroxy/amine/amide multilayer assembly.

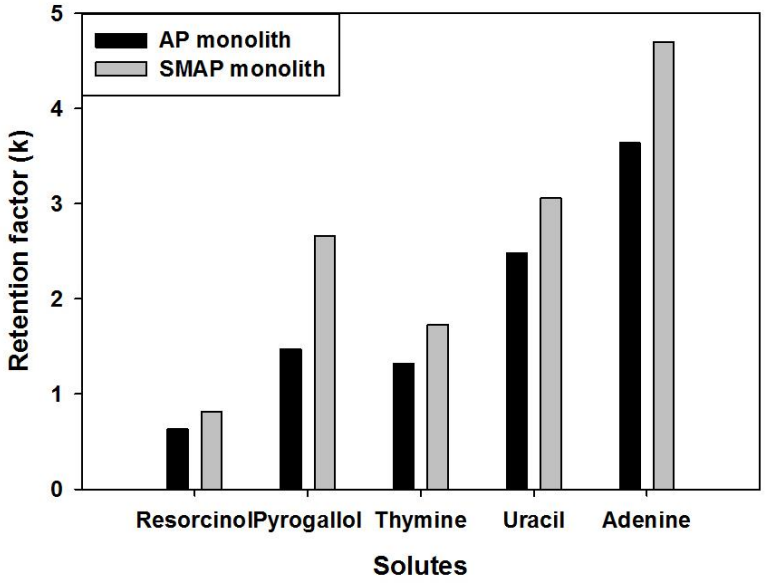


Figure 3. *Comparison of separation performance of the SMAP 2 column before and after modification with maltose. Separation conditions are the same as in Fig. 2.*

Stability and Reproducibility of SMAP Stationary Phase

The SMAP column, which was prepared in a stainless steel HPLC column (10 cm × 4.6 mm id) showed low stability during the HPLC analysis. The backpressure showed a gradual increase with time while peaks showed a decrease in efficiency. Finally, peaks were split into two demonstrating a typical double injection. Around 1 cm of void space

was observed at the inlet after opening the column by removing the end fittings, possibly due to compression of the monolith. The same monolithic column prepared by Gunasena and El Rassi was used in CEC applications without any reported compression problem. Gunasena and El Rassi have used a 100-µm id capillary for their CEC analysis, which relies on the electroosmotic flow (EOF) and not on pumped flow as in HPLC, and consequently compressibility of the packed bed in CEC is avoided. In other words, an EOF in a 100 µm capillary gives no backpressure compared to hydrodynamic flow in a 4.6 mm HPLC column. In addition, the surface silanization during the capillary wall pretreatment process provides a large number of anchoring sites to the monolith allowing strong attachment to the capillary wall thus preventing any monolithic collapse during the analysis. Therefore, although the SMAP stationary phase is suitable for CEC analysis, it is not sturdy enough to withstand the high backpressure in HPLC analysis.

Amine Modified poly(GMA-co-EDMA) Monolith (EGAN monolith)

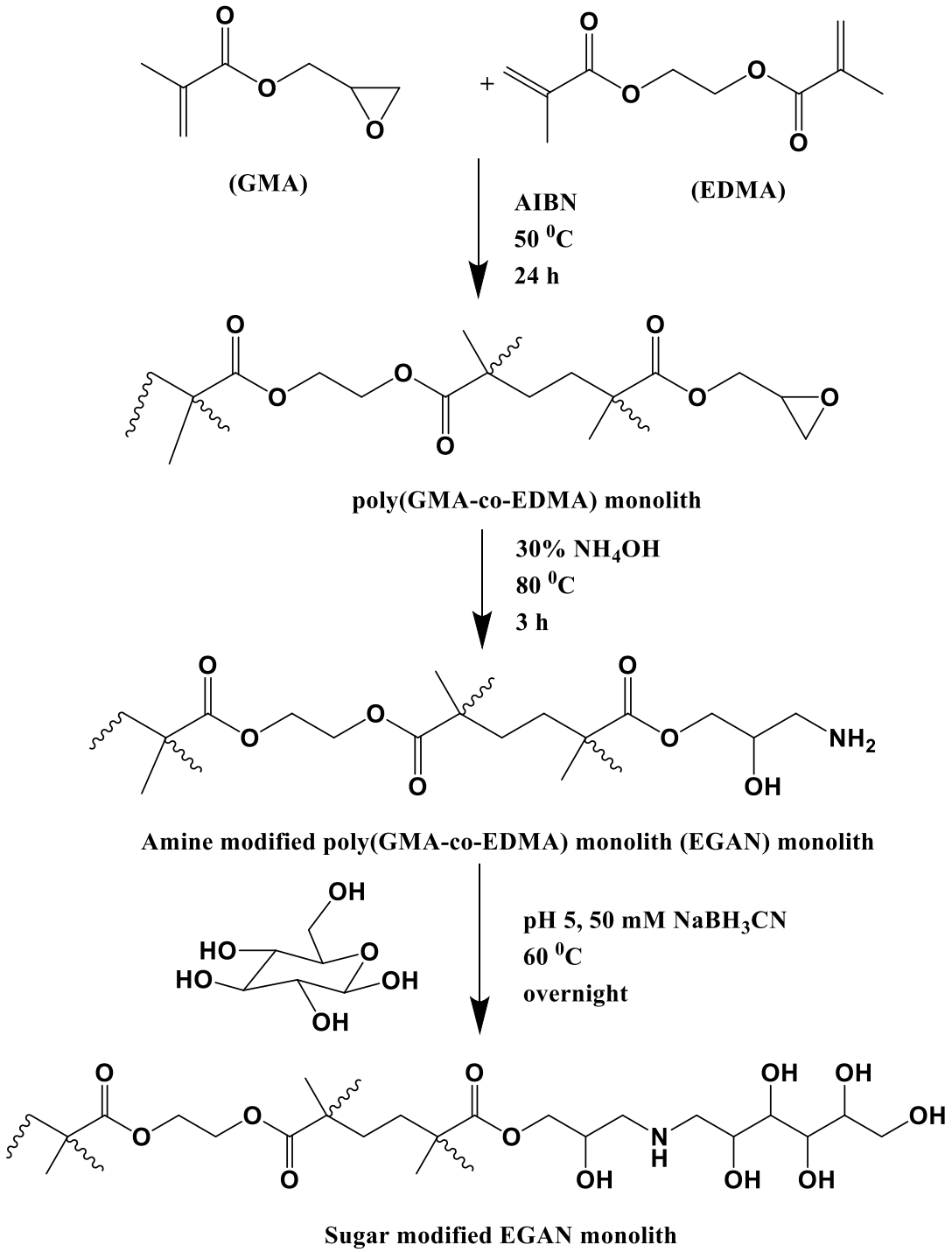
For a second study, an amine based monolithic column referred to as EGAN monolith was prepared, by post polymerization functionalization of the epoxy groups in the poly(GMA-co-EDMA) precursor monolith by reacting it with ammonia [20]. In this regard, a series of monolithic poly(GMA-co-EDMA) columns were prepared to optimize its properties, for the purpose of using it in HILIC separation of small molecules. Generally, this macroporous poly(GMA-co-EDMA) monolith gives broad peaks in small molecule separation leading to poor column resolution due to the lack of small pores, i.e., enough surface area and in turn enough phase ratio [21]. Therefore, an attempt was made to alter the pore properties of this methacrylate monolith to be suitable for small molecule separations in HILIC mode. The variations of pore properties and chromatographic

performance of the poly(GMA-co-EDMA) monolith with minor changes to the polymerization solutions and with polymerization conditions have been extensively studied by Svec et al. and those reports were used as reference for the optimization process [22-24].

After the polymerization was completed, the poly(GMA-co-EDMA) monolith column was heated while continuously pumping ammonia through the column with the aim of maximizing the conversion of epoxide groups into primary amine groups by epoxide ring opening reaction as shown in the Scheme 2. The success of surface functionalization was qualitatively tested using the ninhydrin test, which is a simple test for primary amines. The amine modified poly(GMA-co-EDMA) monolith became blue in color after heating with ninhydrin reagent indicating the successful attachment of amine groups to the monolithic surface.

In order to investigate the influence of crosslinker content in the polymerization solution on the separation performance of the amine modified monolith, three EGAN columns were prepared by increasing EDMA content from 12.0% to 18.0% while keeping the porogenic composition and other conditions the same. Generally, increasing the proportion of crosslinker will decrease the average pore size of the monolith due to early formation of highly crosslinked globules during the polymerization process. This behavior can be seen in the results reported in Table 4, where the average efficiency of the column for the separation of a mixture containing DMF, formamide and thiourea increased from 1470 plates/m to 5460 plates/m by increasing the EDMA content from 12% to 18%. Although the average surface area of the stationary phase increased with the reduced pore size, the retention factors (k) of the polar solutes separated in HILIC mode

showed a decrease, possibly due to the reduced number of functionalities on the monolithic surface resulting from decreasing the functional monomer GMA content.



Scheme 2. *Schematic diagram showing preparation of glucose modified EGAN monolith.*

TABLE 4

RETENTION FACTORS (k VALUES) AND COLUMN SEPARATION EFFICIENCIES FOR SOLUTES OBTAINED ON DIFFERENT AMINE MODIFIED poly(GMA-co-EDMA) MONOLITHS (i.e., EGAN STATIONARY PHASES)

	k Values									Average N plates/m
Column	**DMF**	**Formamide**	**Thiourea**	**Phenol**	**Resorcinol**	**Pyrogallol**	**Thymine**	**Uracil**	**Adenine**	
EGAN 1	0.130	0.600	1.55	0.180	0.690	2.96	1.10	2.17	4.48	1470
EGAN 2	0.0900	0.430	1.25	0.230	0.850	2.87	1.10	1.72	4.20	1590
EGAN 3	0.0800	0.420	1.11	0.260	0.950	2.77	1.10	1.51	3.58	5460
EGAN 4	0.110	0.480	1.24	0.140	0.620	1.61	0.900	1.76	3.20	920
EGAN 5	0.110	0.510	1.39	0.270	1.10	3.93	1.42	2.12	5.52	7950
EGAN 6	0.0200	0.440	1.13	0.210	0.800	2.93	1.03	1.65	3.85	1840
EGAN 7	0.0400	0.670	1.42	0.0500	0.230	0.680	0.420	1.11	1.83	820

Compositions of the EGAN monoliths are given in the Table 2.

A binary porogenic mixture containing cyclohexanol and dodecanol was used for the preparation of poly(GMA-co-EDMA) monolith. The effect of the porogenic composition was next studied by varying the cyclohexanol and dodecanol content while keeping the monomer composition and other conditions the same. As it can be seen from the results for EGAN 1 and EGAN 4 columns, increasing the dodecanol content from 10.5% to 35% resulted in a dramatic decrease of column efficiency from 1470 plates/m to 920 plates/m as well as a decrease in k values for all of the polar solutes analyzed. Dodecanol is less hydrophilic compared to cyclohexanol and it provides lower solubility for the developing methacrylate copolymer system hence leading to larger pores in the final monolith [22]. The monolith with larger through pores and lower surface area leads to decrease in efficiency and retention in HILIC applications. The same behavior can be seen when comparing EGAN 5 to EGAN 6 columns where the average separation efficiency for DMF, formamide and thiourea decreased from 7950 plates/m to 1840 plates/m upon increasing the dodecanol content from 12% to 16%. However, EGAN 5 column containing 58% cyclohexanol and 12% dodecanol gave the best separation performance with average efficiency of 7950 plates/m.

Increasing the polymerization temperature will increase the rate of initiator (AIBN) decomposition and increase the number of precipitating globules at the early stage of polymerization leading to a monolith with smaller pores [22]. Two monolithic columns were prepared at 50 ^{o}C and 70 ^{o}C in order to examine the effect of polymerization temperature towards separation performance. The results obtained from the prepared columns were contradictory to the above theory where EGAN 7 column polymerized at 70 ^{o}C showed poor column efficiency and poor retention in HILIC

separation of smaller polar solutes compared to EGAN 1 which was polymerized at 50 ^{o}C.

The EGAN 2 and EGAN 5 columns, which showed satisfactory separation performance, were tested further in HILIC mode using a wide range of polar solutes. The EGAN 2 monolith with amine functionalities was first used to separate a mixture of small, neutral polar amides containing DMF, formamide and thiourea, which were difficult to separate using reverse-phase chromatography. As it can be seen in Fig. 4A, all three solutes were baseline separated in order of increasing polarity using a mobile phase containing 95% acetonitrile and 5% water, proving the HILIC behavior of the column. Thereafter, a series of phenols were also separated on the EGAN 2 column as shown in Fig. 4B. Although all three solutes were well separated, pyrogallol, which contains 3 hydroxyl groups in its structure, showed a broad peak with around a 3 min peak width at the base.

The EGAN column with primary amine functionalities offers the possibility of electrostatic interactions with analytes carrying negative charges. A series of phenolic acids were then separated on EGAN 2 columns as shown in Fig. 5A. As expected, a mobile phase containing 75% acetonitrile and 25%, 25 mM ammonium acetate was required to elute the anionic phenolic acids from the cationic EGAN column within reasonable time. Moreover, all phenolic acids yielded broad peaks showing the poor efficiency of the cationic EGAN column towards the separation of anionic solutes under HILIC mode. The electrostatic interactions between the cationic EGAN column and the anionic solutes together with the reduced surface area of the stationary phase might lead to this poor separation efficiency [25].

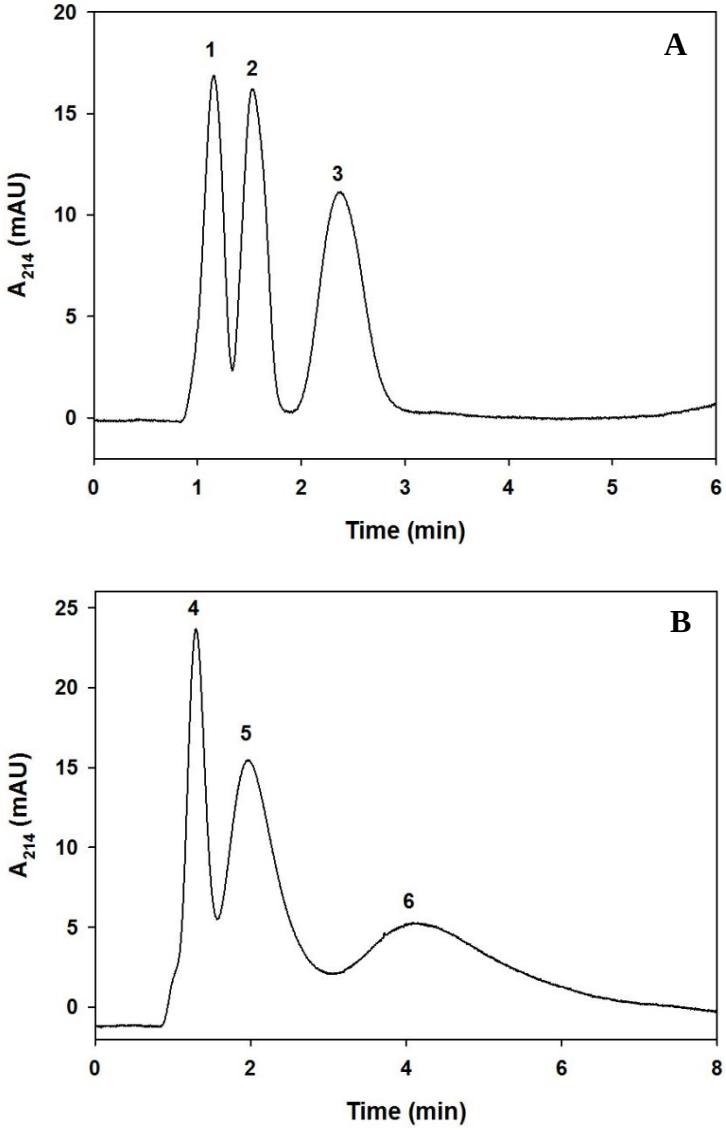


Figure 4. *Chromatograms of three neutral polar compounds (A) and three phenols (B) obtained on the amine functionalized poly(GMA-co-EDMA) monolith (EGAN 2 monolith). Separation conditions; column, EGAN 2 column (10 cm × 4.6 mm id); mobile phase, 95% ACN (v/v), 5% water (v/v); detection, UV at 214 nm; flow rate, 1 mL/min; injection volume, 20 µL; temperature, room temperature. Solutes: 1, N,N-dimethylformamide (DMF); 2, formamide; 3, thiourea; 4, phenol; 5, resorcinol; 6, pyrogallol.*

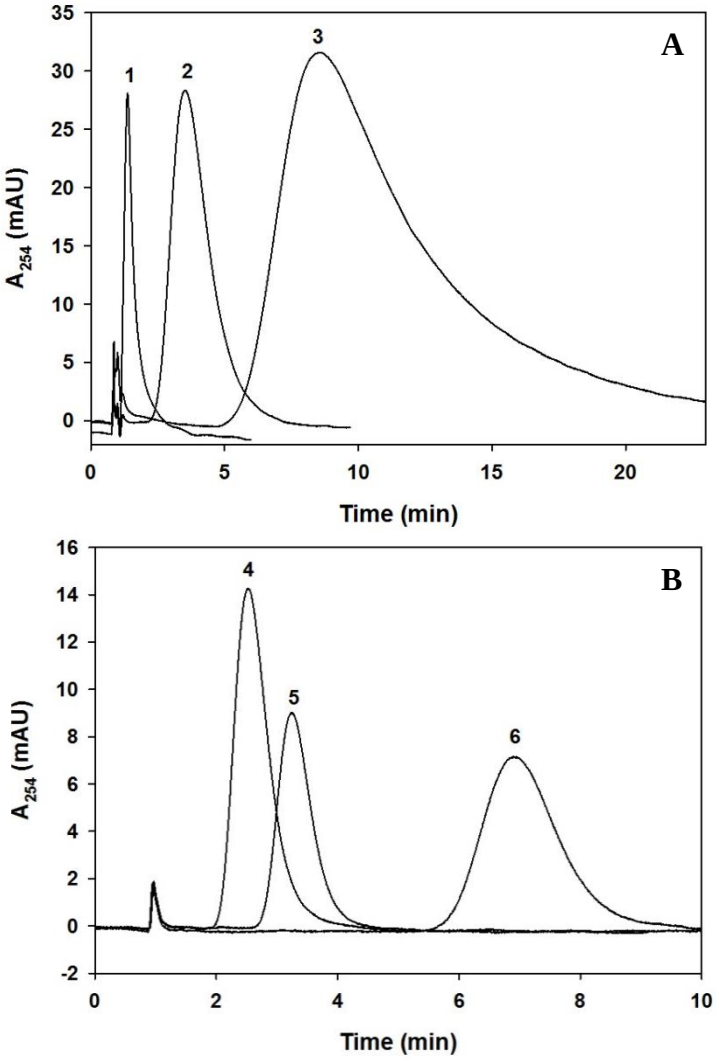


Figure 5. *Chromatograms of three phenolic acids obtained on the EGAN 2 monolith (A) and three nucleobases obtained on the EGAN 5 monolith (B). Separation conditions are the same as Fig. 4A, except that the detection was in the UV at 254 nm. Mobile phases: 75% ACN (v/v), 25% 25 mM ammonium acetate, pH 3 in (A) and 95% ACN (v/v), 5% water (v/v) in (B). Solutes: 1, sinapic acid; 2, vanillic acid; 3, caffeic acid; 4, thymine; 5, uracil; 6, cytosine.*

Thereafter, the EGAN 5 column which gave the best separation efficiency was used to separate nucleobases as shown in Fig. 5B. All analyzed nucleobases showed broad peaks demonstrating the lower separation performance of the column possibly due to the lack of mesopores to provide sufficient surface area for the separation.

Stability and Reproducibility of EGAN Monoliths

The reproducibility and the stability of the EGAN columns were investigated using thiourea as the model compound. Intraday, interday and column-to-column reproducibility for the k values of thiourea were measured and the relative standard deviation (% RSD) were 1.47% (n = 3), 5.46% (n = 2) and 0.45% (n = 2), respectively. Moreover, the columns were used over one month without any significant decrease in column efficiency and without any monolithic compression problem.

Sugar Modified EGAN Monolith

As it was mentioned in the introduction, chromatographic stationary phases with primary amine functionalities may react with aldehydes and many other solutes in the samples leading to unstable retention properties. Therefore, the EGAN monolith containing amine functionalities was further modified with various sugars as shown in Scheme 2, in the aim of deactivating the surface amines as well as for the purpose of obtaining a multi-mode stratified stationary phase with polyhydroxy top layer and secondary amine bottom layer. It was expected to see an improvement in the stationary phase polarity as well as column performance after attachment of the sugar moieties to the amine surface. In this regard, glucose, maltose, *N*-acetylglucosamine (GlcNAc) and maltooligosaccharides, which contain 2 to 7 glucose units with α 1-4 linkages were

covalently attached to the amine surface by using the well known reductive amination reaction. After modifying the EGAN monolith with sugars, significant differences in retention behavior were observed as shown in Fig. 6A, where the k values of the neutral polar amides increased while the k values of the nucleobases decreased with respect to those on unmodified column. The presence of the polar amine, polyhydroxy and amide functionalities on the GlcNAc modified stationary phase might be the reason for the increased k values of the polar amides obtained on the sugar modified column. Moreover, the decrease of the k value for other solutes indicated that the retention behavior was predominantly controlled by the top most sugar layer of the multilayer assembly by establishing different types of interactions with the analytes compared to those on amine surface. This basic principle was further proven when comparing the k values of three different sugar functionalized columns, where k values of phenolic acids increased when increasing the number of glucose units in the attached sugars.

Finally, sugar modified EGAN columns were used for the separation of polar solutes in HILIC mode using a mobile phase containing 95% acetonitrile and 5% water as shown in Fig. 7. As usual, three model compounds namely, DMF, formamide and thiourea were baseline separated with a slight decrease in column efficiency from 1600 plates/m to 1400 plates/m with respect to the unmodified column. Moreover, the separation of phenolic compounds also showed typical HILIC behavior where phenols are eluted in the order of increasing number of hydroxyl groups within the molecule from phenol to pyrogallol, i.e., from 1 to 3 OH.

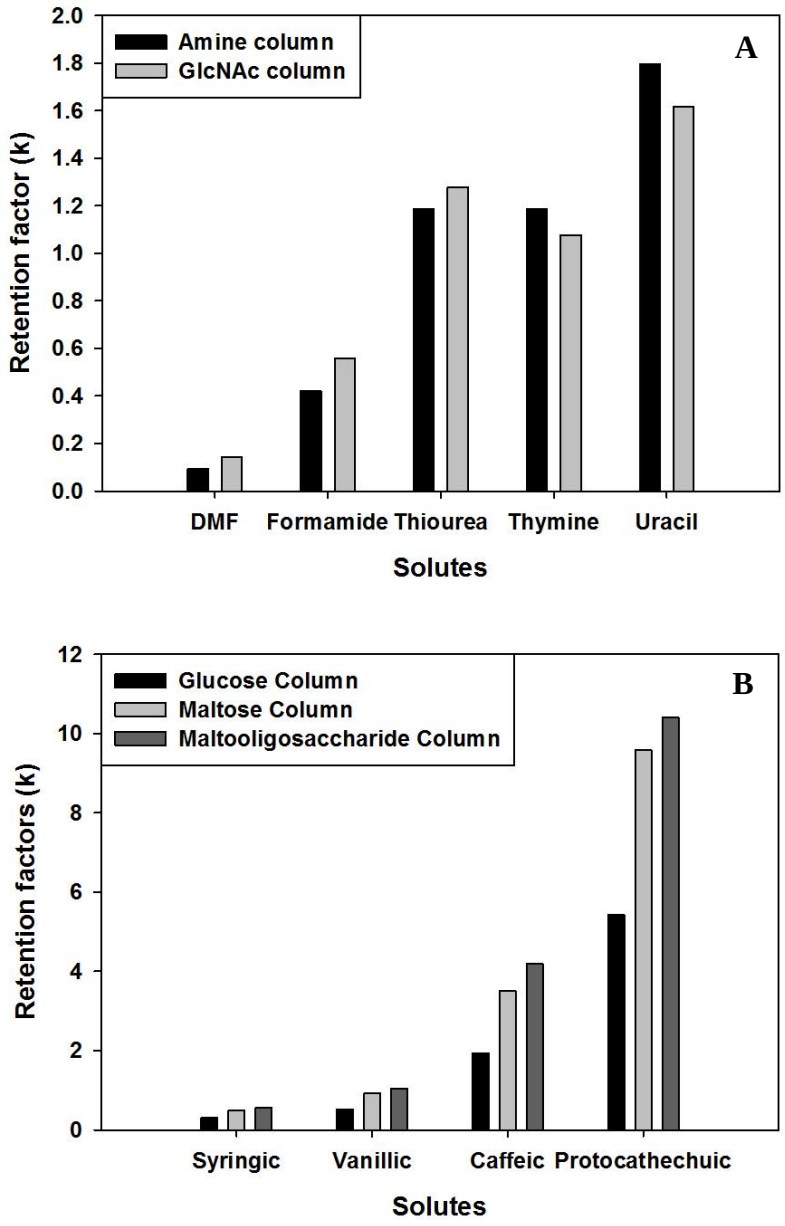


Figure 6. *Comparison of the separations of five polar solutes on EGAN monoliths before and after modification with GlcNAc (A) and comparison of the separations of four phenolic acids on three different sugar modified EGAN columns (B). Separation conditions are the same as Fig. 4A except that the mobile phase was, 95% acetonitrile, 5% 50 mM ammonium acetate, pH 3 in (B); detection, UV at 254 nm for the separation of nucleobases and phenolic acids.*

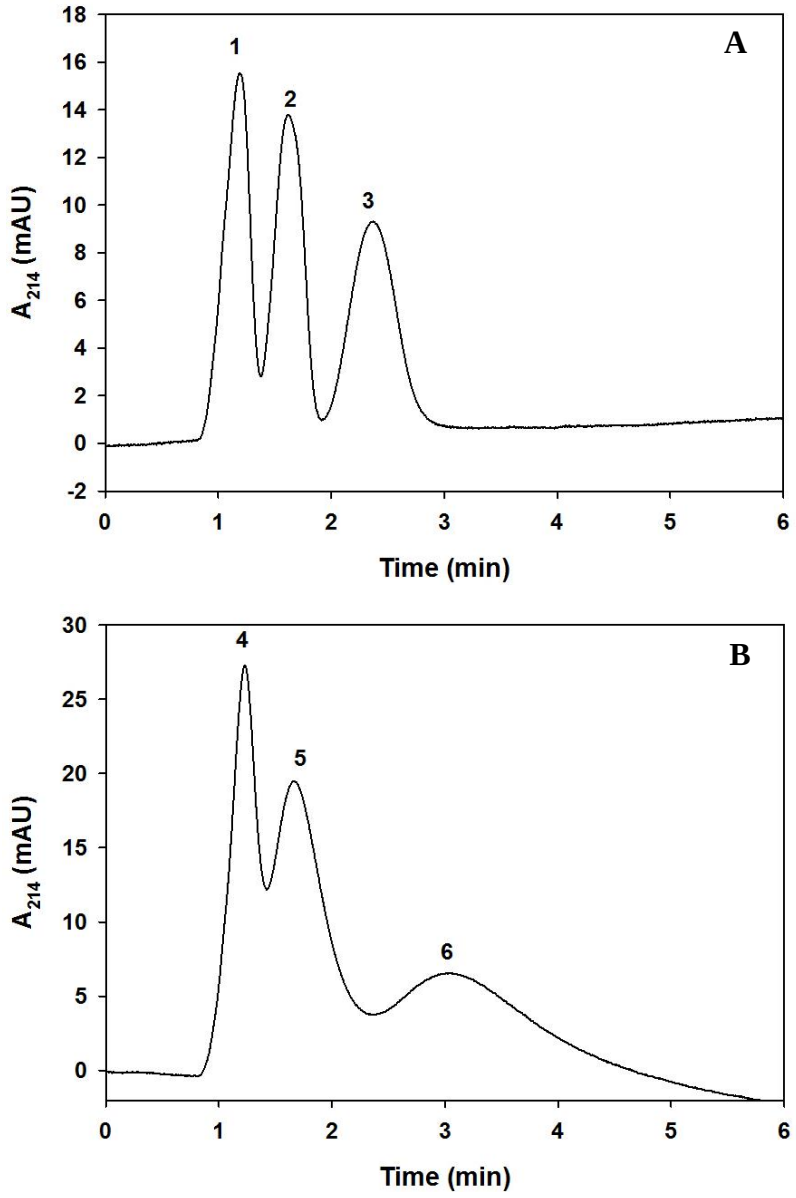


Figure 7. *Chromatogram of the separation of three neutral polar amides (A) and three phenols (B) obtained on GlcNAc modified EGAN 2 monolith. Separation conditions are the same as Fig. 4A. Solutes: 1, DMF; 2, formamide; 3, thiourea; 4, phenol; 5, resorcinol; 6, pyrogallol.*

Conclusions

Although the poly(NAPM-co-EDMA) stationary phase showed satisfactory separation performances in HI-CEC analysis of polar solutes, it is not sturdy enough to withstand higher back pressure in HPLC analysis. The popular precursor monolith, poly(GMA-co-EDMA) monolith can be readily functionalized with ammonia to get the primary amine containing HILIC stationary phases (i.e., EGAN stationary phases). The retention behavior of sugar functionalized EGAN stationary phases are predominantly controlled by the polarity of the top most sugar layer rather than secondary amine sublayer. Although, post polymerization functionalization of the poly(GMA-co-EDMA) precursor monolith with polar functionalities is convenient, the pore properties of the prepared stationary phases are not ideal for small molecule separations. Therefore, all post-polymerization functionalized HILIC stationary phases showed poor separation efficiencies with broad peaks. Further studies have to be done to optimize the pore and surface properties of poly(GMA-co-EDMA) monolith so that it will be useful in small molecules separations under HILIC conditions.

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CHAPTER III

PREPARATION AND APPLICATIONS OF SINGLY AND MULTILAYERED POLAR SILICA STATIONARY PHASES FOR HYDROPHILIC INTERACTION LIQUID CHROMATOGRAPHY

Introduction

Hydrophilic interaction chromatography (HILIC) has gained an increasing interest among separation scientists over the past few decades. In HILIC, a polar (hydrophilic) stationary phase is used with an organic-rich hydro-organic mobile phase in order to separate polar analytes. The use of organic-rich mobile phases in HILIC provides some additional advantages over normal phase chromatography (NPC) and reversed phase chromatography (RPC), making this technique more popular. These advantages include low column backpressure, which allows fast separation of analytes with shorter analysis time and suitability of HILIC to direct coupling with MS detection (e.g., ESI-MS) [1]. As discussed in detail in the previous chapters, hydrophilic partitioning of polar solutes in between the adsorbed water layer on the stationary phase surface and the hydro-organic

mobile phase is considered as the predominant retention mechanism in HILIC separations. However, in addition to the hydrophilic partition, dipole–dipole, ion–dipole, and ion–ion interactions are also involved in the separation of polar solutes on a HILIC column.

Underivatized or bare silica is widely used as a stationary phase in HILIC. Many column manufacturers have designed bare silica columns to be specifically used in HILIC applications (e.g., Zorbax HILIC, Atlantis HILIC). But there are some drawbacks associated with using bare silica columns in HILIC applications including poor stability of bare silica columns at high pH values, acidic surface silanol group interaction with basic compounds which leads to peak tailing, poor reproducibility, low selectivity and poor resolution [2, 3]. Therefore, different types of polar functional groups are bonded onto the bare silica particles as monomeric phases or polymeric phases in order to overcome those drawbacks as well as to increase the hydrophilicity of the separation surface with multiple modes of interactions with solutes, thus broadening the applications range of HILIC. A wide variety of functional groups including amide, cyano, diol, amines, sulfobetaine are used for this purpose and these HILIC stationary phases are categorized into four major groups namely, neutral, cationic, anionic and zwitterionic based on the surface charge of the stationary phase [4]. Other than silica stationary phases, monolithic, TiO_2, ZrO_2 and MgO based stationary phases have been developed and are commercially available as well for use in HILIC applications.

In recent years, grafting a polar polymeric coating onto silica particles in order to synthesize HILIC stationary phases has gained a great interest. These polymeric coatings are more stable and they protect the silica from hydrolytic degradation and specially

provide a thick water enriched layer for HILIC applications. In this regard, Bui et al. have functionalized silica particles with tris(hydroxymethyl)aminomethane (TRIS) by first grafting glycidyl methacrylate (GMA) onto silica particles *via* atom transfer radical polymerization followed by post grafting modification with TRIS [5]. This polymer coated stationary phase with hydrophilic interactions and weak anion exchange mixed mode retention properties was successfully used for the separation of polar neutral, acidic and basic solutes under HILIC conditions. The same research group has reported an analog TRIS acrylamide grafted silica stationary phase containing amide linkages between TRIS functionalities and graft chains of polymer coating, instead of secondary amine linkages employed in the previous work [6]. This TRIS acrylamide grafted silica gel showed different selectivity, substantially higher retention capacity and reduced weak anion exchange properties compared to those of the TRIS amine silica stationary phase. In addition, Peng et al. have prepared a similar TRIS acrylamide functionalized silica stationary phase using a different approach *via* azo immobilized silica gel [7]. The resulting poly *N*-acryloyltris(hydroxymethyl)aminomethane stationary phase showed a mixed mode retention mechanism of hydrophilic and ionic interactions due to the residual amine groups originating from the aminopropyl silica used at the starting material of the synthesis. The novel TRIS acrylamide silica gel was successfully used for the HILIC separation of nucleosides, standard peptides, and β-casein tryptic digest. However, these thick polymeric coatings on the silica surface are known to generate high mass transfer resistance for solutes leading to broad peaks with poor separation efficiencies. Therefore, the synthesis of monomeric TRIS bonded stationary phases is beneficial to achieve high separation efficiencies in HILIC separation of wide range of polar solutes.

Many of the reported HILIC surfaces provide only a single layer of functional groups and limited interactions with the polar solutes, limiting their applications to a narrow range of solutes. Due to the complex nature of the HILIC separation mechanism, having multiple polar layers on the same stationary phase may provide increased hydrophilicity as well as multiple modes of interaction with polar solutes leading to better selectivity and improved separation performance. In this regard, mixed mode chromatographic stationary phases which utilize more than one type of interaction between the stationary phase and the analytes can be readily prepared using the layer by layer approach. The Takeuchi research group has reported a series of multilayer stationary phases prepared by modification of anion exchanger surfaces with "mucopolysaccharides" to be used in ion chromatography [8-12]. The chromatographic performance of these anion exchangers was remarkably altered after modifying them with anionic polysaccharides such as chondroitin sulfate, heparin and dextran sulfate. Moreover, these multilayer stationary phases provided both anion exchange and cation exchange retention behavior with charged solutes depending on the separation conditions.

Here, we are reporting two novel silica based HILIC stationary phases which are synthesized *via* initial attachment of TRIS to the bare silica particles as a monomeric phase. First, the bare silica support was coated with an epoxy active layer *via* the reaction of silica with γ-glycidoxypropyl trimethoxysilane. The activated epoxy silica thus obtained was covered with a layer of TRIS yielding the singly layered silica stationary phase. The TRIS-silica was further coated with a layer of chondroitin sulfate A (CSA) yielding the multi-layered hydrophilic silica stationary phase referred to as CSA-TRIS-silica sorbent. Solid-state infrared was used to evaluate and characterize the various coatings. In addition, an

extensive chromatographic characterization was conducted to assess the extent of each coating step in achieving the singly and multilayered polar coating of the silica microparticles. This included the effect of mobile phase composition, e.g., ACN, buffer and pH on solute retention factor, separation selectivity, separation efficiency and peak resolution. As expected, each coating yielded a unique retention pattern and selectivity towards the polar and slightly polar solutes tested.

