SYNTHESIS OF POLAR POLYMERIC MONOLITHS AND THEIR EVALUATION IN NORMAL PHASE CAPILLARY ELECTROCHROMATOGRAPHY

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SYNTHESIS OF POLAR POLYMERIC MONOLITHS

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CAPILLARY ELECTROCHROMATOGRAPHY

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LIST OF SYMBOLS AND ABBREVIATIONS

α	Selectivity factor
$\Delta\mu_{ep}$	difference in electrophoretic mobilities of two adjacent zones
η	viscosity
k'	chromatographic retention factor
a	radius of a spherical particle
k^*	retention factor of a charged solute in CEC
k^*_{ep}	velocity factor of a charged solute in CEC
k^*_{cc}	peak locator in CEC
μ	electrophoretic mobility
μ_{app}	apparent mobility
μ_{eo}	electroosmotic mobilitu
μ_{ep}	electrophoretic mobility
μ_{eo}^*	apparent electroosmotic mobility in CEC
i_{open}	current observed in open tube
i_{packed}	current observed in packed column
ν_{app}	apparent velocity
ν_{eo}	interstitial electroosmotic velocity
ν_{ep}	electrophoretic velocity
ν	migration velocity

σ_l	standard deviation of the peak in unit length
σ_L^2	peak variance
σ_t	standard deviation of the peak in unit time
E	electric field strength
F_e	electric force
F_f	frictional force
L	total length of capillary
l	effective length
N	efficiency
q	charge of ions
R_s	resolution
t	migration time of a peak
t_0	migration time of a neutral marker
t_M	migration time of analyte
V	applied voltage
w_b	peak width at base
w_h	peak width at half-height
w_i	peak width at the inflection point
2-AB	2-aminobenzamide
ACN	acetonitrile
AIBN	2,2'-azobis(isobutyronitrile)
AMPS	2-acrylamido-2-methyl-1-propanesulfonic acid
APCI	atmospheric pressure chemical ionization

API	atmospheric pressure ionization
CE	capillary electrophoresis
CEA	2-cyanoethyl acrylate
CEC	capillary electrochromatography
CZE	capillary zone electrophoresis
EDMA	ethylene glycol dimethacrylate
EOF	electroosmotic flow
ESI	electrospray ionization
GC	gas chromatography
GMA	glycidyl methacrylate
HPLC	high performance liquid chromatography
IDCN	1H-Imidazole-4,5-dicarbonitrile
LC	liquid chromatography
LIF	laser-induced fluorescence
MALDI	Matrix assisted laser desorption ionization
MS	mass spectrometry
Nano-LC	nano-liquid chromatography
NMR	nuclear magnetic resonance spectroscopy
NP-CEC	normal phase capillary electrochromatography
ODS	octadecyl-silica
OT-CEC	open tubular capillary Electrochromatography
PDA	piperazine diacrylamide
RSD	relative standard deviation

TEA	triethylamine
TEMED	*N,N,N',N'*-tetramethylethylenediamine
TOF	time of flight
VP	1-vinyl-2-pyrrolidinone

CHAPTER I

SOME BASIC PRINCIPLES OF CAPILLARY ELECTROCHROMATOGRAPHY AND SCOPE OF THE STUDY

Introduction

Capillary Electrochromatography (CEC) is a microcolumn separation technique, which involves the use of an electric field as the driving force to transport the mobile phase and the analytes through the capillary column containing a stationary phase. CEC resembles capillary electrophoresis (CE) in the sense that electroosmotic flow (EOF) is the same driving force for bulk flow, but differs from CE because the electrophoretic mobility is not the only factor controlling the migration velocity of charged analytes. For neutral analytes, the differential distribution between mobile phase and stationary phase is the main factor affecting solute's migration velocity as in high performance liquid chromatography (HPLC). We can see from this that CEC is a hybrid technique of CE and HPLC that combines the selectivity of HPLC and the high separation efficiency of CE [1-5]. CEC borrows from HPLC many of the techniques used for stationary phases and column fabrication, but as a requirement for supporting the EOF, some special designs were introduced to develop CEC stationary phases. There are two kinds of stationary phases for packed CEC columns: the particle packed column and monolithic polymeric column. The particle packed columns need high experimental skills and

experience to make stable columns with reproducible properties. One of the challenges is making the frits to retain the packing material and also allow unrestricted flow. In contrast, the monolithic column is fritless since the monolith anchors itself onto the wall of the fused-silica capillary. Thus, monolithic columns avoid the problems of limited stability, insufficient permeability, and susceptibility to bubble formation [6-8]. Currently, monolithic columns emerged as an attractive alternative for particle packed CEC columns. The advantages of this method are the simplicity of the preparation and the virtually unlimited choice of chemistries. Monolithic columns provide a great compatibility for coupling with mass spectrometry (MS) and nuclear magnetic resonance spectroscopy (NMR) [9], thus permitting monolithic CEC to become broadly accepted as a separation technique in life sciences, pharmaceuticals, and environmental analysis.

In this chapter, we will introduce the history of the CEC technique, the various column technologies, the instrument used in this field, and the analytical parameters. Also, an overview of polar monoliths and the rationale of the study will be provided.

Development of Capillary Electrochromatography

The first report on the use of the combination of chromatographic force and electrophoretic force was in 1939 by Strain, who separated several dyes by adsorption chromatography in an alumina column in the presence of an electric field [10]. The early attempt to use EOF in separation was reported by Mould and Synge in the fifties to separate some polysaccharides on collodion membrane [11, 12]. After that, it took a long time to use EOF as pumping action for analytical separation. In fact, it was until 1974

that Pretorius *el al.* suggested EOF as an alternative to pressure driven flow [13]. They demonstrated substantially smaller band broadening as compared to HPLC by driving the mobile phase by EOF through a 1 mm glass tube packed with a particulate stationary phase of 75-125 μm particle diameters. After four decades of the first introduction of the concept of coupling electrophoresis and chromatography, Jorgenson and Lukacs in 1981 proved its feasibility by using 170 μm i.d. glass capillaries packed with 10 μm C_{18}-coated silica (ODS) particles [14, 15]. In the 1980's CEC experienced significant progress and laid its theoretical foundations. To the later 1990's there were more prevalent practice of CEC, and CEC instruments have been developed and marketed for increasing demands in research.

As a hybrid technology, CEC overcomes the flaws of CE and HPLC. Compared with CE, CEC has higher peak capacity and selectivity because of the existence of the stationary phase packed in the column. According to the nature of the analytes it may be necessary to tailor the stationary phases as well as the inner surface of the column. Compared to HPLC, with the plug flow profile of the EOF as the flow driving force, CEC usually has narrower peaks, which yield higher column efficiency. CEC also consumes far less amount of mobile phases, which are normally composed of organic solvents and aqueous buffers. Typical organic solvents used in HPLC are methanol and acetonitrile, which are hazardous to environment and human health.

Theoretical Considerations on EOF

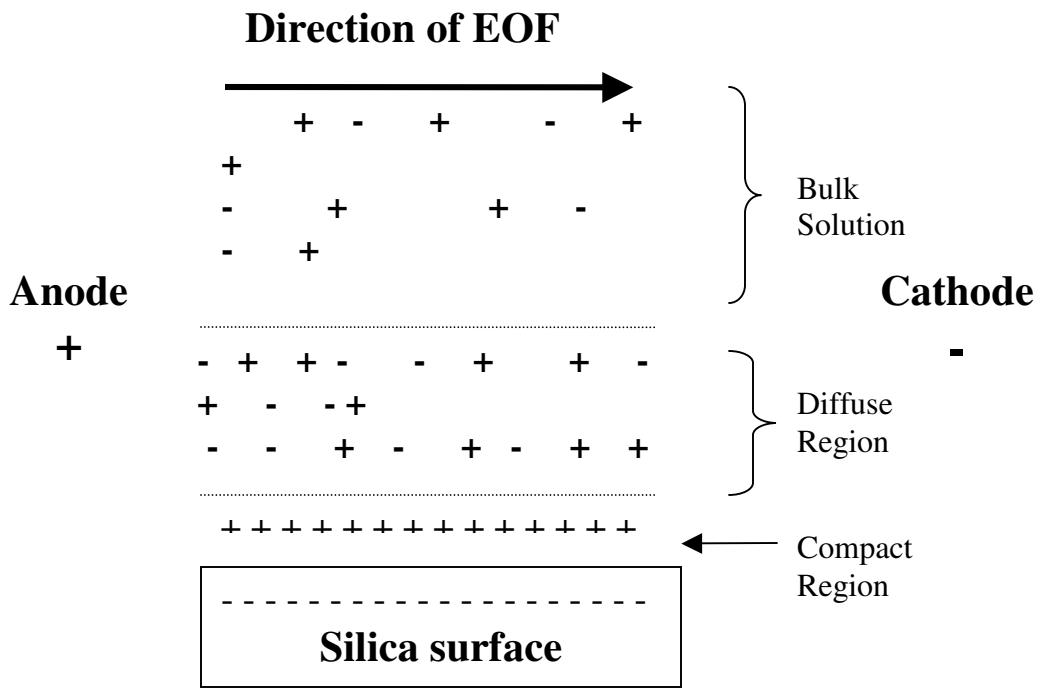

Figure1. *Illustration of the electric double layer formed at a charged surface as well as the generation and direction of EOF.*

Electroosmosis refers to the movement of liquid relative to a stationary charged surface due to an applied electric field [16], which is the "pumping" force of mobile phase in CEC. Figure 1 illustrates the electric double layer in the case of a negatively charged surface (e.g., silica). The electric double layer is composed of a compact region and a diffuse region. The compact region is formed when negatively charged silanol groups on the silica surface electrostatically attract counter ions from the liquid phase. Ions in the compact region are held tightly and are usually not mobile. Due to thermal motion, some of the ions in the compact region move away from the capillary surface, forming the diffuse region, which is mobile. When a potential field is tangentially applied to the electric double layer, the counter ions in the diffuse region will move toward the anode.

Since they are hydrated, these cations will drag the bulk solution with them then forming a flow that is called the EOF.

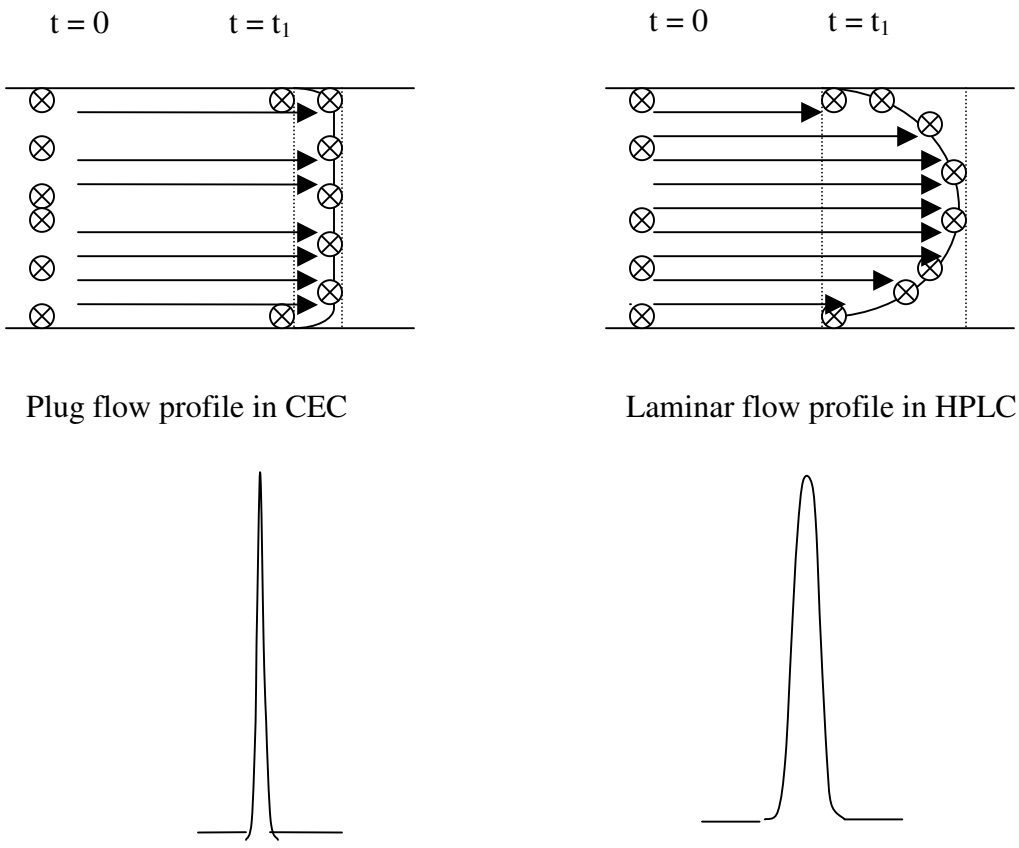

Figure 2. *Plug flow profile (i.e., EOF or electro-driven flow) versus laminar flow profile (i.e., pressure driven flow). Also shown in this illustration the effect of the flow profile on solute bandwidth.*

Since the EOF originates at the liquid-solid interface, it is characterized by a plug like profile, which narrows the width of the zones of the different ions. The narrow zones produce sharper peaks and in turn higher chromatographic peak capacity. In contrast,

HPLC uses high pressure as the flow driving force that exhibits a parabolic flow profile, which usually leads to band broadening. We can see the difference from Figure 2.

