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**CHARACTERIZATION OF ETCHED SILICA COLUMN FOR
CAPILLARY ELECTROCHROMATOGRAPHY**

A Thesis

Presented to

The Faculty of the Department of Chemistry

San Jose State University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

by

Urmi Parkar

May 1998

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ABSTRACT

Characterization of Etched Silica Column for Capillary Electrochromatography

Capillary electrochromatography (CEC) is an important analytical tool that is widely used in chemical separations. A new open tubular approach to CEC was investigated which overcomes most of the problems encountered in the packed capillary format. In this new format, the inner walls of capillaries are etched in order to increase the surface area and then an organic moiety is attached to the etched surface by a silanization-hydrosilation reaction scheme. The organic moiety used in this research was 1-octadecene (C-18) and the performance of this type of capillaries was compared to that of the bare capillaries.

Various mixtures including drugs and proteins were separated using three different types of capillaries. The effects of several parameters on the performance of these capillaries was also studied. Moreover, electroosmotic flows in different types of capillaries were measured and the effect of experimental variables like buffer pH and voltage were investigated.

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1. INTRODUCTION

1.1 Background

Capillary electrochromatography (CEC) is one of the four capillary electroseparation techniques that are being used currently and has the potential of becoming a powerful tool in the analysis of complex mixtures. CEC is a hybrid of two techniques: high performance liquid chromatography (HPLC) and capillary electrophoresis (CE). The separation of solutes is dependent on solute-bonded phase interactions as in HPLC, while the movement of solutes through the capillary takes place by electroosmosis and/or electrophoretic mobility as in CE.

In CE, separation depends on the electrophoretic mobilities of the compounds which is a function of their charge-to-size ratios. Due to this, only charged compounds are separated and the neutral compounds migrate with the electroosmotic flow (EOF) since they do not possess any charge. In contrast, CEC can separate both charged and neutral compounds since migration of compounds is based on their electrophoretic mobilities as well as the interactions of the analytes with the stationary phase. Neutral species can interact with the stationary phase causing them to separate from each other. CEC is therefore preferred in certain cases as all types of compounds can be separated by a single technique which is not possible by using CE as it can only separate charged compounds.

CEC can be performed in open tubes as well as in capillaries packed with conventional packing materials that are used in HPLC. CEC exhibits all of the advantages of a miniaturized separation technique including low sample and solvent volume requirements, high separation efficiencies and low operational costs. Certain

biomolecules like proteins can be denatured or decomposed due to high concentration of organic solvents and high pressures that are used in HPLC. but such a problem is not encountered in CEC. Due to all of these reasons, CEC is evolving as an important and convenient technique in the analysis of all types of chemicals and biomolecules.

1.1.1 History of CEC

The first electrochromatographic experiments were described by Pretorius more than twenty years ago [1]. This field was further developed by Jorgenson and Lukacs some years later [2]. The earlier CEC experiments were carried out using capillary tubes that were packed with stationary phases similar to those used in HPLC. But there are problems associated with CEC in the packed capillary format and these are, bubble formation around the packing material [3-5], packing problems [6,7], the requirement of frits [8,9] and Joule heating. A new open tubular format for carrying out electrochromatographic experiments has been introduced that overcomes most of the problems associated with CEC in the packed capillary format [10,11].

1.1.2 New open tubular format

In this format the inner walls of capillaries are etched [12] and then an organic moiety is attached to the roughened surface. Higher efficiencies (narrow peaks) are observed in this new format compared to those observed in packed capillary columns. Various reactions have been used for the modification of the capillary wall. Some of the reactions that have proven successful for the purpose of bonding a chemical species to

silica surfaces include esterification, organosilanization and chlorination-organometallation [13]. All these approaches have a few disadvantages to them that are not exhibited by the silanization-hydrosilation reaction scheme. This process involves two steps [14-16]. The first step is the reaction of the surface silanol group with triethoxysilane to form a hydride intermediate. This hydride intermediate is then reacted with an organic species containing an unsaturated site like 1-octadecene (C-18) or 7-octene-1,2-diol (diol) in the presence of a suitable catalyst to form a stable Si-C linkage. This reaction scheme is represented below.

Since, there is a single point of attachment for the organic species, the stationary phase formed here resembles the monomeric phase that is formed by all other reactions (mentioned above) except organosilanization (with three reactive sites).