Experimental

Instrumentation

The HPLC system consisted of a quaternary solvent delivery system Q-grad pump from Lab Alliance (State College, PA, USA), a Model 3100 UV–Vis variable wavelength detector from Milton Roy, LDC division (Riviera Beach, FL, USA) and a Rheodyne injector Model 7010 from IDEX Health & Science LLC (Rohnert Park, CA, USA) equipped with a 20 µL loop. Chromatograms were recorded using the clarity version, 3.0.06.589 advanced chromatographic software from Data Apex (Prague, Czech Republic) running on a Dell PC computer. The column performance parameter (e.g., number of theoretical plates) was obtained from the clarity software considering peak width at half height. A constant pressure pump from Shandon Southern Products Ltd. (Cheshire, UK) was used for the slurry packing of the columns. An HPLC system consisting of a Waters Model 590 programmable HPLC pump (Milford, MA, USA) and a Rheodyne injector Model 7010 from IDEX Health & Science LLC (Rohnert Park, CA, USA) equipped with a 2 mL sample loop was used for the modification of the TRIS silica with chondroitin sulfate A.

Reagents and Materials

Zorbax silica with a 5 μm average particle diameter, 75 Å average pore size diameter were donated by BTR separations (Wilmington, DE, USA). Chondroitin sulfate A, adenine, adenosine, uracil, uridine, guanosine, cytidine, cytosine, PNP-α-D maltopentoside, syringic acid, *p*-hydroxybenzoic acid, protocatechuic acid, vanillic acid, uridine 2':3'-cyclic monophosphate (cUMP), adenosine 2':3'-cyclic monophosphate (cAMP), guanosine 2':3'-cyclic monophosphate (cGMP), uridine 5'-monophosphate (UMP), adenosine 5'-monophosphate (AMP), guanosine 5'-monophosphate (GMP), cytidine 5'-monophosphate (5'CMP), cytidine 3'-monophosphate (3'CMP), dansyl-DL-threonine (Dns-thr), dansyl-DL-phenylalanine (Dns-phe), *N*-dansyl-DL-serine (Dns-ser), dansyl-DL-leucine (Dns-leu), *N*-α-dnasyl-DL-tryptophan (Dns-trp), dansyl-DL-methionine (Dns-met), dansyl-DL-aspartic acid (Dns-asp), *N*-dansyl-L-lysine (Dns-lys), dansyl-L-arginine (Dns-arg) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The γ-glycidyloxypropyl trimethoxysilane, tris(hydroxymethyl)aminomethane, *o*-coumaric acid and *p*-anisic acid were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). Urea, phenol and resorcinol were purchased from Fisher Scientific (Fair Lawn, NJ, USA). ACS grade acetonitrile, isopropanol, toluene and were obtained from Pharmco-AAPER (Brookfield, CT, USA). Ammonium acetate and formic acid were obtained from Spectrum Quality Products (New Brunswick, NJ, USA). PNP-α-D-glucopyranoside, PNP-α-D maltoside and PNP-α-D-maltotetroside were obtained from EMD Bioscience Inc. (La Jolla, CA, USA). Inosine was obtained from Calbiochem Corp. (Los Angeles, CA, USA). *N*,*N*-Dimethylformamide, formamide and acetic acid were obtained from EM Science (Gibbstown, NJ, USA). Benzoic acid and pyrogallol were

obtained from J.T Baker Chemical Co. (Phillipsburg, NJ, USA). Thymine was obtained from Nutritional Biochemical Corporation (Cleveland, OH, USA). Phenoxy acid herbicides and esters including (2,4,5-trichlorophenoxy)propionic acid isooctyl ester [silvex ester], (2,4,5-trichlorophenoxy)acetic acid [2.4.5 T], 2,4-dichlorophenoxyacetic acid propylene glycol ester [2.4 DPGI], 2-(3 chlorophenoxy) propionic acid [2.3 CPPA], 2-(4-chloro-2-methylphenoxy)propionic acid [mecoprop] were purchased from Chem Service (West Chester, PA, USA).

Preparation of Epoxy Activated Silica

Typically, 2.5 g of dry Zorbax silica gel were suspended in 30 mL of dry toluene in a round-bottomed flask and the mixture was heated to 95 °C with slow stirring to make a slurry. To this suspension, 2.5 mL of γ-glycidoxypropyl trimethoxysilane were added, and the reaction mixture was stirred for 18 h at 95 °C. The epoxy activated silica thus obtained was rinsed successively with toluene and acetone and then immediately used for the next modification step.

Functionalization of Epoxy Activated Silica with TRIS

The resulting epoxy activated silica (see above) was re-suspended in 30 mL of an aqueous solution of 100 mM tris(hydroxymethyl)aminomethane (TRIS), pH 8, in a round-bottomed flask. The reaction mixture was stirred at 85 °C for 6 h. After the modification, the TRIS silica gel thus prepared was rinsed with water and acetone, and was allowed to dry in the air.

Column Packing

Two grams of TRIS-silica were dispersed in 20 mL of isopropanol to form a 10% (w/v) slurry, which was sonicated for 20 min to eliminate air and ensure homogenization. Then, the slurry was packed into a stainless-steel column (10 cm × 4.6 mm id or 15 cm × 4.6 mm id) with isopropanol as the packing solvent at 6000-7000 psi pressure for 30 min using a constant pressure pump. Thereafter, the columns were equilibrated with the running mobile phase for 30 min before starting the chromatographic analysis.

Modification of the TRIS-Silica Column with Chondroitin Sulfate A

The TRIS-silica column prepared according to the above procedure was washed with distilled water for 30 min at a flow rate of 1 mL/min. Thereafter, 10 mM ammonium acetate, pH 3, was passed through the column at 1 mL/min flow rate for 30 min. The TRIS-silica column was then equilibrated with 50% ACN: 50% of 10 mM ammonium acetate, buffer solution, pH 3, at 1 mL/min flow rate for 30 min. Ten milliliters of 1% (w/v) chondroitin sulfate A prepared in 50:50 ACN/ammonium acetate buffer (10 mM, pH 3) was injected in to the column using the HPLC injector with a 2 mL sample loop. The column was end capped and kept for 2 h at room temperature followed by washing with the 50:50 ACN/ammonium acetate buffer at 1 mL/min flow rate for 30 min.

Chromatographic Conditions for HILIC Separations

Analyses were carried out at 214 nm and 254 nm wavelengths at a 1 mL/min flow rate at room temperature. Solution mixtures containing acetonitrile and ammonium acetate buffer were used as the mobile phase unless otherwise specified. The molarity and the pH of the mobile phases are presented with respect to the aqueous portion. The pH of

the aqueous ammonium acetate buffer was adjusted using formic acid or ammonium hydroxide before mixing it with ACN. All the mobile phases were freshly prepared, filtered through 0.1 Whatman filter papers and sonicated for 30 min before use. The retention factors, k, were determined from the retention time of toluene as the unretained marker (t_0) and solute retention time (t_R).

FTIR Characterization

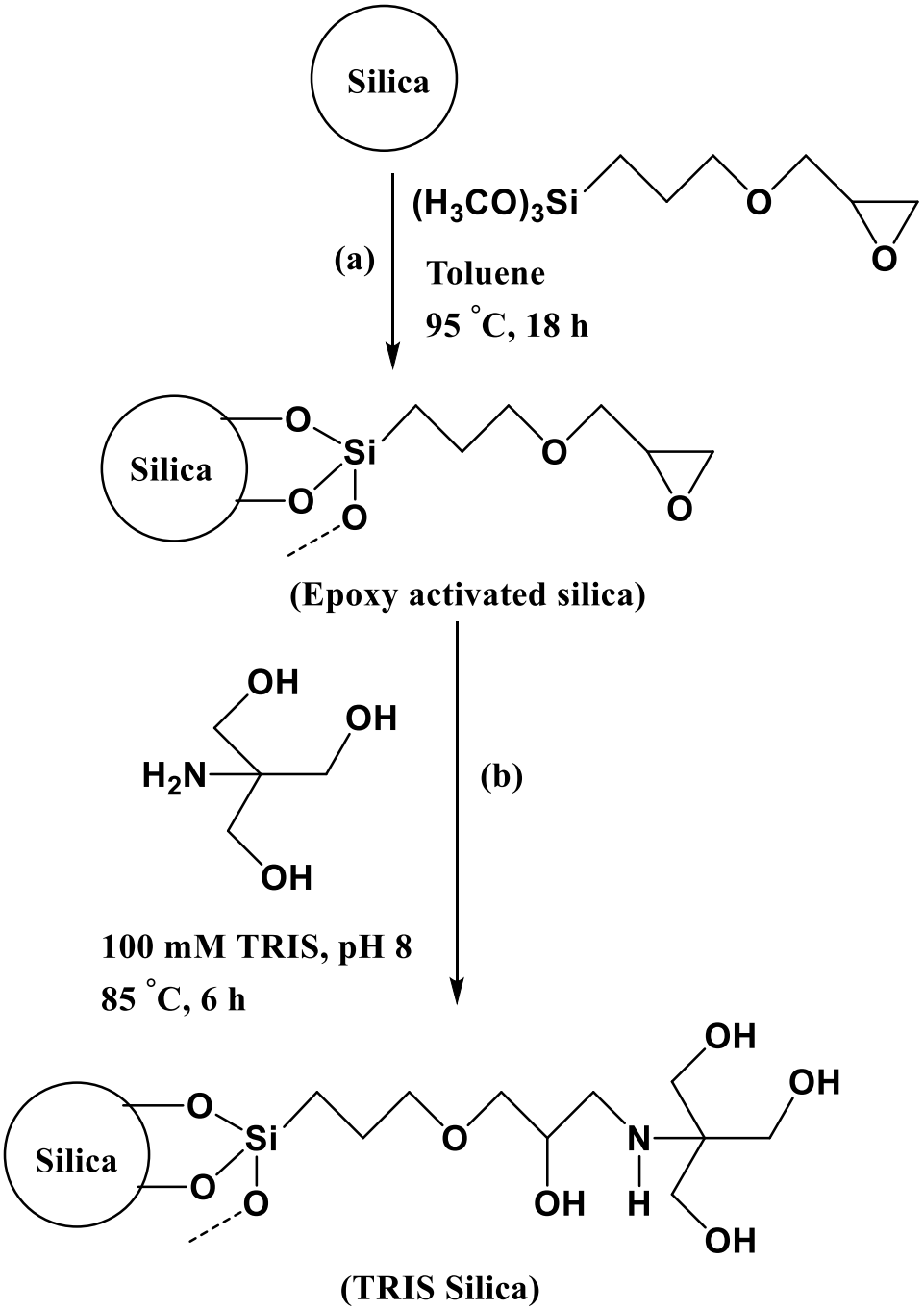
Infrared spectra of the bare silica and TRIS-silica particles were obtained in the 4000-600 cm^{-1} region with the resolution of 4 cm^{-1} by accumulating 32 scans using a Varian 800 FTIR Scimitar series FTIR (Varian Inc., CA, USA). Thin layer of dried silica particles were spread in-between two NaCl window plates and spectra were taken in the transmittance mode.

Results and Discussion

Preparation and Characterization of the TRIS-Silica Stationary Phase

Among the various available chromatographic supports for HILIC applications, silica based supports have some advantages including good mechanical stability, greater hydrophilicity and desirable pore properties [13]. In addition, the high concentration of silanol groups on the silica gel surface allow convenient introduction of many functional groups of interest onto the silica gel surface *via* organosilane coupling agents. The most common silica functionalization methods consist of anchoring of silylated compounds with the desired functional groups, using silanol-alkoxide/halide reaction with the alcohol or hydrohalic acid elimination. The γ-glycidoxypropyl trimethoxysilane (GPTMS) plays

an important role in this regard, as a linker in between the silica particles and many molecules of interest due to its highly reactive oxirane group.



Scheme 1. *Schematic diagram showing (a) attachment of GPTMS to the silica surface and (b) nucleophilic substitution of the epoxy activated silica by TRIS.*

These epoxides readily undergo ring opening reactions with nucleophiles. A reaction of this type is an S_N2 reaction in which the epoxide oxygen serves as the leaving group. In this reaction, the leaving group remains within the same product molecule without departing as a separate entity. In addition, the Si atom in the GPTMS can be considered as trifunctional in terms of methoxy groups and is therefore able to form a very strong link with the silica surface. Therefore, the silica surface is first functionalized with GPTMS in order to obtain the epoxy activated silica surface as shown in the scheme 1a.

The epoxy activated silica thus obtained was then reacted with tris (hydroxymethyl) aminomethane (TRIS) in order to get a polyhydroxyl surface with enhanced hydrophilicity as shown in Scheme 1b. Moreover, the TRIS silica stationary phase contains secondary amine functionalities on its surface giving weak anion exchange properties to the column. The reaction between the epoxy moieties attached to the silica with TRIS molecules is based on typical nucleophilic attack of the basic nitrogen atom of the TRIS to the strained acidic carbon atom of the epoxy ring. Catalyst is not required for this reaction due to the basicity of TRIS.

The modification of the silica particles with polar functionalities was verified by the IR analysis. The FTIR spectra obtained for bare silica and TRIS-silica in the range of 4000 to 600 cm^{-1} are shown in Fig. 1. The main feature of all silica spectra are related to the similarities of peaks associated with the silica backbone such as intense band due to siloxane stretching at 1100 cm^{-1}. The peak at 1620 cm^{-1}on bare silica spectrum is due to the bending vibrations of adsorbed water molecules.

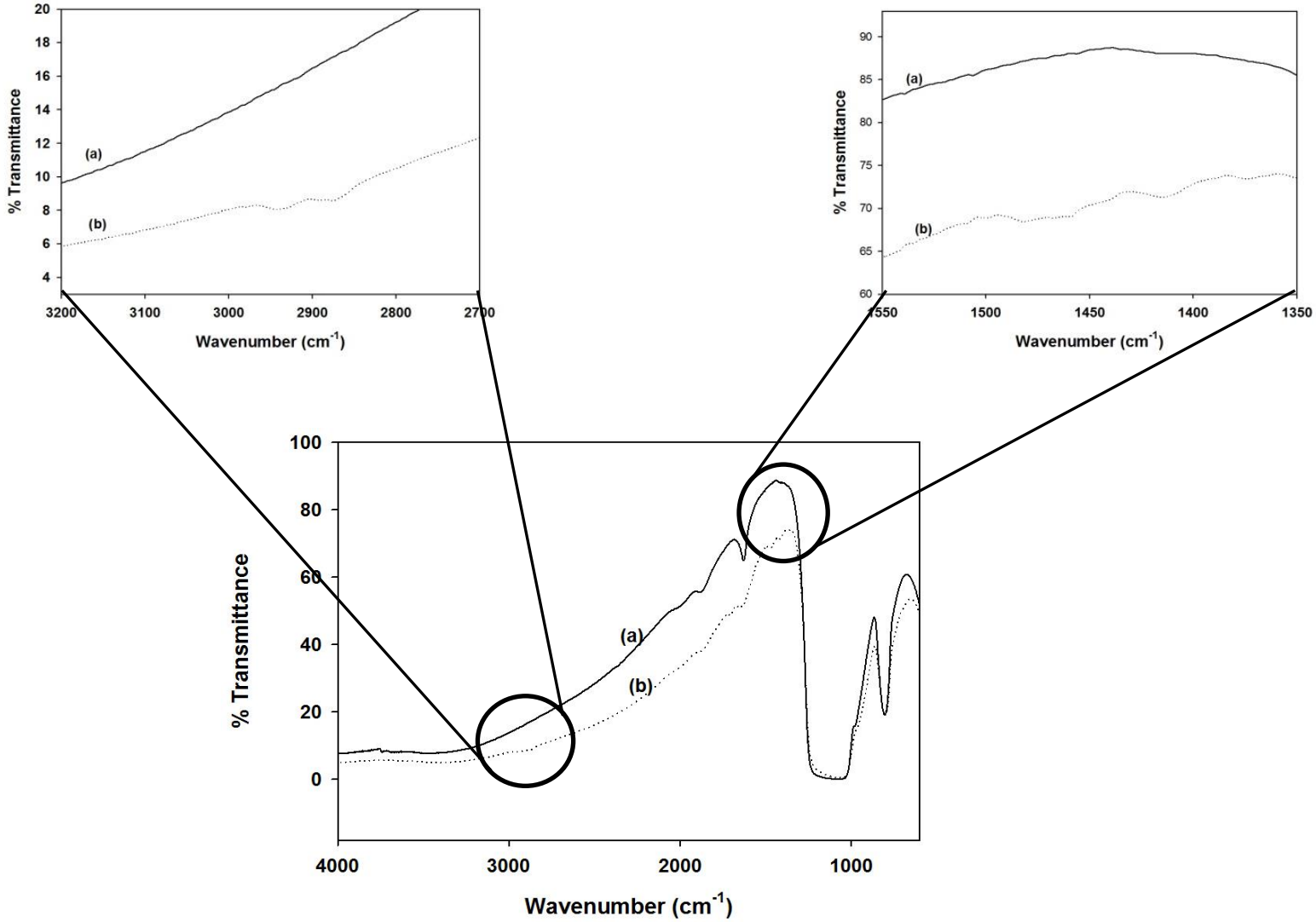


Figure 1. *FTIR spectrum of (a) bare silica and (b) TRIS-silica.*

Moreover, the TRIS-silica spectrum showed less intense characteristic peaks at 1420 cm^{-1} arising from OH bending of alcohol groups, 2800 cm^{-1} and 2900 cm^{-1} arising from CH stretching of alkane groups, and 1465 cm^{-1} arising from CH bending of alkane groups confirming the presence of organic groups immobilized onto the silica surface.

Chromatographic Behavior of the TRIS-Silica Stationary Phase

Similar to RPC, a binary solution containing acetonitrile (ACN) and water is the most commonly used solution as the mobile phase in HILIC. The composition of this hydro-organic mobile phase has a significant effect on the retention factor (k value), selectivity and the column separation efficiency of HILIC separations. However, unlike in RPC the solvent strength of the mobile phase decreases with increasing ACN content in the mobile phase. The effect of the ACN content in the mobile phase on the HILIC solute retention behavior on TRIS-silica stationary phase was evaluated by varying the percentage of ACN in the mobile phase and the results are shown in Fig. 2. As expected, the k values of all analyzed polar solutes increased with increasing the ACN content in the mobile phase showing the typical HILIC behavior. This effect is more significant when the ACN content in the mobile phase is greater than 80%.

The polyhydroxy and secondary amine functionalities on the TRIS-silica surface are responsible for solute hydrophilic interaction (HI) with the stationary phase. In addition, the presence of cationic secondary amine groups on the silica surface may give rise to weak anion exchange properties in the column. Hence, the electrostatic interactions between the stationary phase and the charged solutes may also play an important role in the HILIC separation mechanism of the TRIS-silica stationary phase.

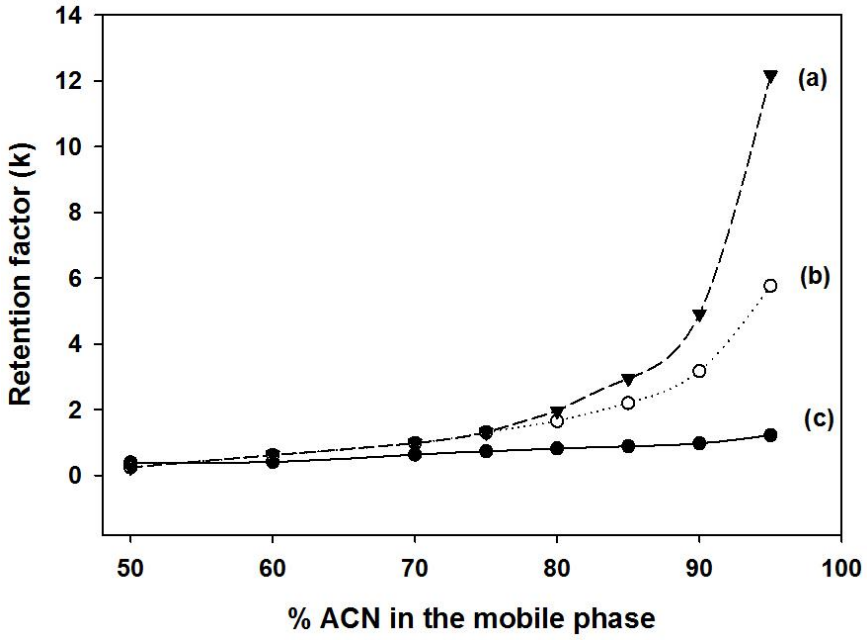


Figure 2. *Plot of the k value vs. % ACN in the mobile phase obtained on TRIS-silica column. Separation conditions; Column, 15 cm × 4.6 mm id; flow rate, 1 mL/min; detection, UV at 254 nm; injection volume, 20 µL; column temperature, room temperature; mobile phase, ACN with varying percentage of 25 mM ammonium acetate, pH 3. Solutes; a, cytosine; b, adenosine, c; uracil.*

Therefore, the effect of salt concentration in the mobile phase on the k values of charged solutes were evaluated by varying the ammonium acetate concentration in the mobile phase while keeping the pH constant at pH 3 and the results are shown in Table 1. The k values of cationic adenosine (pK_a, 3.45) and cytosine (pK_a, 4.6) increased with increasing salt concentration in the mobile phase, while the k values of anionic phenolic acids namely, *p*-anisic acid (pK_a, 4.47) and benzoic acid (pK_a, 4.20) decreased with increasing salt concentration. There are electrostatic repulsions between cationic TRIS-silica surface and cationic analytes. When the ammonium acetate concertation of the mobile phase increased, the acetate ions effectively shield the cationic moieties on the

TRIS-silica surface thus reducing the electrostatic repulsion leading to an increase in the k values. Similarly, the k values of anionic phenolic acids decreased at higher salt concentrations due to the effective shielding of the electrostatic attraction between anionic solutes and the cationic stationary phase. These results indicate the contribution of the electrostatic interactions towards the separation mechanism of the TRIS-silica stationary phase.

TABLE 1

EFFECT OF THE MOBILE PHASE IONIC STRENGTH ON k VALUES OF CHARGED SOLUTES SEPARATED ON TRIS SILICA COLUMN

Solute	**k at 95% ACN: 5% 25 mM Ammonium acetate, pH 3**	**k at 95% ACN: 5% 50 mM Ammonium acetate, pH 3**	**k at 95% ACN: 5% 100 mM Ammonium acetate, pH 3**
Adenosine	6.09 ± (0.0707)	6.49 ± (0.0954)	6.95 ± (0.0919)
Cytosine	12.8 ± (0.00700)	13.5 ± (0.0636)	14.2 ± (0.226)
p-anisic acid	1.57 ± (0.00700)	1.05 ± (0.00910)	0.800 ± (0.0420)
Benzoic acid	2.51 ± (0.0141)	1.65 ± (0.0212)	1.30 ± (0.176)

In order to evaluate the separation efficiency of the TRIS-silica column, the dependence of the HETP on the mobile phase linear velocity or the "Van-Deemter plot" was plotted using cytosine as the test solute. As shown in Fig. 3, TRIS-silica column showed the lowest HETP of 20 μm at the flow rate range of 0.04 cm s^{-1} to 0.06 cm s^{-1} which is slightly higher than expected efficiency for a 5 μm average particle diameter column. The multiple modes of interactions in between the stationary phase and the

charged solutes might be responsible for the above observation. In addition, when the linear velocity is increased by 50% from 0.08 cm s^{-1} to 0.12 cm s^{-1}, the loss of efficiency was only 5.43% indicating lower mass transfer resistance. Therefore, the TRIS-silica column can be operated even at higher flow velocities without any significant loss of the separation performances.

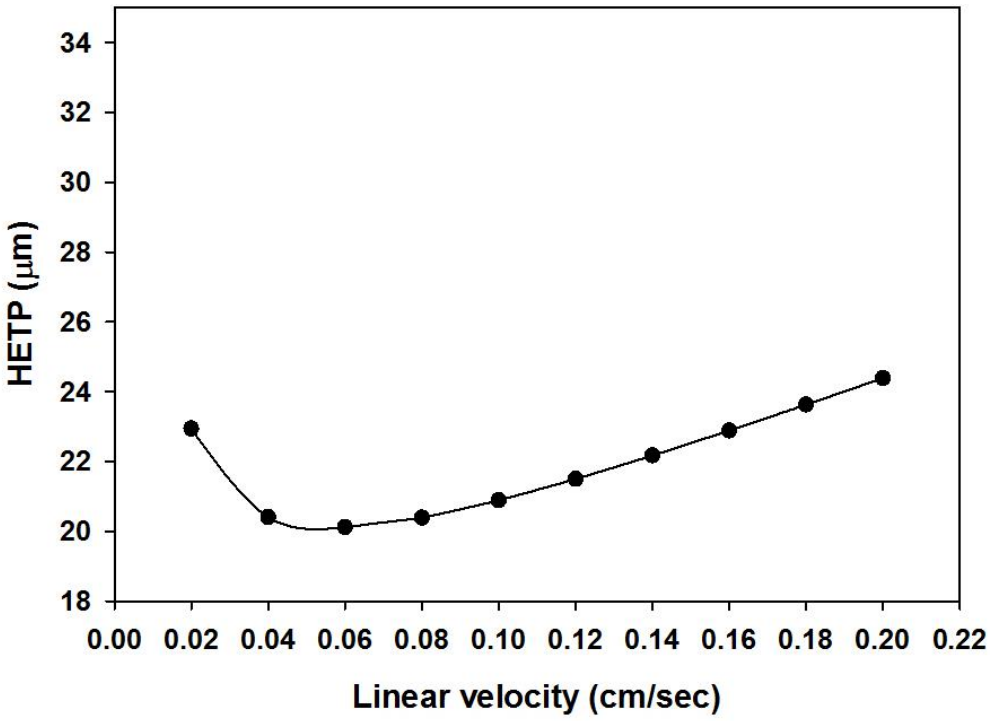


Figure 3. . *Van-Deemter plot obtained for cytosine on a TRIS silica (10 cm × 4.6 mm id) column. Separation conditions are same as in Fig. 2, except that the mobile phase was 95% (v/v) acetonitrile : 5% (v/v) 100 mM ammonium acetate, pH 6.*

The TRIS-silica stationary phase with polyhydroxy and secondary amine functionalities was then used to separate several types of highly and moderately polar neutral, cationic and anionic solutes in order to characterize the column. As discussed earlier in the previous paragraph, HILIC separation of polar solutes on TRIS-silica

stationary phase is believed to be mainly due to the HI and electrostatic interactions. In addition, the hydroxyl functionalities on the TRIS moieties provide the possibility of hydrogen bonding with appropriate analytes as well.

Separation of nucleic acid bases, nucleosides and nucleotides. Separation of nucleic acid bases, nucleosides and nucleotides is important in many areas including, genomics, clinical chemistry, forensic science and pharmaceutical industry. In the current study, a mixture of nine nucleic acid bases and nucleosides was separated on TRIS-silica stationary phase with average peak efficiency of 22,000 plates/m. As shown in Fig. 4, higher resolution was achieved with symmetrical peaks using a simple mobile phase containing only acetonitrile and water. In general, nucleic acid bases and nucleosides eluted in the order of increasing polarity. The conjugation of nucleic acid bases with sugars as in their corresponding nucleoside increased the polar interaction and hence the k values in the HILIC mode. This behavior was observed with uracil/uridine and cytosine/cytidine nucleobase-nucleoside pairs. But the expected retention pattern was reversed with adenine/adenosine pair. This HILIC behavior cannot be attributed to one or two simple mechanisms [14]. Many other types of interactions including electrostatic interactions, hydrogen bonding, ion-dipole interactions or dipole-dipole interaction might also be involved in the separation mechanism of a HILIC stationary phase making it difficult to predict the separation pattern of heterogeneous polar compounds in a typical HILIC separation.

In order to broaden the scope of applications of the TRIS silica column, it was then applied to separate several mixtures containing polar anionic nucleotides including

mixtures of cyclic nucleotides and a mixture of 5’ nucleotide monophosphates as shown in Fig. 5.

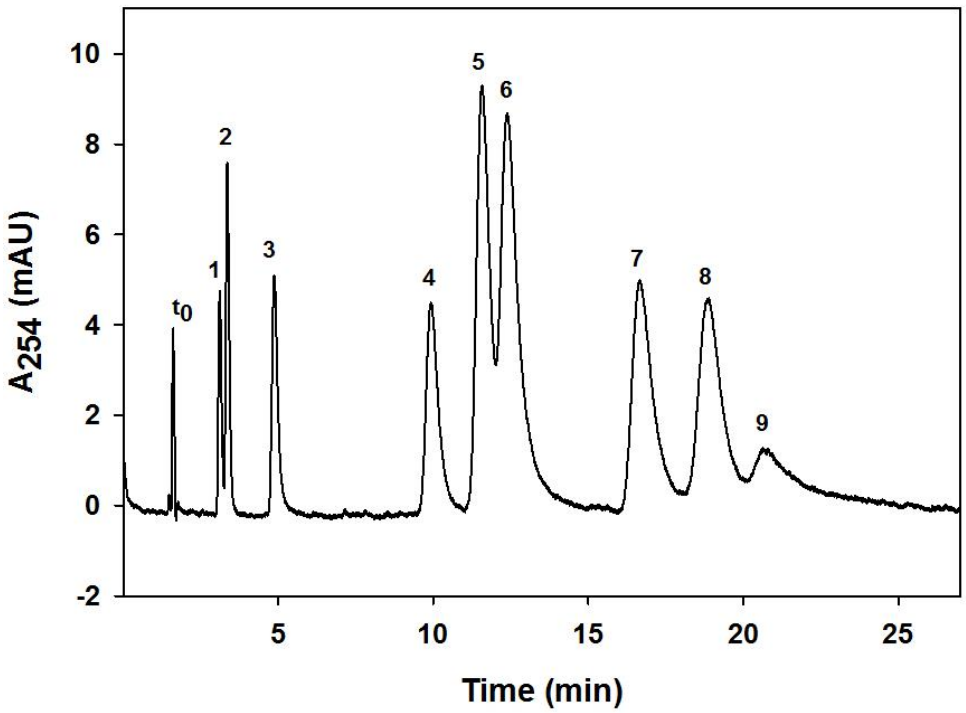


Figure 4. *Chromatogram of nine nucleic acid bases and nucleosides obtained on TRIS-silica stationary phase. Conditions are same as Fig. 2, except that the mobile phase was 95% ACN (v/v) : 5%, water (v/v). Solutes; t_0, toluene; 1, thymine; 2, uracil; 3, uridine; 4, adenosine; 5, inosine; 6, adenine; 7, cytosine; 8, cytidine; 9, guanosine.*

The TRIS silica surface provides both hydrophilic interactions and weak anion exchange possibilities toward the separation of these negatively charged nucleotides. Three cyclic nucleotides were baseline separated using 80% (v/v) ACN: 20% (v/v) 25 mM ammonium acetate, pH 8 mobile phase with an average separation efficiency of 33,000 plates/m. The 5’ nucleotide monophosphates are highly polar due to their phosphate groups and a mobile phase with higher aqueous content is required in order to achieve a successful separation.

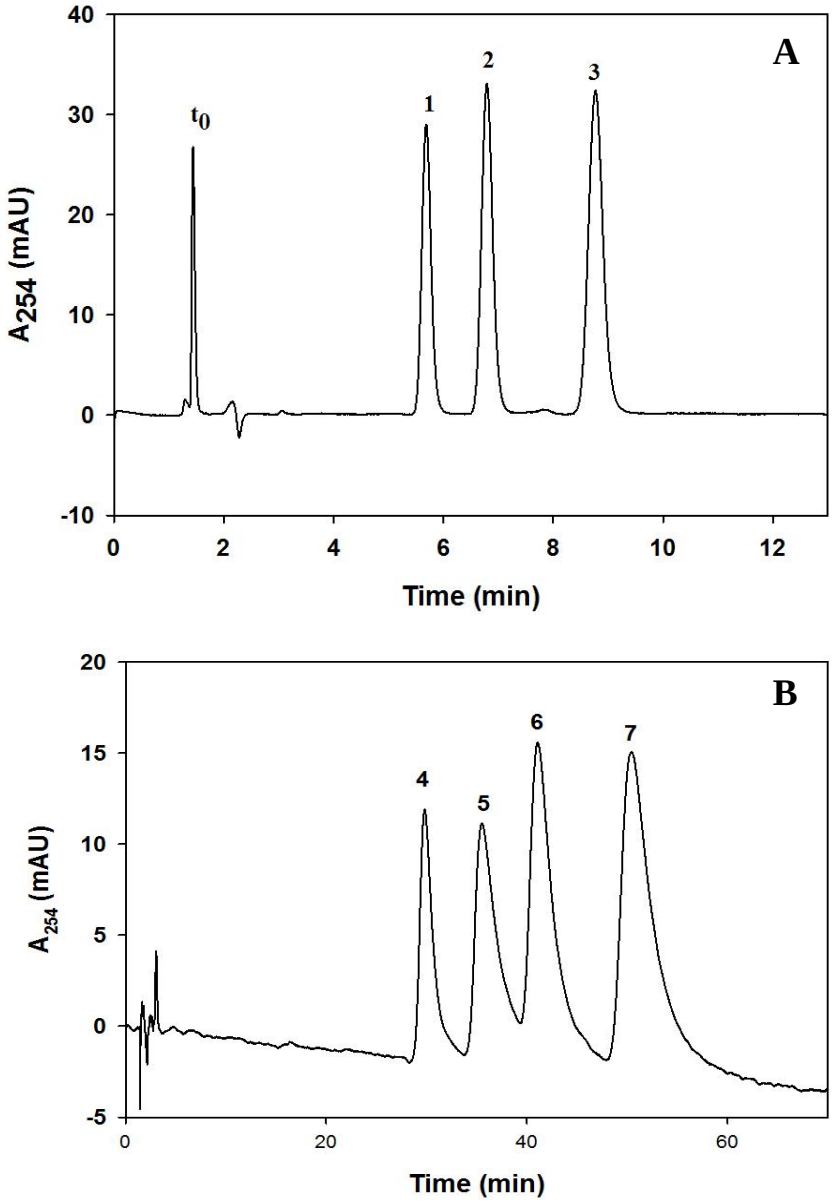


Figure 5. *Chromatograms of three cyclic nucleotides (A) and four 5' nucleotide monophosphates (B) obtained on the TRIS silica stationary phase. Conditions are the same as in Fig. 2 except that mobile phases were 80% (v/v) ACN: 20% (v/v) 25 mM ammonium acetate, pH 8 in (A); 70% (v/v) ACN : 30% (v/v) 25 mM ammonium acetate, pH 3 in (B). Solutes; t_0, toluene; 1, cUMP; 2, cAMP; 3, cGMP; 4, UMP; 5, CMP; 6, AMP; 7, GMP.*

Moreover, the mobile phase pH plays an important role in these nucleotide separations. Nucleotides phosphate group confers a negative charge at pH value above one. But the most nucleic acid bases have pK_a between 3 and 5 and the effective charge on the molecule changes as the base becomes protonated at lower pH. Therefore, 5' nucleotide monophosphate separation was done at pH 3, in order minimize strong electrostatic attraction between cationic stationary phase and the anionic nucleotides. Moreover, a strong mobile phase containing 70% (v/v) ACN: 30%, (v/v) 25 mM ammonium acetate, pH 3 was needed to baseline separate four 5' nucleotide monophosphates within 1 h. The 5' nucleotide monophosphates with pyrimidine bases (uracil and cytosine) eluted early and the nucleotides with purine bases (adenine and guanine) eluted late possibly due to the enhanced hydrophilicity of double ring structure. Nucleotide diphosphates and nucleotide triphosphates were hardly eluted out from the TRIS-silica column, even with mobile phase containing higher aqueous content due to the highly polar anionic phosphate groups.

Two nucleotide isomers differing by the position of the phosphate groups, namely 3' CMP and 5' CMP were separated with a good selectivity as shown in Fig. 6 indicating the important of the position of the phosphate group for the polar interactions between TRIS-silica columns and the nucleotides.

Separation of neutral polar amides and phenolic compounds. A polar, neutral, low molecular weight amide mixture containing *N*,*N*-dimethylformamide (DMF), formamide and urea was separated on the TRIS-silica column as shown in Fig. 7A.