Principles and Parameters of Capillary Electrophoresis and Capillary Electrochromatography

Since CEC is a hybrid separation technique between CE and HPLC, and both chromatographic and electrophoretic effects in the CEC mode govern the separation of charged samples, we will introduce some principles and parameters used in CE in the following sections.

Electrophoretic Migration

When a constant electric field is applied across the column, all ions would experience the electrical force F_e which is proportional to the electric field strength E and the charge q of the ions,

$$F_e = qE \qquad (1)$$

The electric field strength is related to applied voltage V and the total length of the column L as follows

$$E = \frac{V}{L} \qquad (2)$$

The electrical force F_e is the driving force, which causes the migration of the ions toward the oppositely charged electrode. At the same time the ions also encounter the frictional

forces, which counteract the ions' movement. According to Stokes' law, for a spherical particle with radius *a,* the frictional force F_f, is expressed as:

$$F_f = 6\pi\eta a v \qquad (3)$$

Where η is the viscosity of the solution and v is the migration velocity of the ion. The migration velocity of an ion can also be expressed as:

$$v = \mu E \qquad (4)$$

Where μ is the electrophoretic mobility, which is collectively affected by the medium and temperature. When a steady state is reached, the driving force F_e equals frictional force F_f. We can express μ as:

$$\mu = \frac{q}{6\pi\eta a} = \frac{v}{E} \qquad (5)$$

The unit of μ is $cm^2V^{-1}s^{-1}$. It is obvious that the charge and size of the ion and the viscosity of the medium have strong effects on the electrophoretic mobility.

The parameters introduced here are applicable to both CE and CEC for charged solutes. CEC also uses some parameters from HPLC.

Migration Time and Apparent Mobility

Migration Time and Mobility in CE: Migration time refers to the elution time of a peak recorded by the instrument. The observed mobility of a charged analyte is the apparent mobility μ_{app} which is the summation of electrophoretic migration μ_{ep} and electroosmotic flow μ_{eo}.

$$\mu_{app} = \mu_{ep} + \mu_{eo} \qquad (6)$$

Since $v = l/t$, where l is the length of the column from inlet end to the detection point, also called effective length, t is the elution time of the peak, we can express electrophoretic mobility μ as:

$$\mu = \frac{v}{E} = \frac{lL}{tV} \quad (7)$$

The migration time in relation to electrophoretic mobility can be expressed as:

$$t = \frac{v}{E} = \frac{lL}{\mu V} \quad (8)$$

The electroosmotic mobility μ_{eo} is determined by measuring the migration time t_0 of the neutral marker such as acetone or dimethyl sulfoxide. With migration time of the analyte being t_m we have:

$$\mu_{eo} = \frac{v_{eo}}{E} = \frac{lL}{t_o V} \quad (9)$$

$$\mu_{app} = \frac{v_{app}}{E} = \frac{lL}{t_m V} \quad (10)$$

Electrophoretic mobility, μ_{ep}, of a certain analyte can therefore be deduced from the electropherogram as:

$$\mu_{ep} = \mu_{app} - \mu_{eo} = \frac{lL}{V}\left(\frac{1}{t_m} - \frac{1}{t_o}\right) \quad (11)$$

Migration Time and Mobility in CEC In CEC, migration time t_R of a neutral analyte is similar to that in liquid chromatography, which is given by:

$$t_R = t_0(1+k') \quad (12)$$

where k' is the conventional chromatographic retention factor:

$$k' = \frac{(t_R - t_0)}{t_0} \quad (13)$$

As mentioned earlier, charged components in a separation mixture undergo both electrophoretic and chromatographic mechanism when analyzed in CEC [17, 18]. Under these circumstances, k' defined above does not represent the chromatographic partitioning as for neutral solutes; rather it is used as a peak locator. Rathore and Horvath [19, 20] have introduced the retention factor k* to evaluate the migration of charged solutes in CEC. k* is defined as:

$$k^* = \frac{t_m\left(1 + k_{ep}^*\right) - t_o}{t_o} \qquad (14)$$

where the velocity factor, k^*_{ep}, describes the contribution of electrophoretic mobility to the separation of a charged species in CEC and is given by:

$$k_{ep}^* = \frac{\nu_{ep}}{\nu_{eo}} \qquad (15)$$

where ν_{ep} is the electrophoretic velocity of the charged solutes, and ν_{eo} is the interstitial electroosmotic velocity of the mobile phase in the CEC column. For neutral solutes k^*_{ep} is zero. Since ν_{ep} is the same for a charged species in CZE and CEC, its value is usually obtained by running in the CZE mode under the same conditions as the CEC analysis [19, 20]. The value of ν_{eo} is obtained by multiplying the "apparent" electroosmotic mobility ν_{eo}* within the CEC column by the tortuosity factor of the column. The column tortuosity factor is usually estimated by the quotient of the currents observed in the CZE (i_{open}) and CEC (i_{packed}) modes for the same running conditions [21]. Thus, we can express k^*_{ep} as follows:

$$k_{ep}^* = \frac{\mu_{ep}}{\mu_{eo}^* \dfrac{i_{open}}{i_{packed}}} \qquad (16)$$

Because of the presence of k^*_{ep}, k* does not serve as a useful peak locator as its counterpart k' does in chromatography. To facilitate the description of the elution order of charged solutes in CEC, a peak locator, k^*_{cc}, based on chromatographic formalism, has been suggested [19, 20]:

$$k^*_{cc} = \frac{t_m - t_o}{t_o} \qquad (17)$$

Unlike k* and k^*_{ep}, k^*_{cc} is devoid of any mechanistic insight, and so has limited utility [19, 20]. For neutral species, both k* and k^*_{cc} become the true chromatographic retention factor, k'.

Selectivity Factor

In conventional chromatography, selectivity factor α measures the segregation of components between two distinct zones (peaks), and is determined by the ratio of retention factors of the specified components:

$$\alpha = \frac{k_2'}{k_1'} = \frac{\left(\frac{t_2 - t_0}{t_0}\right)}{\left(\frac{t_1 - t_0}{t_0}\right)} = \frac{t_2 - t_0}{t_1 - t_0} = \frac{t_2'}{t_1'} \qquad (18)$$

Where k_1' and k_2' are chromatographic retention factors, t_1 and t_2 are elution times, and t_1' and t_2' are adjusted retention times of respective components.

Equation (18) applies also to estimating selectivity factor for neutral solute in CEC. For charged solute α becomes the ratio of k^*_{cc} calculated by equation (17)

$$\alpha = \frac{k^*_{cc,2}}{k^*_{cc,1}} \frac{\left(\frac{t_{m,2} - t_0}{t_0}\right)}{\left(\frac{t_{m,1} - t_0}{t_0}\right)} = \frac{t_{m,2} - t_0}{t_{m,1} - t_0} \qquad (19)$$

Resolution

Resolution, R_s, expresses the extent of the overlap of two adjacent specified component zones (peaks). It is determined by

$$R_s = \frac{t_2 - t_1}{2(\sigma_1 + \sigma_2)} \qquad (20)$$

where σ_1 and σ_2 are the respective standard deviations of the two neighboring peaks.

In chromatography, resolution can also be expressed by selectivity factor α, efficiency N and retention factor k' as

$$R_s = \frac{\sqrt{N}}{4}\left(\frac{\alpha - 1}{\alpha}\right)\left(\frac{k_2'}{1 + k_2'}\right) \qquad (21)$$

Where k_2' is the retention factor of the more retained peak of the two adjacent peaks, and N is the column efficiency. The resolution is affected by the column efficiency, retention factor and selectivity factor. The selectivity factor has more important influence on the resolution than the other two factors. This equation is applicable for neutral analytes in CEC.

For charged analytes the resolution can be expressed as:

$$R_s = \frac{\sqrt{N}}{4}\left(\frac{\alpha - 1}{\alpha}\right)\left(\frac{k^*_{cc,2}}{1 + k^*_{cc,2}}\right) \qquad (22)$$

where $k^*_{cc,2}$ is the peak locator for the more retarded peak.

In CE, resolution can be expressed by electrophoretic and electroosmotic mobilities as

$$R_s = \frac{\sqrt{N}}{4}\left(\frac{\Delta\mu_{ep}}{\overline{\mu_{app}}}\right) = \frac{\sqrt{N}}{4}\left(\frac{\Delta\mu_{ep}}{\overline{\mu_{ep} + \mu_{eo}}}\right) \quad (23)$$

Where $\Delta\mu_{ep}$ is the difference of the electrophoretic mobilities of two adjacent components, $\overline{\mu_{ep}}$ and $\overline{\mu_{app}}$ are the average electrophoretic mobility and average apparent mobilities of the specified components, respectively. This equation shows that there is no point to quadruple N in order to double R_s. R_s is more easily adjusted by manipulating $\Delta\mu_{ep}$, which is achieved by changing pH and composition of the running electrolyte.

Column Efficiency

In CE and CEC, the number of theoretical plates expresses column efficiency N. Column efficiency is a measure of the dispersivity of migration for a certain analyte. It is expressed as:

$$N = \left(\frac{l}{\sigma_L}\right)^2 \quad (24)$$

Where σ_L is the standard deviation of the peak in unit of length. N can be calculated by the same equation used in liquid chromatography:

$$N = 4\left(\frac{t_M}{w_i}\right)^2 = 5.54\left(\frac{t_M}{w_h}\right)^2 = 16\left(\frac{t_M}{w_b}\right)^2 \quad (25)$$

Where w_i, w_h, and w_b are the peak widths for a Gaussian peak at the inflection point, half height and base, respectively. These equations are valid for both CE and CEC.

Instrumentation

General Aspects of Instrumentation

The instrument used in CEC is a slightly modified version of the instrument design for CE in which the ability to apply gas pressure up to 12 bars on the inlet and /or outlet vials is facilitated. Figure 3 is a schematic representation of a typical instrument used in CEC. Generally, there are five major parts in the instrument: a capillary, a power supply, a detector, a data processor, and sample and buffer containers. The CEC columns are narrow bore fused-silica capillaries of 50-200 µm I.D. containing some type of packing material as the stationary phase or open tubular. The electric field is provided by a power supply that is capable of delivering up to ±30 kV and is connected to the inlet platinum electrode. The outlet electrode is connected to the ground to complete the circuit. A detection window is made by stripping the external polyimide coating of the fused-silica capillary to transmit the detector light. On-column detection for CEC is most commonly accomplished by using a UV-Vis or fluorescence detector. The off column detection can be a mass spectrometry detector or a nuclear magnetic resonance spectroscopy detector.