1.2 Principles of CE and CEC

CE and CEC are both performed using the same equipment. A schematic representation of a CE/CEC system is given in Figure 1 [17]. The capillary, source vial and destination vials are filled with an aqueous buffer solution. The capillary inlet is first placed into the sample vial in order to transfer the sample into the capillary. The capillary inlet is then placed in the source vial and an electric field is applied. The solutes start migrating through the capillary and eventually pass by the detector. The output is then sent to an integrator or computer. The output is displayed in the form of a plot of detector response

versus time, which is called an electropherogram and is represented in Figure 2. The sample components migrate through the capillary at varying rates and hence, pass by the detector at different times which results in the separation of solutes. These separated solutes are represented as peaks with specific migration times (t_1 and t_2 as represented in Figure 2).

Figure 1. Schematic representation of CE/CEC system

Figure. 2 Electropherogram

1.2.1 Electrophoresis

Electrophoresis can be defined as the movement of electrically charged particles in a conductive medium, under the influence of an electric field. Figure 3 shows a schematic representation of electrophoresis [17]. A sample containing a mixture of neutral and electrically charged molecules of different charges and sizes is placed in the anodic end of the capillary and an electric field is applied across the capillary. Due to the electric field, the electrically charged molecules from the sample will start migrating through the capillary in a particular direction and at specific rates depending on their charge-to-size ratios.

Positively charged ions (cations) will migrate towards the negatively charged electrode (cathode) and negatively charged ions (anions) will migrate towards the positively charged electrode (anode). A smaller sized ion will migrate faster than a larger ion of the same charge. An ion with a higher charge will migrate faster than the ion of the same size having a lower charge. Neutral molecules migrate along with the buffer through the capillary due to electroosmosis, which is discussed in the next section. All neutral molecules move at the same rate as the electroosmotic flow (EOF) and do not get separated. Cations move faster than EOF as they get pulled towards the cathode. Anions move slower than the EOF as they get pulled in the opposite direction towards the anode. Hence, the order in which the ions elute is cations, neutral and then anions. The order of migration of ions due to EOF and electrophoretic mobilities can be seen from Figure 4 given below. Moreover, as can be seen from Figure 3, the rates of movement of anions towards the cathode is opposite to that of the cations.

Figure 3. Schematic representation of electrophoresis

Figure 4. Electropherogram showing the order of migration

1.2.2 Electrochromatography

Electrochromatography is carried out in almost the same way as electrophoresis. The main difference is that the capillary that is used in this case is one whose inner surface has been etched and modified or is packed with chemically modified silica particles. When an electric field is applied, the molecules start migrating in different directions and at specific rates depending on their charge-to-size ratios as well as on their partitioning between the stationary and mobile phases. This process in the case of an etched capillary is represented in Figure 5. Neutral molecules that are carried through the capillary by electroosmosis interact with the bonded ligand according to various mechanisms and to variable degrees which in turn leads to their migration at different rates thereby, causing them to separate from each other. Hence, neutral molecules that are not separated by electrophoresis can in fact, be separated by electrochromatography. The order in which the ions elute is generally cations, neutrals and then anions as in electrophoresis. The order of migration of the ions due to EOF, electrophoretic mobilities and solute-bonded phase interactions can be seen in Figure 6 . As can be seen from this figure, the neutral molecules can be separated from each other which cannot be accomplished in electrophoresis as shown in Figure 3.

Figure 5. Schematic representation of electrochromatography

Figure 6. Electrochromatogram showing the order of migration

1.2.3 Electroosmotic flow (EOF)

In CE and CEC systems, buffer solution moves through the capillary under the influence of an electric field. This phenomenon is called electroosmotic flow. The normal direction of EOF is from the anodic end of the capillary towards the cathode. The process of EOF in a capillary is represented in Figure 7 [17].

When a buffer is introduced into the capillary, the inner surface acquires a charge. The silanol (Si-OH) groups on the inner surface of fused silica capillaries become ionized to negatively charged silanoate (Si-O⁻) groups at pH ≥ 3 . These negatively charged Si-O⁻ groups attract positively charged ions from the buffer and a layer of cations is formed here. This layer is tightly held by the Si-O⁻ groups and is called the fixed layer. The density of these cations from the inner layer is not sufficient to neutralize all the negative charges at the surface and so another layer of cations is formed. This second layer of cations is far away from the Si-O⁻ groups, is not tightly held and is referred to as the mobile layer. These two layers (fixed and mobile) of cations form a diffuse double layer. When an electric field is applied, the mobile layer of cations gets pulled towards the cathode. Since, these cations are solvated they drag the bulk buffer with them thus causing EOF.

