

POLAR BONDED STATIONARY PHASES  
CONSISTING OF SILICA-BASED MONOLITHS FOR  
HYDROPHILIC INTERACTION CAPILLARY  
ELECTROCHROMATOGRAPHY

By

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## LIST OF SYMBOLS AND LETTERS

$\alpha$	selectivity factor
$\delta$	thickness of electric double layer
$\varepsilon$	dielectric constant
$\kappa$	Debye-Huckel constant
$\eta$	viscosity
$\sigma_L^2$	peak variance in units of length
$\rho$	charge density of the surface
$\mu_{app}$	apparent electrophoretic mobility
$\mu_{eo}$	electroosmotic mobility in CZE
$\mu_{ep}$	electrophoretic mobility
$v_{app}$	apparent velocity
$v_{eo}$	electroosmotic velocity in CZE
$v_{ep}$	electrophoretic velocity of solute
$v_m$	migration velocity
$\psi_d$	potential at the interface between compact and diffuse regions
$\psi_o$	potential at the interface between the surface and the solution

$\zeta$	zeta potential
$a$	hydrodynamic radius of analyte
$C$	ion concentration
$D$	diffusion coefficient
$E$	applied voltage
$e$	electronic charge
$I$	ionic strength
$i_{\text{open}}$	current observed in open tube
$i_{\text{packed}}$	current observed in packed column
$k$	Boltzman constant
$k'$	retention factor
$k^*$	CEC retention factor
$k_{\text{cc}}^*$	peak locator in CEC
$k_{\text{ep}}^*$	velocity factor
$L$	total length of the column
$l$	effective length of column to detection point
$N$	number of theoretical plates
$q$	effective ionic charge
$R_s$	resolution
$t_0$	elution time of inert and neutral tracer

$t_m$	migration time of charged species
$t_R$	retention time of neutral species
$V$	applied voltage
$w_b$	width at the base of a Gaussian peak
$w_h$	width at the half height of a Gaussian peak
$w_i$	width at the inflection point of a Gaussian peak
$Z$	ion valence

## LIST OF ABBREVIATIONS

2AB	2-Aminobenzamide
$\gamma$ -GPTS	( $\gamma$ -Glycidoxypentyl)trimethoxysilane
AC	Affinity chromatography
ACN	Acetonitrile
CE	Capillary electrophoresis
CEC	Capillary electrochromatography
CZE	Capillary zone electrophoresis
DMF	<i>N, N</i> - Dimethylformamide
EOF	Electroosmotic flow
HILIC	Hydrophilic interaction liquid chromatography
HPLC	High performance liquid chromatography
IDCN	1 <i>H</i> -Imidazole-4,5-dicarbonitrile
IEC	Ion-exchange chromatography
NP	Normal-phase
NPC	Normal-phase chromatography
NP-CEC	Normal-phase capillary electrochromatography
PEG	Poly(ethylene glycol)

RP	Reversed-phase
RPC	Reversed-phase chromatography
SEC	Size-exclusion chromatography
TMOS	Tetramethylorthosilicate

## CHAPTER I

### SOME BASIC PRINCIPLES OF CAPILLARY ELECTROCHROMATOGRAPHY

#### BACKGROUND AND RATIONALE FOR THE STUDY

##### Introduction

Capillary electrochromatography (CEC) is a hybrid separation technique between capillary electrophoresis (CE) and high performance liquid chromatography (HPLC). CEC has received much attention over the last decade due to its high separation efficiency resulting from the electrophoretic component and unique selectivity arising from the chromatographic stationary phase. Initially, the stationary phases and column fabrication techniques in CEC resembled those used in HPLC. Then it was realized that specially designed stationary phases must be introduced for CEC to support a relatively strong electroosmotic flow (EOF). Recently, a particular attention has been given to developing stationary phases based on monoliths, which are continuous porous beds that avoid the difficulties of particle packing and frit fabrication that are the technical problems in conventional column packing process. Polymerization reaction and sol-gel

method are the two principal processes in monolith generation. The former is good in its one-step formation but has limited range of application due to the property of the monolith formed. The sol-gel method is attractive in that the formation of the sol-gel proceeds at low temperature and porous gel can be obtained. Furthermore, the selectivity of the sol-gel monolithic column can be achieved by subsequently modifying the silica monolith with the desired ligands. The stationary phase integrated with both organic and inorganic components thus provides a better chromatographic selectivity for separation of widely different solutes. Sol-gel based stationary phases have been found to be successful in separation, and it can be expected that sol-gel technology will continue to play an important role in the fabrication of stationary phases for CEC columns.

The subject of this dissertation is to further develop the CEC stationary phases based on silica monoliths produced by the sol-gel process by (i) optimizing the process and (ii) introducing novel polar ligands for normal phase CEC (NP-CEC). The polar ligand investigated in this thesis is the 1H-imidazole-4,5-dicarbonitrile (IDCN). The IDCN is characterized by a secondary amine, a tertiary amine and two cyano functions.

The first chapter of this dissertation is focused on the background and rationale of the study. The second and third chapters are concerned with the development and chromatographic characterization of the polar silica monoliths with various polar solutes.

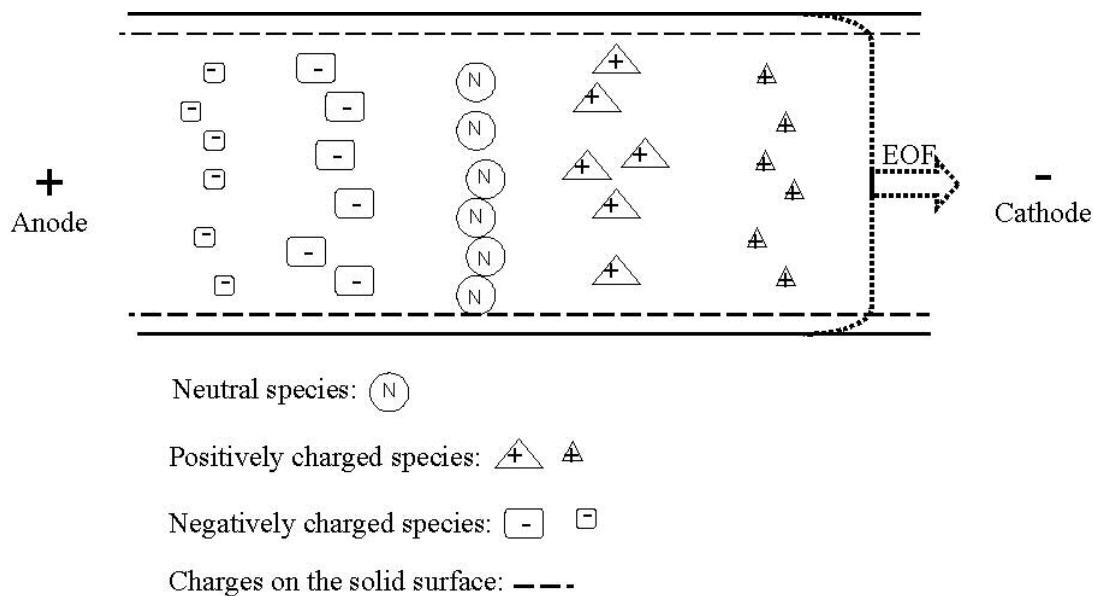
## Background and Some Basic Principles of Capillary Electrochromatography

As stated above, CEC is a hybrid separation technique from HPLC and CE. Capillary electrophoresis is simply the instrumental version of the traditional electrophoresis, which employs fused-silica capillaries of 10-100  $\mu\text{m}$  I.D. to perform efficient electrophoretic separations. Electrophoresis was first introduced by Arne Tiselius in 1937 [1,2], and 30 years later Stellan Hjerten initially demonstrated capillary electrophoresis using 3 mm I.D. glass tubes [3]. In 1981, Jorgenson and Lukacs used 75  $\mu\text{m}$  I.D. fused-silica capillaries for CE separation [4]. Their success in the fast and excellent separations on the amino acids with column efficiencies of over 400,000 plates/meter becomes a landmark in the development of CE [4,5]. Electrophoresis is a separation technique based on rate processes. It is the transport of charged species in a supporting electrolyte (i.e., background electrolyte) under the influence of a direct-current electric field. Components of mixture are separated based on their different kinetic properties. Factors affecting the migration rate of the analytes include the strength of the electric field, electric charge of the analyte, the shape and size of the analyte, and the surrounding of the analyte, i.e., background electrolyte. These factors determine the electrophoretic mobility and electroosmotic mobility or electroosmotic flow (EOF).

The electrophoretic mobility results from the electrostatic attraction between the charged species and the electrode of opposite sign. It is a physical characteristic of the



analytes and therefore has a different value for different analytes. The electroosmotic mobility is the flow of the bulk electrolyte solution under the influence of an electric field, and thus influences the migration velocity of all the analytes to the same extent (see Figure 1).



**Figure 1.** Scheme of the apparent mobilities of species in an electric field in the presence of a cathodal EOF. The smallest positively charged species migrate first followed by the species of larger size. Neutral species migrate after the positively charged species due to the EOF and thus could be used as EOF marker. Neutral species are not separated. Smallest negatively charged species elute last due to their strongest electrostatic interaction with the anode. (Ref.[6]).

### Electrophoretic Migration

In an electric field, the charged analyte moves at a steady electrophoretic migration velocity. The electrophoretic velocity ( $v_{ep}$  in cm/s) per unit of the electric field strength ( $E$  in V/cm) is called the electrophoretic mobility ( $\mu_{ep}$ , in  $\text{cm}^2/\text{V.s}$ ):

$$\mu_{ep} = \frac{V_{ep}}{E}$$

The electrophoretic mobility is dependent on the charge, size and shape of the analyte and the viscosity of the medium. It is given by the following equation [7]:

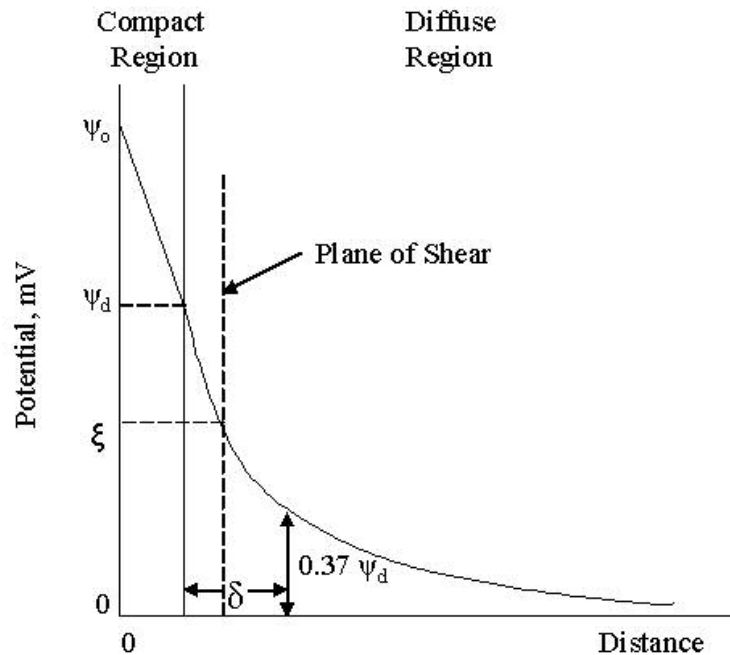
$$\mu_{ep} = \frac{q}{6\pi\eta a}$$

where, q is the effective ionic charge,  $\eta$  is the viscosity of the medium and a is the hydrodynamic radius of the analyte (i.e., radius of the solvated ion). The equation indicates that the electrophoretic mobility increases with decreasing viscosity and size of the analyte and increasing charge-to-mass ratio.

#### Electroosmotic flow

EOF is the bulk flow of the electrolyte solution in a non-conducting capillary tube under the influence of an applied electric field. In CE, the inner wall of the fused silica capillary is negatively charged when pH of the bulk solution is above 3.5 due to the ionization of silanol groups. The sign of the charge on the capillary inner wall can be changed by the covalent attachment of charged functional groups or by dynamic coating with charged surface active agents, e.g., charged surfactants, polyamines, etc. In the case of bare fused-silica capillary, the electrolyte counterions are attracted and held tightly to the charged surface of the inner wall because of the electrostatic attraction. These immobile counterions form the compact region termed the stern layer of the electric double layer while the co-ions are repelled from the surface (see Figure 2).

Due to thermal motion, some of the counterions in the compact region leave the surface and diffuse into the solution farther away from the capillary surface and thus forming a diffuse region of the electric double layer. This distribution of ions creates an electric



**Figure 2.** Stern-Gouy-Chapman model of the electrical double layer (Ref. [8])

potential  $\psi_o$  at the solid surface. The electric potential decreases linearly in the stern layer and exponentially in the diffuse region. The electric potential at the interface of the compact and diffuse region is denoted  $\psi_d$ . When an electric potential is applied to the electrolyte solution, the counterions in the diffuse region migrate to the electrode of opposite charge. Since they are solvated, the movement of the counterions drags the bulk solution and thus producing a liquid flow, which is called EOF ( $\mu_{eo}$ ) (see Figure 1). The plane of shear refers to where the electrolyte solution flows tangentially to the

surface of the capillary. The potential at the plane of shear is termed zeta potential ( $\zeta$ ).

EOF is given by [7]:

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

where  $\zeta$  is given by

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

In this equation,  $\varepsilon$  is dielectric constant,  $\rho$  is the charge density of the surface and  $\delta$  is the thickness of the electric double layer.  $\delta$  can be calculated from the Debye-Huckel constant:

$$\delta = \frac{1}{\kappa}$$

where

$$\kappa = \sqrt{\frac{8\pi e^2 I}{\varepsilon k T}}$$

where  $k$  is the Boltzman constant,  $e$  is the electronic charge and  $I$  is the ionic strength.  $I$  is given by:

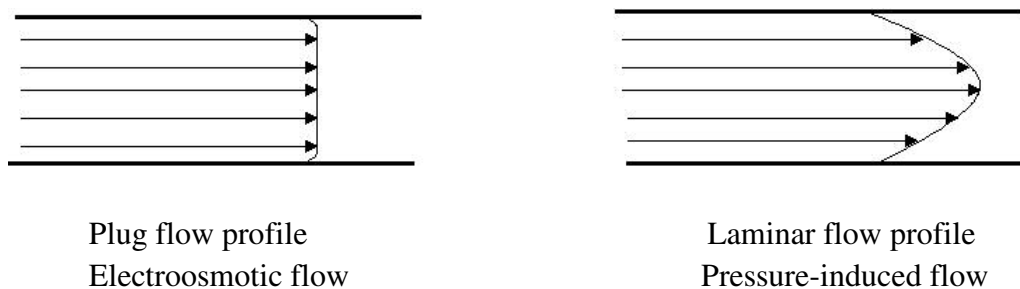
$$I = \frac{1}{2} \sum C_i Z_i^2$$

where  $Z$  is the ion valence and  $C$  is the ion concentration. Thus,

$$\mu_{eo} = \frac{V_{eo}}{E} = \frac{4\pi\rho}{\eta\kappa} \propto \frac{1}{\sqrt{I}}$$

According to the above equation, the EOF velocity increases with increasing the potential applied and decreases with increasing ionic strength and viscosity. EOF is denoted as cathodal when the flow is from anode to cathode. A cathodal EOF indicates that the capillary surface is negatively charged. The migration of positively charged

species is therefore augmented by the EOF, while neutral solutes migrate together with EOF without separation and negatively charged species are swept with the EOF and elute last. When the flow is in the reverse direction, EOF is referred to as anodal which indicates that the capillary surface is positively charged. With an anodal EOF, negatively charged species elute first followed by neutral species and finally positively



**Figure 3.** Profiles of electroosmotic flow and pressure-induced flow in capillary tubes.

charged species. The electric double layer is so thin compared to the diameter of the capillary that we can assume that the EOF originates on the surface of the capillary or solid particles. This explains why the flow profile in CE is a plug like instead of the laminar flow profile obtained with pressure driven flow (Figure 1 & 3).

### Analytical Parameters in CE

Migration Time and Apparent Mobility: The apparent mobility in CE ( $\mu_{app}$ ) is the sum of electrophoretic mobility ( $\mu_{ep}$ ) and electroosmotic mobility ( $\mu_{eo}$ ) as follows [7]:

$$\mu_{app} = \mu_{ep} + \mu_{eo}$$

Accordingly, the apparent velocity  $\mu_{app}$  is the sum of electrophoretic velocity  $v_{ep}$  and electroosmotic velocity  $v_{eo}$  as follows:

$$V_{app} = V_{ep} + V_{eo}$$

The electroosmotic mobility is the electroosmotic velocity per unit of voltage applied (E) as follows:

$$\mu_{eo} = \frac{v_{eo}}{E} = \frac{lL}{t_o V}$$

where V is the applied voltage,  $t_o$  is the migration time of a neutral specie, L is the total length of the capillary and l is the length from the outlet to the detection window of the capillary. Similarly,

$$\mu_{app} = \frac{v_{app}}{E} = \frac{lL}{t_m V}$$

where,  $t_m$  is the migration time of a charged specie.