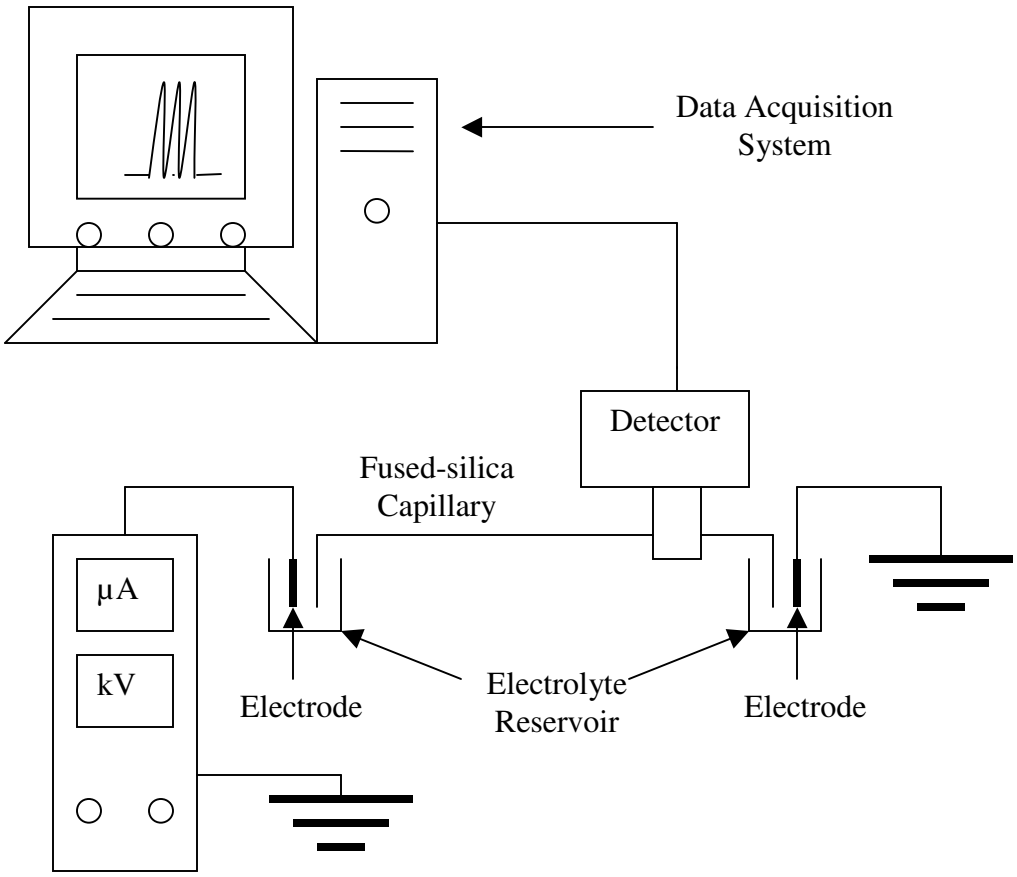

Figure 3. *Schematic illustration of an instrument used in CE/CEC*

The output of the detector is connected to a data acquisition station, where the software displays and integrates the data. Modern instruments have the auto samplers, control over the column and sample tray temperature and the ability to apply high gas pressure to the inlet or outlet vials or both vials [22, 23].

Sample Injection

In CE and CEC, the introduction of a very small amount of the sample with high precision is very important to ensure reproducible quantitative and qualitative analysis. Generally, there are two types of injection: hydrodynamic and electrokinetic mode. Hydrodynamic injection is carried out either by applying a positive pressure at the inlet end or by applying a negative pressure at the outlet end. The limitation of hydrodynamic injection for a packed column is the high back pressure required to drive the mobile phase through the column, which inhibit the injection through a very narrow bore size column. In the electrokinetic injection mode, sample is introduced by combining electroosmosis flow and electrophoresis mobility. Under electrophoretic mobility analytes with the same charge will be introduced while analytes of opposite charge will be eliminated. The composition of the injected solution is likely to be different from the original sample solution. Sample injected by electroosmosis only would not bring about discrimination among injected analytes. So the charge of individual analytes and the nature of the sample matrix are very important for the amount of sample loaded. In this case, quantitative analysis requires that both standards and samples are rum in a homogeneous closely defined matrix. Currently, electrokinetic injection is used in most CEC analyses. The relative standard deviation for retention times and peak heights between electrokinetic and hydrodynamic injection are very small [24].

Detection

On-column detection in CE/CEC is based mostly on absorbance and fluorescence. UV-Vis absorbance is the primary mode of detection due to its simplicity. However, the

concentration sensitivity is generally poor because of the short optical path length (usually 50-100 μm) and light scattering for the curved surface of the capillary [23, 25, 26]. Fluorescence detection has the merit of higher sensitivity and improved detection selectivity. So the sample must either exhibit native fluorescence or be labeled with a fluorophore [27-30]. Laser is used to offer sufficient excitation for enhanced sensitivity. With laser induced fluorescence (LIF), detection limit can be as low as 10^{-12} M [31, 32], while typical limit of detection of absorbance mode is in the range of 10^{-5} M to 10^{-6} M [33, 34]. With on-line preconcentration approach, the detection limit of absorbance mode may be lowered to 10^{-7} M [33].

Mass spectrometry (MS) and nuclear magnetic resonance (NMR) have also been used as detectors for CE/CEC because of their ability to provide additional structural information for the analytes. Compatibility of CE/CEC with MS is facilitated in part by the very low bulk flow rate (<< 1 μL/min) that arises from the EOF in the capillary. Atmospheric pressure ionization (API) methods, including electrospray ionization (ESI) [35] and atmospheric pressure chemical ionization (APCI) [36] are currently popular in the analysis of proteins, peptides [37-39], fatty acids [40, 41], oligosaccharides[42-44], carbohydrates [37, 42, 44], metabolites [45, 46], and even enantiomers [47, 48] from others. NMR detector can provide additional structural information of the analytes without requirement of phase transfer, which is a non-destructive detection technique compared with MS. This was first introduced by Pusecker *et al*. in 1998 [49, 50] for the study the metabolites of paracetamol from human urine extract [51] and analgesic mixture containing caffeine, acetaminophen and acetylsalicylic acid [52] by using

coupled isocratic and gradient CEC-NMR. Gradient CEC-NMR can provide higher separation efficiency in shorter analysis time[50].

Summary and Conclusion

Currently, most CE/CEC equipment has been sufficiently used in isocratic mode of CEC separation, but there are still some challenges for the instrument, such as the absence of gradient delivery system, the limitation of the voltage (above 30 kV), the poor concentration sensitivity of on-column UV-Vis absorption detection, and the accurate temperature control [22]. The improvement in these fields will provide the CEC technique a broader scope of applications.

Column Technologies

Capillary column is the most essential part of the CEC technique, and advanced column technologies have been introduced to produce a variety of stationary phases for the analysis requirement. Generally, there are three main formats of column design: open tubular (OT), particle packing and monolithic columns.

Open Tubular Columns

Open tubular CEC (OT-CEC) is usually performed in a capillary with narrow inner diameter (ca. 10 μm I.D.) having a stationary phase coated onto the capillary inner

walls [53]. In 1982, Tsuda and co-workers demonstrated the first effective OT-CEC separation with chromatographic interactions [54]. Later, a series of experiments were developed to prove the chromatographic effects in the separation of optical isomers [55-58]. Currently, most research on OT-CEC is directed toward the improvement of two fundamental problems of this technique: the long distance for the analytes to interact with the bonded moiety and the low capacity of the column. Chemical etching of the inner wall of the capillary is the most frequent way to circumvent these problems. The etching process to the inner capillary wall can increase the overall surface area of the capillary as much as 1000-fold [59], and also favor the ligand attachment to the capillary inner surface. At the same time the distance of the solute to interact with the stationary phase will be decreased by the radial extension formed from the dissolution and redeposition of the silica material during etching.

Fabrication of these columns is relatively simple and they provide very high separation efficiencies [60-62]. The most commonly used stationary phases in OT-CEC are chemically bonded, organic polymer-based, sol-gel coated and physically or dynamically adsorbed. Chemically bonded phases involve bonding of an organic moiety to the etched surface *via* silanization or hydrosilation process [63]. Organic polymer-based and sol-gel coated involve column fabrication by the polymerization of organic polymers [64] (organic polymer-based) and inorganic alkoxides [65, 66] (sol-gel coated). These processes are congruent with the methods used for the generation of monolithic columns, except the process is limited to the coating of the walls with a porous layer as opposed to generating a continuous bed that occupies the entire volume of the capillary. The adsorbed stationary phases make use of the electrostatic interactions between the

silica surface and the desired ligand, such as surfactants and proteins [67]. Physically adsorbed phases involve stronger interactions compared to dynamically adsorbed ligands, and so addition of the adsorbing agent to the mobile phase for the latter [68]. These columns proved to promote the most retention due to an increase in surface area, while the adsorbed columns are the easier to produce.

OT-CEC can successfully avoid the problems associated with packed columns, such as bubble formation and strong adsorption of basic compounds on the support material. One of the primary advantages of OT-CEC is the efficient separation of basic compounds in biological and pharmaceutical analysis. Compared with HPLC, OT-CEC can obtain good symmetrical peaks with reasonable efficiency [69, 70] for a variety of tetracycline mixtures. OT-CEC can provide a more reproducible analysis than packed column CEC because of less adsorption effect.

Particle Packed Columns

Capillary electrochromatography with particle packed columns is the mostly widely used format of CEC because it overcomes the limitations imposed by the low sample capacity and small surface area encountered in OT-CEC. The most commonly used chromatographic media are the spherical reversed-phase particles with 1.5-10 μm diameters [71]. The media should have adequate charge density to generate EOF and have the functional group to offer chromatographic selectivity. To satisfy these requirements, the majority of applications involve the use of the uncapped, silica-based materials [72]. In order to increase the EOF velocity some columns may incorporate

segments packed with bare silica particles, known as segmented capillaries [73]. Some research groups have used mixed-mode stationary phases that incorporate ionic exchange sites [74-77] to avoid EOF dependence on pH. This approach can provide strong EOF as a result of the fixed charges on the surface while still exhibiting good retention.

Usually, particle packed columns consist of two segments: a packed segment and an open segment, which are used for separation and detection, respectively. The packed segment requires retaining frits at each end to keep the stationary phase particles in place. The most common frit involves the sintering of the packing material or bare silica at elevated temperature in an attempt to fuse the particles to each other and the capillary wall. Silicate polymerization [78], photopolymerization of organic polymer solution [79] and some other approaches to eliminate the frits altogether by incorporating external or internal tapers and restrictors are also used as alternatives for frit fabrication [80]. In all cases improved frit technology is very important for increasing reproducibility, avoiding bubble formation and diminishing fragility of the column.

Pressure packing is the most common method to pack capillary columns for CEC. The columns are packed at elevated pressure (>5000 psi), by connecting the capillary to a slurry reservoir (containing the packing material suspended in an organic solvent), which is connected to a high-pressure pump. In order to get a more stable separation bed, the procedure has been modified by using supercritical CO_2 as the transporting media [81]. Maloney *et al.* used centripetal force to pack capillaries by placing the slurry reservoir in the center of a rotating packing apparatus [82]. Using this packing procedure, capillaries can be packed in 5 min at 2000 rpm. Pseudoelectropacking is also used to pack column because of the advantage of the inherent charges fixed on the packing material [83]. Stol

et al. used a high electric field in conjunction with a hydrodynamic flow to produce well-packed columns [83].

Monolithic Columns

The term "monolith" was first introduced in 1993 to describe a single piece of functionalized cellulose sponge used for the protein separation [84]. Monolithic columns are rigid macroporous polymers prepared by bulk polymerization in a closed mold. Compared with the particle packed columns, the monolithic columns do not require retaining frits to keep the bed in specific location. The monolith itself is anchored to the wall of the fused-silica capillary, which avoids bubble formation and can easily reach high permeability. Not only is the preparation of the columns simplified but also the reproducibility of the columns is improved. The monolithic columns also have other advantages over particle-packed column. The structure and pore size of the polymer can easily be controlled by adjusting the proportion of the monomers or the porogens. According to different separation requirement one can conveniently vary the nature of the attached ligands the surface of the monolithic column.

Monolithic columns can be divided into two general categories: silica-based monolithic column and organic polymer-based monolithic columns. They are prepared by so-gel technology [85, 86] and vinyl polymerization [87, 88], respectively. Since our research focused on the organic polymer-based monoliths, we will just introduce different type of polymers used in organic-polymer based monoliths.

Polyacrylamide The polyacrylamide-based monolithic columns were modified from the separation medium used for electrophoresis into a form suitable for chromatography in late 1980s by Hjerten [89]. In 1995, Hjerten and co-workers published the first report about the monolithic columns for CEC prepared by polymerization of mixtures containing acrylamide. They used the polymer mixture of the aqueous solution of acrylamide, piperazine diacrylamide (PDA) and vinylsulfonic acid and the polymerization was then initiated by *N,N,N',N'*-tetramethylethylenediamine (TEMED) to form persulfate radicals. Later the same group reported another method for the preparation of monolithic capillary column for CEC gradient separation of proteins [90]. Despite the success of the aqueous-based polymerization systems, there are some limitations for this method. The typical nonpolar monomers such as stearyl methacrylate and butyl methacrylate used for reverse-phase CEC are insoluble in aqueous solution. Palm and Novotny simplified the incorporation of highly hydrophobic monomers into acrylamide-based matrices [91]. The overall concentration of the monomers was kept constant at the level of 5% throughout the study and the composition of the mixed buffer/methyl formamide solvent varied with the type of alkyl methacrylate used. With this type of monolithic columns, Novotny and co-workers extended the range of potential analytes to sugars, oligopeptides, steroids, and bile acids [91-93]. They also constructed and optimized a sample deposition device for interfacing CEC columns to MALDITOF mass spectrometry with this type of column [94].

