CAPILLARY COLUMN COATING AND PACKING FOR MICROCOLUMN SEPARATION BY CAPILLARY ELECTROPHORESIS AND CAPILLARY ELECTRO-CHROMATOGRAPHY

By

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LIST OF SYMBOLS AND ABBREVIATIONS

c^*	conductivity of packed column and empty column
c_b	conductivity of empty column
CE	capillary electrophoresis
CEC	capillary electrochromatography
CIEF	capillary isoelectric focusing
CSM	cationic surfactant moiety
CZE	capillary zone electrophoresis
D	diffusion coefficient of the analyte
DMF	*N,N*-dimethylformamide
DMSO	dimethyl sulfoxide
d_p	particle size of the stationary phase
d.p.	degree of polymerization
E	applied electric field strength
EBHPC	epoxybutane derivative of HPC
EDHPC	epoxydecane derivative of HPC
EOF	electroosmotic flow
F_e	electrical force
F_f	frictional force to a spherical particle
GC	gas chromatography

GlcNAc	*N*-acetylglucosamine
GlcUA	glucuronic acid
HPC	hydroxypropyl cellulose
HSA	human serum albumin
ITP	isotachophoresis
k'	chromatographic retention factor
k_c'	retention factor for charged analyte in CEC
k_e'	retention factor in CE
l	length from inlet end to the detection point
L	total column length
LC	liquid chromatography
LOD	limit of detection
MECC	micellar electrokinetic capillary chromatography
N	column efficiency
ODAC	*n*-octadecyldimethyl [3-trimethoxysilylpropyl] ammonium chloride
ODAS	*n*-octadecyldimethyl [3-trimethoxysilylpropyl] ammonium modified silica
ODS	octadecyl-silica
OT-CEC	open tubular CEC
p*I*	isoelectric points
PVA	polyvinyl alcohol
q	charge of the ion
Rs	chromatographic resolution
SDS	sodium dodecyl sulfate

t_0	migration time of a neutral marker
t_1', t_2'	adjusted retention times of components *1* and *2*, respectively
TAC	*N*-trimethoxysilylpropyl-*N,N,N*-trimethyl ammonium chloride
t_R	retention time in CEC
V	applied voltage
v	migration velocity of the ion
v'	average velocity for pressure-driven flow
w_i, w_h, w_b	peak widths for a Gaussian peak at the inflection point, half-height and base, respectively
δ	thickness of the electric double layer
ζ	zeta potential (the electric potential at the interface between plane of shear and bulk solution)
ψ_d	electric potential at the interface of the mobile and immobile region of the double layer
ψ_o	electric potential at the silica-solution interface
Δp	pressure drop over the column
α	selectivity factor
ε	dielectric constant of the medium
ε'	porosity of the stationary phase
ε_o	permitivity of the vacuum
η	viscosity of the bulk solution
μ	electrophoretic mobility
μ_{app}	apparent mobility

μ_c	mobility of a charged analyte in CEC
μ_{eo}	electroosmotic flow
μ_{ep}	electrophoretic migration
$\overline{\mu}_{app}$	average apparent mobility
$\overline{\mu}_{ep}$	average electrophoretic mobility
σ_1 , σ_2	standard deviations of the peaks *1* and *2*, respectively
σ_L	standard deviation of the peak in unit of length
ζ_p	zeta potential on the stationary phase particle
ζ_w	zeta potential of the double layer

CHAPTER I

INTRODUCTION AND SOME BASIC ASPECTS OF CAPILLARY ELECTROPHORESIS AND CAPILLARY ELECTROCHROMATOGRAPHY

Introduction

Capillary electrophoresis (CE) and capillary electrochromatography (CEC) are electrically driven microcolumn separation techniques characterized by high separation efficiency and high resolution. In addition, both techniques require minute amounts of samples and reagents which make them suitable for the analysis of samples of biological and environmental origin. The high separation efficiency achieved by CE and CEC is attributed to the presence of a bulk liquid flow with a plug profile called electroosmotic flow (EOF). EOF is by definition the movement of a liquid inside a capillary under the influence of an electric field.

While separation in CE is based primarily on differences in charge-to-mass-ratio among the analytes, in CEC separation is in addition influenced by the differences of solute partitioning between the mobile phase and stationary phase as in chromatography. In chromatography a pressure driven flow is used to produce differential migration. In certain cases, CE uses electrolyte systems that contain additives (e.g., micelles, cyclodextrins, crown ethers, charged or uncharged soluble polymers, etc.) which are capable to associate with the solutes and modify their mobilities, thus bringing about a

unique selectivity. Such electrolyte systems are viewed as composed of an aqueous phase and a pseudo stationary phase leading to a host of CE methods that are collectively called capillary electrokinetic chromatography (EKC). Under EKC conditions and in the presence of an EOF, CE resembles to CEC with one difference in that both the mobile and stationary phases in EKC are moving at different velocities while only the mobile phase is moving in CEC.

Thus, the aim of this chapter is to provide the reader with the basic concepts of CE and CEC, and to delineate the difference and resemblance between both techniques in order to facilitate the understanding of the various studies conducted within the framework of this dissertation. In addition, this chapter provides the basic relationships that were used in calculating the values of some essential parameters throughout this dissertation.

The major resemblances and differences between CE and CEC are further delineated in Chapter II which provides an account for the basic experimental aspects of both techniques. Furthermore, Chapter II describes some of the advances that have been made in the surface modification of the capillary wall and solid supports for separation by CE and CEC, respectively.

Surface modification in the aim of providing separation media for CE and CEC of a wide range of compounds is a major part of the investigation of this dissertation. In fact, Chapter III describes the introduction of a novel capillary wall coating to reduce solute-wall interaction for the separation of proteins by CE. Chapter IV introduces partially modified octadecyl silica (ODS) specifically designed for CEC separations. The partially modified ODS was evaluated over a wide range of elution conditions and its potential was demonstrated in the CEC of carbohydrates. To provide new ways for

controlling the magnitude of the EOF in CEC, a series of investigations were performed on the design of segmented capillaries in Chapter V. As the name implies, segmented capillaries involved the packing of the CEC column with two different stationary phases to produce two end-to-end connected segments where one of them plays the role of separation and the other functions as the EOF accelerator to speed up the separation. Other investigations aiming at the control of the magnitude and direction of the EOF in CEC are the subject of Chapter VII. In this chapter, a novel surface modification that yielded a reversed-phase stationary phase with a positively charged sublayer was designed. After all, the significance of an investigation resides in its potential in separating important compounds. This is the subject of Chapters VI and VII which provided the optimum conditions for separating some pesticides widely applied in agricultural setups. Furthermore, for the determination of pesticides by CEC at low concentration levels, on-line and off-line preconcentration approaches were introduced in Chapters VI and VII.

Finally, besides the general backgrounds provided in Chapters I and II, specific backgrounds that are pertinent for the various investigations conducted within the framework of this dissertation are summarized in Chapters III, IV, V, VI and VII. The rationale of each study is clearly stated in the introductory part of each of the Chapter. In addition, each of the chapters ends with important concluding remarks.

Historical Background and Development of Capillary Electrophoresis and Capillary Electrochromatography

Capillary electrophoresis (CE) has a relatively long history if column electrophoresis is considered as its early format. Over 30 years ago, Hjertén [1] and

Catsimpoolas [2] were among the first to practice column electrophoresis. The diameter of the column at the early stage was 1-3 mm, and later on 200-400 μm [3]. In 1971, Evraerts conducted capillary zone electrophoresis (CZE) in 200 μm Teflon capillaries with on-column UV detection [4]. In 1981, Jorgenson and Lukacs used 75 μm fused silica capillaries and on-column fluorescence detection for the analysis of mixtures of dansyl and fluorescamine derivatives of amino acids, dipeptides and simple amines [5]. This work was believed to have demonstrated the analytical potentials of CE and marked the beginning of tremendous advancement in the area of CE. Today, CE is perhaps the most rapidly developing area in separation science.

Since the 1980's, CE has derived into a number of separation modes. In addition to CZE, these modes include isoelectric focusing [6], gel electrophoresis [7], and more importantly, micellar electrokinetic capillary chromatography (MECC) [8]. MECC is based on the differential partitioning of analytes into a charged micelle which acts as a separation carrier. The introduction of separation carriers, such as sodium dodecyl sulfate (SDS) [9, 10], has provided a selective media which allow simultaneous separation of neutral, cationic, and anionic analytes by MECC. This has greatly expanded the applications of CE. The late 1990's have seen the advent of a new platform of electrophoresis, CE on a microchip [11]. The development of microchip CE was endowed by integrated circuit technology of the electronic industry. In microchip CE, electrophoresis channels are etched on surface of glassic wafer to promote fast, automated, miniaturized and multiplexed assays, thus meeting the needs of the pharmaceutical industry in drug development. As a complementary technique to gas chromatography (GC) and high performance liquid chromatography (HPLC), CE has been particularly useful in applications such as DNA-sequencing, protein analysis, and

carbohydrate analysis [12-15].

Capillary electrochromatography (CEC) is a newly introduced microscale separation technique which uses electroosmosis to form a plug flow profile to drive the mobile phase through the capillary column. Separation in CEC is based on the partitioning of analytes between a mobile phase and a stationary phase. The history of CEC may be dated back to the late 1930's [16] when Strain first demonstrated the use of electroosmotic flow (EOF) in chromatography. However, EOF as a pumping mechanism for analytical separation was not demonstrated until the work of Pretorius *et al* in the mid 70's [17]. Early works in electrically driven chromatography were performed on relatively large columns (> 1 mm) [18]. Later, capillaries were more popularly adapted due to more efficient dissipation of Joule heat by microbore columns. CEC experienced significant progress in the 1980's and its theoretical foundations were laid. Research on CEC ever since have addressed practical issues such as the importance of pressurization to subdue bubble formation in packed columns, and factors (e.g. column material, electrolytes, voltage, and current) which influence the EOF [19-25]. The late 1990's have seen more prevailed practices in CEC. CEC instruments are being developed and marketed for increasing demands in research. So far, the operation modes of CEC can be derived into two main categories: packed column CEC (packed-CEC) and open tubular CEC (OT-CEC) [26]. In packed-CEC, columns are either packed with particles of, in most cases, reversed-phase silica, with particle sizes ranging from 1.5 to 10 μm, or filled with immobilized polymers. Packed-CEC is discussed in more details in Chapter II. On the other hand, OT-CEC uses open capillaries with inner diameter 25 μm or less, and total length 50 cm or longer. The stationary phase is attached to the inner wall of the column. Microchip CE may also be classified as OT-CEC by this definition.

CEC is considered to be a hybrid of HPLC and CE. As usual, this hybrid has advantages over its parents. Compared with HPLC, CEC consumes far less amount of mobile phases, which is normally composed of organic solvents and aqueous buffers. Typical organic solvents used in HPLC are methanol and acetonitrile, which are hazardous to environment and human health. More importantly, as compared with HPLC, CEC usually has narrower peaks which yield higher plate counts, or column efficiency due to the plug flow profile of the EOF. Compared with CE, CEC has higher peak capacity and selectivity because of the existence of the stationary phase packed in the column. Moreover, the stationary phases as well as the inner surface of the CEC column may be tailored according to the nature of the analytes to be handled. Surface modification of silica for CEC is largely based on the experiences gained from silica modification for HPLC as far as selectivity is concerned. In CEC, besides providing selectivity, silica modification has one more thing to assure, which is a strong EOF to drive the mobile phase through the column.

While silica modification to improve selectivity has been relatively well understood due to extensive practices in HPLC, silica modification to produce and control the EOF in packed column has not been well documented [27]. A handful of references addressed this issue [28-34]. Apparently the understanding of EOF in packed column is critical for the performance of CEC. The dynamics of the mobile phase and the chromatographic selectivity are therefore major topics in the current research of CEC.

Both CE and CEC are compatible with powerful detection tools such as mass spectrometry (MS) and nuclear magnetic resonance spectroscopy (NMR). This will certainly boost their applications in a wider range of separation yet to be exploited.

The aim of this chapter is to introduce CE and CEC from a theoretical point of

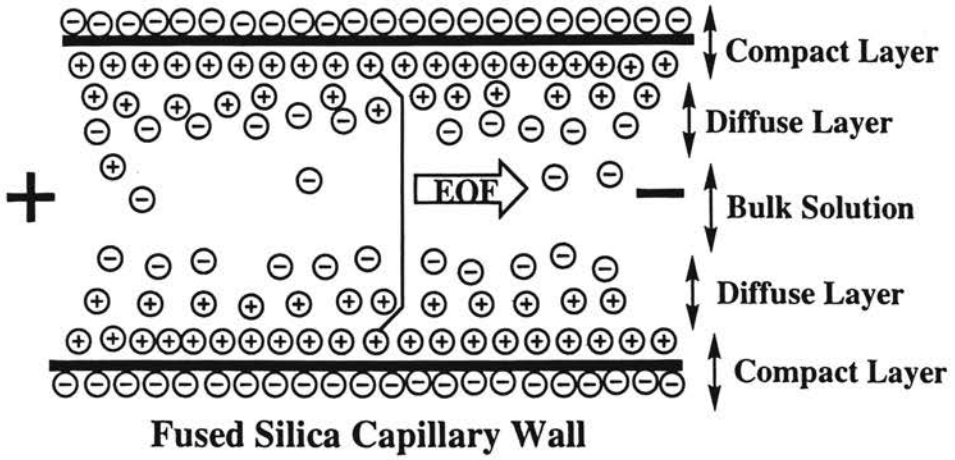
view, with emphasis on the origin of EOF, factors that potentially influence EOF, and parameters to be involved in the separations with CE and CEC.

Theoretical Considerations on EOF

The Origin of Electroosmosis

Electroosmosis refers to the movement of liquid relative to a stationary charged surface due to an applied electric field [35]. The generation of electroosmosis can be explained by Gouy-Chapman model in the revised version proposed by Stern [36-38]. The essence of this model is the electric double layer that is formed at the solid phase/liquid phase interface. The electric double layer formed in the case of capillary electrophoresis is illustrated in Fig. 1. In this model, an electric double layer is composed of a compact region and a diffuse region. The compact region is formed when negatively charged silanol groups on the silica surface electrostatically attract counter ions from the liquid phase. As its name implies, ions in the compact region are held tightly and are usually not moveable. Due to thermal motion, some of the ions in the compact region move away from the capillary surface, forming the diffuse region which is moveable. When a potential field is applied, the counter ions (cations in this case) in the diffuse region will move toward the anode. Hydrated, these cations will drag the bulk solution with them, forming a flow that is called the EOF. The EOF is usually strong enough to sweep all the species whether cationic, anionic or neutral, toward the same electrode, although due to their electrophoretic mobilities, the ions may separate in multiple zones. Because the EOF is originated at the interface, it is characterized by a plug like profile, which narrows the width of the zones of the different ions. The narrow zones will produce sharper peak and in turn higher chromatographic peak capacity. In fact the plug

a.



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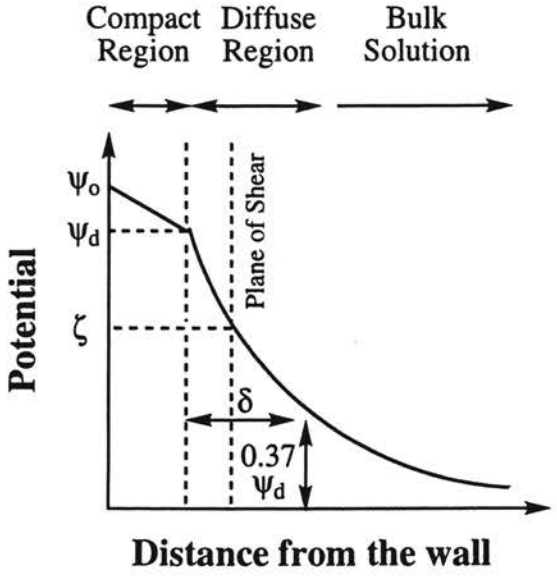

Figure 1. (a) Schematic illustration of the electric double layer and plug flow profile established in CE. (b) Gouy-Chapman model which explains the potential distribution in the electric double layer and vicinity region.

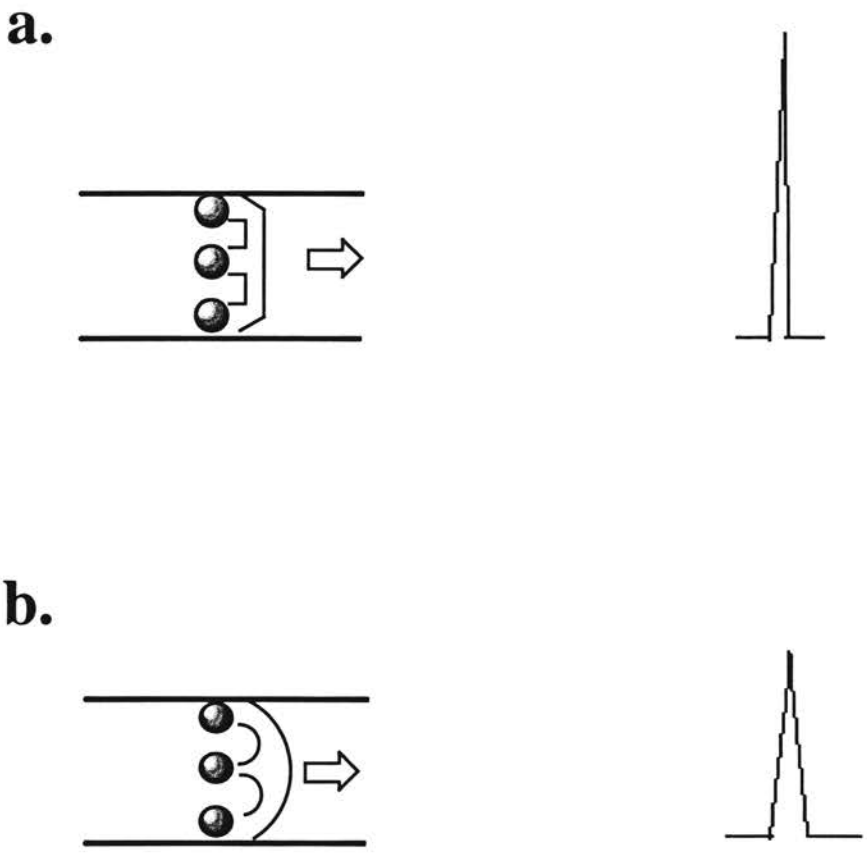

Figure 2. Illustration of flow profiles and their impacts on column efficiency. (a) Plug flow profile in CEC yielding higher efficiency (sharper peak), as shown on the right hand side. (b) Laminar flow profile usually observed in HPLC yielding lower efficiency (broader peak), as shown on the right hand side.

flow profile of EOF has imparted CE with high column efficiency, a major reason for CE to prevail as a separation technique. In contrast, a laminar flow profile is observed in conventional HPLC, see Fig. 2, which usually leads to band broadening.

As shown in Fig. 1b, due to the spatial distribution of ions, an electric potential ψ_o is established at the silica-solution interface. The value of ψ_o decreases linearly in the compact region and decreases exponentially in the diffuse region. At the interface between the compact and diffuse regions of the electric double layer the potential is ψ_d. At the interface between plane of shear and bulk solution, the value of the electric potential is called the zeta potential ζ. The distance out in solution from the interface between the compact and diffuse region to a point where $\psi = 0.37\ \psi_d$ is conventionally called the thickness of the electric double layer δ. The plane of shear is where the bulk solution flows tangentially relative to the surface. This plane of shear is not at the surface of the capillary because the ions in the compact region are associated with solution spheres that are held stationary.

EOF in Open Column

EOF originated in CE is a case of electroosmosis originated in open tube. This type of EOF can be quantitatively described by von Smoluchowski equation [39],

$$u_w = -\frac{\varepsilon \varepsilon_o \zeta_w E}{\eta} \qquad (1)$$

where ε is the dielectric constant of the medium, ε_o is the permitivity of the vacuum, E is the applied electric field and η is the viscosity of the bulk solution. ζ_w is the zeta potential of the electric double layer. Smoluchowski's equation was originally intended to handle EOF produced on a flat and uniformly charged surface. This equation is

applicable to CE due to the dimension of the inner diameter of the capillary relative to that of the thickness of the electric double layer. According to Eq. 1 the EOF in CE can be varied by adjusting the viscosity and ionic strength of the mobile phase, and by adjusting the strength of the applied electric field. Among these three experimental factors, the zeta potential (ζ_w) tends to be more significant, since it can be effectively controlled by monitoring the sign and density of the surface charge on the capillary wall. This is usually done by chemically modifying the capillary wall. When electrophoretic separation is in concern, surface modification on silica becomes liable, in addition to offering the EOF of appropriate polarity and magnitude, to preclude solute-wall interaction and to provide suitable chromatographic selectivity. Specifically, Chapter III described a project on this matter.

EOF in Packed Column

CEC differs from CE in that the former uses packed column while the latter is normally performed with open tubes. The stationary phase in CEC is usually a silica-based material. As a result, the EOF in CEC column is collectively originated from the wall of the capillary and the stationary phase. The charge density on the stationary phase is higher than that on the capillary wall, since the surface area of the granular silica stationary phase is much larger than the surface area of the capillary wall. From this point of view, the magnitude of EOF generated by the stationary phase seems to be more significant than that generated by the capillary wall. Thus far, there are no widely accepted theories which quantify the contributions of the capillary wall and the stationary phases to the overall magnitude of EOF generated in CEC column [40]. In one theory [34], the EOF generated on the capillary wall is called the 'wall effect'. According to this

theory, the wall effect is radially distributed and exponentially decreases toward the center of the capillary due to the existence of stationary phase. Neglecting the wall effect, the EOF generated by the stationary phases in CEC column may be viewed as generated by a bundle of open tubes which obey Smoluchowski's equation. Conductance of CEC column is reduced due to the stationary phase, as compared with an open column in CE. Based on this assumption, Overbeek's model [41] was derived. The average velocity of EOF, u_p, generated in CEC by the stationary phases is given by

$$u_p = -\frac{\varepsilon\varepsilon_o\zeta_p E}{\eta}\left(\frac{c^*}{c_b}\right) \qquad (2)$$

The symbols in Eq. 2 have the same meaning as in Eq. 1. ζ_p is the zeta potential on the stationary phase particle, c^* and c_b are conductivities of packed column and empty column, respectively. Compared with Eq. 1, Eq. 2 has the ratio of conductivities as one more factor to affect the EOF. It is noted that Eq. 2 does not include particle size as a factor to influence the magnitude of EOF in CEC. This may suggest that particles of very small size ($\leq$ 1 μm) may be used without reducing the EOF. This seems to have overcome the difficulty of using small particles which is usually encountered in HPLC. Kozeny-Carman equation [42], Eq. 3, shows the factors influencing the average velocity v' for pressure-driven flow, which is the case in HPLC,

$$v' = \frac{\varepsilon'^2}{180(1-\varepsilon')^2}\frac{d_p^2}{\eta}\frac{\Delta p}{\mathrm{L}} \qquad (3)$$

where ε' is the porosity, d_p is particle size of the stationary phase, Δp the pressure drop over the column, and L is the column length. Equation 3 shows that the average flow velocity is proportional to the square of particle size. Under a given set of conditions,

when smaller particles are packed in the column, the pressure drop (Δp) will increase as a $1/d_p^2$ function. With smaller particle size, porosity of the column will also decrease. Therefore, the particle size of stationary phase influences the flow velocity in more than one way in HPLC. However, smaller particles are favored in chromatographic separation when high separation efficiencies are concerned, since smaller particles will decrease analyte-stationary phase mass transfer and in turn improve chromatographic efficiency.

Despite its being omitted in Eq. 2, the particle size has its limit in the practice of CEC. This limit arises when the particle size falls within 10 times of the thickness of double layer [43], which is usually at the level of nanometers. Within this range, double layers on the surface of particles overlap and cancel each other, resulting in a dramatic decrease in the EOF. The limit of CEC particle size is predicted to be 0.5 μm [44].

Electroosmosis of the Second Kind

According to Eq. 2, the EOF velocity in CEC is linearly proportional to the strength of electric field. This is valid for routine CEC practice. However, an exponential proportionality between the electric field and EOF has also been reported [45]. This is called by Dukhin [46] as "electroosmosis of the second kind" in order to distinguish the first kind of electroosmosis that obeys Eq. 2. Dukhin attributed the second kind EOF to the induction of bulk charge and the enhanced local electric field strength by the induced bulk charge, which in turn enhanced the EOF. Figure 3 shows the induction of this effect. Two factors suffice to induce this electroosmosis: (1) very high electric field strength and (2) conductivity of the stationary phase being higher than the electrophoretic medium. In Dukhin's model, this phenomenon was thought to result from an enhanced double layer due to the tangential movement of the ions on the surface of the

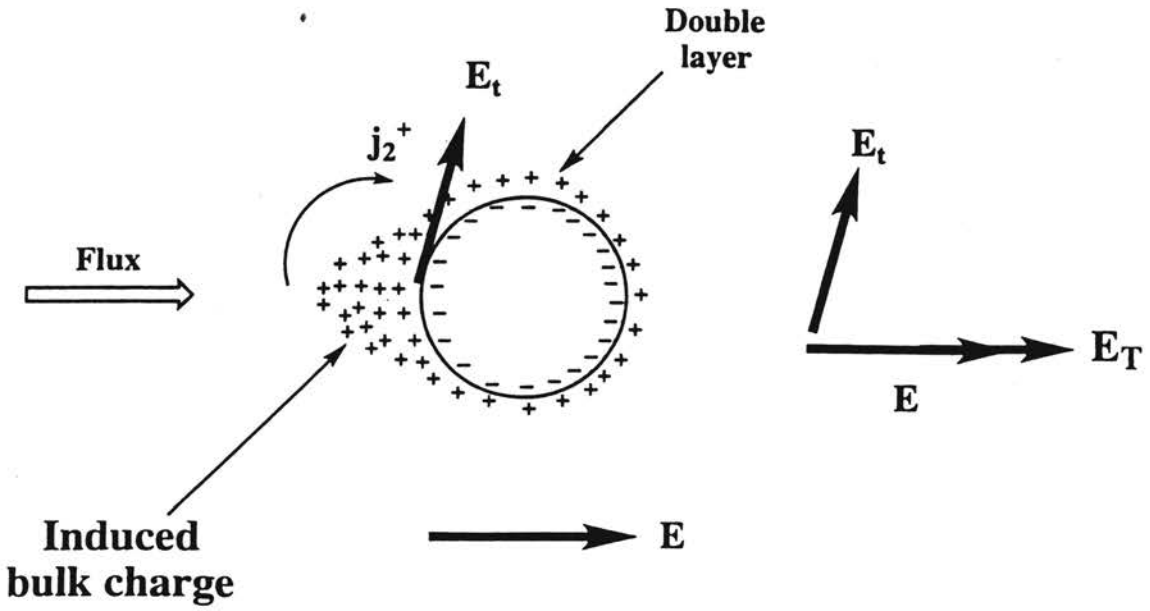

Figure 3. Schematic illustration of the induction of the second kind electroosmosis as explained by Dukhin's model. Under very high voltage (strong electric field), bulk charge is built up on the left side of the particle which faces the flux of the mobile phase. The tangential movement of the ions induces a local electric field E_t, which sums up with external electric field E to form a strengthened overall electric field E_T, as shown by the vectors on the right side of the particle. E_T is believed to be responsible for the induction of the second kind EOF.

stationary phase under very high voltage. The tangential movement of ions also induces a local electric field at the stationary phase particles which sum up to increase the overall electric field strength. Dukhin's rationale provides clues to induce stronger EOF when flow velocity is at stake.

Principles and Parameters of Capillary Electrophoresis and Capillary Electrochromatography

Electrophoretic Migration

When a constant electric field is applied across a capillary, ions in the capillary, whether positively or negatively charged, would experience electrical force F_e which is proportional to the electric field strength E and the charge q of the ion,

$$F_e = qE \qquad (4)$$

The electric field strength, on the other hand, is given by

$$E = \frac{V}{L} \qquad (5)$$

where V is the applied voltage and L is the total length of the capillary. This driving force (i.e., F_e) causes migration of the ion toward a certain electrode. In migration, the ion also experiences frictional forces which counteract the ion's movement. The frictional force F_f to a spherical particle with radius a is expressed by Stokes' law as

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

where η is the viscosity of the solution and v the migration velocity of the ion. The migration velocity of an ion is further expressed as

$$v = \mu E \qquad (7)$$

where μ is the electrophoretic mobility, a characteristic property of the ion collectively affected by the medium and temperature.

When steady state is reached, driving force F_e equals frictional force F_f. Combining Eqs. 4, 5, 6, and 7, μ can be expressed as

$$\mu = \frac{q}{6\pi\eta a} = \frac{v}{E} \qquad (8)$$

The unit of μ is $cm^2V^{-1}s^{-1}$. It can be seen that electrophoretic mobility is controlled by charge and size of the ion, and by the viscosity of the solute medium.

The above description of electrophoretic migration is applicable to both CE and CEC for charged solutes. The major difference is that in CEC the packing may introduce some additional friction to the migration of the solutes.

Migration Time and Apparent Mobility

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

$$\mu_{app} = \mu_{ep} + \mu_{eo} \qquad (9)$$

Since $v = l/t$, where l is the length from inlet end to the detection point, also called the effective length, and t the elution time of the peak, electrophoretic mobility in Eq. 8 can be expanded as

$$\mu = \frac{v}{E} = \frac{lL}{tV} \qquad (10)$$

Upon rearranging Eq. 10, the migration time in relation to electrophoretic mobility can be expressed as

$$t = \frac{v}{E} = \frac{lL}{\mu V} \qquad (11)$$

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

$$\mu_{eo} = \frac{v}{E} = \frac{lL}{t_0 V} \qquad (12)$$

$$\mu_{app} = \frac{lL}{t_M V} \qquad (13)$$

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

$$\mu_{ep} = \mu_{app} - \mu_{eo} = \frac{lL}{V}\left(\frac{1}{t_M} - \frac{1}{t_0}\right) \qquad (14)$$

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

$$t_R = t_0(1 + k') \qquad (15)$$

where k' is the conventional chromatographic retention factor, $k' = (t_R - t_0)/t_0$.

For charged analyte, the migration time is influenced collectively by electrophoresis, electroosmosis, and retardation resulting from the stationary phase retentivity. The mobility of a charged analyte in CEC, μ_c, is expressed as [47]

$$\mu_c = \left(\frac{1}{1+k'}\right)(\mu_{ep} + \mu_{eo}) = \frac{\mu_{ep} + \mu_{eo}}{1+k'} \qquad (16)$$

where t_R and t_0 are retention times of the analyte and the neutral marker, respectively. Combining Eqs. 15 and 16, the retention time of a charged analyte in CEC is expressed as

$$t_R = t_0\left(\frac{\mu_{ep} + \mu_{eo}}{\mu_c}\right) \qquad (17)$$

It can be seen that when μ_{ep} is zero (case of neutral solutes), Eq. 17 yields back to Eq. 15, which is for conventional HPLC.

Retention Factor

Retention factor is the ratio of the separative and non-separative virtual migration lengths. Non-separative virtual migration length is determined by a neutral marker. According to this definition, retention factor k_e' in CE is expressed as

$$k_e' = \frac{\mu_{ep}}{\mu_{eo}} \qquad (18)$$

Taking both electrophoretic mobility and chromatographic retention into account, the retention factor for a charged analyte in CEC, k_c', is expressed as

$$k_c' = k' + k'k_e' + k_e' \qquad (19)$$

For neutral solutes ($k_e' = 0$), Eq. 19 shows that $k_c' = k'$, which is the case of pure chromatography.

Selectivity Factor

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

$$\alpha = \frac{k_2'}{k_1'} = \frac{\left(\frac{t_2 - t_0}{t_0}\right)}{\left(\frac{t_1 - t_0}{t_0}\right)} = \frac{t_2 - t_0}{t_1 - t_0} = \frac{t_2'}{t_1'} \qquad (20)$$

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

While Eq. 20 applies to neutral analytes in CEC, the selectivity factor for charged analytes in CEC is expressed by

$$\alpha = \frac{k_{c2}'}{k_{c1}'} = \frac{k_2' + k_2' k_{e2}' + k_{e2}'}{k_1' + k_1' k_{e1}' + k_{e1}'} \qquad (21)$$

According to its definition, selectivity factor in CE is expressed as

$$\alpha = \frac{k_{e2}'}{k_{e1}'} = \frac{\mu_{ep2}}{\mu_{ep1}} \qquad (22)$$

Resolution

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

$$R_s = \frac{t_2 - t_1}{2(\sigma_1 + \sigma_2)} \qquad (23)$$

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

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

$$R_s = \frac{\sqrt{N}}{4}\left(\frac{\alpha - 1}{\alpha + 1}\right)\left(\frac{\overline{k'}}{1 + \overline{k'}}\right) \qquad (24)$$

where $\overline{k'}$ is the average chromatographic retention factor of the two adjacent components,

and N is the column efficiency. It can be seen that resolution is controlled by column efficiency, retention factor, and selectivity factor. The separation factor seems to influence the resolution to a greater extent than the other two. This equation is applicable for neutral analytes in CEC.

For charged analytes in CEC, similarly, the resolution can be expressed as

$$R_s = \frac{\sqrt{N}}{4}\left(\frac{\alpha-1}{\alpha+1}\right)\left(\frac{\overline{k_c'}}{1+\overline{k_c'}}\right) \qquad (25)$$

On the other hand, resolution in CE can be expressed by electrophoretic and electroosmotic mobilities as

$$R_s = \frac{1}{4}\sqrt{N}\frac{\Delta\mu_{ep}}{\overline{\mu}_{app}} = \frac{1}{4}\sqrt{N}\frac{\Delta\mu_{ep}}{\overline{\mu}_{ep}+\mu_{eo}} \qquad (26)$$

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

Column Efficiency

In chromatography, column efficiency N is expressed in number of theoretical plates. Column efficiency is a measure of the dispersivity of migration for a certain analyte. It is expressed by the following equation,

$$N = \left(\frac{l}{\sigma_L}\right)^2 \qquad (27)$$

where σ_L is the standard deviation of the peak in unit of length. N can be calculated by

the same equation used in liquid chromatography:

$$N = 4\left(\frac{t_M}{w_i}\right)^2 = 5.54\left(\frac{t_M}{w_h}\right)^2 = 16\left(\frac{t_M}{w_b}\right)^2 \qquad (28)$$

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

In CE, under ideal conditions, longitudinal diffusion can be considered as the only contribution to standard deviation σ_L. According to Einstein's law

$$\sigma_L{}^2 = 2Dt_M = \frac{2DlL}{\mu_{app}V} \qquad (29)$$

where D is the diffusion coefficient of the analyte. By substituting Eq. 29 into Eq. 27, column efficiency can be expressed as

$$N = \frac{\mu_{app}Vl^2}{2DlL} = \frac{\mu_{app}Vl}{2DL} \qquad (30)$$

When $l = L$ (as in the cases of electrochemical detection and mass spectrometry detection) column efficiency N in CE under ideal condition can be expressed as

$$N = \frac{\mu_{app}V}{2D} \qquad (31)$$

Eq. 31 shows that in this case, N is independent of the capillary length. By taking into account that $\mu_{app} = \overline{\mu}_{ep} + \mu_{eo}$, resolution in Eq. 26 can be further expressed as

$$R_s = \frac{1}{4}\sqrt{\frac{\mu_{app}V}{2D}}\left(\frac{\Delta\mu_{ep}}{\overline{\mu}_{ep} + \mu_{eo}}\right)$$

$$= 0.177\,\Delta\mu_{ep}\sqrt{\frac{V}{D\left(\overline{\mu}_{ep} + \mu_{eo}\right)}} \qquad (32)$$

R_s reaches infinity when $\overline{\mu}_{ep} = -\mu_{eo}$. Thus R_s can be readily manipulated by varying μ_{ep}

(via pH) and/or μ_{eo} (via surface coating). Increasing R_s by increasing the voltage V has certain limit above which high Joule heat will be generated, causing band broadening and reducing R_s.

Band Broadening Factors in CE

Band broadening and resultant reduction in resolution can arise from several contributing factors. In CE, these factors include initial zone width caused by sample injection, molecular diffusion, electrodispersion, Joule heating and adsorption [48]. The observed band broadening expressed as plate height H will be

$$H_{obs} = h_{inj} + h_{diff} + h_{cond} + h_{Joule} + h_{ads} \qquad (33)$$

To minimize band broadening, initial sample zone must be kept as small as possible. The starting zone length should not exceed 5% of the total capillary length [49]. Band broadening due to axial diffusion should be reduced by shortening analysis time. This can be done by using high voltage and short capillary. Using capillaries with smaller inner diameter is the key to reduce temperature and mobility gradient induced by Joule heat across the tube radius. The use of buffer additives and chemical modification of the capillary wall can serve to reduce the band broadening caused by wall adsorption.