Therefore,  $\mu_{ep}$  can be calculated from the electropherogram by the equation:

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

Separation Efficiency: Column separation efficiency is expressed as the plate number

N which is given by [7]:

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

Where  $\sigma_L^2$  is the statistical equivalence of the variance of the migrating zone width:

$$\sigma_L^2 = 2Dt_m = \frac{2DL}{(\mu_{ep} + \mu_{eo})V}$$

where D is the diffusion coefficient of the analyte. When L = l (when detection is at the tip of the capillary such as in mass spectrometric detection), we have

$$N = \frac{l^2}{\sigma_L^2} = \frac{(\mu_{ep} + \mu_{eo})Vl}{2DL} = \frac{(\mu_{ep} + \mu_{eo})V}{2D}$$

This equation suggests that N increases with increasing  $\mu_{app}$  and decreasing D.

As in chromatography, N can also be calculated from the electropherograms using the following equation:

$$N = \frac{t_R^2}{\sigma_t^2} = 16 \left( \frac{t_m}{w} \right)^2 = 5.54 \left( \frac{t_m}{w_h} \right)^2 = 4 \left( \frac{t_m}{w_i} \right)^2$$

where w,  $w_h$  and  $w_i$  are the widths of the peak at base, half height and at inflection point, respectively.

Resolution and Selectivity: Resolution of two adjacent peaks is given by:

$$R_s = \frac{1}{4} \sqrt{N} \frac{\Delta\mu_{ep}}{\bar{\mu}_{app}} = \frac{1}{4\sqrt{2}} \Delta\mu_{ep} \sqrt{\frac{Vl}{DL(\bar{\mu}_{ep} + \mu_{eo})}}$$

$R_s$  can be calculated from the electropherograms as well by the equation:

$$R_s = \frac{2(t_{m2} - t_{m1})}{w_1 + w_2}$$

where  $t_{m1}$  and  $t_{m2}$  are the migration times of solute 1 and solute 2,  $w_1$  and  $w_2$  are the widths of the peaks of solute 1 and solute 2.

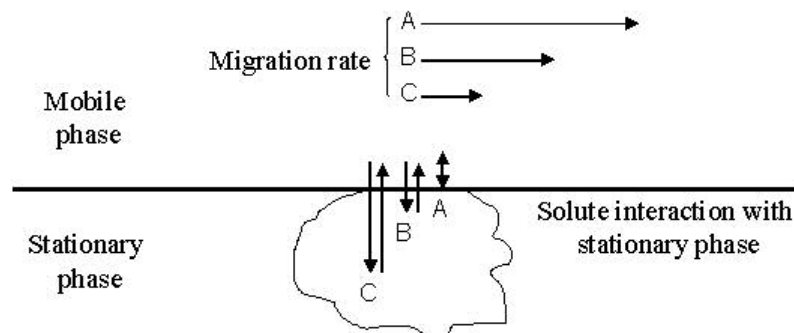
Selectivity factor  $\alpha$  of two adjacent zones is given by:

$$\alpha = \frac{\Delta\mu_{ep}}{\overline{\mu}_{app}} = \frac{\Delta\mu_{ep}}{\overline{\mu}_{ep} + \mu_{eo}}$$

where  $\Delta\mu_{ep}$  is the difference of electrophoretic mobility between solute 1 and solute 2,  $\overline{\mu}_{ep}$  is the average of electrophoretic mobility of solute 1 and solute 2.

### Capillary Electrochromatography (CEC)

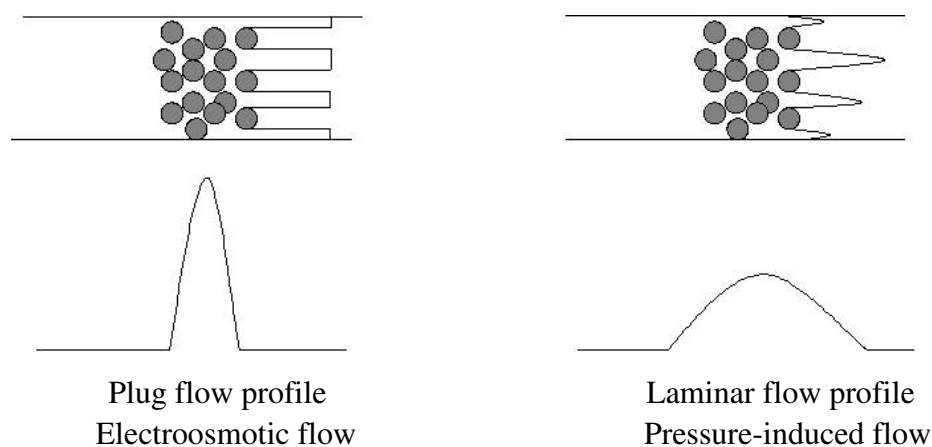
The origin of EOF in CEC is the same as that in CE. Neutral analytes experience the same interaction with the stationary phase and the same elution order as those in HPLC, i.e., the stronger the interaction, the more retained by the stationary phase (Figure 4), except that the flow driven force is from EOF instead of the pressure driven force in HPLC. The use of EOF was first demonstrated by Pretorius in 1974 [9]. Jorgenson and Lukacs produced the first CEC column packed with 10  $\mu\text{m}$  octadecyl-silica particles in a glass capillary of 170  $\mu\text{m}$  I.D. in 1981 [10]. In CEC, the retention and migration of the charged analytes are complicated due to the fact that EOF, electrophoretic migration



**Figure 4.** *Interaction of neutral species with the stationary phase and the relative migration rate in chromatography (Ref.[6]).*



and interaction with stationary phase participate in influencing the migration rate of the charged analytes. In this case, considerations need to be put on the selection of the stationary phase and the proper electrolyte solution to achieve the desired separation. Because of the mixed mode of chromatography and electrophoresis, CEC has the merit of unique selectivity from stationary phases in HPLC and that of high separation efficiency from electroosmotic flow in CE. The different flow profiles in CEC and HPLC are shown in Figure 5.



**Figure 5.** Profile of electroosmotic flow in CEC and pressure-induced flow in micro-HPLC.

In CEC, for a neutral analyte, the retention is evaluated by the retention factor  $k'$  as in chromatography:

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

where  $t_R$  is the retention time of the analyte and  $t_o$  is the retention time of an unretained species (i.e., EOF marker).

Selectivity factor  $\alpha$  is calculated by:

$$\alpha = \frac{t'_{R2}}{t'_{R1}} = \frac{k'_2}{k'_1}$$

where,  $t'_{R1}$  and  $t'_{R2}$  are the adjusted migration time of analyte 1 and analyte 2,  $t'_{R1} = t_{m1} - t_o$ ,  $t'_{R2} = t_{m2} - t_o$ ;  $k'_1$  and  $k'_2$  are the retention factors of analyte 1 and analyte 2.

In CEC, since the migration of a charged analyte is dependent on both its electrophoretic mobility and chromatographic partitioning, the migration rate ( $v_m$ ) is demonstrated by the sum of the rate of the mobile phase ( $v_{eo}$ ) and the electrophoretic rate of the analyte ( $v_{ep}$ ), multiplied by the retardation factor  $1/(1+k^*)$ , as follows:

$$v_m = \frac{v_{ep} + v_{eo}}{1 + k^*} = \frac{v_{eo}(1 + k_{ep}^*)}{1 + k^*}$$

Where  $k^*$  is retention factor in CEC,  $k_{ep}^*$  is the velocity factor, as follows:

$$k_{ep}^* = \frac{v_{ep}}{v_{eo}}$$

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

Where  $t_m$  is the migration time of the analyte,  $t_o$  is the migration time of EOF marker.

$k^*$  and  $k_{ep}^*$  are measured in the mode of CEC,  $v_{ep}$  is measured in the mode of CZE with the same conditions used in CEC. If the analyte is neutral,  $k_{ep}^* = 0$ , and therefore  $k^* = k'$ . Peak locator  $k_{cc}^*$  is used in CEC to describe the elution order of charged solutes due to the different mechanism of migration resulting from electrophoretic process in CEC:

$$k_{cc}^* = \frac{t_m - t_o}{t_o}$$

where  $t_m$  is the migration time of the analyte,  $t_o$  is the migration time of a neutral tracer in

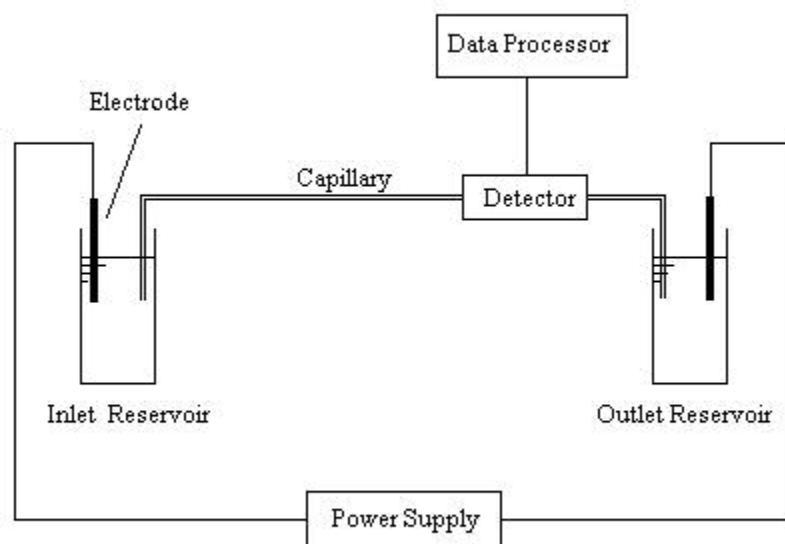
the CEC system (see Chapter III for more details about the above equations).

### Instrumentation

The instrumentation of CEC is the same as that of CE. The CEC columns are fabricated with capillaries of 50-200  $\mu\text{m}$  I.D. packed with particulate or monolithic stationary phases or with capillaries of 50  $\mu\text{m}$  I.D. or less when the stationary phase is coated on the wall (i.e., open tubular).

The principal components of a CE/CEC instrument are a capillary, an autosampler, a high-voltage power supply, a detector, and a data processor. Figure 6 shows a typical scheme of the structure of a CE/CEC instrument. The power supply can provide the capillary with an electric field under which analytes migrate from the anode to the cathode or in the reverse direction. The applied voltage range is 1.0 kV-30 kV with a maximum of 30 kV at 250  $\mu\text{A}$ . The detectors used in CE/CEC are mainly UV-Vis absorbance, fluorescence, mass spectrometric, conductivity, amperometric, radiometric and refractive index [11], among which, the UV-Vis absorbance detector is the most widely used. The UV source of a UV-Vis absorbance detector is a deuterium lamp with a wavelength ranging from 190 nm to 380 nm. An autosampler has two rotating trays to hold vials of buffer solutions and samples. The capillary is held in a cartridge with its optically transparent section lying in the detection window of the cartridge. The injection of sample is achieved by applying a relatively low (1 - 10 kV) voltage or a

pressure. The data handling system of CE/CEC is similar to those used in HPLC or GC. The plotting of detector response versus time is called electrochromatogram, which provides the basis for quantitation.



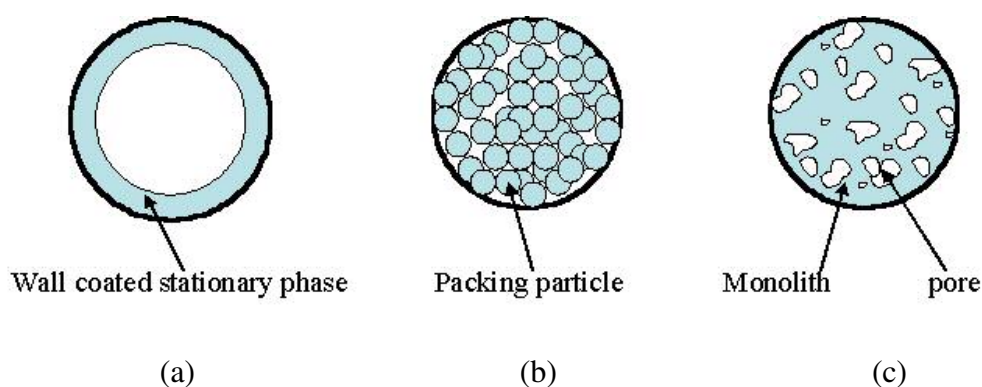
**Figure 6.** *Scheme of a CE/CEC instrument.*

### Column Technology

The columns used in CEC are of three kinds: open tube, particle packing columns and monolithic columns (Figure 7). The stationary phases in CEC must on one hand provide chromatographic retention and selectivity, and on the other hand provide EOF to lead to differential migration and elution.

The open tube column is usually a capillary of small inner diameter (50  $\mu\text{m}$  I.D. or

less) with the stationary phase adhered to its inner wall. Open tube has the merit of simplicity in column making but on the other hand has the limitation of low phase ratio, and consequently low retention and selectivity. The particle packed capillaries are usually prepared by packing the stationary phase particles in capillaries at high pressure and thus involves the problem of frit making. The ligands of the stationary phases in open tubes and particle packed columns are alike. In other words, the chemically bonded stationary phase and physically or dynamically adsorbed stationary phase is the same, and the only difference is that the ligand in one case is immobilized on the capillary walls and in the second case is attached to the particle surface. The third type of packed column is the monolithic column which usually consists of either the organic polymer-based monolith or the inorganic silica-based monolith as the stationary phase media.



**Figure 7.** Different column types in CEC. a, open tube; b, packed column; c, monolithic column.

Monolith is a continuous piece of macroporous material usually created by the *in situ* polymerization of monomers. Compared with the particle packed capillary columns, the

monolithic columns have the following features:

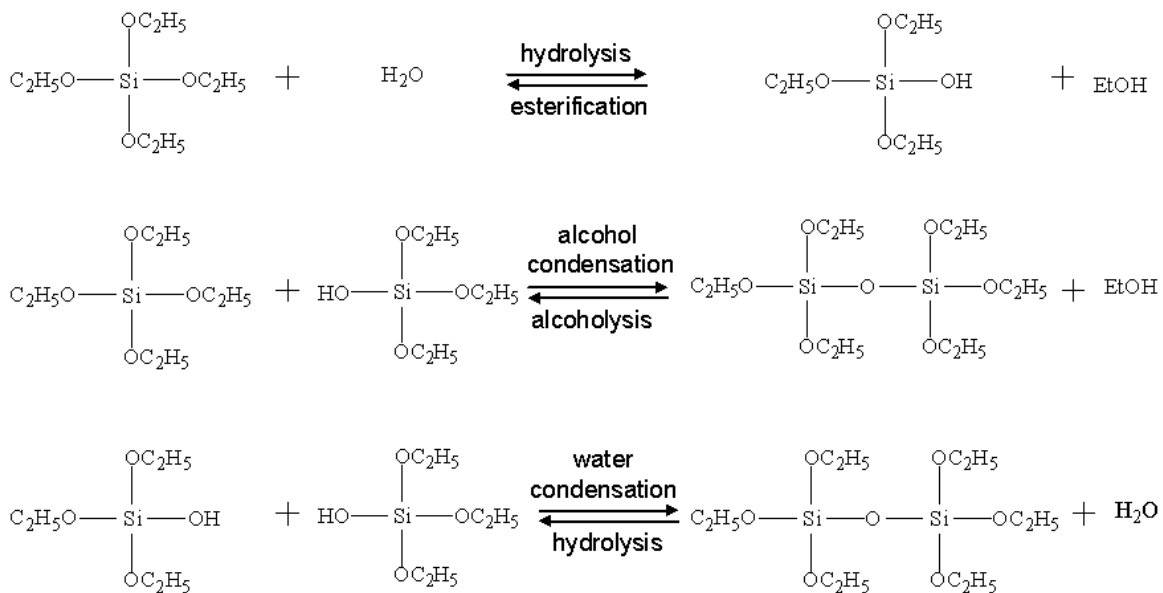
- Simplicity of their *in situ* preparation
- Column packing and frit are not required
- No bubble formation
- The size and structure of the pores which affect the column efficiency within the monolith can be easily controlled
- The ligands attached to the surface of the monolith can be modified easily to satisfy different separation problems

There are mainly two ways to fabricate silica-based monoliths. One is the technique of particle fixing which involves agglomerations of silica microparticles by thermal treatment or entrapment of particles using sol-gel [12,13]. Another is the polymerization of transition metal alkoxide precursors using sol-gel process [14,15,16], which is under investigation by this research.