Polystyrene Polystyrene-based polymer was first introduced by Svec and Frechet in 1990s for the separation of proteins by reversed-phase HPLC [95]. The monomers

used were styrene and divinylbenzene with dodecyl alcohol as the porogen. The reaction was initiated by AIBN thermally at 70 ˚C for 24 hrs. Later in 1994 toluene was used in the porogen for the separation of the small molecules as alkylbenzenes. In 1999, Gusev *et al.* first reported the preparation of polystyrene-based porous rigid monolithic capillary columns for CEC [96]. Later they also reported the preparation of a porous polymer monolith for the separation of proteins and peptides by CEC [97]. Recently Jin et al. demonstrated the excellent separation of a diverse series of neutral and ionic samples [98]. One of the advantages of the polystyrene-based polymers is the wide pH range, which is a great improvement over the conventional LC packing materials. The polymer itself can be used as reversed-phase directly and the surface also can be modified for the separation of smaller molecules.

Polymethacrylates The polymers formed from the glycidyl esters of methacrylic acid were first investigated by Svec in 1978, but the earliest research of methacrylates based monolithic columns in HPLC appeared in early 1990s by Svec and Frechet [99]. Due to the simplicity of column preparation and variety of functional monomers available, a number of modification reactions have been developed for methacrylate polymers [100]. Li *et al.* developed a new separation media for ionizable samples to overcome the problem of irreversible adsorption and electrostatic interaction of biopolymers with the stationary phase, and also achieved high EOF for high speed separations [101]. This column showed fast separation and remarkable stability [101]. The Svec group also investigated the UV initiated polymerization monolithic column for CEC with polymers shells to shield functionalities in the lower layer from unwanted interaction with the

analytes [102]. Bedair and El Rassi studied the neutral poly(glycidyl methacrylate-co-ethylene dimethacrylate) and the cationic poly(glycidyl methacrylate-co-ethylene dimethacrylate-co-[2-(methacryloyloxy)ethyl]trimethyl ammonium chloride) monoliths for affinity chromatography [103]. Preinerstorfer optimized the poly(glycidyl methacrylate-co-ethylene dimethacrylate) monoliths with epoxide groups into thiol groups for the successful separation of 3,5-dinitrobenzoylleucine enantiomers [104].

Polar Monoliths Used in CEC

Currently, monolithic stationary phases for CEC is a growing research field, but compared with reversed-phase monolithic CEC, the development of polar monolithic stationary phases is still limited despite the increasing demands for separation media for biomolecules, such as carbohydrates, amino acids, peptides, and proteins.

There are two types of polar monolithic stationary phases in CEC: silica-based and polymer-based monoliths. The sol-gel technology was first discovered in the late 1800s, but it was not until 1996 that Tanaka *et al.* used the first uniform porous silica rods for reversed-phase liquid chromatography [105]. Currently, the silica-based monoliths have a wider use in normal-phase CEC than polymer-based monoliths. For example, in our research group Allen and El Rassi have reported the synthetic routes for the preparation of hydrophilic silica-based monoliths possessing surface-bound cyano functions. They developed routes to yield the phase as CN-monolith or CN-OH-monolith. The CN-OH-monolith column was demonstrated as a normal-phase CEC for the separation of various polar compounds [106]. Based on the same method Zhong also

developed the silica-based 2CN-OH-monolith and the column showed excellent results in the separation of polar compounds such as basic drugs and peptides [107]. More information on applications and fabrications of polar silica-based monolithic columns used as polar stationary phases in CEC can be found in recent reviews [85, 100, 108].

In the following section we will give a review of the recent developments in the polar polymeric monoliths used in CEC. Que and Novotny [92] introduced aminoalkyl and cyano groups to the polyacrylamide based monolithic stationary phase to increase the polarity of the columns. The amino stationary phase was prepared from 3-amino-1-propanol vinyl ether and (2-(acryloyloxy)ethyl)-trimethylammonium methyl sulfate to get the normal phase monolithic column. This column was coupled with negative-ion ESI-MS for the detection of bile acids. More recently, the same group developed another type of hydrophilic monolithic columns based on a mixture of the acrylamide, 2-cyanoethyl acrylate, *N,N*-methylenebisacrylamide and vinylsulfonic acid monomers to separate neutral saccharide mixtures. The hydrogen-bonding and dipole-dipole interactions between the hydroxyl groups of sugars and the stationary phase showed a strong hydrophilic partition. This column was also coupled with tandem mass spectrometry for further evaluation [42]. Hoegger and Freitag [109] developed rigid, porous polymers with hydrophilic monomers in aqueous phase and tested the influence of polymer morphology from three main synthesis conditions, such as initial monomer concentration, cross-linker concentration, and the addition of a lyotropic salt. The elution sequence of polar aromatic compounds with hydroxyl groups under pure organic mobile phase proved the hydrophilic domination in this separation. Recently, Freitag demonstrated the porous monoliths based on N,N-dimethylacrylamide (DMAA) or

methacrylamide (MAA) and showed the hydrophilic interactions was the basis for the retention by comparing the elution sequence of neutral samples between nano-HPLC and CEC mode. For the charged samples such as amino acids, electrostatic interaction also contributed to the retention [110]. Lammerhofer *et al.* [111] reported hydrophilic macroporous weak and strong anion-exchange monoliths formed from the copolymerization of 2-(N,N-dimethylamino)ethyl methacrylate, 2-hydroxyethyl methacrylate, and ethylene dimethacrylate. This type of monolith was used to separate neutral and basic compounds in normal phase mode. Besides the good separation it also showed very high column efficiency with 231,000 plates/meter. Pyell's [112] group reported a novel synthetic route to amphiphilic acrylamide-based monolithic stationary phases for CEC employing water-soluble cyclodextrins as solubilizing agents. The amphiphilic stationary phases were synthesized by free radical copolymerization of the bisacrylamide-cyclodextrin host-guest complexes with hydrophilic monomers and an additional hydrophilic cross-linker in aqueous solution. The elution sequence of the polar neutral solutes tested on this column proved the normal-phase mode.

Rationale of the Study

Compared with the numerous publications on the various types of monolithic columns including reversed-phase, chiral, size-exclusion, ion exchange and affinity monoliths, the reports on polar polymeric monoliths in CEC are scarce. This fact provided the rationale to this dissertation for pursuing the development of novel polar

monoliths to fill in this gap. In this regard, we have investigated a few polar monomers and ligands in the formation of polar monolithic columns for normal phase CEC. The resulting polar monoliths are expected to find general use and enlarge the scope of applications of CEC.

Conclusions

In this chapter, the basic concepts and principles of CEC have been reviewed and the historical and recent developments in CEC have been summarized. In addition, the various parameters and essential equations used in the evaluation of the electrochromatographic systems were provided. Other background information including the instrument and column technologies in CEC were discussed in order to provide the reader of this dissertation with the necessary elements to understand the rationale of our investigations.

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CHAPTER II

INVESTIGATION OF NOVEL POLAR POLYMERIC MONOLITHS IN NORMAL PHASE CAPILLARY ELECTROCHROMATOGRAPHY

Introduction

Among the various column technologies currently used in CEC, the monolithic columns constitute one of the most rapidly growing area [1]. This is due primarily to the simplicity of the *in situ* preparation of monolithic capillary columns [2], which avoid the problems associated with fabrication and column packing. Furthermore, when compared with the particle-packed columns, the monolithic columns have higher reproducibility. Although the open tubular column (OTC) format is a straight forward solution to eliminate the problems associated with frits, e.g., bubble formation, OTC lacks enough surface area for providing the sample capacity and retention necessary for the isolation and separation of sample components.

Currently, most research on organic-based monolithic stationary phases for CEC focused on the use of the variety of organic materials to form the stationary phases with the desired ligand to solve the given separation problems. Polar monolithic columns have been reported for the separation of enantiomeric compounds [3-7], biochemical analysis [8-13], pharmaceutical applications [14-19], and analysis of industrial and environmental

samples [20-23]. In this chapter we investigated a few polar monomers in the formation of polar monolithic column for the CEC of polar neutral and charged species.

Experimental

Instrumentation

The instrument used for CEC experiments was a P/ACE 2200 capillary electrophoresis system from Beckman Instruments (Fullerton, CA, USA) equipped with a UV detector. Electrochromatograms were recorded with a personal computer running the P/ACE Station software. All samples were injected electrokinetically under various time and voltages. An Isotemp refrigerated circulator (Model 910) for the *in situ* polymerization and an Isotemp oven (Model 615G) for the column incubations were from Fisher Scientific (Fair Lawn, NJ, USA).

Reagents and Materials

1-Vinyl-2-pyrrolidinone (VP), glycidyl methacrylate (GMA), ethylene glycol dimethacrylate (EDMA), 2-acrylamido-2-methyl-1-propanesulfonic acid (AMPS), 2,2'-azobisisobutyronitrile (AIBN), 2-aminobenzamide (2-AB), 1-dodecanol, glycidol, diethylenetriamine, 3-(trimethoxysilyl)propyl methacrylate, glucose, maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose, maltoheptaose and analytical grade acetone were purchased from Aldrich Co. (Milwaukee, WI, U.S.A). Cyclohexanol was

purchased from J.T. Baker (Phillipsburg, NJ, U.S.A). Ethylene glycol and HPLC-grade methanol and acetonitrile were obtained from Fisher Scientific (Fair Lawn, NJ, U.S.A). 2-Cyanoethyl acrylate (CEA) was from Polysciences, Inc (Warrington, PA, U.S.A). 1H-Imidazole-4,5-dicarbonitrile (IDCN) was from Nippon Soda Co. Ltd (Chiyoda-ku, Tokyo, Japan). Fused-silica capillaries with an internal diameter of 100 µm and outer diameter of 360 µm were from Polymicro Technology (Phoenix, AZ, U.S.A).

Column Pretreatment

The columns were pretreated by the method reported by Bedair and El Rassi [24]. A 37 cm fused-silica capillary with 100 µm I.D. was treated with 1.0 M sodium hydroxide for 30 min, rinsed with 0.10 M hydrochloric acid for 30 min, and then flushed with water for another 30 min. The capillary then was ready to react with a solution of 50% (v/v) of 3-(trimethoxysilyl)propyl methacrylate in acetone for 12 hrs to vinylize the inner wall of the capillary [25-27]. Finally, the capillary was rinsed with acetone and water and dried with a steam of nitrogen.

In Situ Polymerization

During this research we investigated a few different monomers and ligands to develop a useful stationary phase for normal-phase CEC (NP-CEC). The monomers and the ligands used are listed in Figure 1. The compositions of porogens are listed in Table 1.

Polar monoliths Since the VP and CEA monomers possess polar functional groups, the poly (CEA-co-EDMA) and poly (VP-co-EDMA) monoliths were prepared *in situ* in a single step polymerization and did not require any subsequent functionalization to transform them to polar monoliths. The two monoliths were formed *in situ* from polymerization mixture solutions according to ratios listed in Table 1, which were first sonicated to get rid of bubbles. Thereafter, the pretreated capillary columns were filled with the polymerization solutions up to 30 cm by immersing the inlet of the capillary in the solution vial and applying vacuum to the outlet. The capillary ends were plugged with GC septa and the capillary was submerged in a 50 °C water bath for 17 hrs for the CEA columns and in a 60 °C water bath for 17 hrs for the VP columns.

Thereafter, the monolithic capillary columns were washed with 80:20 (v/v) acetonitrile: water mixture using an HPLC pump. A detection window was made at the end of the polymer bed by using a thermal wire stripper. The final column was cut to a total length of 37 cm with the effective length of 30 cm.

Neutral monoliths In this section, a neutral monolith bearing epoxy functions on its surface was prepared for subsequent functionalization with polar ligands to yield polar monoliths. The neutral monolith consisted of the poly (glycidyl methacrylate-co-ethylene dimethacrylate) [poly (GMA-co-EDMA)]. The poly (GMA-co-EDMA) monolith was further modified by an in-column surface functionalization process. This method can avoid the effect on pore structure from the changes in the composition of the polymerization mixture and the difficulty for incorporating different chromatographic moieties by using the same polymers. Once the porous structure of the monolith has been

optimized it can be used as substrate for various dedicated chromatographic ligands under the same porous structure and morphology. This technique was in particular the method for the silica-monoliths [28-31], but now has also been introduced in organic polymer monoliths with the epoxide [32-37], azlacton [38], or chloromethyl [39] groups.