Band Broadening Factors in CEC

Several factors contribute to the band broadening in column chromatography, these include: (1) eddy diffusion, (2) axial molecular diffusion, (3) resistance to mass transfer in the mobile and stationary phases, and (4) extra column effects (such in injector, connector, detector, etc.). Eddy diffusion is caused by flow-velocity differences [29]. Capillary electrochromatography is less affected by (1) and (3), in comparison with

pressure-driven liquid chromatography, due to electroosmosis. This is believed to be the major reason of increased chromatographic efficiency in CEC. An extra factor of band broadening in CEC is Joule heat. Similar to Eq. 33, the observed band broadening in CEC expressed as plate height will be

$$H_{obs} = h_{eddy,diff} + h_{a,diff} + h_{trans} + h_{extra} + h_{Joule} \qquad (34)$$

To subdue the band broadening induced by heat, inner diameter of the capillary should be kept at less than 200 μm, buffer concentration at less than 10 mM, and applied voltage at less than 50 kV [50].

Conclusions

This chapter discussed parameters that are involved in the origin of EOF, migration of solutes in CE/CEC, and factors influencing resolution, selectivity and efficiency in CE, CEC and HPLC. It can be seen that intimate connections exist among these three separation approaches. Theoretical models of CEC are based on those that are derived from CE and/or HPLC and still need to be substantiated by more extensive experiments.

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CHAPTER II

EXPERIMENTAL ASPECTS OF CAPILLARY ELECTROPHORESIS AND CAPILLARY ELECTROCHROMATOGRAPHY

Introduction

Capillary electrophoresis (CE) and capillary electrochromatography (CEC) are essentially microscale separation techniques. The amount of injected sample is usually at the level of nanoliters or less, making CE and CEC capable of handling sample as little as a single cell [1]. Instrumentation for CE and CEC is as sophisticated as in the more established separation techniques such as HPLC. The design of CE or CEC instrument encounters challenge in features including power supply, sample injection, temperature control, and detection. As in other platform of separations, column technology is fundamental and may even determine the fate of the emerging technique. This chapter is intended to offer some introduction on the aforementioned aspects.

Instrumentation for CE and CEC

General Description

A wide variety of commercial CE instruments are available, from simple modular systems consisting of a power supply, detector, and strip chart recorder to fully integrated automated systems monitored by computer software package. These instruments, with a

little modification, can be adapted for CEC research. Figure 1 is a schematic illustration of the major components in CE/CEC instrument.

Typically, the instrument contains a power supply capable of delivering precise and constant voltage up to 30 kV with switchable polarities. Most systems also offer constant current operation for up to 300 μA. Constant current may be desirable in systems without adequate temperature control or for special applications.

Both CE and CEC require the introduction of a very small amount of sample at the column inlet end with high precision to ensure reproducible quantitative analysis. All commercial instruments offer electrokinetic injection mode and at least one type of displacement injection. Electrokinetic injection is performed when the column inlet end is immersed in the sample solution and a certain voltage is applied for a brief period

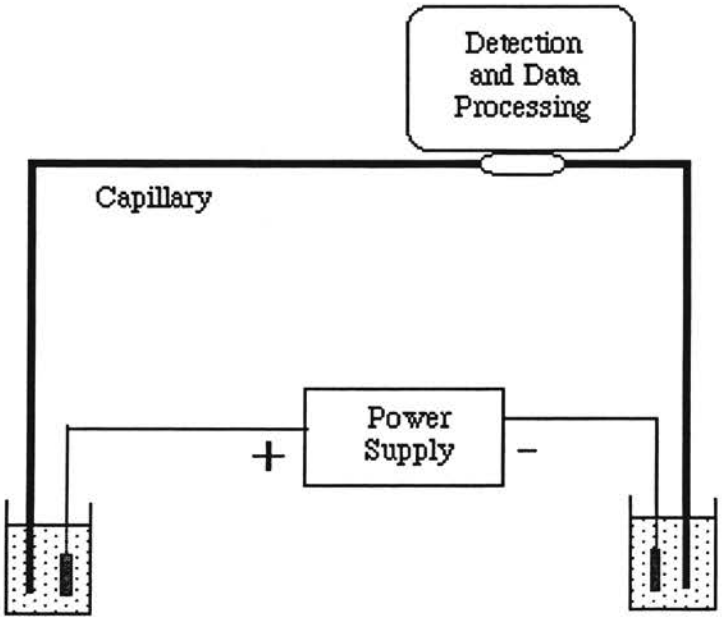


Figure 1. Schematic illustration of instrument set-up for CE/CEC.

(typically a few seconds for CE and one minute or greater for CEC).

In electrokinetic injection mode, sample is introduced by either electroosmosis or electrophoresis, or by both. When sample is injected only by electrophoresis, analytes with the same charge will be introduced while analytes of opposite charge will be eliminated, thus simplifying the separation. But electrophoretic injection has the disadvantage in that the injected sample amount is dependent on the electrophoretic mobility of the analytes, and as a result the composition of the injected solution is likely to be different from the original sample solution. Sample injection by electroosmosis only would not bring about discrimination among injected analytes. Despite the aforementioned limitations, electrokinetic injection is preferred when displacement injection is ineffective due to the existence of less permeable media such as gel or solid stationary phases in the column. For CEC and capillary gel electrophoresis, electrokinetic injection appears to be the most suitable choice.

Displacement injection in commercial CE instrument is carried out either by applying a positive pressure at the inlet end or by applying a negative pressure at the outlet end. In manual instrument, siphoning is the injection mode in addition to the electrokinetic mode. In automated systems, high precision in displacement injection may be achieved by programmed injection according to a constant time-pressure product.

Temperature control is essential for CE and CEC due to the effect of Joule heat on band broadening and migration time. In addition to being constant, operation temperature may be desired to be higher or lower than ambient temperature in special applications such as kinetic studies. For CEC, operation temperature is usually lower than room temperature to subdue bubble formation. Temperature control in CE and CEC systems can be achieved by circulating air flow or liquid coolant inside the capillary compartment.

Liquid coolant is more efficient in temperature dissipation than air flow because of the higher heat capacity of the liquid. However, the air flow is convenient for operation and instrument design.

Detection in CE/CEC is based mostly on absorbance and fluorescence. A vast majority of CE/CEC systems uses absorbance detection. Ultraviolet (UV) or ultraviolet-visible (UV-Vis) absorbance is the primary mode of detection. This is due to the simplicity of UV-Vis detection. Fluorescence detection has the merit of higher sensitivity and improved detection selectivity. This detection mode requires that the compounds to be detected either exhibit native fluorescence or be derivatized with a fluorophore. As a result, derivatization of the sample is usually desirable for this detection mode. To ensure acceptable sensitivity, laser is used to offer sufficient excitation. The use of laser arises from the light coherence and intensity at the detection point. With laser induced fluorescence (LIF), detection limit can be as low as 10^{-12} M [2, 3], while typical limit of detection of absorbance mode is in the range of 10^{-5} M – 10^{-6} M [4, 5]. With on-line preconcentration approach, the detection limit of absorbance mode may be lowered to 10^{-7} M [4].

On-line detection is most frequently seen in CE/CEC systems. In this approach, a section of the protection layer of the capillary is removed at the detection point and this section of capillary is acting itself as a detection cell. Because of the small capillary inner diameter, the light path of detection is short, yielding limited detection sensitivity for this mode. Strategies have been developed to increase signal intensity by increasing the light path of the detection cell. The detection cell can be engineered to have 'bubble' or 'Z' configuration. For example, the Z-cell can increase light path from 50 μm (I.D. of the

capillary) to 1 mm, thus lowering the detection limit by up to 20 folds [6, 7].

On-line coupling of CE and CEC with mass spectrometry (MS) and nuclear magnetic resonance spectroscopy (NMR) have been reported. These couplings were intended for structural determination of the separated components. Compatibility of CE [8] and CEC [9] with MS is facilitated in part by the very low bulk flow rate (<< 1 μL/min) that arises from the EOF in the capillary [5]. Fast-atom bombardment (FAB) and electrospray ionization (ESI) are commonly used in interfacing CE with MS [10]. CEC is more compatible with NMR than CE due to the higher sample capacity of CEC [11].

Some Special Features of CEC Instrumentation

The majority of CEC experiments reported to date are performed on adapted CE instruments, using isocratic mobile phases. Some automated systems have CEC mode incorporated with pneumatic pressure devices which can apply pressure on one end or both ends of the CEC column. The application of pressure is intended to suppress bubble formation and decrease elution time without a significant loss in resolution. The mode with pneumatic pressure applied on one end of the column is called pressurized CEC (pCEC) and has the advantage of allowing the separation of charged species [12].

As in HPLC, the peak capacity of CEC can be increased by performing gradient elution. Two types of gradient CEC have been reported: (1) using a conventional gradient HPLC pump to deliver the changing mobile phase across the CEC column entrance followed by column intaking the mobile phase through EOF [13], and (2) the use of two high-voltage power supplies to control the EOF velocity in two different mobile phases which mix and then enter the CEC column [14]. The use of a LC pump can

precisely control the mobile phase composition but will also result in waste of solvent. Composition of mobile phase delivered by EOF can not be known but waste of solvent can be avoided. These examples, however highlighted the potential of true continuous gradient CEC.

Separation Modes of CE

Separations in CE can be based on charge, molecular size or shape, isoelectric point, or hydrophobicity. These lead to a variety of modes in CE separation, which can be carried out with the same CE instrument. Separation modes in CE include capillary zone electrophoresis, capillary isoelectric focusing (CIEF), isotachophoresis (ITP), and micellar electrokinetic capillary chromatography (MECC).

Capillary zone electrophoresis (CZE) is the most common format of CE, in which the sample is injected as a thin plug at the inlet end of capillary. The two buffer reservoirs and the capillary are filled with the same background electrolyte. Components of the sample migrate toward the detection point and are eventually separated into discrete zones based on their charge-to-mass ratio. In CZE, with uncoated capillary, the EOF generated by the capillary is another parameter that affects the observed mobility of the analytes. MECC is a modification of CZE to allow the simultaneous separation of cationic, neutral and anionic analytes in a single run. MECC may be considered as a chromatographic technique where separation is based on partitioning between two phases, the micellar pseudo stationary phase and the aqueous phase. Usually, the buffer solution in MECC contains a charged surfactant at a concentration above its critical micelle concentration (CMC). At CMC, surfactant monomers are in equilibrium with micelles. Separation in MECC [15] is obtained by taking advantage of electrophoretic mobility,

hydrophobicity (interaction of the components with the micelle), and hydrophilicity of the components of the sample. MECC usually uses uncoated capillary and alkaline buffer to generate a high EOF. The most widely used surfactant in MECC is sodium dodecylsulfate (SDS). Later, the concept of MECC has been extended with the use of cyclodextrin (CD) in chiral separations [16].

Both CIEF and ITP use wall coated capillaries which diminish the EOF. In CIEF, a stable pH gradient is formed in the capillary using carrier ampholytes, and the sample components (proteins) become focused in the gradient at their isoelectric points (p*I*). The desired pH gradient formed by the carrier ampholytes should cover the p*I* range of the components to be separated. ITP is a moving boundary electrophoretic technique in which sample is injected into the capillary between a leading buffer (with mobility greater than the rest of all components) and a terminating buffer (with mobility less than the rest of all components). Zones migrate at equal velocity as a "displacement train" toward the detection point and are detected with zone length proportional to concentration.

Column Technologies for CE and CEC

Fused-silica is by far the dominant material for the making of CE and CEC columns, due to its features for being: (1) easy to fabricate into capillaries, (2) electrically resistant, (3) optically transparent, (4) mechanically strong, and (5) abundant and inexpensive. On average, the surface of fused-silica has 8 $\mu mol/m^2$ of silanol groups which behave as a weak acid, ionizing within the pH range from 3.5 to 9 [17]. The presence of silanol groups is largely unfavorable in CE for two reasons: (1) upon ionization, the anionic silanol groups induce cathodic EOF, which is usually undesirable, and (2) the anionic silanol groups would adsorb positively charged species, causing peak

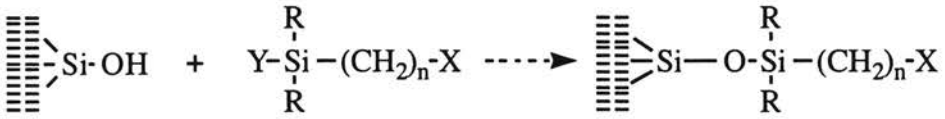
tailing, prolonged or even infinite elution. Several strategies have been developed to reduce solute-wall interaction. These include manipulating the buffer pH [18], dynamic modification of the surface by additives [19-22] in the buffer solution, and chemical modification of the silica surface [23]. Although the various strategies yielded acceptable results, chemical modification of fused-silica/silica appears to be a more promising approach, due to the relatively higher durability of the coating and the possibility of further incorporation of dynamic coating with the existing chemically bonded functions [24]. With these incorporations, the surface coating can be monolayer or multilayer.

Chemical Modification for CE Capillary

Organic moieties can be bonded to the silica surface through a single silicon atom or multiple silicon atoms. The bonding through single silicon atom was referred to by Regnier [25] as modification with *simple silanes*, as opposed to the modification through multiple sites which was referred to as *polymeric coating*. Polymeric coating can be considered as a further step from simple silane coating, since polymeric coating is formed by cross-linking simple coatings to each other or to the other binding sites on the silica surface. Because of the multiple binding sites, polymeric coating is more durable than simple silane coating. Most coatings are bound to the silica surface through siloxane (Si-O-Si) bond [26], although binding through silicon-carbon (Si-C) bonds has also been reported [27]. Siloxane bonded coating suffers the drawback of being instable at pH higher than 8, because of the hydrolysis of the organosilane at such a pH [28]. The erosion of organic silane at high pH may be limited if the organic part of the coating is either less water-wettable or interacts physically with the surface.

The most common simple silane coating is made by bonding halide or methoxy

silanes to the surface silanols, as shown in the following reaction,



where Y is a halide or alkoxy group, R can also be an alkoxy group, and X is either a glycidoxy [29] or amino group [30]. The size of R group and the length of alkyl chain determine the bulkiness of the function, and in turn determine the surface coverage of the organosilane.

After the simple coating is formed, the polymer coating can be made by incorporating the simple silanes into a polymer layer at the silica surface. Polyacrylamide [31], polyvinyl-pyrrolidone [32], and epoxy [33] polymer coatings have been prepared by this approach. Among the reported polymer coatings, polyacrylamide is one of the most popular and successful for protein separations.

Polyacrylamide coating is obtained by first bonding a "linker" such as 3-methacryloxypropyl-triethoxysilane to the capillary surface followed by bonding the linker with acrylamide. The choosing of a suitable linker [34] or a modified acrylamide monomer [35, 36] may serve to prolong the lifetime and hydrophilic stability of the polyacrylamide coating. For example, *N*-substituted acrylamide as monomer has steric protection to the amino group which is believed to be capable of strengthening the hydrophilic stability of the polyacrylamide coating.

Hydrophilic coatings other than polyacrylamide have also been reported. These include polyethers [31, 37, 38], and (bonding through different linking agents) dextrans [39], triblock poly(ethylene oxide)-poly(propylene oxide) [38, 40], and poly(vinyl

alcohol) [41]. These coatings form a hydrophilic layer which diminishes surface charge and in turn the EOF, and block solute-wall interaction.

Coatings with charged moieties are completely different from the above mentioned coatings. The surface charge introduced by the coating is intended to control the magnitude and polarity of the EOF [43, 48]. One of these coating approaches is immobilizing macrocyclic ligands which can form complexes with alkali and alkali-earth metal ions. Upon complexation, the capillary surface will be positively charged [42]. Other coatings can be synthesized with sulfonic acid or quaternary amine groups [43] which provide more stable EOF over a certain pH range.

CEC Column Fabrication

Capillary electrochromatography can be performed in open tube with the stationary phase attached to the capillary wall, but because of its very limited sample capacity, CEC with packed column is the most widely used approach. Figure 2 shows the typical configuration of a CEC column. Figure 2a shows the configuration of a partially packed column. This configuration has a detection window opened at the empty section. Detection by this configuration may be considered as post-column. Figure 2b shows the configuration of a fully packed column. Detection by this configuration is on column, as compared with the configuration in Fig. 2a. Apparently, partially packed column with post-column window has higher detection sensitivity than fully packed column, which has more light scattering due to the stationary phases at the detection point. But partially packed column suffers higher tendency of bubble formation due to its segmented configuration, in which flow velocity is not homogeneous. The flow turbulence at the interface of packed and open segments is easier to bring about bubble formation, which is

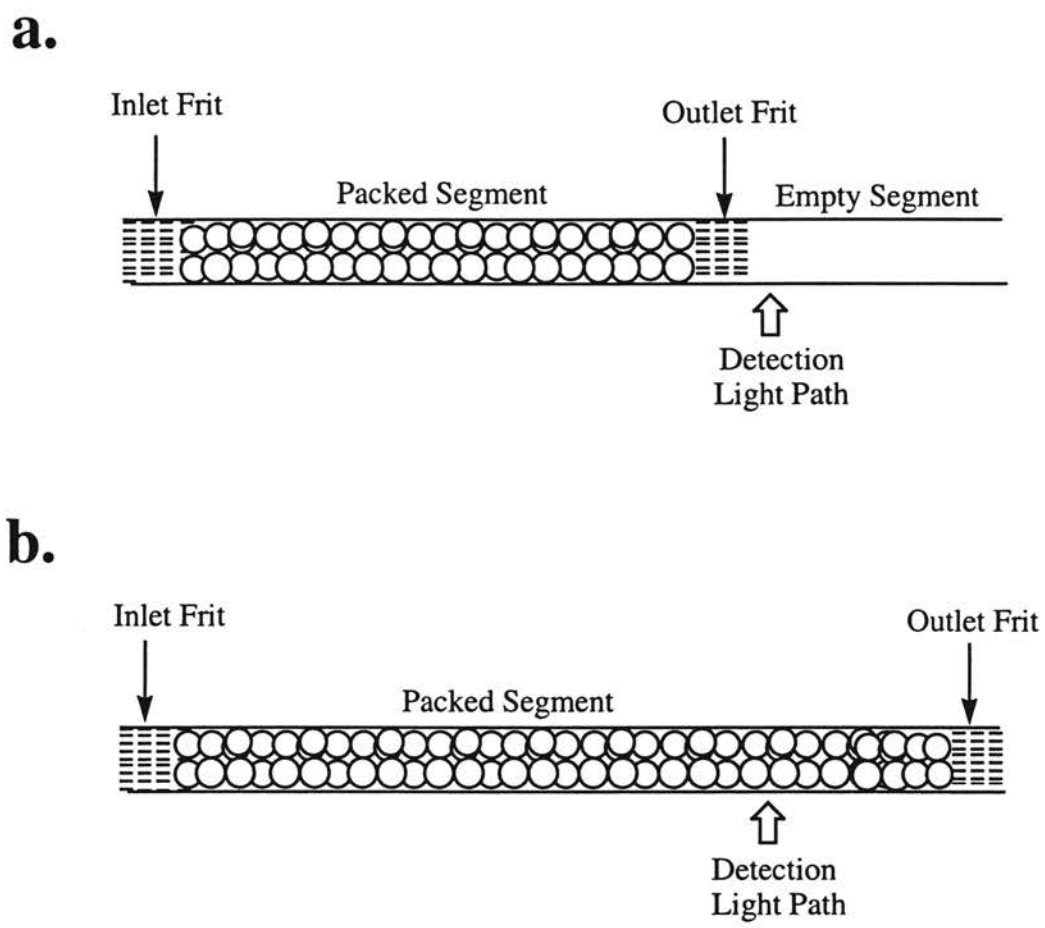


Figure 2. Configurations of CEC column. (a) Partially packed column with post-column detection window. (b) Fully packed column with on column detection window.

a major problem in CEC practice.

The making of CEC columns has proved to be critical for column performance in terms of reproducibility, efficiency, peak shape and resolution. A CEC column usually has the following components: (1) capillary, (2) retaining frits, (3) stationary phase, and (4) on line detection window. Currently, most of the first three components are made of silica based material, although columns packed with polymer (monolithic columns) have also been reported [44]. Capillaries made for CE are used directly for packing CEC columns. Typical inner diameter of a CEC column is 100 μm. Before packing the column, a retaining frit should be made at one end (outlet end) of the capillary. Fabrication of frits is of key importance, since frits should be durable to retain the stationary phase and at the meantime offer high permeability to minimize band broadening. The most common way to construct frits for CEC column is by sintering silica material [45]. Frits made by sintering pure silica wetted with water appeared to be satisfactory in showing stable baseline and current [46]. The frit at the inlet end is made in the same way as the outlet frit after the column is packed.

At least five approaches for packing CEC column have been reported. The first approach is similar to the method used for packing ordinary HPLC columns, in which columns are packed under high pressure delivered by a pneumatic amplification pump [47]. The second approach is referred to as electrokinetic packing [48]. In this approach, a slurry is subjected to an appropriate voltage, and charged silica particles are driven electrokinetically into the capillary which has a frit at one end. Columns packed in this way were reported to give consistently better results and have more reproducible performance than pressure packed columns with the same stationary phase [49, 50]. The third approach uses supercritical CO_2 as the slurrying solvent and pressure packs the

column in a way similar to the first approach [51]. The fourth approach uses *in situ* polymerization in making the CEC columns, which is called 'monolithic' column. More details of monolithic columns will be discussed in the following section. The fifth approach involves the use of centripetal forces to sendiment particles into the capillary with an end frit [52]. Performance of the column so prepared was reported to be similar to those packed electrokinetically. In general, a packing approach should aim to bring about homogenous distribution of the stationary phase, so as to generate reproducible column configuration and to minimize the velocity difference within the column.

Fritless CEC columns have been designed to overcome the difficulties encountered with frits, which are fragile but critical for column performance. Several strategies were developed for making fritless columns. These include homogenous polymerization (monolithic columns), entrapping the silica stationary phase with silicate material [53], or using tapered end instead of the frit to retain the stationary phase [54]. Tapered end is effective to retain stationary phase that can not be sintered in making a frit. Fritless columns have significantly improved mechanical robustness over columns having frits.

Column Packing Materials. The earlier experimenters of CEC used stationary phases commercially available for HPLC, with particle sizes ranging from 3 μm to 5 μm, and pore sizes of 8-10 nm [55]. Octadecyl-silica (ODS) were the most common bonded phase. These materials were not optimized to ensure adequate EOF for CEC. More recently, work with stationary phase manufacturers has led to the development of new particle sizes and stationary phase coatings specially designed for CEC [56]. Several research reports have also addressed these issues [57, 46]. In general, stationary phases for CEC should have coatings to meet the following requirements: (1) the coating should

have adequate charge density to generate EOF, and the sign of surface charge (positive or negative) would control the EOF polarity, (2) the coating should has the functional group to offer chromatographic selectivity. For CEC, since EOF is not a pressure generated flow, very small packing particles can be used. Particle size of the silica stationary phase can be 1 μm or less in CEC. With the stationary phases specially designed for CEC, column efficiencies were usually reported at 100,000 plates per meter. Extremely high efficiencies (> 40 million) were also reported [58]. By using a stationary phase such as sulfonic acid, a constant EOF can be obtained across a wide pH range from 2.5 to 10 [46].

The packing materials for fabricating monolithic column can be silica [59] or non-silica [44, 60-63]. Column fabrication by *in situ* polymerization is relatively more convenient and does not require inlet and outlet frits. Tedious packing process is also avoided. In addition, pore size and charge density may be independently and precisely controlled through adjusting the porogenic solvent and charged functionalities in the polymerization mixture, respectively. CEC column so prepared can have tailored properties in chromatographic selectivity and EOF monitoring. For example, the stationary phase may be polymerized to possess reversed-phase behavior which mimics octadecyl-silica (ODS) [62, 63], or be made with molecular imprinting technique to have predetermined exclusive selectivity to the mother molecule [44].

Conclusion

Although CE and CEC differ significantly in the column design, they share in common the EOF to drive the mobile phases through the column. The stationary phase imparted CEC more capability as a microscale separation technique. At the moment,

most of the hardware in the CEC experimental aspects (such as sample introduction, detection, etc.) is still based on those of CE. Development in these aspects should serve to strengthen the potential of CEC.

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CHAPTER III

CAPILLARY ZONE ELECTROPHORESIS OF PROTEINS WITH FUSED-SILICA CAPILLARIES HAVING POLYMERS AND SURFACTANTS ADSORBED ONTO SURFACTANT MOIETIES THAT ARE PREVIOUSLY COVALENTLY BOUND TO THE CAPILLARY COLUMN SURFACE*

Introduction

The success of protein separations by CZE depends primarily on the availability of fused silica capillaries with hydrophilic coatings which diminish protein-wall interaction and in turn allow high separation efficiency and reproducible analysis to be realized. There are three approaches to shielding the silica surface from protein adsorption to the negatively charged surface silanols: (i) physical adsorption of hydrophilic polymers such as cellulose acetate [1], chitosan [2] and poly(vinyl alcohol) [3] just to name a few, (ii) covalently attached hydrophilic coatings typically consisting of polyether [4], multilayered neutral and charged coatings [5,6], etc. and (iii) a combination of both chemically and physically adsorbed coatings (i.e., hybrid coating) [7-9]. Although approach (i) is simple and can alleviate the adsorption problem and allows high

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separation efficiency to be achieved, the physical coating (i.e., dynamic coating) is rather unstable at pH > 5.0 in the case of PVA [3] and at pH >7.5 in the case of cellulose acetate [1]. This is because the hydrogen bonding which ensures the adsorption of hydroxylic polymers to the fused silica surface becomes weaker as the ionization of silanols increases at higher pH. Approach (ii) is superior to (i) in terms of migration time reproducibility and separation efficiency, but is not flexible as far as changing the nature of the coating is concerned. In other words, since each type of covalently attached coating will be suitable to some proteins and not others, several coated capillaries should be available thus multiplying the number of hardware for achieving the desired separations. This problem can be overcome by the hybrid coating which combine the flexibility of approach (i) to the highest efficiency of (ii). For more details about the existing various strategies of capillary coatings, see recent reviews [10-12].

In this report, we are concerned with approach (iii) to provide novel capillary coatings for protein separations. Our laboratory has previously made several contributions to the area of capillary coating technology [4, 6, 13, 14] by introducing hydrophilic coatings with reduced, bidirectional, constant and zero EOF as well as schemes for controlling the EOF with coated capillaries [15-17]. The present report is thus a continuation to our recent efforts in the area of capillary coatings and EOF control.

Materials and Methods

Instruments

The capillary electrophoresis instrument includes a Model MJ30P0400 and a Model MJ30N0400 high-voltage power supplies with positive and negative polarities

from Glassman High Voltage (White House Station, NJ, USA), and a Linear (Reno, NV, USA) Model 200 UV-Vis variable-wavelength detector equipped with a cell for on column detection. The electropherograms were recorded with a Shimadzu (Columbia, MD, USA) Model CR601 computing integrator.

Materials

Lysozyme from turkey egg white, cytochrome c from horse heart, ribonuclease A and α-chymotrypsinogen A, both from bovine pancreas, β-lactoglobulin A and B from bovine milk, α-lactalbumin from human milk, and human serum albumin were purchased from Sigma (St. Louis, MO, USA). Boron trifluoride etherate, epoxybutane and epoxydecane were obtained from Aldrich (Milwaukee, WI, USA). Reagent grade sodium phosphate monobasic, sodium hydroxide, hydrochloric acid, and the EOF tracer dimethyl sulfoxide were obtained from Fisher Scientific (Pittsburgh, PA, USA). *n*-Octadecyl-*N*-[3-(trimethoxysilyl)propyl)]-*N,N*-dimethyl ammonium chloride was from United Chemical Technologies, Inc. (Bristol, PA, USA). Hydroxypropyl celluloses (HPC) of molecular weight 55,000-77,000, 150,000 and 400,000 were purchased from TCI America (Portland, OR, USA), Brij 35 was obtained from Pierce Chemical Company (Rockford, IL, USA). Fused silica capillary was obtained from Polymicro Technology (Phoenix, AZ, USA).

Capillary Coating

Fused silica capillary (I.D. 50 μm) was etched with 1 M NaOH for 15 min, then rinsed with deionized water, 0.1 M HCl and deionized water, respectively, each for 5 min.

The capillary was then rinsed thoroughly with molecular sieve dried DMF for 15 min. After this pretreatment, the capillary was first filled with commercial *n*-octadecyl-*N*-[3-(trimethoxysilyl)propyl]-*N,N*-dimethyl ammonium chloride (1:1 v/v methanol solution), and then was put in the oven for 30 min at 125 °C. This step was repeated 3 times, and each time the capillary was refilled with the organosilane to obtain efficient silanization. This type of coating was previously introduced by our laboratory for the preparation of HPLC columns for micellar liquid chromatography [18]. The silanized capillary was then rinsed with DMF and methanol. The structure of the CSM coated capillary is shown in Fig. 1.

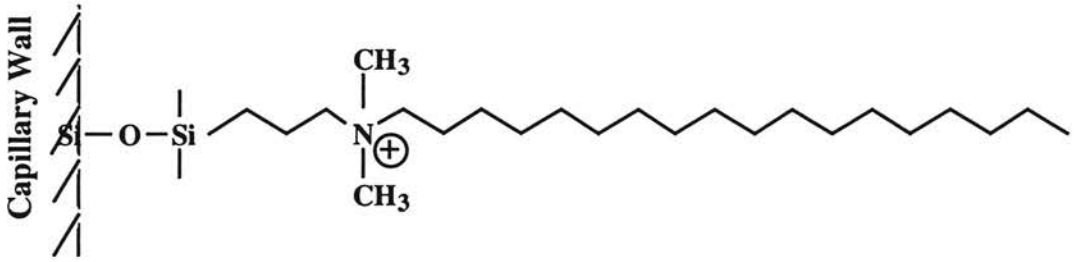
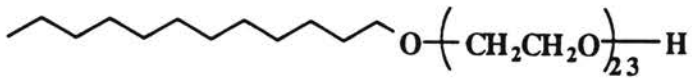


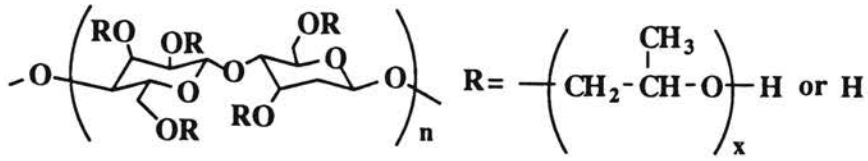
Figure 1. Structure of the capillary surface having covalently bound CSM moieties.

The modified or native hydroxypropyl cellulose (HPC) coating was applied by filling the CSM coated capillaries with 1% (w/w) of the native or modified HPC solutions and equilibrating statically for 1 h. For convenience, this can be left for overnight for next day's use. The excess HPC was driven out by rinsing the capillary with deionized water for 5 min. Similarly, the hyaluronic acid coating can be applied by filling the capillary with 1% (w/w) hyaluronic acid and equilibrating statically for 20 min. The modified HPC and hyaluronic acid coating layer can be removed by rinsing the capillary with methanol. The structures of the various coatings are shown in Fig. 2.

Brij 35

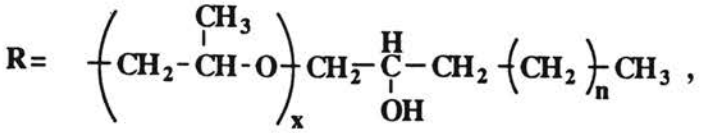


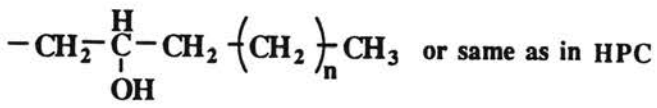
HPC



Epoxyalkyl modified HPC

Same repeating units as in HPC with partially substituted R





For EBHPC, n = 0; for EDHPC, n = 6

Hyaluronic acid

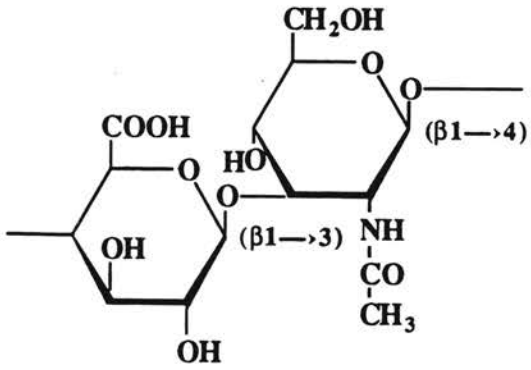


Figure 2. Structures of the various species used as adsorbed layers in the CSM coated capillaries.

HPC Modification

Epoxybutane modified HPC (EBHPC). HPC (0.208 g) was dissolved in 5 mL of molecular sieve dried DMF. To this solution was added 0.0182 g (0.25 mmol) of epoxybutane, the mixture was well stirred, and 10 μL of boron trifluoride etherate was added while stirring. After stirring for 1 h, a second 10 μL portion of boron trifluoride etherate was added to the mixture. The mixture was further stirred for 5 hs. The whole process was done at room temperature. The clear solution thus obtained was dried overnight in a speed vac. The product collected was then dissolved in deionized water to make 1% (w/w) solution. To examine the effect of the size of the coating polymer, HPC's with molecular weights of 55,000-77,000, 150,000 and 400,000 were modified with epoxybutane. The structures of the EBHPC are illustrated in Fig. 2.

Epoxydecane Modified HPC (EDHPC). HPC (MW 55,000-77,000) modification with epoxydecane was carried out in the same way as with EBHPC. In this process, 0.0548 g (0.33 mmol) of epoxydecane was reacted with 0.208 g of HPC (MW 55,000-77,000). For structure of EDHPC, see Fig. 2. The modification of higher molecular weight HPC (i.e., 150,000 and 400,000) with epoxydecane produced EDHPC with poor water solubility.

Other Operational Parameters

Protein solutions of 1.0 mg/mL were introduced into the capillary by siphoning for a fixed time of 5 seconds at a height of 15 cm. Between each run of protein separation, the column was rinsed with running buffer for 1 min. All analyses were run at room temperature without temperature control.

Results and Discussion

Analysis of the Electroosmotic Flow

To assess the effect of each coating on the electroosmotic flow (EOF), the EOF was measured over a wide range of pH on bare fused-silica and CSM coated capillaries as well as on CSM coated capillaries to which neutral and charged hydrophilic polymers were adsorbed. The results are shown in Fig. 3 in terms of electroosmotic mobility of dimethyl sulfoxide, an EOF tracer, versus pH of the running electrolyte. Compared with bare fused-silica capillary, the EOF of the CSM coated capillary is bidirectional changing from anodic at pH < 4.5 to cathodic at pH > 4.5 passing through zero at pH of ca. 4.5. Thus, the isoelectric point of the capillary wall is around pH 4.5 where the negatively charged silanol groups (i.e., residual or unreacted silanols) are balanced by the permanently positively charged quaternary ammonium groups of the CSM coating. At pH < 4.5, the ionization of silanols is partial, and the net charge of the surface is positive. At pH > 4.5, the ionization of the silanols is increased thus leading to a surface with a net negative charge. As expected, adsorbing Brij 35 (see Fig. 4a for a schematic of Brij 35 adsorption) from a 0.01 % buffer solution led to a further decrease in the EOF of the CSM coated capillary without altering the p*I* of the surface and the bidirectional pattern of the EOF was conserved, see Fig. 3. The adsorption of Brij 35 to the CSM coated capillary makes the wall more viscous and also shields the charged surface, and as a result the EOF is slowed down [9, 19]. By adsorbing a layer of HPC of MW 55,000-77,000 to the CSM coated capillary, the EOF was further decreased [8] while the bidirectional pattern of the EOF and the p*I* of the capillary surface were not altered. Upon adsorbing to the CSM coated capillary a layer of higher MW HPC (i.e., HPC of MW 150,000 or

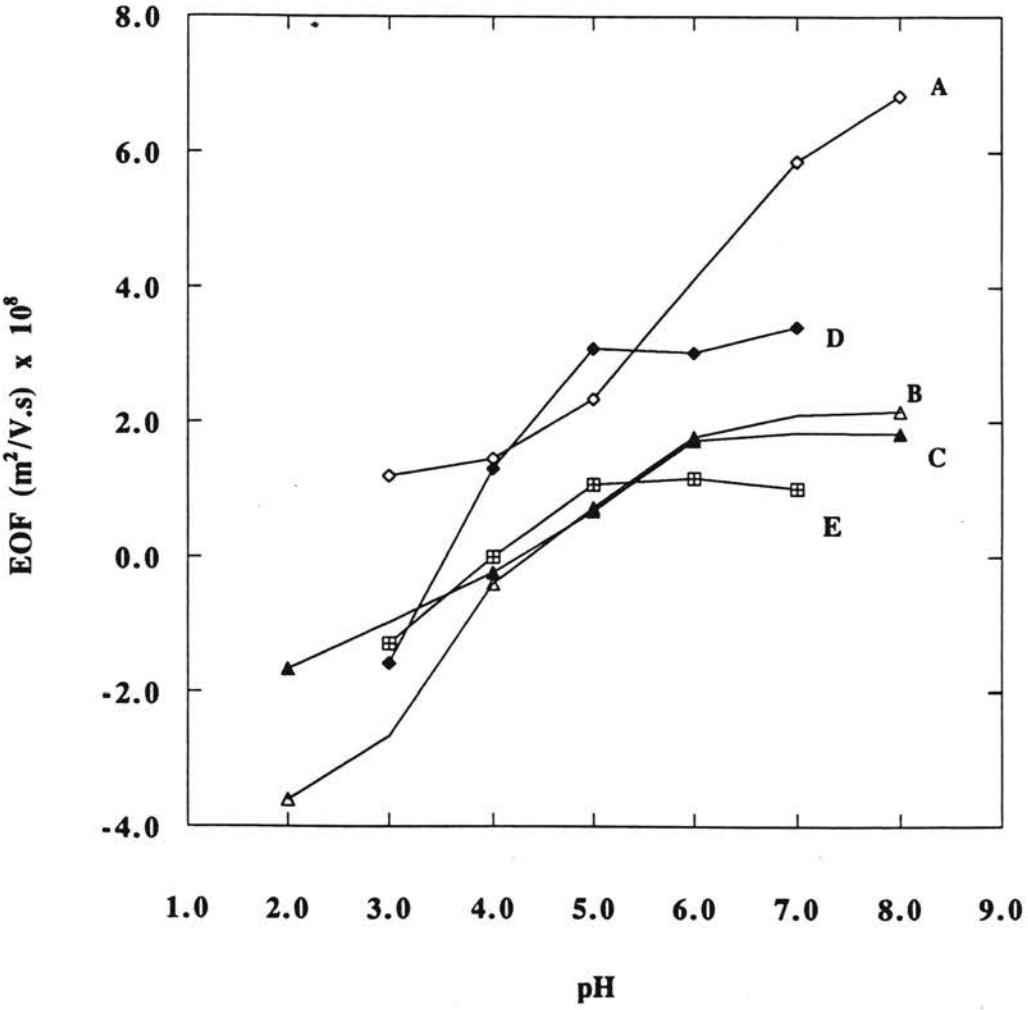


Figure 3. Dependence of electroosmotic mobility on pH for (A) bare fused-silica capillary; (B) CSM coated capillary; (C) CSM coated capillary with 0.01% Brij 35 in buffer; (D) CSM coated capillary coated with an adsorbed layer of hyaluronic acid; (E) CSM coated capillary with an adsorbed layer of HPC (MW 55,000-77,000).