The sol-gel column technology produces an organic-inorganic hybrid stationary phase which provides an alternative interaction between the stationary phases and the analytes and thus yields better separations. The subsequent chemical bonding modifications of the stationary phases make it possible to achieve a high selectivity either in the mode of reversed-phase or normal-phase by introducing a wide range of modifying ligands. In the sol-gel process, a porous continuous bed is formed *in situ* in a capillary and acts as the stationary phase. This bonded stationary phase can be formed from a modified sol-gel matrix or bare sol-gel solution followed by a subsequent ligand

modification.

Sol-gel process usually involves three steps, i. e. hydrolysis, condensation, and successive polycondensation (Figure 8). The hydrolysis of Si-OR in water produces Si-OH which condenses with each other subsequently to produce a poly-condensed species with a Si-O-Si linkage. Successive condensation yields oligomers which finally turn into a gel network [18]. Concentrations of water and catalyst, usually an acid, play



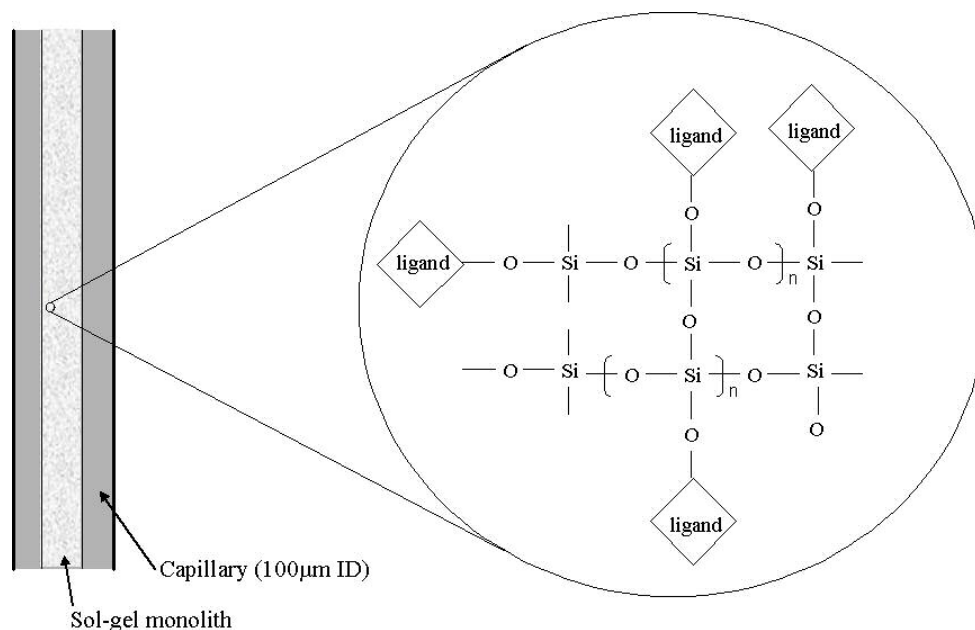
**Figure 8.** Reactions involved in the sol-gel process (Adapted from [17])

important roles in the kinetics of the reactions, which involve the rate of development of the gel network. Since the morphology of the sol-gel network is dependent mostly on the composition of the solution, efforts to vary the starting composition can be made to yield a gel skeleton with the desired structure.

The process of gel formation includes a hydrolysis of alkoxide, gelation and phase separation, aging, solvent exchange, drying and heating treatment. The alkoxides of

TMOS and TEOS are the most often used sol-gel precursors [19-23]. Water soluble surfactants and polymers are used as additives to induce the phase separation. PEG was used as the additive in this investigation based on the fact that glycol and silanol can form strong hydrogen bonds and that results in the formation of PEG-silica oligomers which is more soluble in water. The ratio of the alkoxide, additive and water plays an important role in the morphology of the gel skeleton [19]. The water becomes pore space after drying. Gel formation was carried out at a freezing temperature to diminish the significant influence of the heat from the reaction on the morphology of the gel skeleton. The sol solution needs to be transferred to a vessel (a capillary in the case of the in situ monolith formation) and allowed for gelation and aging at a temperature between 40-80 °C [18]. Aging leads to the gel shrinkage with a concurrent exclusion of a pore forming phase from a bulk gel. The thus formed gel is then subjected to the strengthening or tailoring treatment of the micro- and mesopores with an alcohol, an acid or a base. Ammonium hydroxide is a good pore tailoring reagent to control the mesopore distribution and surface area. It was found that high temperature and high pH help to build large mesopores [24]. The continuous rod with the desired micropores and mesopores undergoes a heating treatment to get rid of volatile and organic components. The monolith obtained is then subjected to the surface modification to meet specific separation requirement. Figure 9 shows the scheme of the sol-gel network in a monolithic column.





**Figure 9.** Scheme of sol-gel network (Ref. [25])

## Polar Bonded Stationary Phases for Hydrophilic Interaction Capillary

### Electrochromatography

The surface of the silica monolith can be modified with polar or non-polar ligands to produce separations in the mode of normal-phase (NP) or reversed-phase (RP), respectively. Similar to chromatography, in CEC, nonpolar ligands are usually employed in the resolution of solutes with low polarity in reversed-phase separation while polar ligands are designed for the resolution of polar solutes in normal-phase separation. Normal phase separation is complementary to reversed-phase separation, and it is more powerful in the separations of polar and charged solutes especially chiral molecules due to its unique polar surface which provides more interaction sites for polar

analytes. Traditional normal-phase separation employs an organic mobile phase with a relatively low polarity compared to that of the polar functional groups on the stationary phase surface. Hydrophilic interaction liquid chromatography (HILIC) is simply a modification of normal-phase chromatography (NPC) where the mobile phase is not a purely organic phase but a mixture of aqueous and organic phases. The term hydrophilic interaction chromatography has been introduced first by Alpert in 1990 [26] to describe all forms of chromatography driven by polar (hydrophilic) interactions involving a wide variety of polar stationary phases, e.g., bare silica gels, cyano, diol, amino silica bonded phases, etc. HILIC has been applied in the separation of polar solutes in HPLC [27], while its application in CEC was first reported by Ye et al. in 2001 [28]. In HILIC, a mobile phase rich in organic solvent (e.g., acetonitrile) and a hydrophilic stationary phase are used to separate polar compounds. As in NPC, in HILIC the solutes are eluted from the stationary phase in the order of their increasing hydrophilicity, that is, the more polar the solute the more it is retained by the stationary phase. The hydrophilic interaction can be promoted by increasing the concentration of the organic solvent in the mobile phase. HILIC which allows the use of aqueous solution may be more suitable than the traditional normal-phase chromatography in the separations of polar or charged solutes [29].

In contrast to the substantial productions of non-polar stationary phases such as C<sub>18</sub> phases since 1980s when chemists started making significant progress in CEC [13,30-32], limited studies were conducted for the generation of polar stationary phases in CEC

[15,16,33,34]. Similar to the polar ligands used in HPLC, cyano, diol and amino are the usual functional groups employed in the production of the polar stationary phases in CEC. Since 1997 when Maruška et al. first demonstrated the production of polar stationary phases for silica gel and cellulose-based packing materials [35], the developments of polar stationary phase ligands utilized for open tubular [36,37], packed columns [35,38] and monolithic columns [39,40] have been reported, however, only a few of investigations involved the introduction of silica-based monolithic columns [16,21,33]. As stated above, in addition to their common merits as monoliths, the sol-gel monolithic column has its own unique property and the merits over the polymer-based monolithic column. Therefore, specially designed polar stationary phases for silica-based monolithic columns should be developed to expand the application of NP-CEC. Various information on sol-gel stationary phases in general as well as on the fabrication and applications of the silica-based monolithic columns and the polar stationary phases for capillary electrochromatography can be found in recent reviews [41-43]. In the following sections, a brief review of the recent developments in the polar stationary phases in CEC with a focus on polar sol-gel monolithic columns will be presented.

To date, in CEC the stationary phase media involved in the fabrication of polar stationary phases are porous beads (e.g., silica, cellulose beads), polymer-based (usually polyacrylamide-based and polymethacrylated-based) monoliths and silica-based monoliths. Bare silica column is the most common normal-phase column in HPLC, and it is now employed in the fabrication of polar stationary phase in CEC. Lai and

Dabek-Zlotorzynska demonstrated the use of a bare silica packed column operated in the mode of normal phase CEC to separate theophylline, caffeine and related drugs. A mobile phase containing isopropanol/hexane/1 mM Tris (52:40:8, pH 8) was employed to separate eight compounds with good resolution and an efficiency of 63,000 plates/meter [44]. Wei et al. reported using a 3- $\mu$ m silica particle packing column to separate the basic compounds of berberine, jatrorrhizine, ephedrine, codeine, cocaine and thebaine etc. The separation mechanism was found to be multifunctional, and it varied with the composition of mobile phase [45]. Maruska and Pyell used a bare silica Polygosil 100-10 packed column to separate methanol, benzene, phenol, resorcinol, 2-aminophenol and 1, 2-diaminobenzene. The non-aqueous mobile phases (acetonitrile, methanol, hexaethanol-methanol) produced a high EOF and stable separations were obtained [35]. Vickers and Smith successfully separated the chiral analytes of brendroflumethiazide, Trogers base, warfarin and benzoin using a spherical silica-packing column with normal phase solvent systems such as 1:1 hexane:IPA by the addition of small amount of water [46]. Packed bare silica column was also reported to be successful in the separation of basic compounds of theophylline, caffeine, acetaminophen, hydrochlorothiazide etc. in the mode of normal phase separation by Lai and Dabek-Zlotorzynska [47].

Through an in-column derivatization, Suzuki et al. prepared a chemically bonded silica gel column by pumping an ethanolic solution of 3-aminopropyltrimethoxysilane into a capillary packed with bare silica particles. The column showed high efficiency and high reproducibility. Good separation was achieved for the

1-phenyl-3-methyl-5-pyrazolone derivatives of aldopentoses on the 3-aminopropylsilica column [48]. Krause et al. prepared silica gel packed columns modified by covalent attachment of poly-N-acryloyl-L-phenylalanine ethylester or by coating with cellulose tri(3,5-dimethylphenylcarbamate) for enantioseparations of chiral compounds such as  $\beta$ -blockers, benzodiazepines, diuretics, etc.[49]. Packed columns with polysaccharide stationary phases of Chiralcel OD, LJ, and Chiralpak AD, AS were used by Mangelings et al. for the chiral separations of tetramisole, warfarin, praziquantel, acenocoumarol, pindolol and oxazepam [50]. Some other polar ligands were also found useful in the case of silica-based packed columns for different separation purposes, e.g., Kato et al. used (S)-*N*-3,5-dinitrobenzoyl-1-naphthylglycine and (S)-*N*-3,5-dinitrophenylaminocarbonylvaline to modify the packed silica stationary phase to separate amino acids [51]; Wistuba and Schurig introduced the permethylated  $\beta$ -cyclodextrin to a packed silica stationary phase for the separation of barbituric acids, benzoin, carprofen and ibuprofen [52].

Polar polymer-based monolithic stationary phases to achieve the separations of polar solutes by normal phase CEC were also introduced. Que and Novotny used 2-cyanoethyl acrylate and 3-amino-1-propanol vinyl ether to prepare acrylamide-based polar stationary phases. The stationary phase containing amino- or cyano- groups was efficient in the separation of glycan mixtures with the use of mobile phases containing acetonitrile and ammonium buffer solution. The separation mechanism is essentially a hydrophilic partition involving hydrogen-bonding and dipole-dipole interactions between the

hydroxyl groups of sugars and the polar stationary phase. A wide range of carbohydrates including mono-, oligosaccharides with the intact reducing end, saccharide alditols and even the anomers formed due to mutarotation could be separated by the polymeric monolithic columns [34]. Wahl et al. attempted a novel synthetic route to produce amphiphilic acrylamide-based monolithic stationary phases employing water-soluble cyclodextrins as solubilizing agents. The investigators synthesized the amphiphilic stationary phases via free radical copolymerization of the bisacrylamide-cyclodextrin host-guest complexes with hydrophilic monomers and an additional hydrophilic cross-linker in aqueous solution. These columns showed sufficiently high EOF, high reproducibility and a plate number as high as 200,000 plates/meter [53]. Hoegger and Freitag synthesized a *N,N*-dimethylacrylamide-piperazine diacrylamide-based monolithic stationary phases bearing sulfonic acid groups for the separation of positively charged amino acids and peptides. *N*, *N*-dimethylacrylamide, methacrylamide, 2-hydroxyethyl methacrylate, and 2-hydroxyethyl acrylate were the functional monomers used in the stationary phase synthesis. The columns exhibited both electrostatic and hydrophilic interactions, which could be varied by the amount of the charged monomer and the hydrophilic property of the monomers [54].

Polar methacrylate-based monolith is another polymer-based monolith employed in the normal-phase column fabrication. Lammerhofer et al. produced a monolith *via* a polymerization of 2-hydroxyethyl methacrylate, ethylene dimethacrylate, and

2-(dimethylamino)ethyl acrylate in the presence of a binary porogenic mixture of dodecanol and cyclohexanol. The tertiary amino functionalities were used to afford strong anion-exchangers. The hydroxyl groups of the polymerized 2-hydroxyethyl methacrylate repeat units provided hydrophilic interaction sites for the neutral phenolic compounds and basic aromatic amines. Column efficiency of 231,000 plates/meter was achieved for the separation of a mixture of 2-substituted propionic acid drugs [40]. A cationic acrylic monolithic column was synthesized by Zhang et al. through an in situ copolymerization of glycidyl methacrylate, methyl methacrylate and ethylene glycol dimethacrylate with propanol and formamide as porogens. *N*-Ethylbutylamine was employed to modify the surface of the monolith in order to form fixed tertiary amino functions with ethyl- and butyl-chains. A hydro-organic mobile phase containing acetonitrile and sodium phosphate buffer (pH 2.5 %) was used for the separation of a mixture of ribonuclease A, insulin,  $\alpha$ -lactalbumin and myoglobin [55].

Similar to bare silica particle packed column, bare silica monolithic column is used in the mode of normal-phase separation, and the silica monolith could be modified with polar ligands to generate a polar surface in order to improve separation. As stated above, silica-based monoliths have a few attractive features including (i) the monolith could be produced at room temperature, (ii) the surface modification is easy to perform, and (iii) the organic and inorganic components provide different interaction sites with solutes, and thus the columns produce better selectivity toward analytes.

Bare silica monoliths were recently reported in the separation of basic

pharmaceuticals of  $\beta$ -blocker and alkaloids extracted from traditional Chinese medicines by Xie et al. The silica-based monolith was prepared by a sol-gel process in which TMOS and PEG were employed with an acetic acid as a catalyst. Separation was conducted on the column after the monolith was treated for mesopore tailoring and drying. The column separation efficiency was as high as 250,000 plates/meter and separation was completed within 10 min. The retention of the basic pharmaceutical on the silica monolith was found mainly contributed by a cation-exchange mechanism. Normal- and reversed-phase retentions of the basic pharmaceuticals were also observed. The investigation indicated that the monolithic silica columns provided different selectivity from bare capillary columns in the separation of basic compounds, and the monolithic columns showed excellent stability with a RSD of 1.12% for the migration time of the alkaloids [56].

Dulay et al. prepared a porous photopolymerized sol-gel monolith bonded with aminopropyl. The parent monolith was prepared at room temperature with a precursor stock solution of methacryloxypropyltrimethoxysilane and a hydrochloric acid as a catalyst. The reagent n-propylaminotriethoxysilane to be bonded to the monolith was introduced into the monolithic column and the in situ reaction was allowed to proceed with the monolithic surface for not longer than 60 min at room temperature. The columns were found to be more stable at a pH of 4 or below compared to their parent monoliths, and to have column efficiencies of over 180,000 plates/meter. The bonded phases altered the hydrophobicity of the sol-gel continuous bed and were effective in the



separations of biological and pharmaceutical molecules such as peptides, nucleosides, taxol and its precursors [33].