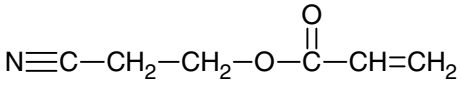
TABLE 1. THE COMPOSITION OF DIFFERENT MONOLITHIC COLUMNS

Column	Monomers	Porogens	AIBN
CEA	CEA 49.5%, EDMA 49.5%, AMPS 1%	Cyclohexanol 30%, dodecanol 70%	1% of monomer
VP	VP 49.5%, EDMA 47.5%, AMPS 3%	Ethylene glycol 13.2%, cyclohexanol 83.2%, water 3.6%	
GMA (neutral monolith)	GMA 60%, EDMA 40%	Cyclohexanol 50%, dodecanol 50%	

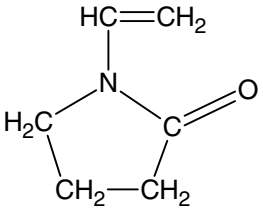
All the percentages in this table are wt%. The monomers and porogens ratios are 30: 70 for CEA and VP monolith, 40: 60 for GMA monolith.

The GMA contains 2,3-epoxypropyl groups, which can be used as the substrate for the immobilization of any suitable anchor. Currently, the *in situ* prepared monolithic

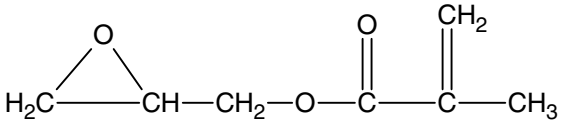
poly (glycidyl methacrylate-co-ethylene dimethacrylate) (poly (GMA-co-EDMA)) capillary columns are actively used in research involving monolithic columns [34, 35, 40]. Researchers modified the epoxy group on the surface with affinity ligands [41, 42], enantioselectors [43, 44], or metal chelates [45]. In this research, the surface of GMA-co-EDMA monolith was functionalized with IDCN ligands bearing 2 CN groups and diethylenetriamine ligands possessing three amine groups.



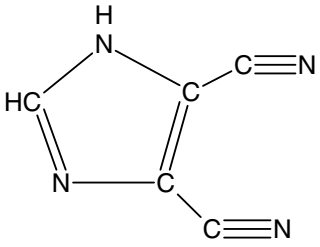
2-Cyanoethyl acrylate (CEA)



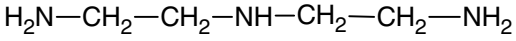
1-Vinyl-2-pyrrolidinone (VP)



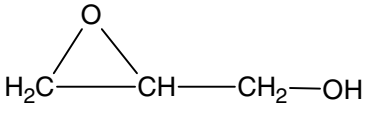
Glycidyl methacrylate (GMA)



1H-Imidazole-4,5-dicarbonitrile (IDCN)



Diethylenetriamine



Glycidol

Figure 1. *Structures of the monomers and ligands used in the preparation of the various monolithic capillary column*s.

The procedure and conditions for preparing the basic neutral GMA-co-EDMA monolith are the same as the polar VP monolith. The composition of the monomer and porogen is listed in Table 1.

Bonding of the Polar Ligands to the Surface of the Neutral Monolith

IDCN Column: The GMA-co-EDMA monolithic column was rinsed with a 0.2 M IDCN solution (adjusted to pH 9 by NaOH solution) for 30 min and then incubated in an oven at a temperature of 70 °C for 3 hrs. This step was repeated twice and the column was incubated for an additional 12 hrs. After that the IDCN column is ready to be used.

Amine Column: The GMA-co-EDMA monolithic column was rinsed with a 0.2 M diethylenetriamine solution for 30 min and thereafter incubated in an oven at 70 °C for a total 15 hrs. For the first 3 hrs, the column was rinsed with a 0.2 M diethylenetriamine solution after every hour. Then the column was treated with a solution of 10% (v/v) glycidol dissolved in 0.1 M Na_2CO_3 solution and incubated in an oven at 70 °C for a total of 15 hrs. The column was flushed with the 10% (v/v) glycidol solution after every hour for the first 3 hrs. After that the column is ready to be used.

Sugar Derivatization

Since carbohydrates lack chromophores in their structures, we used the UV-absorbing (also fluorescing) derivatizing agent 2-amino benzamide (2-AB) to label the

sugar samples used in this study. This precolumn derivatization [46-49] can greatly increase the detection sensitivity. The derivatization reaction consisted of a solution of 0.25 M 2-AB dissolved in 50% (v/v) methanol and water solution titrated to pH 5 with acetic acid. Thereafter, sodium cyanoborohydride was added to the 2-AB/sugar-solution at the concentration of 0.375 M just prior to the addition of the reducing carbohydrate. The reaction mixtures were incubated at 70 °C for 24 hrs. Figure 2 illustrates the reaction scheme.

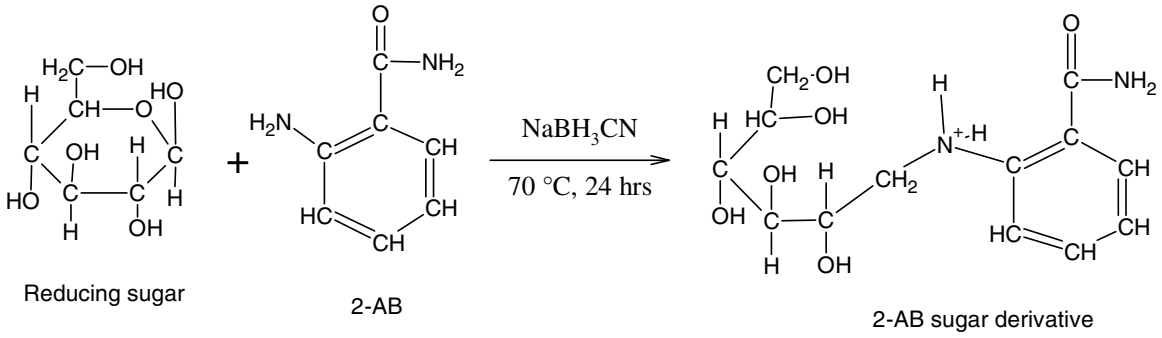


Figure 2. *Reaction scheme for the sugar derivatization*

Results and Discussion

Evaluation of the Different Monolithic Columns

In search for the most polar monolithic capillary column with the optimal electroosmotic flow (EOF) velocity that would yield the maximum selectivity and resolution, the performance of the different monolithic columns was evaluated under the same elution conditions. The test mixture was composed of phenol, catechol, and

pyrogallol, and the results are shown in Table 2 in terms of k' values for the three different phenolic solutes. Under the pH condition of the mobile phase (pH 6.5), the phenols are neutral analytes and their retentions are evaluated by the retention factor k' as in chromatography.

TABLE 2. VALUES OF RETENTION FACTORS OF PHENOLS OBTAINED ON DIFFERENT COLUMNS UNDER THE SAME ELUTION CONDITIONS

	k'			
	CEA	VP	IDCN	Amine
Phenol	0.032	0.068	0.066	0.397
Catechol	0.042	0.110	0.079	0.330
Pyrogallol	0.067	0.205	0.200	0.370

Conditions: monolithic capillary column, 30/37 cm x 100 μm ID; mobile phase, hydro-organic solution made up of 10% v/v of 2 mM TEA (pH 6.5) and 90% v/v acetonitrile; wavelength, 214 nm; column temperature, 25 °C.

CEA Column As can be seen in Table 2, the CEA column provided a relatively good selectivity factor ($\alpha = k'_2/k'_1 = 1.31$ for the solute pair catechol/phenol and 1.60 for the solute pair pyrogallol/catechol). However, the crucial problem with the CEA column was the proper choice of the porogenic solvent for the *in situ* polymerization, which can afford a monolith with good permeability. Various porogenic solvents were investigated

but without significant enhancement in the permeability of the CEA column. This may indicate that the presence of the CEA did not produce a favorable phase separation of the solid polymer from the liquid porogen regardless of the nature of the porogen. In other words, the presence of CEA monomer has a leveling effect that rendered the various porogens to behave as microporogens which have good solvency for the resulting polymer aggregates, a fact that led to the formation of monoliths with very small pores which prohibited pressure driven flow through the CEA monolithic columns even by using an HPLC pump.

TABLE 3. VALUES OF RETENTION TIMES AND RETENTION FACTORS OF PHENOLS OBTAINED ON THE CEA COLUMN UNDER DIFFERENT ACETONITRILE CONCENTRATIONS IN THE MOBILE PHASE

ACN %	90%		95%		97.5%	
solute	t_R	k'	t_R	k'	t_R	k'
toluene	2.977		2.797		2.723	
hydroquinone	3.037	0.020	2.960	0.058	2.890	0.061
resorcinol	3.057	0.027	3.013	0.077	3.067	0.126
phenol	3.073	0.032	2.970	0.062	2.987	0.097
catechol	3.103	0.042	3.057	0.093	3.053	0.121
pyrogallol	3.177	0.067	3.210	0.148	3.483	0.279

Conditions: as in Table 2.

The EOF velocity across the CEA column using a hydro-organic mobile phase (10% v/v of 2 mM TEA, pH 6.5, and 90% v/v acetonitrile) at 30 kV is 1.68 mm/s, which is relatively low for an applied field strength of 1 kV/cm. This is another indication of the presence of micropores in the CEA monolith where extensive double layer overlap would exist thus reducing the magnitude of the overall EOF velocity. The EOF velocity is calculated from the length of the column and retention time of the unretained solute, which is in this case toluene see Table 3 which lists the retention of toluene and other phenols at various acetonitrile concentrations in the mobile phase.

As shown in Table 3, the k' values of the various phenols are increased with increasing acetonitrile concentration in the mobile phase. Despite the fact that this behavior is typical of a polar stationary phase, the CEA column is of limited practical utility due to the low permeability and EOF velocity.

On the basis of the above results, the CEA monolithic column was not investigated further. This prompted us to consider the design of another more useful polar monolith, which is the subject of the next section.

VP monolith: In order to provide a more useful polar monolith with improved flow characteristics, the VP monomer was evaluated in the formation of VP-co-EDMA monolith. From the results shown in Table 2, the values of retention factor obtained with the VP monolith are almost double those obtained on the CEA monolith under the same elution conditions. In addition, the selectivity factors α for the solute pairs catechol/phenol and pyrogallol/catechol are 1.62 and 1.86, respectively, as compared to 1.31 and 1.60 with the CEA monolith.

Although the permeability of the VP monolith was slightly higher than that observed with the CEA column, the EOF velocity as measured from the retention of toluene is 1.70 mm/s, which is about the same as that obtained on the CEA column. This is an indication that while the VP monolith possesses some domains of large flow through pores, which facilitate pressure driven flow, it still has much more domains of micropores.

TABLE 4. VALUES OF RETENTION TIMES AND RETENTION FACTORS OF PHENOLS OBTAINED ON THE VP COLUMN UNDER DIFFERENT ACETONITRILE CONCENTRATIONS IN THE MOBILE PHASE

ACN %	90%		95%		97.5%	
solute	t_R	k'	t_R	k'	t_R	k'
toluene	2.944		3.003		3.510	
phenol	3.143	0.068	3.217	0.071	3.883	0.106
hydroquinone	3.213	0.091	3.490	0.162	4.306	0.227
catechol	3.267	0.110	3.537	0.178	4.328	0.233
resorcinol	3.293	0.119	3.640	0.212	4.937	0.407
pyrogallol	3.547	0.205	3.953	0.316	5.657	0.612

Conditions as in Table 2.

To further improve the permeability and EOF velocity of the VP capillary column, the effect of % AMPS in the polymerization solution was investigated. Increasing the %

AMPS from 1% to 2%, increased the permeability of the column for pressure driven flow and the EOF velocity from 1.7 mm/s to 2.0 mm/s (i.e., an 18% increase).

Increasing the %AMPS monomer in the polymerization solution resulted not only in improving the flow characteristics of the VP monolith, but also increased its polar character. The AMPS monomer is a negatively charged monomer carrying a sulfonic acid group. It is usually added in small amount to provide the EOF and move the mobile phase across the column [24]. As shown in Figure 3, increasing the %AMPS increased the polarity of the monolith as manifested by the increase of the k' values of three 2-AB derivatized saccharides, namely glucose, maltose and maltotriose. In summary, the VP monolith can be conveniently tailored to yield the desired retentivity and flow characteristics by adjusting the % AMPS in the polymerization solution which would result in adjusting the polar surface of the monolith.