An electrical imbalance is created at the plane between the fixed and the mobile layers which is the potential difference across the layers and is called the “zeta potential”. The EOF is directly proportional to the zeta potential. This zeta potential (ζ) can be calculated by the following equation:

$$\zeta = 4\pi\delta e/\epsilon \quad (1)$$

where δ = thickness of the diffuse double layer, e = charge per unit surface area and ϵ = dielectric constant of buffer.

1.2.3a Electroosmotic flow velocity

The EOF velocity (v_{eof}) is given by the following equation:

$$v_{eof} = \epsilon \zeta E / 4 \pi \eta \quad (2)$$

where ϵ = dielectric constant of the buffer, ζ = zeta potential,

E = applied electric field (volts/cm) and η = viscosity of buffer.

1.2.3b Electroosmotic flow mobility

EOF mobility (μ_{eof}) is given by the following equation:

$$\mu_{eof} = \epsilon \zeta / 4 \pi \eta \quad (3)$$

where ϵ = dielectric constant of the buffer, ζ = zeta potential

and η = viscosity of buffer.

Figure 7. Representation of electroosmotic flow in a capillary

1.2.3c Methods for measurement of EOF

EOF plays a major role in electrophoretic and electrochromatographic separations and hence, its measurement is important for the complete characterization of a system. Various methods have been developed over the years for measuring EOF in different types of capillaries. One of the methods, known as the traditional method, involves the use of a neutral compound that is carried through the capillary by the buffer after application of voltage. The migration time of the neutral compound is recorded and EOF velocity is calculated using the equation: $v_{\text{eof}} = l/t_m$

where l = effective length of capillary,

and t_m = migration time.

From this value, EOF mobility is calculated by using $\mu_{\text{eof}} = v_{\text{eof}}/E$ where

E = electric field = voltage/total length.

This method has certain disadvantages like the requirement for true neutrality and inertness of the compound towards the wall of the capillary. Moreover, when modified capillaries are being used, EOF is very low or even completely eliminated. In this case, it takes a long time for the neutral marker to reach the detector, if at all. Thus, measuring EOF using this method is very time consuming if it is even possible.

Altria and Simpson proposed another method called the gravimetric method for measuring EOF in various types of capillaries [18]. This method was carried out by weighing the amount of buffer coming out of a capillary after the application of voltage for a specific period of time. This is basically an off-line method that requires the use of a

balance and there is also a possibility of solvent evaporation that poses major problems in true measurements.

A new method has been proposed by Sandoval and Chen [19] that has overcome most of the problems associated with the older methods. This method is very fast and can be performed in a simple and on-line fashion. This new accelerated method is described in the Experimental section and the EOF measurements obtained using this method are discussed in the Results and Discussion section.

1.3 Types of sample injection

In CE/CEC systems, samples can be introduced into a capillary by hydrodynamic or electrokinetic injection. These two methods of injections are discussed below.

1.3a Hydrodynamic injection

This process can be done using pressure or gravity. Pressure injections involve placing the inlet of a capillary into the sample vial and then applying pressure to the vial. Another approach to pressure injections involves placing the inlet of the capillary in the sample vial and then applying a vacuum to the destination vial. This type of injection is represented in Figure 8a. In this case, care has to be taken to maintain the same liquid levels in the sample and destination vials in order to minimize siphoning of the sample or buffer during the injection process. Siphoning of the sample or buffer can cause unexpected variations in injection volumes that can lead to lack of reproducibility in peak areas and peak heights. Moreover, since the injection volume is dependent on the applied pressure or vacuum, it is important to maintain a constant pressure or vacuum in order to obtain reproducible results. Gravity injections can be made by placing the inlet of the capillary into the sample vial which is then raised above the level of the destination vial in order to allow the sample to siphon into the capillary.