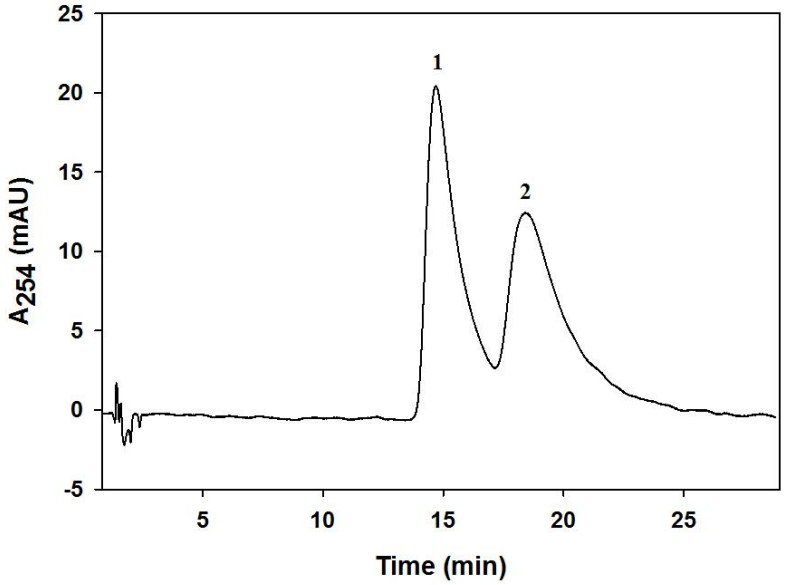


Figure 6. *Chromatogram of two nucleotide monophosphate isomers obtained on TRIS-silica stationary phase. Conditions are the same as in Fig. 2 except that mobile phase was 60% (v/v) ACN : 40% (v/v) 25 mM ammonium acetate, pH 3. Solutes; 1, 5' CMP; 2, 3' CMP.*

The separation of the amide mixture demonstrated the typical HILIC retention behavior where the most polar urea eluted last while the nonpolar toluene eluted at the column dead time t_0. In addition, all other solutes eluted in order of increasing polarity.

To further evaluate the HILIC behavior of the TRIS silica stationary phase, a mixture of phenolic compounds including phenol, resorcinol and pyrogallol was baseline separated as shown in Fig. 7B. As expected, the k values of the solutes increased with the increasing number of hydroxyl groups in the molecules.

Separation of phenolic acids and benzoic acid derivatives. In order to demonstrate the importance of the mobile phase pH on the separation of polar solutes, anionic solute

mixtures containing benzoic acid and some other phenolic acids were separated on the TRIS-silica stationary phase as shown in Fig. 8.

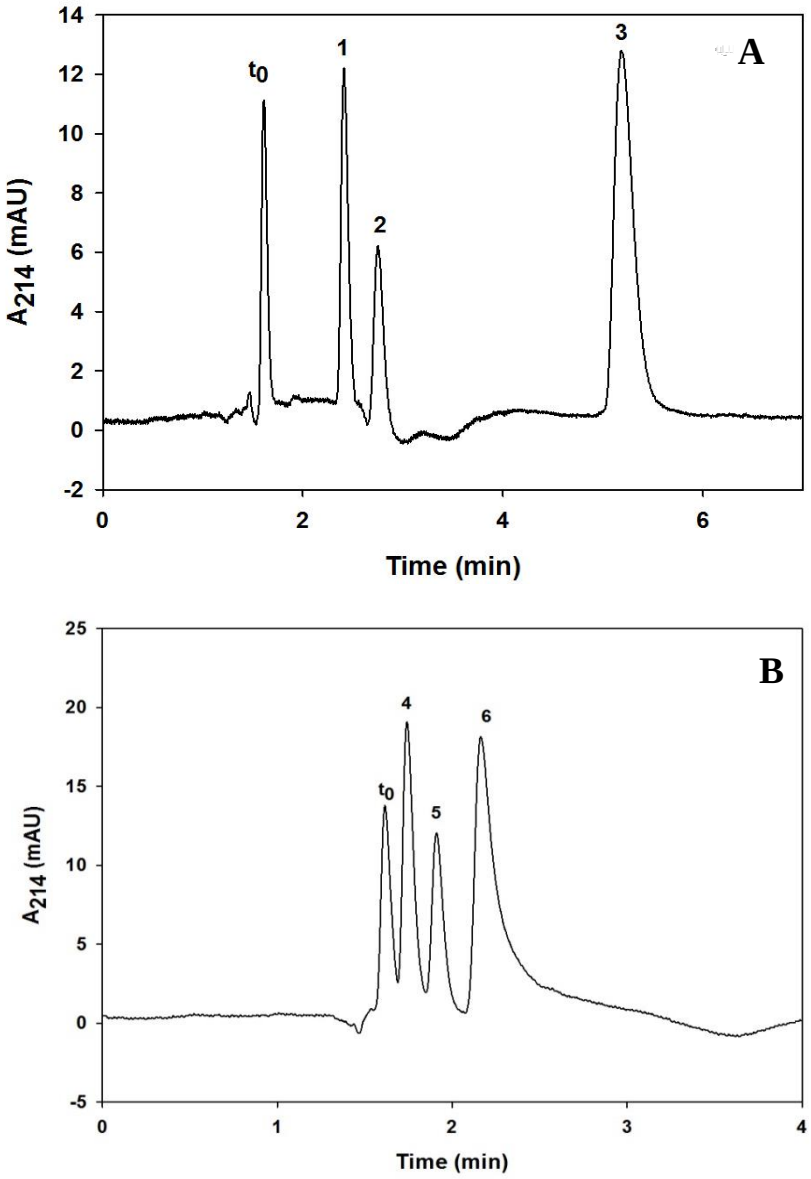


Figure 7. *Chromatogram of three neutral polar compounds (A) and three phenols (B) obtained on the TRIS-silica stationary phase. Conditions are the same as in Fig. 2 except that the mobile phase was 95% (v/v) ACN : 5% (v/v) water; detection, UV at 214 nm. Solutes; t_0, toluene; 1, N,N-dimethylformamide (DMF); 2, formamide; 3, urea; 4, phenol; 5, resorcinol; 6, pyrogallol.*

The retention of acidic solutes on the TRIS-silica column is in major part pH dependent. At higher pH conditions, all benzoic and phenolic acids become deprotonated and acquire negative charges. Apart from a greater hydrophilicity, due to the ionization there is a significant electrostatic attraction between the negatively charged solutes and TRIS-silica surface at higher pH. Therefore a mobile phase containing 95% ACN : 5% of 25 mM ammonium acetate, pH 3, was selected for this analysis. Five phenolic acids were baseline separated under low pH conditions within 8 min as shown in Fig. 8A. Moreover, the k values of the three selected benzoic acid derivatives namely *p*-hydroxybenzoic acid, protocatechuic acid and gallic acid increased with the increasing number of hydroxyl groups in the molecules as shown in Fig. 8B showing the typical HILIC behavior.

Separation of derivatized sugars. The separation of sugars using chromatographic techniques is highly important and challenging task in many areas including glycomics and proteomics. Because of the inherent hydrophilic nature of the carbohydrates, HILIC can be considered as an ideal technique for analyzing sugars. Moreover, many carbohydrate species do not possess chromophores and need to be derivatized with UV or fluorescent tags in order to be analyzed by UV detection and/or fluorescence detection. Four *p*-nitrophenyl (PNP) derivatized sugars were analyzed on TRIS silica column under un-buffered conditions, using a mobile phase containing only acetonitrile and water as shown in Fig. 9. The logarithm of the retention factors (log k) of sugars increased linearly with the increasing number of glucosyl units indicating typical HILIC behavior and the suitability of TRIS silica column for carbohydrate analysis.

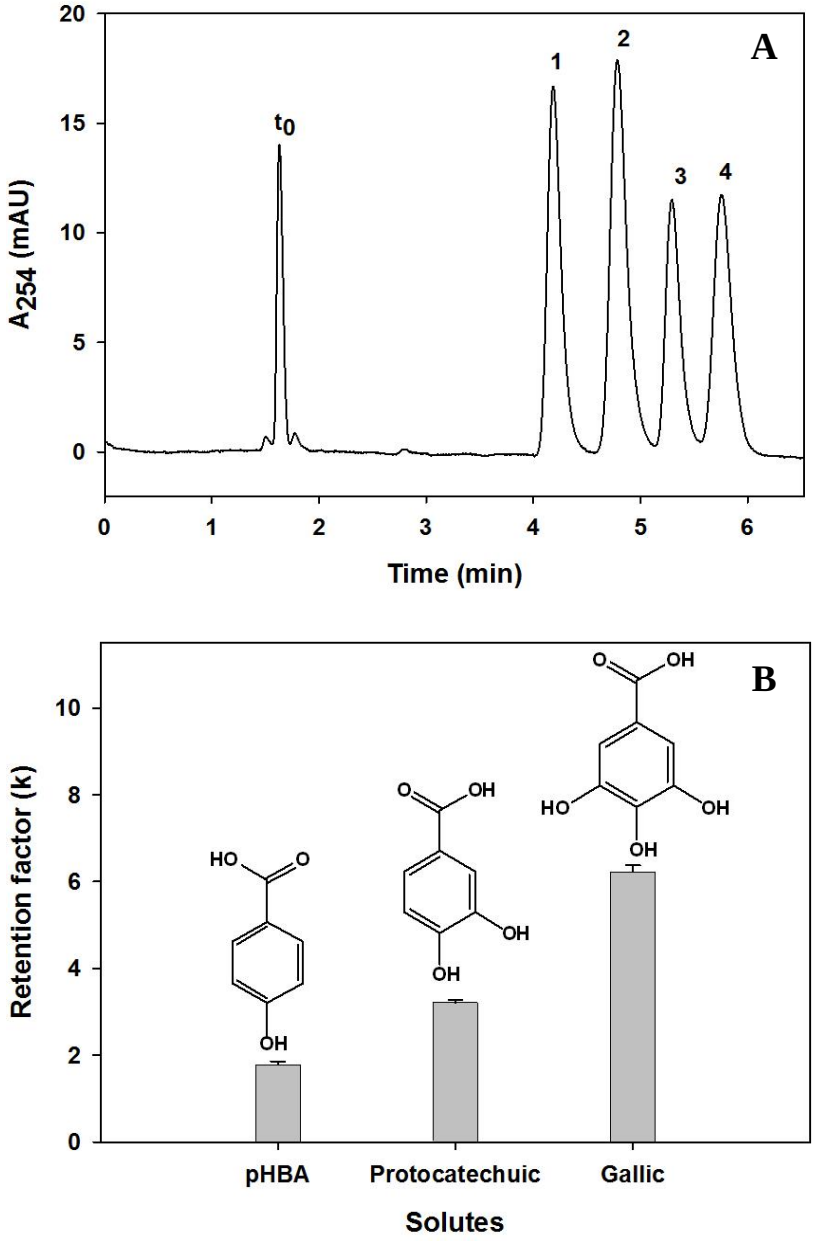


Figure 8. *Chromatogram of phenolic acids (A) and plot of k vs the number of hydroxyl groups in the benzoic acid derivatives (B) obtained on TRIS-silica stationary phase. Conditions are the same as in Fig. 2 except that mobile phase was 95% (v/v) ACN : 5% (v/v), 25 mM ammonium acetate, pH 3. Solutes; t_0, toluene; 1, p-anisic acid; 2, o-coumaric acid; 3, vanillic acid; 4, benzoic acid.*

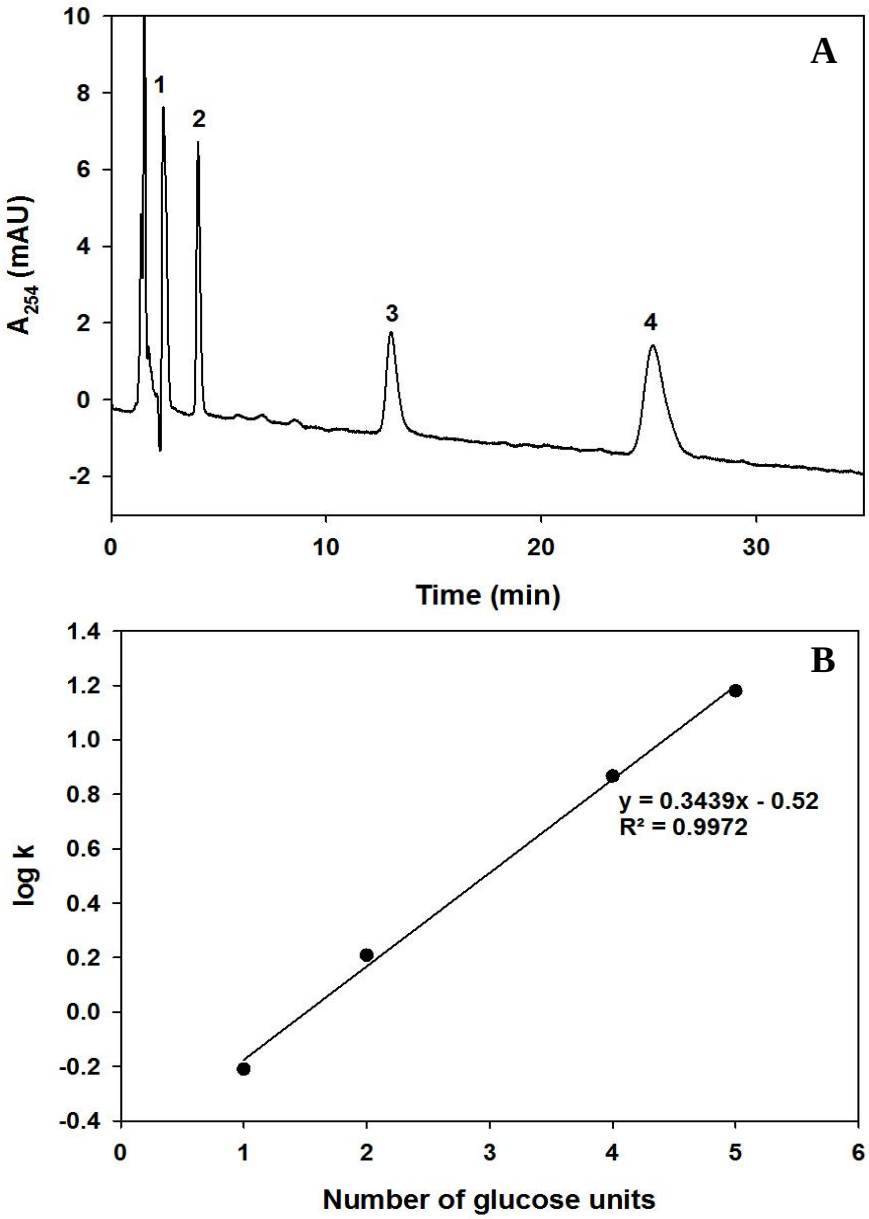


Figure 9. *Chromatogram of four PNP-derivative sugars (A) and plot of log k vs. number of glucosyl units in PNP-derivative sugars (B) obtained on TRIS-silica stationary phase. Conditions are the same as in Fig. 2 except that the mobile phase was 85% (v/v) ACN : 15% (v/v), water. Solutes; t_0, toluene; 1, PNP-α-D-glucopyranoside; 2, PNP-α-D-maltose; 3, PNP-α-D-maltotetroside; 4, PNP-α-D-maltopentoside.*

Separation of amino acid derivatives and phenoxy acid herbicides. A group of Dns-AA with the chemical formula $(CH_3)_2NC_{10}H_6SO_2NH$-AA (where NH-AA denotes the amino acid moiety of the derivative) was separated using the TRIS-silica stationary phase under investigation. Besides the ionizable carboxylic acid group of the amino acid moiety, the dimethylamino group of the dansyl moiety is a weak base with a pK_a between 3.0 and 4.0, and this value is largely independent of the ionic properties of the side chain of the amino acid [15]. In addition, Dns-AA contains the dansyl nonpolar group in their structures [15-17]. The net charge of Dns-AA acid is pH dependent. In order to induce interactions with the TRIS-silica stationary phase, the Dns-AAs were separated using a mobile phase containing ammonium acetate, pH 6, as the aqueous component. At higher pH, the dimethylamino group of the dansyl moiety was fully deprotonated giving a less polarity to the Dns-AAs making it difficult to analyze them under HILIC conditions. Five Dns-AAs were baseline separated at pH 6 on TRIS-silica column as shown in Fig. 10A. The retention order was clearly independent of the polarity of the AAs side chain where the polar serine eluted before the less polar tryptophan probably due to the complex nature of interactions between these solutes and the HILIC column.

In order to broaden the application range of the TRIS silica column, it was then applied to separate mixture containing phenoxy acid herbicides and their esters as shown in Fig. 10B. Three phenoxy acid herbicides and two of phenoxy acid herbicide esters were separated using a mobile phase containing 95% acetonitrile and 5% of 10 mM ammonium acetate, pH 6. The pK_a of phenoxy acid herbicides are around 3.5 and the majority of these solutes are anionic in the mobile phase containing ammonium acetate at

pH 6. Therefore, the TRIS-silica column provides both hydrophilic and electrostatic interactions towards the retention of these phenoxy acid herbicides.

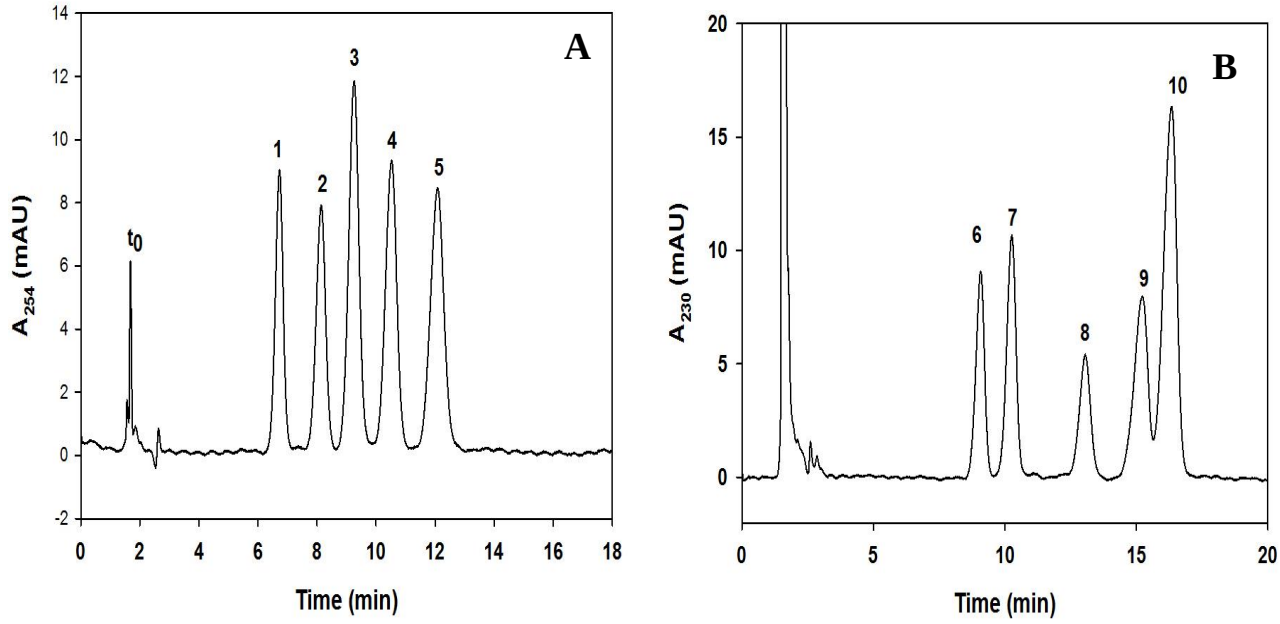
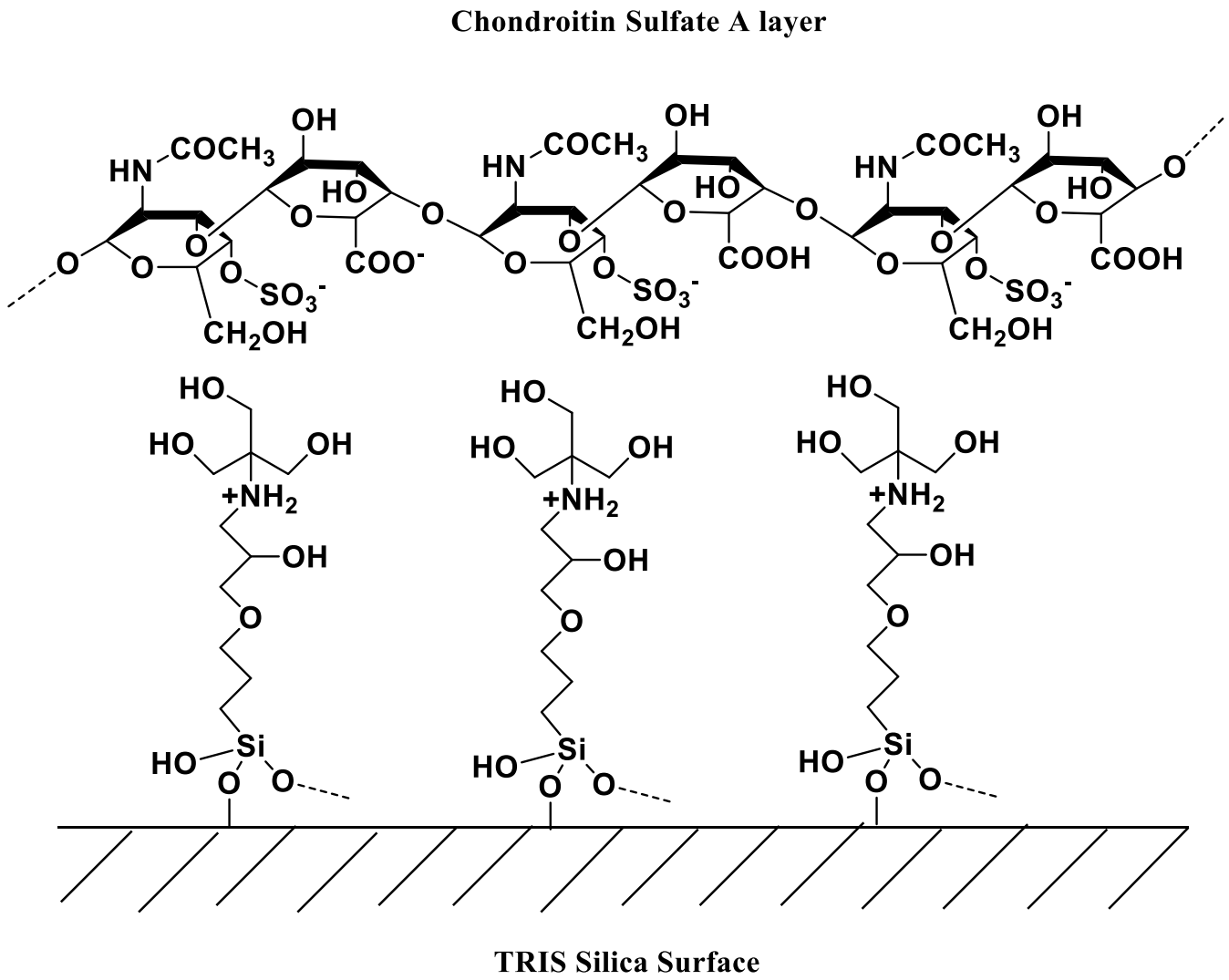


Figure 10. *Chromatograms of five Dns-AAs (A) and five phenoxy acid herbicides and their esters (B) obtained on TRIS-silica column. Conditions are the same as in Fig. 2 except the mobile phases were 95% (v/v) ACN : 5% (v/v) 10 mM ammonium acetate, pH 6; detection, UV at 254 nm in (A); UV at 230 nm in (B). Solutes; 1, Dns-thr; 2, Dns-phe; 3, Dns-ser; 4, Dns-leu; 5, Dns-trp; 6, (2,4,5-trichlorophenoxy)propionic acid isooctyl ester; 7, (2,4,5-trichlorophenoxy)acetic acid; 8, 2,4-dichlorophenoxyacetic acid propylene glycol ester; 9, 2-(3 chlorophenoxy)propionic acid; 10, 2-(4-chloro-2-methyl phenoxy)propionic acid.*

Chondroitin Sulfate A Modified TRIS-Silica Stationary Phase (CSA-TRIS-Silica)

The TRIS-silica surface was further coated with a layer of chondroitin sulfate A (CSA) yielding a multi-layered hydrophilic silica based stationary phase referred to as CSA-TRIS-silica sorbent. The additional hydrophilic layer on the TRIS-silica surface is thought to provide enhanced hydrophilicity and multi-mode interactions with polar solutes leading to different retention behavior and resolution compared to the singly layered TRIS-silica stationary phase. Chondroitin sulfate A is a sulfated mucopolysaccharide composed of a chain of alternating sugars *N*-acetylgalactosamine and glucuronic acid with average molecular weight of 40-100 kDa and it contains carboxylic groups with pK_a around 3.3 and sulfate groups with a pK_a around -1 [18]. Therefore, the sulfate groups of CSA are deprotonated even at very low pH and carboxylic acid groups are deprotonated above pH 4.5 giving a strong negative charge to the chondroitin sulfate A molecules. These anionic functionalities in the CSA electrostatically attract the cationic secondary amine groups on the TRIS-silica surface yielding a physically anchored CSA layer onto the TRIS-silica surface leading to the formation of the multi-layered HILIC stationary phase as shown in Scheme 2. Moreover, the hydroxyl and acetamide functionalities on the CSA can establish hydrogen-bonding interactions with the TRIS-silica surface, and consequently further strengthening the attachment of CSA to the TRIS-silica. Due to the higher density of anionic functionalities of the CSA layer, it was expected to see a net negative charge on the multi-layered CSA-TRIS-silica sorbent. To evaluate the influence of mobile phase pH and the ionic strength towards the HILIC retention mechanism of the multilayered column, two acidic solutes



Scheme 2. *Schematic diagram of multilayered chondroitin sulfate A modified TRIS-silica stationary phase.*

TABLE 2

EFECT OF MOBILE PHASE IONIC STRENGTH AND pH ON THE SEPARATION OF CHARGED SOLUTES ON CSA-TRIS-SILICA SORBENT

Solute	**k at 95% ACN: 5%, pH 6, Ammonium acetate**			**k at 95% ACN: 5%, pH 4.5, Ammonium acetate**			**k at 95% ACN: 5%, pH 3, Ammonium acetate**		
	10 mM	**25 mM**	**50 mM**	**10 mM**	**25 mM**	**50 mM**	**10 mM**	**25 mM**	**50 mM**
p-Hydroxyl benzoic acid	7.73 ± (0.00630)	8.83 ± (0.0134)	9.96 ± (0.0190)	6.45 ± (0.0212)	6.85 ± (0.0212)	7.40 ± (0.00700)	1.93 ± (0.00630)	1.61	1.51 ± (0.0516)
Syringic acid	11.9 ± (0.0473)	13.2	14.0 ± (0.0494)	11.5 ± (0.0424)	11.5 ± (0.0141)	11.7 ± (0.106)	3.62 ± (0.0565)	2.87 ± (0.0127)	2.28 ± (0.0579)

namely, *p*-hydroxybenzoic acid (PHB) and syringic acid were separated on the CSA-TRIS-silica sorbent under three different ionic strengths and three different pH conditions and these results are summarized in the Table 2.

The CSA polysaccharide layer does not completely cover the entire TRIS-silica surface. Therefore, some exposed residual cationic secondary amine moieties can also be present on the CSA-TRIS-silica sorbent. Similarly, all available anionic functionalities of the CSA layer are not involved in electrostatic interactions with the TRIS-silica surface. Hence, a substantial portion of these anionic groups are available for the interactions with the polar solutes. As a result, the net charge of this multilayered stationary phase is dependent on the pH value of the mobile phase. At high pH, both the sulfate and carboxylic groups of the CSA are fully deprotonated. Moreover, the residual silanol groups of the silica surface might also be ionized giving additional negative charges to the column. Therefore, the CSA-TRIS-silica sorbent acquires a net negative charge at pH 6 and pH 4.5. The analyzed PHB (pK_a = 4.54) and syringic acid (pK_a = 4.33) are also deprotonated under these pH conditions. Consequently, the k values of the phenolic acid derivatives increase with increasing the ionic strength of the mobile phase as shown in Table 2, possibly due to the shielding of electrostatic repulsion between the anionic stationary phase and the anionic analytes.

Although, the sulfate groups are deprotonated, the carboxylic groups provide a lesser contribution to the negativity of the CSA layer at low pH conditions. In contrast, the secondary amine groups of the TRIS moieties are fully protonated at low pH. These cationic amine groups can neutralize a significant portion of the negative charge on CSA-TRIS-silica sorbent and this may lead to a net positive charge at very low pH conditions

as well. In addition, the phenolic acid derivatives are slightly deprotonated under low pH conditions. Consequently, the k values of phenolic acid derivatives slightly decreased with increasing the ionic strength of the mobile phase at low pH conditions, possibly due to the shielding of electrostatic attractions between the slightly negative phenolic acids and the positively charged residual secondary amine groups of the TRIS moieties. These results suggest the pH switchable cation exchange/anion exchange behavior of the CSA-TRIS-silica sorbent.

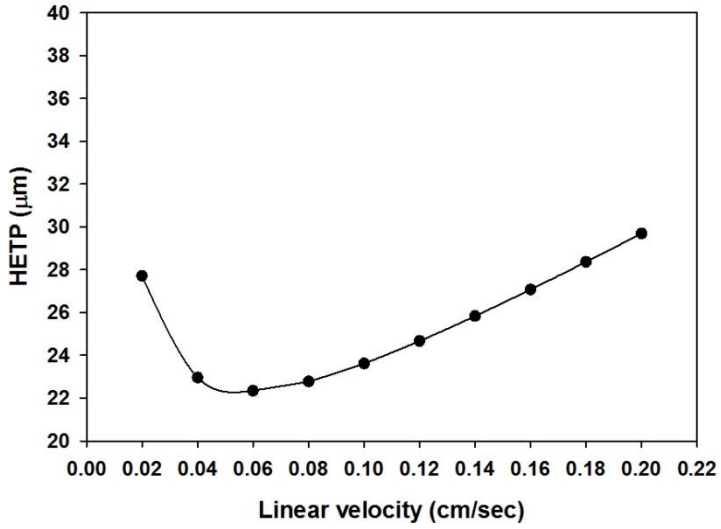


Figure 11. *Van-Deemter plot obtained for cytosine on CSA-TRIS-silica sorbent. Separation conditions; column, 10 cm × 4.6 mm id; flow rate, 1 mL/min; detection, UV at 254 nm; injection volume, 20 µL; column temperature, room temperature; mobile phase 95% (v/v) acetonitrile: 5% (v/v) 100 mM ammonium acetate, pH 6.*

The Van-Deemter plot obtained for cytosine shown in Fig. 11 indicate the lowest HETP equal to 22 µm at the flow rate range of 0.04 cm s^{-1} to 0.06 cm s^{-1} which was slightly higher than that obtained on the TRIS-silica column. In addition, when the linear flow velocity is increased by 50% from 0.08 cm s^{-1} to 0.12 cm s^{-1}, the loss of efficiency

was 8.30% which is also higher than that of the TRIS-silica column. These results indicate the higher mass transfer resistance for solutes in the multilayered stationary phase, possibly due to the thick CSA polysaccharide layer.

Chromatographic Evaluation of the CSA-TRIS-Silica Sorbent

Separation of nucleic acid bases and nucleosides. The CSA-TRIS silica sorbent was first used to separate a mixture containing nine nucleic acid bases and nucleosides as shown in Fig. 12. The selectivity of the modified stationary phase towards nucleic acid bases and nucleosides was the same as that of the TRIS-silica stationary phase except for the late eluting solutes namely, cytosine, cytidine and guanosine indicating the different selectivity of the CSA-TRIS-silica column due to the presence of the CSA layer. The k values for the all nucleic acid bases and nucleosides were significantly increased on the multilayered stationary phase compared to those obtained on the TRIS-silica column and a gradient elution was needed to elute all the nine solutes within 30 min. Moreover, the increase of the k values was much more significant for the later eluting solutes (cytosine) than that of early eluting solutes (thymine). The modification of TRIS-silica stationary phase with CSA gives an additional polar layer to interact with the solutes, thus increasing the overall hydrophilicity of the column.

Separation of neutral polar amides and phenols. Neutral polar amide mixture containing DMF, formamide and urea was then separated on the CSA-TRIS-silica column as shown in Fig. 13A. All solutes were eluted in the order of increasing polarity from toluene to urea showing the typical HILIC behavior of the CSA-TRIS-silica sorbent. The k values of the all amides were greater on CSA-TRIS-silica column than

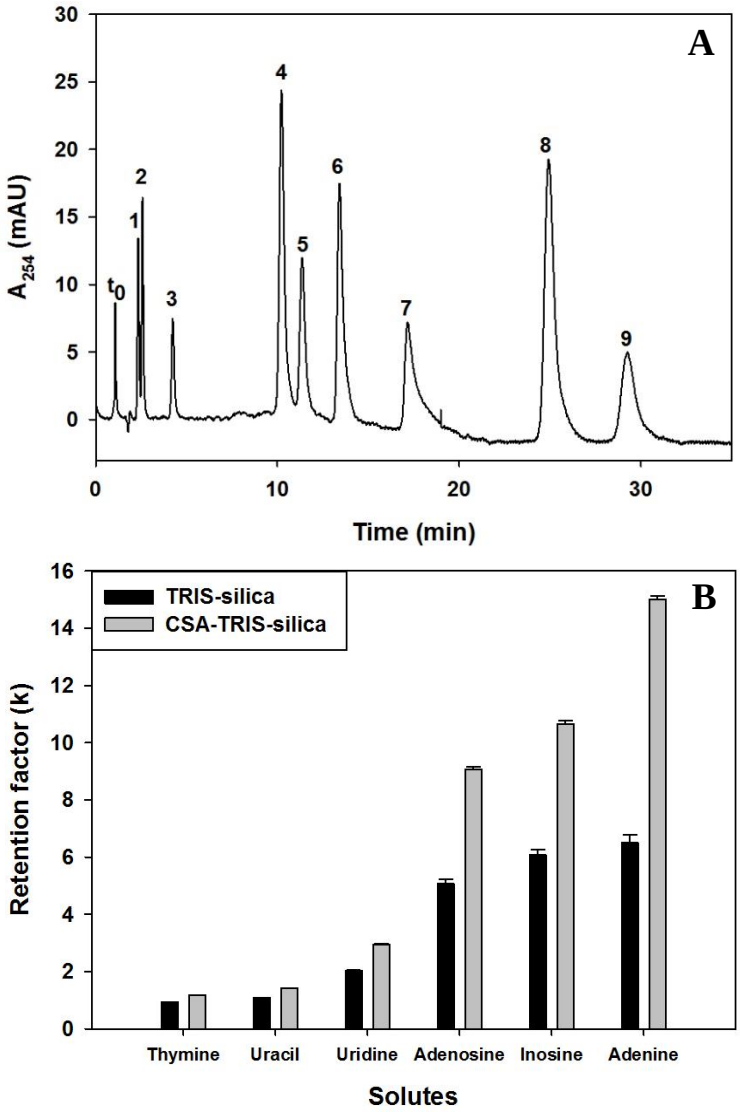


Figure 12. *Chromatogram of nine nucleic acid bases and nucleosides obtained on CSA-TRIS-silica sorbent (A) and comparison of k values of nucleic acid bases and nucleosides obtained on different HILIC columns (B). Separation conditions are the same as in Fig.11 except using a gradient elution with mobile phases A, 100% ACN; mobile phase B, 100% water, 0-5 min, isocratic with 95% A : 5% B; 5-20 min, gradient from 95%-85% A in B; 20-40 min, isocratic with 85% A : 15% B. Solutes; t_0, toluene; 1, thymine; 2, uracil; 3, uridine; 4, adenosine; 5, inosine; 6, adenine; 7, guanosine; 8, cytidine; 9, cytosine.*

that on TRIS-silica column showing the greater hydrophilicity of the multilayered HILIC stationary phase.

Similarly, a mixture of phenols including phenol, resorcinol and pyrogallol was baseline separated on the CSA-TRIS-silica sorbent as shown in Fig. 13B. The retention factors (k values) of the analyzed solutes increased with the increasing number of hydroxyl groups on the molecule. Moreover, the k values obtained on CSA-TRIS-silica column were greater than that obtained on the TRIS-silica column.