As shown in Figure 4, the VP monolith formed a polymerization solution with 1% AMPS required a mobile phase having a relatively high acetonitrile content to bring about higher retention and selectivity and achieve baseline separation among the component of the injected mixture. On the other hand, the VP monolith formed from a polymerization solution with 2% AMPS yielded not only a faster separation (due to the higher EOF velocity) but an improved selectivity and a baseline resolution (see Figure 5) under otherwise the same elution conditions.

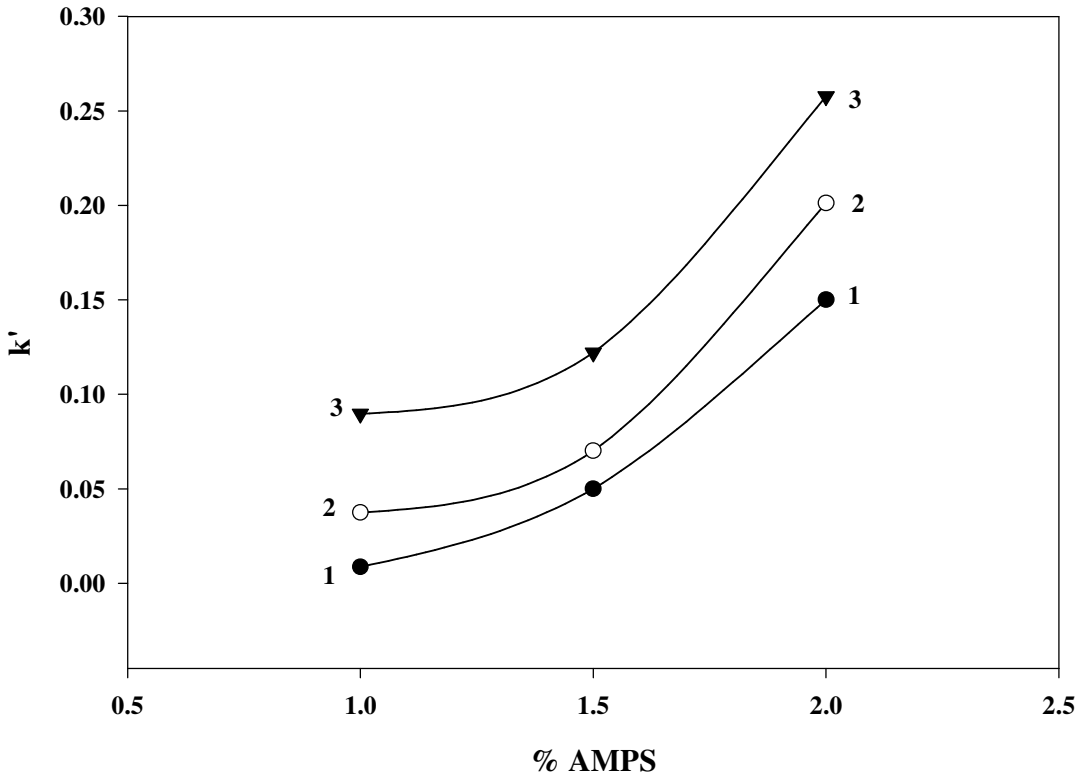


Figure 3. *Plots of retention factor of 2-AB derivatives of glucose (1), maltose (2) and maltotriose (3) versus wt% of AMPS in the polymerization solution used in the preparation of the various VP monoliths. Mobile phase, 2 mM triethylamine phosphate, pH 6.5 at 85% (v/v) acetonitrile, running voltage, 30 kV. Capillary column, 30 cm effective length, 37 cm total length with 100 μm I.D.*

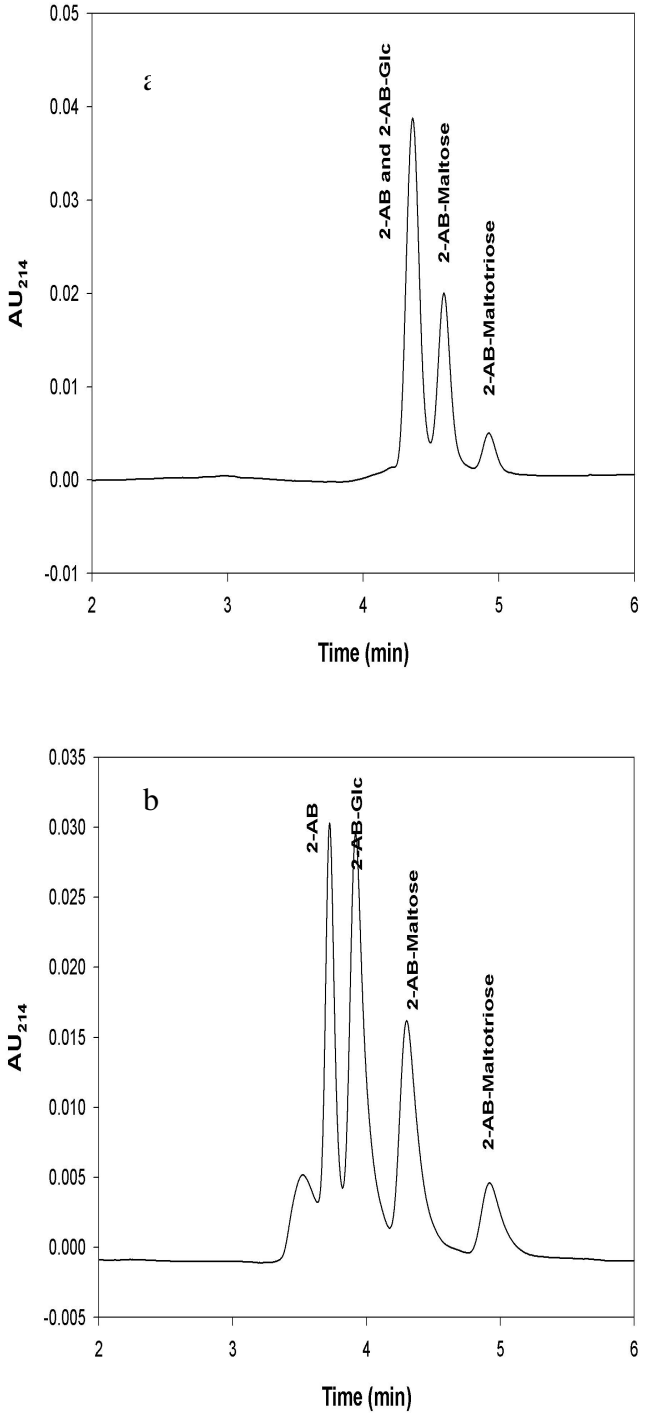


Figure 4. *Electrochromatograms of 2-AB derivatives of glucose, maltose and maltotriose obtained on VP column (1% AMPS) under different mobile phase composition.*

Conditions are the same as in Fig 2 except the mobile phase has 85% ACN (v/v) in (a) and 90% ACN (v/v) in (b).

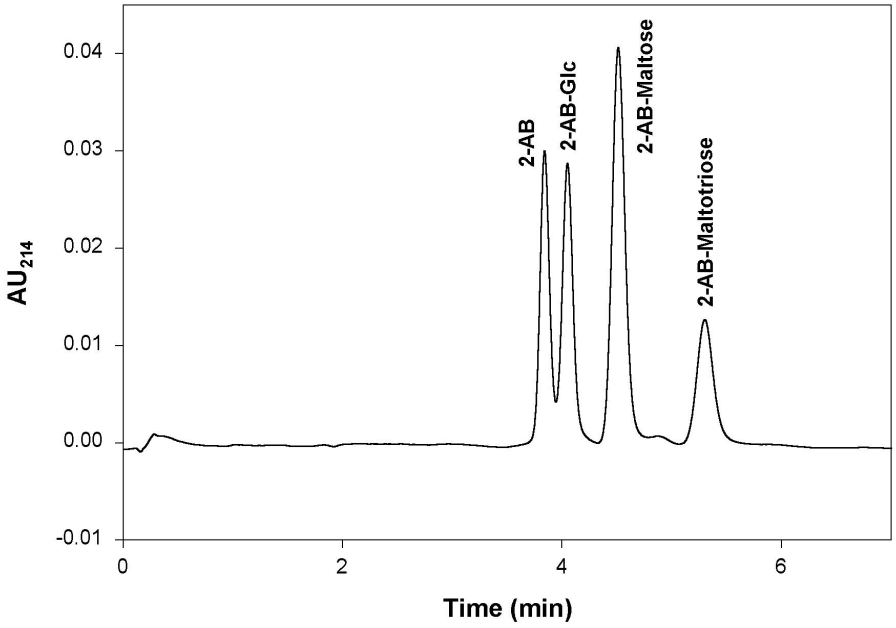


Figure 5. *Electrochromatogram of 2-AB derivatives of glucose, maltose and maltotriose obtained on VP column (2% AMPS). Conditions: monomers, VP 49.5%, EDMA 48.5% and AMPS 2%; porogen, ethylene glycol 13.2%, cyclohexanol 83.2%, water 3.6%; AIBN 1%; 30/37 cm x 100 µm ID; voltage, 30 kV; wavelength, 214 nm; column temperature, 25 ˚C; mobile phase, hydro-organic solution made up with 10% v/v 2mM TEA (pH=6.5) and 90% v/v acetonitrile.*

In summary, despite the fact that the VP monolith is a true polar stationary phase as manifested form the increase in retention factor with increasing the percent acetonitrile in the mobile phase (see Table 4) and yielded better results than the CEA monolith, the search for other polar monoliths with more polar character and improved selectivity was pursued in our studies. This constitutes the subject the next section.

Other Polar Monoliths: Very recently, IDCN ligands were immobilized on a monolithic silica and yielded a polar stationary phase that proved superior to a monolithic silica stationary phase having immobilized ligands with a single cyano function [50]. This finding prompted us to explore the utility of IDCN ligands in polymeric monoliths, e.g., GMA-co-EDMA. In general, the basic GMA-co-EDMA monoliths exhibited a relatively high permeability [32, 42, 43, 51]. On this basis, the GMA-co-EDMA monolith was further modified with IDCN ligands, which possess 2-CN groups per ligand, a fact that should in principle yield a more polar surface. Returning to Table 1, one can see that IDCN offers more or less similar retention and selectivity than the VP-monolith under otherwise the same elution conditions. The EOF velocity on the IDCN column is 2.47 mm/s, which about 1.5 times higher than that obtained on the VP and CEA monolith. This in major part is attributed to the macroporous structure of the basic GMA-co-EDMA monolith. However, the initial results on the IDCN monolith show that the retention mechanism is rather complicated and does not follow a normal-phase mechanism in the sense that the k' of polar solutes such as phenol, catechol and pyrogallol did not increase with increasing the acetonitrile concentration in the mobile phase as shown in Figure 6. In fact the column loses selectivity at 97.5 % acetonitrile

and the three solutes co-elute. The IDCN column will need further investigation before any definite conclusion can be made on its utility and the underlying retention mechanism. The IDCN monolith was not studied further in the rest of this chapter.