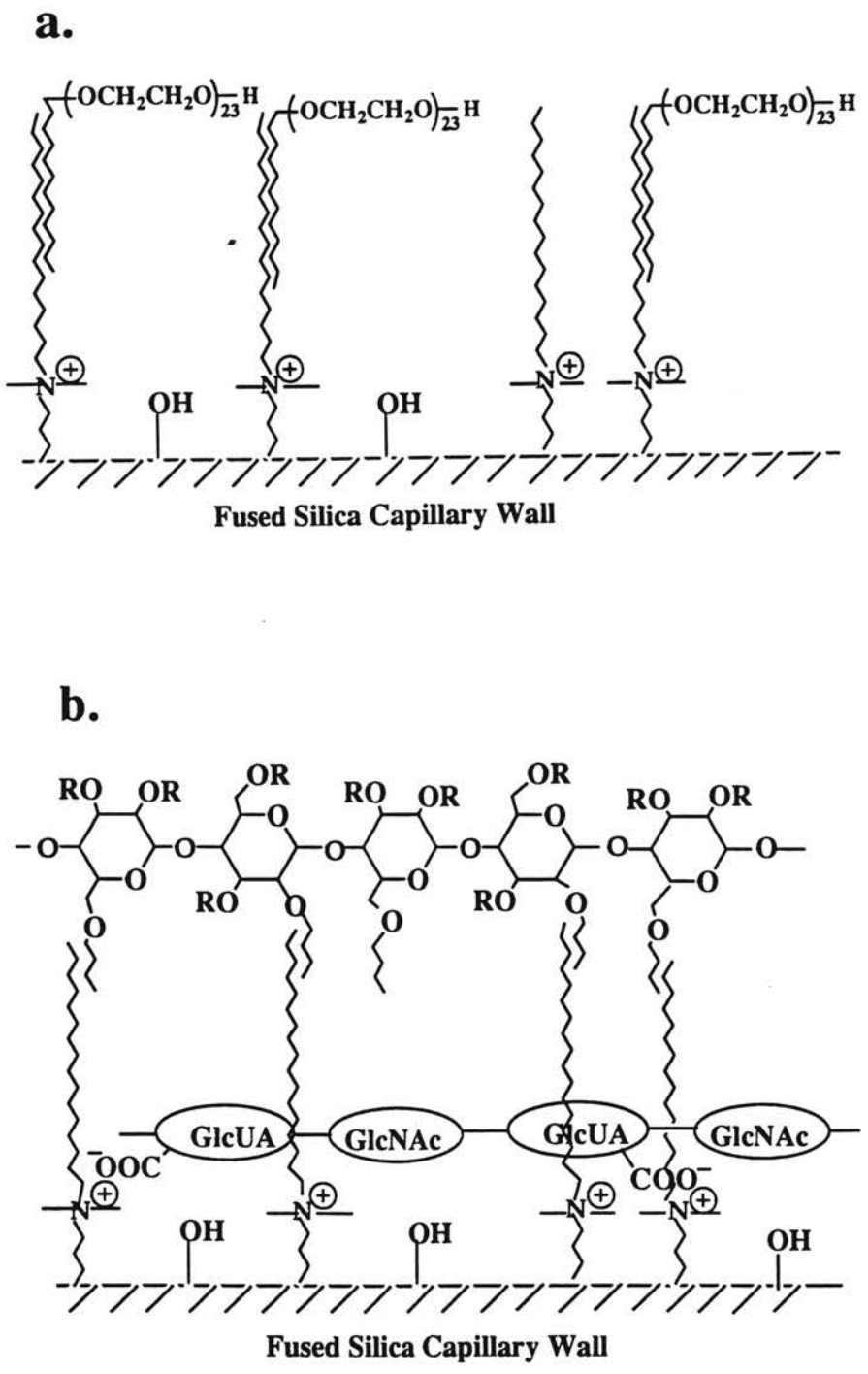


Figure 4. Schematic illustrations of the CSM coated capillaries with adsorbed layers of Brij 35 in (a) and HPC or modified HPC and/or hyaluronic acid in (b). The hyaluronic acid is symbolized by its glucuronic acid (GlcUA) and N-acetylglucosamine (GlcNAc) repeating monosaccharide residues.

400,00), the EOF was not measurable. Also, when a neutral polymer layer of EBHPC (see Fig. 4b for a schematic illustration of the adsorption of native and modified HPC) was adsorbed, the EOF was not detectable, regardless of the molecular weight of the parent HPC molecule. The adsorbed layer, consisting of relatively large molecular weight HPC or EBHPC, provided more shielding of the surface silanols and the quaternary ammonium groups and at the same time increased the viscosity of the layer at the solid-solution interface, thus leading to the suppression of the EOF [19]. On the other hand, adsorbing a negatively charged polymer layer such as hyaluronic acid to the CSM coated capillary increased the EOF with respect to other adsorbed species and in a certain pH range (i.e., 4.5-5.5) an even stronger EOF than on the untreated fused-silica was observed. With adsorbed hyaluronic acid, the p*I* of the capillary surface decreased to 3.5, which corresponds to a decrease of one p*I* unit when compared to CSM-Brij 35 or CSM. Due to electrostatic attraction between the oppositely charged hyaluronic acid and the quaternary ammonium functions on the capillary wall, it is believed that the hyaluronic acid layer was adsorbed closer to the wall than the HPC and modified HPC layer (see Fig. 4b).

When the CSM coated capillary column surface is saturated with adsorbed surfactant or polymer, the EOF tends to remain constant. This is shown by the EOF study using CSM coated capillaries with an adsorbed Brij 35 layer (see Fig. 5a). At pH 7.0 the cathodic EOF decreased as the amount of Brij 35 in the buffer was increased up to 0.02% (w/w), and then stabilized in the concentration range of 0.02 to 0.1% (w/w). A similar pattern was obtained for the anodic flow at pH 3.0. When electrophoresing proteins at pH 5.0, the migration time of some basic proteins decreased first at low Brij 35 concentration (0.002 to 0.01%) and then remained almost the same at higher Brij 35

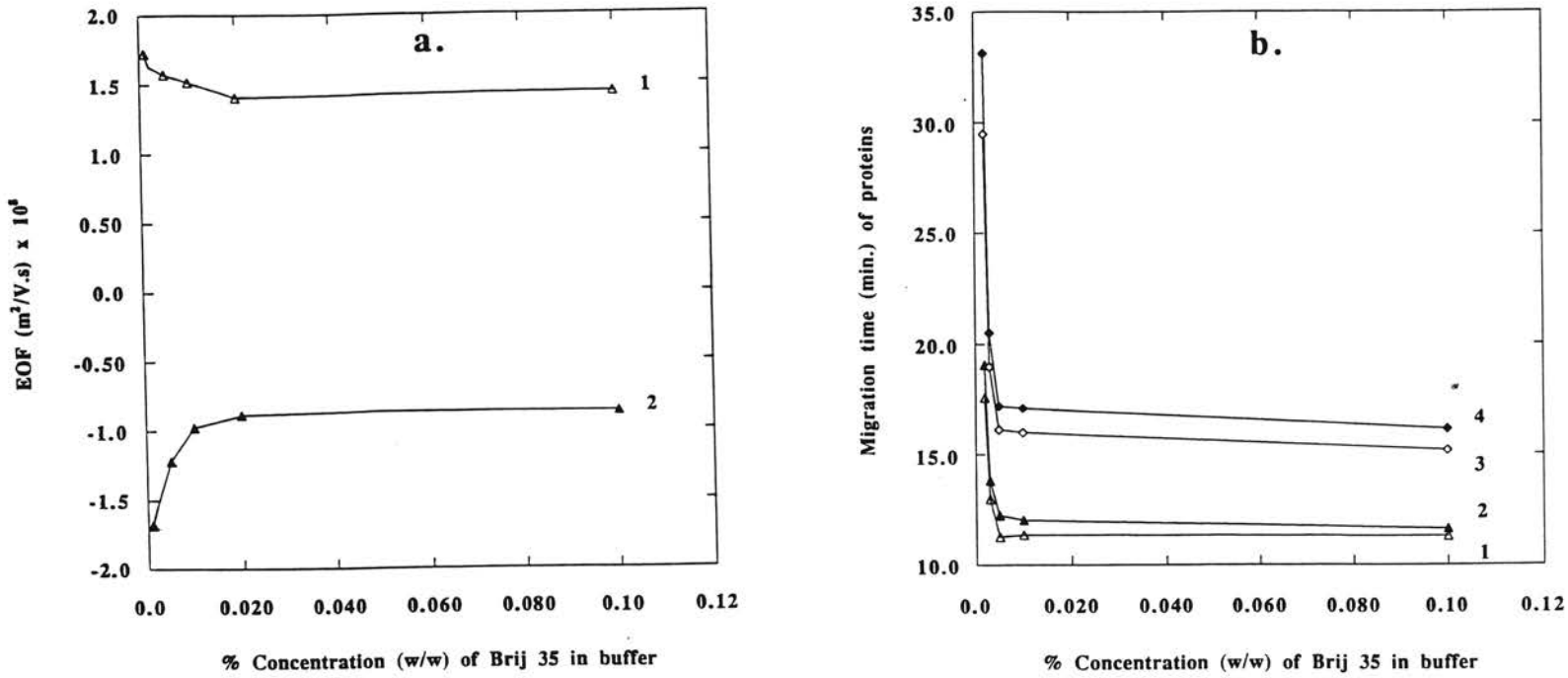


Figure 5. (a) Electroosmotic flow versus percentage concentration (w/w) of Brij 35 in buffer. pH were fixed at 7.0 in (1) and at 3.0 in (2). (b) Migration time of basic proteins (1) lysozyme, (2) cytochrome c, (3) ribonuclease A, and (4) α-chymotrypsinogen A versus percentage concentration (w/w) of Brij 35 in buffer. Conditions: 0.1 M NaH_2PO_4, pH 5.0; detection wavelength, 210 nm; +21 kV, 15 μA; L = 85 cm, l = 50 cm, 50 μm I.D..

concentration in the running electrolyte (0.02 to 0.10%) (see Fig. 5b). At pH 5.0, the EOF is very small in magnitude, and the decrease in migration time of the proteins under investigation with increasing Brij 35 concentration in the running electrolyte can be attributed to the decrease in hydrophobic interaction between the proteins and the octadecyl surface as the amount of adsorbed Brij 35 is increased. It is therefore concluded that a threshold concentration exists for a given adsorbed coating, which is believed to be the concentration that saturates the column surface. It is further observed that reproducible migration times for proteins in CZE can only be obtained when the column surface is saturated by the adsorbed surfactant or polymer. For protein CZE on a CSM coated capillary with an adsorbed layer of EBHPC, 1% (w/w) of EBHPC was determined to be a concentration higher than the threshold value. Therefore 1% (w/w) is the concentration for HPC and modified HPC used throughout this study.

Reproducibility and Coating Stability

The CSM coated capillary with an adsorbed layer of EBHPC was very durable for performing the CZE of some basic proteins at pH 3.0-6.0. As long as the CSM coating remains on the column surface, the EBHPC coating can be very stable due to strong hydrophobic interactions between the polymer and the covalently attached CSM. At pH 3.0-6.0, the covalently attached CSM layer can sustain continuous electrophoretic runs for at least 100 hs, and the adsorbed EBHPC layer can hold for a whole day (8 hs) operation without being replenished. Under such circumstances, no EBHPC is needed as buffer additive and the capillary still shows reliable reproducibility and high column efficiency. Column reproducibility can be restored and maintained by reloading the column with EBHPC and equilibrating statically for 1 h or longer. As shown in Table I, the

reproducibility of migration time of some basic proteins, namely lysozyme, cytochrome c, ribonuclease A and α-chymotrypsinogen A, as expressed by %RSD is quite good from run-to-run, day-to-day and column-to-column. The CSM coated capillary with adsorbed EBHPC and hyaluronic acid layers showed the same behavior. However, when large acidic proteins are electrophoresed at pH 7.0 on CSM coated capillaries with adsorbed layers of both EBHPC (the parent HPC has MW = 400,000) and hyaluronic acid, the coating was contaminated by adsorbed proteins as manifested by significant band broadening and prolonged protein migration time after seven electrophoretic runs. In the event of contamination, the column performance can be restored by rinsing with methanol and reloading hyaluronic acid and EBHPC (HPC MW = 400,000). Table II summarizes

TABLE I. Percent relative standard deviation (%RSD) of the migration time of some basic proteins on CSM coated capillary with an adsorbed layer of EBHPC.

	EBHPC (55,000-77,000)			EBHPC (150,000)			EBHPC (400,000)		
Protein	run-to-run	day-to-day	column-to-column	run-to-run	day-to-day	column-to-column	run-to-run	day-to-day	column-to-column
Lysozyme	0.44	0.85	1.5	0.33	0.65	1.45	0.36	0.63	1.32
Cytochrome c	0.58	1.88	2.32	0.50	1.76	2.23	0.48	1.45	2.22
Ribonuclease A	1.20	2.00	2.51	1.20	1.86	2.42	1.18	1.81	2.36
α-Chymotrypsinogen A	1.56	2.54	3.03	1.35	2.33	2.85	1.33	2.26	2.68

Conditions: 0.1 M sodium phosphate, pH 6.0; + 21 kV; 30 μA; capillary, L= 85 cm, l = 50 cm, I.D. = 50 μm. In all cases, n = 6.

the migration time for some acidic proteins on a CSM coated capillary with adsorbed EBHPC (HPC MW = 400,000) and hyaluronic acid at pH 7.0. The %RSD of migration times before and after the column treatment with methanol are about the same, indicating the column performance is restorable with such a treatment.

TABLE II. Average migration time and %RSD of some acidic proteins obtained on a CSM coated capillary with adsorbed EBHPC (HPC MW = 400,000) and hyaluronic acid.

	Fresh column (n = 5)		Contaminated column after treatment with methanol (n = 5)	
Protein	Average migration time (min)	%RSD	Average migration time (min)	%RSD
HSA	18.72	1.23	18.26	1.32
β-Lactoglobulin A	19.20	1.40	19.17	1.15
β-Lactoglobulin B	21.45	2.15	21.20	1.92
α–Chymo-trypsinogen A	30.47	3.02	30.23	2.55

Conditions: 0.05 M sodium phosphate, pH 7.0; -21 kV; 30 μA; Capillary, L = 85 cm, l = 50 cm x 50 μm.

Protein CZE

As can be seen in Table III, CSM coated capillaries with an adsorbed layer of Brij 35 gave the shortest migration time for the four basic proteins under investigation. This is simply due to the relatively higher EOF in the presence of adsorbed Brij 35. Brij 35 saturated the column surface at concentration of 0.05% w/w. The hydrophobic tail of Brij 35 undergoes non-polar interaction with octadecyl groups of the CSM coating on the capillary column surface, but this interaction is not strong enough for Brij 35 to hold for

long, as was also reported by Regnier and coworkers [9]. Brij 35 has to be replenished from the running electrolyte which contains Brij 35 as an additive.

By adsorbing unmodified HPC instead of Brij 35, the migration time becomes longer. It was observed that a 1% HPC solution was sufficient to saturate the column

TABLE III. Migration time of some basic proteins using different coatings.

Protein	Migration time (min)					
	0.01% Brij 35 as buffer additive	1% HPC (55,000-77,000)	1% EBHPC (55,000--77,000)	1% EBHPC (150,000)	1% EBHPC (400,000)	1% EDHPC (55,000-77,000)
Lysozyme	11.36	13.64	13.51	14.13	14.27	17.1
Cyto-chrome C	12.24	14.59	14.67	15.80	16.49	18.48
Ribonu-clease A	16.14	21.20	21.13	23.67	27.12	28.28
α-Chymo-trypsino-gen A	17.23	23.36	23.00	26.10	30.39	31.65

Conditions: 0.1 M sodium phosphate, pH 5.0; +21 kV; 15 μA; Capillary, L = 85 cm, l = 50 cm x 50 μm.

surface as was manifested by the column constancy in terms of migration time and efficiency. Due to its polymeric nature with multiple sites for interaction, HPC (MW = 55,000-77,000) exhibited stronger hydrophobic interaction with the octadecyl groups of the CSM capillary coating than did Brij 35. Compared with Brij 35, HPC (MW = 55,000-77,000) concentration as buffer additive can be as low as 0.001% to maintain good column reproducibility.

The CSM coated capillary with an adsorbed layer of HPC (MW = 55,000-77,000) has satisfactory performance with basic proteins in the pH range 3.0-5.0, in which the average column efficiency can easily exceed 125,000 plates. This column efficiency is significantly higher than that of the CSM coated capillary with an adsorbed layer of Brij 35 surfactant. The latter usually showed an average column efficiency lower than 75,000 plates. The results are summarized in Table IV.

TABLE IV. Average column separation efficiency of some basic proteins at pH 5.0 and 6.0.

pH	Average separation efficiency, N x 10^{-3}					
	0.01% Brij 35 as buffer additive	1% HPC (55,000-77,000)	1% EBHPC (55,000--77,000)	1% EBHPC (150,000)	1% EBHPC (400,000)	1% EDHPC (55,000-77,000)
pH 5.0	71.5	140.5	151	140	132.5	190.5
pH 6.0	CNM	CNM	85.5	105.5	56	CNM

CNM = cannot be measured. Other conditions: 0.1 M sodium phosphate; +21 kV; capillary, CSM coated having different adsorbed layers, L = 85 cm, l = 50 cm x 50 μm.

CSM coated capillaries with an adsorbed layer of HPC (MW = 55,000-77,000) exhibited almost the same migration time as with adsorbed EBHPC derived from HPC with MW = 55,000-77,000. However, increasing the size of HPC in the epoxybutane derivatives of HPC (EBHPC) to a MW of 150,000 and 400,000 increased the migration time by about 13% and 30%, respectively. Because of their larger sizes, the EBHPC derived from HPC of MW 150,000 and 400,000 shielded the surface better and provided a more viscous wall so that the EOF is practically eliminated. In addition, these derivatives are large polymers so that they can undergo some weak hydrophobic

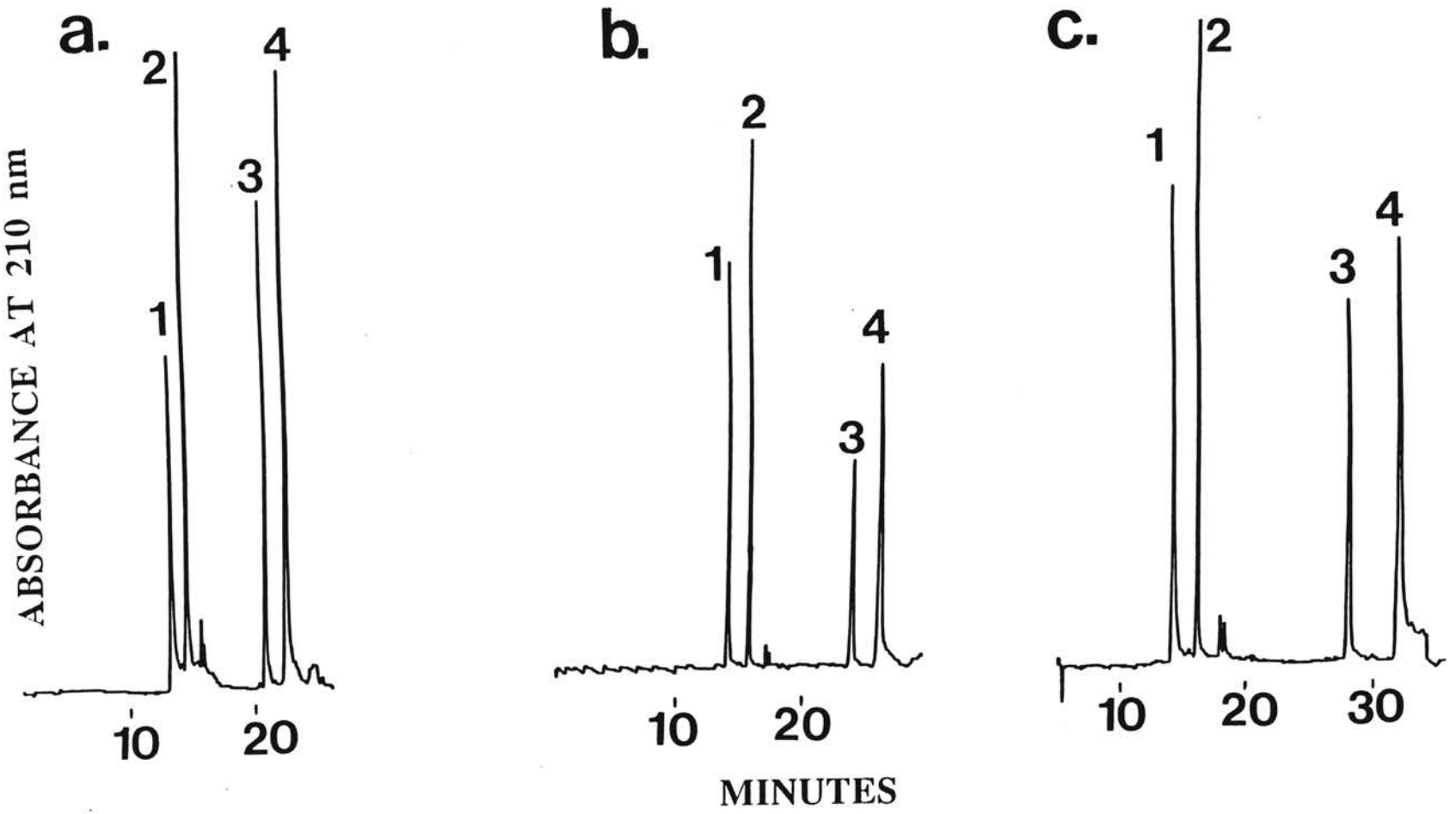


Figure 6. Electropherograms of four basic proteins on a CSM coated capillary with an adsorbed layer of EBHPC derived from HPC with molecular weights of (a) 55,000-77,000, (b) 150,000 and (c) 400,000. Conditions: 0.1 M NaH_2PO_4, pH 6.0; detection wavelength, 210 nm; +21 kV, 30 μA. Capillary, L = 85 cm, l = 50 cm, 50 μm I.D.. Peak assignment same as in Fig. 5.

interaction with the proteins, a phenomenon that leads to a longer migration time (see Fig. 6). Due to its stronger hydrophobic character, the adsorbed EDHPC layer (i.e., epoxydecane derivative of HPC of MW 55,000-77,000) on the CSM coating exhibited stronger interactions with the proteins than with its counterpart EBHPC. This was manifested by the longer analysis time (37%). It should be noted that epoxydecane modified HPC (EDHPC) resulted in products with poor solubility in water, probably due to the stronger hydrophobic character imparted by the substitution with epoxydecane. Capillary coating with EDHPC using the parent HPC of molecular weights of 150,000 and 400,000 was therefore not conducted.

The various coated capillaries were evaluated in terms of separation efficiency under the same operating conditions at pH 5.0 and 6.0. The results are listed in Table IV. The CSM coated capillary with an adsorbed layer of Brij 35 showed the lowest separation efficiency. At pH 6.0, Brij 35 coating as well as HPC (MW = 55,000-77,000) and its EDHPC derivative were not efficient in shielding the surface of the capillary as was manifested by strong protein adsorption to the capillary surface, and no peaks were observed. On the other hand, when the epoxybutane derivatives of HPC (i.e., EBHPC) of MW = 55,000-77,000, 150,000 or 400,000 were adsorbed, the CSM coated capillaries yielded relatively high separation efficiencies as shown in Table 4 and Fig. 6.

To accelerate the EOF, and consequently affect a faster separation of basic proteins, a layer of hyaluronic acid was adsorbed onto the EDHPC capillary. As can be seen in Fig. 7 the migration time was decreased by almost 50% in the presence than in the absence of hyaluronic acid. Adding the hyaluronic acid did not have an adverse effect on the separation efficiency. In fact, the average plate counts were 193,000 and 173,000 in the absence and presence of hyaluronic acid, respectively.

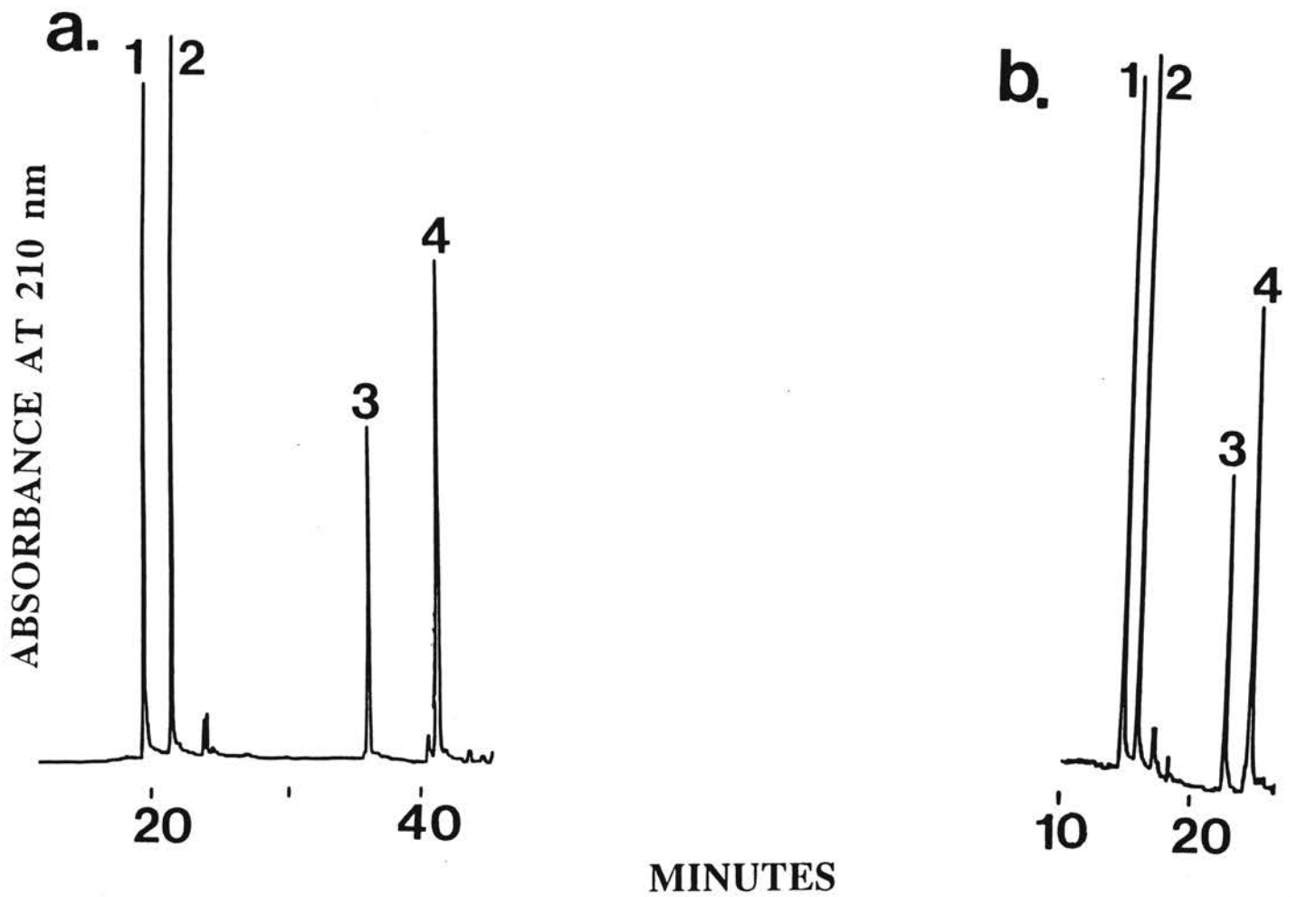


Figure 7. Electropherograms of basic proteins on CSM coated capillary with an adsorbed layer of EDHPC derived from HPC of MW 55,000-77,000. (a) Capillary was not flushed with 1% hyaluronic acid; (b) Capillary was flushed with 1% hyaluronic acid. Conditions: 0.1 M NaH_2PO_4, pH 5.0; detection wavelength, 210 nm; +18 kV, 10 μA; L = 85 cm, l = 50 cm, 50 μm I.D. Peak assignment same as in Fig. 5.

Adsorbing hyaluronic acid to the CSM coated capillaries followed by adsorbing a layer of EBHPC yielded almost the same migration times for acidic proteins regardless of the size of the EBHPC (see Fig. 8). Conversely, the separation efficiency seems to be affected by the size of the EBHPC, and the average plate counts as measured with β-lactoglobulin A and B and α-lactalbumin peaks increases from 35,000 to 67,500 and then somewhat decreases to 57,000 when going from EBHPC with a MW of 55,000-77,000 to 150,000 and to 400,000, respectively.

Finally, using the EBHPC derived from HPC of MW 400,000 allowed the separation of eight acidic and basic proteins at pH 3.0 with a relatively high separation efficiency (141,500 plates), see Fig. 9.

Conclusion

We have shown that fused silica capillaries with hybrid coating are very suitable for the separation of proteins by CZE. Hybrid coating refers to the presence of a covalently attached layer (i.e., the primary layer) to the capillary wall to which a layer of neutral or charged hydrophilic polymer is adsorbed. This configuration allows the replacement of the adsorbed layer by another species more suitable for a given separation problem by simply washing the previously adsorbed species and reloading the capillary wall with the desired species or a mixture of species. This kind of surface manipulation proved easy to perform and allowed the separations of both acidic and basic proteins. In addition, the EOF of the capillary can be varied conveniently by changing the nature of the adsorbed layer.

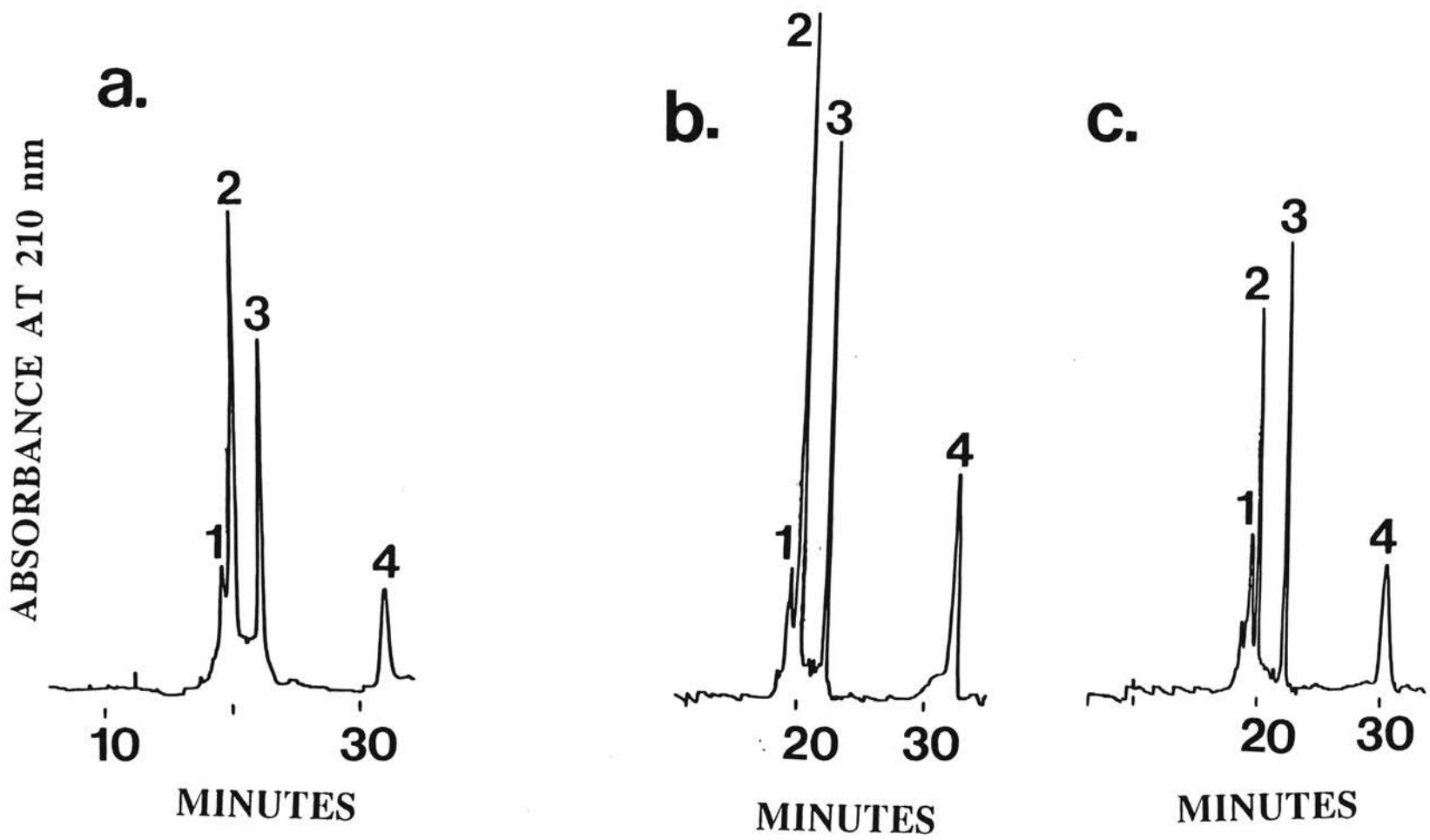


Figure 8. Electropherograms of four acidic proteins on CSM coated capillary with EBHPC derived from HPC of molecular weights of (a) 55,000-77,000, (b) 150,000 and (c) 400,000. Capillary was flushed with 1% hyaluronic acid. Other conditions: 0.05 M NaH_2PO_4, pH 7.0; detection wavelength, 210 nm; -21 kV, 30 μA; L = 85 cm, l = 50 cm, 50 μm I.D.. Peak assignment: (1) albumin, (2) β-lactoglobulin A, (3) β-lactoglobulin B and (4) α-lactalbumin.