Separation of derivatized sugars. Four *p*-nitrophenyl (PNP) derivatized sugars were baseline separated on CSA-TRIS-silica column under un-buffered conditions, using a mobile phase containing only ACN and water as shown in Fig. 14. As expected, all the separated sugars showed higher k values on CSA-TRIS-silica column compared to the TRIS-silica column showing the enhancement of the hydrophilicity of TRIS-silica stationary phase after modification with an additional hydrophilic layer. The logarithm of the retention factors of sugars increase linearly with the increasing number of glucosyl units in the molecule indicating typical HILIC behavior. This behavior is in agreement with the separation model $\log k = (\log \alpha)n_{Glc} + \log \beta$ where α represents the glucosyl selectivity factor between two adjacent sugar molecules separated on the HILIC stationary phase and β represents the k value of the UV tag PNP. The CSA-TRIS-silica sorbent showed a higher selectivity of $\alpha = 2.6$ for separating adjacent sugars compared to that of $\alpha = 2.2$ obtained on the TRIS-silica column. These results indicate that the multilayered CSA-TRIS-silica stationary phase provides different retention, selectivity and efficiency compared to the singly layered TRIS-silica stationary phase.

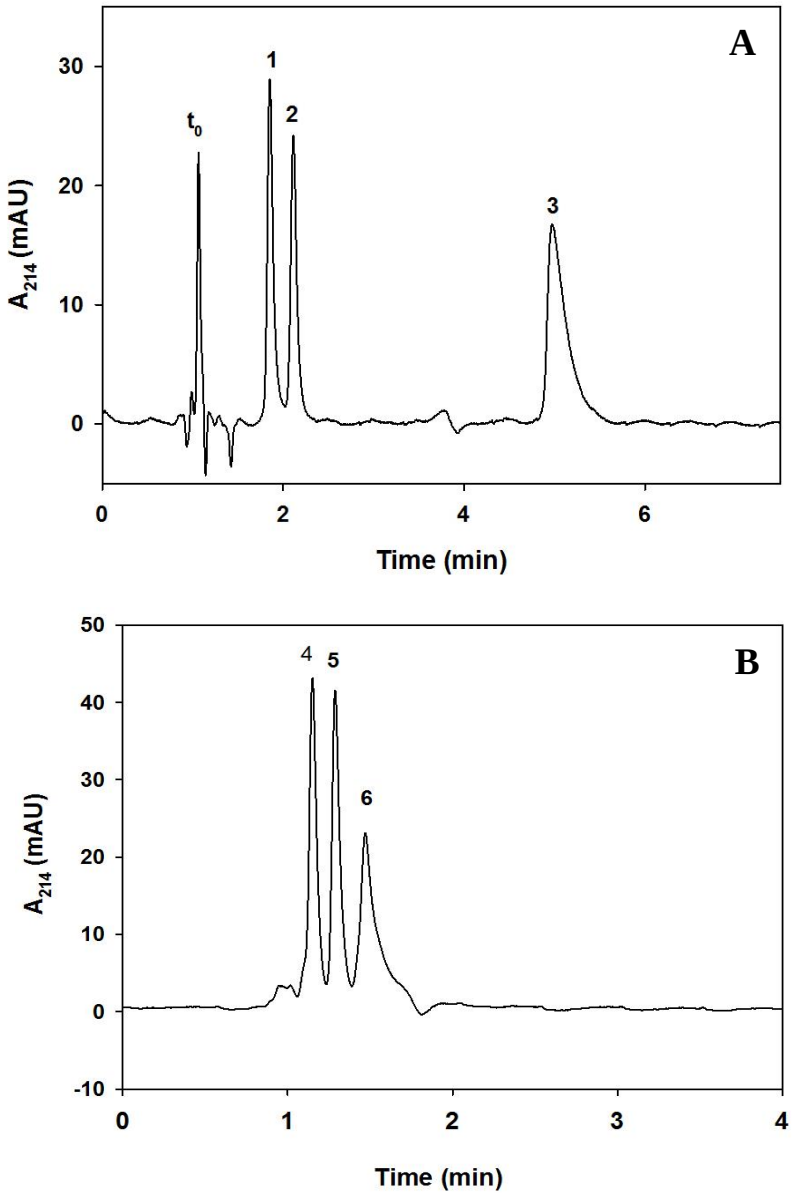


Figure 13. *Chromatograms of three neutral polar compounds (A) and three phenolic compounds (B) obtained on the CSA-TRIS-silica sorbent. Conditions are the same as in Fig. 11 except that the mobile phase was 95% (v/v) ACN : 5%, (v/v) water; detection, UV at 214 nm. Solutes; t_0, toluene; 1, N,N-dimethylformamide (DMF); 2, formamide; 3, urea; 4, phenol; 5, resorcinol; 6, pyrogallol.*

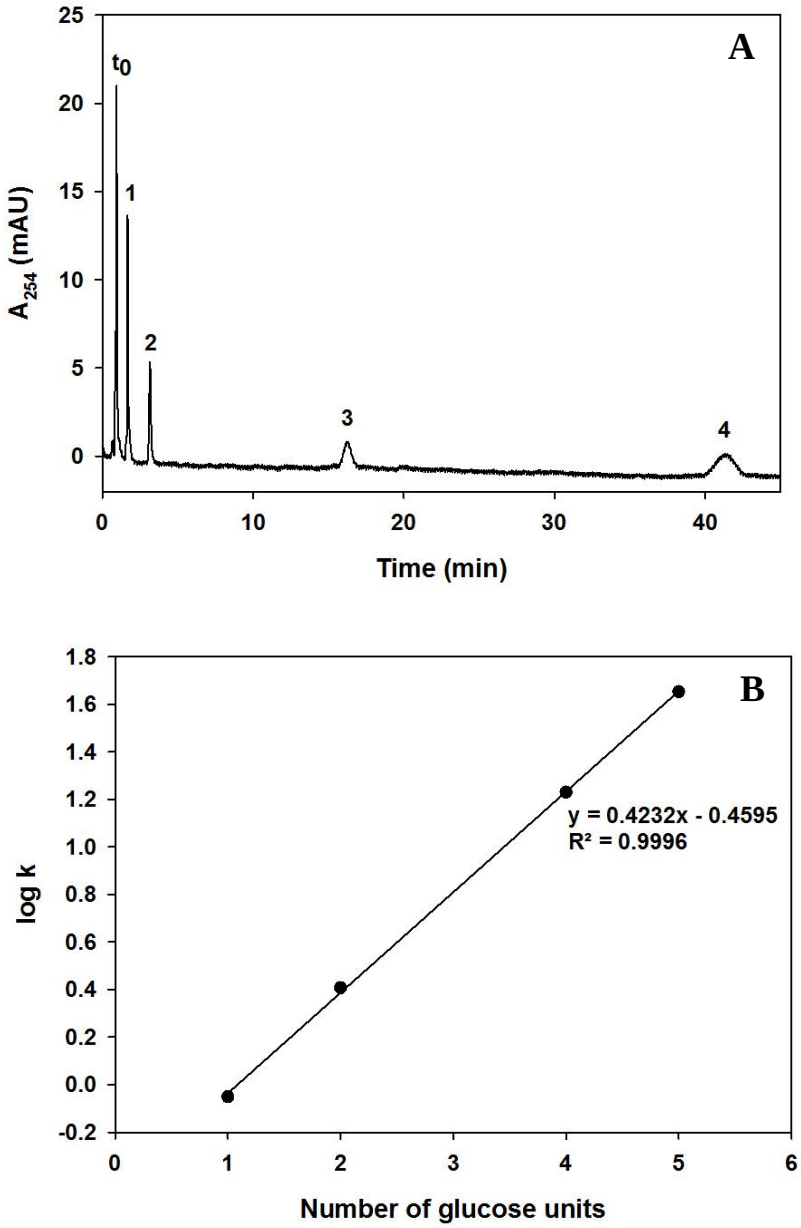


Figure 14. *Chromatograms of four PNP-sugar derivatives (A) and plot of log k vs. number of glucosyl units in the PNP- sugar derivatives (B) obtained on CSA-TRIS-silica sorbent. Conditions are the same as in Fig. 11 except the mobile phase was 85% (v/v) ACN : 15% (v/v) water. Solutes; t_0, toluene; 1, PNP-α-D-glucopyranoside; 2, PNP-α-D-maltose; 3, PNP-α-D-maltotetroside; 4, PNP-α-D-maltopentoside.*

Separation of cyclic nucleotides and phenoxy acid herbicides. The CSA-TRIS-silica sorbent was then applied to separate polar anionic cyclic nucleotides as shown in Fig. 15A. The k values for the anionic nucleotides were significantly decreased on CSA-TRIS-silica column compared to TRIS-silica column under the same conditions due to the repulsion of anionic phosphate group by the anionic CSA layer, as shown in Fig. 15B.

In order to broaden the application range of CSA-TRIS-silica sorbent it was then applied to separate a mixture containing phenoxy acid herbicides and their esters as shown in Fig. 16A. Three phenoxy acid herbicides and two of phenoxy acid herbicide esters were separated using a mobile phase containing 95% ACN and 5% of 10 mM ammonium acetate, pH 6. Since the pK_a of phenoxy acid herbicides are around 3.5, the majority of them are anionic at pH 6. The k values for the anionic herbicides obtained on CSA-TRIS-silica sorbent were lower than that on the TRIS-silica column as shown in Fig. 16B, probably due to the electrostatic repulsion between the anionic herbicides and the anionic CSA layer.

Separation of amino acid derivatives. Seven Dns-AA derivatives were baseline separated under gradient elution conditions using a mobile phase containing ACN and 10 mM ammonium acetate, pH 3as shown in Fig. 17. At this low pH condition, the dimethylamino group of the dansyl moiety is protonated giving a net positive charge to the molecules. Dns-AA were eluted in the order of increasing polarity of the side chain at pH 3, where nonpolar leucine was eluted first, the polar non charged AAs namely, methionine, tryptophan, threonine and serine were eluted next and polar charged AAs namely, aspartate (pK_a = 3.65), lysine (pK_a = 10.53) and arginine (pK_a = 12.48) were eluted last.

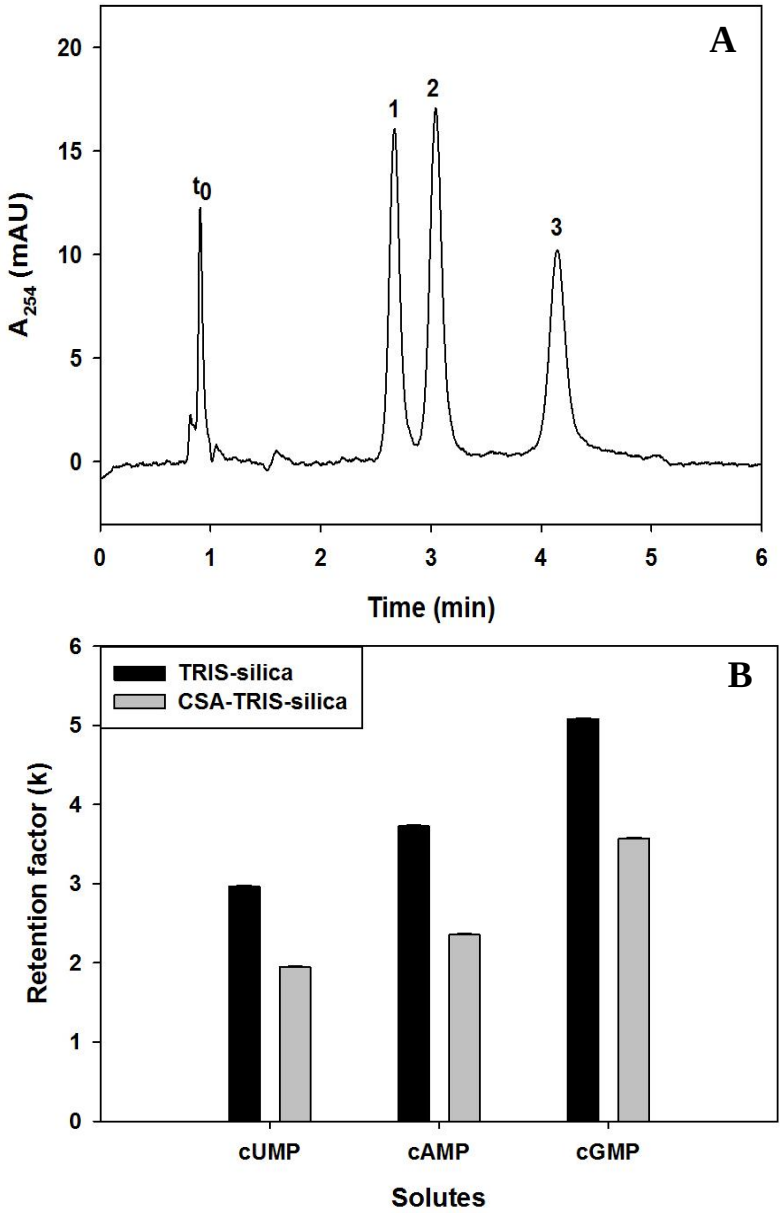


Figure 15. *Chromatograms of three cyclic nucleotides (A) obtained on the CSA-TRIS-silica sorbent and comparison of the k values of cyclic nucleotide obtained on different HILIC columns (B). Conditions are the same as in Fig. 11 except that the mobile phase was 80% (v/v) ACN : 20% (v/v) 25 mM ammonium acetate, pH 8. Solutes; t_0, toluene; 1, cUMP; 2, cAMP; 3, cGMP;*

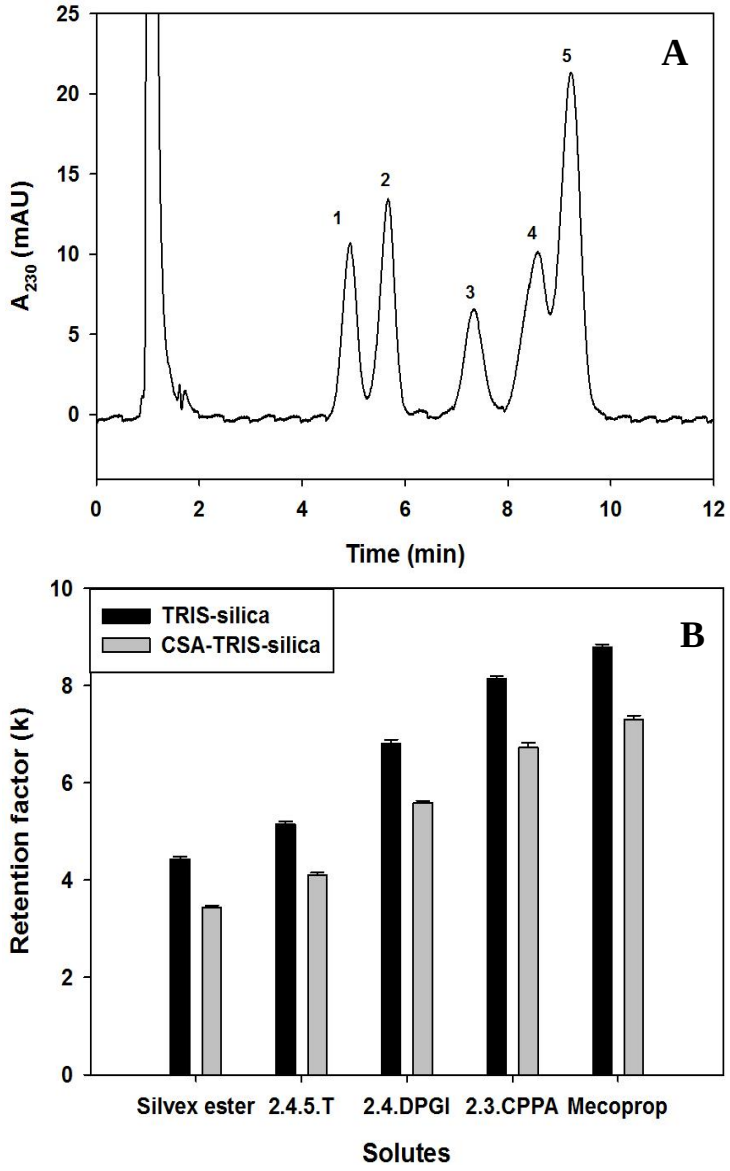


Figure 16. *Chromatogram of five phenoxy acid herbicides and their esters (B) obtained on the CSA-TRIS-silica sorbent and comparison of the k values of phenoxy acid herbicides obtained on the different HILIC columns (B). Conditions are the same as in Fig. 11 except that the mobile phase was 95% (v/v) ACN : 5% (v/v) 10 mM ammonium acetate, pH 6; detection, UV at 230 nm. Solutes;* $t_{0,}$ *toluene; 1, (2,4,5-trichlorophenoxy)propionic acid isooctyl ester; 2, (2,4,5-trichlorophenoxy)-acetic acid; 3, 2,4-dichlorophenoxyacetic acid propylene glycol ester; 4, 2-(3 chlorophenoxy) propionic acid; 5, 2-(4-chloro-2-methyl phenoxy) propionic acid.*

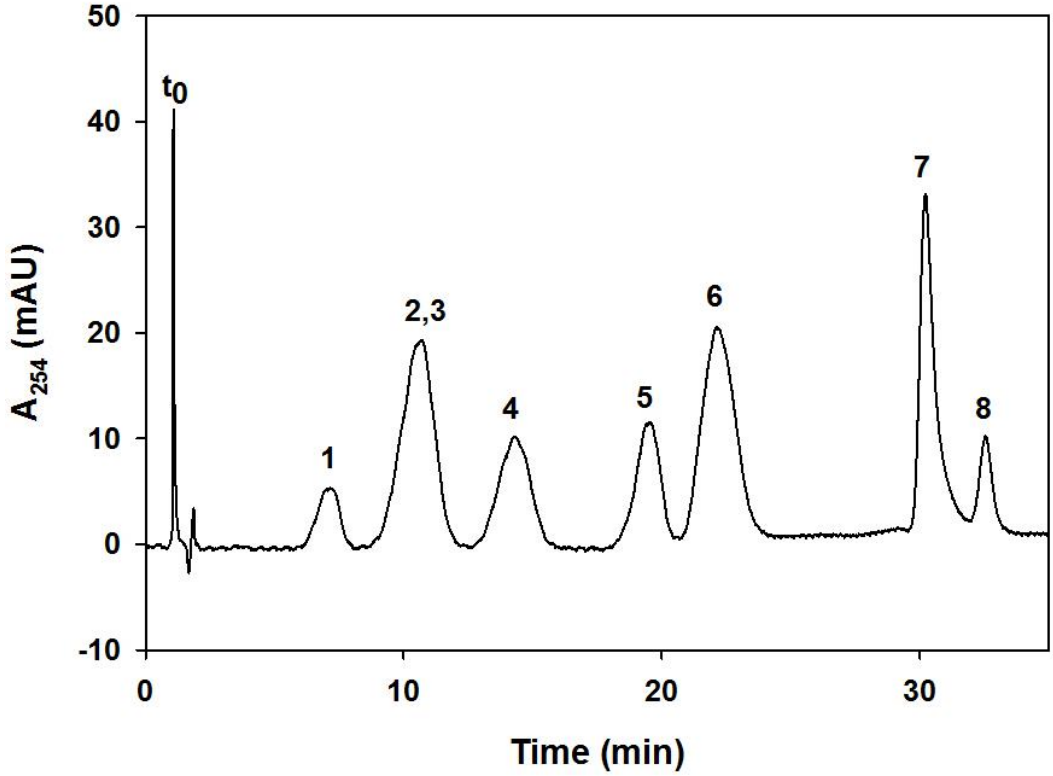


Figure 17. *Chromatograms of Dns-AAs obtained on the CSA-TRIS-silica sorbent. Conditions are the same as in Fig .11 except that the gradient elution with mobile phase A, 100% ACN; mobile phase B, 100% 10 mM ammonium acetate, pH 3; 0-15 min, isocratic with 95% A : 5% B; 15-20 min, gradient from 95%-90% A in B; 20-40 min, isocratic with 90% A : 10% B. Solutes; t_0, toluene; 1,Dns-leu; 2, Dns-met; 3, Dns-trp; 4, Dns-thr; 5, Dns-ser; 6, Dns-asp; 7, Dns-lys; 8, Dns-arg.*

When comparing the retention of different solutes, on the CSA-TRIS-silica column with those obtained on the TRIS-silica column under identical conditions, it was clear that the k values of neutral and cationic solutes was significantly higher on the CSA-TRIS silica column due to the enhanced hydrophilicity of the multilayered HILIC surface. However, anionic solutes showed lower k values on CSA-TRIS-silica column due to the electrostatic repulsion of the CSA layer.

Reproducibility and Stability of the TRIS-Silica Stationary Phases

TRIS-Silica Column. The reproducibility and chemical stability of the TRIS-silica column was investigated using adenosine as the model compound. Intraday and interday reproducibility of the retention factors (k) for adenosine were evaluated and the relative standard deviation (% RSD) were 0.67% (n = 4) and 1.5% (n = 3), respectively. Moreover the % RSD for the k values of adenosine measured on two different TRIS-silica batches (batch-to-batch reproducibility) was 0.78% (n = 2) demonstrating good reproducibility of the TRIS-silica columns.

CSA-TRIS-Silica Column. The stability of the non-covalently attached CSA coating was investigated by considering the % RSD values of the k values for the separation of nucleic acid bases and nucleosides on the CSA-TRIS-silica sorbent. The intraday average %RSD for six nucleic acid bases and nucleosides was 0.810% (n = 3). Moreover, the CSA coating was strongly attached and did not leach out with the mobile phases as shown in the Fig. 18, when using an unbuffered mobile phase. However, the CSA layer was slightly washed off with mobile phases containing high salt content and needed to be re-loaded after approximately 3 days of analysis to get the original separation performance again. The reproducibility of coating procedure (re-loading) was evaluated using DMF, formamide and urea as model compounds. The average % RSD for those solutes was 3.88% (n = 5), indicating the reproducibility of the coating.

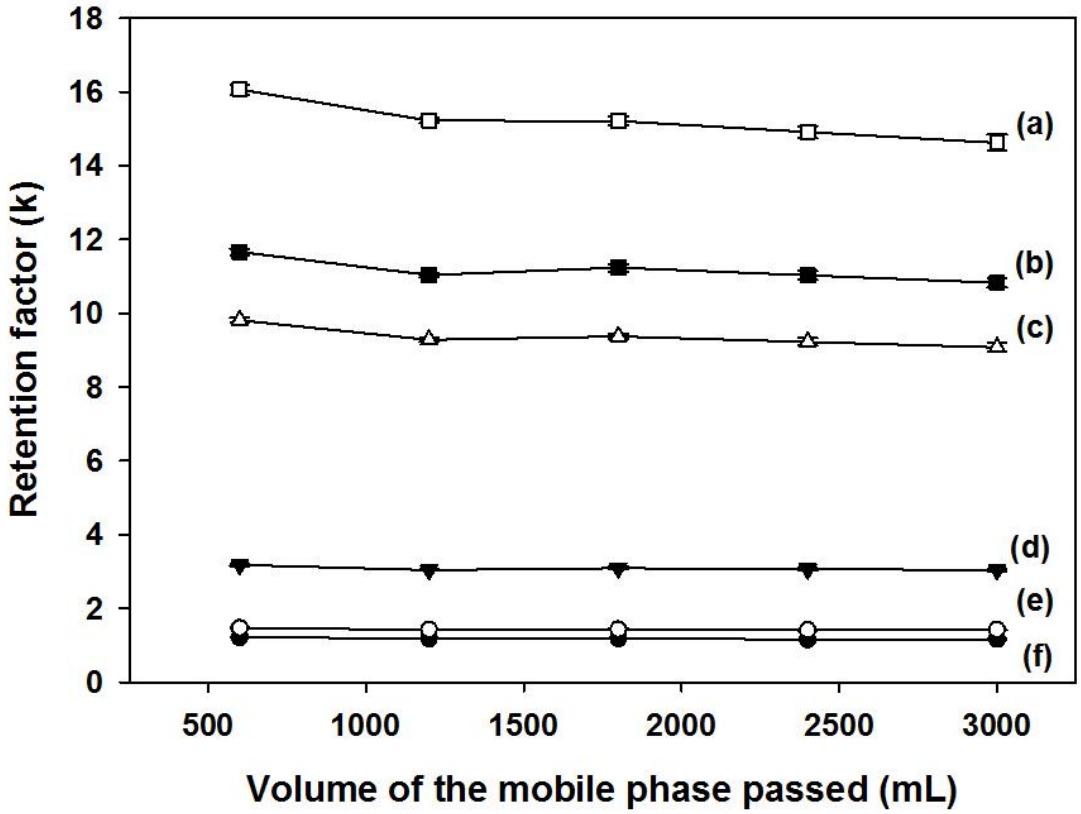


Figure 18. *Coating stability of non-covalently attached CSA layer. Separation conditions are the same as in Fig. 11 except that the mobile phase was 95% ACN : 5% water. Solutes; a, adenine; b, inosine; c, adenosine; d, uridine; e, uracil; f, thymine.*

Conclusions

The TRIS-silica stationary phase with polyhydroxy and amine functionalities showed hydrophilic interaction/weak anion exchange interaction mixed mode retention behavior during the HILIC separation of various polar solutes. Electrostatic interactions between cationic TRIS-silica stationary phase and charged solutes play an important role in HILIC separation mechanism of the TRIS-silica column in addition to the hydrophilic partitioning. Further functionalization of the TRIS-silica surface with anionic mucopolysaccharide chondroitin sulfate A (CSA) yielded the CSA-TRIS-silica composite phase with sulfate, amide, amine and polyhydroxy functionalities. The CSA-TRIS-silica sorbent having a net positive charge at lower pH conditions and net negative charge at higher pH conditions offered different HILIC selectivity and retentivity towards various polar charged and neutral solutes compared to those on the TRIS-silica column.

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CHAPTER IV

SUGAR FUNCTIONALIZED NEUTRAL POLAR SILICA-BASED STATIONARY PHASES FOR HYDROPHILIC INTERACTION LIQUID CHROMATOGRAPHY

Introduction

Hydrophilic interaction liquid chromatography (HILIC) is an alternative chromatographic technique for the separation of polar analytes that may be difficult to analyze using reversed phase chromatography (RPC) or normal phase chromatography (NPC) [1]. Since in depth details about HILIC technique and the HILIC separation mechanism were discussed in the previous chapters, only brief additional background relevant to the research project reported in this chapter is included below.

A wide range of polar functional groups including amino [2], amide [3], diol [4], saccharides [5, 6], sulfoalkyl-betaine [7], and cyano [8] have been bonded to the bare silica surface in order to develop HILIC stationary phases with increased hydrophilicities and different separation selectivity. Among them, the stationary phases with amide, diol, cyano, and saccharide functional groups are considered as polar but neutral stationary

phases because they cannot be charged in the pH range typically used in HILIC. Therefore, these neutral stationary phases undergo little to no electrostatic interaction with charged solutes leading to higher separation efficiencies and different separation selectivity compared to those of charged HILIC surfaces.

Some biomolecules such as carbohydrates, proteins, peptides, amino acids naturally contain multiple polar groups (e.g., amine, carbonyl, polyhydroxy and amide) in their structures. Hence, biomolecule bonded silica can be considered as ideal stationary phases for HILIC separations [9]. In this regard, carbohydrates which are widely used both as chromatographic supports and chemically bonded stationary phases in chromatography have gained an increasing attention as HILIC separation media during the last decade. These mono and oligosaccharides contain a high density of exposed hydroxyl groups and the great hydrophilicity of these multiple hydroxyl groups makes these saccharides attractive as HILIC stationary phases. In this regard, Persson et al. have grafted sorbitol methacrylate onto the silica and this stationary phase was used for the separation of nucleic acid bases under HILIC chromatographic conditions [6]. In addition, several attempts have been made to immobilize saccharides on solid supports *via* "click chemistry" in recent years [10, 11]. HILIC stationary phases with immobilized maltose [5, 12, 13], glucose [14], β-cyclodextrin [14, 15], chitooligosaccharides [16], chondroitin sulfate [17] have been reported in the recent literature as well. However, in most of these reported derivatization procedures, the sugar molecules were modified with some sort of anchoring group in advance so that it can react with the functional groups on the silica surface, which is very inconvenient to perform during stationary phase preparation process. Moreover, some of these sugar functionalized HILIC stationary

phases showed strong ionic interactions with ionic solutes due to the charged functional groups or the charged coupling agents present on the silica surface, leading to poor separation performance during the analysis of ionic compounds. Therefore, the development of neutral, polar, robust sugar functionalized silica based HILIC stationary phases remains a challenge.

On the other hand, it is well known that the specific surface area and the number of silanol groups on the silica surface is decreased by increasing the pore size of the porous silica particles. Additionaly, the rates of the mass transfer diffusion of analytes within the pores towards the separation surface are also affected by increasing the pore size. In this regard, the impact of the silica gel pore and particle sizes towards HPLC column efficiency and resolution on sugar grafted chiral stationary phases have been reported [18, 19]. The results of the above studies showed that the selectivity factor for chiral separations increases with the pore size possibly due to the better accessibility of chiral selector to the racemic compounds as the pore size increases. Similar systematic studies focusing on the effect of the pore size of polar bonded silica stationary phases towards HILIC separation performance is highly beneficial for separation scientists to choose the best column for their HILIC applications.

Here, we are reporting an alternative synthetic pathway for the functionalization of silica with various sugars including maltose (MAL) and sorbitol (SOR) by modifying our previous attempt to functionalize zirconia with PEG 2000 [20]. The anchoring groups used in this synthetic route do not possess any charged groups. Therefore, the attachment of the sugars yielded neutral but polar stationary phases for HILIC separation of a wide range of analytes without undesirable electrostatic interactions. Moreover, the effect of

the mobile phase composition, e.g., ACN, buffer and pH on the solute retention factor was studied to understand the separation mechanism of the novel sugar silica matrices. In addition, each column was characterized by separating a large number of polar solute mixtures under HILIC conditions. Finally, the effect of the silica gel pore size on HILIC separation performance was investigated by using Zorbax silica gel with different average pore diameters (75 Å and 300 Å).

Experimental

Instrumentation

Most HPLC separations were performed on a Waters Alliance 2690 separation module (Milford, MA, USA), equipped with an in-line degasser, a quaternary solvent pump, an auto sampler and a thermostated column compartment. Detection was performed using a PDA detector (W2996). The sample and the column compartments were maintained at ambient temperature for all chromatographic separations. In addition, an HPLC system consisting of a quaternary solvent delivery system Q-grad pump from Lab Alliance (State College, PA, USA), a Model 3100 UV–Vis variable wavelength detector from Milton Roy, LDC division (Riviera Beach, FL, USA) and a Rheodyne injector Model 7010 from IDEX Health & Science LLC (Rohnert Park, CA, USA) equipped with a 20 µL loop was occasionally used. Data acquisition was carried out using Empower 2 (Build 2154) software (Waters Chromatography) or using the clarity version, 3.0.06.589 advanced chromatographic software from Data Apex (Prague, Czech Republic). A constant pressure pump from Shandon Southern Products Ltd. (Cheshire, UK) was used for the slurry packing of the columns.

Reagents and Materials

Zorbax silica, with a 5 μm average particle diameter and 75 Å average pore diameter was donated by BTR separations (Wilmington, DE, USA). Zorbax silica, with 5 μm average particle diameter and 300 Å average pore diameter was obtained from E.I. du Pont de Nemours and Company Inc. (Wilmington, DE, USA). Adenine, adenosine, cytosine, uracil, uridine, guanosine, cytidine, PNP-α-D maltopentoside, maltotriose, maltotetrose, maltopentose, maltohexose, maltoheptose, gentisic acid, syringic acid, *p*-hydroxybenzoic acid, uridine 2':3'-cyclic monophosphate (cUMP), adenosine 2':3'-cyclic monophosphate (cAMP), guanosine 2':3'-cyclic monophosphate (cGMP), uridine 5'-monophosphate (UMP), inosine 5'-monophosphate (IMP), guanosine 5'-monophosphate (GMP), cytidine 5'-monophosphate (CMP) and thiourea were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The γ-glycidyloxypropyl trimethoxysilane (GPTMS), 2-aminobenzamide (2-AB), toluic acid, ferulic acid and *o*-coumaric acid were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). Maltose, D-sorbitol and urea were purchased from Fisher Scientific (Fair Lawn, NJ, USA). ACS grade acetonitrile, isopropanol, toluene and methanol were obtained from Pharmco-AAPER (Brookfield, CT, USA). Ammonium acetate and formic acid were obtained from Spectrum Quality Products (New Brunswick, NJ, USA). Sodium cyanoborohydride was purchased from Acros Organic (New Jersey, USA). BF_3·etherate was obtained from TCI America (Portland, OR, USA). PNP-α-D-glucopyranoside, PNP-α-D maltoside and PNP-α-D-maltotetroside were obtained from EMD Bioscience Inc. (La Jolla, CA, USA). Inosine was obtained from Calbiochem Corp. (Los Angeles, CA, USA). *N*,*N* - Dimethylformamide and acetic acid were purchased from EM Science

(Gibbstown, NJ, USA). Benzoic acid was obtained from J.T Baker Chemical Co. (Phillipsburg, NJ, USA). Thymine was obtained from Nutritional Biochemical Corporation (Cleveland, OH, USA).

Preparation of Epoxy Activated Silica

Typically, 2.5 g of dry Zorbax silica gel was suspended in 30 mL of dry toluene in a 3-neck round-bottomed flask connected to a reflux condenser. Initially, this mixture was heated to 95 °C with slow stirring to make a slurry. To this suspension, 2.5 mL of γ-glycidoxypropyl trimethoxysilane was added, and the reaction mixture was stirred for 18 h at 95 °C. The epoxy activated silica thus obtained was rinsed successively with toluene and acetone and then immediately used in the next modification step.

Functionalization of Epoxy Activated Silica with Sugars

The resulting epoxy activated silica was rinsed once with DMF and re-suspended in 30 mL of either 100 mM maltose (MALT) in DMF or 100 mM sorbitol (SOR) in DMF in a round-bottomed flask. This mixture was stirred at room temperature for 30 min to make homogeneous slurry. Fifty microliter aliquots of BF_3·etherate catalyst were then added to this reaction mixture 4 times, at 2 h time intervals. The reaction mixture was stirred at room temperature for 18 h. The resulting MALT-silica/ SOR-silica gels were rinsed with DMF, water and acetone, and were allowed to dry in air.

Column Packing

Two grams of either MALT-silica or SOR-silica were dispersed in 20 mL of isopropanol to form a 10% (w/v) slurry, which was sonicated for 20 min to eliminate air

and to ensure homogenization. The slurry was then packed into a stainless-steel column (10 cm × 4.6 mm id) with isopropanol as the packing solvent under 6000-7000 psi pressure for 30 min using a constant pressure pump. Then, the columns were equilibrated with the running mobile phase for 30 min before starting the chromatographic analysis.

Chromatographic Conditions for HILIC Separations

Analyses were carried out at 214 nm or 254 nm wavelengths under 1 mL/min flow rate at room temperature. Solution mixtures containing ACN and ammonium acetate buffer was used as the mobile phase unless otherwise specified. Molarity and the pH of the mobile phases are presented with respect to the aqueous portion. The pH of the aqueous ammonium acetate buffer was adjusted using formic acid or ammonium hydroxide, before mixing it with ACN. All the mobile phases were freshly prepared, filtered through 0.1 Whatman filter papers and sonicated for 30 min before use. The retention factors, k, were determined from the retention time of toluene as the unretained marker (t_0) and the solute retention (t_R).

Precolumn Derivatization of Sugars with 2-Aminobenzamide (2-AB)

The 2-AB derivatization was performed according to a previously published reductive amination procedure [21, 22]. Briefly, a solution of 200 µL of 0.25 M 2-AB prepared in 1:1 methanol/water pH 5.0 (pH adjusted with acetic acid) was mixed with 30 µL of 1.0 M $NaBH_3CN$ in a 0.3 mL reaction vial. To this solution, 40 µL of 1.4 M aqueous saccharide solution were added and the content was mixed well. The vials were sealed and put in a dry-bath. The derivatization was allowed to proceed at 72 °C for 24 h.

FTIR Characterization

Infrared spectra of the bare silica and SOR silica particles were obtained in the 4000-600 cm^{-1} range with a resolution of 4 cm^{-1} by accumulating 32 scans using a Varian 800 FTIR Scimitar series FTIR (Varian Inc., CA, USA). A thin layer of dried silica particles were spread in-between two NaCl window plates and spectrum were taken in transmittance mode.