A few other investigations reported that the polar bonded silica monolithic columns with the stationary phase ligands such as cyclodextrin and amino groups (e.g. (s)-*N*-3,5-dinitrophenylaminocarbonylvaline, L-phenylalaninamide complexed with  $\text{Cu}^{2+}$ ) were successful in the separations of enantiomers. Chen et al. described a monolithic silica column modified with L-prolinamide to separate dansyl amino acids and DL-*p*-hydroxyphenyllactic acids [21]. The monolithic silica matrix was prepared *via* an in situ sol-gel process, in which TMOS, PEG and acetic acid were employed. Two steps were involved in the modification. A spacer arm 3-glycidoxypropyltrimethoxysilane was bonded to the surface prior to the linkage with L-prolinamide conditioned by  $\text{CuSO}_4$ . The EOF was dependent on the pH, the applied voltage and the mobile phase. The column showed a relatively high enantioselectivity toward the solutes which have large substituent groups such as phenyl and indole ring at the chiral  $\alpha$ -carbon of dansyl amino acids and hydroxyl acids. The column efficiency was 13,200 plates/meter. Also, the same research group attempted linking the silica monolith with L-phenylalaninamide complexed with  $\text{Cu}^{2+}$  [20] to separate dansyl amino acids, and L-hydroxyproline complexed with  $\text{Cu}^{2+}$  to resolve dipeptides, dansyl amino acids, free amino acids, hydroxyl acids such as DL-*p*-hydroxyphenyllactic acid [57]. A mobile phase containing acetonitrile/0.50 mM  $\text{Cu}(\text{Ac})_2$ -50mM  $\text{NH}_4\text{Ac}$  (7:3) adjusted to pH 5.5-6.5 was used in the separation. Other ligands such as permethylated  $\beta$ -cyclodextrin were also reported in

the synthesis of the chiral monolithic columns for the separations of mephobarbital, hexobarbital, benzoin and carprogen [58].

Another silica monolith produced by sol-gel process and having a polar stationary phase with a cyano functional group was reported by Allen and El Rassi [16]. The preparation of the silica backbone was similar to the method described by Ishizuka et al. [59]. The column denoted CN-monolith was generated by a one step reaction with 3-cyanopropyldimethylchlorosilane. The column denoted CN-OH-monolith was produced through a reaction with  $\gamma$ -glycidoxypopyltrimethoxysilane and a subsequent reaction with 3-hydroxypropionitrile. The functional groups of –OH and –CN on the CN-OH-monolith provided more polar interactive sites for the polar analytes than the single –CN functional group on the CN-monolith did. Thus, the CN-OH-monolith column gave higher selectivity for the separations of polar compounds including nucleosides, nucleic acids, bases, phenols and chloro-substituted phenols, and nitrophenyl derivatives of mono- and oligosaccharides. Retention of the solutes could be adjusted by the organic content of the mobile phase. The separation could be completed within 5 min due to the strong EOF over a wide range of mobile phase composition.

### Rationale and Significance of the Study

It is apparent from the above overview of the recent advances in the design of polar stationary phases for NP-CEC that the search for new polar ligands is still in full swing,

and especially the polar ligands for bonded silica monoliths. The design of monolithic polar stationary phases for CEC that exhibit sufficient retention and selectivity for a wide spectrum of polar solutes requires overcoming the inherent low surface area of monoliths which leads to relatively low phase ratio, and consequently low retention and selectivity. On this basis, the polar ligands that were previously tested in HPLC may not be very useful in CEC simply because HPLC uses porous microparticles of much higher surface area and consequently a much higher phase ratio for the same chromatographic ligands. To overcome the problem of low surface area, the monoliths must be bonded with much stronger polar ligands than one normally uses for porous particle-based bonded phases. As stated above, Allen and El Rassi from our laboratory have already introduced ligands with dual polar groups such as the CN-OH-monolith, which proved useful for the separation of polar compounds by NP-CEC.

In this dissertation, we propose a novel polar ligand to further the development of NP-CEC to solve a wider range of separation problems. In this regard, 1*H*-imidazole-4,5-dicarbonitrile (IDCN) ligand, which has 4 polar functions namely two cyano and two basic functions, was bonded to silica-based monoliths confined into narrow capillaries. The relatively strong polar property of IDCN makes this ligand superior to the 3-hydroxypropionitrile bonded phases (denoted as CN-OH-monolith) previously described by Allen and El Rassi [16]. In fact, and as will be shown in Chapters II and III, the silica monolith with surface bound IDCN (referred to as 2CN-OH-monolith) provided higher retention and selectivity in the separation of polar solutes such as

carbohydrates, peptides and basic compounds when compared to CN-OH-monolith. The higher retention and selectivity offered by the 2CN-OH-monolith are attributed the presence of more polar sites on the surface of the stationary phase for interaction with the solutes to be separated. This represents a major advance in the design of polar stationary phase not only for CEC but also for HPLC with monolithic columns.

### Conclusion

This chapter summarized some basic concepts and principles of capillary electrophoresis and capillary electrochromatography. The sol-gel technology and its application in the fabrication of monolithic columns were outlined. Also, the recent advances in the design and application of the polar bonded stationary phases for hydrophilic interaction capillary electrochromatography were reviewed. Finally, an account for the rationale and significance of the present study has been well asserted.

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## CHAPTER II

# SYNTHESIS OF SILICA-BASED MONOLITHIC POLAR BONDED STATIONARY PHASES AND THEIR ELECTROCHROMATOGRAPHIC CHARACTERIZATION

### Introduction

Monolithic columns have the merits over the traditional packing columns in that monolith offers, among other things, higher separation efficiency and permeability than packed column and avoids the problems of column packing and frit-making.

To exploit the full potential of monolithic columns in CEC, researchers are currently focusing their efforts on the surface modification of monoliths to immobilize novel chromatographic ligands in order to achieve CEC separation by various chromatographic modes, e.g., normal-phase chromatography (NPC), reversed-phase chromatography (RPC), ion-exchange chromatography (IEC), size-exclusion chromatography (SEC) and affinity chromatography (AC). While some non-polar ligands, which were successfully used in HPLC, e.g., octyl and octadecyl bonded phases, have been successfully implemented in column modification in CEC [1-8], much less attention has been paid to bonding polar ligands to silica-based monoliths [9-11] for use

in normal phase CEC (NP-CEC). Thus, it is the aim of the present investigation to introduce silica-based monoliths bonded with a novel polar ligand, namely 1*H*-imidazole-4,5-dicarbonitrile (IDCN), and the resulting stationary phase is denoted by 2CN-OH monolith. As will be shown in this chapter and in Chapter III, the new 2CN-OH column was found to have higher retentivity and higher selectivity than previously described cyano column [11] toward the solutes tested under otherwise the same elution conditions. The 2CN-OH monolithic column was applied to the separations of carbohydrates, peptides and basic compounds with satisfactory separation efficiency (see Chapter III also).

## Experimental

### Instruments

The instrument for the CEC studies was a P/ACE 5010 capillary electrophoresis system from Beckman Instruments (Fullerton, CA, USA) equipped with a UV detector and a data handling system comprised of an IBM personal computer and P/ACE Station software. In the process of column fabrication, a Sigma 3 gas chromatograph from Perkin-Elmer (Norwalk, CT, USA) was used for the temperature programming, and an Isotemp refrigerated circulator (Model 910) and an Isotemp oven (Model 615G) for heat treatment and incubations were from Fisher Scientific (Fair Lawn, NJ, USA).

## Reagents and Materials

Fused silica capillaries having 100  $\mu\text{m}$  I.D. and 375  $\mu\text{m}$  O.D. were purchased from Polymicro Technologies (Phoenix, AZ, USA). Poly(ethylene glycol) (PEG) with an average MW of ca. 10,000, tetramethylorthosilicate (TMOS), 3-hydroxypropionitrile and boron trifluoride diethyl etherate were purchased from Aldrich Chemical Co., Inc (Milwaukee, WI, USA). Hydrochloric acid, sulfuric acid, toluene and acetonitrile were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Ammonium hydroxide, ethanol and DMF were purchased from Pharmco (Brookfield, CT, USA). Acetic acid was obtained from EM Science (Gibbstown, NJ, USA).  $\gamma$ -Glycidoxypyltrimethoxysilane was purchased from Hüls America Inc. (Bristol, PA, USA). 1*H*-Imidazole-4,5-dicarbonitrile was purchased from Nippon Soda Co. Ltd (Chiyoda-ku, Tokyo, Japan).

## Column Fabrication

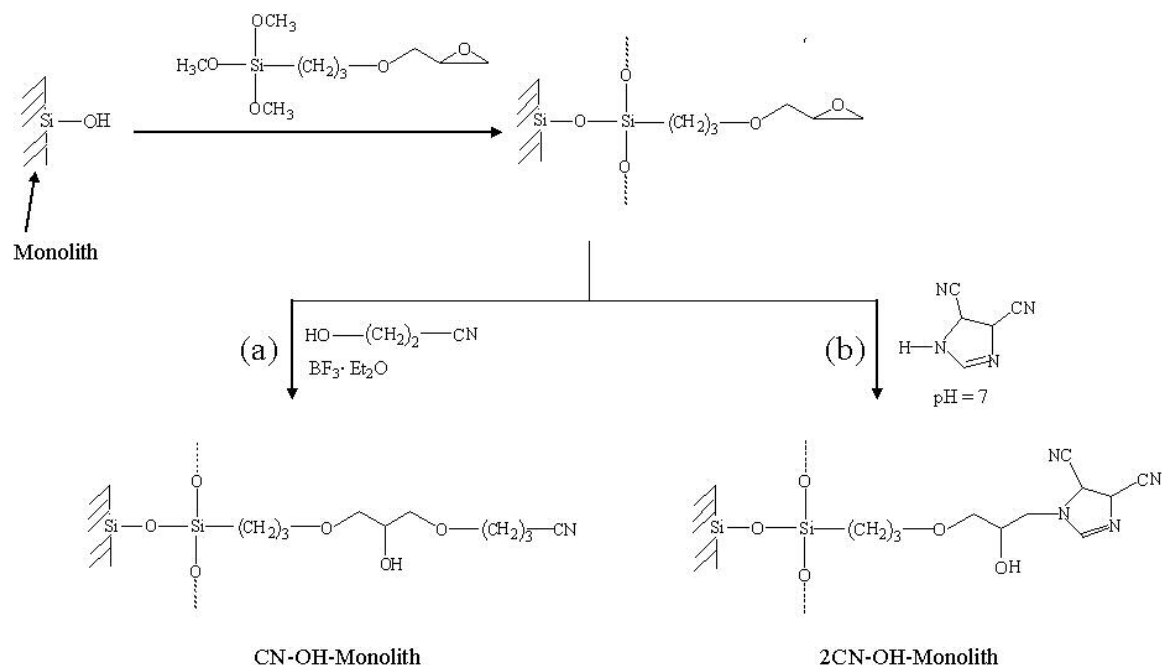
The method used to generate the monolith is similar to the one reported by Ishizuka et al. [6] and slightly modified by Allen and El Rassi [8,11,15]. A segment of 100  $\mu\text{m}$  I.D. fused-silica capillary with 37 cm in length was pretreated by rinsing and filling with 1.0 M NaOH followed by 0.2 M HCl for 30 min, respectively. The capillary was ready for use after being rinsed with water and dried with helium. A sol-gel solution was prepared by dissolving 198.9 mg PEG in 1250  $\mu\text{L}$  of 0.01 M acetic acid solution with stirring at room temperature for 2 min and then at 0  $^{\circ}\text{C}$  for 10 min, and the subsequent mixing with 1250  $\mu\text{L}$  of TMOS. The mixture was stirred at 0  $^{\circ}\text{C}$  for 45 min. Then the

sol-gel solution was introduced into the pretreated capillary leaving 12-cm segment at one end empty. The two ends were connected with a piece of Teflon tubing. The capillary was then put in an Isotemp refrigerated circulator at a temperature of 40 °C for 14 h. The monolith thus formed was rinsed with water and then with 0.01 M NH<sub>4</sub>OH before it was subjected to the heating treatment at 120 °C for 1 h. After rinsing with water and ethanol and then drying with helium for 30 min, the column was subjected to a gradient heating treatment by a GC temperature programmed oven, where temperature was increased at a rate of 2.5 °C/min from an initial temperature of 30 °C (which was held for 2 min) to 180 °C (held for 1 h) and finally to 330 °C (held for 21 h).

#### Bonding of the Polar Ligand to the Surface of the Silica Monolith

Figure 1 illustrates the various reaction steps involved in the preparation of a polar silica monolithic capillary column. The silica monolithic column, prepared as described in the preceding section, was filled with a solution of  $\gamma$ -glycidoxypolytrimethoxysilane in toluene (1:10 v/v) and incubated in an oven at 110 °C for 2 h. This step was repeated three times. The column was then rinsed with toluene, DMF, H<sub>2</sub>O and finally a solution of 0.1 M 1*H*-imidazole-4,5-dicarbonitrile adjusted with a 2.0 M NaOH solution to have a pH of 7.0. The column was then incubated in an oven at the temperature of 70 °C for 14 h, and was treated twice in the same way for 3 h. After rinsing with water, a detection window was made in the empty segment at 12 cm from the inlet. The monolithic column bonded with 2CN-OH was then ready to be used. In the case of modifying the monolith with CN-OH, the column

treated with  $\gamma$ -glycidoxypyrtrimethoxysilane was rinsed with toluene, then DMF and finally a solution containing DMF (200  $\mu$ L)/3-hydroxypropionitrile (20  $\mu$ L)/boron trifluoride diethyl etherate (3  $\mu$ L). The column obtained was incubated at room temperature for 14 h, and was treated twice in the same way for 3 h. The column was ready for use after subsequent rinsing with acetonitrile and water [11].



**Figure 1.** Reaction schemes for the bonding of polar ligands to the silica monoliths. (a) CN-OH-monolith (b) 2CN-OH-monolith

## Results and Discussion

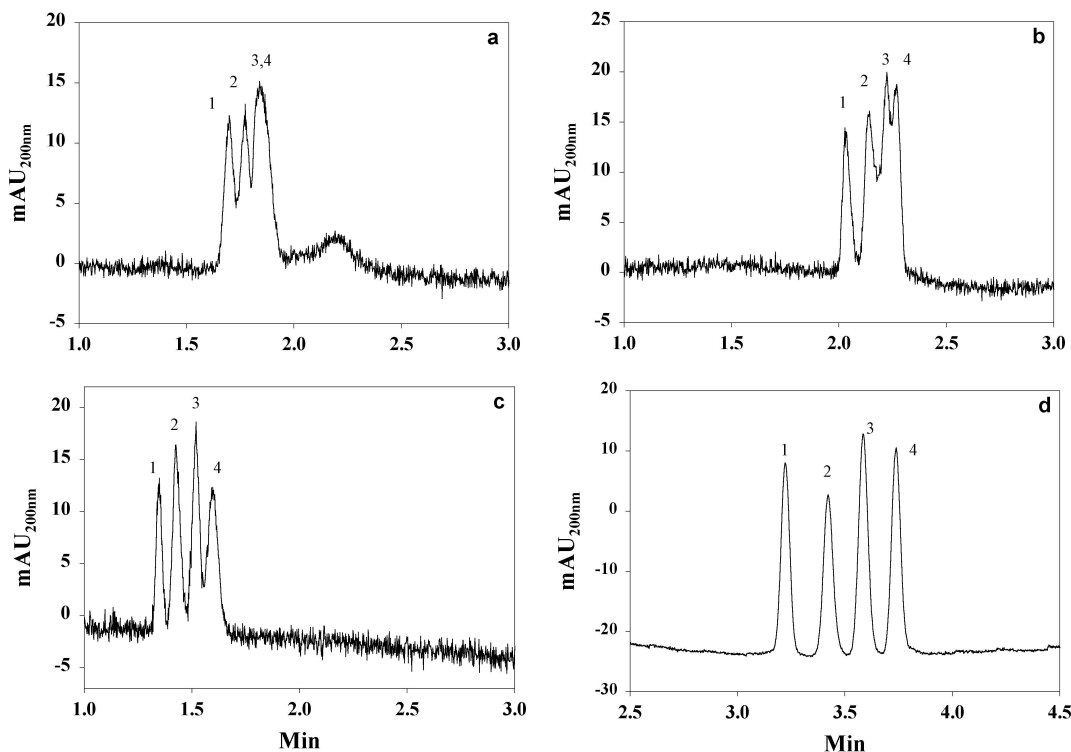
### Optimization of Column Fabrication

In the formation of the monolith, the nature of solvent and the amount of silica and PEG affect the structure of the monolith [12]. PEG, which forms hydrogen bonds

with the silanols, was used as an additive to induce the phase separation and to generate macropores along the sol-gel network. Ammonium hydroxide, the pore-tailoring reagent, creates mesopores on the surface of the gel network. Solvent water, which is the fluid phase, is believed to leave behind pore space after drying.

Effects of TMOS/PEG/H<sub>2</sub>O Ratio. In this investigation, the effect of the ratio of TMOS: PEG: H<sub>2</sub>O at a constant ammonium hydroxide post treatment time of 1 h on the resulting monolith was studied. Figure 2 shows the different separations corresponding to different ratios of TMOS: PEG: H<sub>2</sub>O. It was found that increasing the ratio of the sol-gel precursor to the porogen or the solvent could effectively lower EOF, promote retention and at the same time improve column efficiency (see Figure 2 and Table 1). Excessive sol-gel precursor might lead to the problems of low current and poor flow. The amount of the sol-gel precursor was best when the EOF was 5 - 6 cm/min with a mobile phase containing 5% v/v of 5 mM NH<sub>4</sub>Ac (pH 6.0) and 95% v/v acetonitrile using a running voltage of 20 kV.