1.3b Electrokinetic injection

This method involves placing the inlet of the capillary in the sample vial with the anode and then applying voltage for a precise period of time. This type of injection is represented in Figure 9a. Due to the application of voltage, the solutes (ions) start

migrating into the capillary due to EOF and electrophoretic mobility. The amount of sample injected into the capillary depends on the rate of EOF, electrophoretic mobilities of solute ions, applied voltage, solute concentration and capillary dimensions. Since the amount of solutes injected into the capillary depends on the electrophoretic mobilities, a sample bias is introduced with electrokinetic injections. Larger quantities of solutes (ions) that have high mobilities are injected into the capillary as compared to lower quantities of solutes (ions) with lower mobilities. Representation of an electropherogram obtained from a sample that is injected electrokinetically is represented in Figure 9b. As can be seen from this figure, smaller peaks are obtained from later eluting solutes that have lower mobilities. The electropherogram obtained from hydrodynamic injection of the same sample mixture is represented in Figure 8b. As can be seen from this figure, there is no sampling bias in this case and hence all the peaks obtained exhibit same peak areas and peak heights.

Figure 8a. Hydrodynamic injection

Figure 8b. Electropherogram obtained from hydrodynamic injection of sample

Electrokinetic

Figure 9a. Electrokinetic injection

Figure 9b. Electropherogram obtained from electrokinetic injection of sample

1.4 Some important parameters useful in the characterization of electrophoretic and electrochromatographic systems

1.4a Efficiency (N)

The efficiency is expressed as the number of theoretical plates and is related to peak width. It is given by, $N = 16(t/w)^2$ or $N = 5.54(t/w_{1/2})^2$ (4)

where, t = migration or retention time

w = peak width measured at the base of the peak

$w_{1/2}$ = peak width measured at half peak height.

As can be seen from the above formula, a narrower peak leads to higher efficiencies and therefore, better separation of closely migrating analytes is obtained.

1.4b Separation factor (α)

This parameter is a measure of the extent of separation and depends on the distance between the peaks. It is given by, $\alpha = t_2/t_1$ (5)

where, t_2 and t_1 = migration or retention times of

the second and first eluting compounds respectively.

1.4c Peak symmetry

This factor is a measure of the peak symmetry calculated at 10% peak height. It is given by, peak symmetry = A/B where, A and B are as represented in figure 10. Thus, the closer the number is to 1, the more symmetric is the peak.

Figure 10. Representation of peak width, peak height and peak symmetry.

1.5 Comparison of CEC to CE and HPLC

1.5a Comparison of CEC to CE

CE can be used in the separation of charged compounds but, it cannot be used in the analysis of neutral compounds. On the other hand, CEC can be used in the analysis of both charged and neutral compounds. The reasons for these statements have been discussed in section 1.2.2. CE has higher efficiencies than CEC because the peaks obtained in an electropherogram are much narrower than those obtained in electrochromatograms [20]. Sample and reagent volume requirements are the same in both CE and CEC. In all other facets, CEC and CE are very similar to each other.

1.5b Comparison of CEC to HPLC

HPLC can be used in the analysis of almost all of those compounds that can be analyzed using CEC. An important advantage of CEC over HPLC is the relatively small amounts of sample and reagents (buffers) used in the analysis of various compounds. Only a few nanoliters are required in CEC because of the small size of the capillaries, as opposed to a few microliters of sample required for analysis by HPLC. Moreover, in HPLC, typical flow rates are in the order of 1-2 mL/min that require 500 to 1000 mL of mobile phase per day whereas, only a few mL's of buffer are required per day in CEC.

Another advantage of CEC over HPLC is that it has higher efficiencies than HPLC. Narrower peaks result from the flat profile of EOF compared to pumped or laminar flow in HPLC [21]. EOF and pumped flow profiles are represented in Figures 11a and 11b respectively. In CEC systems, because of frictional drag, EOF at the capillary

wall is relatively slower than that in the rest of the capillary. This reduction in EOF is negligible since the area at the wall is very small and hence, it subtracts very little from the flat profile. Due to this flat profile, all of the sample components, regardless of their position in the capillary, are governed by the same velocity component caused by EOF and therefore, elute as narrow bands. Hence, the efficiencies are relatively higher than those seen in HPLC. On the other hand, due to the pumped flow in HPLC, the solutes present in the center of a tube move faster than those present near the wall giving rise to broader bands. Hence, the efficiencies that are a measure of the peak width are relatively lower than those of CEC.

Figure 11a and 11b. EOF and pumped flow profiles

1.6 Goal of the present research

From the above discussion it can be seen that CEC can provide separation of neutral compounds which is not offered by CE. One of the goals of this research was to investigate the ability of CEC in separating charged as well as neutral compounds. Several capillaries were etched and modified according to the new open tubular approach to CEC. The etching was done at two different conditions and the effect of this etching process on the surface morphology was studied. The etched capillaries were then modified using the silanization-hydrosilation reaction scheme as described earlier and the chemical species bonded to the surface in this case was C-18.