Results and Discussion

Preparation and Characterization of Sugar Bonded Silica Based HILIC Stationary Phases

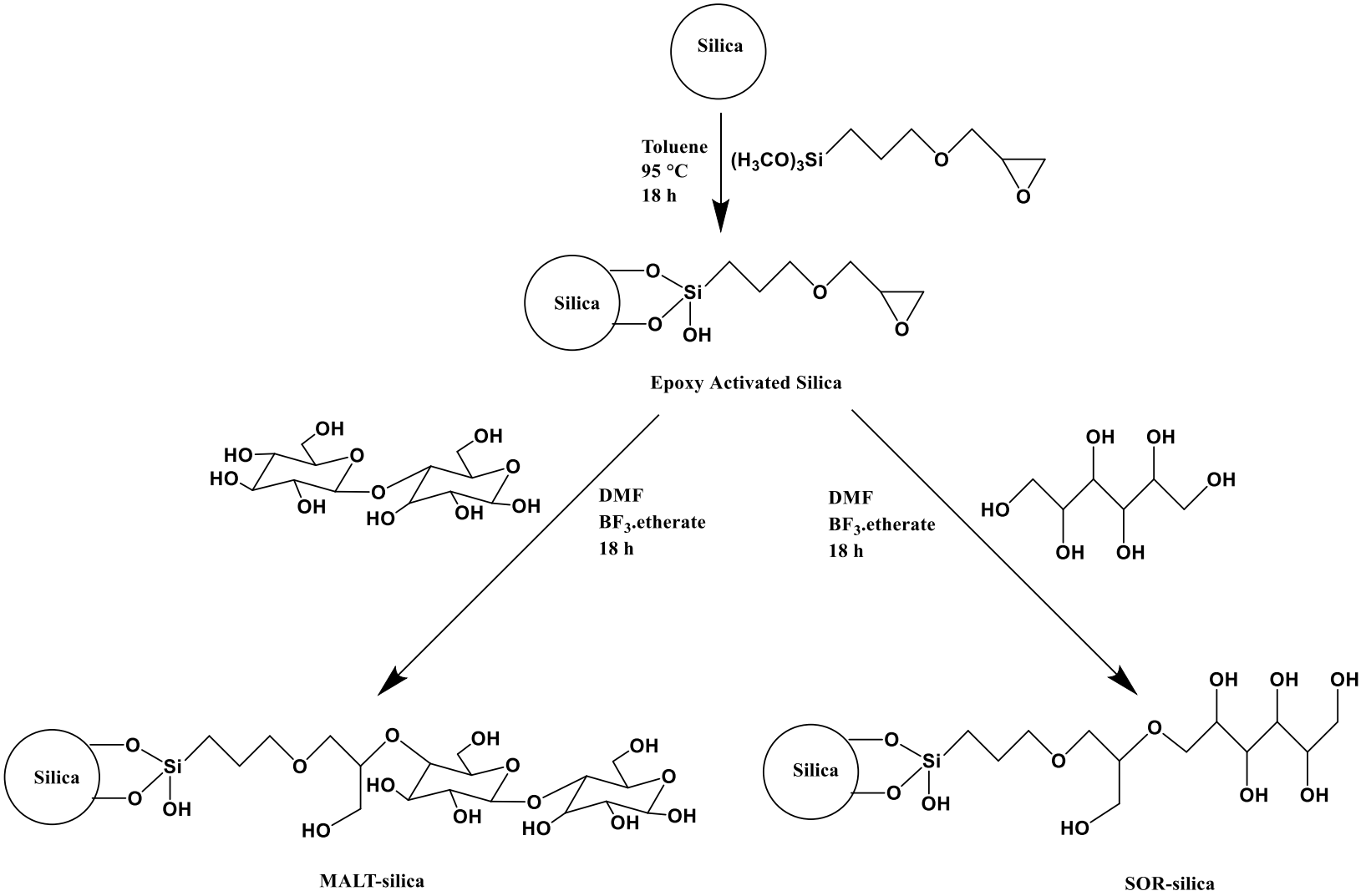
As it is mentioned earlier, neutral HILIC stationary phases do not undergo electrostatic interactions with charged solutes leading to a different selectivity compared to anionic or cationic stationary phases. Therefore, silica microparticles were functionalized with two different saccharides in order to utilize the great hydrophilicity of multiple hydroxyl groups in HILIC separations under neutral conditions. In this regard, sorbitol which is a linear hexose sugar alcohol and maltose [*O*-α-D-glucopyranosyl-(1→4)-D-glucopyranose] which is a common glucosyl-glucose disaccharide produced by enzymatic hydrolysis of starch were attached to the silica surface *via* an epoxy coupling agent.

The organosilane γ-glycidoxypropyl trimethoxysilane (GPTMS) is useful for covalently attaching reactive epoxy groups to the silica surface for grafting a chromatographic ligand of interest. GPTMS contains both trimethoxysilane groups which can condense with the silica surface to form a stable anchor as well as an epoxide group

which can undergo a ring opening reaction with a wide range of nucleophiles for the attachment of the desired functionality to a silica surface. Therefore, the bare silica surface was first activated to an epoxy surface by reacting it with GPTMS as shown in Scheme 1.

Sorbitol or maltose was then attached to the epoxy activated silica *via* nucleophilic ring-opening reaction using a Lewis acid namely BF_3·etherate as the catalyst according to the Scheme 1. Usually, this reaction is carried out in an aprotic solvent such as dioxane [23-25]. Since these polar sugars showed limited solubility in the relatively nonpolar dioxane, DMF was selected as a polar aprotic medium for the epoxide-sorbitol reaction.

The attachment of polar sugar functionalities to the silica particles was assessed by subjecting the bonded silica to IR analysis. The IR spectra obtained for bare silica and SOR-silica are presented in Fig. 1. The main feature of all the silica spectra are related to the similarities of peaks associated with the silica backbone such as an intense band due to siloxane stretching at 1100 cm^{-1}. These intense peaks mask most of the characteristic peaks, which can be used to confirm the attachment of sugar functionalities onto the silica surface. However, SOR-silica spectra showed characteristic peaks at 1410 cm^{-1} arising from OH bending of alcohol groups, 1480 cm^{-1} arising from CH bending of alkane groups and at 2850 cm^{-1} and 2950 cm^{-1} arising from C-H stretching of alkane groups confirming the presence of organic groups immobilized onto the silica surface. MALT-silica also showed similar results in IR analysis.



Scheme 1. *Schematic diagram showing the preparation of sugar functionalized silica.*

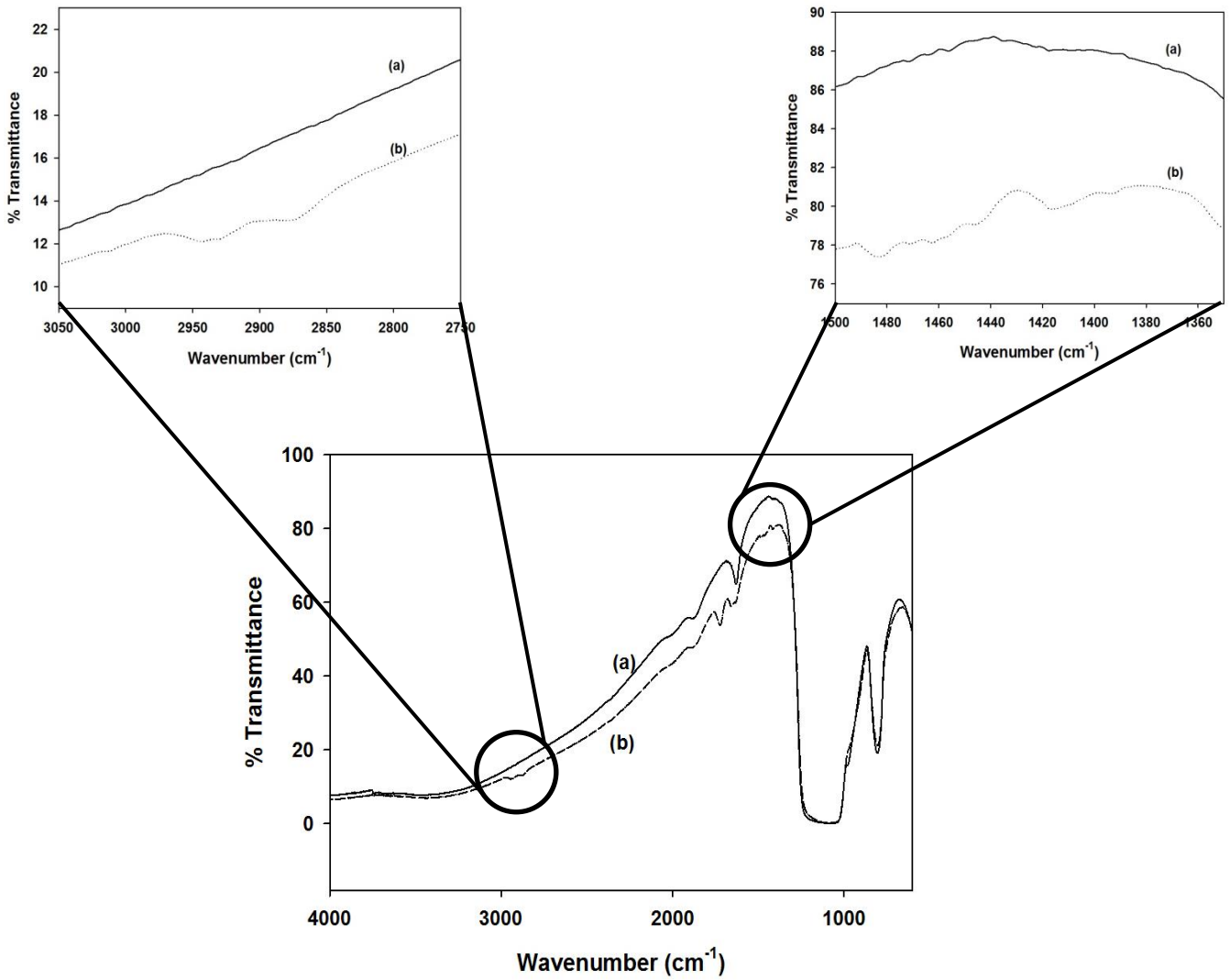


Figure 1. *FTIR spectrum of (a) bare silica and (b) SOR-silica.*

Chromatographic Behavior of Sugar Bonded Silica Stationary Phases

The retention of polar solutes on HILIC stationary phases is largely dependent on the solvent strength or the relative polarity of the mobile phase. Acetonitrile is commonly used for the preparation of hydro-organic mobile phase in HILIC due to its excellent miscibility with water at all proportions. Since, ACN is the weaker solvent in HILIC, increasing the ACN content in a hydro-organic mobile phase will decrease its relative polarity and the elution power leading to increasing the solute retention time. Therefore, the effect of the ACN content on the retention behavior of polar solutes on sugar bonded silica stationary phases was studied by varying the percentage of ACN in the mobile phase. As shown in Fig. 2, the retention factor, k, of polar solutes separated on SOR-silica and MALT-silica stationary phases increased with increasing ACN content in the mobile phase showing the typical HILIC separation behavior of sugar bonded stationary phases.

Moreover, the steep increase in the k values of the polar solutes above 85% ACN in the mobile phase indicated that the polar solutes are extremely sensitive to even smaller variations of the % ACN under the HILIC conditions in that region. Therefore, special attention must be paid to this fact during HILIC method development.

The plots of log retention factor (log k) vs. % buffer (v/v) content in the mobile phase obtained on sugar functionalized silica stationary phases showed completely opposite behavior compared to a reversed-phase column where the log k decreases with increasing water content in the mobile phase confirming the hydrophilic interaction behavior. Moreover, these plots are not perfectly linear (R^2 around 0.900) indicating that

the separation mechanism is not solely based on hydrophilic partitioning. As expected, the slopes of the log k *vs* % water (v/v) content of the mobile phase for two different nucleobases, namely cytosine and uracil, showed different values. Lastly, the slope of log k vs. % water (v/v) content in the mobile phase for adenosine obtained on the MALT-silica column showed a higher value (-0.0526) than that obtained on the SOR-silica column (-0.0396) under identical conditions indicating the greater hydrophilicity of the MALT-silica column possibly due to the greater number of exposed hydroxyl groups.

In addition, the mobile phase ionic strength and pH also play an important role in HILIC separations. In this regard, two acidic analytes [syringic acid and *p*-hydroxybenzoic acid (PHB)], one nucleobase (cytosine) and one nucleoside (adenosine) were separated on the sugar-bonded stationary phases under three different ionic strengths and two different pH conditions in order to evaluate the possible mobile phase effects on HILIC retention and the results are summarized in tables 1 and 2.

The choice of the salt for maintaining the ionic strength in HILIC applications are largely limited by the poor solubility of salts in ACN-rich mobile phases. Therefore, ammonium acetate was used for this purpose given the fact that it has a greater solubility even in hydro-organic mobile phases containing high percentage of ACN.

Even if the grafting of sugars onto the silica surface is successful, there can be some exposed silanol groups on the bonded silica surface. These silanol groups may ionize at high pH conditions giving cation exchange characteristics to the column. Therefore, acidic solutes at high mobile phase pH conditions may experience interplay of electrostatic repulsion from the stationary phase and hydrophilic partitioning.

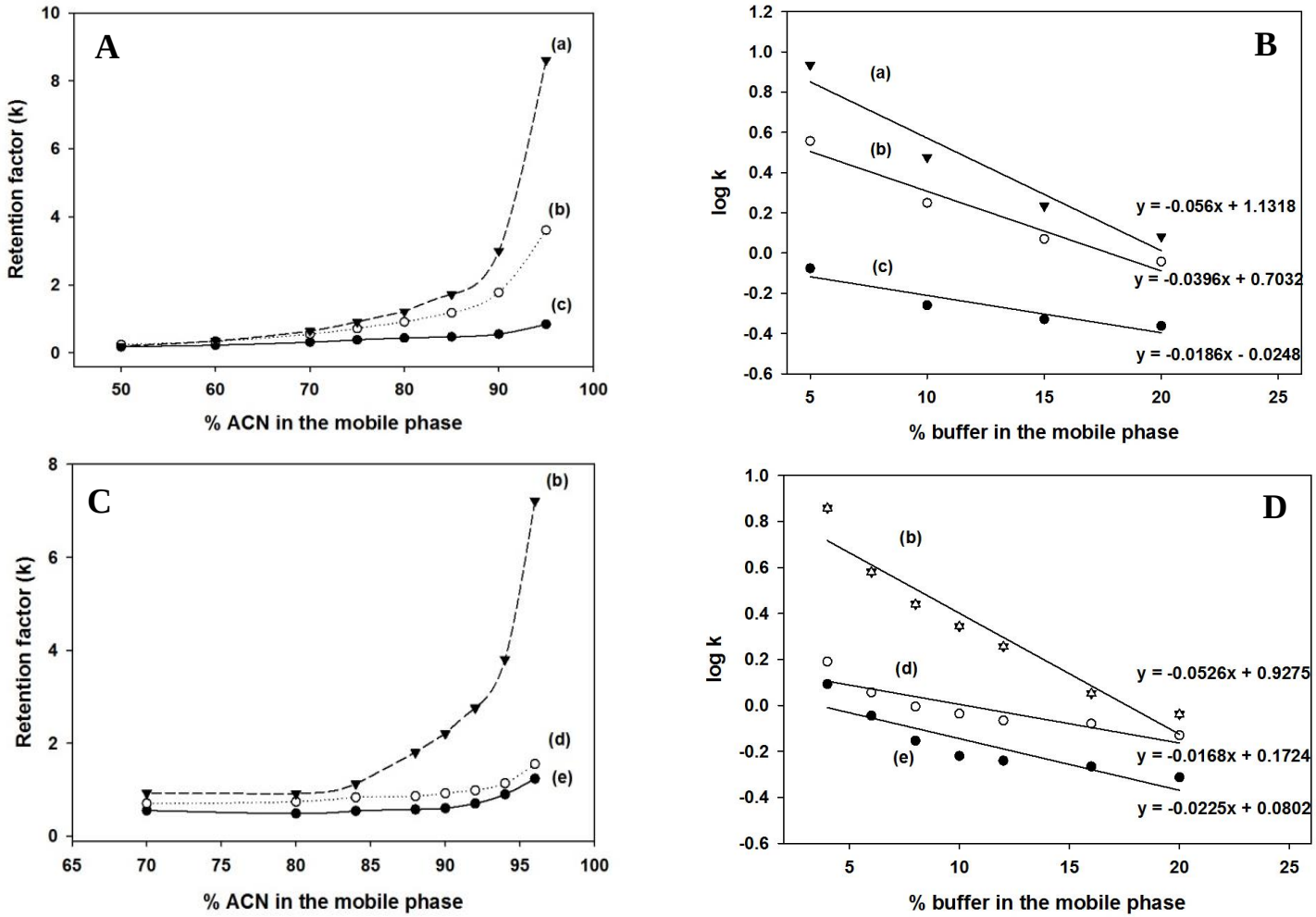


Figure 2. *Plots of k values vs. %ACN (v/v) in the mobile phase obtained on SOR-silica (A) and MALT-silica (C) and plots of log k vs. % water (v/v) in the mobile phase obtained on SOR-silica (B) and MALT-silica (D) columns. Separation conditions; column, 10 cm × 4.6 mm id; flow rate, 1 mL/min; detection, UV at 254 nm; injection. volume, 20 µL; column temperature, room temperature; mobile phase, ACN with varying % (v/v) of 25 mM ammonium acetate, pH 3. Solutes; a, cytosine; b, adenosine; c, uracil; d, uridine; e, PNP-α-D-glucopyranoside.*

TABLE 1

EFFECTS OF MOBILE PHASE IONIC STRENGTH AND pH
ON THE k VALUES OF CHARGED SOLUTES –
SORBITOL SILICA COLUMN

Solute	**k at 95% ACN : 5%, pH 7, Ammonium acetate**			**k at 95% ACN : 5%, pH 3, Ammonium acetate**		
	25 mM $NH_4^+Ac^-$	**50 mM $NH_4^+Ac^-$**	**100 mM $NH_4^+Ac^-$**	**25 mM $NH_4^+Ac^-$**	**50 mM $NH_4^+Ac^-$**	**100 mM $NH_4^+Ac^-$**
Adenosine	3.57 ± (0.00700)	3.59 ± (0.0127)	3.76 ± (0.00700)	3.94 ± (0.0197)	4.01 ± (0.0325)	4.27 ± (0.127)
Cytosine	8.00 ± (0.00700)	8.06 ± (0.0210)	8.34 ± (0.00700)	9.17 ± (0.0120)	9.23 ± (0.0310)	9.77 ± (0.253)
p-Hydroxyl benzoic acid	3.18 ± (0.0440)	4.51 ± (0.0430)	6.00 ± (0.00700)	-	-	-
Syringic acid	5.03 ± (0.0282)	6.63 ± (0.0120)	8.30 ± (0.0636)	1.15 ± (0.0127)	1.13 ± (0.00630)	1.20

TABLE 2

EFFECTS OF MOBILE PHASE IONIC STRENGTH AND pH
ON THE k VALUES OF CHARGED SOLUTES –
MALTOSE SILICA COLUMN

Solute	**k at 95% ACN : 5%, pH 7, Ammonium acetate**			**k at 95% ACN : 5%, pH 3, Ammonium acetate**		
	25 mM $NH_4^+Ac^-$	**50 mM $NH_4^+Ac^-$**	**100 mM $NH_4^+Ac^-$**	**25 mM $NH_4^+Ac^-$**	**50 mM $NH_4^+Ac^-$**	**100 mM $NH_4^+Ac^-$**
Adenosine	4.40 ± (0.0272)	4.09 ± (0.0171)	4.42 ± (0.0079)	3.74 ± (0.00390)	3.85 ± (0.00170)	3.59 ± (0.00110)
Cytosine	8.86 ± (0.0750)	8.15 ± (0.0285)	9.18 ± (0.0170)	7.68 ± (0.00560)	7.34 ± (0.00790)	6.11 ± (0.00230)
p-Hydroxyl benzoic acid	2.50 ± (0.0428)	3.08 ± (0.0358)	5.03 ± (0.0477)	0.380 ± (0.00780)	0.420 ± (0.00170)	0.450 ± (0.0006)
Syringic acid	4.16 ± (0.0604)	4.84 ± (0.0533)	7.36 ± (0.0658)	0.590 ± (0.0447)	0.670 ± (0.0388)	0.650 ± (0.00450)

Consequently, the k values should be decreased with increasing pH. In contrast to the above basic principle, syringic acid (pK_a = 4.33) and PHB (pK_a = 4.54) showed longer retention at pH 7 than at pH 3 on both sugar functionalized silica columns as shown in the Tables 1 and 2. This indicates that the electrostatic repulsion between anionic acidic solutes and anionic silica surface is less significant compared to the hydrophilic interaction between the charged analytes and the attached sugar layer indicating an effective shielding of the residual silanols by the surface bonded sugar layer. As expected, the k values of acidic solutes increased with increasing ionic strength of the mobile phase at pH 7 due to the effective masking of the exposed charged functionalities (i.e., residual surface silanol groups) on the polar bonded silica surface by the salt molecules.

The analyzed adenosine (pK_a = 3.4) and cytosine (pK_a = 4.6) are slightly protonated at pH 3, acquiring net positive charges. These analytes however did not show any significant change in their retention factors with increasing salt concentration in the mobile phase indicating the neutral polar HILIC surface of the sugar functionalized silica columns at lower pH conditions as expected. With the neutral polar HILIC stationary phases under investigation, the high salt concentration in the mobile phase should in principle lead to more salt ions into the water layer adsorbed to the HILIC surface yielding an increase in the volume and the hydrophilicity of that layer [1]. As a result, the k values of all analyzed solutes showed in general slight increase with increasing ionic strength of the mobile phase at pH 3.

Separation of nucleosides and nucleic acid bases. Since the retention properties of a HILIC stationary phase is not solely related to the polarity itself, it is difficult to compare and evaluate the relative hydrophilicity of different stationary phases by only considering their polar structures as discussed in the previous chapters. Moreover, unlike alkylbenzenes in RPC there is no available commonly accepted probe analytes that can be used to compare the relative hydrophilicity of different stationary phases due to the complexity of the HILIC retention mechanism [26]. Therefore, the relative hydrophilic interaction behavior of SOR-silica column and MALT-silica column was compared by means of common chromatographic parameters such as k values, selectivity and separation efficiency, using nucleic acid bases and nucleosides as test probes and the resulting chromatograms are shown in Fig. 3. According to the chromatograms in Fig. 3 and the summarized data in Table 3, the solutes separated on the MALT-silica column showed apparently greater k values than those obtained on SOR-silica column possibly due to the enhanced hydrophilicity imparted by the greater number of hydroxyl groups within the maltose molecule. This behavior is more significant for the late eluting solutes (guanosine and adenine) from the MALT-silica column confirming the greater hydrophilicity of the column compared to the SOR-silica stationary phase.

Although the selectivity values of the adjacent nucleic acid bases and nucleosides separated on the sugar functionalized silica columns showed comparable values, the MALT-silica column showed significantly greater affinity towards cytosine and its nucleoside cytidine and a mobile phase with higher percentage of water was required to elute these two analytes out from the column. Moreover, cytidine which was eluted

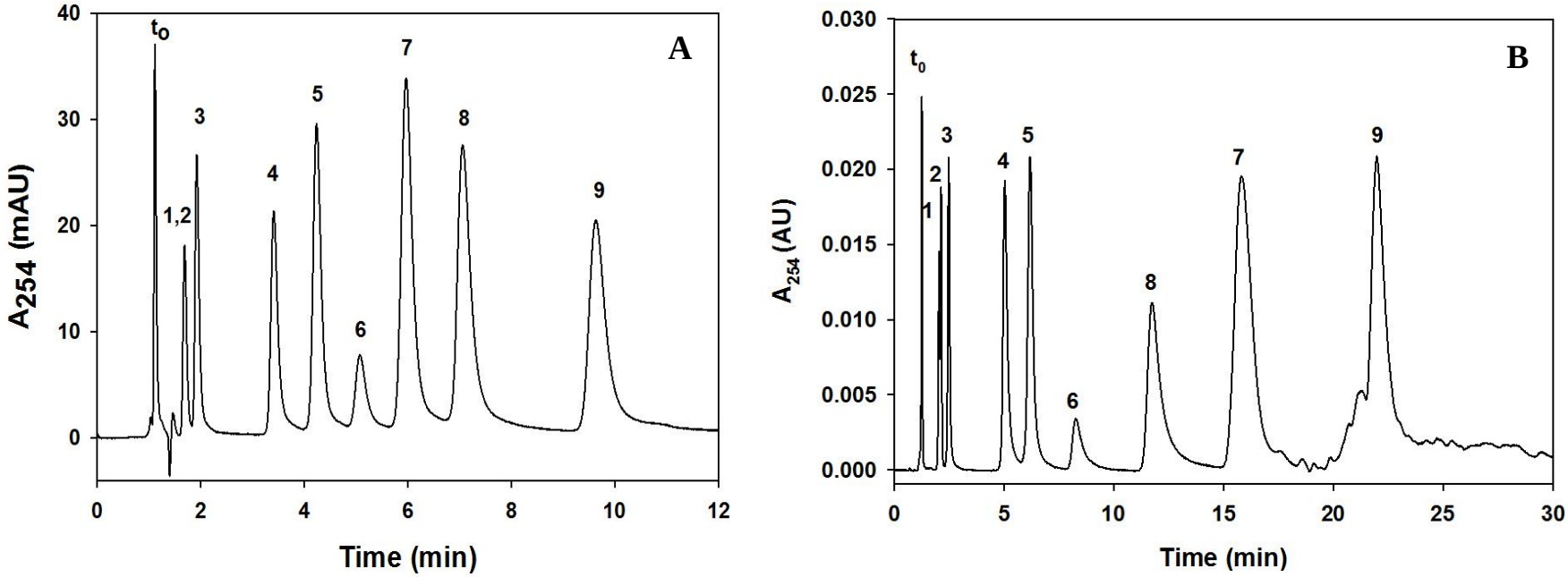


Figure 3. *Chromatograms of nine nucleic acid bases and nucleosides obtained on the SOR-silica stationary phase (A) and the MALT-silica stationary phase (B). Separation conditions; column, 10 cm × 4.6 mm id; flow rate, 1 mL/min; detection, UV at 254 nm; injection volume, 20 µL; column temperature, room temperature. Mobile phases in A, 95% (v/v) ACN : 5% (v/v) water; mobile phase in B gradient elution with mobile phases I, 95% (v/v) ACN : 5% (v/v) water; mobile phase II, 100% water, 0-12 min, isocratic with 100% I; 12-20 min, gradient from 100%-85% I in II; 20-30 min, isocratic with 85% I: 15% II. Solutes; $t_{0,}$ toluene; 1, thymine; 2, uracil; 3, uridine; 4, inosine; 5, adenosine; 6, guanosine; 7, cytidine; 8, adenine; 9, cytosine.*

TABLE 3

CHROMATOGRAPHIC PARAMETERS OF THE TEST SOLUTES OBTAINED ON SOR-SILICA AND MALT-SILICA COLUMNS

Column	**SOR-silica**			**MALT-silica**		
Analytes	**Retention Factor (k)**	**Selectivity (α)**	**Efficiency (N/m)**	**Retention Factor (k)**	**Selectivity (α)**	**Efficiency (N/m)**
Thymine	0.508		18739	0.614		51840
		1.12			1.10	
Uracil	0.571		26570	0.678		57760
		1.27			1.40	
Uridine	0.723		22345	0.949		38027
		2.84			3.07	
Inosine	2.05		31176	2.91		34177
		1.36			1.31	
Adenosine	2.79		33665	3.82		33785
		1.27			1.41	
Guanosine	3.54		25629	5.40		22380
		1.50			1.48	
Adenine	5.30		35221	8.00		18705

in between guanosine and adenine on the SOR-silica column was eluted after the adenine on the MALT-silica column showing a different selectivity. Since the separation efficiency values are greater for solutes separated on the MLAT-silica column, thymine and uracil which co-eluted on the SOR-silica column were well separated on the MALT-silica column although they show similar selectivity on both columns.

In order to evaluate column efficiency and factors responsible for band broadening in detail for each column, the dependence of HETP on the mobile phase linear velocity was plotted as shown in Fig. 4.

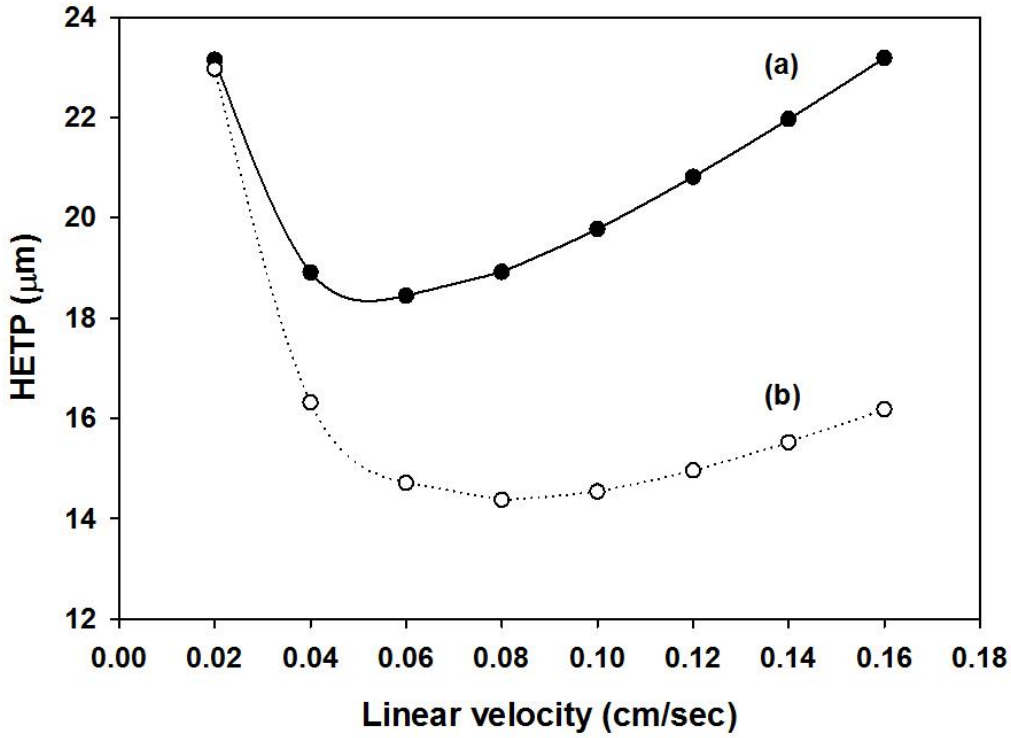


Figure 4. *Van-Deemter plot obtained for cytosine on the SOR-silica column (a) and on the MALT-silica column (b). Separation conditions are the same as in Fig. 3A, except that the mobile phase was 95% (v/v) ACN : 100 mM ammonium acetate, pH 6 and the plate counts were calculated at different flow velocities.*

According to the Van-Deemter plots, the MALT-silica column showed the lowest HETP at 0.08 cm s^{-1} to 0.12 cm s^{-1} flow velocity range, which is the most common flow rate range used in HPLC analysis. In addition, even the linear flow velocity is increased by 50% from 0.08 cm s^{-1} to 0.12 cm s^{-1}, the loss in separation efficiency is only 4.1%. In contrast, the SOR-silica column showed much lower separation efficiency with optimum

flow velocity around 0.04 cm s^{-1} to 0.06 cm s^{-1}, which is much slower than that used for a routine chromatographic analysis. Moreover, when the linear flow velocity is increased by 50% from 0.08 cm s^{-1} to 0.12 cm s^{-1}, the loss in separation efficiency on the SOR-silica column is around 10%, which is much larger than that observed on the MALT-silica column. This larger rate of band broadening at higher flow velocities on the SOR-silica column may indicate the greater mass transfer resistance within the pores of the SOR silica particles compared to that of the MALT-silica particles. The higher mass transfer resistance within the SOR-silica may arise from a thicker hydration layer on the SOR-silica than that on the surface of the MALT-silica.