Also, Figure 2 and Table 1 show that increasing the amount of solvent or PEG lead to the formation of larger pores, which result in higher EOF and lower retention. While TMOS was required for the sol-gel monolith formation, too much TMOS resulted in a significant decrease in column permeability and consequently flow problems, which are indicative of the presence of narrow pore space in the silica network. In this investigation, the ratio of TMOS/PEG/H<sub>2</sub>O of 863  $\mu$ L/198.9 mg/1875  $\mu$ L was found to yield good EOF, retention and selectivity, see Figure 2d.



**Figure 2.** Electrochromatograms of test mixture on columns with different ratio of TMOS/PEG/H<sub>2</sub>O. Conditions: monolithic capillary column, 20/27 cm × 100 μm ID; mobile phase, hydro-organic solution made up of 5% v/v of 5 mM NH<sub>4</sub>Ac (pH 6.0) and 95% v/v acetonitrile; voltage, 20 kV; wavelength, 200 nm; column temperature, 20 °C. TMOS(μL)/PEG(mg)/H<sub>2</sub>O(μL): a. 750/238.7/2250, b. 825/218.8/1875, c. 750/198.9/1875, d. 863/198.9/1875. Solutes: 1, toluene; 2, DMF; 3, formamide; 4, thiourea.

Pore Tailoring. The effect of ammonium hydroxide treatment was carried out with the monolith obtained with the TMOS/PEG/H<sub>2</sub>O ratio of 750 μL/198.9 mg/1875 μL. The diluted ammonium hydroxide solution with a pH of 8.0 was used to generate the mesopores within the skeleton of the silica monolith. The dissolution of the sol-gel network originated by the slightly basic ammonium hydroxide solution leads to the formation of the mesopores. Mesopores with proper size could be achieved by varying the temperature and the treatment time with NH<sub>4</sub>OH [8,13,14]. In this investigation, the temperature was set at 120 °C while the effect of treatment time was studied. Figure 3 shows the electrochromatograms of the test sample on the monolithic columns treated



with ammonium hydroxide for different time periods. As shown in Table 2, the EOF, retention factor  $k'$  and selectivity factor  $\alpha$  varied as the reaction time changed from

TABLE 1.  
EFFECT OF RATIO OF TMOS/PEG/H<sub>2</sub>O ON COLUMN PERFORMANCE

Column	EOF (cm/min)	$k'$		
		DMF	Formamide	Thiourea
a	11.79	0.04	0.08	0.08
b	9.87	0.06	0.09	0.12
c	14.84	0.06	0.13	0.19
d	5.04	0.10	0.21	0.30

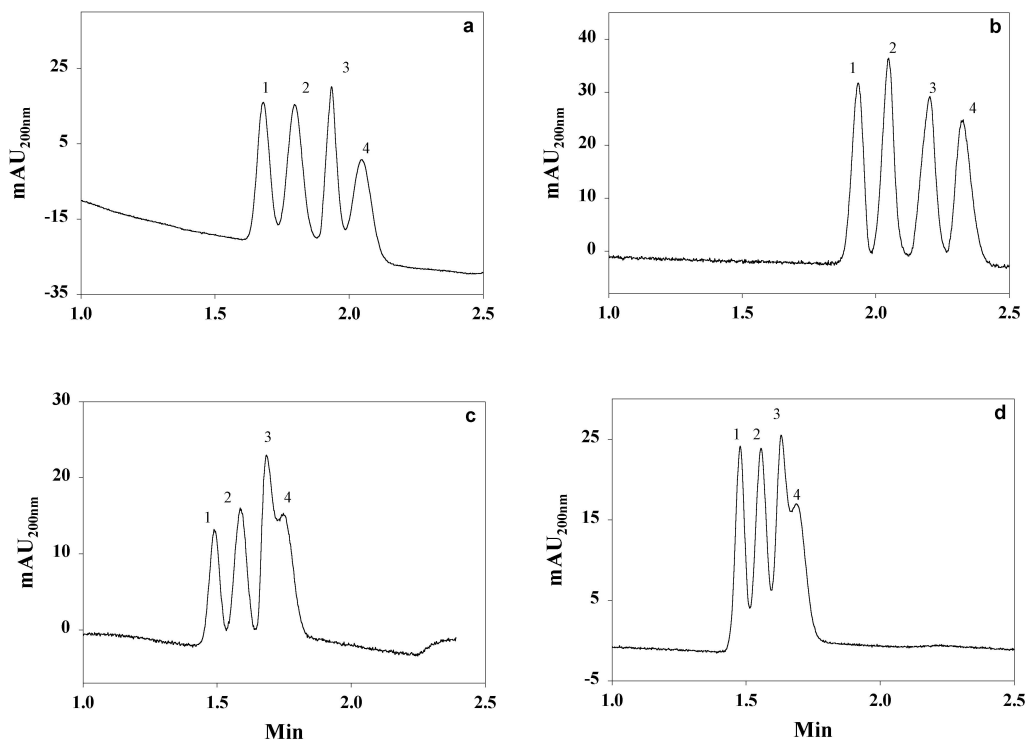
*Conditions: monolithic capillary column, 20/27 cm  $\times$  100  $\mu$ m ID; mobile phase, hydro-organic solution made up of 5% v/v of 5 mM NH<sub>4</sub>Ac (pH 6.0) and 95% v/v acetonitrile; voltage, 20 kV; wavelength, 200 nm; column temperature, 20 °C. TMOS( $\mu$ L)/PEG(mg)/H<sub>2</sub>O( $\mu$ L): a. 750/238.7/2250, b. 825/218.8/1875, c. 750/198.9/1875, d. 863/198.9/1875.*

TABLE 2.  
EFFECT OF TREATMENT TIME OF NH<sub>4</sub>OH ON COLUMN PERFORMANCE

Treatment Time (hr)	EOF (cm/min)	$k'$			$\alpha_{\text{FA/DMF}}$	$\alpha_{\text{TU/FA}}$
		DMF	Formamide (FA)	Thiourea (TU)		
0.5	11.90	0.07	0.15	0.22	2.25	1.44
1	10.34	0.06	0.14	0.20	2.39	1.47
1.5	13.44	0.07	0.13	0.17	2.00	1.29
2	13.53	0.05	0.10	0.14	2.02	1.36

*Conditions: monolithic capillary column, 20/27 cm  $\times$  100  $\mu$ m ID; mobile phase, hydro-organic solution made up of 5% v/v of 5 mM NH<sub>4</sub>OH (pH 6.0) and 95% v/v acetonitrile; voltage, 20 kV; wavelength, 200 nm; column temperature, 20 °C. a. 0.5 h, b. 1.0 h, c. 1.5 h, d. 2.0 h.*

0.5 h to 2 h. As the treatment time of  $\text{NH}_4\text{OH}$  increased, retention time declined. This can be attributed to the low phase ratio resulting from the larger pores as a consequence



**Figure 3.** *Electrochromatograms of a test mixture on columns with different pore-tailoring time with ammonium hydroxide. Conditions: monolithic capillary column, 20/27 cm  $\times$  100  $\mu\text{m}$  ID; mobile phase, hydro-organic solution made up of 5% v/v of 5 mM  $\text{NH}_4\text{OH}$  (pH 6.0) and 95% v/v acetonitrile; voltage, 20 kV; wavelength, 200 nm; column temperature, 20  $^\circ\text{C}$ . a. 0.5 h, b. 1.0 h, c. 1.5 h, d. 2.0 h. Solutes as shown in Fig. 2.*

of longer treatment time. The enlargement of the pore also resulted in the increase of EOF velocity. This may be explained by the decrease in the extent of electric double layer overlap as the pores are being transformed from micro to mesopores by prolonged  $\text{NH}_4\text{OH}$  treatment. In other words, an EOF can be established within the mesopores as the size of these pores increases. An exception to this trend was observed when the treatment time was shorter than 1 h where the EOF decreased slightly from 11.9 cm/min

to 10.3 cm/min as the treatment time was increased from 0.5 h to 1 h. This may be explained by a better accessibility of the silanols to react with the organosilane thus reducing the amount of free silanols and in turn the amount of ionizable groups to support the EOF.

The highest selectivity was achieved when the treatment time was 1 h. It was also found that the mesopore size was too big when the treatment time was longer than 1 h. This is manifested by a higher EOF, lower retention and lower selectivity factor  $\alpha$  (Figure 3c and 3d), and consequently less satisfactory component separation. The column treated for 0.5 h showed slightly stronger retention but higher EOF than that treated for 1 h (Figure 5a and b). The optimum condition was found to be 863  $\mu$ L TMOS, 198.9 mg PEG, 1875  $\mu$ L H<sub>2</sub>O and a reaction time of 1 h for NH<sub>4</sub>OH, under which the column obtained has an acceptable EOF and a maximum selectivity factor, see Table 2.

#### Comparison of CN-OH-Monolith and 2CN-OH-Monolith

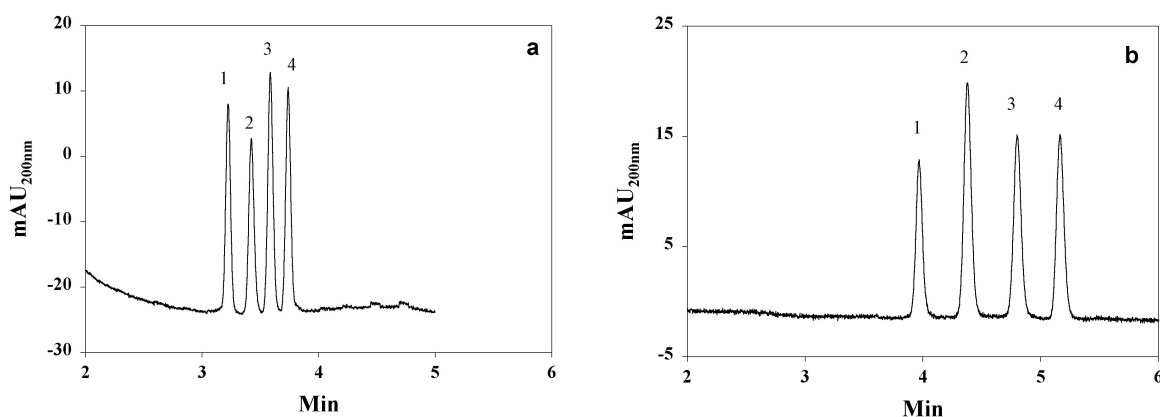
In order to assess the performance of the 2CN-OH monolith with respect to the previously described polar cyano phases such as the CN-OH monolith [11], the 2CN-OH and CN-OH-monolithic columns were produced using the same sol-gel method described in the Experimental, and their CEC performance was compared under the same elution conditions. A test mixture consisting of toluene, DMF, formamide and thiourea was used to test the column separation efficiency and retention characteristics. Figure 4 and Table 3 show the separation, EOF velocity and the  $k'$  values of the test solutes on the two columns. The 2CN-OH-monolithic column exhibited stronger retention ( $\sim$  double) when

TABLE 3.

COMPARISON OF RETENTION FACTOR, PLATE NUMBER AND EOF OF  
CN-OH-MONOLITH AND 2CN-OH-MONOLITH

Ligand	EOF (cm/min)	N (plates/meter)	k'		
			DMF	Formamide	Thiourea
CN-OH	6.21	144,000	0.06	0.11	0.16
2CN-OH	5.04	133,000	0.10	0.21	0.30

*Conditions: monolithic capillary column, 20/27 cm  $\times$  100  $\mu$ m ID; mobile phase, hydro-organic solution made up of 5% v/v of 5 mM  $\text{NH}_4\text{Ac}$  (pH 6) and 95% v/v acetonitrile; voltage, 20 kV; wavelength, 200 nm; column temperature, 20  $^\circ\text{C}$ .*

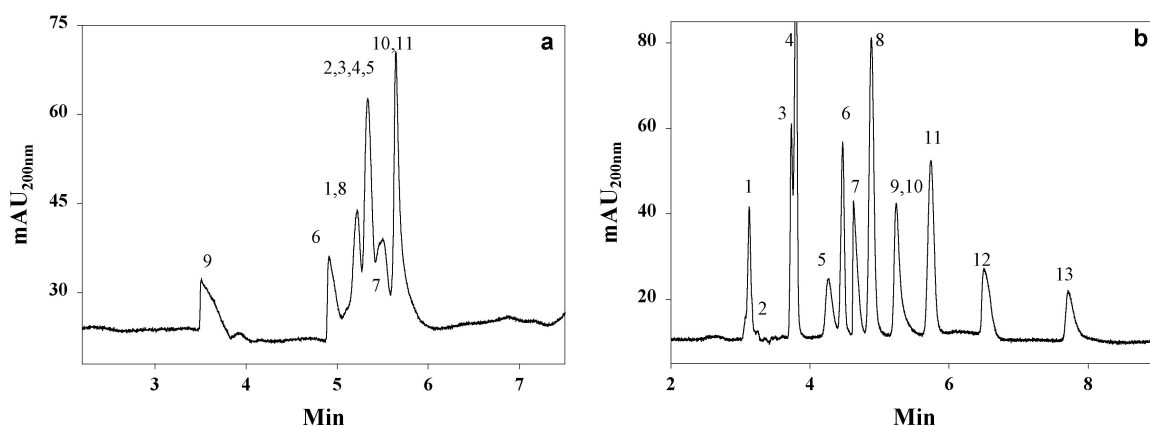


**Figure 4.** *Electrochromatograms of a test mixture on two monolithic columns. Conditions: monolithic capillary column, 20/27 cm  $\times$  100  $\mu$ m ID; mobile phase, hydro-organic solution made up of 5% v/v of 5 mM  $\text{NH}_4\text{Ac}$  (pH 6) and 95% v/v acetonitrile; voltage, 20 kV; wavelength, 200 nm; column temperature, 20  $^\circ\text{C}$ . a. CN-OH-monolithic column; b. 2CN-OH-monolithic column. Solutes as shown in Figure 2.*

compared to the CN-OH-monolithic column for all of the solutes tested with a lower EOF velocity. The stronger retention of the 2CN-OH monolith indicates a higher polar phase ratio arising from the presence of 2 polar cyano groups and 2 basic functions for each

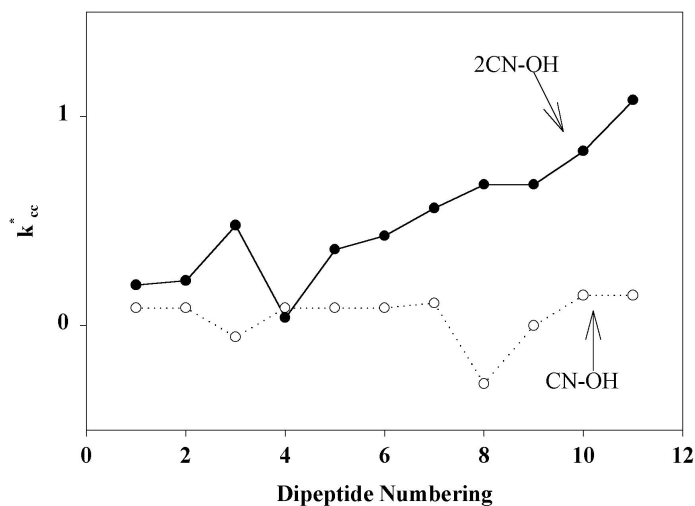
immobilized IDCN ligand, and may indicate a greater extent of reaction of the silica monolith with the IDCN (2CN-OH-monolith) than with the hydroxy propionitrile (CN-OH-monolith). The higher extent of surface modification may also explain the weaker EOF exhibited by the 2CN-OH-monolith since this will yield a surface with a lower free silanol density and less charge to support a strong EOF.

In order to better assess the difference between the two cyano monolithic phases, another test mixture consisting of 11 dipeptides was used to compare the two columns. The dipeptides showed different retention times on the 2CN-OH-monolithic column and this resulted in the fact that all of the solutes were separated from each other, while on the CN-OH-monolithic column, solutes showed similar retention and some of the peaks overlapped, see Figure 5. Figure 6 shows the values of peak locator  $k_{cc}^*$  on the two different columns. More details on the retention parameters  $k_{cc}^*$  will be discussed in the next chapter (Chapter III). Again, the significantly higher retention and selectivity of the



**Figure 5.** Electrochromatograms of dipeptides on two monolithic columns. Conditions: monolithic capillary column, 20/27 cm  $\times$  100  $\mu$ m ID; mobile phase, hydro-organic solution made up of 25% v/v of 5 mM  $\text{NH}_4\text{Ac}$  (pH 3.9) and 75% v/v acetonitrile; voltage, 20 kV; wavelength, 200 nm; column temperature, 20  $^\circ\text{C}$ . . a. CN-OH-monolithic column; b. 2CN-OH-monolithic column. Solutes: 1, toluene; 2, Phe-Pro-1; 3, Leu-Phe-1; 4, Leu-Phe-2; 5, Phe-Pro-2; 6, Ala-Phe; 7, Gly-Leu; 8, Gly-Phe; 9, Gly-Trp; 10, Gly-Lys;

11, *Gly-Tyr*; 12, *Gly-Ala*; 13, *Met-Glu*.