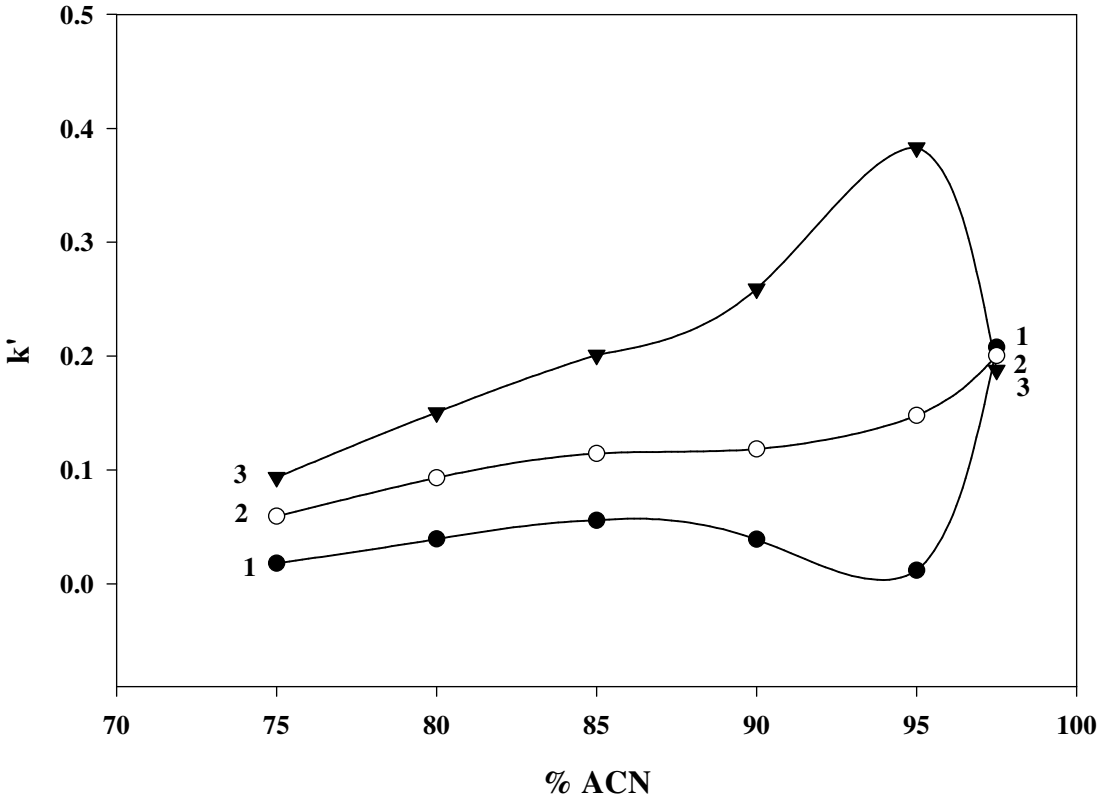


Figure 6. *Plots of retention factors of phenolic compounds, e.g., (1) phenol, (2) catechol, and (3) pyrogallol, versus percent different acetonitrile concentrations. Mobile phase, 2mM triethylamine phosphate, pH 6.5 at various percentages (v/v) of acetonitrile, running voltage, 30 kV. Capillary column, 30 cm effective length, 37 cm total length with 100 µm I.D.*

Another ligand that was worth investigating is the diethylenetriamine. This ligand has two primary amine functions and one secondary amine function, which upon

immobilization will have two secondary amine functions and one primary amine function. Due to the reactivity of primary amine, the surface was further reacted with glycidol to accomplish two goals: (i) convert the remaining primary amine to a secondary amine and (ii) give the surface more polar character *via* the glycidol moiety which would yield diol functionalities upon immobilization to the amine surface.

Returning to Table 1, one can see that the amine monolith yielded higher k' values for the test solutes but did not exhibit enough selectivity. The EOF velocity is 1.40 mm/s, which is the lowest among all the other monoliths investigated in this study. However, the amine monolith exhibited high permeability as its counterpart the IDCN monolith due to the fact that both are made from the same basic monolith the GMA-co-EDMA monolith, which is known for its good permeability for pressure driven flow. The weak EOF can be explained by the extensive binding of mobile phase counter-ions, which were in this case phosphate ions.

Despite the relatively weak EOF of the amine monolith, the column proved useful for the separation of peptides as will be shown below. Overall, the VP monolith and to a lesser extent the amine monolith were further investigated and characterized as shown in the next section.

Characterization of VP Monolith over a Wide Acetonitrile Concentration

To further characterize the VP monolith and better understand the underlying retention, the performance of the VP column was evaluated over a wide range of acetonitrile concentration in the mobile phase. The results are shown in Figure 7 in terms

of k' values versus the percent acetonitrile (v/v) in the mobile phase. While the k' values of 2-AB, which is a relatively less polar than the 2-AB derivatives of saccharides and uracil, decreased rapidly between 10 and 40% acetonitrile, the polar solutes showed virtually no retention in this percent range. This behavior shows that the VP monolith has also a non polar character and behaves as a reversed phase packing toward less polar solutes at low acetonitrile concentration. Conversely, at high acetonitrile concentration (> 70%), the column behaves as a normal phase packing toward polar solutes such as 2-AB glucose, maltose, maltotriose and to a lesser extent uracil. In fact, the retention of these polar solutes increases with increasing acetonitrile concentration at > 70% acetonitrile and very rapidly in the percent range 90 – 97.5%. The dual retention mechanism (i.e., reversed-phase and normal phase at low and high acetonitrile concentration, respectively) is attributed to the composite nature of the monolith, whose acrylic backbone is relatively less polar than the pyrrolidinone ligand. In addition, the charged AMPS monomer introduces polar and negatively charged sulfonic groups.

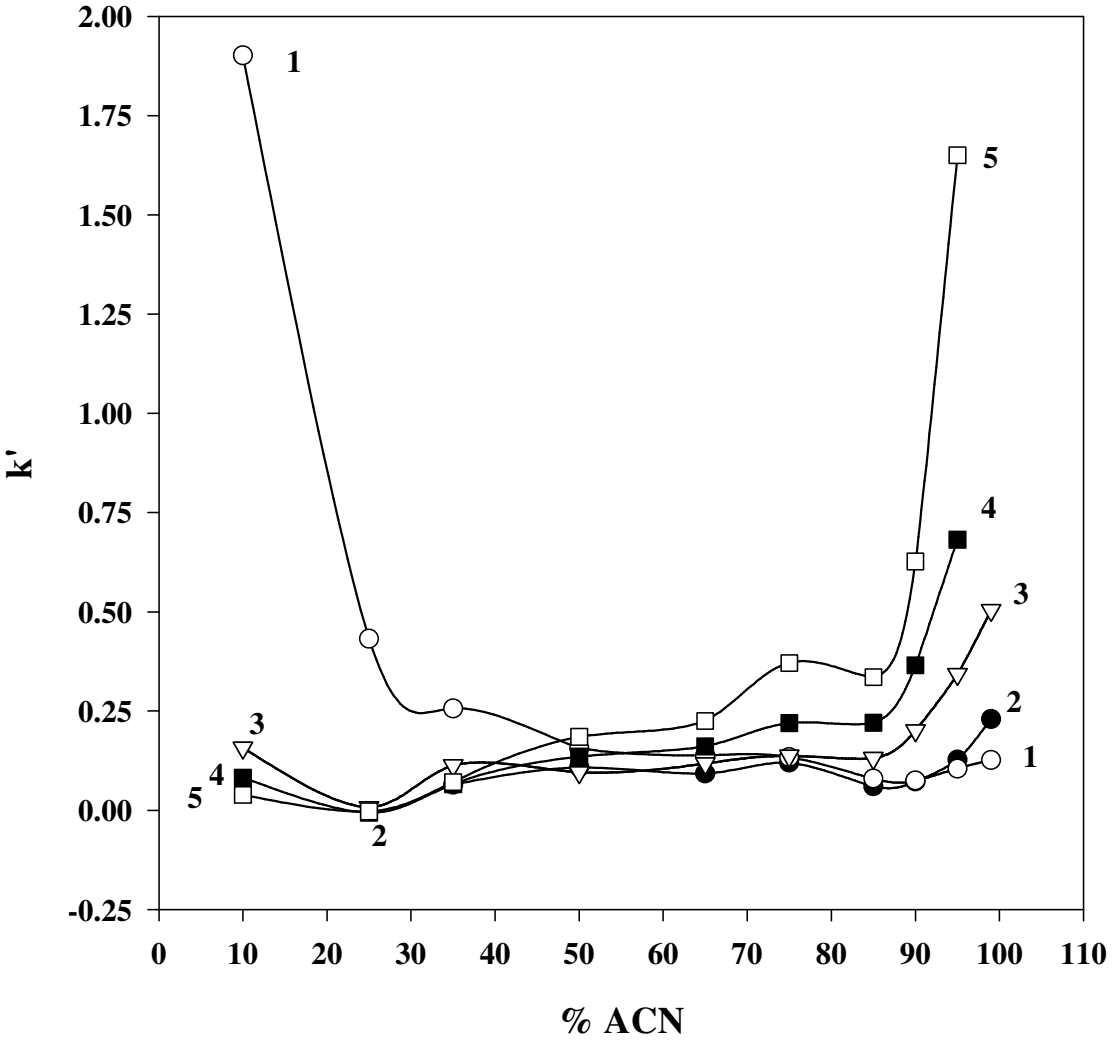


Figure 7. *Effect of acetonitrile concentrations on the retention factor k'. Conditions: monomers, VP 49.5%, EDMA 49.5%, AMPS 1%; porogen, ethylene glycol 13.2%, cyclohexanol 83.2%, water 3.6%; AIBN 1%; column, 30/37 cm x 100 µm ID; voltage, 30 kV; wavelength, 214 nm; column temperature, 25 ˚C; mobile phase, hydro-organic solution made up with 2mM TEA (pH 6.5) at various percent acetonitrile. Samples: 1, 2-AB; 2, uracil; 3, glucose; 4, maltose; 5, maltotriose.*

Reproducibility of Monolithic Columns

Since VP monoliths and to a lesser extent amine monoliths were the most useful, some reproducibility data for the two monoliths are reported in this section. The reproducibility of the VP column was estimated in terms of the relative standard deviation (%RSD) for the solute retention and separation efficiency using the 2-AB derivatives of saccharides including glucose, maltose and maltotriose as model solutes. The observed %RSD from column-to-column (n = 3) was 2.56 and 11.7 for retention and separation efficiency, respectively, while the % RSD from run-to-run (n = 3) for retention and separation efficiency was 0.83 and 26.58, respectively. On the other hand, the %RSD for solute retention on the amine column form run-to-run was 3.59% (n = 4).

Typical Separations on VP and Amine Monoliths

Separation of 2-AB Derivatized Saccharides A series of 2AB-derivatized maltooligosaccharides were electrochromatographed on the VP monolithic column to assess the utility of this column in NP-CEC. Carbohydrates are typical polar compounds and their polarity increase with increasing their degree of polymerization. Due to the strong retention of the homologous, the best separation was achieved by step gradient elution with mobile phases containing 2 mM TEA (pH 6.5) at different percentage of acetonitrile. The step gradient elution resulted in shortening the analysis time and sharpening the peaks of retarded saccharides as shown in Figure 8.

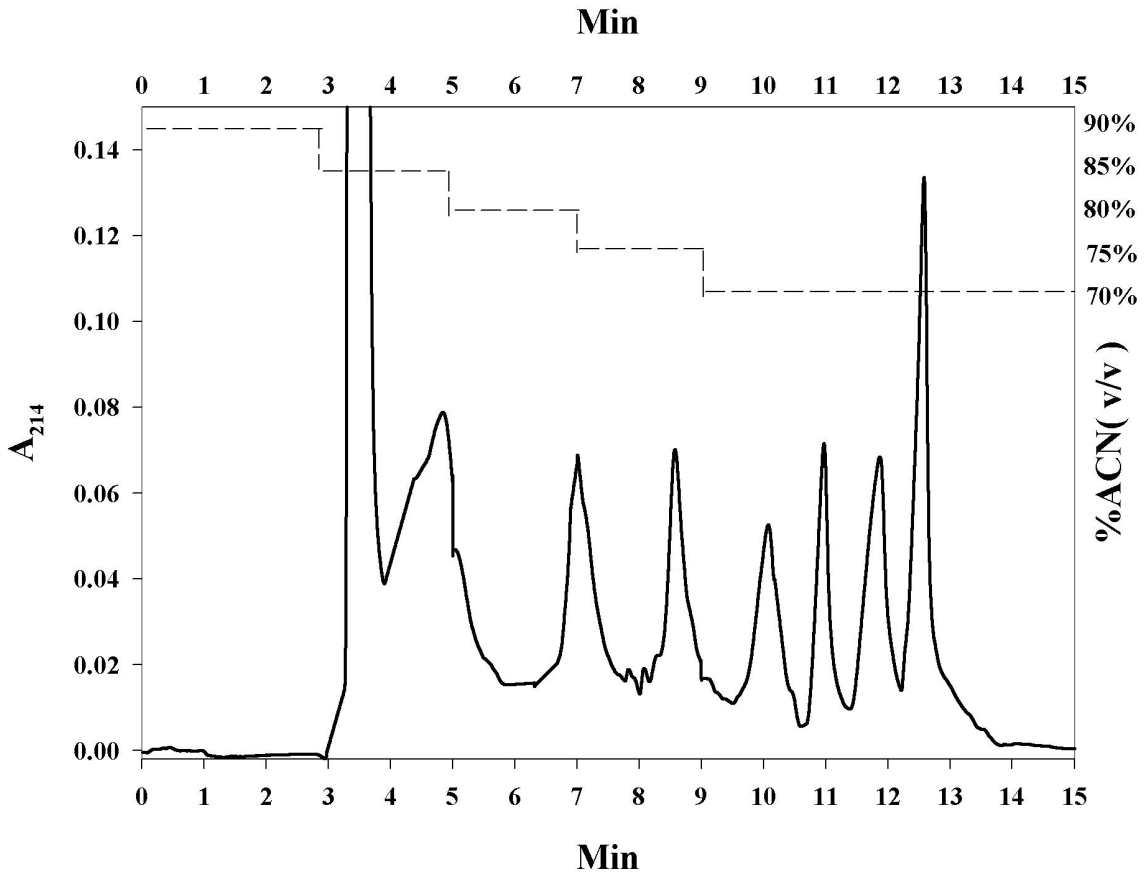


Figure 8. *Electrochromatogram of the 2-AB derivatized maltooligosaccharides on VP monolithic column. Conditions: monomers, VP 49.5%, EDMA 47.5%, AMPS 3%; porogen, ethylene glycol 13.2%, cyclohexanol 83.2%, water 3.6%; AIBN 1%; columns, 30/37 cm x 100 µm ID; voltage, 30 kV; wavelength, 214 nm; column temperature, 25 ˚C; mobile phase, hydro-organic solution made up of 2 mM TEA (pH 6.5) and various percent of acetonitrile, which involved 90% ACN for 3 min, followed by 85% ACN, 80% ACN, and 75% ACN each for 2 min, and ended with 70% ACN for another 6 min. The*

samples separated from left to the right are: 2-AB, glucose, maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose and maltoheptaose.