Separation of derivatized sugars. Due to the inherent hydrophilic nature of carbohydrates, HILIC can be considered as an ideal technique for separating them. To evaluate the separation performance of sugar functionalized silica columns, four PNP-derivatized sugars were separated on MALT-silica column as shown in Fig. 5. In addition, a mixture of seven 2-AB-derivatized sugars and another mixture of four PNP-derivatized sugars were also separated on SOR-silica column under unbuffered mobile phase composition as shown in Fig. 6. In all cases, logarithm of the k values of the separated sugars increased linearly with the number of glucosyl units in the molecule indicating typical HILIC behavior. This behavior is in agreement with the separation model, $\log k = (\log \alpha)n_{Glc} + \log \beta$ where α represents the glucosyl group selectivity of each stationary phase and β represents the k value of the UV tag. Although, both stationary phases provided comparable selectivity (α) for the separation of PNP-derivatized sugars under identical conditions, the MALT-silica column showed higher k values and better separation efficiencies (N_{av}/m = 50,000 plates) compared to those of

the SOR-silica column (N_{av}/m = 20,000 plates). A mobile phase containing higher water content (85% ACN : 5% water) was needed to elute out all analyzed sugars from the

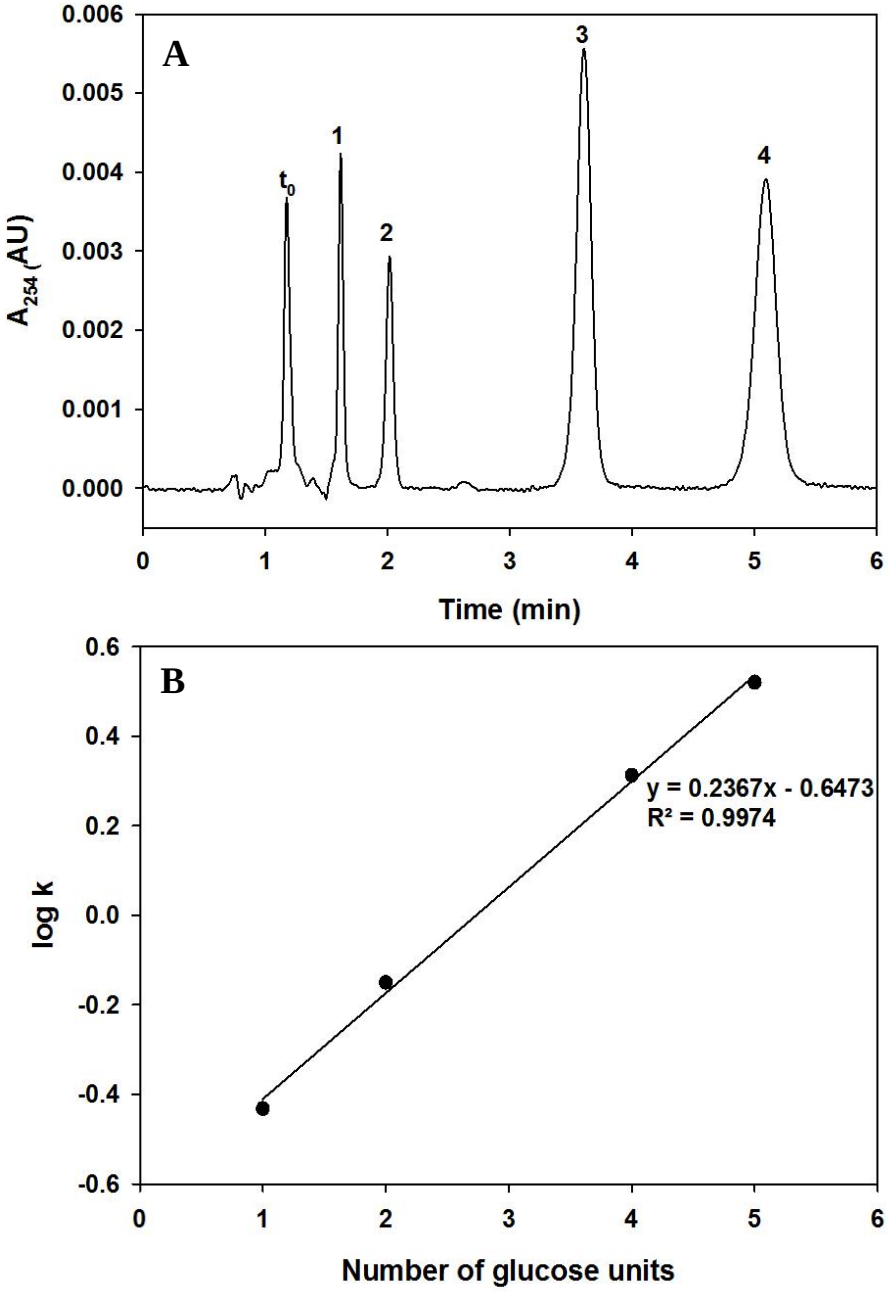


Figure 5. *Chromatogram of four PNP-sugar derivatives (A) and plot of log k vs. number of glucosyl units in the PNP-sugar derivatives (B) obtained on the MALT-silica column. Conditions are the same as Fig. 3A, except that mobile phase was 85% (v/v) ACN : 15% (v/v) water. Solutes; t_0, toluene; 1, PNP-α-D-glucopyranoside; 2, PNP-α-D-maltose; 3, PNP-α-D-maltotetroside; 4, PNP-α-D-maltopentoside.*

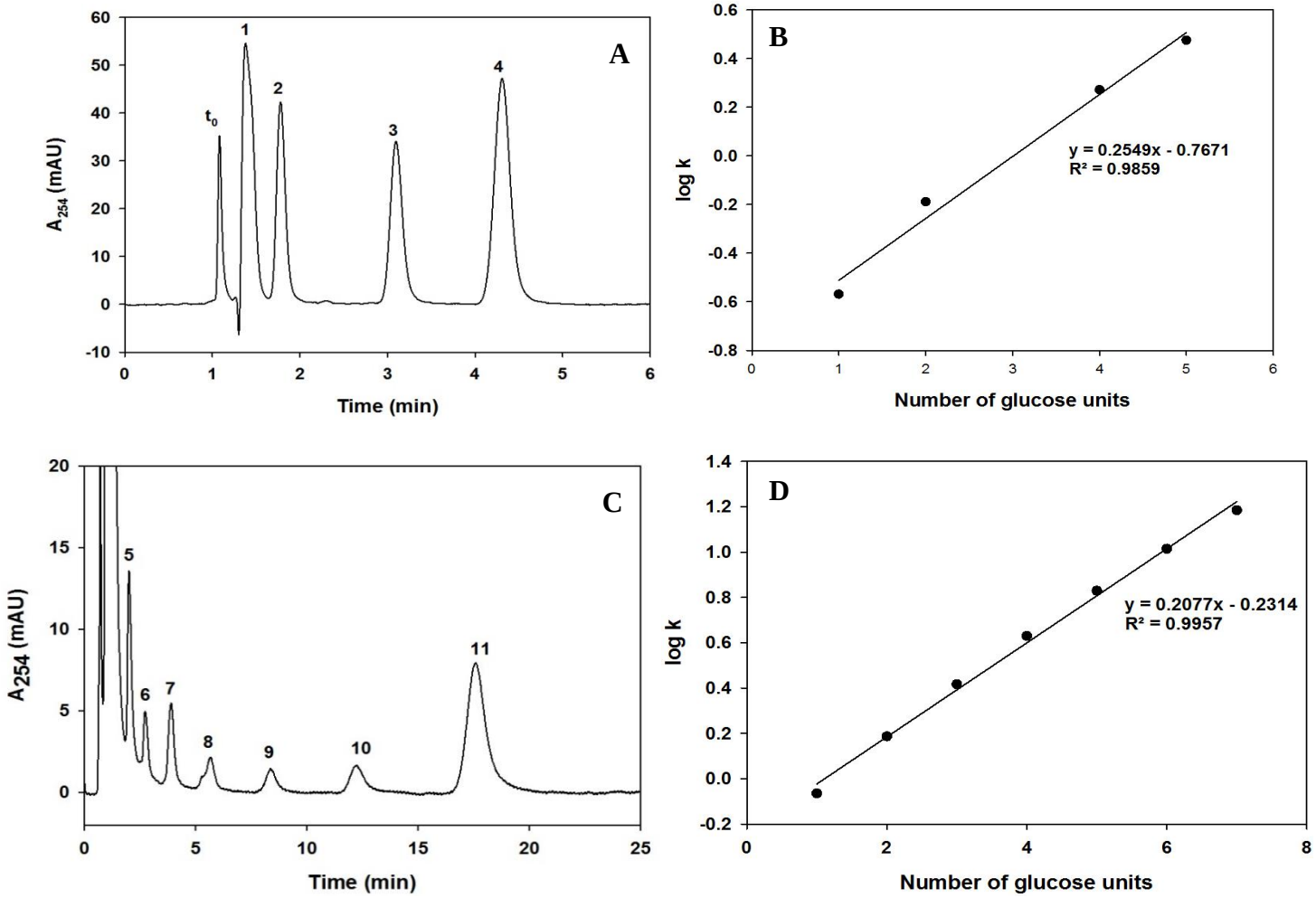


Figure 6. *Chromatograms of four PNP-derivative sugars (A); plot of log k vs. number of glucosyl units in the PNP-sugar derivatives (B); chromatogram of seven 2-AB-sugar derivatives (C); plot of log k vs. number of glucosyl units in the 2-AB-sugar derivatives (D) obtained on the SOR-silica column. Conditions are the same as in Fig. 5. Solutes; t_0, toluene; 1, PNP-α-D-glucopyranoside; 2, PNP-α-D-maltose; 3, PNP-α-D-maltotetroside; 4, PNP-α-D-maltopentoside; 5, 2-AB-glucose; 6, 2-AB-maltose; 7, 2-AB-maltotriose; 8, 2-AB-maltotetrose; 9, 2-AB-maltopentose; 10, 2-AB-maltohexose; 11, 2-AB-maltoheptose.*

columns indicating stronger interactions between the columns and the sugars. This is possibly due to the effect of hydrogen bonding in between hydroxyl groups of the stationary phase and the analytes in addition to other hydrophilic interactions.

Separation of phenolic acids. The effect of the mobile phase pH on the separation of phenolic acids was discussed in the previous section. Since the sugar modified silica stationary phase is neutral and the phenolic acids are only slightly anionic at pH 3, all analyzed acids were eluted unresolved close to the t_0. Therefore, an ammonium acetate containing mobile phase at pH 7 was used to baseline separate the acid derivatives taking advantage of their greater polarity in deprotonated state. Six acid derivatives were baseline separated on the MALT-silica column with high separation efficiency (N_{av}/m = 35,000 plates) as shown in Fig. 7A. The SOR-silica column showed less resolving power towards the acid derivatives and only three of them were separated on SOR-silica column as shown in Fig. 7B (N_{av}/m = 32000 plates).

Separation of cyclic nucleotides and 5' nucleotide monophosphates. A mixture of three cyclic nucleotides and 5' nucleotide monophosphates were baseline separated on the sugar functionalized silica columns as shown in Fig. 8. The MALT-silica column showed broad peaks during the separation of 5' nucleotides possibly due to the increased hydrogen bonding interaction with the polyhydroxy stationary phase.

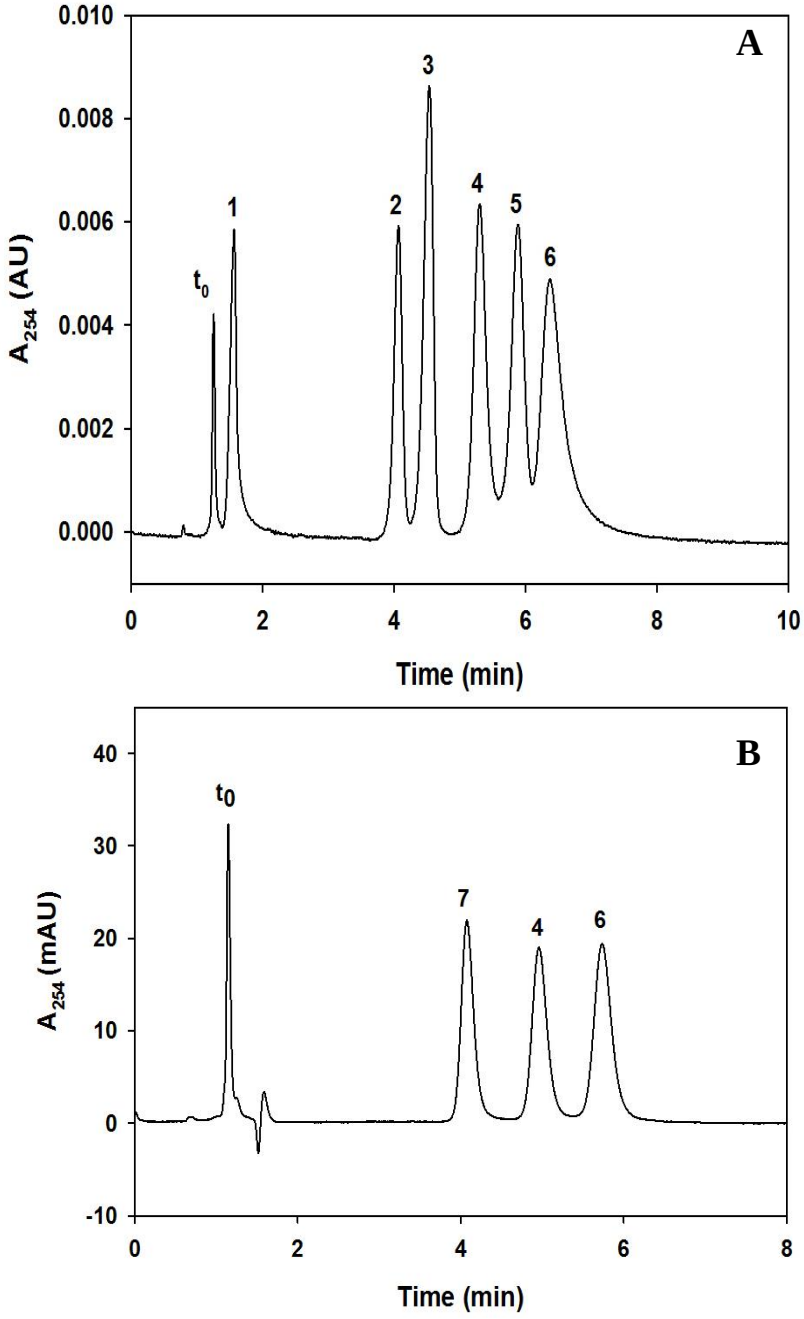


Figure 7. *Chromatograms of phenolic acids obtained on the MALT-silica stationary phase (A) and the SOR-silica stationary phase (B). Separation conditions are the same as Fig. 3A, except that mobile phase was 95% (v/v) ACN : 5% (v/v) 25 mM ammonium acetate, pH 7. Solutes; t_0, toluene; 1, gentisic acid; 2, toluic acid; 3, benzoic acid; 4, ferulic acid; 5, o-coumaric acid; 6, syringic acid; 7, p-hydroxybenzoic acid.*

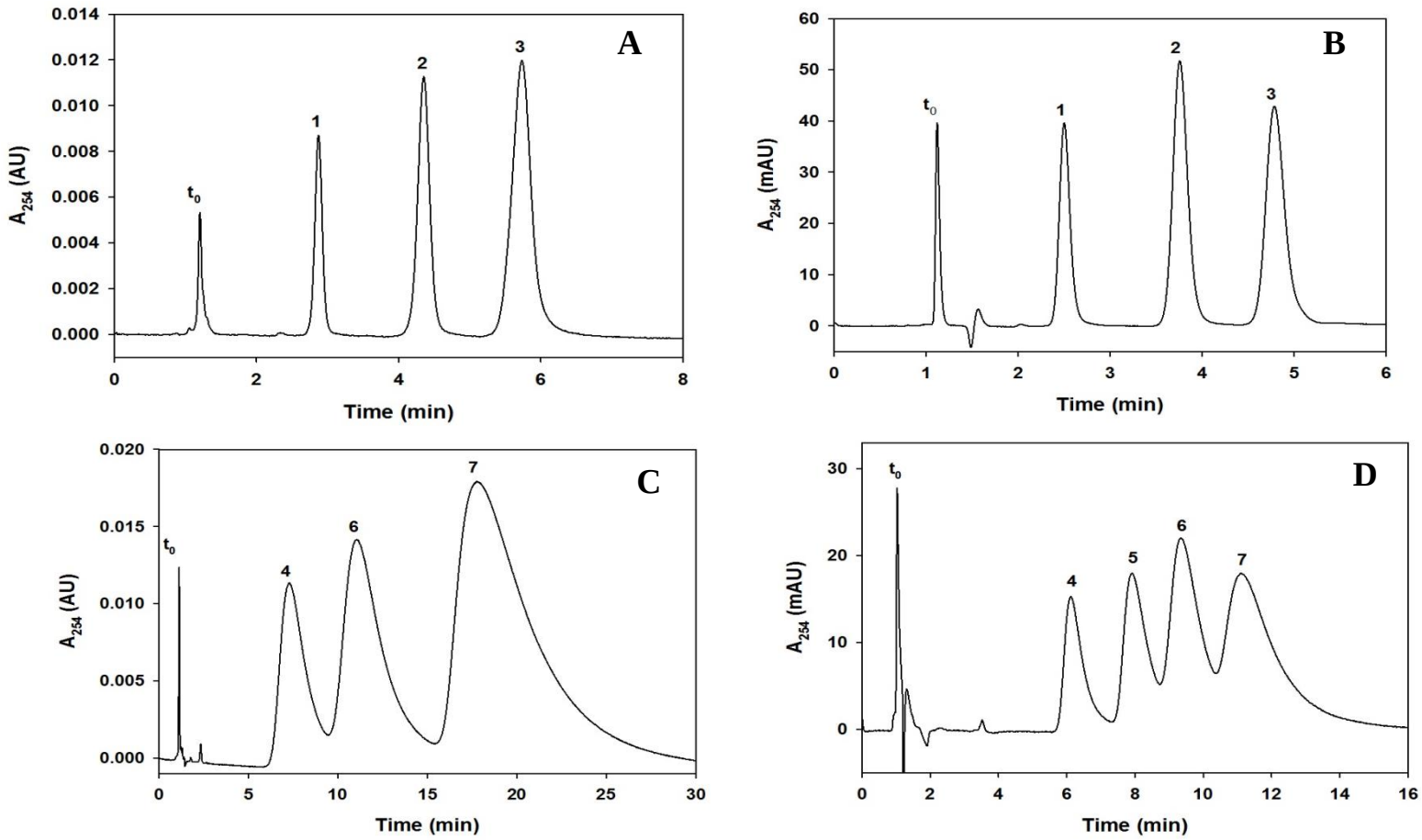


Figure 8. *Chromatograms of cyclic nucleotides obtained on the MALT-silica column (A), the SOR-silica column (B) and separation of 5' nucleotide monophosphates obtained on the MALT-silica column (C), the SOR-silica column (D). Conditions are the same as in Fig.3A, except that mobile phases were 90% (v/v) ACN : 10% (v/v) 25 mM ammonium acetate, pH 8 in A and B; 80% (v/v) ACN : 20% (v/v) 25 mM ammonium acetate, pH 3 in C and D. Solutes; t_0, toluene; 1, cUMP; 2, cAMP; 3, cGMP; 4, UMP; 5, IMP; 6, GMP; 7, CMP.*

Separation of neutral polar compounds. A mixture of neutral polar amides including DMF, thiourea and urea was separated on sugar modified silica stationary phases as shown in Fig. 9. Although thiourea has higher polarity compared to DMF, it was eluted before DMF, close to the t_0 on both columns. Thiourea does not have a polar amide linkage within the molecule. Therefore, it forms less hydrogen bonding interactions compared with the other two solutes, which eluted out in order of increasing polarity. This observation indicates that hydrogen bonding interactions play an important role in the HILIC separation mechanism of sugar modified silica stationary phases in addition to the hydrophilic partitioning.

Reproducibility and Stability of SOR-Silica and MALT-Silica Columns

The reproducibility and chemical stability of MALT-silica and SOR-silica columns were investigated using the k values of adenosine as the model compound. Interday and intraday reproducibility were evaluated in terms of percentage relative standard deviation (% RSD). The intraday and interday % RSD of k values of adenosine obtained on MALT-silica column were found to be 1.29% (n = 8) and 1.29% (n = 2), respectively. Similarly, the intraday and interday % RSD of k values of adenosine obtained on SOR-silica column were found to be 0.43% (n = 4) and 2.13% (n = 3), respectively. Moreover, both columns were used daily for more than 2 months of time period without any apparent loss in separation resolution.

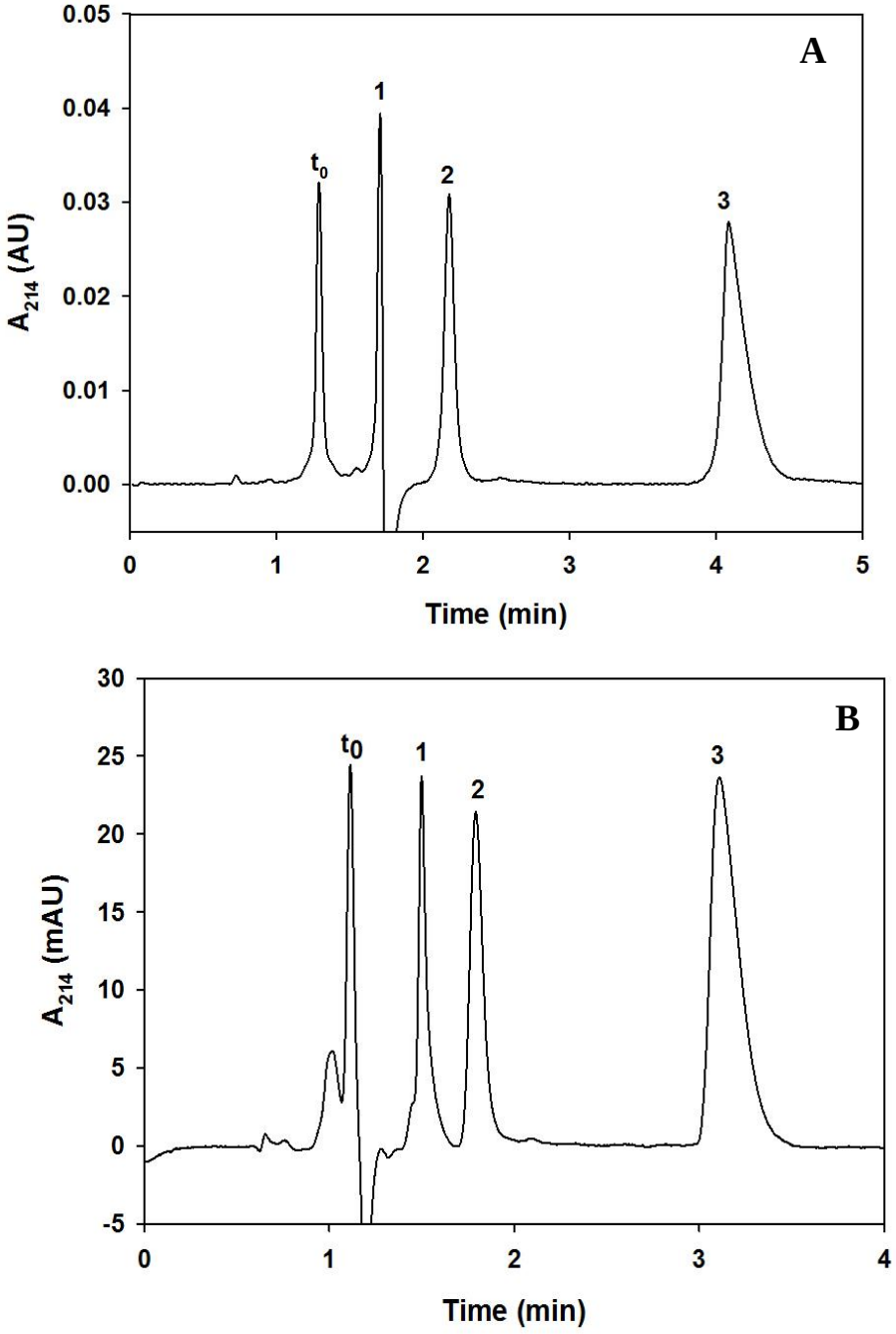


Figure 9. *Chromatograms of some neutral polar amides obtained on the MALT-silica column (A) and the SOR-silica column (B). Separation conditions are the same as Fig. 3A, except that detection was UV at 214 nm; mobile phases, 95% (v/v) ACN : 5% (v/v), water. Solutes; t_0, toluene; 1, thiourea; 2, DMF; 3, urea*

The Influence of Silica Particle Pore Size on Solute Retention and Separation Efficiency of the Column

With the advancement of chromatographic materials, silica particles are available with a wide range of average particle diameters and pore diameters. The choice of the silica particles with suitable particle and pore diameter is highly important to achieve maximum resolution in chromatographic analysis. Therefore, the influence of silica particle average pore diameter on separation efficiency and solute retention was studied using Zorbax silica with two different pore diameters, namely 75 Å and 300 Å.

The separation of nucleic acid bases and nucleosides on the MALT-silica stationary phase with 300 Å average pore diameter (300 Å silica) showed apparently lower k values compared to that of the MALT-silica stationary phase with 75 Å average pore diameter (75 Å silica) as shown in Table 4 and Fig. 10A. It is well known that the specific surface area of the silica surface is decreased with increasing pore size of the silica micro-particles. Therefore, the number of available silanol groups for the modification step was also decreased with the 300 Å silica, resulting in significantly lower amounts of bound maltose on the silica surface. Due to the lower relative hydrophilicity of the 300 Å MALT-silica column, it provided poor retention to the polar solutes compared to that of 75 Å MALT-silica column.

The same behavior was observed during the separation of phenolic acids as shown in Fig. 10B. The separation efficiency on the 300 Å silica column also exhibited low values in addition to the lower k values. In general, the mobile phases within the pores are stagnant or stationary. Analytes should diffuse through the stagnant mobile phase to

reach the surface of the stationary phase. When the pore size increases, mass transfer resistance in stagnant mobile phase is also increased leading to band broadening.

TABLE 4

CHROMATOGRAPHIC PARAMETERS FOR THE TEST SOLUTES OBTAINED ON MALT-SILICA COLUMNS WITH TWO DIFFERENT AVERAGE PORE DIAMETERS

Column	**MALT-silica (75 *Å* pore diameter)**			**MALT-silica (300 *Å* pore diameter)**		
Analytes	**Retention Factor (k)**	**Selectivity (α)**	**Efficiency (N/m)**	**Retention Factor (k)**	**Selectivity (α)**	**Efficiency (N/m)**
Uridine	0.61		38027	0.23		7284
		6.26			3.47	
Adenosine	3.82		33785	0.80		7820
		1.41			1.43	
Guanosine	5.40		22380	1.15		6550
		-			-	
Genitisic	0.393		12641	0.00		3484
		6.10			-	
Toluic	2.40		50173	0.58		5897
		1.83			1.60	
Syringic	4.39		19802	0.93		5137

Together with lower k values and lower separation efficiencies, the 300 Å MALT-silica stationary phase exhibited apparently lower resolution towards the PNP-sugar derivatized and cyclic nucleotides as shown in Fig. 11.

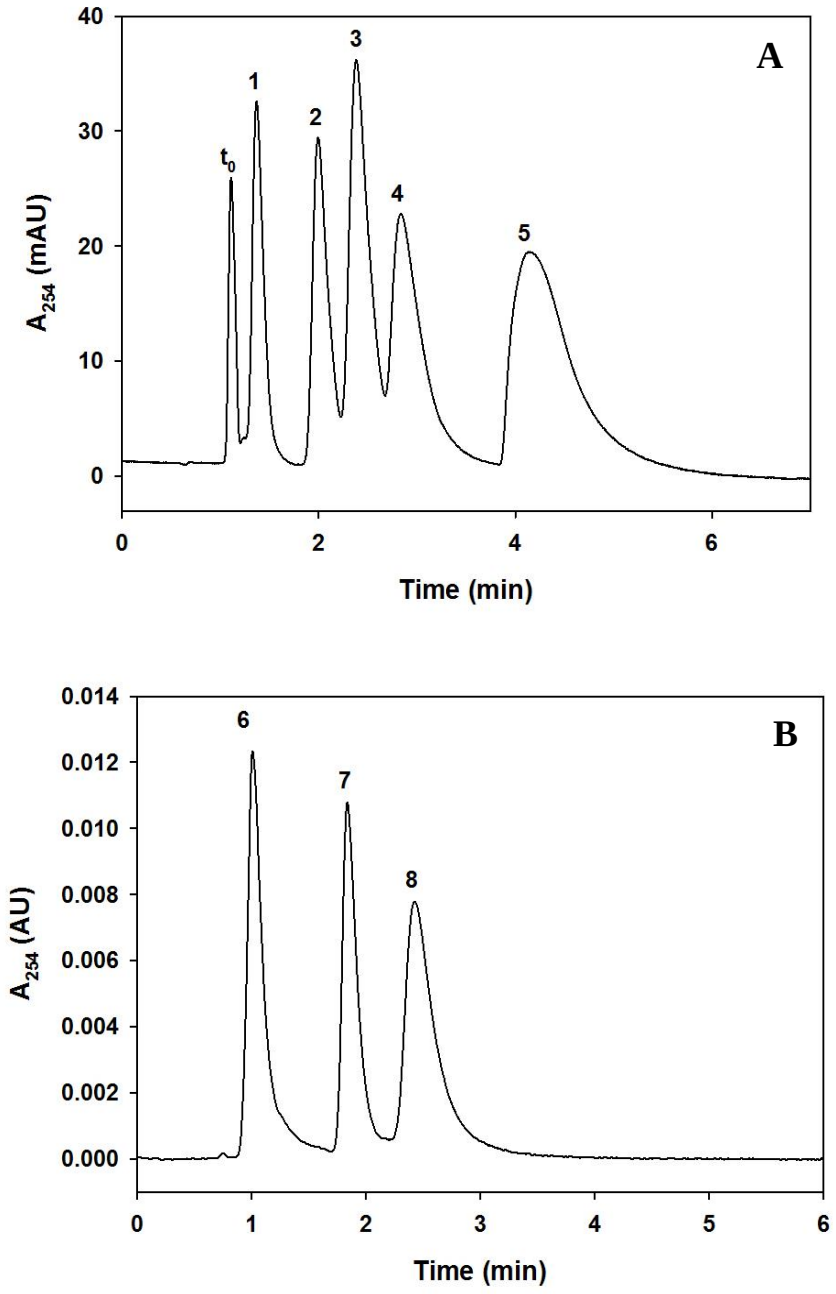


Figure 10. *Chromatograms of nucleic acid bases and nucleosides (A) and phenolic acids (B) obtained on the 300 Å average pore diameter MALT-silica column. Separation conditions for A are the same as in Fig. 3 A and separation conditions for B are the same as in Fig. 7. Solutes;1, uridine; 2, adenosine; 3, guanosine; 4, cytidine; 5, cytosine; 6, gentisic acid; 7, toluic acid; 8, syringic acid.*

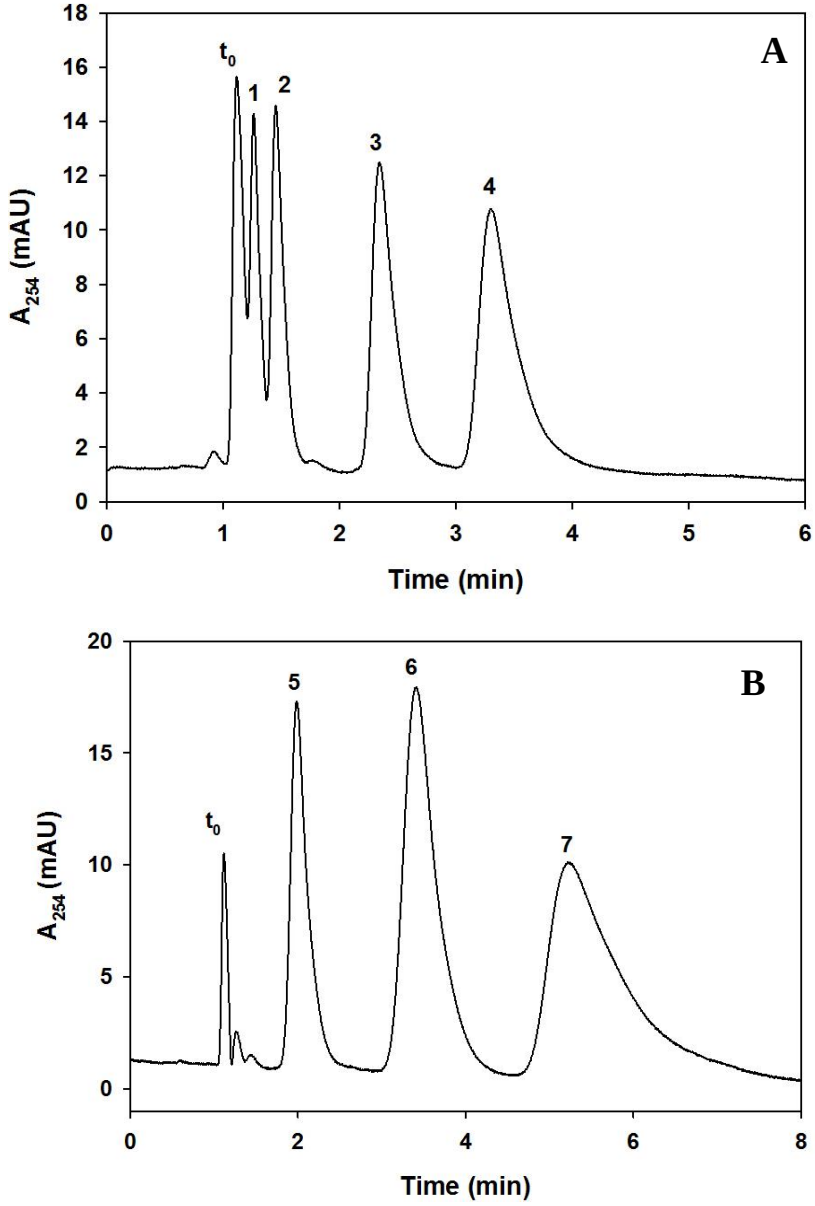


Figure 11. *Chromatograms for separation of PNP-sugar derivatives (A) and cyclic nucleotides (B) obtained on the 300 Å average pore diameter MALT-silica column. Conditions are the same as in Fig. 3A, except that mobile phases were 90% (v/v) ACN : 10% (v/v) water in A; 95% (v/v) ACN : 5% (v/v) 25 mM ammonium acetate, pH 8 in B. Solutes; t_0, toluene; 1, PNP-α-D-glucopyranoside; 2, PNP-α-D-maltose; 3, PNP-α-D-maltotetroside; 4, PNP-α-D-maltopentoside; 5, cUMP; 6, cAMP; 7, cGMP.*

Conclusions

SOR-silica and MALT-silica polyhydroxylated (sugar) neutral polar stationary phases offer unique HILIC selectivity and retention for the separation of a wide range of polar solutes. These stationary phases may become negatively charged at higher pH conditions due to the ionization of residual silanol groups. In addition to the hydrophilic interactions, various other specific and nonspecific interactions may also contribute to the separation mechanism of these sugar functionalized HILIC stationary phases. Therefore, the mobile phase pH and the ionic strength play an important role in the resolution of the HILIC of polar solutes. Polar analytes separated on MALT-silica column showed apparently higher k values and separation efficiencies compared to those obtained on SOR-silica stationary phase due to the higher number of hydroxyl groups on the separation surface of the former column compared to the latter. The amount of bonded maltose, surface coverage, relative hydrophilicity, retention and separation efficiency increased with decreasing the silica gel average pore size.

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CHAPTER V

DEVELOPMENT AND APPLICATIONS OF SURFACE BOUND MALTOSE SILICA-BASED STATIONARY PHASES FOR AFFINITY CHROMATOGRAPHY UNDER REDUCED NON-SPECIFIC INTERACTIONS

Introduction

The in-depth proteomic analysis of human serum for the discovery of novel biomarkers in disease studies and drug development has been a major challenge over many decades. The protein content of serum spans a wide dynamic concentration range extending over ten orders of magnitude and the identification of protein or peptide biomarkers of interest is often hindered by 6-8 high abundance proteins present in biological fluid at comparatively larger concentrations. The removal of these high abundance proteins in a specific, selective and reproducible manner will lead to the discovery of hundreds of lower abundance proteins, which are potential candidates as biomarkers. The various available depletion strategies for high abundance proteins including immobilized dyes e.g., Cibacron Blue (CB) suffers from a lack of

specificity and they may remove some other proteins which are useful as biomarkers in addition to the targeted molecules [1]. In this regard, immunoaffinity purification techniques based on the antibody-antigen interactions that allow a high degree of molecular selectivity have proven to be unique tools in the depletion of high abundance proteins [2]. Moreover, glycosylation is one of the most important post translational modifications of proteins and more than 50% of all human serum proteins are glycosylated [3]. Therefore, the complexity of human serum proteome can be effectively addressed by using lectin affinity chromatographic (LAC) techniques. Lectins are proteins of non-immune origin that specifically interact with carbohydrates and they can assist in the purification, isolation and separation of glycoconjugates in complex matrices such as human serum [4, 5].

In this regard, the bio-specificity of affinity ligands could be coupled with the high speed and resolution obtained in high performance liquid chromatography (HPLC) to achieve high performance affinity chromatography (HPAC) for the fast and efficient purification and isolation of potential biomarkers in human serum. The HPAC is a liquid chromatographic method in which a biologically active affinity ligand is immobilized on a given solid support to yield an affinity stationary phase for the HPLC purification and analysis of samples [6, 7]. In other words, in HPAC one of a pair of interacting molecules is immobilized onto a rigid, high performance solid support and packed into a column to be used as the stationary phase. The complex biological mixture is then introduced into the column and the molecule of interest will be specifically captured by the affinity ligand whereas the extraneous components will pass through with the loading mobile phase. In a later step, elution of the adsorbed compounds can be achieved by disturbing

the interactions between the affinity pair by using different means such as changing pH or ionic strength.

Along with the affinity ligand, the choice of the chromatographic support is equally important when developing a given HPAC stationary phase. Various different properties must be considered when choosing a chromatographic support in HPAC including its mechanical strength under fast flow rates, hydrophilicity, low levels of nonspecific interactions with the sample components, permanent porosity, moderate back pressure, stability to a wide range of solvents, ease of ligand immobilization, stable chemical bonding with the ligand, biocompatibility, larger surface area, low cost and reusability [7, 8]. Although various chromatographic matrices have been introduced as the support in HPAC, an ideal chromatographic support, which fulfills all of these requirements, is still not available. For instance, the matrices of natural polymers (agarose, cellulose, and dextran) have been widely used as affinity chromatographic supports over many decades due to their distinct advantages including biocompatibility, lower nonspecific interactions and stability over a wide pH range [9, 10]. Furthermore, these carbohydrate based stationary phases are inexpensive and they contain large number of hydroxyl groups for activation and ligand coupling under mild conditions. However, the poor mechanical stability, severe shrinkage and compressibility of these low performance supports limit their applications in HPAC, which operates with high flow rates and pressure.

In contrast, inorganic matrices such as silica have been successfully used as chromatographic supports for immobilizing affinity ligands in the field of HPAC [11].

Silica supports are well known as HPLC support media due to their excellent mechanical stability and they can be used with higher flow rates than the low-pressure polysaccharide-based stationary phases. Moreover, these inorganic silica particles are available in a broad range of particle and pore sizes, providing flexibility to choose the desired chromatographic support according to the intended requirements. These silica microparticles can be also easily derivatized to introduce functional groups *via* silanizing coupling agents. However, acidic silanol groups on the silica surface could establish various nonspecific interactions such as electrostatic interactions with proteins, peptides and other biomolecules. It is well known that nonspecific adsorption of the affinity chromatography matrix severely decrease the selectivity and performance of the separation and this factor limits the applicability of silica supports in affinity chromatography separations. Moreover, silica supports are unstable at extreme pH conditions and this further limits the usage of certain buffers as mobile phases in HPAC.