The goal was to investigate the separating abilities of these etched-modified capillaries and compare the separations obtained on these capillaries to those obtained by bare capillaries. Various mixtures, proteins, drugs and antidepressants were analyzed using bare and etched-modified capillaries. A detailed study was carried out to see if the etching and modification processes improved the separation process. Another goal was to measure EOF in different types of capillaries and also to study the effect of various parameters on EOF and various separations.

2. EXPERIMENTAL

2.1 Reagents and materials used

Deionized water for rinsing the capillaries and preparing all the buffers was obtained from a Milli-Q water purification system. Three buffers were prepared:

1. pH 2.14 buffer: Composition was 0.3 M phosphatate (phosphate from 85% H_3PO_4) + 0.19 M Tris [tris(hydroxymethyl)aminomethane].
2. pH 3.0 buffer: Composition was 0.3 M Citric acid + 0.25 M β -Alanine.
3. pH 4.41 buffer: Composition was 300 mM acetic acid + 375 mM GABA (γ -aminobutyric acid).

Acetic acid was purchased from J. T. Baker Chemical Co. (Phillipsburg, NJ) and β -Alanine and GABA were purchased from Sigma Chemical Co. (St. Louis, MO).

Methanol, acetone, diethyl ether were also obtained from Sigma Chemical Co. Ammonia was purchased from J. T. Baker Chemical Co. Tetrahydrofuran (THF) and toluene were used as washing solvents in the modification of the capillaries and were bought from J. T. Baker Chemical Co. and EM Industries Inc. respectively. Triethoxysilane (TES) and dioxane were used to prepare the hydride intermediate and were purchased from Aldrich Co. Inc. (Milwaukee, WI). 1-octadecene (C-18) which was attached to the inner walls of the capillaries was obtained from Aldrich Co. Inc. Hexachloroplatinic acid and 2-propanol were used to prepare Spiers catalyst and were bought from Aldrich Co. Inc. and J. T. Baker Chemical Co. respectively.

Dimethyl sulfoxide (DMSO) from Sigma Chemical Co. was used as a marker for EOF measurements. Antidepressants that were analyzed using bare and modified capillaries were purchased from Aldrich Co. Inc. and are listed in Table 1. A drug mixture containing five components was obtained from Sigma Chemical Co. and was separated using a C-18 capillary. These components are listed in Table 2. Amino acids, lysozymes, angiotensins, Cytochrome C, Ribonuclease A, and Myoglobin were obtained from Sigma Chemical Company. These amino acids and proteins are listed in Tables 3 and 4 respectively.

Melatonin, serotonin and tryptamine were purchased from Aldrich Co. Inc. and were used without further purification. The structures of melatonin, serotonin and tryptamine are represented in Table 5. Fused silica capillaries with a 50 μm I. D. were used for all the experiments. These capillaries were purchased from Polymicro Technologies Inc.(Phoenix, Az).

2.2 Instrumentation

A gas chromatography (GC) oven was used for controlling the reaction temperatures during the etching and modification processes. The GC oven was modified in order to accomodate more than one capillary at a time through its inlet and outlet for etching. This modified GC oven is shown in Figure 12. Heavy walled glass tubes were used as reservoirs for holding solvents and reagents used in the etching and modification of the inner walls of the capillaries. These glass tubes had swagelok fittings to facilitate the application of nitrogen pressure that was required to force the solvents and reagents

from one end of the capillary to the other. Small glass vials were used to collect the solvents and reagents that were forced out of the ends of the capillaries.

All the analyses and separations were done using two HPCE instruments. Part of the experimental work was done on a Spectra PHORESIS 500Tm system and the remainder of the experiments were done using an Applied Biosystems 270A-HT Capillary Electrophoresis System.