**Figure 6.**  $k'_{cc}$  of dipeptides on CN-OH-monolithic column and 2CN-OH-monolithic column. Solutes from left to right: 1, *Leu-Phe-1*; 2, *Leu-Phe-2*; 3, *Gly-Leu*; 4, *Phe-Pro-1*; 5, *Phe-Pro-2*; 6, *Ala-Phe*; 7, *Gly-Phe*; 8, *Gly-Lys*; 9, *Gly-Trp*; 10, *Gly-Ala*; 11, *Met-Glu*.

2CN-OH-monolithic column when compared to the CN-OH-monolithic column was due to the different modifying ligands and more specifically the number of their polar functional cyano groups and basic functions. The 2CN-OH ligand exhibited more polarity and hence the polar solutes were retained longer on the column.

## Conclusions

Silica-based monoliths can be produced *via* the sol-gel technology, which employs silica alkoxide as the sol-gel precursor and PEG as the porogen with acetic acid as the catalyst. Ammonium hydroxide can be used to adjust the size of the mesopores. Through an epoxide ring opening reaction, the surface of the silica monolith can be modified with the desired chromatographic ligand to solve different analytical problems. This investigation has yielded a novel polar silica-based monolith suitable for NP-CEC of

polar species. Indeed, the 2CN-OH monolithic silica column exhibited higher retentivity and selectivity than the monofunctional CN-OH monolithic silica column. This represents a major advance in the design of polar monolithic silica columns for normal phase chromatography in general and for NP-CEC in particular.

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## CHAPTER III

### STUDY OF THE ELECTROCHROMATOGRAPHIC BEHAVIORS OF POLAR COMPOUNDS ON THE 2CN-OH-MONOLITHIC SILICA

#### Introduction

The 2CN-OH-monolithic silica is a viable stationary phase for performing normal phase CEC (NP-CEC) which is a CEC mode involving the use of mobile phases that are less polar than the stationary phase. For a bonded normal phase monolithic column to be useful in CEC, the stationary phase must not only have a good pore size distribution but also the proper polar chromatographic ligand that can yield sufficient retention and selectivity. This requirement is ensured by the unique selectivity and strong polar character of the 2CN-OH-monolithic column. In fact, the IDCN polar ligand has two cyano groups and two basic functions through which polar and selective solute interaction can be readily established. As will be shown in this chapter, the 2CN-OH-monolithic column was useful in the NP-CEC separation of carbohydrates, peptides and basic compounds.

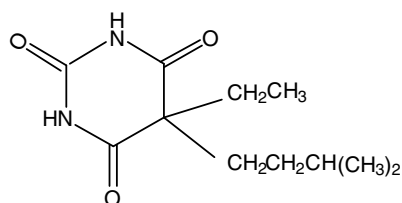
## Experimental

### Instruments

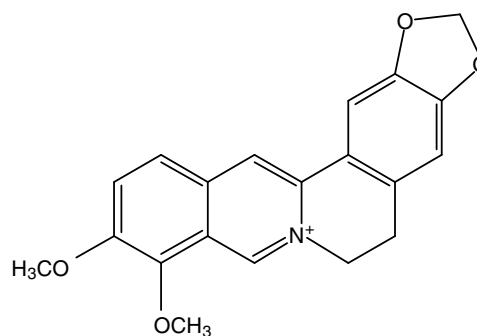
The instrument for the CEC studies was a P/ACE 5010 capillary electrophoresis system from Beckman Instruments (Fullerton, CA, USA) equipped with a UV detector. The data handling system was comprised of an IBM personal computer and P/ACE Station software. The dry-bath for the derivatization of saccharides was the Thermolyne Type 17600 (Dubuque, IA, USA).

### Reagents and Materials

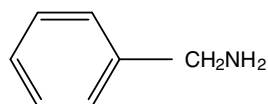
Gramine and 2-aminobenzamide (2AB) were purchased from Aldrich Chemical Co., Inc. (Milwaukee, WI, USA). Glucose, mannose, fucose, N-acetylglucosamine (GlcNAc), N-acetylgalactosamine (GalNAc), sodium cyanoborohydride, Leu-Phe, Met-Glu, Phe-Pro, Gly-Gly-Ala, Gly-Gly-Gly, Gly-Gly-Leu, Gly-Phe-Phe, Gly-Gly-Gly-Gly, Gly-Pro-Gly-Gly, Phe-Gly-Gly-Phe, procainamide, sulfanilamide and theophylline (see Figure 1 for structures) were obtained from Sigma-Aldrich Fine Chemicals (St. Louis, MO, USA). Gly-Ala, Gly-Tyr, Gly-Leu, Gly-Phe, Gly-Pro, Gly-Trp, Ala-Phe were purchased from Mann Research Laboratories (New York, USA). Maltooligosaccharides were purchased from Pfanstiehl Laboratories, Inc. (Waukegan, IL, USA). Diphenhydramine was purchased from Acros Organics (New Jersey, USA). Other reagents are as in Chapter II.



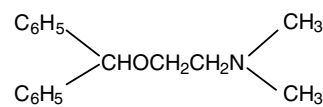
Amobarbital,  $pK_a=7.94$



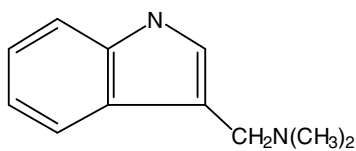
Berberine



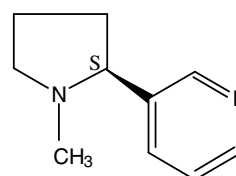
Benzylamine,  $pK_a=9.40$



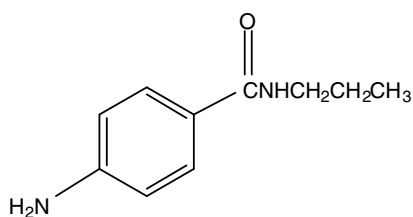
Diphenhydramine,  $pK_a=8.76$



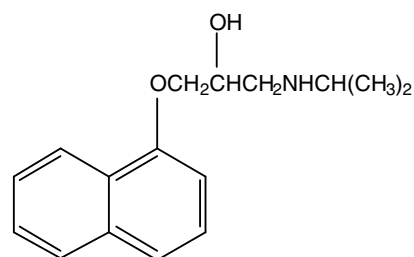
Gramine,  $pK_a=9.67$



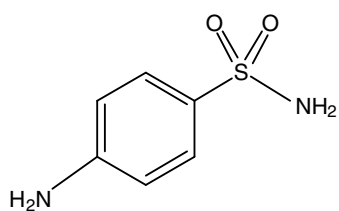
Nicotine,  $pK_a=8.00$



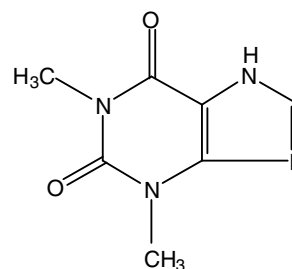
Procainamide,  $pK_a=9.86$



Propranolol,  $pK_{a1}=9.14$ ,  $pK_{a2}=13.84$



Sulfanilamide,  $pK_{a1}=1.85$ ,  $pK_{a2}=10.10$



Theophylline,  $pK_{a1}=1.05$ ,  $pK_{a2}=8.60$

**Figure. 1.** Structures of basic drugs used in this study.

### Derivatization of Saccharides with 2AB

A solution of 200  $\mu\text{L}$  of 0.25 M 2AB prepared in 1:1 methanol: water pH 5.0 (pH adjusted with acetic acid) was added and mixed with 30  $\mu\text{L}$  of 1.0 M  $\text{NaBH}_3\text{CN}$  in a 0.3-mL reaction vial. To this was added 40  $\mu\text{L}$  of 1.4 M aqueous saccharide solution and the reaction was mixed well. The vials were sealed and put in a Dry-bath. The derivatization was allowed to proceed at 72  $^{\circ}\text{C}$  for 24 h.

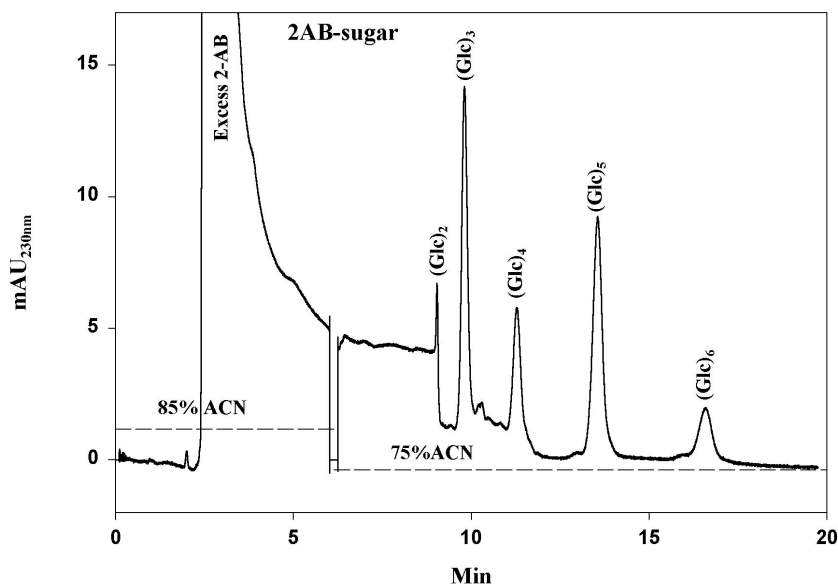
## Result and Discussion

### Separation of Mono- and Oligosaccharides

The CEC behavior of the 2AB-derivatized maltooligosaccharides was examined on the 2CN-OH-monolithic column in order to assess the utility of this polar column in NP-CEC of polar compounds. Carbohydrates are typical polar compounds. As can be seen in Figure 2, good separation according to the degree of polymerization was achieved by stepwise gradient elution using mobile phases containing 5mM  $\text{NH}_4\text{Ac}$  (pH 6.0) at various concentrations of acetonitrile. The step gradient elution involved 85% ACN during 6 min followed by 75% ACN. The net effect of the step gradient was to shorten retention time and compress the peaks thus facilitating the detection of the oligosaccharides with high degree of polymerization.

Using isocratic elution with a mobile phase containing 75% (v/v) ACN, the retention factor  $k'$  of the 2AB-derivatized maltooligosaccharides on the 2CN-OH-monolithic

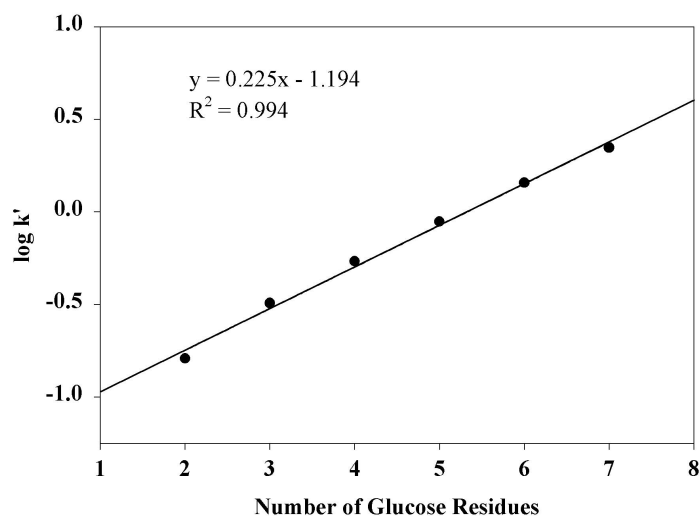
column increased as expected with increasing the number of glucose residues in the sugar molecule. As can be seen in Figure 3,  $\log k'$  increased linearly with increasing the number of glucose residues in the maltooligosaccharides yielding a line with a slope of 0.2248 and a correlation coefficient  $R^2$  of 0.9942.



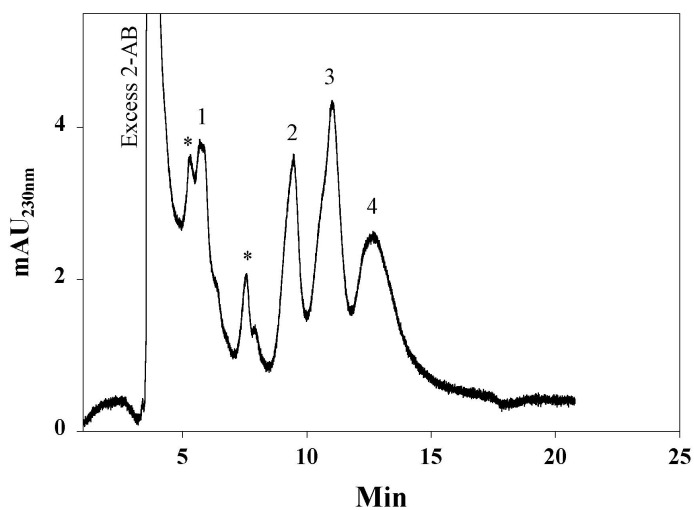
**Figure 2.** *Electrochromatogram of 2AB derivatized maltooligosaccharides on 2CN-OH-monolithic column. Conditions: monolithic capillary column, 20/27 cm  $\times$  100  $\mu$ m ID; mobile phase, hydro-organic solution made up of 15% - 25% v/v of 5 mM  $\text{NH}_4\text{Ac}$  (pH 6.0) and 75% - 85% v/v acetonitrile; voltage, 20 kV; wavelength, 200 nm; column temperature, 20  $^\circ\text{C}$ . Solute concentration: 0.018M.*

The retention behavior of the 2AB derivatized monosaccharides, including fucose, GalNAc, mannose and GlcNAc were also examined on the 2CN-OH-monolithic column with a mobile phase containing 5 mM  $\text{NH}_4\text{Ac}$  aqueous solution at various acetonitrile concentrations in the range 91% - 97% (v/v) ACN. A typical electrochromatogram is shown in Figure 4. As shown in Figure 5, logarithmic retention factor ( $\log k'$ ) is a linear relationship with the percentage of acetonitrile in the mobile phase, yielding a slope of

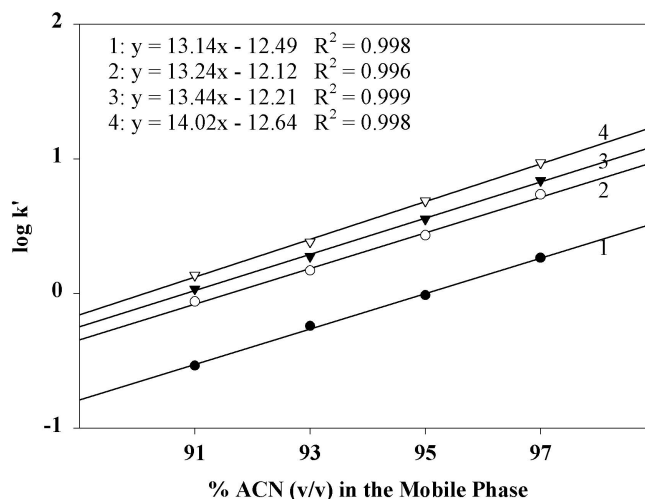
13.14, 13.24, 13.44 and 14.02 for 2AB-fucose, 2AB-GalNAc, 2AB-mannose and 2AB-GlcNAc, respectively. The slope is indicative of the polarity of the solute and reflects the magnitude of the polar contact area between the solute and the stationary phase surface.



**Figure 3.** Dependence of  $\log k'$  on the number of glucose residues.



**Figure 4.** Electrochromatogram of monosaccharides on the 2CN-OH-monolithic column. Conditions: monolithic capillary column, 20/27 cm  $\times$  100  $\mu$ m ID; mobile phase, hydro-organic solution made up of 5% v/v of 5 mM  $\text{NH}_4\text{Ac}$  (pH 6.0) and 95% v/v acetonitrile; voltage, 20 kV; wavelength, 230 nm; column temperature, 20  $^\circ\text{C}$ . Solute concentration: 0.018 M. Solutes: 1, 2AB-fucose; 2, 2AB-GalNAc; 3, 2AB-mannose; 4, 2AB-GlcNAc; \*, unknown.