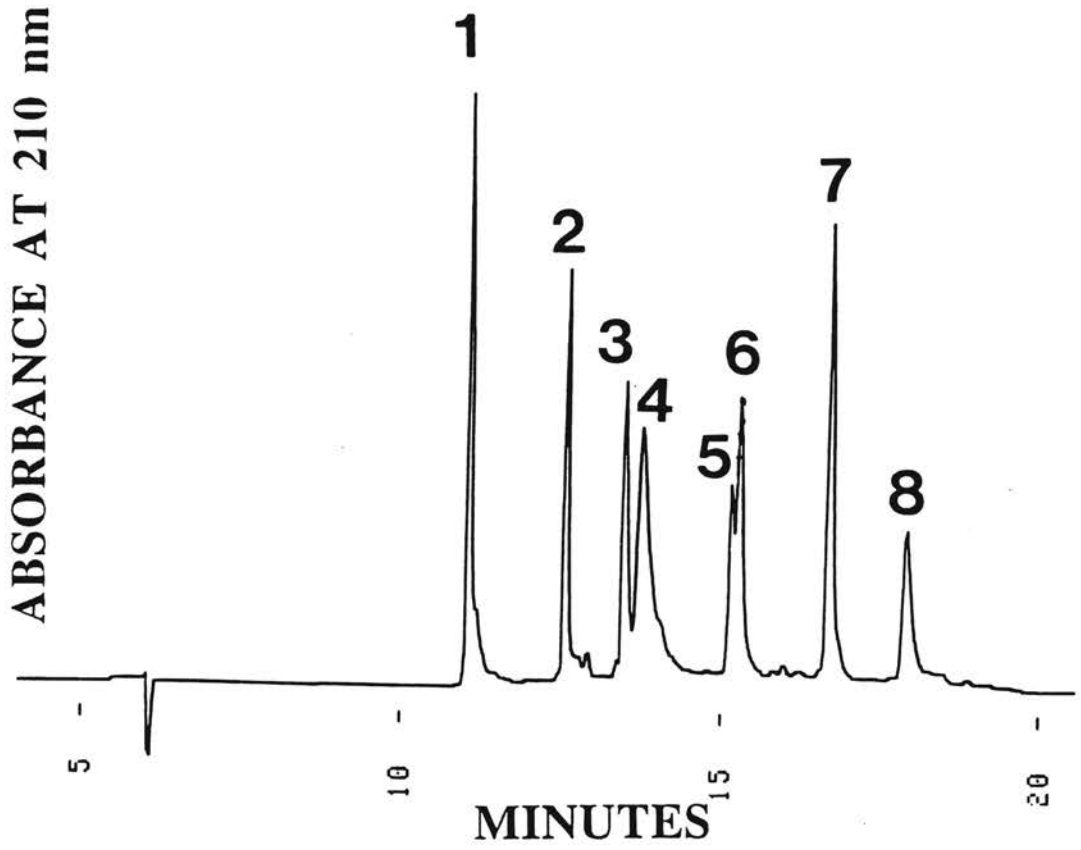


Figure 9. Electropherogram of some acidic and basic proteins on a CSM coated capillary with an adsorbed layer of EBHPC derived from HPC of MW 400,000 at pH 3.0. Other conditions: 0.05 M NaH_2PO_4, pH 3.0; detection wavelength, 210 nm; +21 kV, 30 μA; *L* = 85 cm, *l* = 50 cm, 50 μm I.D.. Peak assignment: (1) cytochrome c, p*I* = 10.2; (2) lysozyme, p*I* = 11.0; (3) β-lactoglobulin A, p*I* = 5.1; (4) conalbumin, p*I* = 6.0; (5) hemoglobin, p*I* = 5.6; (6) ribonuclease A, p*I* = 9.3; (7) α-chymotrypsinogen A, p*I* = 9.2; (8) trypsin inhibitor, p*I* = 4.2.

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CHAPTER IV

CAPILLARY ELECTROCHROMATOGRAPHY OF DERIVATIZED MONO- AND OLIGOSACCHARIDES WITH A SPECIALLY DESIGNED OCTADECYL-SILICA STATIONARY PHASE*

Introduction

Capillary electrochromatography (CEC) whose potentials were first demonstrated in 1974 by Pretorius *et al.* [1], is currently making major advances toward becoming a microcolumn separation technique [2-4] which can rival micro-liquid chromatography (μ-LC). The usefulness of the CEC technique stems from the fact that it is a hybrid of high performance liquid chromatography (HPLC) and capillary electrophoresis (CE). In fact, CEC combines the analyte differential interactions (e.g., partitioning, adsorption, etc.) between two immiscible phases (e.g., in HPLC) to the electroosmotic mobility and differential electrophoretic mobility caused by the applied electric field as in CE. The hybrid nature of CEC provides a unique selectivity that can not be achieved by either HPLC or CE alone. This sound feature has just started to be exploited in some applications [4, 5], and the technique is still far from being applied to the separations of a wide range of charged and uncharged species of varying molecular sizes. This is due

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primarily to the lack of (1) stationary phases that are specially designed to suit CEC and (2) commercial equipment that will allow gradient elution to be performed in CEC. Although some investigations have been reported concerning approaches for gradient elution [6, 7] and stationary phases making [3, 8, 9], there is still much more room for improvement so that CEC will gain increased popularity.

Thus, the aim of this report is to enlarge the scope of applications of CEC by introducing the technique to the fascinating area of carbohydrate analysis. To achieve this goal, a specially designed octadecyl-silica (ODS) stationary phase for CEC at relatively moderate electroosmotic flow (EOF) was developed. Recently, the importance of the EOF and its origin in CEC has been summarized in a review article [10].

As will be shown in this article, the unique selectivity of CEC is suitable for the separation of closely related carbohydrates, namely *p*-nitrophenylglycosides of some monosaccharides and some maltooligosaccharides. As in other separation techniques, a prerequisite for achieving the separation and detection of carbohydrates by CEC with ODS capillary columns, is to derivatize the sugar analytes with fluorophores or chromophores to yield preferably neutral derivatives. Neutral sugar derivatives can be rapidly electrochromatographed on ODS capillary columns exhibiting moderate electroosmotic flow velocity. Negatively charged sugar derivatives may not be transported through the capillary column within reasonable analysis time under moderate EOF velocity in packed ODS capillary columns. On the other hand, positively charged derivatives may undergo silanophilic interaction with unreacted silanols on the surface of ODS stationary phase, a phenomenon that may lead to band broadening and loss of resolution between the separated analytes. The rationale for carbohydrate derivatization is two folds: (1) to increase the sensitivity of the detection of carbohydrates and (2) to

confer the hydrophobicity necessary for reversed-phase CEC.

Materials and Methods

Chemicals

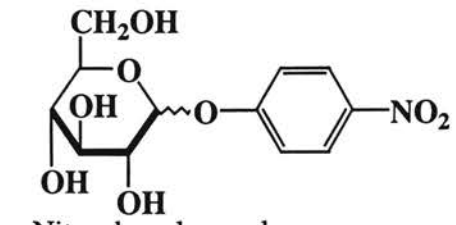
Sodium phosphate monobasic was from Mallinckrodt (St. Louis, MO, USA). HPLC grade acetonitrile and isopropanol were from Baxter (McGaw Park, IL, USA). HPLC grade methanol and analytical grade acetone were from Fisher Scientific (Fair Lawn, NJ, USA). *p*-Nitrophenyl modified monosaccharides and oligosaccharides were from Sigma (St. Louis, MO, USA). The structures of the sugar derivatives are shown in Fig. 1. Benzene, alkylbenzenes, and thiourea were from Aldrich (Milwaukee, WI, USA). Fused-silica capillaries with an internal diameter of 100 μm and an outer diameter of 360 μm were from Polymicro Technologies (Phoenix, AZ, USA).

Instrumentation

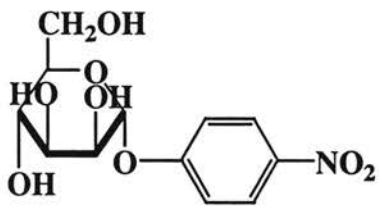
The instrument for the CEC studies was a P/ACE 5010 capillary electrophoresis system from Beckman Instruments Inc. (Fullerton, CA, USA) equipped with a UV detector and a data handling system comprised of an IBM personal computer and P/ACE Station software. The capillary columns were packed with a Shandon column packer from Keystone Scientific (Bellefonte, PA, USA).

Stationary Phase

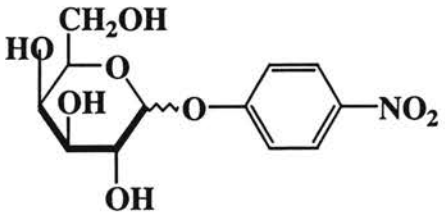
Zorbax PSM 150 microspherical silica, with a mean particle diameter of 5 μm, mean pore diameter of 150 Å and specific surface area of 147 m^2/g, was used as support.



p-Nitrophenyl-α and
β-D-glucopyranoside

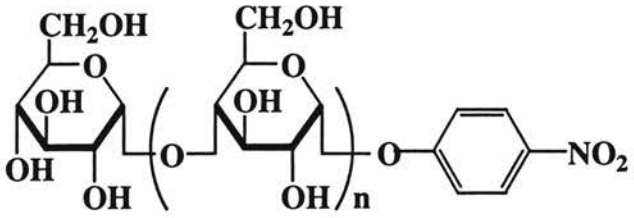


p-Nitrophenyl-α-D-
mannopyranoside



p-Nitrophenyl-α and
β-D-galactopyranoside

p-Nitrophenyl-*N*-acetyl-α and
β-D-glucosaminide



p-Nitrophenyl-α-D-maltooligosccharides:
n = 1, *p*-Nitrophenyl-α-D-maltoside
n = 2, *p*-Nitrophenyl-α-D-maltotrioside
n = 3, *p*-Nitrophenyl-α-D-maltotetraoside
n = 4, *p*-Nitrophenyl-α-D-maltopentaoside

Figure 1. Structures of *p*-nitrophenyl derivatives of some mono- and oligosaccharides.

The Zorbax was converted in house to octadecyl-silica (ODS). The surface coverage in octadecyl ligands was determined from elemental analysis to be 2.1 μmoles/m^2.

Column Fabrication, Packing and Conditioning

Before packing the capillary column, a retaining frit on one end of the capillary was made by dipping that end into wet bare silica of 5 μm average particle size, followed by sintering the end in a Bunsen burner for 1 to 2 min. Detection window of 3 mm wide was made by peeling off the polyimide protection layer with a wire stripper from Teledyne Electronic Technologies (San Diego, CA, USA). The open end was then attached to a stainless steel slurry reservoir of 3 cm x 4.6 mm I.D. by an Upchurch (Temecula, CA, USA) finger tight capillary fitting, and mounted on the column packer. The slurry was made in acetone, and isopropanol was used as the pumping solvent. Packing pressures of 2000, 3000, 4000 and 5000 psi were chosen to investigate the effect of packing pressure on column efficiency. After rinsing the capillary column with deionized water, the column was cut to the desired length and was sintered on the other end to make the retaining inlet frit, in a similar way as the outlet frit was made.

In order to investigate the light scattering effect of the packing material on detection sensitivity, a CEC column with an empty section was fabricated with detection window being opened on the empty section. Column was first fully packed as above, and then the outlet frit was made between the inlet frit and the end of the column with the wire stripper. The outlet frit was made when the column was being rinsed by water under a pressure not exceeding its packing pressure. To form the frit, the wire stripper blade was set at range 3, which corresponds to ca. 300 °C. The heating was continued for 2 to 3 min and resulted in a frit that was comparable to the one made by the Bunsen burner. In

this study, the outlet frit was made 20 cm from the inlet frit. Total column length was 27 cm, and the empty section was 7 cm long. The detection window was made at 5 mm from the outlet frit and was on the empty section.

Before the column cartridge was mounted on the Beckman capillary electrophoresis system, the capillary column was filled with acetonitrile with the assistance of a micro-syringe that was manually pressurized with the screw of an in-house built device that resembles a Hoffman tubing clamp. After mounting the column cartridge on the CE instrument, the column was equilibrated with the running buffer at 3 and 10 kV for 40 and 30 min, respectively. Abrupt changes in voltage were carefully avoided to minimize the likelihood of air bubble formation. Since silica particles are charged and tend to move under the influence of an electric field, the column equilibration process would, in the mean time, homogenize the compactness of the packing by dissipating the packing gradient that arises from pressure packing. Thus, conditioning the column is essential to obtain reproducible resolution and separation efficiency. This effect is most significant when the column is newly packed by pressure.

Results and Discussion

Fabrication of Frits and Columns

The retaining frits of a CEC column must ensure good column permeability and allow high separation efficiency to be achieved. Also, the frits should be strong enough to withstand the packing pressure, be reasonably durable and allow reproducible column performance. There are at least three approaches for frit fabrication [11]. In one type, the frit is formed by in situ polymerization of potassium silicate solution with formamide

according to the method of Cortes et al. [12]. Usually, the material is completely polymerized after 1 h at 120 °C. In a second approach, the frit is made by tapping the capillary tip into silica wetted with potassium silicate solution, and the frit is sintered using an electrically heated hot iron [11]. In a third type, the frit is formed by sintering water-wetted silica. In this work, we chose to make the frits by heating water-wetted bare silica in a Bunsen burner commonly used in a routine chemistry laboratory. Since the flame temperature of the Bunsen burner is moderately hot, an evenly sintered frit is obtained by heating the wet silica for 1 to 2 minutes. CEC columns with frits thus made have shown merits in strength, durability and reproducibility.

Generally, two types of columns are currently used in CEC. In one type of packed capillary, the column is composed of a segment that is packed with a given stationary phase and a segment that is empty. This kind of column is referred to as a partially packed capillary column. One of the retaining frits (the outlet frit) is fabricated at the interface of the two segments and a detection window is opened on the empty side very close to the interface [13-15]. In this configuration, the packed segment is less conductive than the open one. Thus, for the same current across the whole capillary, the electric field strength in the packed segment is higher. Also, the Joule heating in the open segment is higher than in the packed segment. Furthermore, the intrinsic EOF in the open segment is higher than in the packed segment because of column porosity, reduced number of free surface silanols of the packing and lack of orientation between the capillary axis and the interconnected channels. However, the mass conservation law dictates that the volumetric flow-rate of the mobile phase to be the same in the open and packed segments. Thus, an average flow across the capillary will be established. Under these conditions, there will be an acceleration on the intrinsic flow-rate in the packed

segment exerted by the higher intrinsic EOF in the open segment, and the EOF may partially degenerate to viscous flow. This degeneration of EOF to a viscous flow and the difference in joule heating between the open and packed segments are believed to be the principal contributors to the formation of air bubble in partially packed columns, i.e., columns with a packed segment and an empty segment.

The second type of CEC columns is fully packed with the stationary phase [3, 8]. We chose to pack columns of this type in our work. Fully packed capillary columns are more homogeneous in terms of conductance, Joule heating and EOF. These properties reduced air bubble formation when compared to partially packed column. The drawback of fully packed columns is that detection sensitivity was reduced due to light scattering by the packing material at the detection window [5, 11].

To evaluate the amount by which the detection sensitivity is decreased when going from a partially to a fully packed column, two columns of 27 cm total length each (separation distance 20 cm) were fabricated with a detection window on the empty section and on the packing, respectively. For an accurate estimate of the detection sensitivity on both columns, extreme care was taken to introduce the same amount of sample on both columns. Because of the difference in column permeability, fixed pressure injection will not introduce equal amount of sample on both columns. On the other hand, electrokinetic injection at the same volatge will not introduce an equal amount either, due to the difference in the EOF between both columns. Thus, the EOF velocity on each column was carefully measured using thiourea as the EOF marker. From these EOF velocity measurements, the electrokinetic injection time was set according to the ratio of EOF velocities with both columns.

With the partially packed capillary, where the window is free from packing, the

signal is about 2.8 times higher (in terms of peak height) than that obtained with fully packed column under otherwise the same operating conditions including the applied field strength. In terms of peak areas, the signal was about 1.5 times higher with the partially packed capillary column. For a more meaningful comparison, the applied field strength was adjusted so that the EOF was the same in both the partially and fully packed columns. Under these conditions, the peak height decreased further for the fully packed column, and the signal became about 4.3 times lower with the fully packed column when compared to the partially packed column. Also, the peak area decreased further and became 3.2 times lower when compared to the partially packed capillary column. Going from an EOF velocity (u_{EOF}) of 0.38 mm s^{-1} to an EOF velocity of 0.62 mm s^{-1} for the fully packed column to match the EOF in the partially packed column, the peak area decreased by a factor of 0.48, which corresponds to following the true dependence of peak area with $1/u_{EOF}$ for a concentration dependent detector [16]. In terms of peak height, the decrease is by a factor of about 0.65 which is in accordance with the dependence of peak height on $\leq u_{EOF}^{-0.2}$ for a concentration dependent detector [16].

Characterization of Columns with Benzene and Alkylbenzenes

A mixture of benzene, toluene, ethylbenzene, propylbenzene, butylbenzene and amylbenzene was injected on three ODS capillary columns packed at 2000, 3000 and 5000 psi to evaluate the effect of packing pressure on separation efficiency. Within the pressure range studied, i.e., from 2000 to 5000 psi, separation efficiency does not change dramatically. In fact, the separation efficiency per meter of packing was 110000, 112000 and 118000 at 2000, 3000 and 5000 psi, respectively. Using packing pressure greater

than 6000 psi yielded columns which were more difficult to condition. It was difficult to drive mobile phase into the column (packed at 6000 psi) for the purpose of clearing air bubbles within the column, due to the decreased permeability which resulted from high packing pressure. Columns packed at 2000 psi were easier to handle, but the column lifetime was short as far as column performance reproducibility was concerned because the stationary phase was not sufficiently compact under relatively low packing pressure. Packing pressure in the range 3000 to 4000 psi was found to be a good compromise, thus providing columns that are sufficiently permeable and durable.

Capillary columns packed and fabricated as explained above were first evaluated with benzene and alkylbenzenes as typical test solutes customarily used to evaluate the performance of ODS stationary phases in reversed-phase liquid chromatography (RPC). Figure 2 shows a representative electrochromatogram of benzene and alkylbenzenes obtained on an ODS capillary column. The stationary phase was intentionally designed to have a relatively low octadecyl ligand density (ca. 2.1 μmoles/m^2) to allow a relatively high EOF velocity. The linear velocity of the EOF, as measured from the peak of thiourea, was relativley high (1.16 mm/s) under the conditions used for the CEC separation of alkylbenzenes, Fig. 2. The relatively low ligand density corresponds to reacting 25% of silanol groups to form bonded octadecyl ligands while leaving 75% of the silanols intact. Although the surface coverage of octadecyl ligands was relatively low on the silica gel surface, the stationary phase exhibited reversed-phase behavior as is shown in Fig. 3 by the linear plots of the logarithmic capacity factor (log k') of these solutes versus various percentages of organic solvent in the mobile phase. Thiourea has been used by several researchers [2, 14, 17] as an inert EOF marker, i.e., an unretained solute marker. As will be shown in the following article [18], we have found

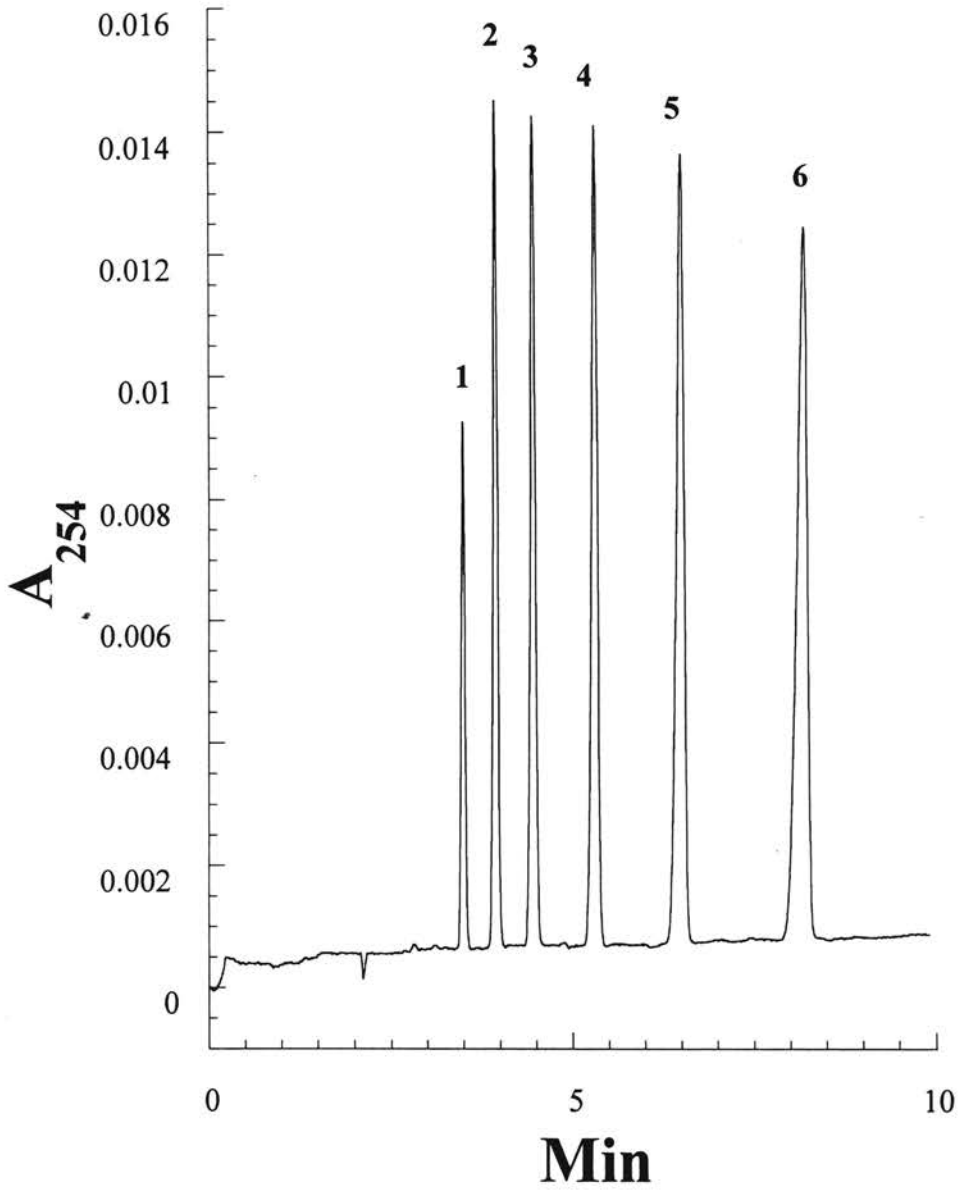


Figure 2. A typical electrochromatogram of benzene and alkylbenzenes. Conditions: capillary column, 20 cm/27 cm x 100 μm I.D., column packed with ODS under 3000 psi for 30 minutes; mobile phase, hydro-organic solution made up of 20% (v/v) of 5 mM NaH_2PO_4 (pH 6.0) and 80% acetonitrile (v/v); volatge, 20 kV; detection window at 20 cm from column inlet; detection wavelength, 254 nm; column temp., 15 °C. Solutes: 1, benzene; 2, toluene; 3, ethylbenzene; 4, propylbenzene; 5, butylbenzene; 6, amylbenzene.

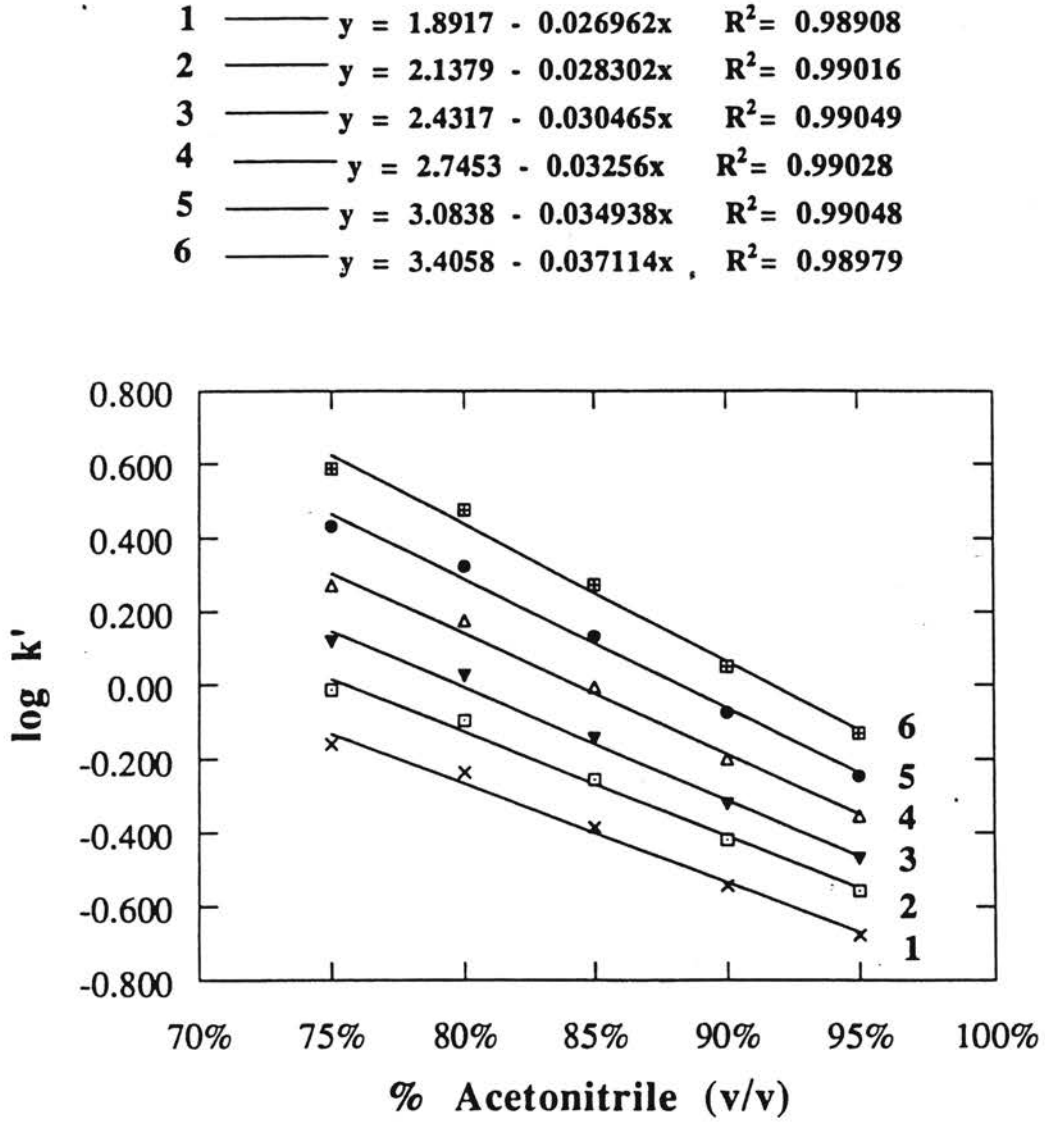


Figure 3. Plots of logarithmic capacity factor (log k') of benzene and alkylbenzenes vs. percent acetonitrile (v/v) in the mobile phase. Mobile phases, 5 mM NaH_2PO_4 (pH 6.0), mixed with acetonitrile in different proportions. Solutes and other conditions are the same as in Fig. 2.

that thiourea does not provide the correct measurement of the EOF which is in accordance with an earlier report [14]. Other investigators used vitamin B_{12} [3, 8] and ethanol [9] as inert EOF markers or unretained solute markers. As in HPLC, there is no universal inert marker for the retention time of an unretained species, and the inertness of the substance used must be established experimentally or at least the nature of the inert marker must be indicated.

CEC of Carbohydrates

Figure 4 shows the CEC electrochromatograms of *p*-nitrophenyl-α-D-glucopyranoside and *p*-nitrophenyl-α-D-maltooligosaccharides. The percentage of acetonitrile (v/v) in the mobile phase was changed in order to determine the optimum mobile phase composition for rapid elution time and high separation efficiency. Satisfactory separation was obtained with a mobile phase at relatively low acetonitrile content (20% v/v) and low electric field strength (370 V/cm). Under these circumstances, the reproducibility of the electrochromatographic system in terms of retention time is very good with %RSD of ≤ 0.55, see Table I. In addition, the column separation efficiency was relatively high varying between 72,000 to 152,000 plates/m for the different derivatives. The elution order of the glycosides is *p*-nitrophenyl-α-D-maltopentaoside, -maltotetraoside, -maltotrioside, -maltoside, and -glucoside. This elution order is the same as that observed in RPC [19], and is believed to be determined by the hydrophobicity of the glycosides as well as by organic induced conformational change of the glycosides. It has been shown that the homologous of degree of polymerization (d.p.) 2-5 are increasingly more soluble in water due to the decreasing effect of the *p*-nitrophenyl

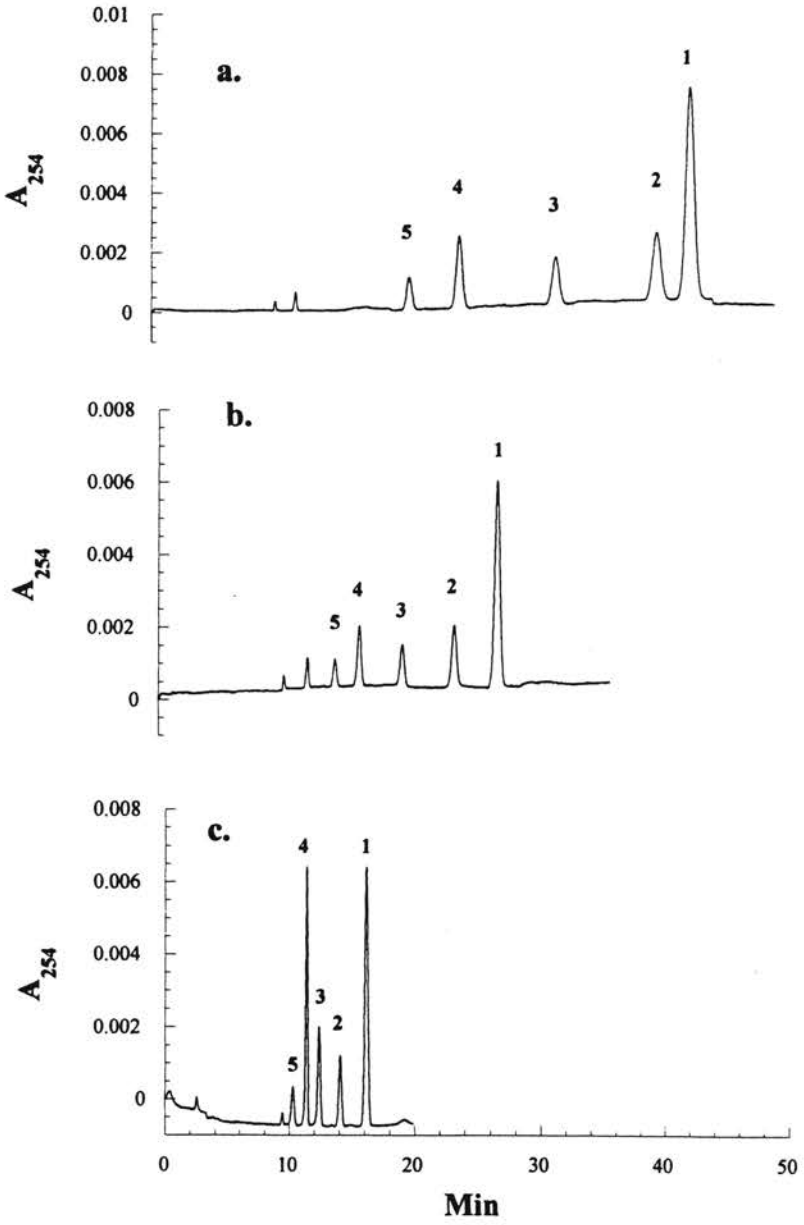


Figure 4. Electrochromatograms of *p*-nitrophenyl-α-D-glucopyranosides and maltooligosaccharides. Mobile phases: (a), 40% (v/v) of 5 mM NaH_2PO_4 (pH 6.0), 40% (v/v) H_2O and 20% (v/v) acetonitrile; (b), 42.5% (v/v) of 5 mM NaH_2PO_4 (pH 6.0), 42.5% (v/v) H_2O and 15% (v/v) acetonitrile; (c), 45% (v/v) of 5 mM NaH_2PO_4 (pH 6.0), 45% (v/v) H_2O and 10% (v/v) acetonitrile; voltage, 10 kV; other conditions are the same as in Fig. 2. Solutes: 1, *p*-nitrophenyl-α-D-glucopyranoside; 2, *p*-nitrophenyl-α-D-maltoside; 3, *p*-nitrophenyl-α-D-maltotrioside; 4, *p*-nitrophenyl-α-D-maltotetraoside; 5, *p*-nitrophenyl-α-D-maltopentaoside.

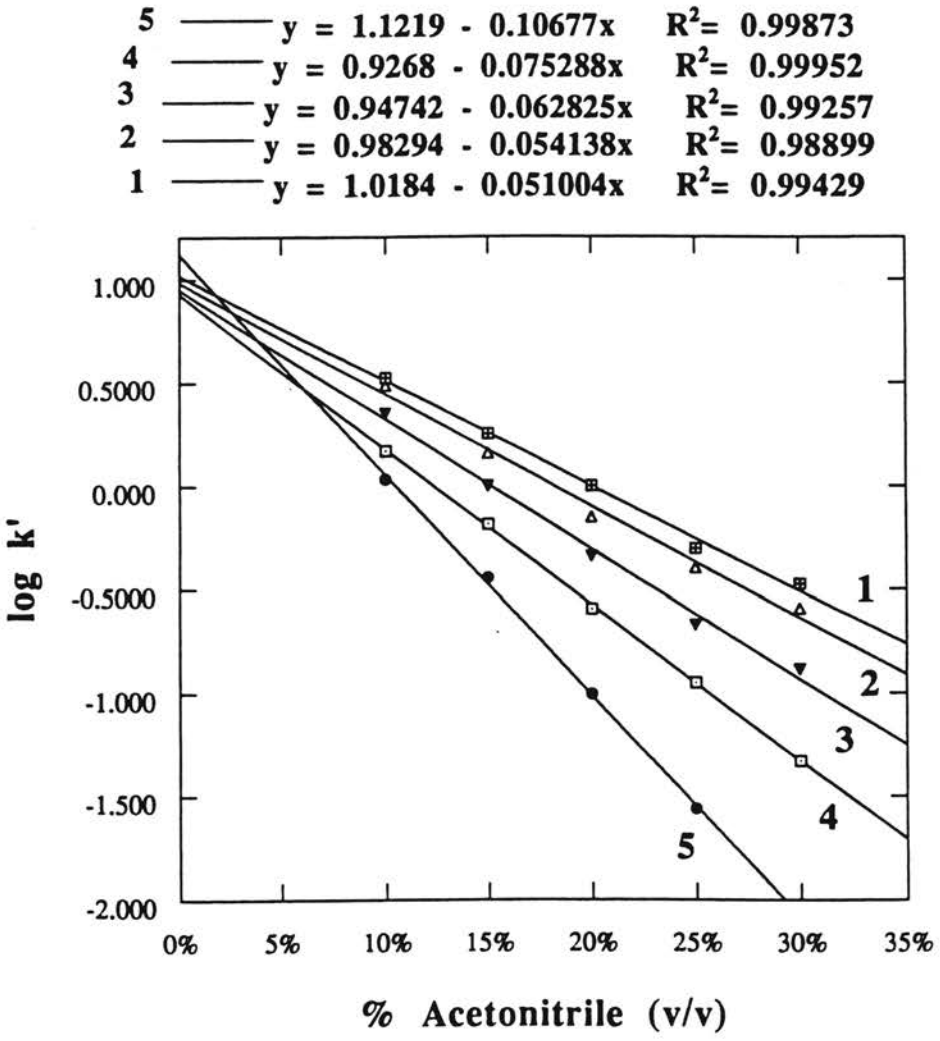


Figure 5. Plots of logarithmic capacity factor (log k') of *p*-nitrophenyl-α-D-glucopyranosides and maltooligosaccharides vs. percent acetonitrile in the mobile phase. Solutes and conditions are the same as in Fig. 4.

residue [19]. This means that the polarity of the solute increases when going from a d.p. of 2 to a d.p. of 5. Figure 5 shows linear plots for the dependence of log k' on % acetonitrile in the mobile phase in the range of 10 to 30% (v/v). These plots show different patterns when compared to those of the alkylbenzene homologous series in the sense that at low % organic and plain water (see extrapolated curves, Fig. 5), the log k' values of the former are distributed over a narrow numerical range while the k' values of the later are distributed over a wider numerical range, compare Fig. 3 to Fig. 5. By extrapolating to 0% acetonitrile in the mobile phase, the curve of the *p*-nitrophenyl-α-D-maltopentaoside intersects the y-axis at a log k' value higher than that of glucose, and the

Table I. Column efficiency (plates/m) and retention time reproducibility (%RSD).

p-Nitrophenyl derivative of	Retention time (%RSD)	Column efficiency (plates/m)
Galactose	0.54	95,000
Glucose	0.47	92,000
N-Acetylglucosamine	0.21	83,000
Mannose	0.31	84,000
Maltose	0.55	95,000
Maltotrioside	0.23	85,000
Maltotetraoside	0.49	152,000
Maltopentaoside	0.26	72,000

Mobile phase: 20:80% (v/v) acetonitrile: 3.34 mM sodium phosphate, pH 6.0; capillary column: 20 cm (27 cm total length) x 100 μm I.D. packed with 5 μm ODS; 10 kV; λ = 254 nm; pressure injection at 20 psi for 10 sec.

order of elution becomes maltotetraoside, maltotrioside, maltoside, glucose and maltopentaoside. These irregularities in the elution order when changing the organic content of the mobile phase have been observed previously in RPC of carbohydrates [19, 20]. The change in the elution order of the *p*-nitrophenyl-α-D-maltopentaoside may be attributed to organic solvent induced conformational change [20].