Based on the above considerations, the better mechanical stability of silica can be coupled with the chemical inertness, good stability and biocompatibility of carbohydrates to form sugar-silica composite chromatographic matrices for HPAC. These types of stationary phases provide better shielding for the surface silanol groups, thus protecting analytes from nonspecific interactions with the silica surface. In addition, these composite materials protect the silica matrix against chemical attacks by mobile phases, and consequently increase the lifespan of the column [12]. So far, several attempts have been made to coat silica supports with many different carbohydrates including, agarose [13], dextran [14-17], cellulose [18], chitosan [19, 20], glucose [21] and maltose [22]. In most of these reports, carbohydrate layers were coated on the silica surface by using non-

covalent approaches leading to leaching out of the sugar layer with the attached affinity ligands, and in turn compromising the reusability of the HPAC stationary phase. Furthermore, the original porous structure of the silica gel might be altered after some of the above-mentioned coating steps, which may hinder the accessibility of affinity ligands to the porous surface and also increasing the mass transfer resistances of the analytes. In addition, it was reported that a substantial proportion of silanol groups remained unreacted even after these coating steps indicating the requirement of novel alternative pathways for the functionalization of silica with different carbohydrates.

In this work, we are reporting a novel synthetic pathway for the functionalization of silica microparticles with the disaccharide maltose (MALT). The polar MALT-silica stationary phase was further functionalized by converting it first to aldehyde-activated MALT-silica *via* treatment with sodium periodate. The aldehyde-activated sorbent thus obtained served as the initial and starting hydrophilic support for immobilizing various antibodies, e.g., anti-human serum albumin (HSA), anti-transferrin and lectin affinity ligands, e.g., concanavalin A. These affinity stationary phases, which comprise a neutral polar maltose sublayer ensured minimum nonspecific interactions with the solutes. These immunoaffinity and lectin affinity stationary phases were successfully used to deplete high abundance proteins as well as to enrich low abundance proteins in human serum.

Experimental

Instrumentation

Most HPAC separations were performed on a Waters Alliance 2690 separation module (Milford, MA, USA), equipped with an in-line degasser, a quaternary solvent pump, an auto sampler and a thermostated column compartment. Detection was performed using a PDA detector (Model W2996). The sample and the column compartments were maintained at ambient temperature for all chromatographic separations. In addition, an HPLC system consisting of a quaternary solvent delivery system Q-grad pump from Lab Alliance (State College, PA, USA), a Model 3100 UV–Vis variable wavelength detector from Milton Roy, LDC division (Riviera Beach, FL, USA) and a Rheodyne injector Model 7010 from IDEX Health & Science LLC (Rohnert Park, CA, USA) equipped with a 20 μL loop was occasionally used. Data acquisitions were carried out using Empower 2 (Build 2154) software (Waters chromatography) or using the clarity version, 3.0.06.589 advanced chromatographic software from Data Apex (Prague, Czech Republic). A constant pressure pump from Shandon Southern Products Ltd. (Cheshire, UK) was used for the slurry packing of the column. A syringe pump from Cole-Parmer (Vernon Hills, IL, USA) was used to pump the derivatizing agents and protein solution for immobilization through the column at appropriate slow flow rates.

Reagents and Materials

Zorbax silica, with 5 μm average particle diameter, 300 Å average pore diameter was obtained from E.I. du Pont de Nemours and Company Inc. (Wilmington, DE, USA). Human serum albumin (HSA), bovine serum albumin (BSA), horse skeletal muscle

myoglobin, human transferrin, bovine pancreas ribonuclease A, bovine pancreas ribonuclease B, ovalbumin, asialofetuin from fetal calf serum, anti-albumin antibody produced in rabbits, anti-transferrin antibody produced in goats, concanavalin A (Con A) from *Canavalia ensiformis*, peroxidase from *Arthromyces ramosus*, *p*-nitrophenyl (PNP)-α-D-galactopyranoside, methyl-α-D-mannopyranoside (Me-α-Man), silver nitrate, calcium chloride and Sigma Marker™ were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The γ-glycidyloxypropyl trimethoxysilane, tris(hydroxymethyl)aminomethane and manganese chloride were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). Maltose, sodium periodate, sodium acetate, sodium azide, urea, sodium thiosulfate, sodium carbonate and *N,N'*-methylene-bis-acrylamide were purchased from Fisher Scientific (Fair Lawn, NJ, USA). The *N,N*-dimethylformamide (DMF), sodium chloride, sodium monohydrogen phosphate and acetic acid were purchased from EMD Chemical Inc. (Gibbstown, NJ, USA). Glycine, acrylamide and bromophenol blue and sodium dodecyl sulfate (SDS) were obtained from Bio-Rad Laboratories (Hercules, CA, USA). PNP-*a*-D-glucopyranoside and PNP-*a*-D-mannopyranoside were purchased from EMD Biosciences Inc. (La Jolla, CA, USA). BF_3·etherate was obtained from TCI America (Portland, OR, USA). Sodium cyanoborohydride was purchased from Acros Organics (New Jersey, USA). Disease free human serum was purchased from Bioreclamation (Jericho, NY, USA).

Preparation of Epoxy Activated Silica

Typically, 2.5 g of dry Zorbax macroporous silica particles were suspended in 30 mL of dry toluene in a 3-neck round-bottomed flask connected to a reflux condenser. Initially, this mixture was heated to 95 °C with slow stirring to make slurry. To this

suspension, 2.5 mL of γ-glycidoxypropyl trimethoxysilane was added, and the reaction mixture was stirred for 18 h at 95 °C. The epoxy activated silica thus obtained was rinsed successively with toluene and acetone and then immediately used in the next modification step.

Preparation of MALT-Silica

The resulting epoxy activated silica was then rinsed one more time with DMF and re-suspended in 30 mL of 100 mM maltose in DMF in a round-bottomed flask. This mixture was stirred at room temperature for 30 min to make a homogeneous slurry. Fifty microliter aliquots of BF_3·etherate catalyst were then added into this reaction mixture 4 times, with 2 h time intervals. The reaction mixture was stirred at room temperature for 18 h. The resulting MALT-silica gel was rinsed with DMF, water and acetone, and was allowed to dry in the air.

Column Packing

Two grams of MALT-silica were dispersed in 20 mL of 0.1 M NaCl solution to make 10% (w/v) slurry, which was sonicated for 20 min to eliminate air and to ensure homogenization. The slurry was then packed into a stainless-steel column (10 cm × 4.6 mm id) with 0.1 M NaCl as the packing solvent under 6000-7000 psi pressure for 30 min using a constant pressure pump. The column was then equilibrated with water before subjecting it to the periodate oxidation.

Periodate Oxidation

The 10 cm long MALT-silica columns were allowed to react with freshly prepared 0.2 M $NaIO_4$ for 2 h at room temperature in order to oxidize the diol groups to aldehyde groups. The columns were rinsed with water for 15 min and the proteins were immobilized on the rinsed columns immediately [10, 23].

Immobilization of Polyclonal Antibodies onto the MALT-Silica Column

The covalent immobilization of anti-HSA polyclonal antibodies onto the aldehyde activated MALT-silica surface was done on-column *via* reductive amination by passing a 5 mL of solution containing 2 mg/mL of anti-HSA antibody in 0.10 M sodium acetate, pH 6.4, and 50 mM sodium cyanoborohydride at room temperature for 12 h [2, 24]. The immobilization of anti-transferrin was carried out in the same way except that the immobilization solution was made up of 5 mg/mL of anti-transferrin antibody in 0.10 M sodium acetate, pH 6.4, and 50 mM sodium cyanoborohydride. In a last step, all antibody immobilized columns were scavenged from any remaining aldehyde groups by reductive amination using a solution containing 0.40 M Tris/HCl, pH 7.2, and 50 mM sodium cyanoborohydride, which was pumped through the column at room temperature for 3 h. Finally, the column was rinsed with water and stored in 0.01% NaN_3 at 4 °C.

Immobilization of Lectins on to the MALT-Silica Column

The covalent immobilization of Con A onto the aldehyde activated MALT-silica surface was done on-column by passing a solution of 10 mg of Con A in 5 mL of 0.1 M sodium acetate, pH 6.4, containing 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 0.1 M Me-α-D-Man and 50 mM sodium cyanoborohydride through the column at room temperature

for 12 h. The scavenging of any remaining aldehyde groups was done by reductive amination by passing a solution of 0.4 M Tris/HCl, pH 7.2, and 50 mM sodium cyanoborohydride for 3 h at room temperature. These immobilized lectin columns were stored with a mobile phase containing 20 mM Tris/HCl, 100 mM NaCl, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM $CaCl_2$, pH 6.0 and 0.01% NaN_3 at 4 °C [25, 26].

Chromatographic Conditions for Immunoaffinity Chromatography

Successive adsorption / desorption of the analytes on the antibody immobilized affinity column was accomplished by changing the mobile phase from a loading buffer to an elution buffer. The loading buffer used for the anti-HSA column contained 0.05 M Na_2HPO_4, 0.5 M NaCl, pH 7.4, and elution buffer was 0.05 M Na_2HPO_4, 0.5 M NaCl, pH 7.4 with 5 M urea. The loading buffer used for the anti-transferrin column was 0.05 M Na_2HPO_4, 0.25 M NaCl, pH 7.4, and elution buffer was 0.05 M Na_2HPO_4, 0.25 M NaCl, pH 7.4 with 5 M urea. Before injecting the sample, the column was allowed to equilibrate with 10 column volumes of the loading buffer. After injection, the loading buffer was passed through the column for 10 min and then switched to the elution buffer. All samples were prepared and diluted in the loading buffer and injected *via* a 20 μL loop. Flow rates of the loading and elution buffers were maintained at 1 mL/min. All chromatograms were recorded at 280 nm wavelength. All chromatographic experiments were performed at ambient temperature.

Chromatographic Conditions for Lectin Affinity Chromatography

The Con A immobilized MALT-silica column was first equilibrated with 10 column volumes of the binding mobile phase consisting of 20 mM Tris/HCl containing

250 mM NaCl, 1 mM $MgCl_2$, 1 mM $MnCl_2$ and 1 mM $CaCl_2$, pH 7.4. After injection of standard proteins or diluted human serum samples, the unbound proteins were washed away with the binding mobile phase at 1 mL/min for 10 min. The bound fraction to the Con A column was then eluted using the eluting mobile phase consisting of hapten sugar 0.1 M Me-α-D-Man in the binding mobile phase. The lectin column was again equilibrated with 20 column volumes of the binding mobile phase to prepare it for the next injection. The experiments were conducted at ambient temperature at a flow rate of 1.0 mL/min and the baseline was monitored at λ= 280 nm.

Serum Depletion

In the case of MALT-silica based affinity columns, and unless otherwise indicated, serum samples were 1:3 diluted and injected into the chromatographic system. The pass through and bound fractions to the affinity columns were collected into Eppendorf micro centrifuge tubes and diluted up to 2 mL with the binding mobile phase.

TGA Analysis

The coating of maltose on the bare silica surface was verified by thermogravimetric analysis (TGA) using a TA instruments Q-50 thermogravimetric analyzer (TA instruments, New Castle, DE, USA). Samples were heated from 20 °C to 700 °C at a heating rate of 20 °C/min under 40 mL/min of continuous air flow.

1D SDS PAGE Analysis

The SDS-PAGE analysis of the anti-HSA bound fraction and pass through sample was performed on 8% SDS polyacrylamide home cast gels (14 cm × 16 cm, 1.5 mm

thickness) for 180 min at 100 V. After electrophoresis, the gels were rinsed 3 times with DI water, and stained using a silver staining protocol reported elsewhere [27]. Briefly, the gels were fixed with 50% methanol/12% acetic acid for 1 h. After several changes of 50% methanol and water, the gels were sensitized by incubating in 0.2 g/L sodium thiosulfate followed by several washes with DI water. The gels were incubated in 2 g/L silver nitrate for 20 min followed by thorough rinsing with DI water. The gels were developed in 0.05% formalin in 60 g/L sodium carbonate and the reaction was terminated with 5% acetic acid.

Results and Discussion

Development of the Affinity Stationary Phase

The matrix plays an important role in the preparation of stationary phases for affinity chromatography by contributing to the reduction of non-specific interactions with the analytes of interest as well as with the other sample components. Usually, HPLC stationary phases for small molecules separation consist of silica particles with 60 Å to 100 Å pore diameters. On the other hand, silica particles with larger average pore diameter are required in affinity chromatography to provide proper accessibility to the higher molecular weight affinity ligands, as well as to facilitate unhindered diffusion of relatively large sample molecules such as proteins. Although, the specific surface area and therefore the number of bound affinity ligands decreases with increasing pore size, the totally porous Zorbax silica with 5 μm average particle diameter and 300 Å average pore diameter was chosen as the chromatographic support in the current work based on the above considerations.

The disaccharide maltose was chosen to coat the silica microparticles in order to cover the acidic silanol groups, which often cause strong and irreversible adsorption of proteins. Maltose [*O*-α-D-glucopyranosyl-(1→4)-D-glucopyranose] is a common glucosyl-glucose disaccharide which is produced by enzymatic hydrolysis of starch. This biocompatible and stable sugar contains a high content of hydroxyl groups available for the activation and ligand coupling. Although many other carbohydrates can also be used for this purpose, it was reported that small molecules such as glucose did not result in a significant reduction of the protein-surface interaction [21]. The use of polysaccharides such as agarose or cellulose is largely limited due to their low solubility in the aprotic solvents used in the current coupling procedure. Moreover, polysaccharides can alter the inner pore structure of the silica particles giving some undesirable properties to the HPAC matrix. Therefore, it is believed that the coupling of maltose to glycidoxypropylsilyl-silica will give an effective shielding to the silica surface.

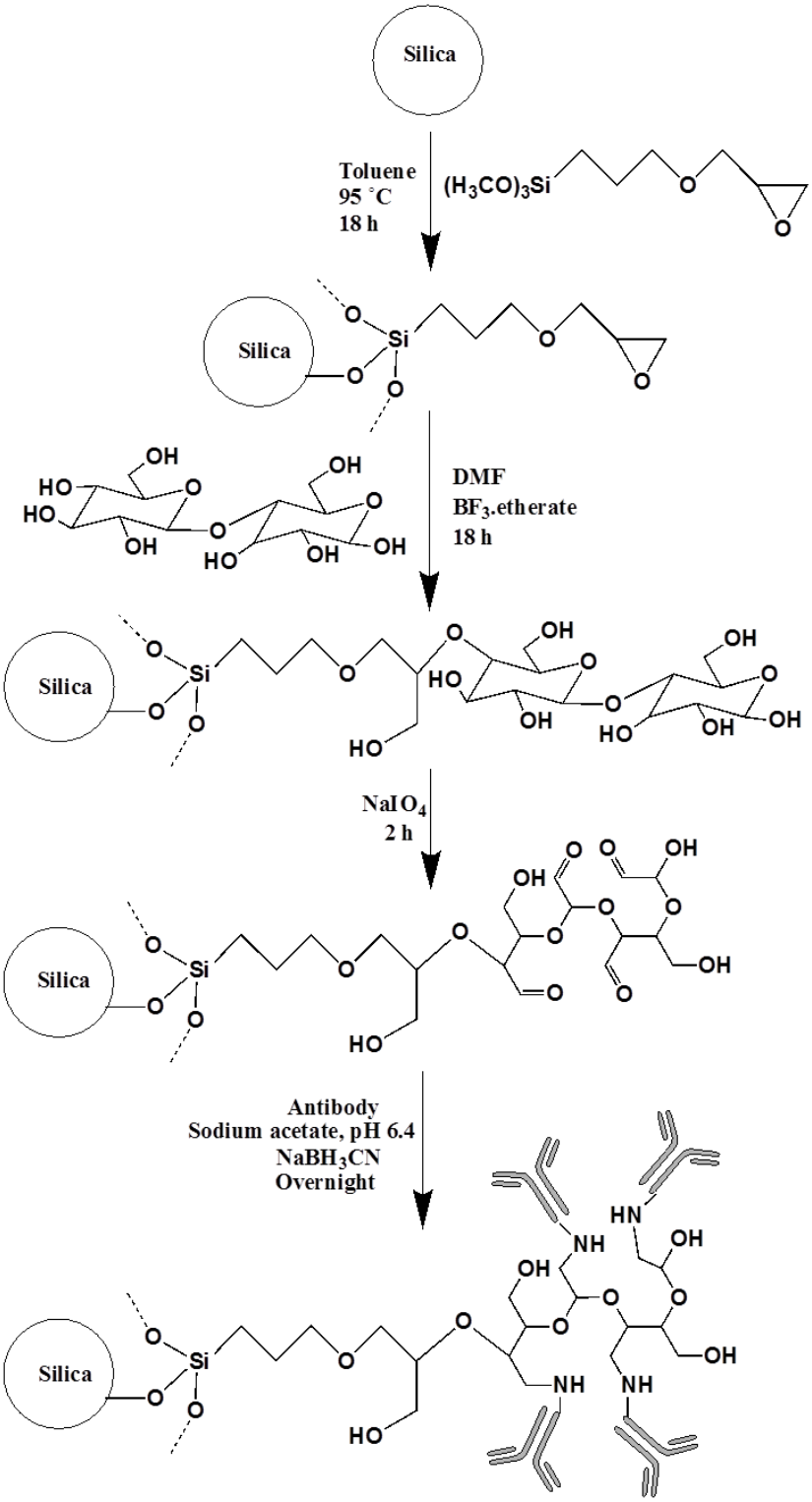
The bare silica surface was first activated to an epoxy coated surface by reacting the bare silica with γ-glycidoxypropyl trimethoxysilane (GPTMS). The maltose was then attached to the epoxy activated silica *via* nucleophilic epoxide ring-opening reaction using a Lewis acid namely BF_3.etherate as the catalyst as shown in Scheme 1. DMF was chosen as the aprotic solvent for this reaction considering the solubility of maltose.

The diol groups on the maltose layer can be easily modified for the immobilization of proteins or other affinity ligands *via* Schiff base method. In this regard, the sodium periodate oxidation of vicinal diol groups of the maltose yields a pair of aldehyde groups at the each monosaccharide ring. These dialdehyde functionalities then react with amine groups of the proteins and form Schiff bases as shown in Scheme

1. These Schiff bases are unstable and they can be easily dissociated to the aldehyde and the proteins back. Therefore, the aldimine functionalities are reduced using mild reducing agents such as sodium cyanoborohydride to form a stable amine linkage between the immobilized protein and the maltose layer. The remaining unbound aldehyde groups can later be scavenged using TRIS/HCl.

Characterization of the MALT-Silica Stationary Phase

The thermogravimetric analysis (TGA) were performed on MALT-silica epoxy activated silica and unmodified silica particles (bare silica) in the range of 20 °C to 700 °C. Based on the thermogram provided in Fig. 1, it can be seen that bare silica showed only 1% weigh loss at temperatures below 100 °C, probably due to the removal of physically adsorbed water. On the other hand, the epoxy activated silica showed a distinct thermal event in the range of 200 °C to 450 °C. This 5% weight loss is attributed to the thermal decomposition of organic moieties, which are attached to the silica surface by the Si-C bond of the siloxane species. In addition, MALT-silica showed two thermal events, one occurring in the range of 200 °C – 400 °C and other in the range of 500 °C – 600 °C. The first of these two regions was very similar to the decomposition of the organic moieties in the silanizing coupling agent of epoxy silica proving that the maltose was indeed attached to the coupling agent. The second larger weight loss about 4.5% was attributed to the thermal decomposition of maltose. These results proved the successful attachment of γ-glycdoxypropyl trimethoxysilane to the silica surface and the attachment of maltose onto the epoxy activated surface.



Scheme 1. *Schematic diagram showing the preparation of the MALT-silica stationary phase and the subsequent immobilization of antibodies on it.*

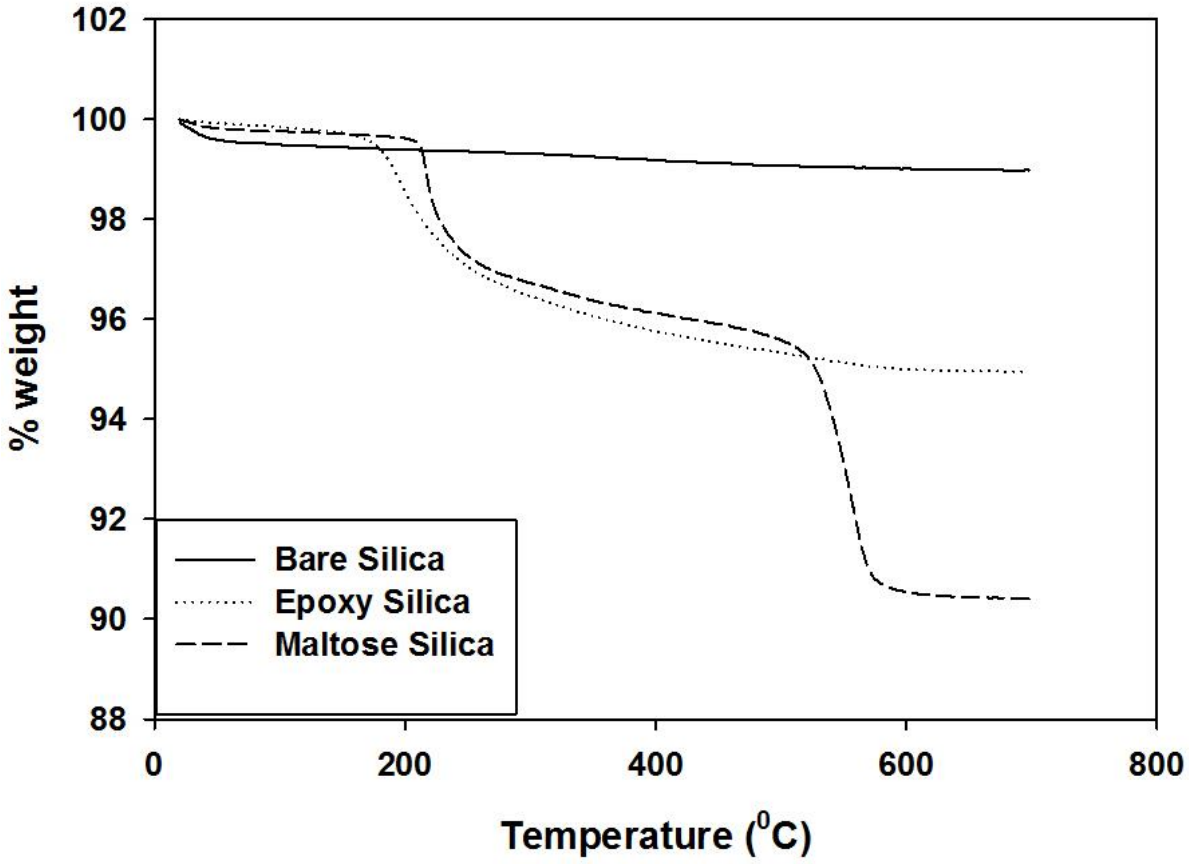


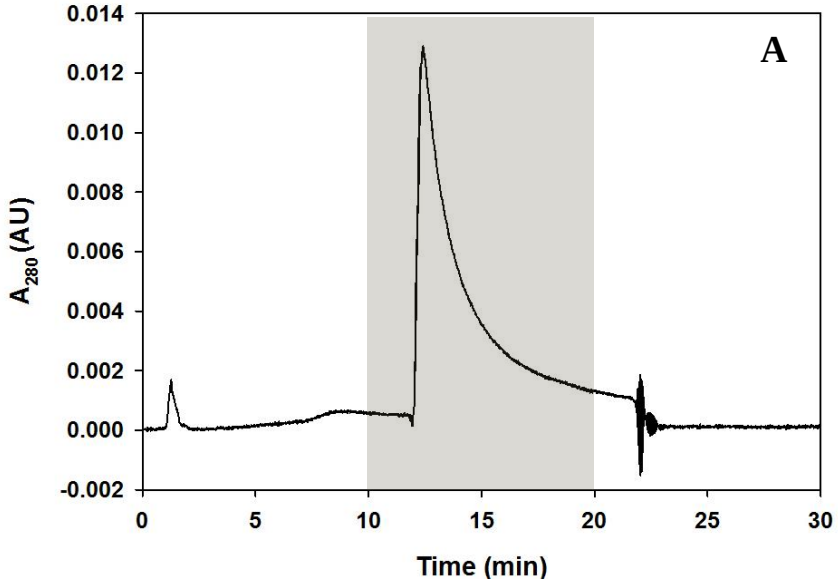
Figure 1. *The TGA curves of bare-silica, epoxy activated silica and MALT-silica.*

Evaluation of Anti-HSA Immunoaffinity Stationary Phase

Albumin is the most abundant protein in human serum which accounts for 50% of the serum protein mass. The concentration of HSA in adult human serum in the fasting state was found to be 35-50 mg/mL [2, 28]. Therefore, polyclonal anti-albumin antibodies were immobilized onto the MALT-silica column for the highly specific, highly efficient fast depletion of HSA under reduced nonspecific interactions.

The establishment of the suitable chromatographic conditions for the binding and elution of the target molecules in immunoaffinity chromatography is an empirical process. A 50 mM phosphate buffer solution, pH 7.4, containing 0.5 M NaCl which mimics physiological conditions was chosen as the binding mobile phase in order to achieve maximum binding of the albumin to the anti-HSA column. The choice of the eluting mobile phase, which is sufficiently strong to remove the proteins completely and also sufficiently gentle to preserve the biological activity of the antibody, is a challenging task. In principle, the eluting mobile phase should disrupt the antigen-antibody complex without permanently damaging the immobilized antibodies. There are several ways of achieving this including raising or lowering the pH, addition of chaotropic ions, such as perchlorate, iodate and thiocyanate or addition of certain compounds such as urea which are known to disrupt the hydrogen bonding interactions between molecules. A series of buffer solutions containing urea at different concentrations ranging from 2 M to 5 M were examined as the eluting mobile phase in the current study. Low eluting strength mobile phases such as 2 M urea lead to broad peaks due to the slow release of the protein from the antibody antigen complex. The eluting buffer containing 5 M urea leads to sharp elution profiles with higher separation efficiencies. However, the use of eluting mobile phases containing even higher concentrations of urea was avoided in order to minimize the permanent denaturation of the antibodies by disrupting their secondary and tertiary structures. The column was re-equilibrated with the binding mobile phase for about 20 min after each elution step in order to regenerate the affinity surface without any significant activity loss.

The most important step in the design of an affinity stationary phase is to evaluate both its specific and nonspecific interactions with the sample of interest. In fact, the amount of maltose coverage on the silica surface is one of the most important parameters, which determines the specificity of the stationary phase in the current study. To assess that the adsorption/desorption of the substrate is solely based on affinity specific interactions and not on nonspecific interactions, HSA, myoglobin and bovine serum albumin (BSA) standards were injected onto the anti-HSA immobilized MALT-silica column under investigation and the results are shown in Fig. 2. As can be seen in this figure, HSA was strongly retained on the affinity column while all other injected proteins were eluted close to the breakthrough of the column (pass through fraction) indicating the presence of minimum nonspecific interactions between analytes and the stationary phase.



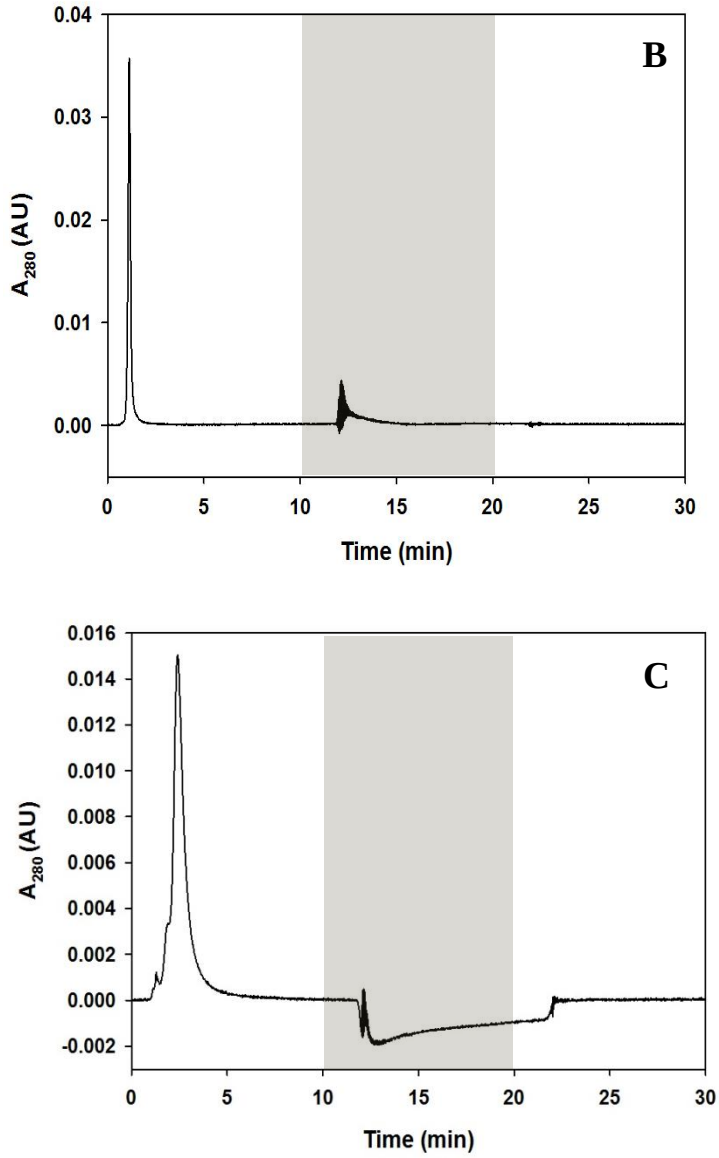


Figure 2. *Chromatograms of standard proteins obtained on the anti-HSA immunoaffinity column (5 μm particle and 300 Å pore diameter). Separation conditions; column 10 cm × 4.6 mm id; flow rate, 1 mL/min; detection, UV at 280 nm; injection volume, 20 μL. Mobile phase: 0.05 M Na_2HPO_4, 0.5 M NaCl, pH 7.4 (0-10.0 min), and 5 M urea in 0.05 M Na_2HPO_4, 0.5 M NaCl, pH 7.4 (10.0-20.0 min). Proteins; (A) HSA, (B) BSA, (C) myoglobin. The gray regions indicate the eluting mobile phase application.*

Since, one of the major objectives of this study was to develop an immunoaffinity column capable of effectively deplete the most abundant housekeeping protein in human serum, it was important to determine the sample loading capacity of the prepared anti-HSA immobilized MALT-silica column. In order to determine the sample loading capacity, standard solutions of HSA at different concentrations were injected into the anti-HSA column and the peak heights of the proteins were measured with the aid of chromatographic software. The peak heights were then plotted against the concentration of each protein standard solution as shown in Fig. 3.

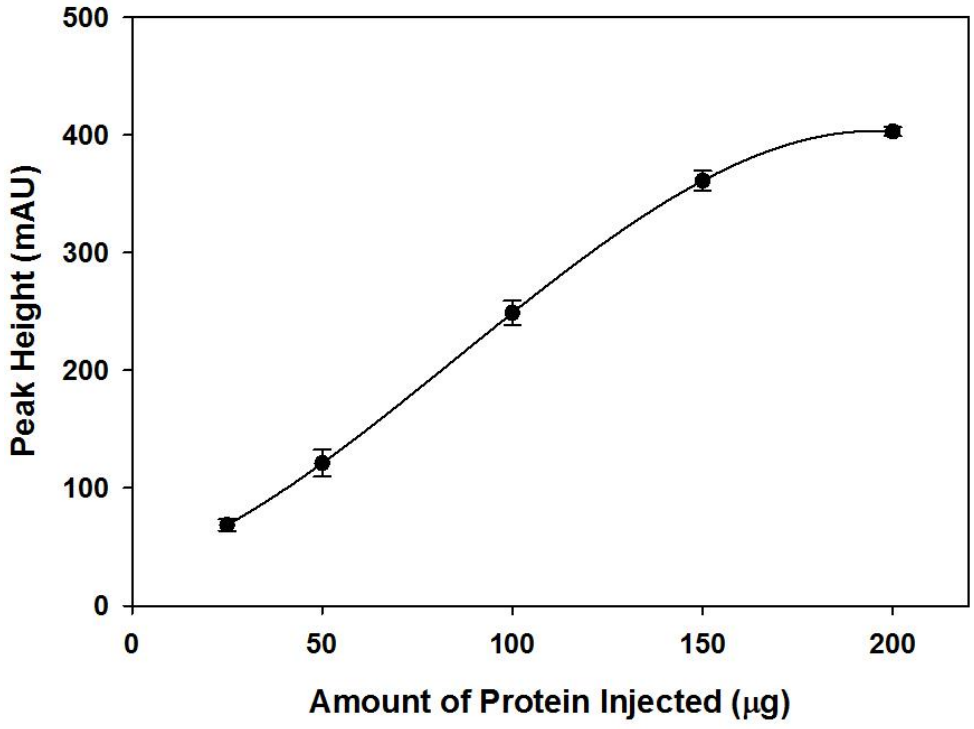


Figure 3. *Plot of peak height versus amount of protein injected on the anti-HSA immunoaffinity column. The separation conditions are the same as in Fig. 2.*

The plot of peak height versus the amount of HSA injected was linear up to a certain concentration and leveled off at high concentrations. At large concentrations, most of the available binding sites on the affinity surface should be occupied and the excess of the injected proteins would be spilled out from the column. The above observation was further confirmed by the appearance of a peak at the breakthrough time on the chromatograms at higher concentrations of injected HSA, which was possibly due to the spilled out fraction of the protein. Based on the above considerations, the value of the sample loading capacity of the anti-HSA column was estimated to be 150 μg per column. The sample loading capacity was defined as the concentration where the slope of the curve started to decline rapidly.

Depletion of human serum albumin by anti-HSA immunoaffinity column A 1:3 diluted human serum sample was injected into the anti-HSA column in order to evaluate the depletion efficiency of HSA by the column. This process is important for the removal of albumin in order to facilitate the profiling and identification of low abundance proteins in human serum. The nonspecific interactions are believed to be lower in the anti-HSA column as shown in the previous section due to the effective covering of the silica surface by the maltose layer. Moreover, it was reported that certain low molecular weight proteins may bind to the depletion column *via* the captured albumin in a so-called "sponge" effect caused by albumin as was reported in some other depletion strategies [1]. Highly specific antibody-antigen interactions were used in the current anti-HSA column in order to minimize other undesired binding effects during serum depletion. Figure 4 is a chromatogram obtained when a 20-μL aliquot of 1:3 diluted human serum was injected into the anti-HSA column.

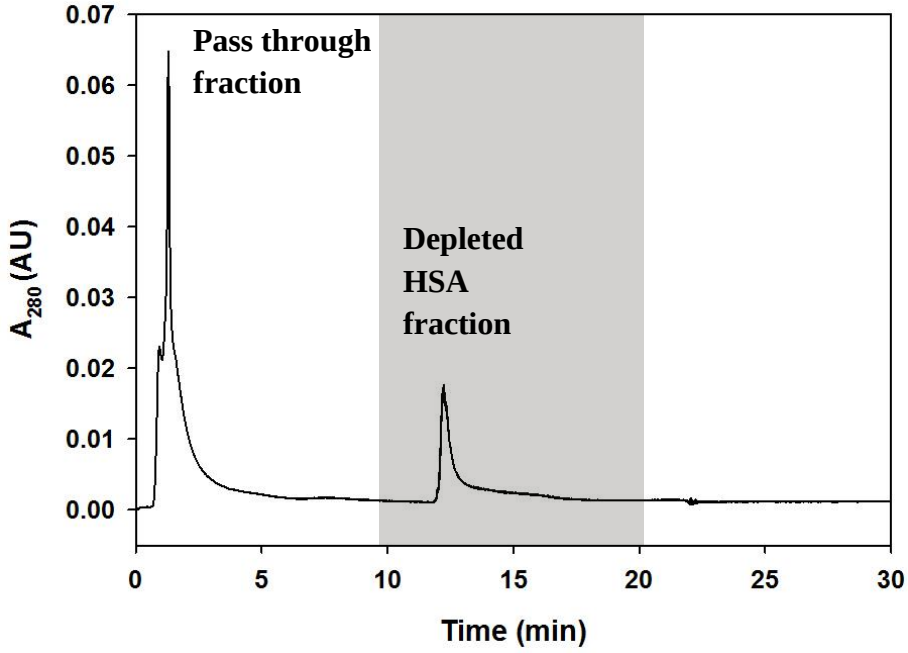


Figure 4. *Chromatogram of 1:3 diluted human serum obtained on the anti-HSA immunoaffinity column. Separation conditions are the same as in Fig. 2.*

To confirm that the depletion has occurred, the pass through fraction, depleted HSA fraction (collected fraction), 1:300 diluted human serum sample and HSA standard were analyzed using SDS-PAGE, and the results are shown in Fig. 5. As expected, the pass through fraction showed all protein bands in the human serum except the protein band corresponding to HSA confirming the high specific depletion of HSA from the human serum by the anti-HSA column. Moreover, the depleted HSA fraction showed only a single protein band in the SDS-PAGE, proving the lower nonspecific adsorption of the proteins onto the anti-HSA immunoaffinity stationary phase and demonstrating the applicability of the MALT-silica matrix in proteomics.