Table 1. Antidepressants used in this research

Antidepressants	Structures
Doxepin
Clomipramine
Desipramine
Nortriptyline
DL-propanolol
Imipramine

Table 2. Components of drug mixture

Drug name	Molecular formula
Barbital	$C_8H_{12}N_2O_3$
Butethal	$C_{10}H_{16}N_2O_3$
Amobarbital Na^+	$C_{11}H_{17}N_2O_3Na$
Pentobarbital	$C_{11}H_{13}N_2O_3$
Secobarbital	$C_{12}H_{17}N_2O_3$

Table 3. Types of amino acids used

Amino acids	Structures
Asparagine	$ \begin{array}{c} \text{COO}^- \\ \\ \text{H}-\text{C}-\text{CH}_2-\text{C} \\ \quad \quad \quad // \\ \text{NH}_3^+ \quad \quad \quad \text{O} \\ \quad \quad \quad \quad \quad \quad \backslash \\ \quad \quad \quad \quad \quad \quad \text{NH}_2 \end{array} $
Glutamine	$ \begin{array}{c} \text{COO}^- \\ \\ \text{H}-\text{C}-\text{CH}_2-\text{CH}_2-\text{C} \\ \quad \quad \quad \quad // \\ \text{NH}_3^+ \quad \quad \quad \text{O} \\ \quad \quad \quad \quad \quad \quad \backslash \\ \quad \quad \quad \quad \quad \quad \text{NH}_2 \end{array} $
Valine	$ \begin{array}{c} \text{COO}^- \quad \text{CH}_3 \\ \quad \quad / \\ \text{H}-\text{C}-\text{CH} \\ \quad \quad \backslash \\ \text{NH}_3^+ \quad \text{CH}_3 \end{array} $

Table 4. Proteins used in this work

Protein	Organism	Tissue	Mol. wt.(k Da)
Lysozyme	Turkey	Egg white	13.9
Lysozyme	Chicken	Egg	13.9
Cytochrome C	Horse	Heart	13.4
Myoglobin	Horse	Skeletal muscle	16.9
Ribonuclease A	Bovine	Pancreas	13.8

Table 5. Structures of melatonin, serotonin and tryptamine

Name	Structure
Melatonin	<chem>CC(=O)NCCc1c[nH]c2cc(OC)ccc12</chem>
Serotonin	<chem>NCCc1c[nH]c2cc(O)ccc12</chem>
Tryptamine	<chem>NCCc1c[nH]c2ccccc12</chem>

Figure 12. Modified GC oven

2.3 Etching and modification of capillaries

2.3.1 Etching of the inner walls of a 50 μm I.D capillary

Six bare 50 μm I.D capillaries were filled with concentrated HCl and after sealing the two ends, they were heated overnight at 80⁰C. After opening the ends, the capillaries were washed successively with distilled water, acetone and diethyl ether. The capillaries were then dried with nitrogen for one hour. A 5% solution of ammonium hydrogen difluoride in methanol was prepared and stirred overnight. The capillaries were then filled with the ammonium hydrogen difluoride solution and left to stand for one hour. Nitrogen was passed through the capillaries for 10 minutes to remove the methanol from the capillaries. Five of the capillaries were then sealed and heated at 300⁰C for 2 hours and then at 400⁰C for 2 hours. One capillary was sealed and heated at 400⁰C for 4 hours. After this process the capillaries were washed with methanol and dried with nitrogen for half an hour.

2.3.2 Preconditioning of capillaries

The capillaries were treated with 6mM ammonia for 20 hours at room temperature. After this they were rinsed with deionized water for 1 hour followed by flushing with a 0.1 M HCl solution for 4 hours. They were again washed with deionized water for 2 hours and then dried with nitrogen at 100⁰C for at least 20 hours.

2.3.3 Modification of capillaries

2.3.3a Formation of hydride intermediate

The capillaries were washed with dioxane and then treated with TES solution (in dioxane) at 90°C for 90 minutes. After this process, the hydride modified capillaries were first washed with THF/water (50:50) for 2 hours and then with THF for two hours at room temperature. The capillaries were then dried with nitrogen for one-half hour.

2.3.3b Attachment of 1-octadecene

The hydride modified capillaries were first rinsed with dry toluene and then flushed with the 1-octadecene solution at 100°C for 45 hours. The 1-octadecene solution was prepared by mixing 2 ml of 1-octadecene with 70 µl of 10 mM Spiers catalyst (hexachloroplatinic acid in 2-propanol) and heating the mixture at 60-70°C for 1 hour. After flushing the capillaries with the 1-octadecene solution they were first washed with toluene and then with THF for one hour. At the end of this process, the capillaries were dried with nitrogen overnight at 100°C.

2.4 Electrophoresis and electrochromatography experiments

Various experiments were performed using bare and etched-modified capillaries. The results of these experiments were compared to determine which capillary gave better performance. The ability of C-18 capillaries to separate a variety of mixtures, drugs and proteins was also investigated.