Figure 6 shows the electrochromatograms of *p*-nitrophenyl-α-D-glycoside derivatives of monosaccharides, e.g., galactose, *N*-acetylglucosamine, glucose and mannose. As can be seen in Fig. 1, glucose and galactose differing only by the orientation of the OH on C4 (equatorial vs. axial), and glucose and mannose differing by the orientation of the OH on C2 (equatorial vs. axial) separated very well on the ODS capillary column. For mannose, the axial OH at C2 can interact with the axial glycosidic linkage. Under these conditions, the hydroxyl group at C2 in mannose will confer less polar character to the molecule than the OH at C2 in glucose. This may explain the higher retention time of mannose. The axial OH in galactose at C4 confers more hydrophilic character to the molecule than the equatorial OH on the C4 of glucose because the OH group on C4 of galactose may not engage in a significant interaction with other OH groups in the molecule of galactose. This reasoning may explain why galactose is less retained than glucose. Finally, because of the presence of the *N*-acetyl group (slightly hydrophobic), the *N*-acetylglucosamine is more retarded than glucose.

The retention behavior of α and β anomers of *p*-nitrophenyl-*N*-acetyl-glucosaminide, *p*-nitrophenyl-galactopyranoside, and *p*-nitrophenyl-glucopyranoside was investigated. By adding borate to the hydro-organic mobile phase, base-line resolution can be obtained for α and β anomers of *p*-nitrophenyl-galactopyranoside and *p*-

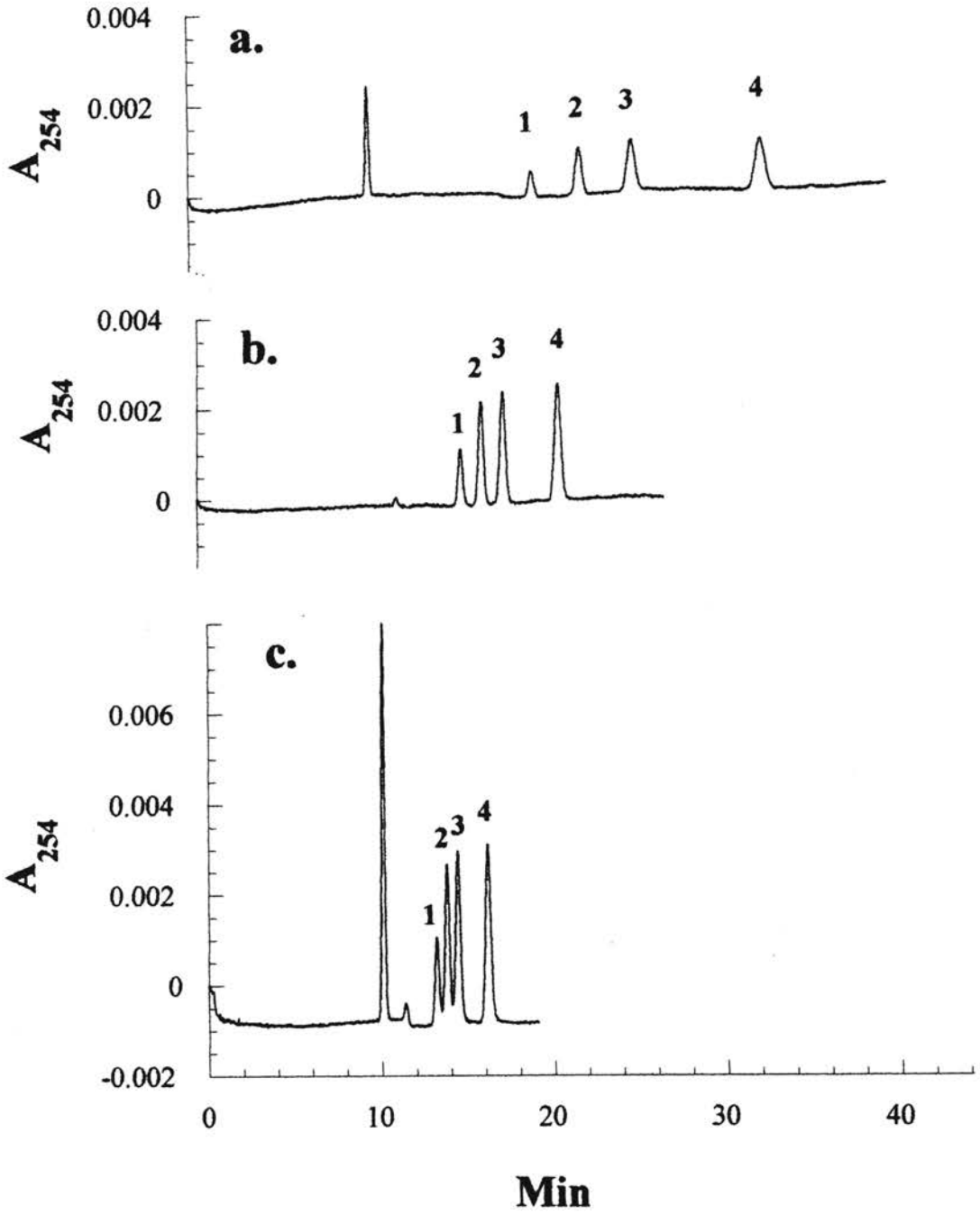


Figure 6. Electrochromatograms of *p*-nitrophenyl-α-D-glycoside derivatives of monosaccharides. Mobile phases: (a), 42.5% (v/v) of 5 mM NaH_2PO_4 (pH 6.0), 42.5% (v/v) H_2O and 15% (v/v) acetonitrile; (b), 40% (v/v) of 5 mM NaH_2PO_4 (pH 6.0), 40% (v/v) H_2O and 20% (v/v) acetonitrile; (c), 37.5% (v/v) of 5 mM NaH_2PO_4 (pH 6.0), 37.5% (v/v) H_2O and 25% (v/v) acetonitrile. Solutes: 1, *p*-nitrophenyl-α-D-galactopyranoside; 2, *p*-nitrophenyl-α-D-glucopyranoside; 3, *p*-nitrophenyl-*N*-acetyl-α-D-glucosaminide; 4, *p*-nitrophenyl-α-D-manopyranoside. Other conditions are the same as in Fig. 4.

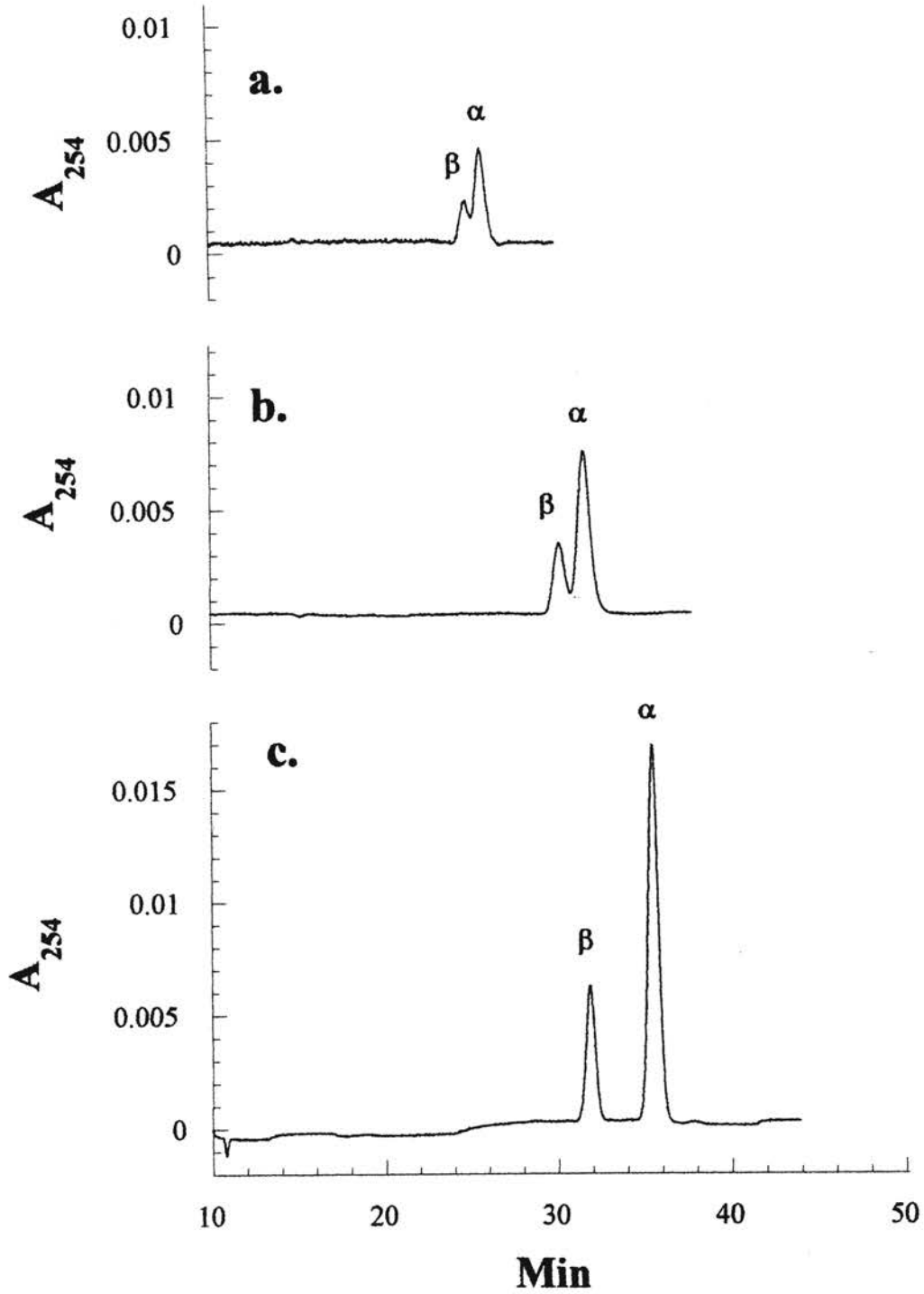


Figure 7. Electrochromatograms of β and α anomers of *p*-nitrophenyl-glucopyranoside. Mobile phase: (a), 42.5% (v/v) of 30 mM H_3BO_3 (pH 7.0), 42.5% (v/v) H_2O and 15% (v/v) acetonitrile; (b), 42.5% (v/v) 50 mM H_3BO_3 (pH 7.5), 42.5% (v/v) H_2O, and 15% (v/v) acetonitrile; (c) 45% (v/v) of 50 mM H_3BO_3 (pH 7.5), 45% (v/v) H_2O and 10% (v/v) acetonitrile; Detection wavelength, 280 nm; other conditions are the same as in Fig. 4. Elution order: β-anomer first and α-anomer second.

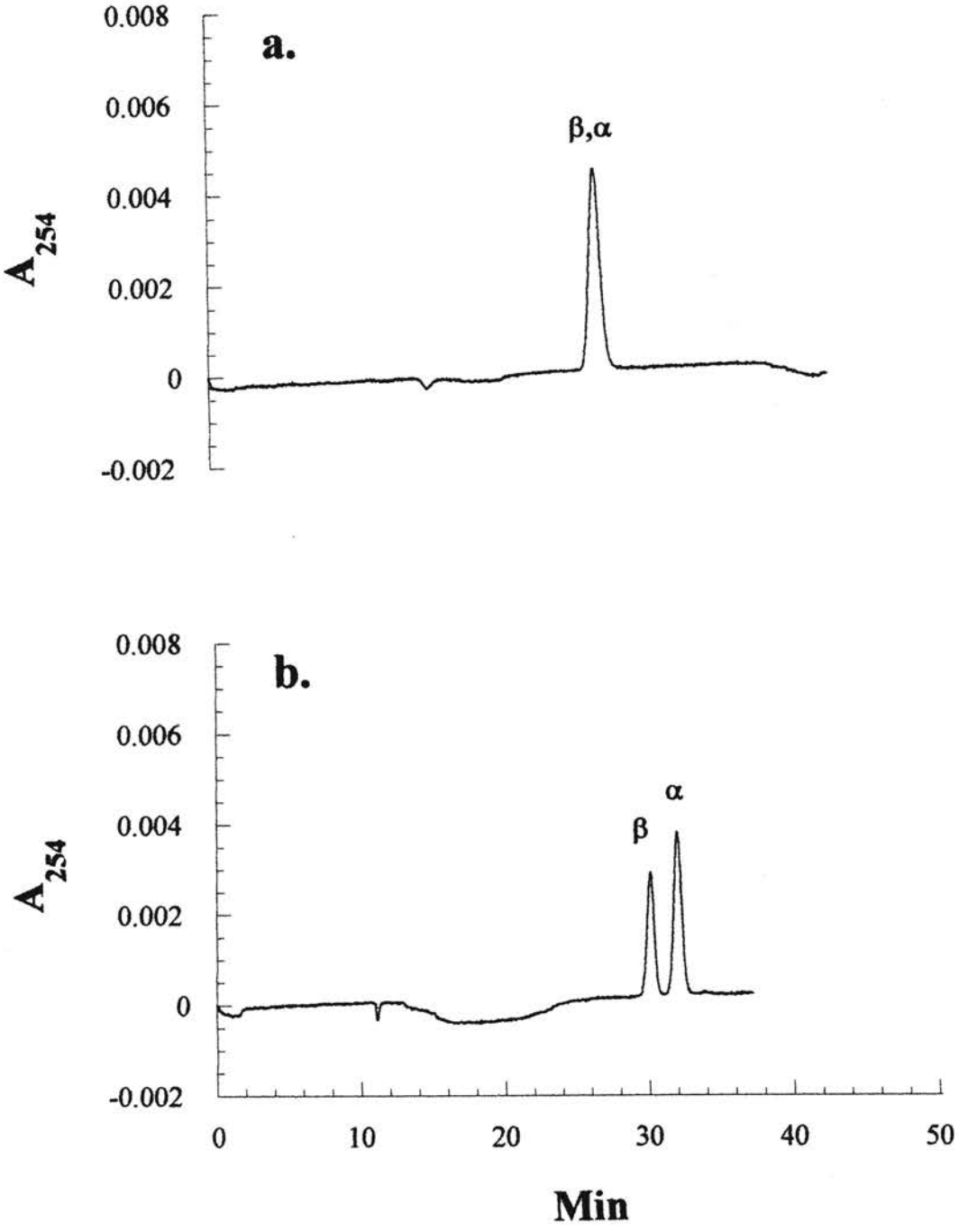


Figure 8. Electrochromatograms of β and α anomers of *p*-nitrophenyl-galactopyranoside. Mobile phases: (a), 42.5% (v/v) of 5 mM NaH_2PO_4 (pH 6.0), 42.5% (v/v) H_2O and 15% acetonitrile; (b), 45% (v/v) of 50 mM H_3BO_3 (pH 7.5), 45% (v/v) H_2O and 10% (v/v) acetonitrile; detection wavelength, 280 nm; other conditions are the same as in Fig. 4. Elution order: β-anomer first and α-anomer second.

nitrophenyl-glucopyranoside, as shown in Figs 7 and 8. β-Anomer eluted earlier than α-anomer, because β-anomer complexed stronger than α-anomer with borate [21]. Under this condition, the borate complex of the β-anomer is more negatively charged than the borate complex of the α-anomer, and consequently the former is less retained than the latter. In α-anomer, the glycosidic *p*-nitrophenyl group occupies an axial position and will interact strongly with the hydrogen atoms on C3 and C5 thus destabilizing the borate complex. This is not the case for the β-anomer where the *p*-nitrophenyl substituent occupies an equatorial position, and consequently is free from strong non-bonding interaction. The separation of α and β anomers of *p*-nitrophenyl-*N*-acetyl-glucosaminide was not successful under these conditions.

Conclusions

This work has shown the usefulness of CEC in the analysis of derivatized carbohydrates, thus enlarging the scope of applications of this newly emerging microseparation technique. The ODS stationary phase of light surface coverage produced relatively high EOF velocities which allowed the rapid separation of *p*-nitrophenylglycosides using hydro-organic eluents. Reversed-phase CEC offered high selectivity toward the *p*-nitrophenyl derivatives of monosaccharides thus permitting the separation of monosaccharides differing only by the orientation of OH (e.g. axial and equatorial). In addition, upon adding small amounts of borate to the hydro-organic eluent, the separation of the α and β anomers of the *p*-nitrophenyl derivatives of some monosaccharides was possible.

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CHAPTER V

CAPILLARY ELECTROCHROMATOGRAPHY WITH SEGMENTED CAPILLARIES FOR CONTROLLING ELECTROOSMOTIC FLOW*

Introduction

Capillary electrochromatography (CEC) is emerging as an important member of the class of differential migration methods. To achieve a differential migration (i.e., relative or separative displacement) a liquid flow (i.e., bulk displacement) is needed. The flow in CEC is generated under the influence of an electric field, and is referred to as the electroosmotic flow (EOF). Recently, the importance of the EOF and its origin in CEC have been summarized in a review article by Rathore and Horváth [1]. As in all flow-based differential migration methods (e.g., HPLC, GC), the flow rate and flow profile are essential factors for the time scale and efficiency of separations, respectively. The flat flow profile of EOF is very attractive for achieving high separation efficiencies by CEC. However, to exploit the full potential of EOF in CEC separations, ways for controlling and manipulating the EOF are needed.

Generally, two types of packed columns are currently used in CEC. In one type of packed capillary, the column is composed of a segment packed with a given stationary

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* *The content of this Chapter has been published in Electrophoresis, 1999, 20, 18.*

phase and of another segment that is empty. This type of column is referred to as a partially packed column. One of the retaining frits (the outlet frit) is fabricated at the interface of the two segments and a detection window is opened on the empty side very close to the interface [2-4]. The other type of packed CEC column is the fully packed column [5-7], with or without retaining frits. Unlike the partially packed columns, fully packed capillary columns are axially homogeneous in terms of conductance, Joule heating and EOF [7]. These properties reduce air bubble formation when compared to partially packed columns [7]. For a detailed study on the axial nonuniformities of partially packed CEC columns, the reader is advised to consult Ref. [8]. The drawback of fully packed columns is that the detection sensitivity is reduced by about 3 times in terms of peak area due to light scattering by the packing material at the detection window [7]. However, this column configuration opens up ways to manipulate the EOF. As will be shown in this report, a fully packed column can be designed to contain a segment packed with the desired stationary phase (e.g., ODS) to accomplish separation (i.e., separation segment) and another segment packed with bare silica serving as the EOF accelerator segment (see Fig. 1). This type of column is referred to as segmented capillary. Bare silica produces higher EOF than ODS due to the availability of more silanol groups on the surface of bare silica than on octadecyl-treated silica. The EOF accelerator segment can be packed with bare silicas of various pore size to further manipulate the EOF. In a segmented capillary configuration, Joule heating and conductance can be for all practical purposes considered constant across the capillary column.

In summary, the aim of this report is to introduce segmented capillaries (i.e., fully packed columns containing two packed segments) where one segment serves as the separation segment while the other segment functions as the EOF accelerator segment.

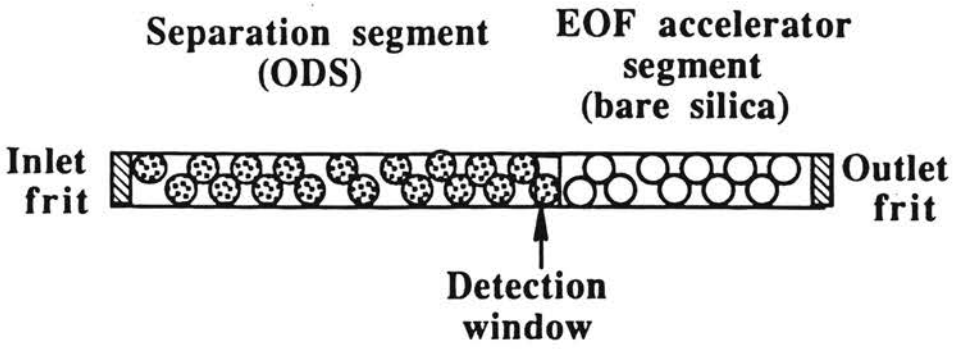

Figure 1. Schematic illustration of the segmented capillary.

The effects of the length of the EOF accelerator segment as well as the nature of the bare silica on the overall EOF and analysis time are described.

Materials and Methods

Instrumentation

The instrument for the CEC studies was a P/ACE 5010 capillary electrophoresis system from Beckman Instruments Inc. (Fullerton, CA, USA) equipped with a UV detector and a data handling system comprised of an IBM personal computer and a P/ACE software. Part of the study was performed with an in-house assembled instrument which consisted of a Model MJ30P0400 and a Model MJ30N0400 high-voltage power supplies with positive and negative polarities from Glassman High Voltage (White House Station, NJ, USA) and a Linear (Reno, NV, USA) Model 200 UV-Vis variable wavelength detector equipped with a cell for on-column detection. Electrochromatograms on the in-house assembled instrument were recorded with a Shimadzu (Columbia, MD, USA) Model CR601 computing integrator. The capillary columns were packed with a Shandon column packer from Keystone Scientific (Bellefonte, PA, USA).

Reagents and Materials

Sodium phosphate monobasic was from Mallinckrodt (St. Louis, MO, USA). HPLC grade acetonitrile and isopropanol were from Baxter (McGaw Park, IL, USA). HPLC grade methanol and analytical grade acetone were from Fisher Scientific (Fair Lawn, NJ, USA). Benzene, alkylbenzenes and thiourea were from Aldrich (Milwaukee, WI, USA). Fused-silica capillaries with an internal diameter of 100 μm and 360 μm

outer diameter were from Polymicro Technologies (Phoenix, AZ, USA). Zorbax silica was obtained from DuPont (Wilmington, DE, USA), Nucleosil silica was purchased from Macherey Nagel (Düren, Germany), and Vydac silica was obtained from The Separations Group (Hesperia, CA, USA).

Stationary Phases

Zorbax PSM 150 microspherical silica was used as the support, with a mean particle diameter of 5 μm, mean pore diameter of 150 Å and specific surface area of 147 m^2/g. The Zorbax was converted in house to octadecyl-silica (ODS) [7]. The surface coverage in octadecyl ligands was determined from elemental analysis to be 2.1 $\mu moles/m^2$. Unmodified Vydac and Nucleosil silicas were used in the packing of the EOF accelerator segment. For Vydac, the mean particle diameter, mean pore diameter and specific surface area are 5 μm, 300 Å and 100 m^2/g, respectively. For Nucleosil silica, the mean particle diameter, mean pore diameter and specific surface area are 5 μm, 1000 Å and 25 m^2/g, respectively.

Column Packing and Fabrication

Fused-silica capillaries of 100 μm I.D. were used in fabricating the CEC columns. Before packing the capillary column, a retaining frit on one end of the capillary was made by dipping that end into wet bare silica of 5 μm average particle size, followed by sintering the end in a Bunsen burner for 1 to 2 min. A detection window, 3 mm wide, was made by peeling off the polyimide protection layer with a wire stripper from Teledyne Electronic Technologies (San Diego, CA, USA). The open end was then attached to a stainless steel slurry reservoir of 3 cm x 4.6 mm i.d. by an Upchurch

(Temecula, CA, USA) finger tight capillary fitting, and mounted on the column packer. The slurry was made in acetone, and isopropanol was used as the pumping solvent. A pressure of 3000 psi was normally used in the packing. After rinsing the capillary column with deionized water, the column was cut to the desired length and was sintered on the other end to make the retaining inlet frit, in the same way as the outlet frit was made. In the case of segmented columns consisting of two segments packed with two different packings (e.g., ODS and bare-silica), the packing process was essentially the same with the difference that two different slurries were used one after another. Also, a temporary retaining frit was first made at the outlet end of the column to hold the bare silica and form the EOF accelerator segment. After packing the remaining length of the column with ODS to form the separation segment, the segmented capillary column was rinsed with deionized water. Thereafter, the temporary retaining outlet frit was cut out at the desired distance and a new permanent retaining frit was sintered in a Bunsen burner. The segmented column was then cut to the desired length on the inlet end and was sintered on that end to make the retaining inlet frit, in a similar way as the outlet frit was made. Figure 1 shows the configuration of segmented capillaries. A clear visual difference in light reflection between the segment packed with ODS and that packed with bare silica allowed the experimenter to locate the interface and to adjust the length of each segment within ±1 mm.

Before the capillary column was mounted onto the capillary electrophoresis instrument, the capillary column was filled with acetonitrile with the assistance of a micro-syringe that was manually pressurized with the screw of an in-house built device that resembles a Hoffman tubing clamp. After mounting the capillary column on the CE instrument, the column was equilibrated with the running buffer at 3 and 10 kV for 40

and 30 min, respectively. An abrupt change of voltage was carefully avoided to minimize the likelihood of air bubble formation.

Results and Discussion

The effect of the length of the EOF accelerator segment on the EOF in segmented capillaries was investigated with capillary columns having a separation segment of a fixed length and an EOF accelerator segment of a variable length. As can be seen in Fig. 2, increasing the length of the EOF accelerator segment increases the EOF mobility, which in turn decreases the analysis time. With an EOF accelerator segment of 28 cm in length, the observed EOF mobility is increased by 63% with respect to the column without an EOF accelerator segment.

Figure 3 shows the effect of the length of the capillary segment packed with bare silica, i.e., EOF accelerator segment, on the analysis time of a mixture of benzene and 5 alkylbenzenes. The EOF velocity with the capillary without the EOF accelerator (i.e., without the segment packed with bare silica) was 0.8 mm/s. This EOF velocity was increased by 25% and 37.5% when the length of the EOF accelerator capillary segment was increased to 6 and 28 cm, respectively. The analysis time was decreased by 28 % from 18.6 min to 13.5 min when changing the segment length for the bare silica from 0 to 28 cm. It is important to note that despite the generation of a viscous flow (see below), the average separation efficiency (plates/m) increased from 101,000 to 108,000 and to 130,000 when going from 0 to 6 and to 28 cm bare silica segment, respectively. This may be due to the decreasing of the residence time in the column which results in less longitudinal diffusion.

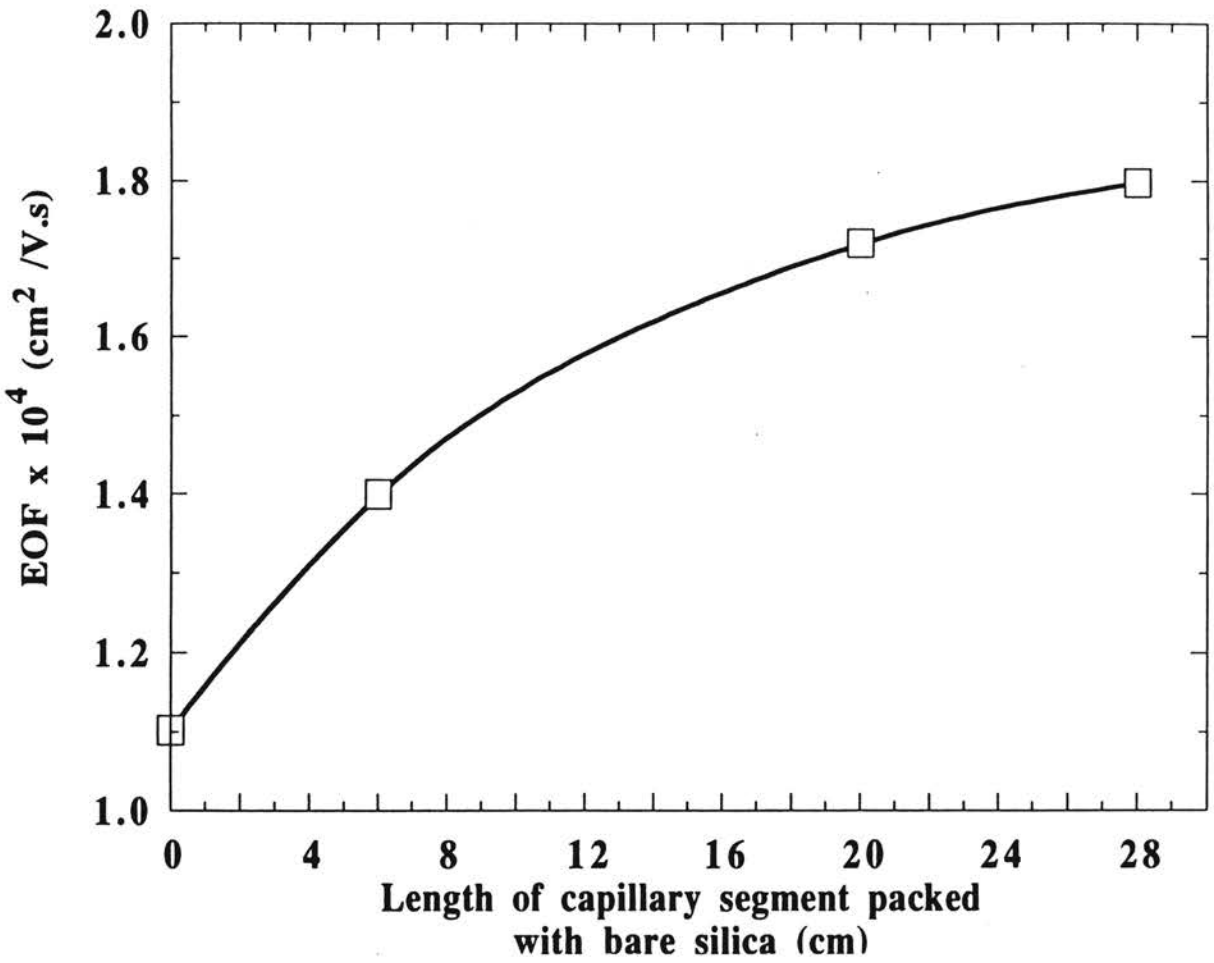

Figure 2. Plot of EOF mobility versus the length of the capillary segment packed with bare silica in the segmented capillary. Capillary, 100 μm I.D. with a 20 cm ODS segment and a variable length bare silica segment of 0, 6, 20 or 28 cm. Mobile phase, 1.25 mM sodium phosphate monobasic, pH 6.0, containing 75 % (v/v) acetonitrile. Applied filed strength, 625 V/cm. EOF tracer, thiourea.

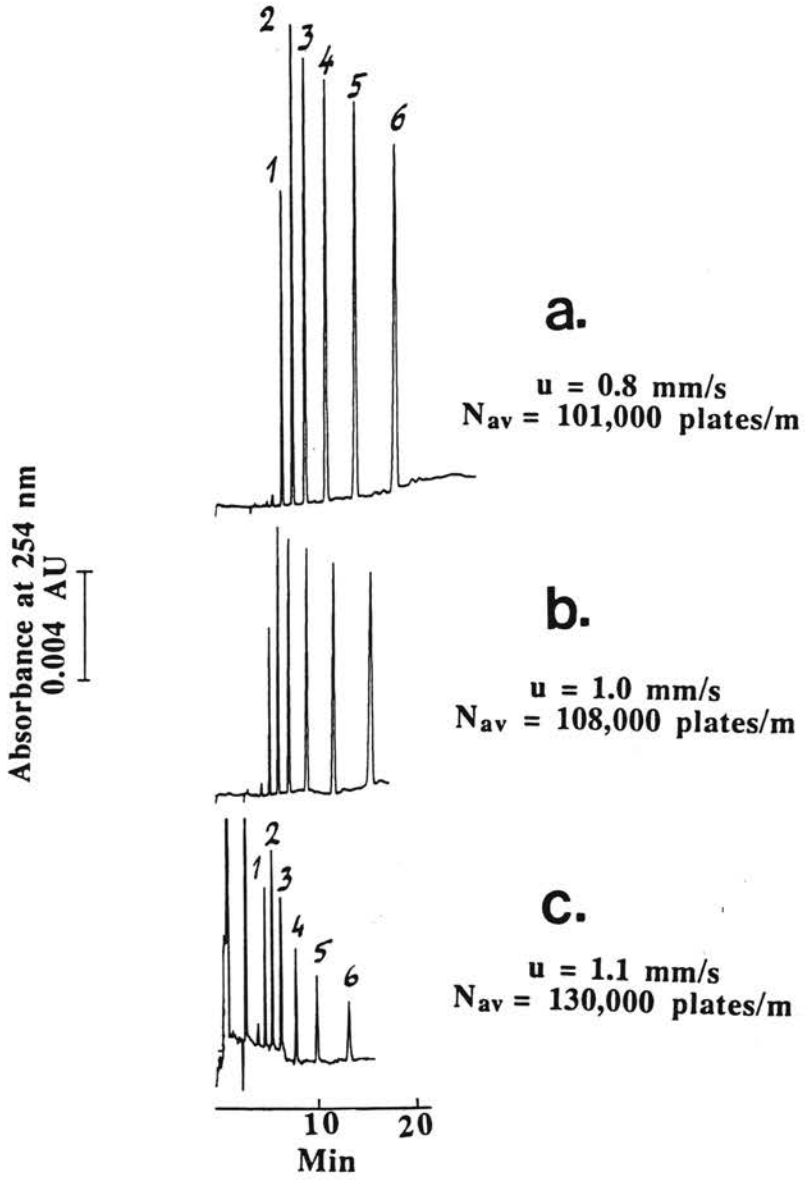

Figure 3. Electrochromatograms exhibiting the effect of the length of the bare silica segment on the separation of benzene and alkylbenzenes. Capillary, 100 μm I.D. with a 20 cm ODS segment and a variable length bare silica segment of 0 cm in (a), 6 cm in (b) and 28 cm in (c). Mobile phase, 1.25 mM sodium phosphate monobasic, pH 6.0, containing 75 % (v/v) acetonitrile. Applied filed strength, 625 V/cm. Detection, UV at 254 nm. Solutes: 1, benzene; 2, toluene; 3, ethylbenzene; 4, propylbenzene; 5, butylbenzene; 6, pentylbenzene.

Table I lists the k' values of benzene and some alkylbenzenes on the various segmented capillary columns with a fixed length separation segment and a variable length EOF accelerator segment. As expected, by extending the length of the bare silica segment, the EOF can be significantly increased without changing the k' values. The detection of the solute is achieved at the end of the separation segment just before it enters the bare silica segment.

TABLE I. k' values of benzene and some alkylbenzenes obtained on segmented capillaries.

	k' values				
Length of bare silica segment (cm)	Benzene	Toluene	Ethyl Benzene	Propyl Benzene	Butyl Benzene
0	0.66	0.92	1.22	1.72	2.41
6	0.69	0.96	1.28	1.84	2.63
28	0.66	0.91	1.21	1.71	2.40

Conditions are same as in Fig. 3.

In a column constructed from two segments (i.e., segmented capillary) each packed with a different stationary phase having its own zeta potential, an average EOF will be established across the whole column in order to satisfy the mass conservation law. In a study involving coupled open capillary tubes for CE, we have shown that the average electroosmotic mobilities, $\mu_{eo,av}$, for two coupled capillary segments is given by [9] [10]:

$$\mu_{eo,av} = \frac{\mu_{eo,1} \times l_1}{l_t} + \frac{\mu_{eo,2} \times l_2}{l_t} \quad (1)$$

where $\mu_{eo,1}$ and $\mu_{eo,2}$ are the electroosmotic mobilities in segment 1 and segment 2,

respectively, when operated individually. l_t, l_1 and l_2 are the total column length, the length of segment 1 and the length of segment 2, respectively. The total column length is $l_t = l_1 + l_2$. Eq. 1 can be rearranged into the following form:

$$\mu_{eo,av} = (\mu_{eo,1} - \mu_{eo,2}) \bullet \frac{l_1}{l_t} + \mu_{eo,2} \qquad (2)$$

According to Eq. 2, a plot of $\mu_{eo,av}$ versus the fractional length of a given segment is linear with a slope equal to $\mu_{eo,1}$ - $\mu_{eo,2}$ and an intercept of $\mu_{eo,2}$. $\mu_{eo,1}$ - $\mu_{eo,2}$ is the range over which the EOF can be varied under a given set of conditions. This range extends between the EOF values generated with the capillary packed with ODS at the low end and that packed with bare silica at the high end. As shown in Fig. 4, the experimental plot of EOF versus the fractional length of the capillary segment packed with bare silica is linear with an R = 0.9986.