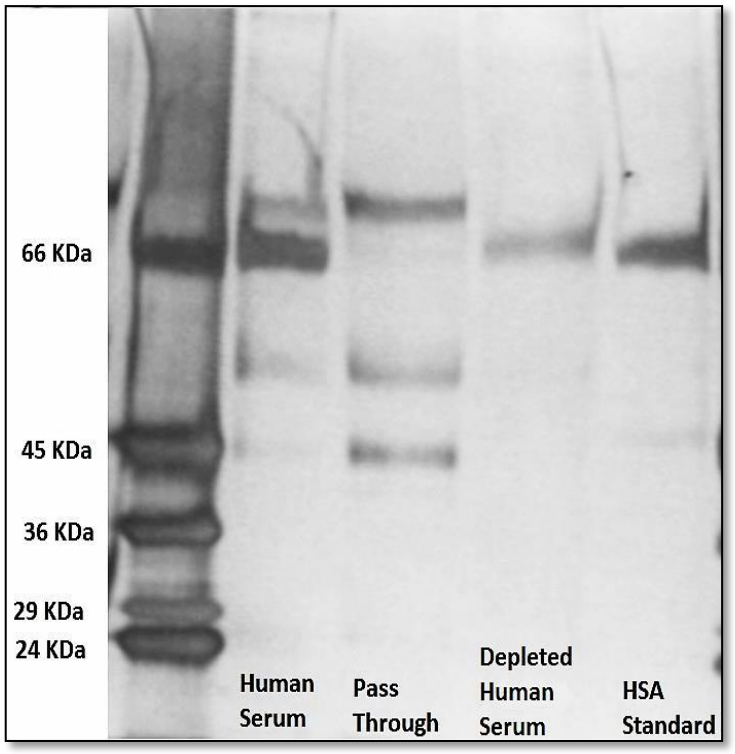


Figure 5. *SDS-PAGE of Sigma Marker^{TM} , diluted serum sample, pass through fraction, depleted human serum fraction and HSA standard. Conditions: 8% SDS polyacrylamide home cast gels (14 × 16 cm, 1.5 mm thickness) for 180 min at 100 V.*

Evaluation of Anti-transferrin Immunoaffinity Stationary Phase

Transferrin (Trf) is an iron-binding serum glycoprotein that is synthesized in the liver and its concentration in adult human serum in the fasting state was found to be 2.2-4 mg/mL [2]. Normal human serum transferrin is present in several isoforms, due to differences in glycosylation, and iron saturated transferrin normally separates into four different isoforms depending on the number of negatively charged sialic acid residues per molecule [29]. Moreover, Trf is one of the preferred biochemical markers for diagnostic analysis of certain diseases because of its abundance in human serum [30]. Therefore, the

development of an anti-transferrin immunoaffinity column is important both to deplete the high abundant Trf from human serum in proteomics as well as for clinical determination of Trf for diagnostic purposes. In the current study, anti-human transferrin polyclonal antibodies were immobilized onto the MALT-silica surface using the Schiff base method described early in order to achieve highly specific depletion of Trf in human serum under reduced nonspecific interactions. Moreover, the effectiveness of the maltose layer in shielding the residual silanol groups of the silica support was studied in depth using anti-transferrin immobilized MALT-silica stationary phase.

Initially, human transferrin was injected into the depletion column with the phosphate buffer loading mobile phase and eluted in a later step using 5 M urea containing eluting mobile phase. The resulting chromatogram is shown Fig. 6. According to the chromatogram, although a part of the injected Trf was successfully retained by the anti-transferrin immunoaffinity column, the major fraction of the Trf eluted out with the pass through fraction without showing any retention. The peak area of both retained and un-retained fractions were increased proportionally with the increasing human Trf concentration indicating the partial affinity behavior of the column. It is clear that the immobilized antibodies were capable of recognizing only certain type/types of Trf isoforms and unrecognized isoforms eluted out with the pass through fraction. More studies need to be done on this anti-transferrin column to immobilize suitable antibodies, which can retain all isoforms of the Trf for the complete depletion of house keeping proteins. However, the successful immobilization of the anti-transferrin antibodies onto the MALT-silica matrix and their active affinity behavior confirmed the reproducibility

of the functionalization protocol as well as the candidacy of the MALT-silica matrix as an affinity chromatographic support.

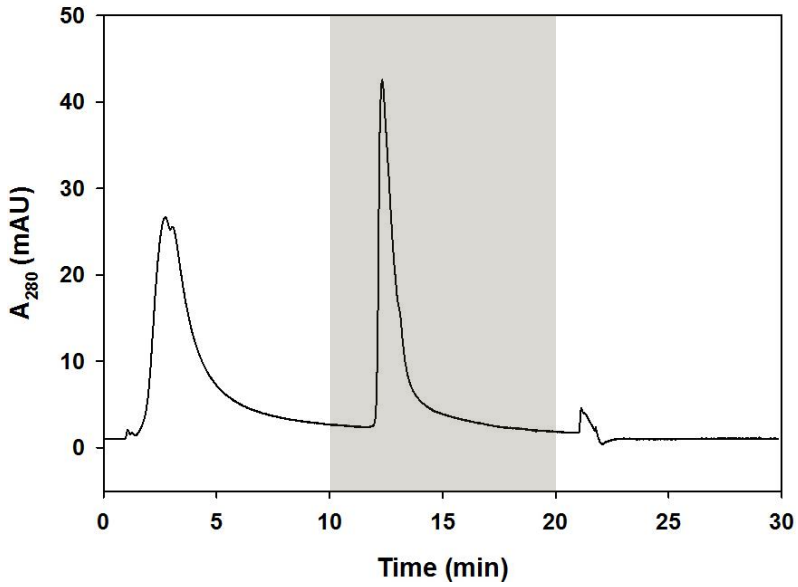


Figure 6. *Chromatograms of human transferrin obtained on the anti-transferrin immunoaffinity column (5 μm particle and 300 Å pore diameter). Separation conditions; column 10 cm × 4.6 mm id; flow rate, 1 mL/min; detection, UV at 280 nm; injection volume 20 μL. Mobile phase: 0.05 M Na_2HPO_4, 0.25 M NaCl, pH 7.4 (0-10.0 min), and 5 M urea in 0.05 M Na_2HPO_4, 0.25 M NaCl, pH 7.4 (10.0-20.0 min). The gray region indicates the eluting mobile phase application.*

In addition, the anti-transferrin stationary phase was tested using several acidic and basic protein standards in order to evaluate the ability of the maltose coating towards reducing the nonspecific interactions between proteins and the silica support. Solutions of HSA (pI, 5.6), BSA (pI, 5.07), myoglobin (pI, 6.5) and ribonuclease A (pI, 9.6) standards

were injected onto the anti-transferrin immobilized MALT-silica column under investigation and the results are shown in Fig. 7.

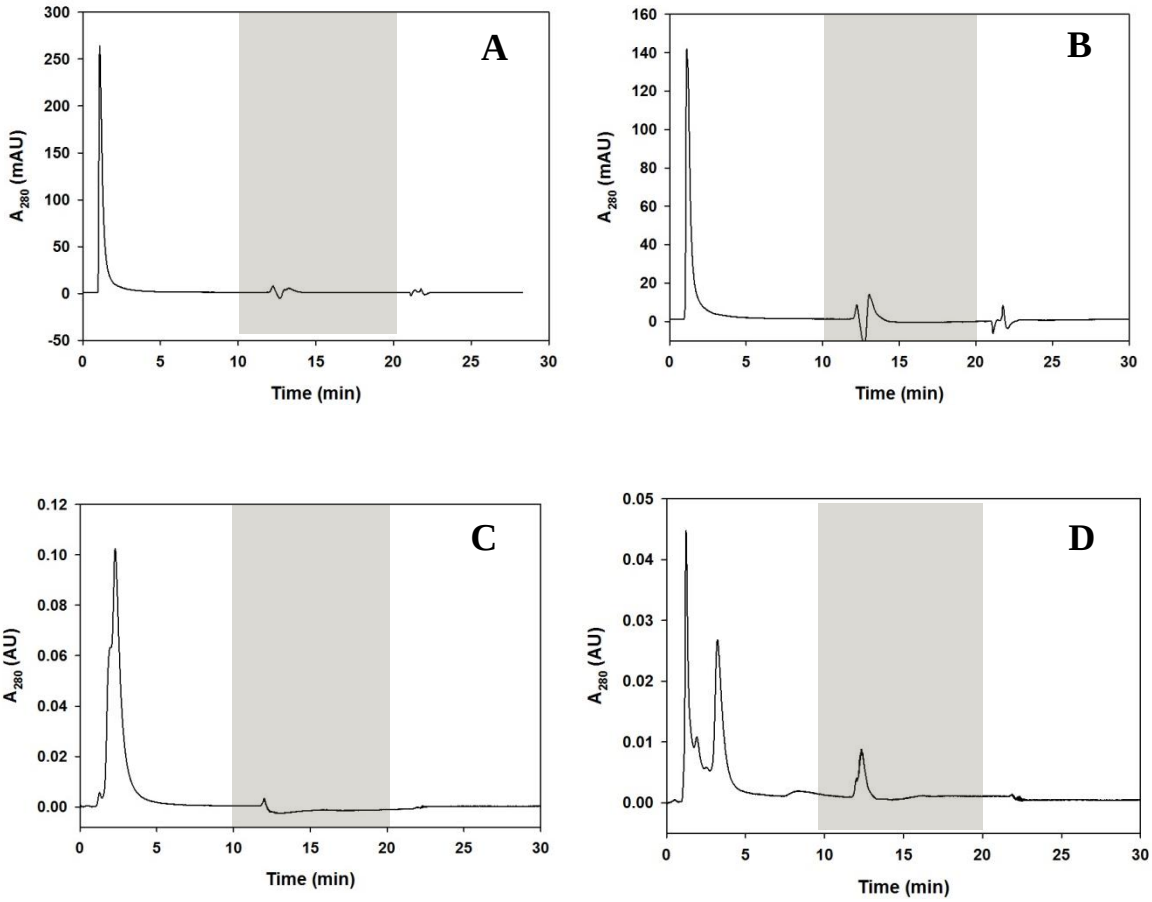


Figure 7. *Chromatogram of standard proteins obtained on anti-transferrin immunoaffinity column. The separation conditions are the same as in Fig. 6. Proteins, (A) HSA; (B) BSA; (C) myoglobin; (D) ribonuclease A.*

As can be seen in Fig. 7, most of the proteins especially all the acidic proteins eluted at the breakthrough of the column indicating the minimum nonspecific interactions between the silica support and the proteins due to the effective shielding effect of the maltose coating. However, the basic protein ribonuclease A showed an abnormal retention by the support and this effect related to the high pI value and the small size of this protein, indicating that some of the silanol groups, especially inside the through pores of the silica may not be completely covered with the maltose coating. In addition, the anti-transferrin column was successfully used for the depletion of Trf in human serum as shown in the Fig. 8.

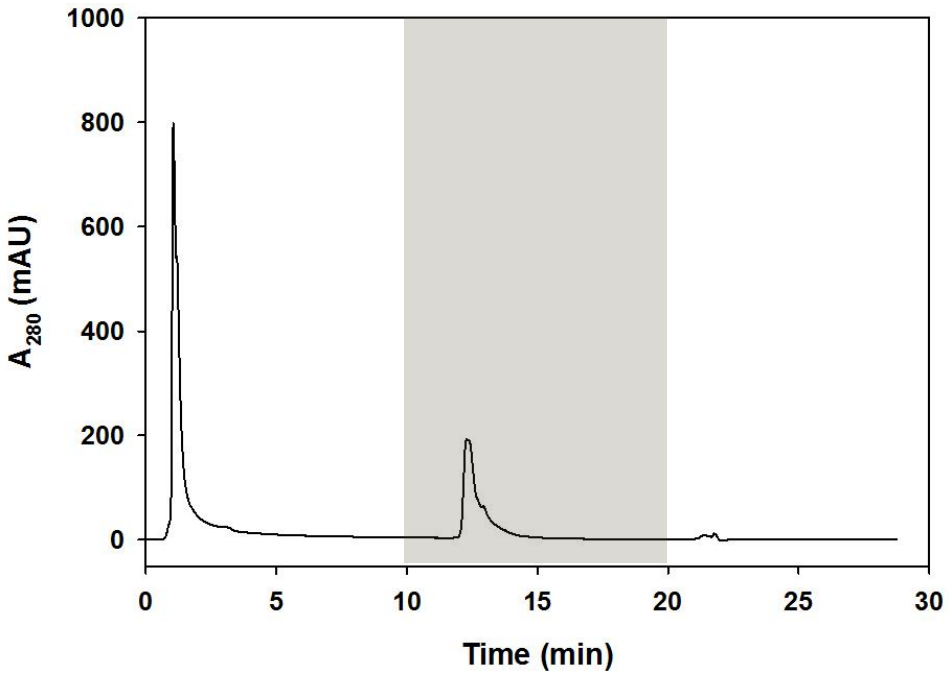


Figure 8. *Chromatogram of 1:3 diluted human serum obtained on the anti-transferrin immunoaffinity column. Separation conditions are the same as in Fig. 6.*

Evaluation of Lectin Affinity Stationary Phase

Lectins, e.g., concanvalin A (Con A), are well known affinity ligands in (bio)affinity chromatography for the purification of a large number of compounds such as carbohydrates, glycoproteins and hormones, due to their ability to specifically bind with mono- and oligosaccharides. Moreover, among hundreds of post-translational modifications, glycosylation is the most common and more than 50% of human serum proteins are glycosylated. Therefore, the development of efficient liquid chromatographic approaches for the fractionation and pre-concentration of proteins based on their glycosylation patterns are beneficial in proteomics for addressing the complexity of human serum before identifying them using common detection techniques such as LC-MS/MS. The chromatographic support used in glycoproteomics is also an important factor for the rapid, specific and selective separation of glycoproteins. Therefore, Con A was covalently immobilized onto the MALT-silica support to evaluate the applicability of the novel affinity support in glycoproteomics for the selective fractionation and enrichment of proteins in human serum.

Concanavalin A is a lectin from jack beans, and its binding sites are most complementary to α-D-mannopyranosyl residues. Any modification or substitution of the hydroxyl groups at carbons 3, 4, and 6 greatly interferes with the binding to Con A. Furthermore, oligosaccharides bearing terminal α-pyranosyl forms of D-glucose and *N*-acetyl-D-glucosamine are known to interact with Con A [31]. It was reported that Con A could interact with oligosaccharides not only with terminal non-reducing α-D-manno- or α-D-gluco-pyranosyl residues but also with internal 2-*O*-substituted α-D-manno- and α-D-gluco-pyranosyl residues.

Initially, the Con A immobilized MALT-silica column was evaluated with respect to its ability to resolve a mixture of sugar derivatives, namely PNP-α-D-galactopyranoside, PNP-α-D-glucopyranoside and PNP-α-D-mannopyranoside. The *p*-nitrophenyl derivatives were chosen for the convenient detection of each sugar using a UV detector. The chromatogram for the above separation is shown in Fig. 9.

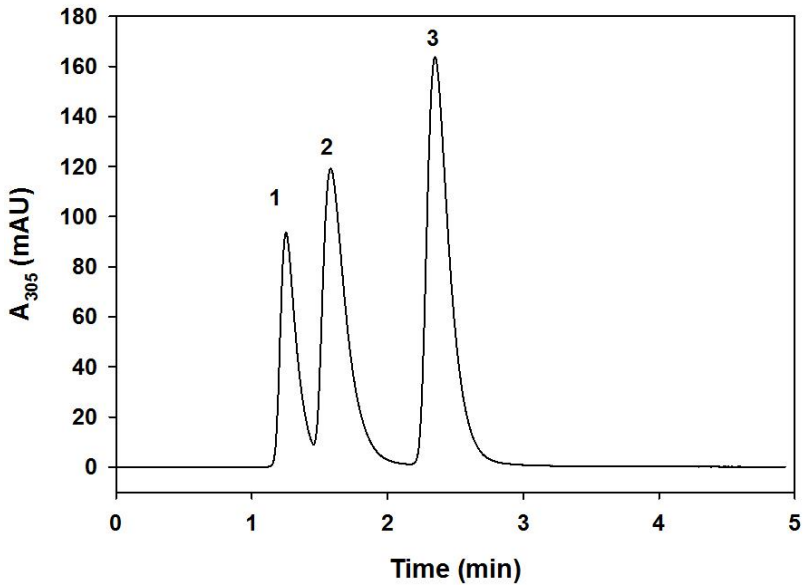


Figure 9. *Chromatograms of three PNP-sugar derivatives obtained on the Con A column. Separation conditions; column, 10 cm × 4.6 mm id; flow rate, 1 mL/min; detection, UV at 305 nm; injection volume, 20 µL; column temperature, room temperature; gradient elution with binding mobile phase, 20 mM Tris containing 250 mM NaCl, 1 mM of* Mn^{2+}, Mg^{2+} *and* Ca^{2+}, *pH =7.4; eluting mobile phase, 0.1 M methyl-α-D-mannopyranoside (Me-α-D-Man) in the binding mobile phase, 0-1 min, isocratic with 100% binding mobile phase; 1-5 min, isocratic with 99% binding mobile phase and 1% eluting mobile phase. Solutes; 1, PNP-α-D-galactopyranoside; 2, PNP-α-D-glucopyranoside; 3, PNP-α-D-manopyranoside.*

The α-D-galactopyranoside, which is known to interact weakly with Con A, eluted first close to the dead volume of the column showing little or no retention. On the other hand, α-D-glucopyranoside showed more retention on the Con A column and α-D-manopyranoside showed the highest retention as expected proving the known selectivity of the Con A immobilized MALT-silica column [31].

To verify that the Con A immobilized MALT-silica column has the ability to specifically capture the N-linked high mannose type and hybrid type glycoproteins under reduced non-specific interactions, it was tested with standard glycoproteins and non-glycoproteins as shown in the Figs. 10 and 11. The injected myoglobin, which is a non-glycoprotein and complex sialic acid type glycoprotein asialofetuin, which is not a Con A binding type glycoprotein were eluted out with the binding mobile phase with very little or no retention as shown in Fig. 10. These results confirmed the reduced levels of nonspecific interactions between the chromatographic support and the analyzed proteins as well as the high specificity of the column towards Con A binding glycoproteins.

The potential of Con A immobilized silica as an affinity stationary phase for glycoproteins was then probed with ribonuclease B, peroxidase and ovalbumin, which are known to interact with Con A [25, 32, 33]. As shown in Fig. 11, ribonuclease B and peroxidase from *Arthromyces ramosus* which are high mannose type glycoproteins were completely retained on the column. They were eluted only in the presence of the haptenic sugar in the eluting mobile phase showing the high specificity of the column towards high mannose type glycoproteins. In this regard, peroxidase contains a protohemin group

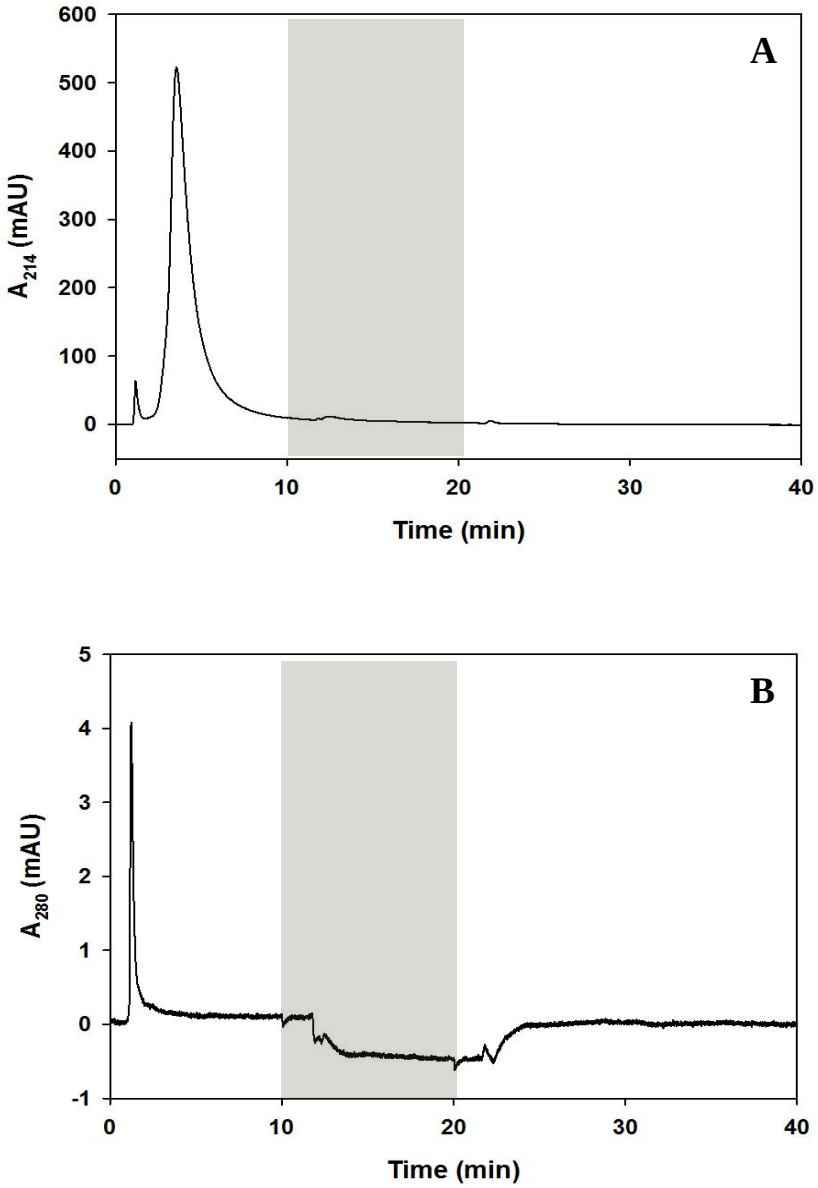


Figure 10. *Chromatogram of myoglobin (A) and asialofetuin (B) injected into the Con A column (10 cm × 4.6 mm ID). Binding mobile phase, 20 mM Tris containing 250 mM NaCl, 1mM of Mn^{2+}, Mg^{2+} and Ca^{2+}, pH = 7.4; eluting mobile phase, 0.1 M methyl-α-D-mannopyranoside (Me-α-D-Man) in the binding mobile phase; flow rate, 1 mL/min; Detection, UV at 280 nm for asialofetuin and UV at 214 nm for myoglobin.*

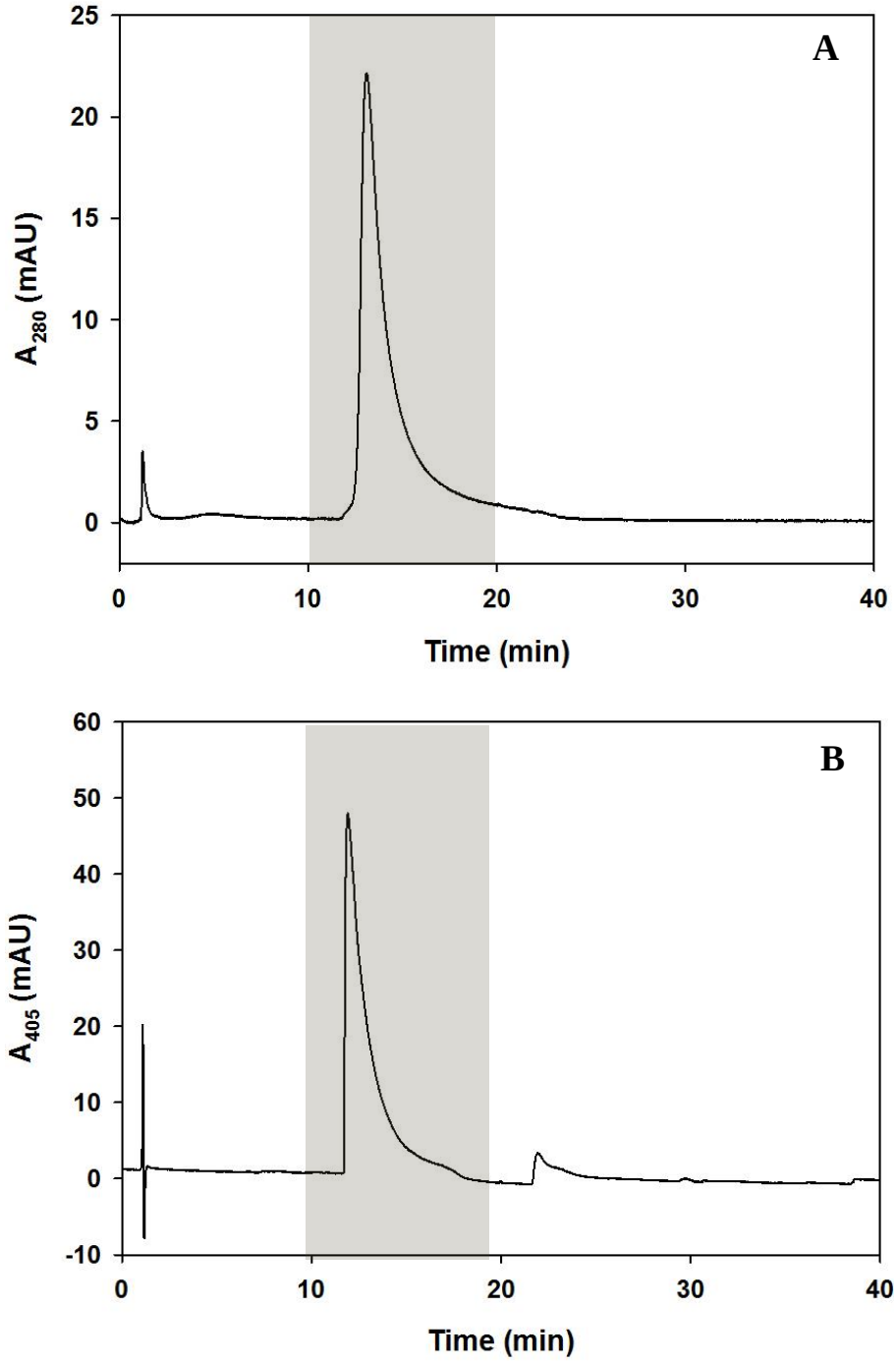


Figure 11. *Chromatograms of ribonuclease B (A) and* peroxidase from *Arthromyces ramosus (B) injected into the Con A column (10 cm × 4.6 mm ID). Conditions are the same as in Fig. 10B except detection at 405 nm for peroxidase.*

which shows strong absorbance at 405 nm, allowing convenient semi-specific detection of the proteins [25]. Therefore, the chromatogram of peroxidase elution was recorded at 405 nm.

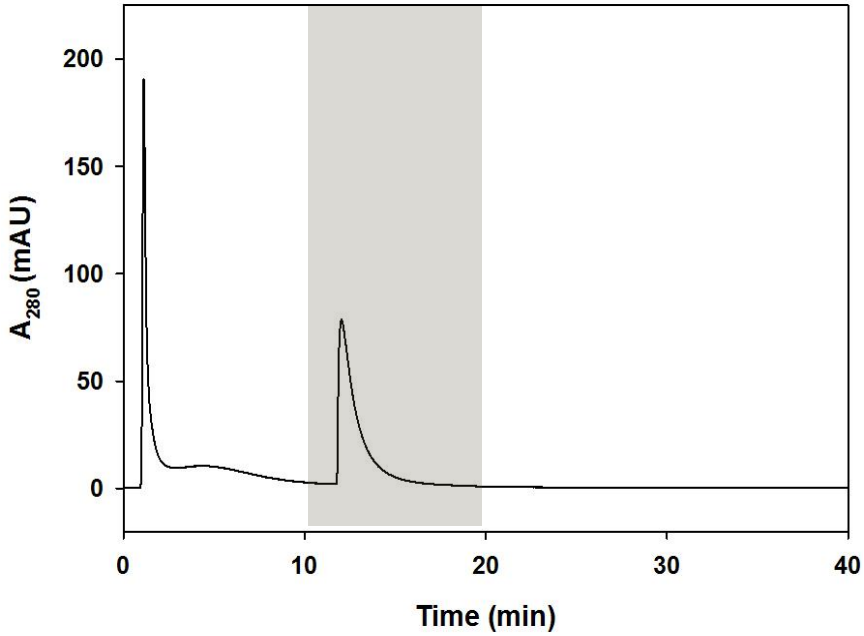


Figure 12. *Chromatogram of ovalbumin injected into the Con A column (10 cm × 4.6 mm ID). Conditions are the same as in Fig. 10B.*

In addition, it was reported that ovalbumin glycans mainly consist of the high mannose [predominantly $(Man)_5(GlcNAc)_2$ and $(Man)_6(GlcNAc)_{2,}$] hybrid and bi- to penta-antennary structures which largely lack the terminal galactose that is common in complex glycans from mammalian systems [34]. Therefore, two major peaks were observed on the Con A affinity chromatogram of ovalbumin as shown in Fig. 12 may be due to the complex nature of the glycosylation patterns.

Finally, 1:3 diluted human serum sample was injected onto the Con A immobilized MALT-silica column and the resulting chromatogram is shown in Fig. 13. A large portion of the unbound proteins was eluted first at the breakthrough of the column. The bound fraction, which contains specifically captured glycoproteins by Con A, may have some of the high mannose type and hybrid type N-linked glycoproteins since they were eluted out later from the column using *Me-α-D-Man* hapten sugar. This demonstrates the typical selective enrichment and fractionation of glycoproteins from the human serum in proteomics studies.

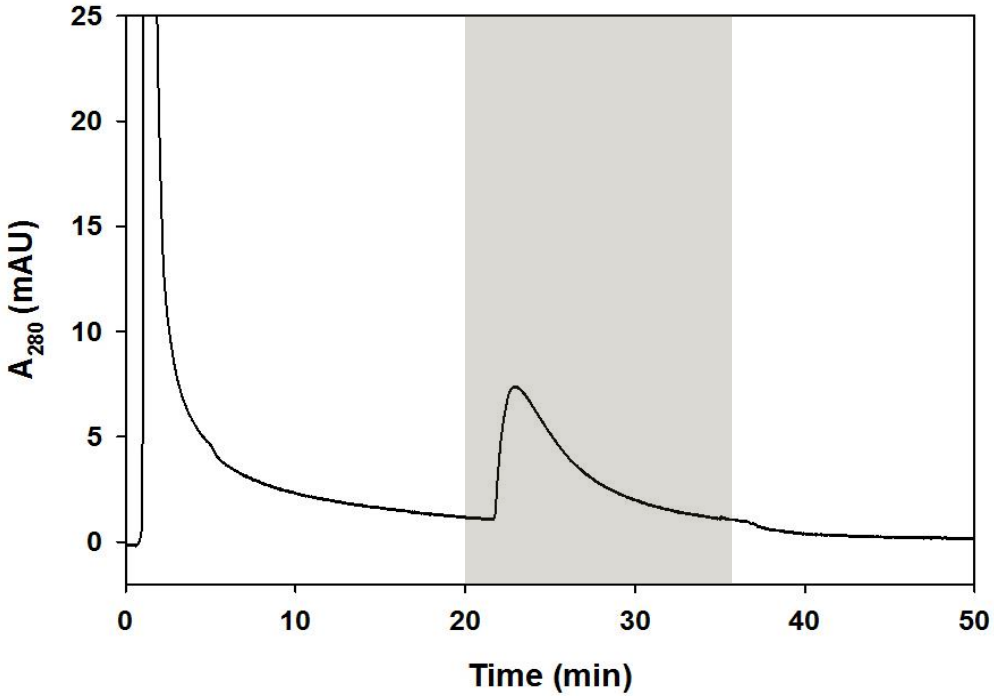


Figure 13. *Chromatogram of 1:3 diluted human serum injected into the Con A column (10 cm × 4.6 mm ID). Conditions are the same as in Fig. 10 except that elution mobile phase was applied at 20 min.*

Stability of the Grafted Affinity Ligands on MALT-Silica Support

The stability of the sugar coating and the strong interaction between affinity ligands and the chromatographic support are important in order to minimize the leaching of the attached ligands with time. In addition the mildness of the chromatographic conditions is equally important to minimize the permanent denaturation of the attached affinity ligands during the operation. These factors are important to achieve an increased lifetime of the affinity column in order to reuse expensive antibodies. The stability of the anti-HSA antibody immobilized MALT-silica stationary phase was evaluated in terms of the reproducibility of its binding capacity and the results are shown in Fig. 14A. A relatively concentrated HSA standard was injected onto the anti-HSA column during the operation of the column for 7 days. As it is shown in the results the binding capacity of the column remained unchanged over 5 liters of binding/eluting mobile phase cycles. The concentrated standard was chosen for the purpose of achieving maximum binding capacity and the low concentration standards also showed similar results (chromatograms are not shown). Similarly, the human serum affinity separation showed reproducible binding capacity and the separation pattern indicated the greater stability of the column for potential affinity chromatographic separation in proteomics analysis. Moreover, the anti-HSA column was stored under proper storage conditions explained earlier for 3 months and the binding capacity of the affinity stationary phase did not show any significant decrease after the 3 months of storage. The column was successfully used for the depletion of HSA in human serum even after 3 months.

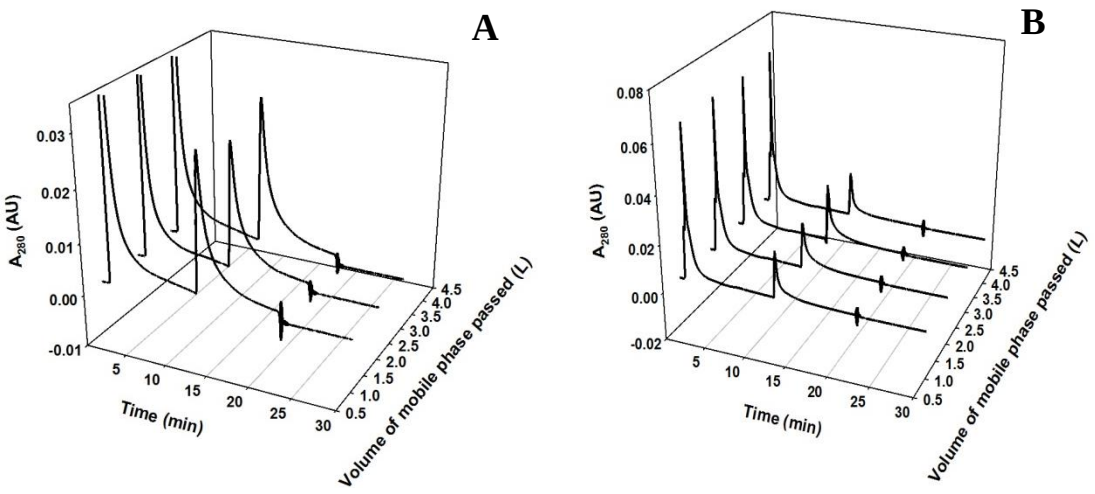


Figure 14. *Monitoring the stability of anti-HSA immobilized MALT-silica column with respect to repeated injection of HSA (A) and human serum (B). Conditions are the same as in Fig. 2.*

Conclusions

Functionalization of bare silica microparticles with maltose combined the advantages of two popular chromatographic supports, namely silica and carbohydrate-based suports. The MALT-silica stationary phase successfully minimized the nonspecific adsorption of proteins with the free silanol groups on the silica support. The sugar layer on the silica allows the convenient coupling of various affinity ligands onto the silica support *via* conventional coupling methods used for polysaccharide-based supports. Considering all of the above facts, the MALT-silica matrix can be considered as an ideal chromatographic support for HPAC for the fast separation and isolation of biomolecules.

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