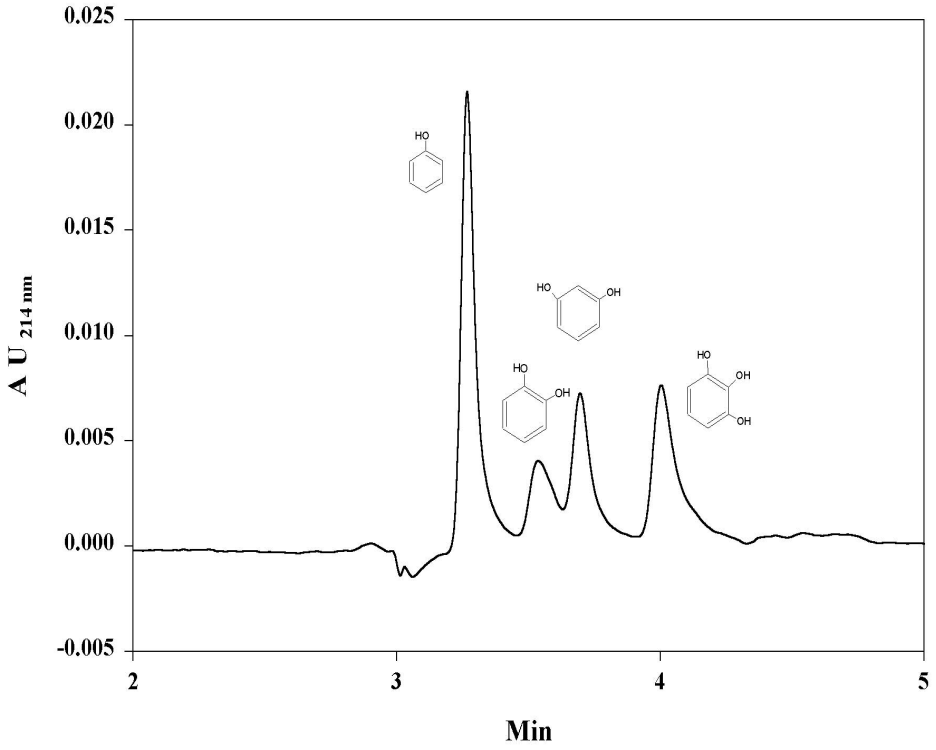


Figure 9. *Electrochromatogram of the phenols on VP monolithic column. Condition: as in Fig 8. Mobile phase, hydro-organic solution made up of 2.5% v/v 2 mM TEA (pH 6.5) and 97.5% v/v of acetonitrile.*

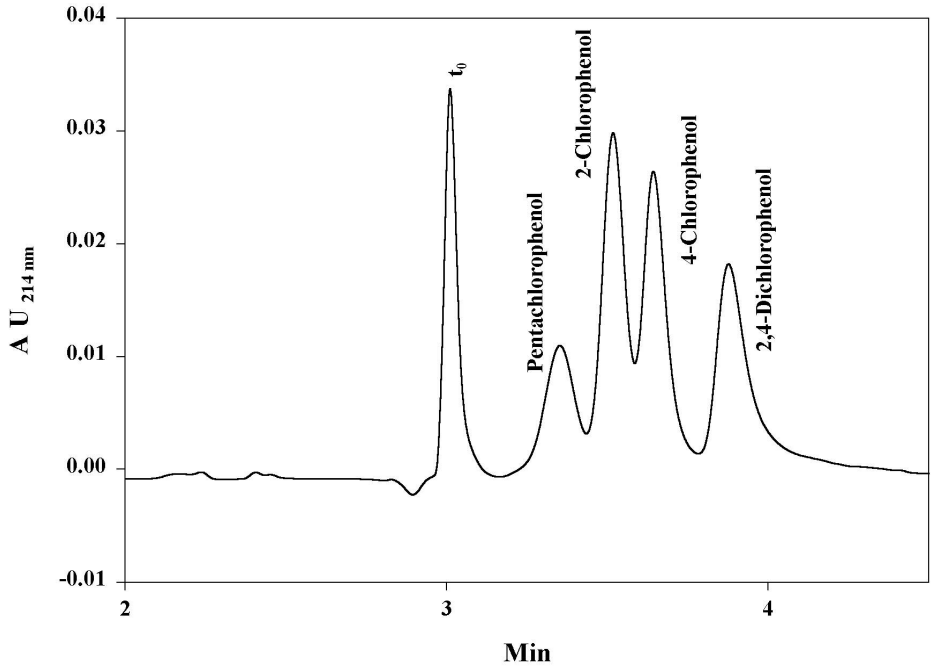


Figure 10. *Electrochromatogram of chlorophenols obtained on VP monolithic column. Conditions as in Fig 8. Mobile phase, hydro-organic solution made up of 2.5% v/v 2mM TEA (pH 4) and 97.5% v/v of acetonitrile*.

Separation of Phenols Phenolic compounds are a class of widespread environmental pollutants due to their industrial applications [52]. Currently, researchers

have used micellar liquid chromatography [53], reversed-phase high-performance liquid chromatography [54, 55], and gas chromatography-mass spectrometry [56-58] to separate the phenolic compounds. Here NP-CEC with VP monolith is introduced to the separation of some phenolic compounds. As seen in Figure 9, the retention time of the phenols increased with the increasing number of the hydroxyl groups in the solute, which shows that the VP monolithic column is indeed a true polar stationary phase. For the chlorophenols (Figure 10), the elution order is more or less in the order of decreasing the number of chlorine groups. The ionization of these weakly acidic compounds decreases with decreasing the number of chlorine groups.

Separations of Peptides As stated above, the amine monolithic column proved useful in the separation of dipeptides as shown in Figure 11. In fact, the column can separate the diastereoisomers of DL-Leu-DL-Phe. The dipeptides are separated on the basis of their difference in polarity with the least polar Leu-Phe eluting first and most polar Gly-Tyr eluting last. Of course, and since the solutes are charged, they also are separated on the basis of differences in electrophoretic mobilities. This represents one of the advantages of CEC in the separation of charged species in that the analytes are separated according to their differential partitioning between mobile and stationary phases and differential electromigration. NP-CEC will certainly play an important role in the analysis of protein tryptic digests especially in the area of emerging proteomics.

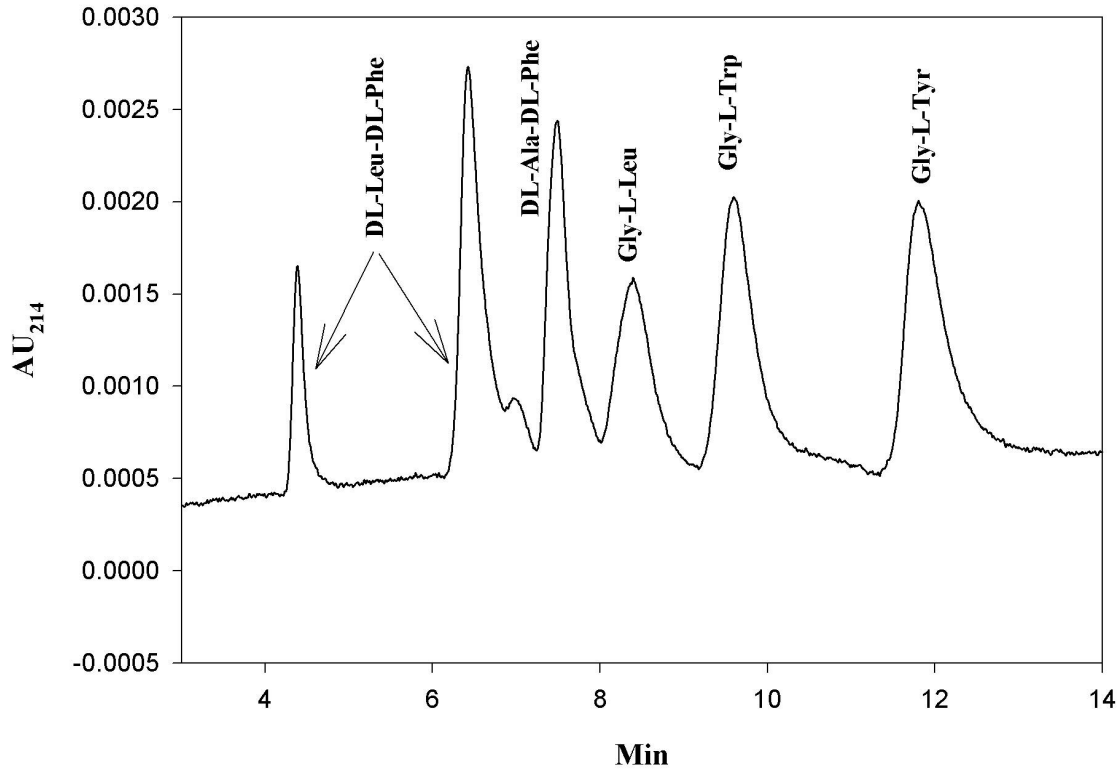


Figure 11. *Electrochromatogram of dipeptides obtained on amine modified GMA-co-EDMA monolithic column. Conditions: monomers, GMA 60%, EDMA 40%; porogen, cyclohexanol 50%, dodecanol 50%; AIBN 1% of the monomer; the ratio of monomer to porogen is 40:60 (wt/wt); column, 30/37 cm x 100 µm ID; voltage, -20 kV; wavelength, 214 nm; column temperature, 25 ˚C; mobile phase, hydro-organic solution made up of 2 mM NH_4AC (pH 6) at 90% v/v acetonitrile.*

Conclusions

This investigation has evaluated four polar monolithic capillary columns in NP-CEC. Two of the monoliths, VP monolith and CEA monolith, were prepared by *in situ* polymerization in a single step process while the two others, IDCN monolith and amine monolith, were obtained from the subsequent functionalization of a GMA-co-EDMA monolith with either IDCN ligands or diethylenetriamine ligands. VP monolith and amine monolith were the most useful stationary phases for the separation of polar compounds including phenols, carbohydrates and dipeptides by normal phase CEC. This study further confirmed that GMA-co-EDMA monolith is a promising precursor for the subsequent functionalization with various polar ligands offering convenience for in-column modification and high permeability.

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VITA

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Scope and Method of Study: This research has focused on the development of polar polymeric monolithic stationary phases for capillary electrochromatography (CEC). This involved novel monolithic stationary phases with polar function groups. They were prepared by the *in situ* copolymerization of ethylene glycol dimethacrylate and 2-acrylamido-2-methyl-1-propanesulfonic acid with either 2-cyanoethyl acrylate or 1-vinyl-2-pyrrolidinone (VP) in different porogenic solvents to produce columns with different functional groups. To further enlarge the scope of applications of monoliths, we modified the surface of monoliths based on the copolymerization of glycidyl methacrylate and ethylene dimethacrylate with 1-H-imidzaole-4,5-dicarbonitrile or diethylenetriamine to increase the polarity of the column.

Findings and Conclusions: Organic polymeric monolithic stationary phases thus developed proved to be useful for the normal phase CEC separations of neutral and charged solutes. The VP monolithic columns exhibited normal-phase retention mechanism toward neutral solutes (e.g. oligosaccharides) and a mixed mode retention behavior with charged solutes, e.g., phenols and chlorophenols. The monolithic columns with surface-bound diethylenetriamine were successfully used to separate dipeptides.

ADVISOR'S APPROVAL: _____