Based on the above results, if each segment was operated individually under otherwise the same conditions, the EOF in the bare silica segment is higher than that in the ODS segment. Once connected, an average flow is established to satisfy the mass conservation law. Thus, the bare silica segment exerts a pull on the volumetric flow while the ODS segment restrains the EOF in the bare silica segment so that a steady volumetric flow is generated along the axis of the column. This will lead to an interfacial pressure at the bare silica-ODS joint [8]. Under these conditions, the interfacial pressure is less than the inlet and outlet pressure in order to yield a steady flow[8]. This generates pressure gradients and partial degeneration of the EOF to viscous flow. The magnitude of the flow degeneration from EOF to viscous flow is greater when the CEC column is constructed of a packed segment and an empty segment (i.e., partially packed columns) due to a much larger difference in the EOF velocities in the individual

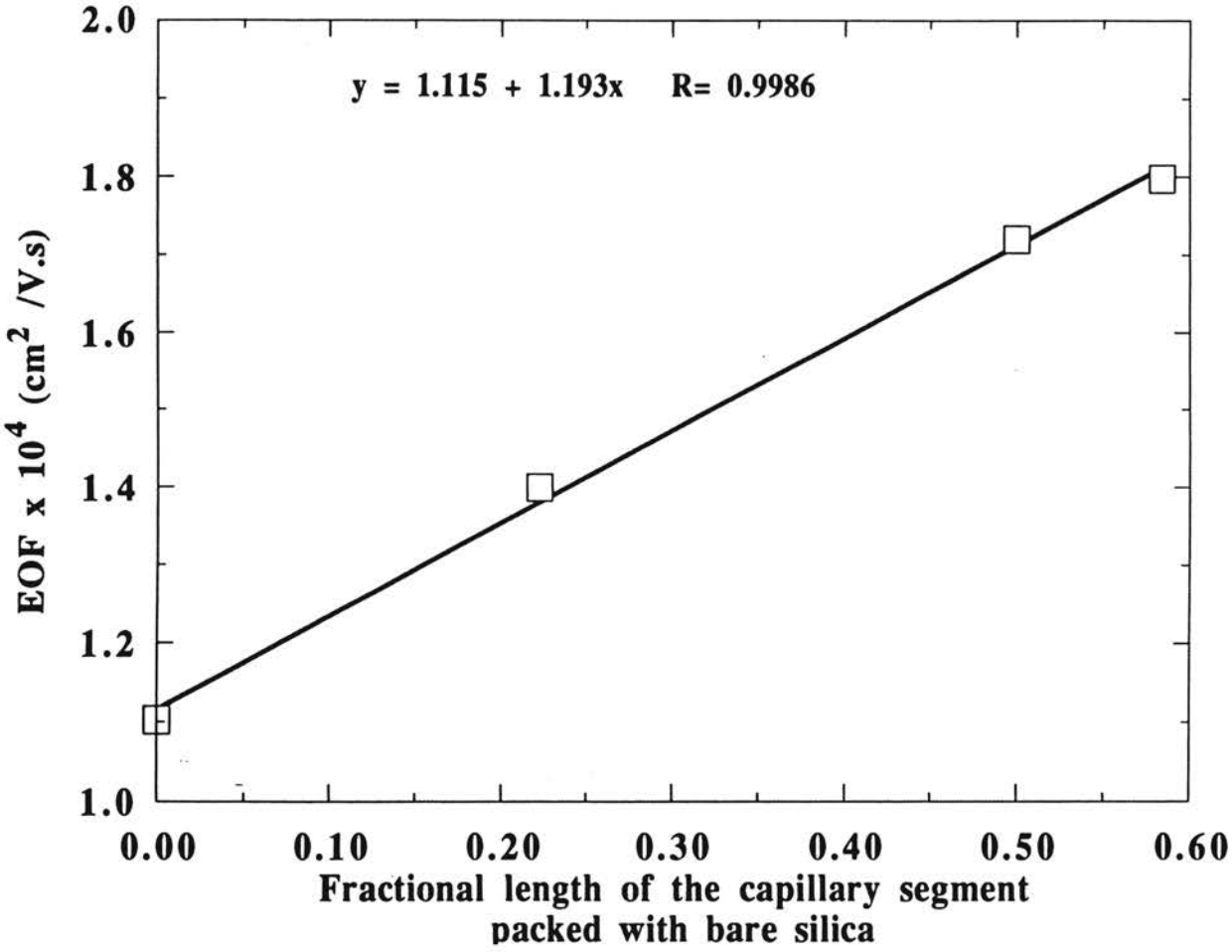

Figure 4. Plot of the EOF mobility as a function of the fractional length of the capillary segment packed with bare silica in the segmented capillary. Conditions are same as in Fig. 2.

packed and open segments than in a packed column with two segments (i.e., segmented capillaries).

TABLE II. Effect of pore size of bare silica on the average flow in segmented capillaries.

Column configuration				EOF	
Length of segment packed with bare silica (cm)	Length of separation segment packed with ODS (cm)	Window position (cm)	Total capillary length (cm)	μ x 10^4 (cm^2/Vs)	u (mm/s)
24 cm Vydac 300 Å	8 cm ODS-Zorbax 150 Å	10 cm	32 cm	1.64	1.0
20 cm Nucleosil 1000 Å	12 cm ODS-Zorbax 150 Å	12.1 cm	32 cm	2.53	1.6

Conditions are same as in Fig. 2.

The effect of the pore size of the bare silica in the EOF accelerator segment on the average flow was investigated. Table II shows the comparison between two bare silicas with different pore sizes. Under the same conditions, Nucleosil (pore size 1000 Å) induced an EOF which is 63% higher than the EOF generated by Vydac 300 (pore size 300Å) despite the fact that the ODS segment and the Nucleosil segment are 4 cm longer and 4 cm shorter than in the first segmented capillary column which consisted of the combination ODS-Vydac. The increase in EOF for the wider pore silica can be the result of decreasing double layer overlap.

In order to assess the difference in the EOF velocities between open tubes and packed tubes with either bare or octadecyl modified silica, the EOF was measured or

calculated for different column packing and configuration. The results are summarized in Table III.

TABLE III. Average EOF in different column configurations.

Nature of the capillary	EOF ($cm^2/V.s$)	% EOF as compared to an open tube
Open tube	4.50×10^{-4}	100%
Unpacked capillary with two retaining frits at both ends	2.54×10^{-4}	56%
Capillary packed with 5 μm bare Vydac silica (300Å)	2.31×10^{-4} *	51%
Capillary packed with 5 μm ODS Zorbax (150Å)	1.12×10^{-4} *	25%

* Values calculated by the equation of the straight line in Fig. 4. Capillary, 100 μm I.D.,20 cm to detection point, 27 cm total length either open or unpacked with two retaining frits, or fully packed. Conditions as in Fig. 2.

As can be seen in this table, the open tube provided the highest EOF followed by the one that is empty with two retaining frits on both ends, then the capillary packed with bare silica and finally the capillary packed with ODS. The retaining frits significantly reduced the EOF by 44% while the reduction of the EOF resulting from packing the column with bare silica amounted for only 5%, which is the difference between 56% and 51%. Packing the capillary with ODS led to a 75% decrease in EOF 31% of which is due to the ODS proper and 44% of which is due to the retaining frits. The zeta potential of the ODS is lower than that of the bare silica due to the reduced number of free surface

silanols as a consequence of the covalent attachment of the octadecyl ligands.

Thus, the inlet and outlet frits in CEC columns are restrictive points slowing the average EOF across the column and causing this flow to degenerate from EOF to viscous flow. The interfacial pressure at the inlet frit-packing interface is less than the pressure at the outlet and inlet of the column, while the interfacial pressure at the packing-outlet frit interface is higher than the pressure at the inlet and outlet to yield a steady average flow. The resistance of frits to flow arises from the sintering process which reduces the number of surface silanols and to a lesser extent the pore size. In addition, sintering reduces the interstitial spaces between the particles in the frits causing double layer overlap that translates into reduced EOF through the frit. On this basis, frits-containing CEC columns exhibit a volumetric flow profile that is not solely based on electroosmosis but contaminated by the viscous flow component.

Conclusions

In this report, we have shown a simple way to control and manipulate the EOF in CEC. It involved the design of segmented capillaries with two different segments. One segment served as the separation segment and was packed with ODS while the other segment was packed with bare silica and functioned as the EOF accelerator segment. This configuration permitted the increase in the average EOF across the whole capillary and yielded shorter analysis time over a certain range. The width of this range seemed to be dependent on the difference of the magnitude of EOF between the two segments. The bare silica segment is not responsible for the CEC separation but only offers EOF pumping forces, this provided flexibility in choosing the particle size and the pore size of the bare silica in order to obtain maximum EOF pumping forces. By increasing the pore

size of the bare silica in the EOF accelerator segment, a further increase in the magnitude of the average EOF was obtained. In addition to the investigation of EOF pumping mechanism, this study has also revealed the influence of retaining frits on the nature and the value of the average flow in CEC.

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CHAPTER VI

ON-LINE AND OFF-LINE PRECONCENTRATION OF UREA HERBICIDES IN CAPILLARY ELECTROCHROMATOGRAPHY

Introduction

As in all capillary-based analytical separation techniques (e.g., capillary electrophoresis and chromatography), in capillary electrochromatography (CEC) the column effluent is monitored on column using optical detectors (e.g., UV-Vis, fluorescence) in order to keep the detection volume at the nL or pL scale. This fact limits the detection path length to the inner diameter of the capillary, i.e., 50 to 100 μm. This short path length leads to a decreased sensitivity, which in turn restricts concentration detection limits, making CEC not directly applicable to environmental contaminants, which are usually found in the ecological system at the concentration level of ppb or less.

Recently, three different approaches have been described to alleviate the problem of on-column detection. In one approach, a 150 μm bubble cell is formed near the end of the capillary. This commercially available capillary configuration is made for capillaries of 50 μm I.D., thus giving a three fold increase in path length [1]. In fact, in a paper from our laboratory we demonstrated that with a bubble cell, the sensitivity is improved by a

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* *The content of this Chapter has been published in Electrophoresis, 1999, 20, in press.*

factor of 2.85 [2]. In a second approach, which is also commercially available, detection is performed on a z shaped cell [3]. In one configuration, the capillary column is bent into a z shaped cell assembly with a path length of 3 mm [3]. In another configuration, the capillary is connected via zero dead volume fittings to a z shaped cell with an extended path length of 1.2 mm [4]. While the later configuration produced 10-fold increase in detection sensitivity over standard capillaries as reported by the manufacturer [4], the former configuration yielded less improvement in detection sensitivity than was expected probably because of inadequate focusing of light and stray light [3]. In a third approach, the path length is increased by designing a multireflection on-column cell [5]. This consists of depositing on the detection point a reflective coating of silver, thus allowing the radiation beam to undergo numerous reflections until it exits the capillary. The multireflective cell, which is not commercially available, was reported to allow a 40-fold improvement in sensitivity.

Although the above approaches improved the detection sensitivity of capillary-based separation techniques, their concentration limits of detection (LOD) are not at the level of environmental samples which are in the low ppb. Thus, efficient trace enrichment processes before CEC separation become necessary. This report is aimed at introducing ways for on-line and off-line preconcentration of dilute samples of pesticides prior to CEC separation.

Materials and Methods

Instrumentation

The instrument was a P/ACE 5010 capillary electrophoresis system from

Beckman Instruments Inc. (Fullerton, CA, USA) equipped with a UV detector and a data handling system comprised of an IBM personal computer and P/ACE Station software. The capillary columns were packed with a Shandon column packer from Keystone Scientific (Bellefonte, PA, USA).

Reagents, Chemicals and Materials

Sodium phosphate monobasic was from Mallinckrodt (St. Louis, MO, USA). HPLC grade acetonitrile and isopropanol were from Baxter (McGaw Park, IL, USA). HPLC grade methanol and analytical grade acetone were from Fisher Scientific (Fair Lawn, NJ, USA). All urea herbicides used in this study were purchased from Chem Service (West Chester, PA, U.S.A.). The structures of these herbicides are shown in Fig. 1. Zorbax PSM 150 microspherical silica was obtained from DuPont (Wilmington, DE, USA), and Nucleosil silica was purchased form Macherey Nagel (Düren, Germany). Fused-silica capillaries with an internal diameter of 100 μm and an outer diameter of 360 μm were from Polymicro Technologies (Phoenix, AZ, USA).

Stationary Phases

The Zorbax silica was converted in-house to an octadecyl-silica (ODS). This silica has a mean particle diameter of 5 μm, a mean pore diameter of 150 Å and a specific surface area of 147 m^2/g. The surface coverage in octadecyl ligands was determined from elemental analysis to be 2.1 $\mu moles/m^2$ [6] [7]. Nucleosil 120-5 silica was also converted in house to ODS in a similar way to that of Zorbax silica, and was used as the stationary phase in on-column preconcentration studies. The Nucleosil silica has a mean particle

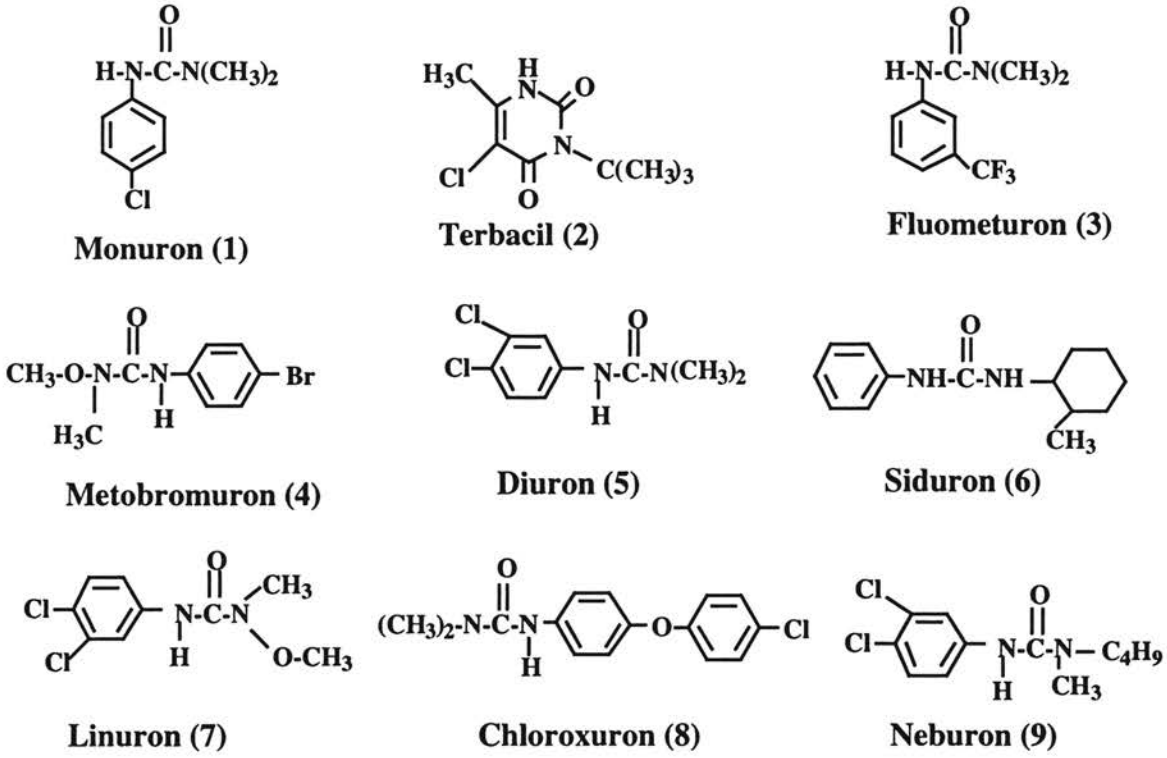

Figure 1. Structures of the urea herbicides used in this study. The numbering in parentheses corresponds to the order of elution of the urea herbicides with methanol containing mobile phases.

diameter of 5 μm, a mean pore diameter of 120 Å and a specific surface area of 200 m^2/g.

Column Packing and Fabrication

CEC columns were packed and handled as previously described [6-9]. The packing pressure was 3000 psi. The columns were fully packed without an open segment.

Sample Preparation

The stock solutions of urea herbicides were prepared by dissolving 3 mg of each of the nine urea herbicides, namely terbacil, monuron, fluometuron, metobromuron, linuron, diuron, siduron, chloroxuron and neburon in 2 mL of a 1:1 (v/v) water:acetonitrile mixture. These stock solutions contained ca. 6 x 10^{-3} M or 1500 ppm of each herbicide. For on-line and off-line preconcentration studies, the stock solutions were made up of metobromuron and neburon, which were considered to be representative solutes based on their elution order. These stock solutions were then diluted accordingly to 10^{-5} M, 10^{-7} M and 10^{-10} M, depending on the procedures the sample was subjected to.

Sample Injection, On-line Preconcentration and Off-line Preconcentration

Samples were injected either by pressure (20 psi) or by voltage, both injection modes are available on the Beckman P/ACE 5010 capillary electrophoresis system. Sample enrichment was performed either by on-line preconcentration or by combining off-line enrichment with on-line preconcentration. Off-line enrichment was done by solid-phase extraction (SPE), which is described in the following paragraph. For on-line preconcentration studies, a plug of plain water was first injected for 20 seconds at 3 kV,

followed by injecting the urea herbicide sample for 90 seconds at 3 kV.

To investigate the combination of off-line SPE and on-line preconcentration, the urea herbicide stock solutions were diluted to the level of 10^{-10} M or 0.1 ppb. A Bakerbond spe C18 column from J.T. Baker (Phillipsburg, NJ, USA) was employed to perform the SPE. Before the SPE, the Bakerbond spe C18 column was conditioned first with 2 mL of 100% methanol and then rinsed with 5 mL of water. 1000 mL of the diluted sample solution was thereafter passed through the extraction column. The enriched urea herbicides were eluted from the column with 5 mL of 100% acetonitrile. The collected acetonitrile solution was vaporized by subjecting the solution to a speed vac, which reduced the volume to 500 μL. Theoretical concentration of the enriched sample was 10^{-6} M, assuming no sample loss during the process. This sample was further enriched on column with the on-line preconcentration described above, and was then analyzed by CEC.

Results and Discussion

CEC of Urea Herbicides

Capillary electrochromatography of 9 urea herbicides was performed using hydro-organic mobile phases which consisted of 5 mM NaH_2PO_4 (pH 6.0), containing either acetonitrile or methanol. Typical electrochromatograms obtained with methanol or acetonitrile containing mobile phases are shown in Fig. 2a and b, respectively. To obtain baseline resolution while keeping approximately the same analysis time, the organic phase was 70% (v/v) for methanol as compared with 50% (v/v) for acetonitrile. This is expected since methanol is more polar and its eluting strength is lower than that of

acetonitrile [10-12]. As can be seen in Fig. 2a and b, the elution time for the last peak (corresponding to neburon) with methanol (34.4 min) was close to that with acetonitrile (34.1 min). It is interesting to note that in comparison to the electrochromatogram obtained with the acetonitrile containing mobile phase, the elution order with the methanol containing mobile phase differs for some urea herbicides. In this case, terbacil switched order with monuron, and diuron switched order with metobromuron.

Table I summarizes column separation efficiencies, N, as a function of the nature of the organic modifier. On the average, N is about 1.5 fold higher with acetonitrile than with methanol. This is may be attributed to higher mass transfer resistances in the pores with the methanol containing mobile phase than with the acetonitrile containing mobile phase. In fact, the viscosity of the mobile phase for 70% methanol is 1.1 cP as opposed to 0.75 cP for 50% acetonitrile [10].

Figure 3 is a plot of log k' of 9 urea herbicides vs. % acetonitrile in the mobile phase. The linear relationship is indicative of the reversed-phase chromatographic behavior of the column, although the surface coverage of the silica with the octadecyl ligands is relatively low (ca. 2.1 μmoles/m^2). Using the same ODS stationary phase, plots of log k' of alkyl benzene homologous series versus percent acetonitrile (v/v) were also linear [6]. The ODS stationary phase was intentionally designed to have a low surface coverage in octadecyl ligands. This allowed the realization of a relatively high EOF velocity, and in turn relatively fast separations.

Preconcentration of Dilute Samples

On-line Preconcentration.

On-column or on-line preconcentration from a dilute

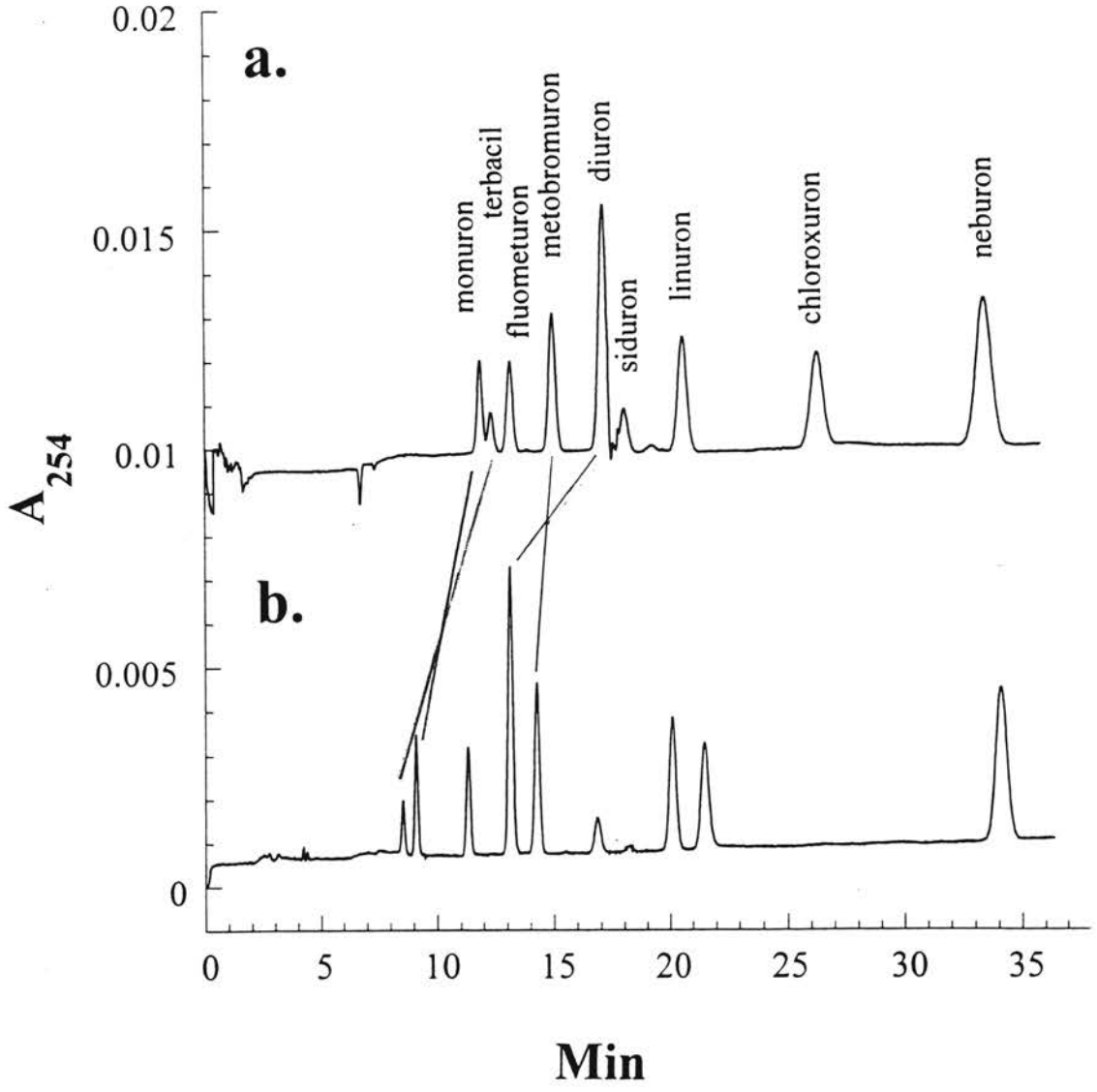

Figure 2. Electrochromatograms obtained with methanol (a) and acetonitrile (b) containing mobile phases. Mobile phases: (a), 30 % (v/v) 5 mM NaH_2PO_4, pH 6.0, mixed with 70% (v/v) methanol; (b) 50% (v/v) 5 mM NaH_2PO_4, pH 6.0, mixed with 50% (v/v) acetonitrile; column, ODS-Zorbax, length 27 cm, detection window at 20 cm; running voltage, 20 kV; pressure injection; 20 sec at 20 psi; temperature, 15 °C; UV detection, 254 nm. Elution order in (a): 1, monuron; 2, terbacil; 3, fluometuron; 4, metobromuron; 5, diuron; 6, siduron; 7, linuron; 8, chloroxuron; 9, neburon; in (b): 1, terbacil; 2, monuron; 3, fluometuron; 4, diuron; 5, metobromuron; 6, siduron; 7, linuron; 8, chloroxuron; 9, neburon.

sample is usually achieved by introducing a large volume of that sample. In CEC, sample introduction can be performed by either pressure or electromigration. Two different urea herbicides, namely metobromuron and neburon, were chosen to demonstrate the capability of CEC in on-column preconcentration. Metobromuron is a typical example of urea herbicides of relatively medium retentivity on the ODS stationary phase, while neburon is an example of urea herbicide with a relatively strong affinity to the ODS stationary phase.

TABLE I. Column efficiencies (plates/m) when using mobile phase containing different organic modifiers.

	Number of plates, N	
	70% (v/v) Methanol	50% (v/v) Acetonitrile
Terbacil	14,000	22,400
Monuron	15,600	22,000
Fluometuron	13,800	22,200
Diuron	15,000	20,000
Siduron	14,200	23,200
Metobromuron	15,400	20,800
Linuron	15,600	20,000
Chloroxuron	15,200	22,400
Neburon	14,400	22,200

See Fig. 2 for conditions.

As can be seen in Fig. 4, due to the relatively strong affinity of metobromuron and neburon for the ODS column, prolonged injections can be performed to enhance their

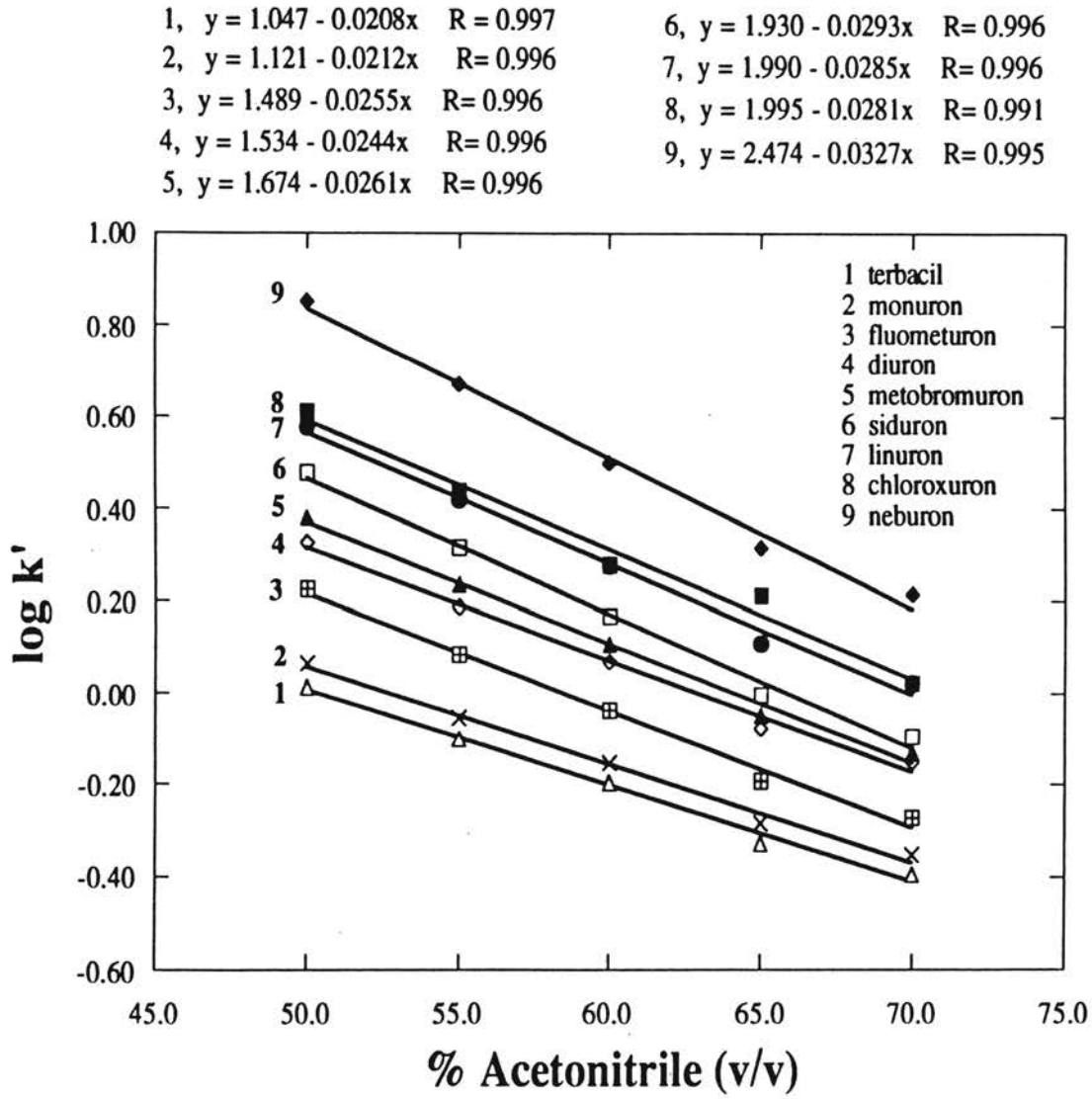

Figure 3. Plots of log k' of urea herbicides vs. % acetonitrile (v/v). Elution order and other conditions are same as Fig. 2.

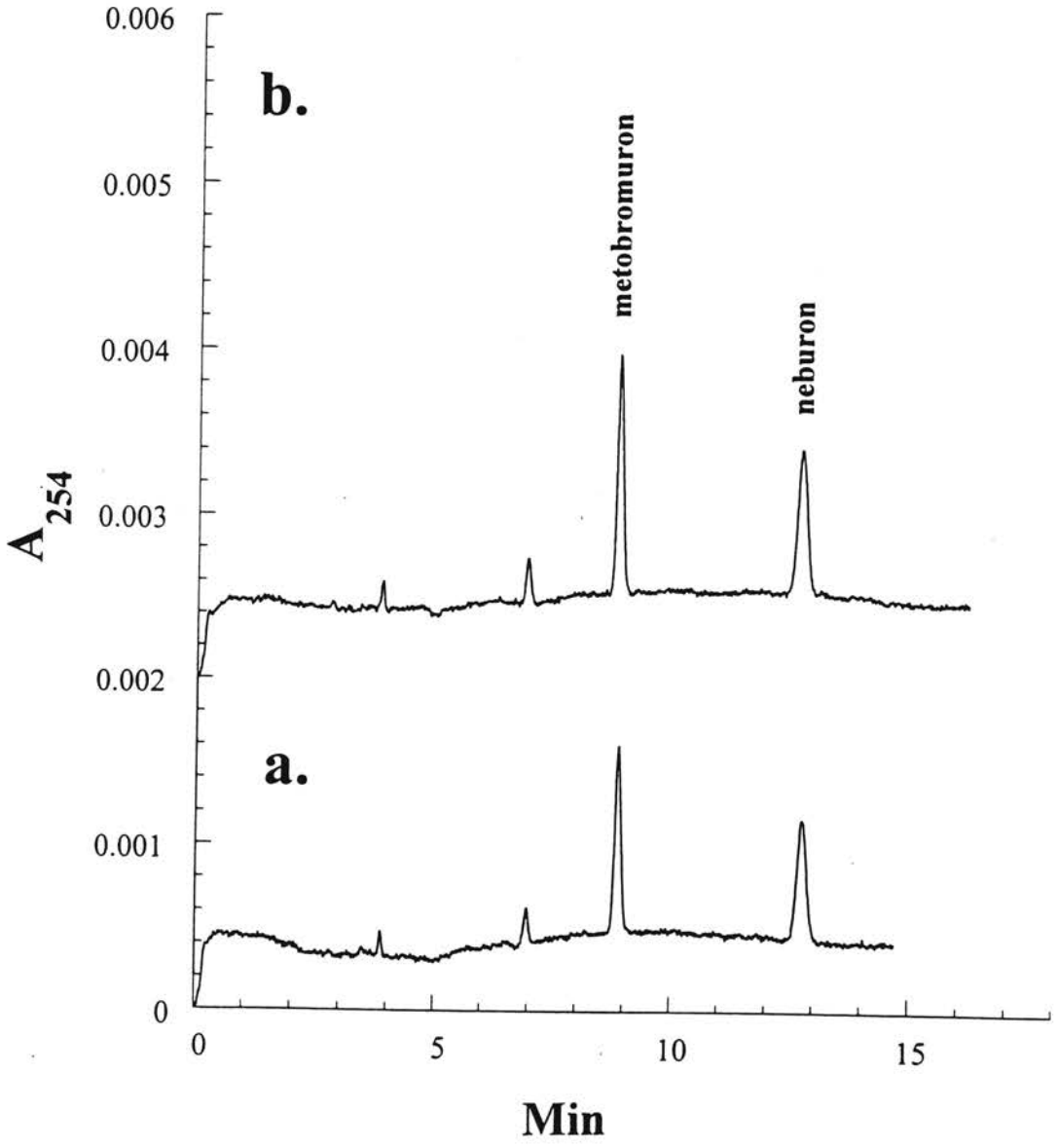

Figure 4. Electrochromatograms of two urea herbicides obtained at two different pressure injection times. Sample was injected by pressure at 20 psi for 30 sec in (a) and 60 sec in (b). Sample concentration, 10^{-4} M; mobile phase: 40% (v/v) 5 mM NaH_2PO_4, pH 6.0, mixed with 60% (v/v) acetonitrile. Other conditions as in Fig. 2.

detectability without sacrificing separation efficiency. This can be viewed as an on-line solid-phase extraction process. In this process, the sample components accumulate on the stationary phase as narrow bands. In fact, the calculated average column efficiency for a 30 s injection was found to be 19,000 plates, while it was 18,000 plates for a 60 s injection. This indicates that in this time range, the injection time did not seem to effect the separation efficiency. The limit of detection (LOD) by the prolonged (90 sec) pressure injection mode was determined to be at the level of 10^{-5} M (15 ppm) for the 2 urea herbicides, as is shown in Fig. 5. Without on-line preconcentration (i.e., short injection times of 10 sec or less) the LOD was found to be at the level of 10^{-4} M.

Similarly to pressure injection, sample injection by electromigration allows the introduction of a large volume from a dilute sample. The major difference is that pressure injection allows sample introduction without discrimination among the sample components, which may otherwise have different electrophoretic mobilities should these components are charged and be injected electrokinetically. Urea herbicides are neutral and therefore pressure or electromigration injections should in principle yield the same results as far as on-column preconcentration is concerned. In fact, the LOD by the prolonged electromigration injection method was determined to be at the level of 10^{-5} M (15 ppm), which is the same as that of the prolonged pressure injection. Because of the ease with which the volume and the amount of the sample introduced by electromigration can be controlled and estimated, sample preconcentration by electromigration injection was used for the remainder of the study.