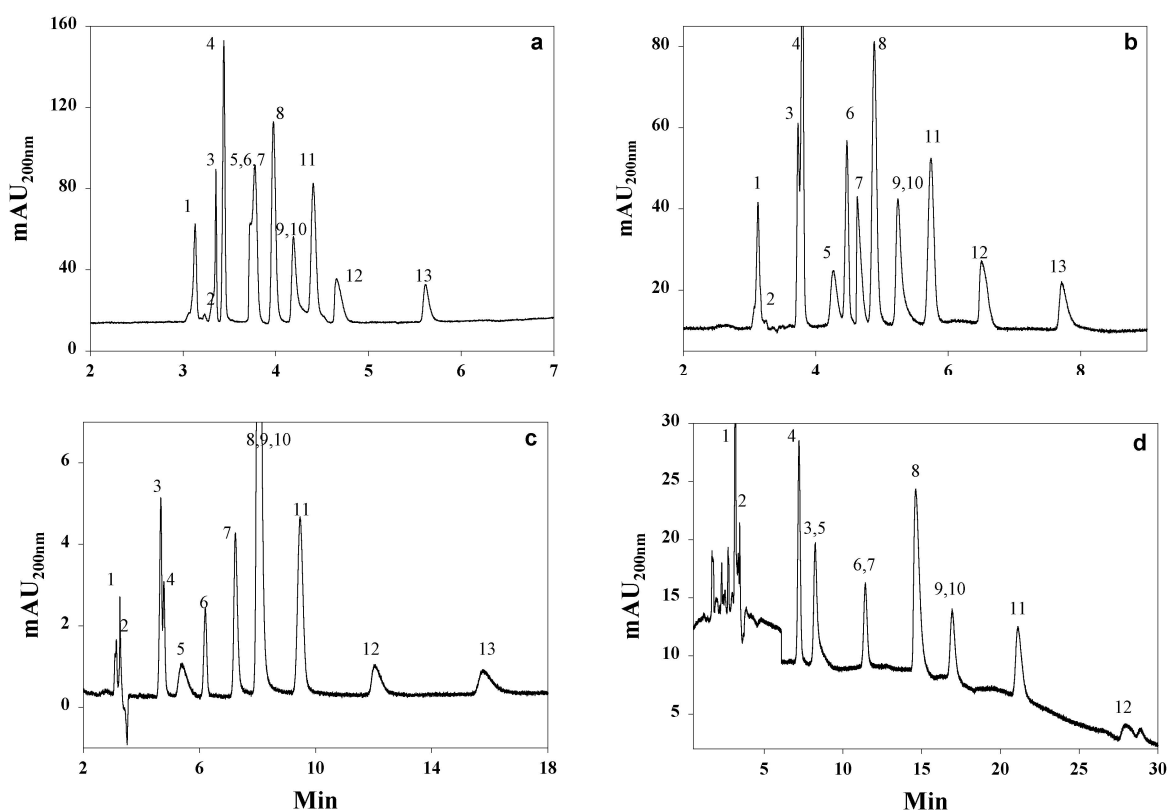
**Figure 5.** Dependence of  $\log k'$  on the concentration of acetonitrile in the mobile phase. 1, 2AB-fucose; 2, 2AB-GalNAc; 3, 2AB-mannose; 4, 2AB-GlcNAc.

### Separation of Peptides

Separation of Dipeptides Although capillary zone electrophoresis (CZE) was reported to be successful in the separation of peptides on the basis of differences in their charge-to-mass ratio [1,2,3], it was found that normal phase chromatography was more powerful to achieve high selectivity [4,5]. To evaluate the usefulness of the 2CN-OH-monolithic column in the NP-CEC of peptides, ten standard dipeptides having different degrees of polarity and charges were selected and electrochromatographed on the 2CN-OH-monolithic column, and for sake of comparison on 1CN-OH-monolithic column using a mobile phase containing acetonitrile and 5 mM  $\text{NH}_4\text{Ac}$  (pH 3.9). Figure 6 shows the electrochromatograms of the separation of the dipeptides with different acetonitrile content in the mobile phase. As the concentration of acetonitrile increased in the range of 70% - 85% (v/v), the dipeptides were more retarded and yielded a higher

selectivity factor  $\alpha$  (Table 1).

To assess the contribution of electrophoretic mobility to the observed migration of dipeptides in CEC, the migration behavior of these dipeptides was examined in the CZE mode using a running electrolyte of 5 mM  $\text{NH}_4\text{Ac}$ , pH 3.9 containing 70%, 75%, 80% and 85% ACN, which is the same as the mobile phase composition used in CEC. Under these conditions and involving different concentration of ACN, the ionization of the



**Figure 6.** Electrochromatograms of dipeptides obtained on the 2CN-OH-monolithic column. Conditions: monolithic capillary column, 20/27 cm  $\times$  100  $\mu\text{m}$  ID; voltage, 20 kV; wavelength, 200 nm; column temperature, 20  $^{\circ}\text{C}$ . ; mobile phase, hydro-organic solution made up of 5 mM  $\text{NH}_4\text{Ac}$  (pH 6.0) and acetonitrile, a. 70% ACN; b. 75% ACN; c. 80% ACN; d. 85% ACN. Solutes: 1, toluene; 2, Phe-Pro-1; 3, Leu-Phe-1; 4, Leu-Phe-2; 5, Phe-Pro-2; 6, Ala-Phe; 7, Gly-Leu; 8, Gly-Phe; 9, Gly-Trp; 10, Gly-Lys; 11, Gly-Tyr; 12, Gly-Ala; 13, Met-Glu.



TABLE 1.

## EFFECT OF % ACN ON SELECTIVITY FACTOR

70%			75%			80%			85%		
Solute	$k_{cc}^*$	$\alpha$	Solute	$k_{cc}^*$	$\alpha$	Solute	$k_{cc}^*$	$\alpha$	Solute	$k_{cc}^*$	$\alpha$
Phe-Pro-1	0.03	2.15	Phe-Pro-1	0.04	5.10	Phe-Pro-1	0.04	13.24	Phe-Pro-1	0.09	14.56
Leu-Phe-1	0.07		Leu-Phe-1	0.19		Leu-Phe-2	0.49		Leu-Phe-2	1.28	
Leu-Phe-2	0.10	1.39	Leu-Phe-2	0.22	1.10	Leu-Phe-1	0.54	1.09	Leu-Phe-1	1.60	1.25
Gly-Leu	0.20	2.07	Phe-Pro-2	0.36	1.69	Phe-Pro-2	0.72	1.34	Phe-Pro-2	1.62	1.00
Phe-Pro-2	0.20	1.00	Ala-Phe	0.43	1.17	Ala-Phe	0.99	1.36	Ala-Phe	2.62	1.61
Ala-Phe	0.20	1.00	Gly-Leu	0.48	1.11	Gly-Leu	1.25	1.26	Gly-Leu	3.11	1.19
Gly-Phe	0.27	1.31	Gly-Phe	0.56	1.17	Gly-Phe	1.34	1.06	Gly-Phe	3.63	1.16
Gly-Lys	0.34	1.25	Gly-Lys	0.68	1.20	Gly-Lys	1.60	1.19	Gly-Lys	4.36	1.20
Gly-Trp	0.34	1.00	Gly-Trp	0.68	1.00	Gly-Trp	1.60	1.00	Gly-Trp	4.36	1.00
Gly-Tyr	0.41	1.20	Gly-Tyr	0.84	1.23	Gly-Tyr	2.06	1.28	Gly-Tyr	5.69	1.30
Gly-Ala	0.49	1.20	Gly-Ala	1.08	1.29	Gly-Ala	2.91	1.41	Gly-Ala	7.88	1.38
Met-Glu	0.79	1.62	Met-Glu	1.47	1.35	Met-Glu	4.36	1.49	Met-Glu	9.93	1.26

Conditions: as in Figure 6.

dipeptides seems to depend on the %ACN present in the mobile phase. At 70% ACN, the dipeptides seem to have a net positive charge as they proceed the EOF marker (toluene) in CZE, with the exception of Met-Glu (net negative charge as it follows the EOF marker) and Phe-Pro (net zero charge, as they co-migrate with the EOF marker). This ionization changes upon increasing the %ACN to 75%. The net positive charge of the dipeptides decreases as the dipeptide Leu-Phe becomes neutral and co-migrates with the toluene while the velocity factor  $k_{ep}^*$  of all peptides decreases, see Table 2. The ionization changes totally at 80% and 85% ACN where all solutes become negatively charged as manifested by the negative values of  $k_{ep}^*$ , see below for details. The effect of organic on the ionization of charged solutes and consequently their electrophoretic migration in CEC is well documented [8]. Under the mode of CZE, the charged molecules were eluted in the order determined by specie size and charge mainly. However, the differences in charge-to-mass ratios among the various peptides were not large enough to warrant their separation as they migrated within a very narrow time window of ~ 35 sec. While in the mode of CEC, in addition to these factors, the polarity of the solute also contributed to the interaction with the stationary phase and therefore affected the elution order to a great extent and brought about improved separation among the various peptides.

In CEC, both the electrophoretic mobility and chromatographic partitioning control the migration of charged solutes such as some of the peptides under investigation. Under this condition, the dimensionless retention factor  $k^*$  for charged solutes is given by the equation [6]:

$$k^* = \frac{t_m(1 + k_{ep}^*) - t_o}{t_o} \quad (1)$$

TABLE 2.

VALUES OF RETENTION PARAMETERS OF DIPEPTIDES UNDER DIFFERENT CONDITIONS

ACN%	70%			75%			80%			85%		
solute	$k_{cc}^*$	$k_{ep}^*$	$k^*$	$k_{cc}^*$	$k_{ep}^*$	$k^*$	$k_{cc}^*$	$k_{ep}^*$	$k^*$	$k_{cc}^*$	$k_{ep}^*$	$k^*$
Leu-Phe-1	0.07	0.05	0.12	0.19	0	0.19	0.54	-0.14	0.27	1.60	-0.035	1.51
Leu-Phe-2	0.10	0.02	0.12	0.22	0	0.22	0.49	-0.14	0.31	1.28	-0.22	0.77
Gly-Leu	0.20	0.10	0.32	0.48	0.06	0.58	1.25	-0.03	1.18	2.62	-0.04	2.95
Phe-Pro-1	0.03	0	0.03	0.04	0	0.04	0.04	-0.06	-0.02	0.09	-0.08	0.00
Phe-Pro-2	0.20	0	0.21	0.36	0	0.36	0.72	-0.06	0.63	1.62	-0.08	1.40
Ala-Phe	0.20	0.02	0.24	0.43	0.02	0.45	0.99	-0.02	0.94	2.62	-0.05	2.44
Gly-Phe	0.27	0.05	0.33	0.56	0.03	0.61	1.34	-0.04	1.24	3.63	-0.08	3.26
Gly-Lys	0.34	0.62	1.17	0.68	0.56	1.61	1.60	0.50	2.91	4.36	0.32	6.06
Gly-Trp	0.34	0.06	0.42	0.68	0.04	0.75	1.60	-0.03	1.53	4.36	-0.05	4.09
Gly-Tyr	0.41	0.03	0.44	0.84	0.02	0.87	2.06	-0.05	1.92	5.69	-0.08	5.16
Gly-Ala	0.49	0.06	0.58	1.08	0.05	1.17	2.91	-0.05	2.72	7.88	-0.07	7.24
Met-Glu	0.79	-0.26	0.32	1.47	-0.27	0.79	4.36	-0.54	1.44	9.93	-0.53	4.12

*Conditions: as in Figure 6.*

where  $k_{ep}^*$  is the velocity factor,  $t_m$  is the migration time of the analyte and  $t_0$  is the migration time of the EOF marker in the CEC column.  $k_{ep}^*$  describes the contribution of electrophoretic mobility to the separation of a charged species in CEC and is given by:

$$k_{ep}^* = \frac{v_{ep}}{v_{eo}} \quad (2)$$

$k^*$  and  $k_{ep}^*$  are measured under conditions used in the CEC experiments. The electrophoretic velocity  $v_{ep}$  is obtained from separate capillary zone electrophoresis (CZE) measurements in an open tube using the same mobile phase and other conditions as in the CEC experiment. The electroosmotic velocity (i.e., mobile phase velocity)  $v_{eo}$ , is the actual “interstitial” EOF velocity in the monolithic column, which is equal to the apparent EOF velocity,  $v_{eo}^*$ , within the CEC column multiplied by the ratio of current in open tube to that in the monolithic or packed column [7]. For neutral solutes,  $k_{ep}^* = 0$ , and consequently  $k^* = k'$ , the retention factor normally encountered in chromatography.

Because of the presence of  $k_{ep}^*$  in eq 1 which reflects the electrophoretic process,  $k^*$  does not serve as a useful peak locator as its counterpart  $k'$  in chromatography. To reflect the elution order of charged solutes in CEC, a peak locator,  $k_{cc}^*$ , based on chromatographic formalism, has been suggested [6]:

$$k_{cc}^* = \frac{t_m - t_0}{t_0} \quad (3)$$

Despite the fact that  $k_{cc}^*$  is devoid of any mechanistic insight, and has limited utility, it proved useful for calculating resolution [209]. Its value increases with the migration time as  $k'$  in chromatography.  $k_{cc}^*$  is negative for components migrating faster than the EOF tracer (e.g., Gly-Leu, Gly-Lys on the 1CN-OH-monolith).

The retention parameters of the dipeptides are shown in Figures 7 and 8 and Tables 2 and 3. As can be seen in Table 3, the 2CN-OH-monolith yielded higher retention parameters  $k^*$  and  $k_{cc}^*$  than the 1CN-OH-monolith under otherwise the same mobile phase composition. This is another quantitative confirmation of the higher polarity and in turn retentivity and selectivity of the 2CN-OH monolith toward polar

TABLE 3.

VALUES OF RETENTION PARAMETERS OF DIPEPTIDES ON TWO DIFFERENT MONOLITHIC COLUMNS.

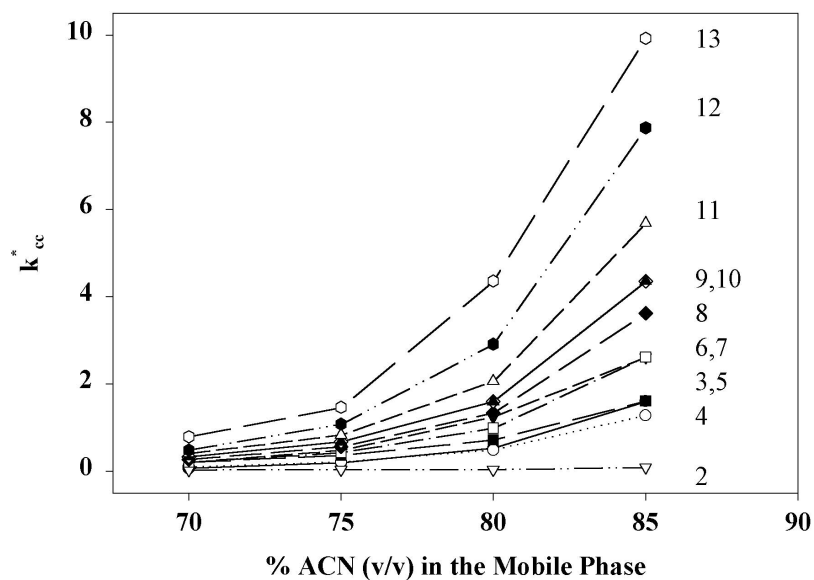
Solute	1CN			2CN		
	$k_{cc}^*$	$k_{ep}^*$	$k^*$	$k_{cc}^*$	$k_{ep}^*$	$k^*$
Leu-Phe-1	$k' = 0.02$	0	$k' = 0.02$	$k' = 0.19$	0	$k' = 0.19$
Leu-Phe-2	$k' = 0.02$	0	$k' = 0.02$	$k' = 0.22$	0	$k' = 0.22$
Gly-Leu	-0.06	0.15	0.09	0.48	0.06	0.58
Phe-Pro-1	$k' = 0.02$	0	$k' = 0.02$	$k' = 0.04$	0	$k' = 0.04$
Phe-Pro-2	$k' = 0.02$	0	$k' = 0.02$	$k' = 0.36$	0	$k' = 0.36$
Ala-Phe	0.09	0.04	0.06	0.43	0.02	0.45
Gly-Phe	0.11	0.07	0.12	0.56	0.03	0.61
Gly-Lys	-0.28	1.33	0.59	0.68	0.56	1.61
Gly-Trp	0	0.11	0.11	0.68	0.04	0.75
Gly-Tyr	0.14	0.04	0.13	0.84	0.02	0.87
Gly-Ala	0.14	0.11	0.20	1.08	0.05	1.17
Met-Glu	NM	NM	NM	1.47	-0.27	0.79
$v_{oc}(\text{cm/min})$		3.70			6.40	

(NM: not measured).