2.5 Study of the effects of different parameters on EOF and separation

Several experiments were performed by changing various experimental conditions and parameters. The effects of buffer pH on EOF, migration times of solutes and separation efficiencies were studied by using buffers with a pH of 2.14, 3.0 and 4.41. Similar experiments were performed by varying the voltage between 5 kV to 30 kV. Experiments were also performed at several dilutions of pH = 4.41 buffer ranging from 1:5 to 1:30.

2.6 Electroosmotic flow measurements

EOF was measured using a new method which is described here. First the capillary was flushed with the buffer (pH = 4.41). After this flushing cycle, a marker was injected. The marker that was used in these experiments was DMSO. The electric field was then applied for a specific period of time to electroosmotically move the marker band along with a dragged plug of electrolyte over a short distance of the capillary. A second injection of DMSO was then made to mark the end of the electrolyte plug. After this, vacuum was applied to take the marker-sandwiched electrolyte plug through the capillary to the detection window. The migration times of the two marker bands were recorded and the EOF was measured using the following equation:

$$\mu_{\text{eof}} = LI/Vt_v * (1-t_1/t_2) \quad (6)$$

where, L = total length of capillary, l = effective length of capillary,

V = applied voltage, t_v = time for which voltage was applied,

t_1 and t_2 = migration times of the two marker bands.

The EOF's in bare, etched-bare, C-18 and diol capillaries were measured using this method. The calculated EOF was then plotted as a function of the applied electric field. These graphs were then used to compare the EOF in all the above mentioned types of capillaries.

3. RESULTS AND DISCUSSION

3.1 Etching of the capillaries

The etching process causes a large increase in the surface area. It also produces finger-like particles that extend radially from the surface. This change in the surface morphology was studied with the help of scanning electron microscopy (SEM) and the results of this study are shown in Figure 13a, b and c [20]. Figure 13a shows the surface of a bare capillary which appears to be smooth at this magnification. Figure 13b shows the inner surface of a capillary that has been etched at 300°C for 3 hours. In this figure protruding particles are seen that are quite large leading to an irregular surface. Figure 13c shows the inner surface of a capillary that has been etched at 400°C for 3 hours. This figure shows that the inner capillary surface becomes more uniform with higher etching temperatures. Previous studies have shown that in the early stages of the etching process, larger spikes are seen protruding out from the surface, but later the surface becomes more uniform.

In this particular research project, two different etching conditions were used. Five capillaries were etched at 300°C for 2 hours followed by 400°C for 2 hours and one capillary was etched at 400°C for 4 hours. Initially the idea was to etch the first set of capillaries at 300°C for 2 hours and compare the surfaces obtained from this set of conditions to those obtained from etching the remaining capillary at 400°C for 2 hours. However, the capillaries were blocked after etching at 300°C for 2 hours and therefore had to be etched once more using a higher temperature in order to get them to flow. The

other capillary was also blocked after etching at 400°C for 2 hours and hence, it was also etched again at 400°C for 2 more hours. Since both sets of capillaries were etched at 400°C for a long time, it is hard to conclude which surface will be more uniform and stable.

The mechanical stability of these etched surfaces was also tested by vibrating the capillaries for 30 minutes. It was observed that the large spikes (finger-like protrusions) were severely damaged due to the mechanical stress of vibration. However, the more uniform surfaces that are produced at higher temperatures and at a later time in the etching process were not damaged due to the mechanical stress of vibration.

a

b

c

Figure 13. SEM pictures of (a) bare capillary, (b) capillary etched at 300°C for 3 hours and (c) capillary etched at 400°C for 3 hours.

3.2 Effect of etching and modification of capillary surfaces on retention of solutes and peak shape

Experiments were performed using Asparagine as a test solute to evaluate the effect of etching and modification on the retention time of solutes and the peak shape. Asparagine (Asn) is a basic amino acid. All amino acids other than tyrosine, tryptophan and phenylalanine do not contain any UV absorbing chromophore (22) and hence it is not possible to use them directly. They have to be derivatized using dansyl chloride or phenylisothiocyanate before they can be analyzed. The phenylthiohydantoin (PTH) derivative of Asparagine (PTH-Asn) was used in this study.