To further enhance the on-column preconcentration of dilute samples in CEC, and consequently enhance the detectability, the following approach was introduced and its

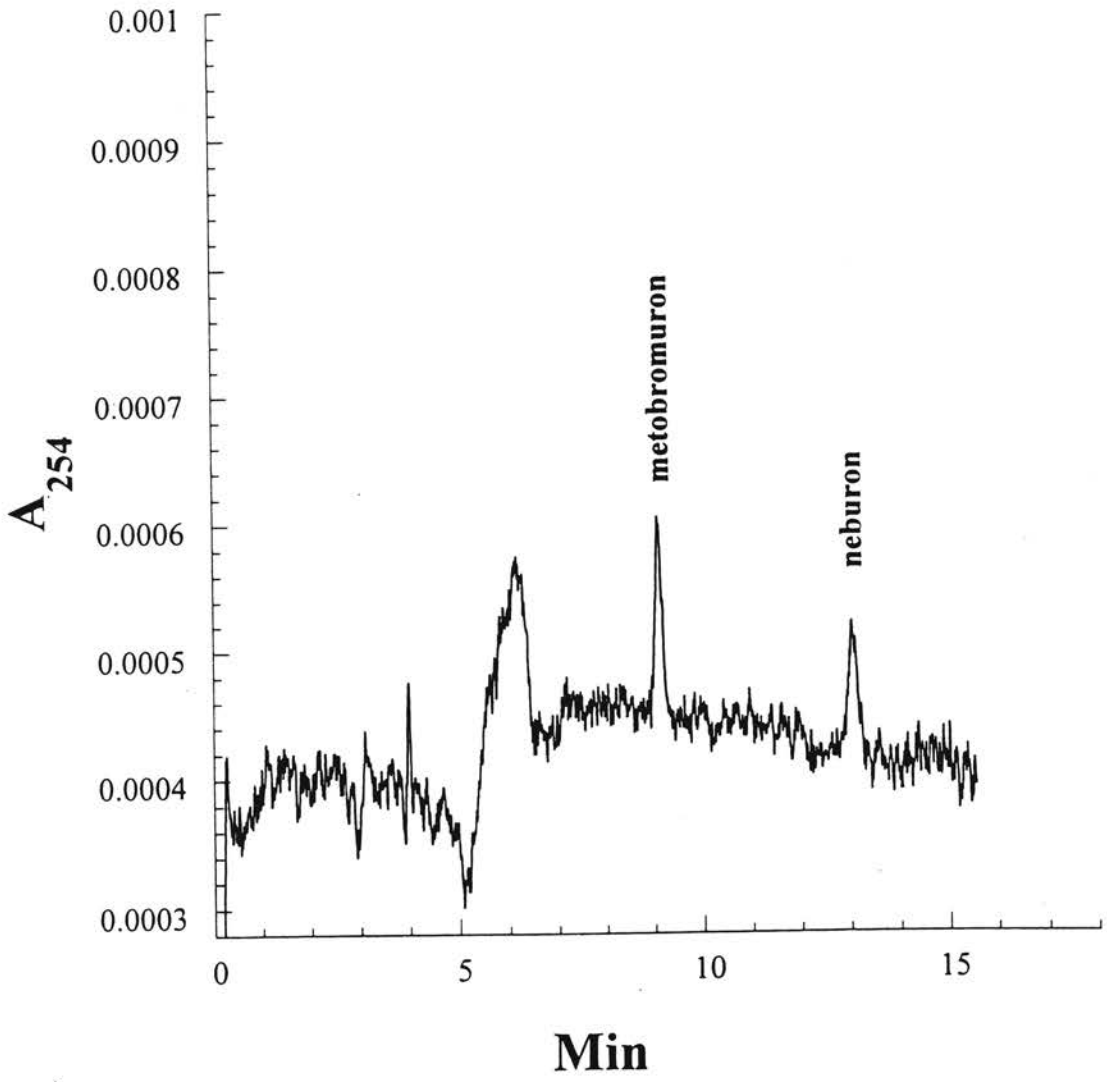

Figure 5. Electrochromatogram of metobromuron and neburon demonstrating the limit of detection when sample is injected by pressure at 20 psi for 90 sec. Sample concentration, 10^{-5} M. Mobile phase is same as in Fig. 4, and all other conditions are the same as in Fig. 2.

effectiveness was evaluated. To allow maximum adsorption of the solutes onto the stationary phase, which was originally equilibrated with a mobile phase containing 60% v/v acetonitrile, a plug of water was injected before sample introduction to render the surface of the stationary phase more retentive toward the sample components. The length of the plugs of the refreshing water and the sample are of concern, since they lead to axial nonuniformities within the CEC column as far as the electric field strength and the EOF are concerned. Above a certain range of plug length, the axial nonuniformities caused the formation of air bubbles under high separation voltage, a fact that impeded the CEC process.

The length of the injected plug (whether water or sample) was determined by measuring the EOF under the injection voltage. The water vacancy peak was observed to measure the EOF. The injection time in sec was then converted to plug length in mm based on the measured EOF velocity. For on-line preconcentration, water was injected for 20 sec and sample solution was injected for 90 sec at 3 kV. This was equivalent to a 3 mm water plug and a 12 mm sample plug. Injections at higher voltage or for a longer time were found to be vulnerable in causing air bubbles. With this injection mode, dilute samples of 10^{-7} M or 150 ppb could be detected, as is shown in Fig. 6. This is about two orders of magnitude lower than the concentration detected by simply using prolonged injection for 90 sec as in Fig. 5.

Combination of an off-line solid phase extraction step with an on-line pre-concentration step. Fig. 7 shows the electrochromatogram obtained by combining off-line and on-line preconcentration, which are essentially solid-phase extraction processes. The initial concentration of the sample used in Fig. 7 was 10^{-10} M. By combining off-line

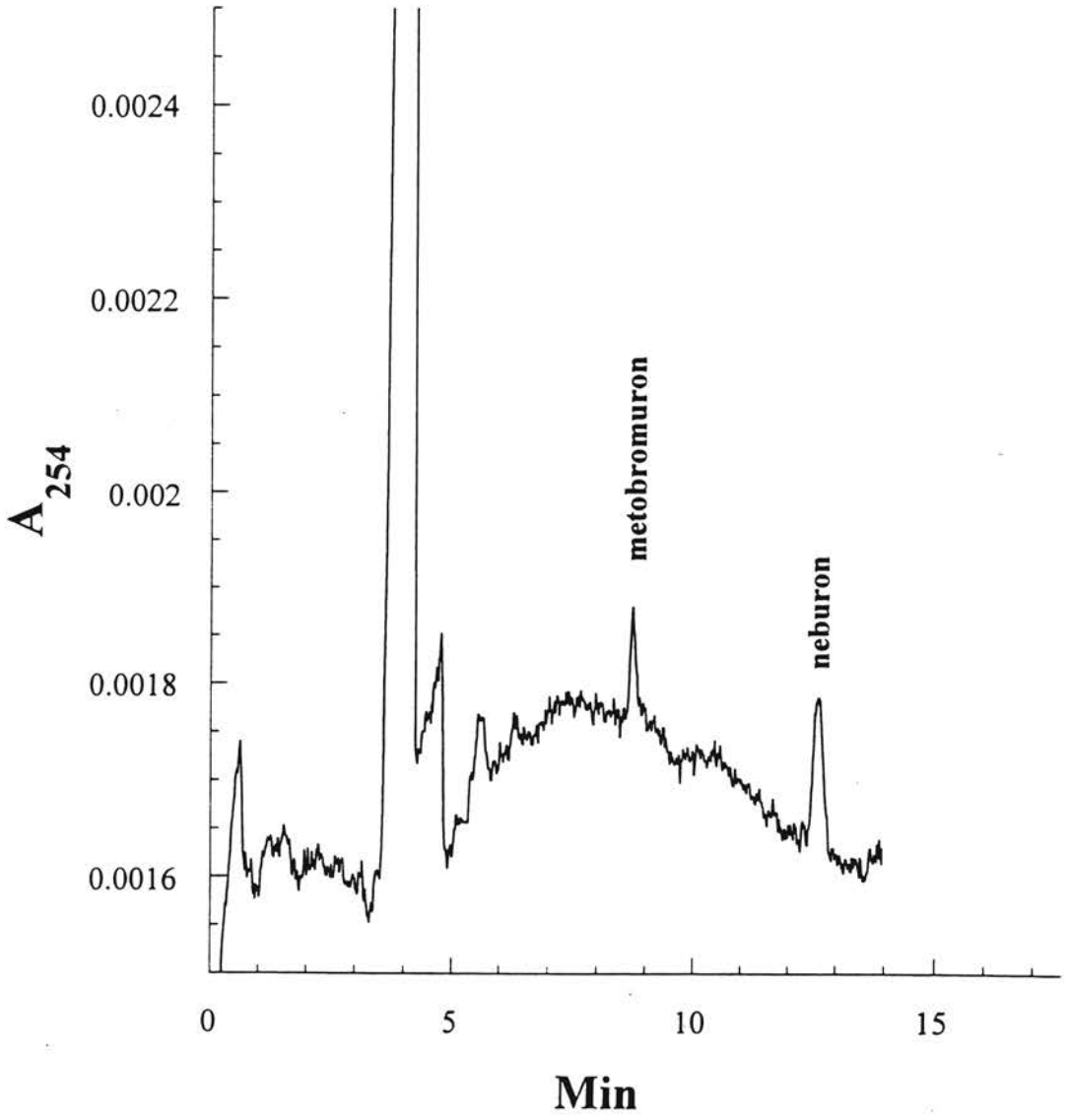

Figure 6. On-line preconcentration of metobromuron and neburon by CEC. Sample was injected for 90 sec at 3 kV preceded by a water injection for 20 sec at 3 kV. Sample concentration, 10^{-7} M or 100 ppb. Conditions same as Fig. 5.

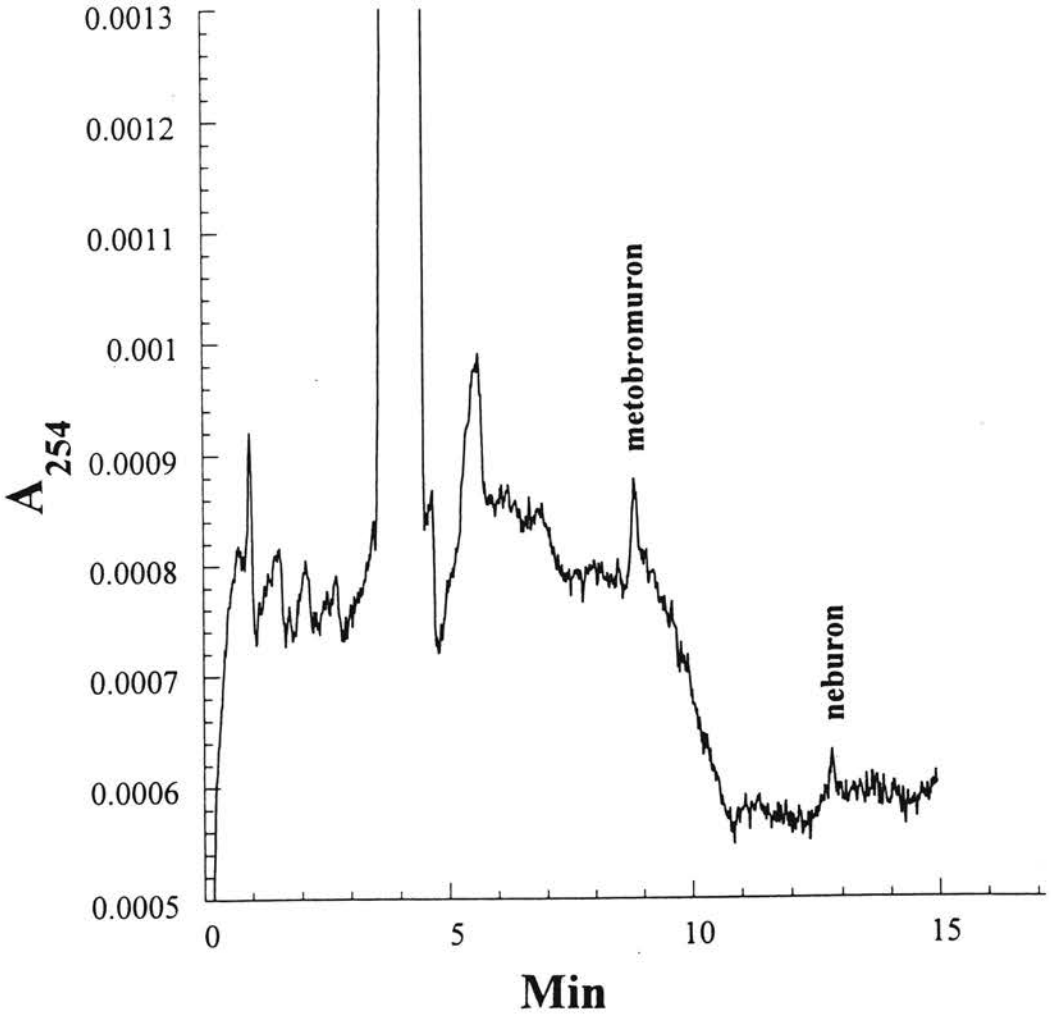

Figure 7. Combination of off-line and on-line preconcentration of dilute samples of metobromuron and neburon. Sample concentration before off-line preconcentration, 10^{-10} M or 0.1 ppb. The off-line enriched sample was subjected to on-line preconcentration, as in Fig. 6.

and on-line preconcentration, the sample is enriched by a relaying process. The off-line preconcentration enriches the sample to a concentration level that is readily determined by the on-line preconcentration process. In other words, to be effective, the off-line preconcentration should raise the sample concentration to a level that is detectable by the on-line preconcentration approach. With off-line preconcentration, the sample was enriched from 10^{-10} M to 10^{-6} M, which is above the concentration that can be determined by the on-line preconcentration (10^{-7} M). This assumes that no sample loss has occurred during the off-line preconcentration process. The combination of off-line with on-line preconcentration allowed the detection of very dilute samples whose concentration is three orders of magnitude lower than the concentration detected by simply using an on-line preconcentration with a plug of water. The results of these different preconcentration processes are shown in Table II.

TABLE II. Limit of detection in CEC and detectable concentrations achieved by on-line and off-line preconcentration of dilute pesticide samples prior to CEC separation.

No preconcentration	Pressure injection for 90 s at 20 psi	20 s water injection followed by 90 s sample injection at 3 kV	Off-line and on-line pre-concentration
10^{-4} M (150 ppm)	10^{-5} M (15 ppm)	10^{-7} M (150 ppb)	10^{-10} M (0.1 ppb)

Conclusions

In this work, we have evaluated the potentials of CEC in the separation of urea herbicides over a wide range of elution conditions as far as the concentration and nature of the organic modifier is concerned. In terms of separation efficiency, the best results

were obtained when acetonitrile was used as the organic modifier instead of methanol. When compared to mobile phases containing methanol, the elution order of the analytes was switched when using acetonitrile containing mobile phases. Furthermore, CEC allowed the determination of dilute herbicide samples (10^{-7} M) by on-line preconcentration which consisted of performing relatively prolonged injections from dilute samples that were preceded by the introduction of a small plug of pure water to enhance the accumulation of the solutes on the ODS stationary phase. When combined with off-line preconcentration of dilute urea herbicide samples, this on-line preconcentration procedure permitted the detection of ultra dilute samples of ca. 10^{-10} M.

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CHAPTER VII

CAPILLARY ELECTROCHROMATOGRAPHY ON NOVEL SILICA BASED STATIONARY PHASES HAVING A SURFACE BOUND SURFACTANT MOIETY

Introduction

In capillary electrochromatography (CEC), the stationary phase has two important roles: (a) to ensure the chromatographic selectivity and (b) to provide the electroosmotic flow (EOF) necessary for differential migration. Therefore, the surface modification of chromatographic media (e.g., silica) for CEC is not a simple transposition to what is already known for producing stationary phases for high performance liquid chromatography (HPLC). We have addressed this issue in Chapters IV and V by designing partially modified ODS stationary phases (Chapter IV) and by introducing the concept of segmented capillary columns (Chapter V). We report in this Chapter a stationary phase which possesses positive surface charges for controlling the magnitude and polarity of the EOF which serves to drive the mobile phases through the column, and at the same time provides selectivity which parallels that of a reversed-phase stationary phase in conventional liquid chromatography. The silica-based stationary phase described in this Chapter is designated as ODAS and consists of a surfactant type ligand chemically bonded to the surface of the silica support. This ligand has an octadecyl moiety and a permanently charged quaternary amine group. This surface modification is

similar to that described in Chapter III for capillary coating for CZE of proteins. The ODAS stationary phase differs from that in Chapter III in that it contains short quaternary amine functions chemically bonded to the surface to ensure a stationary phase with a high net positive charge. In addition, to ensure surface charge homogeneity, the inner wall of the capillary used to pack the CEC column was also modified to carry positive charges. The ODAS stationary phase was characterized over a wide range of elution conditions with several categories of analytes to demonstrate its applicability and to understand its CEC behavior.

Materials and Methods

Instrumentation

The instrument used in this study was an HP ^{3D} capillary electrophoresis system from Hewlett Packard Co. (Wilmington, DE, USA) equipped with a UV diode array detector and a data handling system comprised of a PC computer and ChemStation software. The capillary columns were packed with a Shandon column packer from Keystone Scientific (Bellefonte, PA, USA).

Reagents

Nine urea herbicides, namely terbacil, monuron, fluometuron, linuron, metobromuron, siduron, linuron, chloroxuron, and neburon were from ChemService, Inc. (West Chester, PA, USA). The molecular structures of these urea herbicides are shown in Chapter VI. Another group of five herbicides, namely aldicarb, prometon, prometryne, propazine, and diazinon were also from ChemService, Inc. Figure 1 shows the molecular

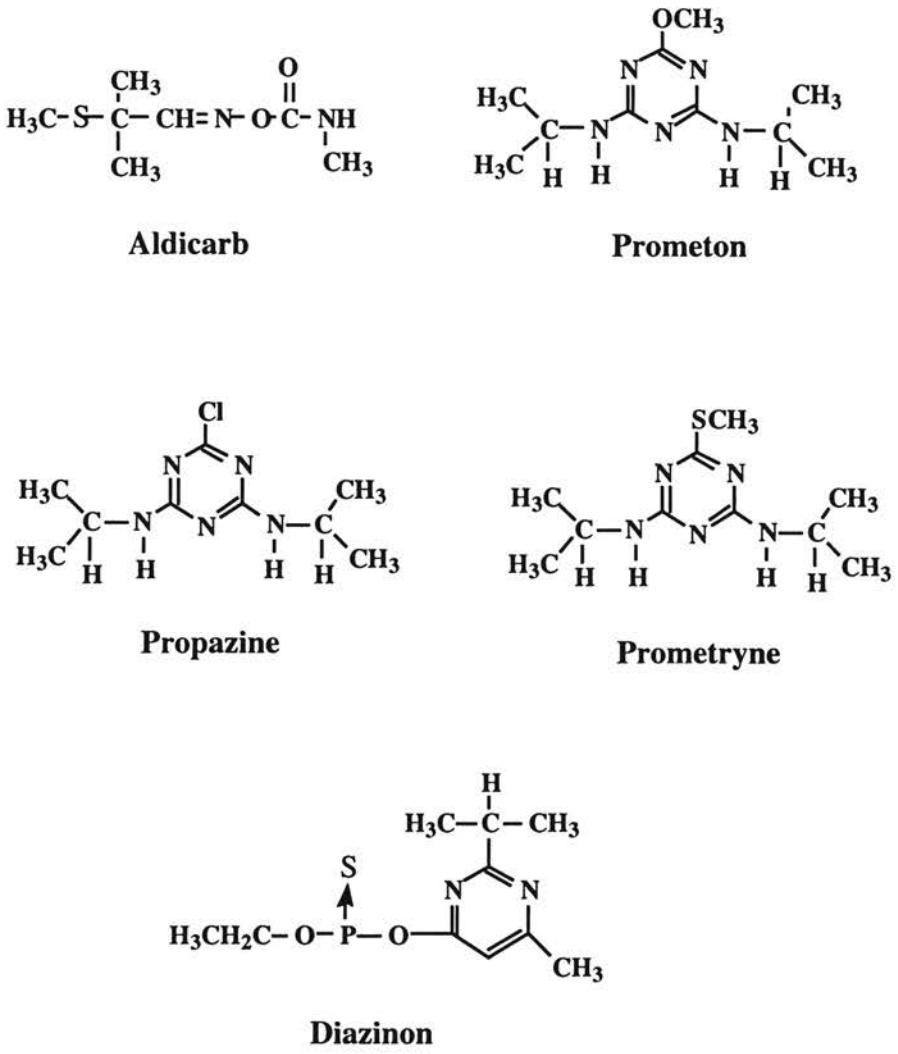

Figure 1. Molecular structures of a group of herbicides investigated in this study.

structures of these herbicides. Sodium phosphate monobasic was from Mallinckrodt (St. Louis, MO, USA). HPLC grade acetonitrile and isopropanol were from Baxter (McGaw Park, IL, USA). HPLC grade methanol and analytical grade acetone were from Fisher Scientific (Fair Lawn, NJ, USA). *n*-Octadecyldimethyl [3-trimethoxysilyl-propyl] ammonium chloride (ODAC) in 50% (v/v) methanol and *N*-trimethoxysilyl-propyl-*N,N,N*-trimethyl ammonium chloride (TAC) in 50% (v/v) methanol were obtained from United Chemical Technologies, Inc. (Bristol, PA, USA). *N,N*-Dimethylformamide (DMF) was obtained from EM Science (Gibbstown, NJ, USA) and was dried with molecular sieve before use. Fused-silica capillaries with an internal diameter of 100 μm and an outer diameter of 360 μm were from Polymicro Technologies (Phoenix, AZ, USA). Water refers to deionized water throughout this Chapter.

Coating of Capillary Wall

A 35-cm segment of open fused-silica capillary was first rinsed with water, and then filled with 0.3 M NaOH to etch the capillary inner surface for 15 min. Thereafter, the capillary was flushed with water, 0.3 M HCl, and water again to restore the neutrality of the capillary wall. Then, the capillary was flushed thoroughly with dry DMF. After being filled with TAC in 50% (v/v) methanol, the capillary was heated in the oven at 120 °C for 30 min. This coating process was repeated for two more times to ensure maximum conversion of silanol groups originally on the capillary surface into quaternary amine ligands. Each time fresh TAC was filled into the capillary. After coating, the capillary was rinsed with DMF to remove the unreacted excess TAC. The coated capillary was ready for packing. Figure 2a illustrates the bonded function on the capillary wall.

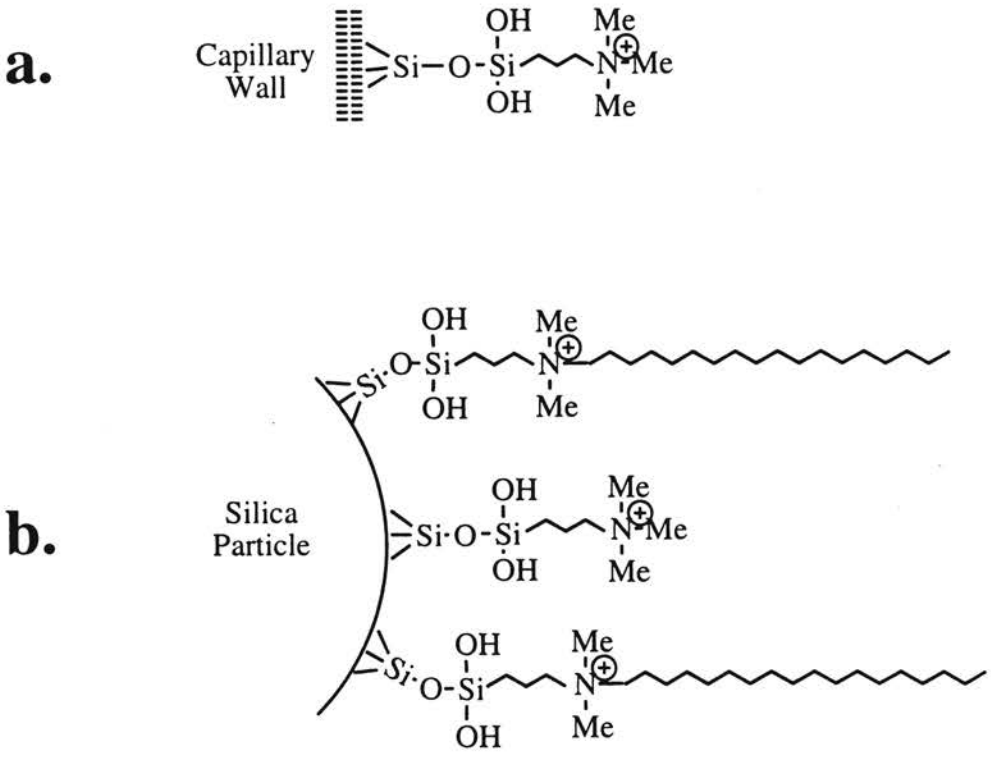

Figure 2. (a), Illustration of bonded function on the fused silica capillary wall. (b), Illustration of bonded functions on the surface of the silica particles.

Stationary Phase

Zorbax PSM 150 microspherical silica from DuPont (Wilmington, DE, USA) was used as support for CEC in this study. This silica has a mean particle diameter of 5 μm, a mean pore diameter of 150 Å and a specific surface area of 147 m^2/g. The Zorbax was first treated with ODAC [1]. Briefly, 2.0 g of Zorbax PSM 150 wwas mixed with 20 mL of dry DMF in a round-bottomed flask, then 7.0 g of ODAC in 50% (v/v) methanol was added. The suspension was stirred with a paddle stirrer and the reaction proceeded at 120 °C for 24 h. After this reaction, the silica was collected by centrifugation, and was rinsed twice with DMF. The silica was then placed in a clean round-bottomed flask, mixed with 20 mL of dry DMF and reacted with 10 g of a 50% (v/v) methanol solution of TAC, in the same way as in the preceding reaction. After this, the silica was collected by centrifugation, rinsed twice with dry DMF and twice with methanol. Finally, the collected silica slurry in methanol was dried in the air. Figure 2b illustrates the bonded functions on the surface of the silica particle. The silica thus obtained is referred to as ODAS.

Column Packing and Fabrication

The CEC columns in this study were packed and handled as previously described [2, 3], see Chapters IV and V. The packing pressure was 3000 psi and in one case 4000 psi. Columns were fully packed without an open segment. For HP ^{3D}CE system, the CEC columns were prepared with a total length of 32 cm. The length between the inlet end and the detection window was 24 cm. Operating temperature was kept constant at 20 °C.

Results and Discussion

Magnitude and Polarity of the EOF

Water was injected in small plugs as an EOF marker for the evaluation of the impact of surface modification of the capillary inner wall and the stationary phase on the magnitude and direction of EOF in open tubes and packed columns for CE and CEC, respectively. Water vacancy peaks observed in CE and CEC were recorded and were used to calculate the EOF in the columns under study. As expected, the EOF exhibited by the uncoated open column can only be observed with positive polarity voltage, and its magnitude was 9×10^{-8} m^2 $sec^{-1}.V^{-1}$. After coating the inner surface of the capillary with TAC, the direction of the EOF in the open column was reversed from cathodic to anodic, and its value was -5×10^{-8} m^2 $sec^{-1}.V^{-1}$. Going from a bare fused-silica capillary to a TAC coated capillary, the magnitude of EOF was cut by approximately 44%. This was attributed to the incomplete conversion of silanol groups on the inner surface of the capillary.

Packed CEC columns in capillaries with walls coated and uncoated with TAC were subjected to EOF studies to investigate the effect of the inner wall of the capillaries on the magnitude of the EOF originated inside the columns. The results are summarized in Fig. 3. As expected, columns with coated walls exhibited higher EOF than columns with uncoated wall over a pH range of 3.0 to 8.0, under otherwise identical conditions. The difference in the magnitude of the EOF wass larger at low pH than at higher pH, as shown in Fig. 3. At pH 3.0, the EOF was 40% higher on the ODAS column with coated walls than on the ODAS column with uncoated walls, while at pH 8.0 the EOF was 14% higher on the former column than on the latter column. The net positive charge density

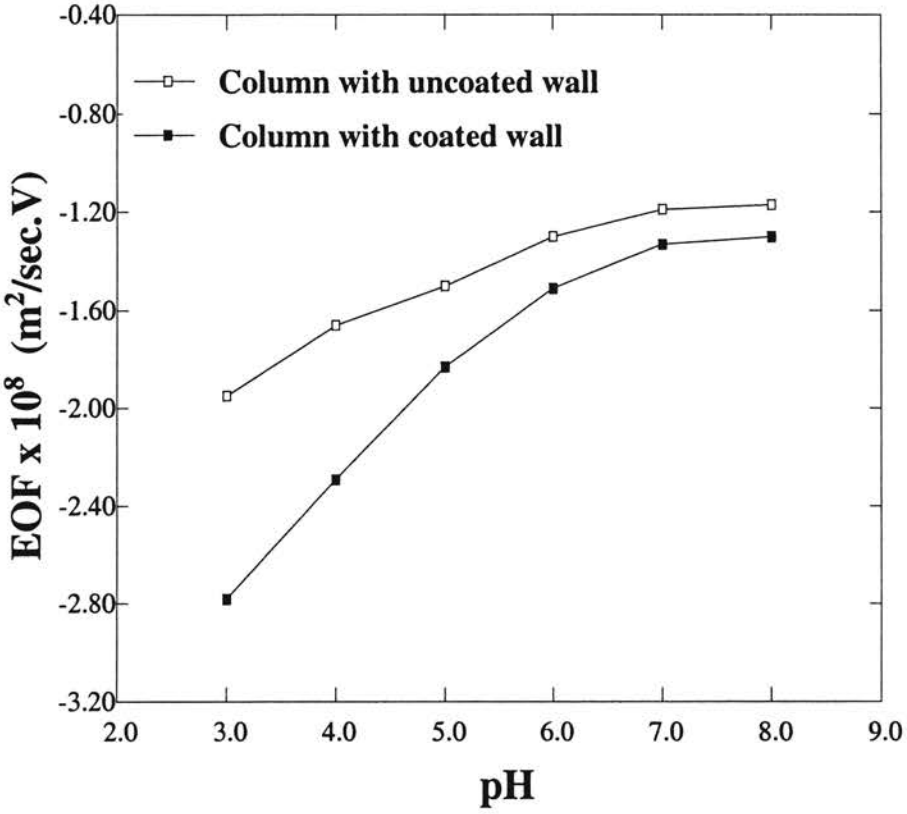

Figure 3. A comparison of the EOF at different pH values obtained on packed columns with uncoated wall and packed columns having TAC coated wall. CEC conditions: mobile phase, 20% (v/v) of 5 mM sodium phosphate at various pH values, and 80% (v/v) acetonitrile; column, 32 cm total length, 24 cm to detection x 100 μm I.D.; temperature, 20 °C; voltage, -20 kV; detection wavelength, 254 nm. Water was used as an EOF marker.

in the ODAS column with TAC coated wall is higher than that in the ODAS column with uncoated wall counter balance the net positive charge on the ODAS particles. In porous media like CEC, this would induce a higher EOF [4]. For both type of columns, the EOF tended to level off toward higher pH due to the complete ionization of the silanol groups on the surface of the capillary wall as well as on the stationary phase surface.

The effects of acetonitrile content in the mobile phase on the EOF observed with columns packed under 3000 psi and 4000 psi with TAC coated and uncoated capillaries are shown in Fig. 4. In all cases, the EOF increased when going from 50 to 90% (v/v) acetonitrile in the mobile phase. This was due to the decrease in the viscosity and the ionic strength of the mobile phase as the acetonitrile content was increased. As shown above in Fig. 3, under the same running conditions, the EOF in columns with TAC coated walls was higher than that in columns with uncoated walls. On the other hand, columns packed at lower pressure (3000 psi) generated higher magnitude of EOF than columns packed at higher pressure (4000 psi). This can be explained by the differences in electric conductivity. More tightly packed columns have lower conductivity. In this case, for columns packed at higher pressure, reduction of conductivity seemed to have overcome the relatively increased surface charges brought about by the increased amount of the stationary phase in the column.

Chromatographic Characterization of the Stationary Phase

In one of our previous investigations [2] (see Chapter VI), silica with partial surface coverage by octadecyl functions, (i.e., ODS), was prepared and evaluated as a CEC stationary phase. This partially modified ODS had negative charges to induce EOF

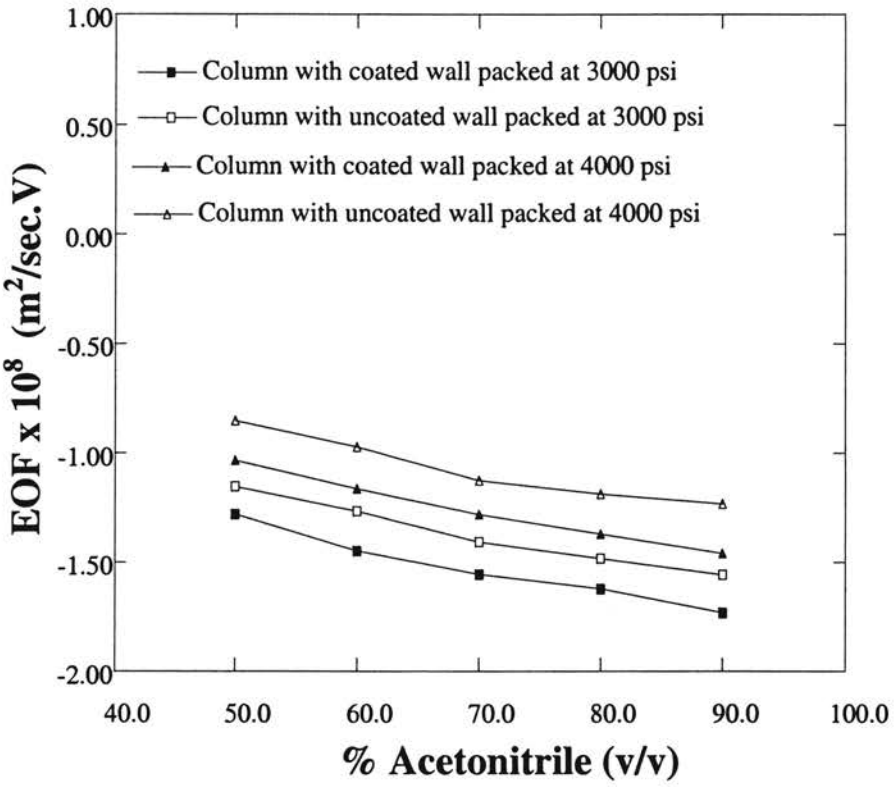

Figure 4. A comparison of the EOF observed with columns packed at two different packing pressures in capillaries with TAC coated and uncoated walls. Mobile phase, Various % (v/v) of 5 mM phosphate at pH 6.0 and various % (v/v) acetonitrile. Water was used as neutral marker. Other conditions are the same as in Fig. 3.

and had octadecyl groups to provide chromatographic selectivity. Silica so designed showed strong EOF and at the same time maintained reversed-phase column behavior [2]. Compared with this partially modified ODS, ODAS modified silica in this study, had a net positive surface charge provided by the quaternary amine groups. The silane reagent ODAC in a three-fold excess to silanol groups on the silica surface was used in the first step of the surface modification (see Materials and Methods), to yield the ODAS stationary phase. A limit of conversion exists due to the bulkiness of the ODAC. This limit was compensated for by a second step of reaction with an excess amount of TAC, which has a shorter carbon chain. TAC is very reactive in modifying silanol groups as was observed when the reagent was used in coating the capillary wall.

In this study, a mixture of six alkylbenzenes was used to investigate the chromatographic behavior of the ODAS stationary phase and to compare the results to those obtained on the partially modified ODS stationary phase. Figure 5a shows an electrochromatogram of the six alkylbenzenes obtained with 20:80 (v/v aqueous:organic) mobile phase. Under this condition, the first three analytes, namely benzene, toluene and propyl benzene, were not separated with a base line resolution; the average column efficiency was 53,000 plates/m with this running condition. The relatively strong EOF enabled the last analyte to be eluted within 10 minutes. With 50:50 (v/v aqueous:organic) mobile phase, the standard mixture was separated within 30 minutes, as shown in Fig. 5b. The average column efficiency is 35,000 plates/m with this running condition. Plots of log k' values of these analytes versus percentage of organic modifier as well as number of carbon on side chains are summarized in Fig. 6a, Fig. 6b, Fig. 7a and Fig. 7b, respectively. These plots indicated that the ODAS stationary phase possessed a reversed-phase behavior. It is noteworthy that log k' values obtained from the ODAS columns

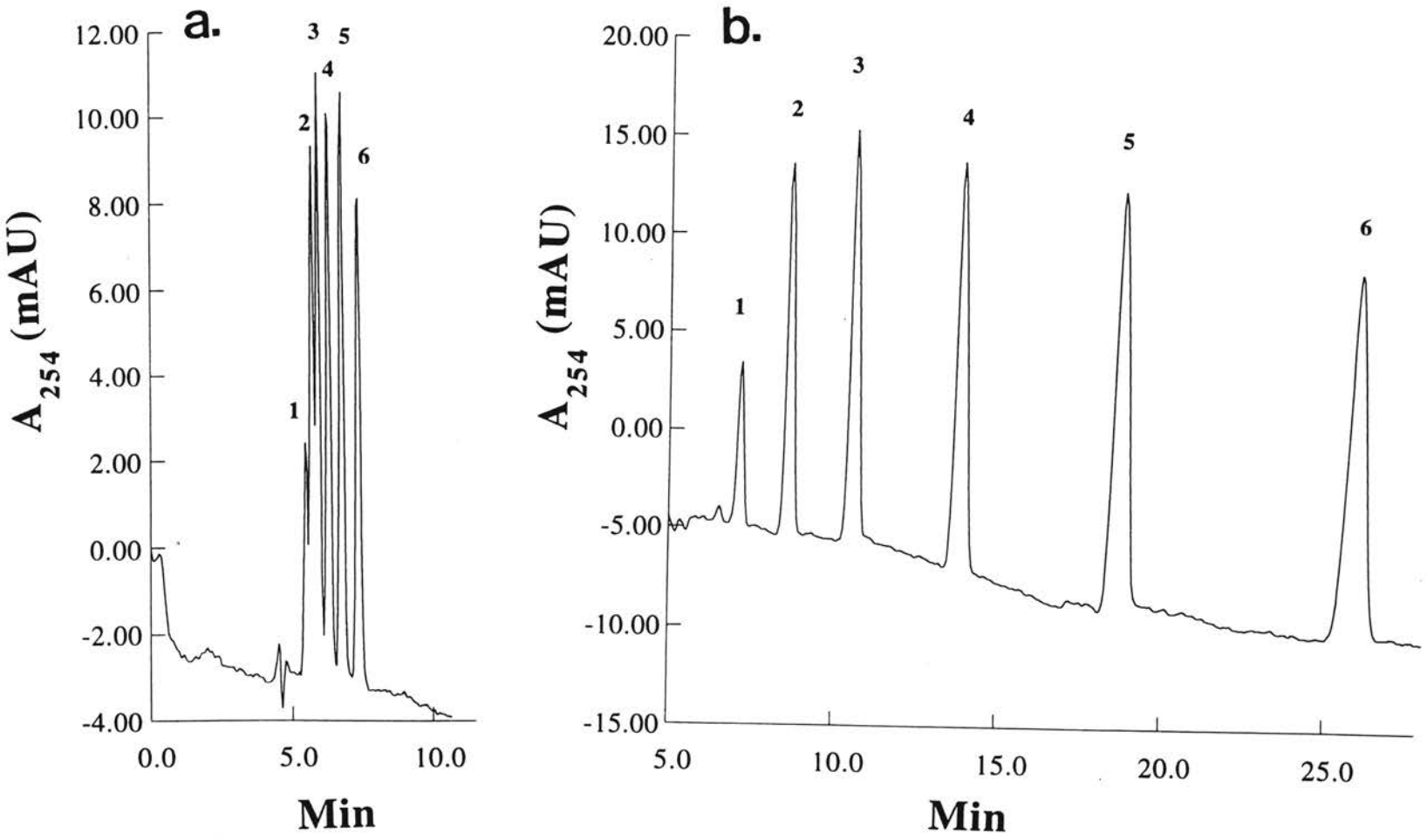

Figure 5. Electrochromatograms of benzene and alkylbenzenes. Mobile phase, 20% (v/v) of 5 mM sodium phosphate, pH 6.0, 80% (v/v) acetonitrile in (a); and 50% (v/v) of 5 mM sodium phosphate, pH 6.0, and 50% (v/v) acetonitrile in (b). Solutes: (1) benzene, (2) toluene, (3) ethylbenzene, (4) propylbenzene, (5) butylbenzene, and (6) amylbenze. Other conditions are the same as in Fig. 3.