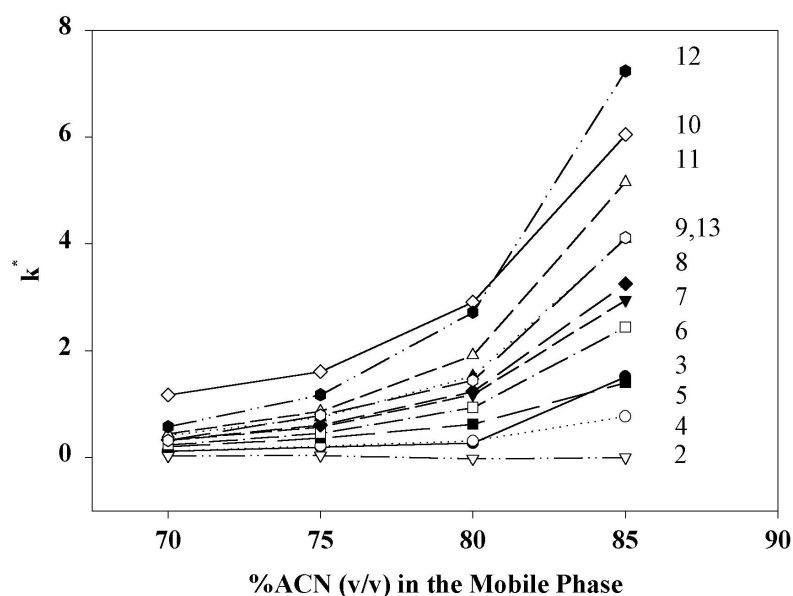
Conditions: monolithic capillary column, 20/27 cm  $\times$  100  $\mu\text{m}$  ID; mobile phase, hydro-organic solution made up of 25% v/v of 5 mM  $\text{NH}_4\text{Ac}$  (pH 3.9) and 75% v/v acetonitrile; voltage, 20 kV; wavelength, 200 nm; column temperature, 20  $^\circ\text{C}$ .

solutes such as the dipeptides. In fact the 2CN-OH-monolith can separate the diastereoisomer forms of Leu-Phe and Phe-Pro. Also, Gly-Lys which is a positively charged solute at the pH of the experiment and has a negative  $k_{cc}^*$  on the 1CN-OH-monolith underwent strong interaction with the 2CN-OH-monolith and its  $k_{cc}^*$  value is  $> 0$ . Furthermore, the 2CN-OH-monolith offers a relatively strong EOF ( $\sim 2$  times that on the 1CN-OH-monolith) and consequently  $k_{ep}^*$  are lower than those obtained on the 1CN-OH-monolith thus bringing about the elution of negatively charged peptides such as Met-Glu.

As shown in Table 2 and in Figures 7 and 8, with only one exception (Phe-Pro) the retention parameters of all dipeptides studied increase with increasing ACN in the mobile phase indicating that the dipeptides undergo strong polar interactions with the stationary phases and the contribution of the electrophoretic component to the overall solute migration is rather limited.



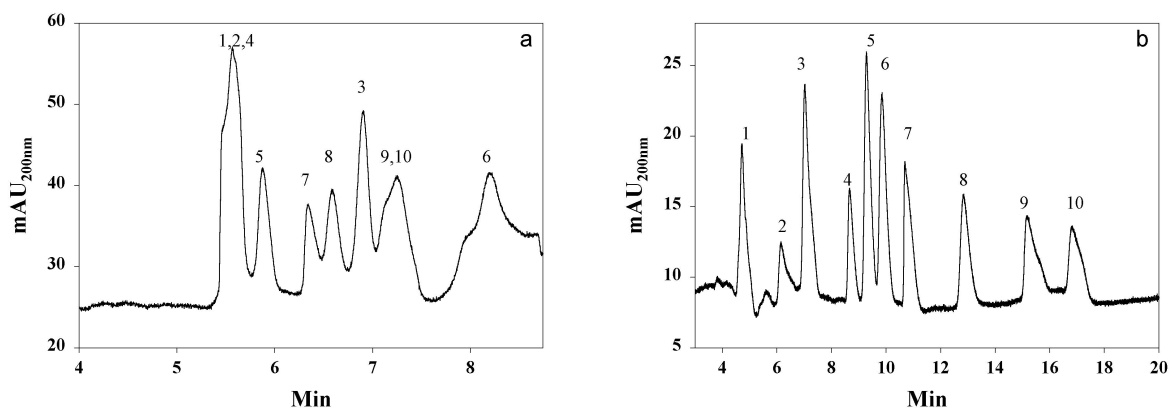
**Figure 7.** Effect of acetonitrile concentration on the retention factor  $k_{cc}^*$  Conditions as in Figure 6. Solutes: 2, Phe-Pro-1; 3, Leu-Phe-1; 4, Leu-Phe-2; 5, Phe-Pro-2; 6, Ala-Phe; 7, Gly-Leu; 8, Gly-Phe; 9, Gly-Trp; 10, Gly-Lys; 11, Gly-Tyr; 12, Gly-Ala; 13, Met-Glu.



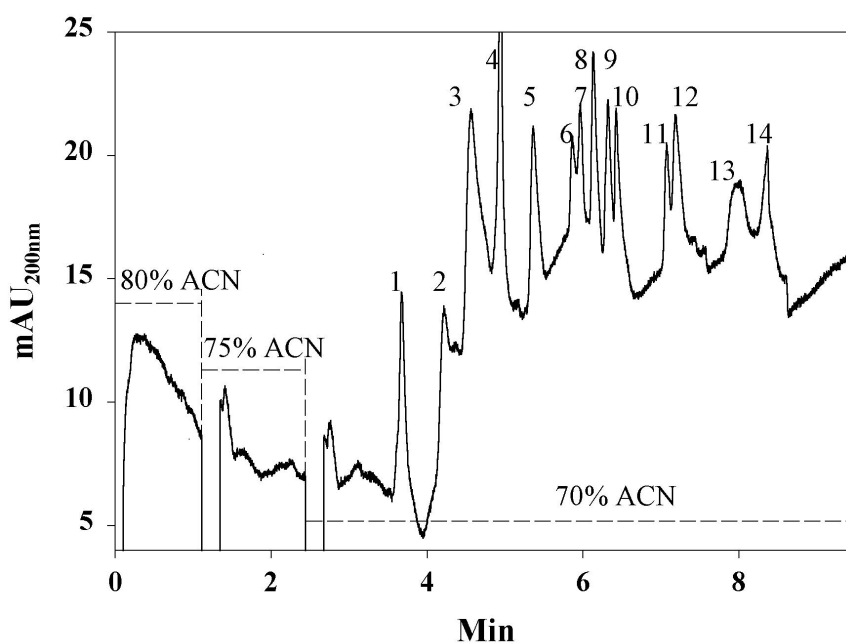
**Figure 8.** Effect of acetonitrile concentration on  $k^*$ . Conditions are the same as in Figure 6. Solutes are the same as in Figure 7.

Separation of Di-, Tri- and Tetrapeptides A mixture of standard di-, tri- and tetrapeptides was separated on the columns under investigation. Again, the 2CN-OH-monolithic column yielded better separation than the 1CN-OH-monolithic column (Figure 9).

Step gradient elution is more efficient for the separation of widely differing peptides than isocratic elution, but this may be at the cost of shift in baseline due to continuous changes in the equilibrium of the column. Figure 10 shows the electrochromatogram of the mixture of peptides with step gradient elution. With a mobile phase containing 5 mM  $\text{NH}_4\text{OH}$  (pH 6.0) and 80% ACN at 0 – 1.1 min, 75% ACN at 1.1 - 2.2 min followed by 70% ACN, the column could separate all of the peptides under investigation with a satisfactory selectivity factor. Note that Gly-Gly-Ala could be



**Figure 9.** *Electrochromatograms of peptides on two monolithic columns. Conditions: monolithic capillary column, 20/27 cm  $\times$  100  $\mu$ m ID; mobile phase, hydro-organic solution made up of 25% v/v of 5 mM  $\text{NH}_4\text{Ac}$  (pH 3.9) and 75% v/v acetonitrile; voltage, 20 kV; wavelength, 200 nm; column temperature, 20  $^\circ\text{C}$ . a. CN-OH-monolithic column; b. 2CN-OH-monolithic column. Solutes: 1, Toluene; 2, Gly-Phe-Phe; 3, Phe-Gly-Gly-Phe; 4, Gly-Gly-Leu; 5, Gly-Tyr; 6, Phe-Gly-Gly; 7, Gly-Ala; 8, Gly-Pro-Gly-Gly; 9, Gly-Gly-Gly; 10, Gly-Gly-Gly-Gly.*



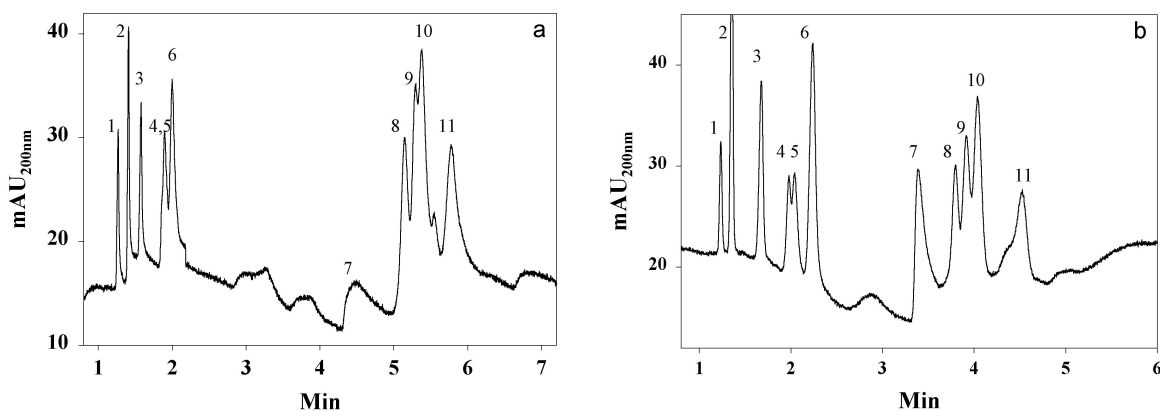
**Figure 10.** *Electrochromatogram of peptides on 2CN-OH-Monolithic column with step gradient elution. Conditions: monolithic capillary column, 20/27 cm  $\times$  100  $\mu$ m ID; mobile phase, hydro-organic solution made up of 20-25-30% v/v of 5 mM  $\text{NH}_4\text{Ac}$  (pH 3.9) and 80-75-70% v/v acetonitrile; voltage, 20 kV; wavelength, 200 nm; column temperature, 20  $^\circ\text{C}$ . Solutes: 1, Toluene; 2, Leu-Phe-1; 3, Leu-Phe-2; 4, Gly-Phe-Phe; 5, Phe-Gly-Gly-Phe; 6, unknown; 7, Gly-Gly-Leu; 8, Gly-Tyr; 9, Phe-Gly-Gly; 10, Gly-Ala; 11, Gly-Gly-Ala; 12, Gly-Pro-Gly-Gly; 13, Gly-Gly-Gly; 14, Gly-Gly-Gly-Gly.*



separated from Gly-Pro-Gly-Gly in step gradient elution while they overlapped in isocratic elution.

### Separation of Basic Compounds

The 2CN-OH-monolithic column was also found to be successful in the separation of basic compounds. The basic compounds, which usually contain amino groups, are positively charged in buffer solutions with a low pH. As shown in Figure 11, the positively charged solutes, berberine, diphenhydramine, gramine, procainamide, propanolol, benzylamine and nicotine, were eluted before the neutral solute, toluene. Table 4 shows the retention parameters of the basic compounds on the two columns investigated. As can be seen in this table, and as expected the 2CN-OH-monolith showed higher retention than the CN-OH-monolith. In both cases, and since the



**Figure 11.** *Electrochromatograms of basic compounds on two monolithic columns. Conditions: monolithic capillary column, 20/27 cm  $\times$  100  $\mu$ m ID; mobile phase, hydro-organic solution made up of 25% v/v of 5 mM  $\text{NH}_4\text{Ac}$  (pH 3.9) and 75% v/v acetonitrile; voltage, 20 kV; wavelength, 200 nm; column temperature, 20  $^\circ\text{C}$ . a. CN-OH-monolithic column; b. 2CN-OH-monolithic column. Solutes: 1, berberine; 2, diphenhydramine; 3, gramine; 4, procainamide; 5, propanolol; 6, benzylamine; 7, nicotine; 8, toluene; 9, amobarbital; 10, sulfanilamide; 11, theophylline.*

TABLE 4.

VALUES OF RETENTION PARAMETERS OF BASIC COMPOUNDS ON TWO DIFFERENT MONOLITHIC COLUMNS

Solute	1CN				2CN			
	$k_{cc}^*$	$k_{ep}^*$	$k^*$	$\alpha$	$k_{cc}^*$	$k_{ep}^*$	$k^*$	$\alpha$
Berberine	-0.75	2.41	-0.16		-0.68	3.34	0.41	
Diphenhydramine	-0.73	2.34	-0.08	1.04	-0.64	3.25	0.52	1.05
				1.05				1.15
Gramine	-0.69	2.63	0.11		-0.56	3.65	1.05	
Procainamide	-0.64	2.32	0.21	1.09	-0.48	3.22	1.20	1.16
				1.00				1.04
Propranolol	-0.64	1.82	0.03		-0.46	2.53	0.90	
Benzylamine	-0.61	2.42	0.33	1.04	-0.41	3.35	1.56	1.12
				4.59				3.77
Nicotine	-0.13	0.65	0.43		-0.11	0.91	0.70	
Amobarbital	$k' = 0.03$	0	0.03	4.75	$k' = 0.03$	0	0.03	3.51
				1.57				2.00
Sulfanilamide	$k' = 0.04$	0	0.04		$k' = 0.06$	0	0.06	
Theophylline	$k' = 0.12$	0	0.12	2.77	$k' = 0.19$	0	0.19	3.10
$v_{eo}(\text{cm/min})$		3.89				5.27		

Conditions: monolithic capillary column, 20/27 cm  $\times$  100  $\mu\text{m}$  ID; mobile phase, hydro-organic solution made up of 25% v/v of 5 mM  $\text{NH}_4\text{Ac}$  (pH 3.9) and 75% v/v acetonitrile; voltage, 20 kV; wavelength, 200 nm; column temperature, 20  $^\circ\text{C}$ .

electrophoretic mobility of the positively charged solutes is in the same direction as the EOF, the values of  $k_{ep}^*$  are  $>0$ .

## Conclusions

This investigation has evaluated the 2CN-OH-monolith with a wide range of polar species whether neutral (carbohydrates), ampholytes (peptides) or positively charged species (e.g., basic drugs) under various mobile phase composition. The study proved that the 2CN-OH-monolith is a viable stationary phase for NP-CEC. This represents a significant contribution to the advance of monolithic stationary phases for use not only in CEC but also in HPLC. Furthermore, the study has contributed to a better understanding of the electrochromatographic behavior of charged species in CEC with organic-rich mobile phases by evaluating the retention parameters under different mobile phase composition as well as polarity of the stationary phase (2CN-OH-monolith vs. CN-OH-monolith).

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## VITA

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Master of Science

Thesis: POLAR BONDED STATIONARY PHASES CONSISTING OF SILICA-BASED  
MONOLITHS FOR HYDROPHILIC INTERACTION CAPILLARY  
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BASED MONOLITHS FOR HYDROPHILIC INTERACTION  
CAPILLARY ELECTROCHROMATOGRAPHY

Pages of Study: 71

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Scope and Method of Study: The aim of this study was to introduce novel polar stationary phases based on silica monoliths for normal-phase capillary electrochromatography (NP-CEC). The silica monoliths were produced by a sol-gel process which was optimized to allow the bonding of the polar ligands. The polar ligand investigated in this study was the 1*H*-imidazole-4,5-dicarbonitrile, which has four strong polar functions that make it possible to compensate for the low monolith surface area thus improving separation and selectivity for polar solutes.

Findings and Conclusions: Silica-based monoliths can be produced *via* the sol-gel technology, which employs silica alkoxide as the sol-gel precursor and PEG as the porogen with acetic acid as the catalyst. Ammonium hydroxide can be used to adjust the size of the mesopores. Through chemical bonding reactions, the surface of the silica monolith can be modified with the desired chromatographic ligand to solve different analytical problems. The 1*H*-imidazole-4,5-dicarbonitrile modified monolithic column, which is denoted 2CN-OH-monolith, exhibited higher retentivity and selectivity than the monofunctional CN-OH-monolithic column toward a wide range of polar species including neutral (carbohydrates), ampholytes (peptides) and positively charged species (basic drugs). This study proved that the 2CN-OH-monolith is a viable stationary phase for NP-CEC. This represents a major advance in the design of polar monolithic silica columns for the normal phase chromatography in general and for NP-CEC in particular.

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