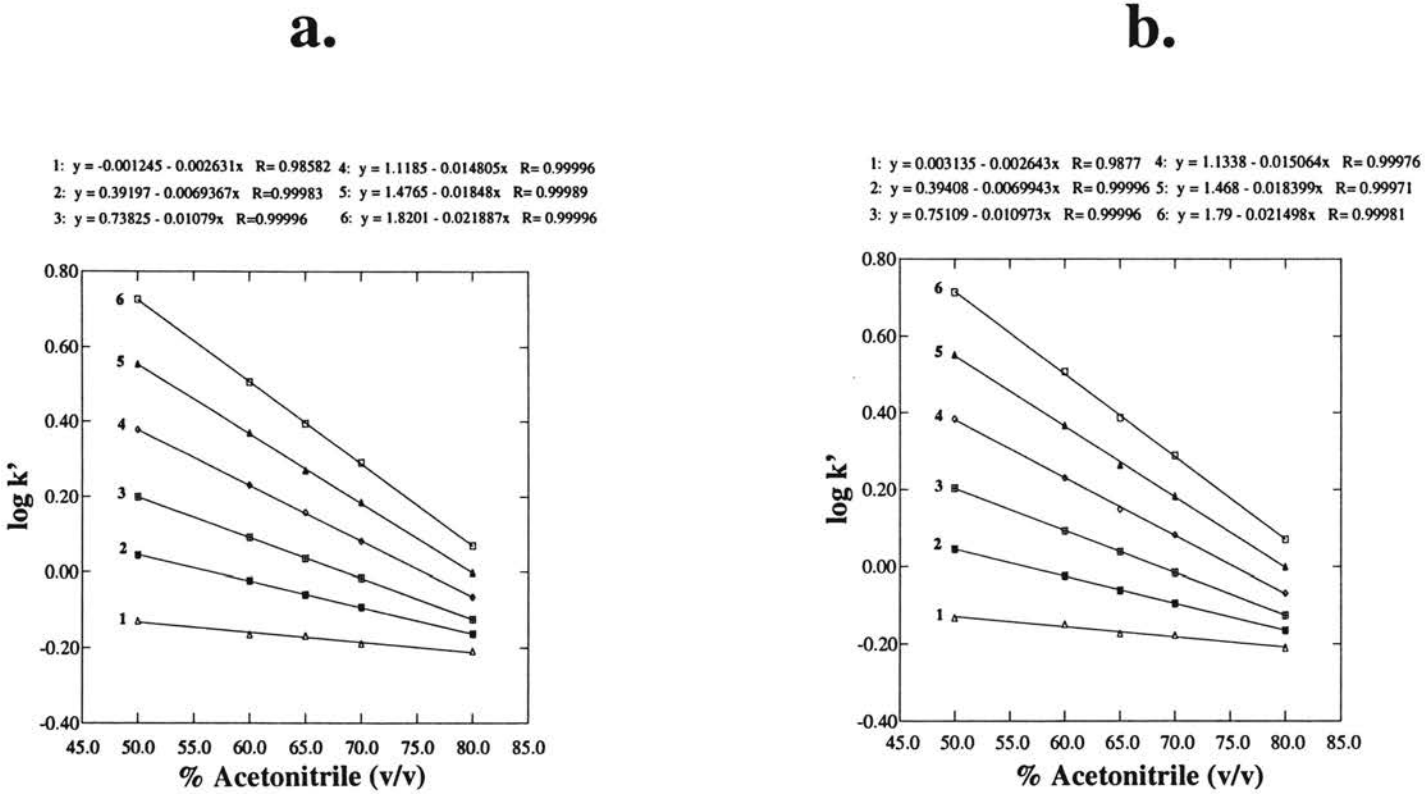

Figure 6. Plots of log k' values vs. the content of acetonitrile in the mobile phase obtained on columns with TAC coated wall in (a), and uncoated wall in (b). Aqueous phase, 5 mM sodium phosphate, pH 6.0. Plots are numbered according to the component numbers in Fig. 5. Other conditions are the same as in Fig. 3.

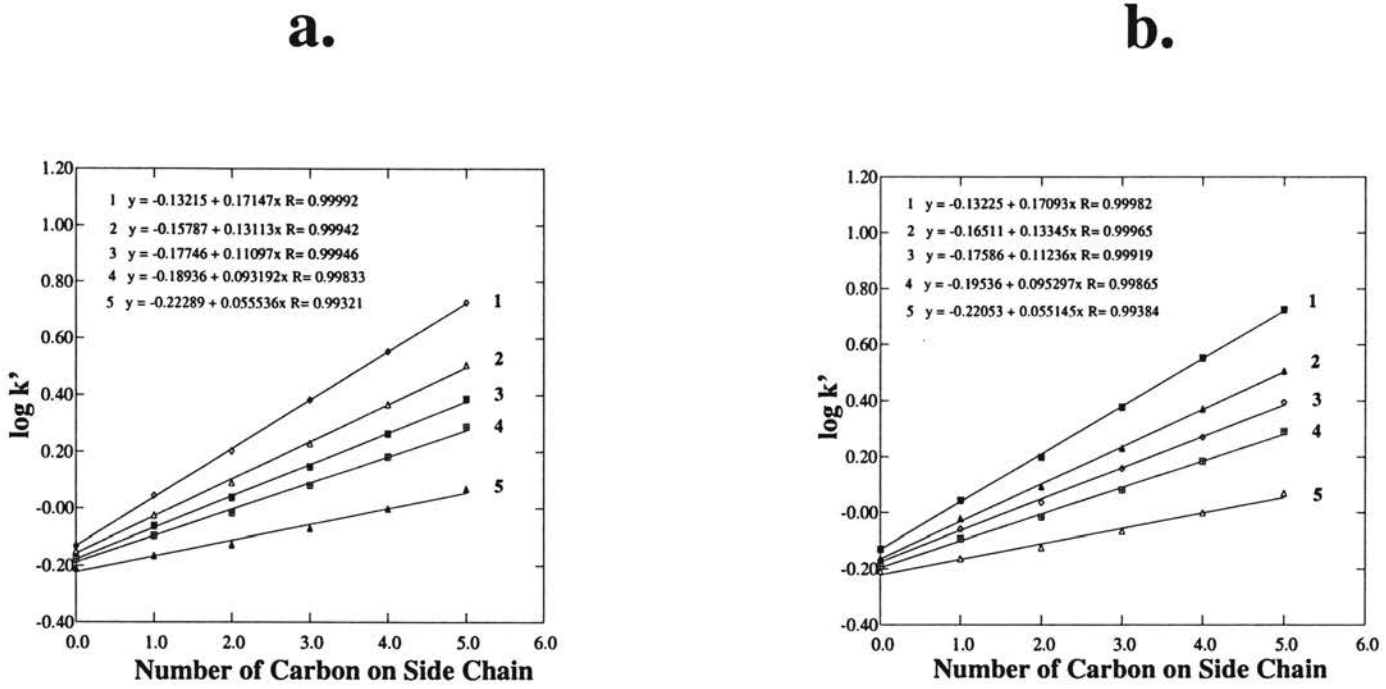

Figure 7. Plots of log k' values vs. the number of carbons in the side chain of alkyl benzenes obtained on columns with TAC coated wall in (a), and uncoated wall in (b). Mobile phase compositions in aqueous:organic (v/v) are: (1) 50:50, (2) 40:60, (3) 35:65, (4) 30:70, and (5) 20:80. Other conditions are the same as in Fig. 5.

with coated wall and uncoated wall are essentially the same, indicating that the retention of analytes are mostly controlled by the stationary phase rather than by the capillary wall.

Table I summarizes the CEC results of the six alkylbenzenes obtained under the same running conditions on the partially modified ODS and the ODAS stationary phase prepared in this study. From these results, the ODAS stationary phase appears to be less retentive to hydrophobic analytes like the alkylbenzenes than the partially modified ODS. As a result, the separation factors of the ODAS stationary phase are smaller. Another difference between ODAS and ODS is that the former exhibited lower column efficiencies than the latter. This may be attributed to the presence of a dual EOF polarity whose overall polarity is anodic. Under this condition, the flow profile may not be a plug like profile.

Applications

CEC of Urea Herbicides. The effectiveness of ODAS was demonstrated in the CEC of a mixture of nine urea herbicides. Separation was obtained with a mobile phase composed of 50% (v/v) 5 mM phosphate at pH 6.0 and 50% (v/v) acetonitrile. The elution of last peak (neburon) was within 15 minutes, as shown in Fig. 8. The CEC results of these urea herbicides obtained with the partially modified ODS in one of our previous works [5] (see Chapter VI) were compared to those obtained on the ODAS column, and are summarized in Table II. Similar to the comparison made in Table I, the ODAS stationary phase is less retentive towards the urea herbicides than the partially modified ODS, yielding less separation factors or selectivity. The lower column efficiencies of the ODAS stationary phase may also be attributed to the dual polarity of

TABLE I. A comparison of column behavior with the ODS stationary phase and the ODAS stationary phase in terms of retention factor (k'), selectivity factor (α) and plate counts (N).

	ODS			ODAS		
	k'	$\alpha=k_2'/k_1'$	N (plates/m)	k'	$\alpha=k_2'/k_1'$	N (plates/m)
benzene	0.6619		104,000	0.8213		65,000
		1.3237			1.0917	
toluene	0.8762		100,000	0.8966		63,000
		1.2826			1.0812	
ethyl benzen	1.1238		100,000	0.9694		50,000
		1.3602			1.1254	
propyl benzene	1.5286		100,000	1.0909		50,000
		1.3738			1.1421	
butyl benzene	2.1		90,000	1.2459		46,000
		1.3832			1.2630	
amyl benzene	2.9048		75,000	1.4425		45,000
			N_{AV} =			N_{AV} =
			95,000			53,000

Standard hydrocarbon mixture was used in the test. CEC conditions: capillary, 32 cm (total length), 24 cm (to the detection point) x 100 μm I.D.; Mobile phase, 20% (v/v) of 5 mM sodium phosphate, pH 6.0, 80% (v/v) acetonitrile; detection wavelength 254 nm; voltage, -20 kV; temperature 20 °C.

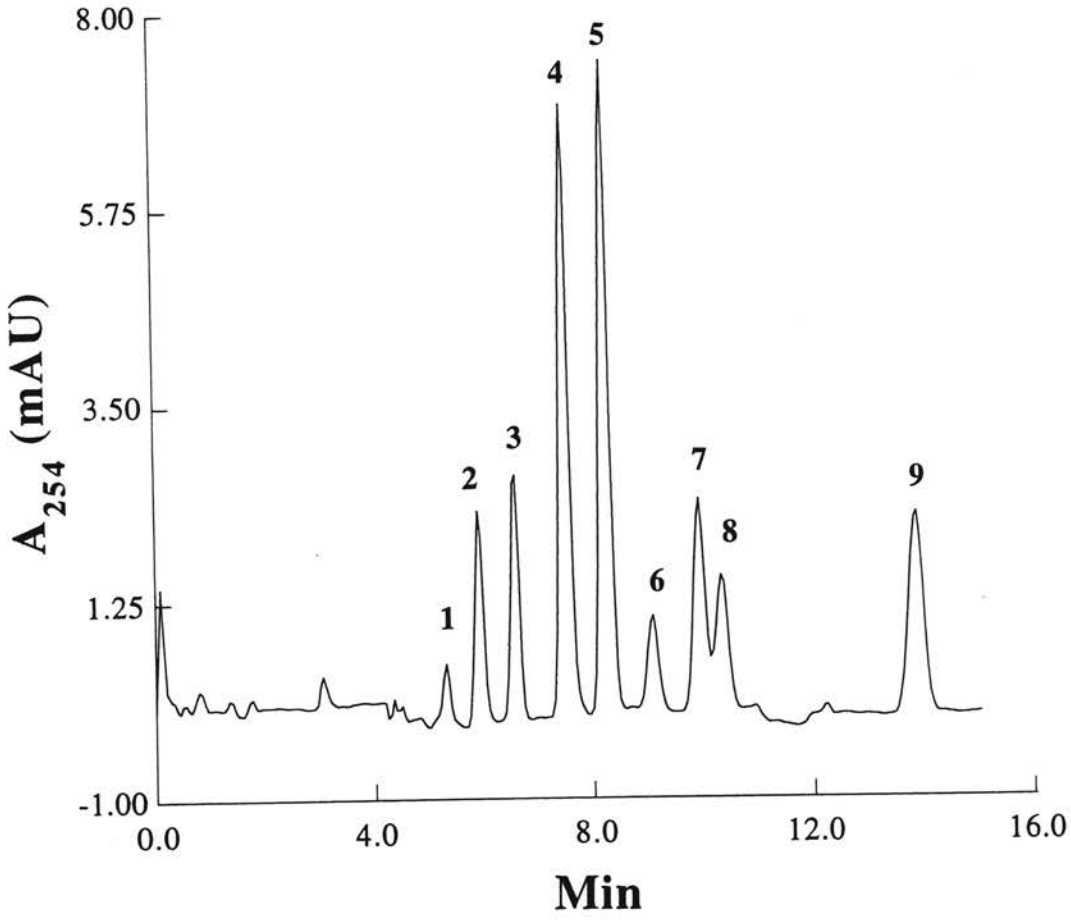

Figure 8. Electrochromatogram of the nine urea herbicides. Mobile phase, 50% (v/v) of 5 mM sodium phosphate, pH 6.0, 50% (v/v) acetonitrile; column with TAC coated wall packed under 3000 psi. Solutes: (1) terbacil, (2) monuron, (3) fluometuron, (4) diuron, (5) metobromuron, (6) siduron, (7) linuron, (8) chloroxuron, and (9) neburon. Detection wavelength, 254 nm. Other conditions are the same as in Fig. 3.

TABLE II. CEC results of urea herbicides obtained from partially modified ODS stationary phase and the ODAS stationary phase in terms of retention factor (k'), selectivity factor (α) and plate counts (N).

	ODS			ODAS		
	k'	$\alpha=k_2'/k_1'$	N (plates/m)	k'	$\alpha=k_2'/k_1'$	N (plates/m)
terbacil	1.0346	1.1308	112,000	0.3333	1.5053	15,000
monuron	1.1699	1.4438	101,000	0.5017	1.3375	33,000
fluometuron	1.6891	1.2611	110,000	0.6710	1.3516	40,000
diuron	2.1301	1.1330	100,000	0.9069	1.2063	40,000
metobromuron	2.4133	1.2526	100,000	1.0940	1.1752	26,000
siduron	3.0229	1.2605	115,000	1.2857	1.1867	15,000
linuron	3.8105	1.0737	106,000	1.5257	1.0612	30,000
chloroxuron	4.0913	1.7455	106,000	1.6190	2.1783	24,000
neburon	7.1412		110,000	3.5267		50,000
			N_{AV} = 107,000			N_{AV} = 30,000

Mobile phase was composed of 50% (v/v) of 5 mM sodium phosphate at pH 6.0, and 50% (v/v) acetonitrile. Other conditions are same as in Table I.

the EOF. Nonetheless, satisfactory separation of these urea herbicides can still be obtained with the ODAS stationary phase.

CEC of Other Herbicides. CEC was also performed on a mixture of aldicarb (sulfur carbamate herbicide), prometon, prometryne, propazine (*s*-triazine herbicides), and diazinon (organophosphorous pesticide) to demonstrate the potentials of the ODAS stationary phase, see Fig. 9. Under the tested conditions, a baseline resolution of prometon, prometryne, and propazine was obtained. These analytes have very similar structures but different pK_a values, 4.20, 1.85 and 4.05 for prometon, prometryne, and propazine, respectively [6]. Separation of these analytes appeared to be due to the differences in electrophoresis and chromatographic partitioning, since the elution order did not follow the order of pK_a values. It was noted that higher aqueous content in the mobile phase did not bring about further separation between prometryne and diazinon. Compared to the separation with 40:60 (v/v aqueous:organic) mobile phase shown in Fig. 9a, prometryne and diazinon co-eluted in 50:50 (v/v aqueous:organic) mobile phase, as shown in Fig. 9b. In these runs, the pH values of the aqueous phases were kept at 6.0, a pH at which the three *s*-triazine herbicides were neutral. However, when the pH of the aqueous phase was lowered to 3, which is below the pK_a values of prometon and prometryne, elution order of these analytes did not change, as shown in Fig. 10.

On-line Preconcentration. Previously, we have demonstrated that on-line preconcentration preceded by injecting a water plug can assist in on-line sample enrichment which in turn allowed the detection of dilute samples [5] (see Chapter VI). In this work, we intended to quantify the effect of the length of the injected water plug on on-line sample enrichment. Water plug and sample were injected electrokinetically at

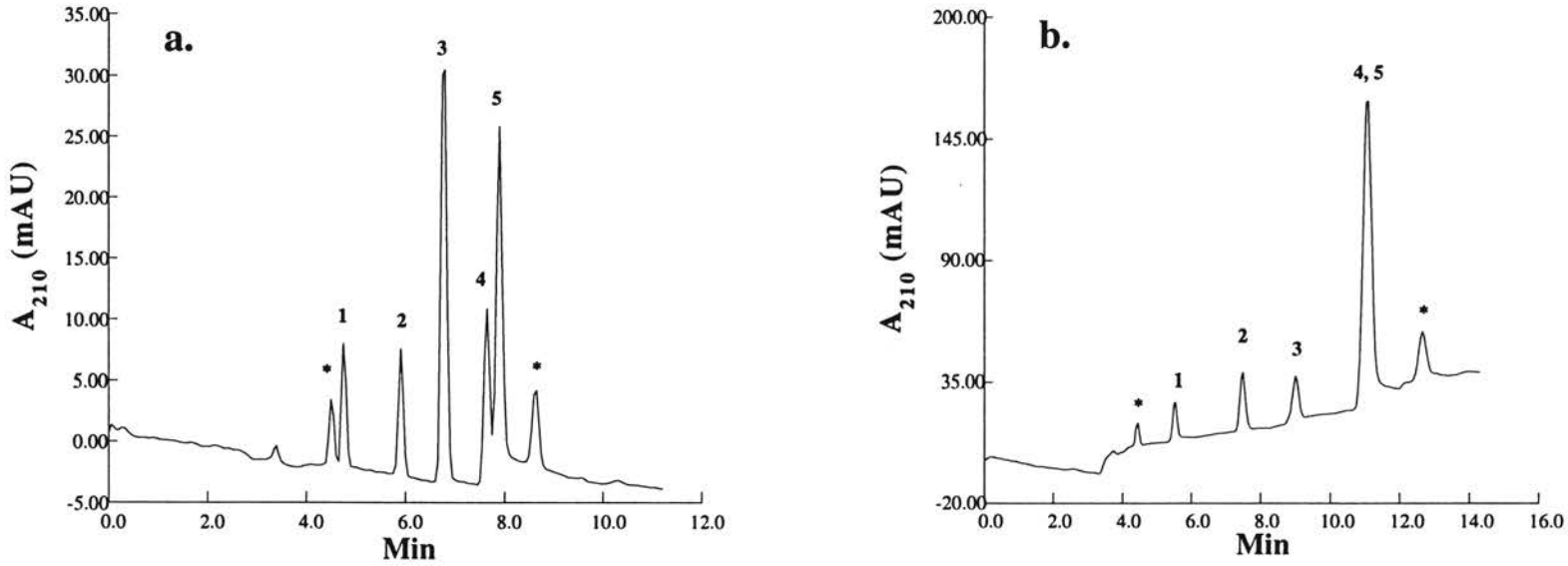

Figure 9. Electrochromatogram of a mixure of 5 herbicides. Mobile phases, 40% (v/v) of 5 mM sodium phosphate, pH 6.0, 60% (v/v) acetonitrile in (a), and 50% (v/v) of 5 mM sodium phosphate, pH 6.0, 50% (v/v) acetonitrile in (b); ODAS column with TAC coated wall packed under 3000 psi. Solutes: (1) aldicarb, (2) prometon, (3) propazine, (4) prometryne, and (5) diazinon. *: impurities from diazinon sample. Detection wavelength, 210 nm. Other conditions are the same as in Fig. 3.

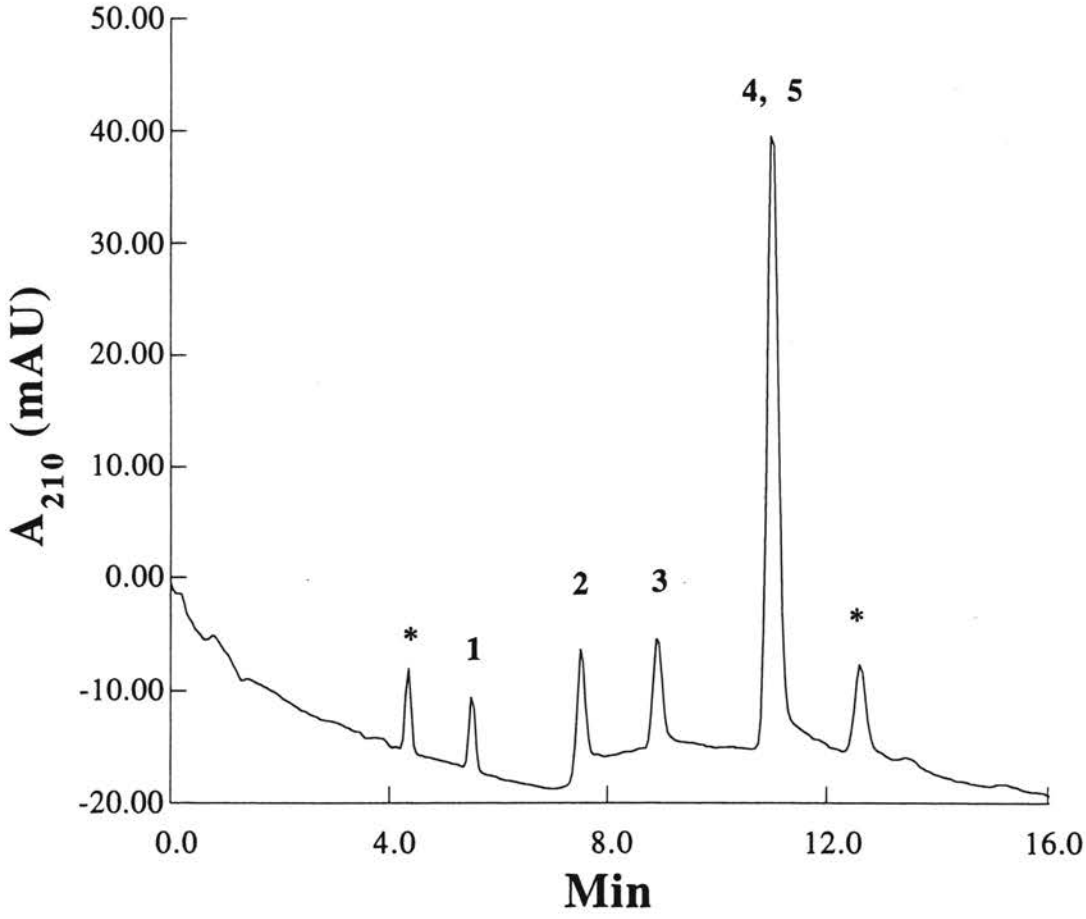

Figure 10. Electrochromatogram of a mixure of 5 herbicides. Mobile phases, 50% (v/v) of 5 mM sodium phosphate, pH 3.0, 50% (v/v) acetonitrile. Other conditions are the same as in Fig. 9.

lower voltage, since slower EOF velocity promotes on-line sample preconcentration without provoking bubble formation. For the neutral analytes investigated in this study, electrokinetic injection will not bring about discriminative introduction among the analytes in the sample.

A representative mixture of aldicarb, propazine, diazinon, and neburon was selected for this study. A mobile phase composed of 20% (v/v) of 5 mM sodium phosphate buffer at pH 6.0 and 80% (v/v) acetonitrile was found to yield fast and base-line resolution. For the ODAS stationary phase, detection limit without water plug was determined to be 10^{-5} M, as shown in Fig. 11a. Then, at the same running conditions, water was injected with varying duration before injecting the dilute sample in order to vary the lengths of the water plug. Signal intensities in terms of the peak area multiplied by baseline band width in seconds (mAU x seconds) were recorded to compare the effect of the length of the injected water plug. Water plugs were injected at -1 kV for 30, 60, 90, and 120 seconds, which corresponded to lengths of 1.9 mm, 3.8 mm, 5.6 mm, and 7.5 mm, respectively. Figure 12 summarizes the signal intensities of diazinon in this series. With the introduction of a water plug, the signal intensity increased, to a maximum, and then declined. For diazinon, signal intensity may be intensified by nearly four times with a water plug than without a water plug. Figure 11b illustrates such a difference. With the introduction of water plug injected at -1 kV for 90 seconds (which corresponds to a water plug length of approximately 5 mm), detection limit at this mobile phase condition (i.e., 20:80 (v/v) = phosphate: acetonitrile) was determined to be 3 x 10^{-6} M.

The effect of water plug in enhancing sample enrichment is apparently due to the difference of analytes partitioning in water and acetonitrile. Before water and sample injection, the surface of the stationary phase was equilibrated with the mobile phase that

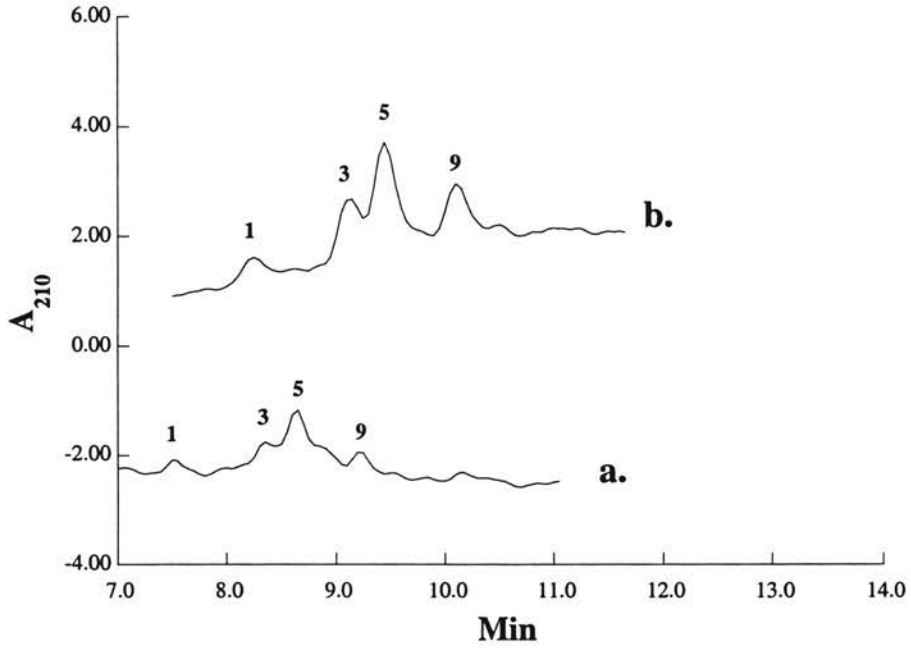

Figure 11. Effect of injecting a water plug on on-line sample enrichment as illustrated by electrochromatograms. Mobile phases, 20% (v/v) of 5 mM sodium phosphate, pH 6.0, 80% (v/v) acetonitrile. Injection modes: electrokinetic sample injection for 180 seconds at -1 kV in (a); electrokinetic injection of water for 90 seconds at -1 kV, followed by sample injection for 180 seconds at -1 kV in (b). Sample concentration was 1 x 10^{-5} M for each of aldicarb (1), propazine (3), diazinon (5), and neburon (9). Detection wavelength, 210 nm. Other conditions are the same as in Fig. 3.

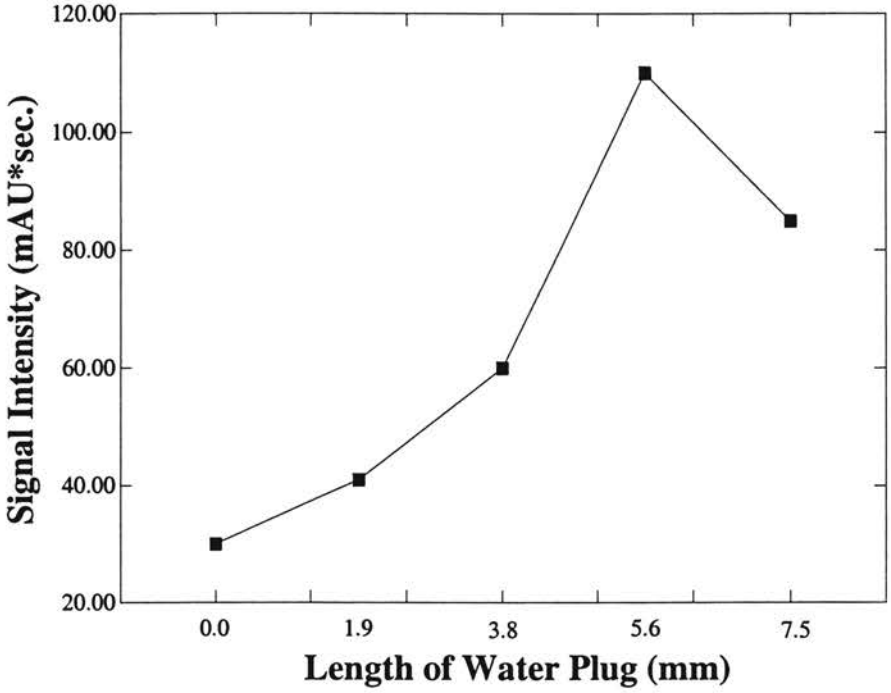

Figure 12. Effect of the length of the injected water plug on on-line sample enrichment as illustrated by changes in signal intensities. Water plug injection, voltage and sample injection are otherwise the same as in Fig. 11b.

had high content of acetonitrile (80% v/v). The injected water would dilute the content of the acetonitrile at the inlet end, creating a zone of higher retentivity on the surface of the stationary phase ahead of the analytes, which will facilitate the zone concentrating of analytes when the sample is subjected to CEC after injection. However, prolonged water injection could reach a point where zone focusing begins to deteriorate due to the decreased EOF, and could ultimately cause bubble formation. In general, the retentivity of the stationary phase plays a major role in on-line preconcentration. Solvents that enforce this retentivity would help to intensify the on-line sample enrichment and in turn allow the detection of dilute samples.

With this rationale, sample enrichment in the presence of mobile phases having 30% and 40% (v/v) of 5 mM sodium phosphate at pH 6.0 were also conducted. The results are illustrated in Fig. 13. As a comparison, mobile phases with aqueous content of 20%, 30%, and 40% (v/v) were found to allow the detection of 3×10^{-6} M, 1×10^{-6} M, and 7×10^{-7} M, respectively. In this case, higher aqueous content has further increased the retentivity of the stationary phase. It is therefore concluded that on-line sample enrichment can be facilitated with a preceding injection of retentivity enforcing solvent, and with mobile phase having higher content of the retentivity enforcing solvent.

Conclusions

The novel ODAS stationary phase proved useful in the CEC separation of neutral pesticides. Due to its net positive charge, the ODAS exhibited an anodic EOF, the magnitude of which was pH dependent. When compared with a partially modified ODS which had a net negative charge and generated a cathodic EOF, the ODAS yielded less

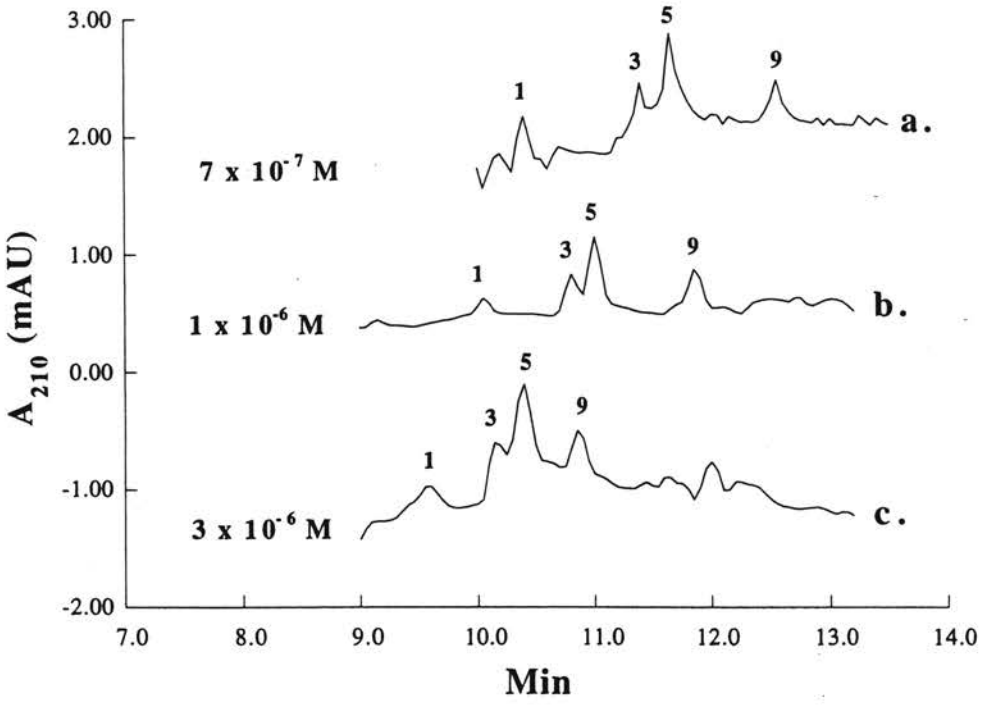

Figure 13. Sample enrichments obtained with mobile phases having different aqueous content. Percentage of 5 mM sodium phosphate at pH 6.0 in the mobile phases were: (a) 40%; (b) 30%, and (c) 20%. Injection modes and other conditions same as in Fig. 11b.

retention toward hydrophobic solutes (e.g., alkylbenzenes and herbicides) and consequently decreased their selectivity factors. The decreased retentivity of the ODAS stationary phase has also led to limited on-line sample enrichment for dilute samples. However, the ODAS column exhibited normal reversed-phase behavior which was shown by the dependence of the retention of hydrophobic solutes on the organic modifier content in the mobile phase. In light of this, the full potential of the ODAS is yet to be exploited in the CEC separation of a wider range of compounds. Such further investigation will shed more light on the electrochromatographic behavior of the new ODAS stationary phase.

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