Figures 14a and b show the electropherograms obtained on bare and C-18 capillaries respectively with PTH-Asn as the test solute. As can be seen from Figure 14a, PTH-Asn elutes in 9.69 minutes on a bare capillary as a narrow and symmetrical peak. When a similar run was carried out on a C-18 capillary under the same conditions of buffer and voltage, an increase in the retention time as well as the peak width was obtained which can be seen in Figure 14b. This result is obtained because the etching and modification processes reduce the number of free silanols on the surface causing a reduction in EOF in the C-18 capillary. Moreover, PTH-Asn took a longer time to migrate through the capillary due to the increased interactions with the C-18 attached to the etched surface and hence, eluted later on the C-18 capillary as compared to the bare capillary. The observed increase in the peak width also supports this fact since an increase in mass transfer effects causes a decrease in the efficiency. Similar results were obtained when PTH-Glutamine (PTH-Gln) was analyzed using bare and C-18 capillaries.

Figure 14. Elution of PTH-Asn on (a) bare capillary ($L = 56$ cm, $l = 30.7$ cm) and (b) C-18 modified etched capillary ($L = 50$ cm, $l = 25$ cm): Detection at 254 nm, $V = 30$ kV on bare capillary and $V = -30$ kV on C-18 capillary, Buffer: citric acid- β -alanine at pH 3.0.

3.3 Analysis of Amino acids

Several PTH and Dansyl derivatized amino acids were analyzed with a C-18 capillary. An interesting observation was made with these two types of amino acids. It was seen that Dansyl-Valine (Dansyl-Val) and other Dansyl-amino acids eluted in a reasonable time (within 10 minutes) with positive voltage. However, the PTH-amino acids did not elute with positive voltage even after 90 minutes. Instead, PTH-Asn and PTH-Gln were observed to elute with reversed polarity. They took around 26-30 minutes to elute with -30 kV which was much longer than Dansyl-amino acids. These results are shown in Figures 15a and b. The structures of Asparagine, Glutamine and Valine are shown in Table 3 of the Experimental section.

Previously, it was thought that the PTH group being more hydrophobic tends to stick to the walls and hence takes much longer than the Dansyl group to elute from a C-18 capillary. But, recently it has been suggested that trapped NH_4^+ or a species like H_2F^+ may be present at the capillary surface causing a change in the surface charge. Due to this change in the charge, the PTH-amino acids tend to migrate in the same direction as the EOF (which is in the opposite direction) causing them to be seen when polarity is reversed. This matter needs to be investigated further in order to make any solid conclusions about the behavior of PTH-amino acids on a C-18 capillary.

Figure 15. Elution of (a) Dansyl-Val and (b) PTH-Gln on a C-18 capillary ($L = 50$ cm, $l = 25$ cm): Detection at 254 nm, $V = 30$ kV for Dansyl-Val and $V = -30$ kV for PTH-Gln, Buffer: citric acid- β -alanine at pH = 3.0.

3.4 Analysis of Angiotensins

Angiotensins were analyzed using bare and C-18 capillaries. Angiotensins are proteins of clinical importance that are related to vasoconstriction [23]. An enzyme found in the lungs and plasma can convert Angiotensin I, which is a decapeptide present in the human body, to Angiotensin II by cleaving off two of its terminal carboxylic groups. The results of the separation of Angiotensins I and II obtained on a bare capillary and a C-18 capillary are represented in Figures 16a and b, respectively. As can be seen from Figure 16b, angiotensins were successfully separated on the C-18 capillary. The order of elution of these angiotensins was the same on both the capillaries with Angiotensin I eluting before Angiotensin II. The angiotensins eluted later on a C-18 capillary compared to that on the bare capillary due to the increased interactions between the angiotensins and the C-18 bonded to the surface.

3.5 Analysis of Antidepressants

Antidepressants are of great clinical importance and hence it is necessary to analyze them. A mixture of six antidepressants (listed in Table 1 of the Experimental section) was analyzed using bare and C-18 capillaries. These results are represented in Figures 17a and b, respectively. These figures show that only four out of six antidepressants could be separated on both the bare and C-18 capillaries. First, all the antidepressants were analyzed individually and their migration times were recorded. Then a mixture was prepared by mixing imipramine, clomipramine, doxepin, desipramine, dl-propanolol and nortryptiline in this particular sequence. Stepwise analysis was carried

out after adding each component. Three peaks were seen with the mixture of imipramine, clomipramine and doxepin confirming that these three were part of the total four components that were separated at the end. No extra peaks (other than the previous three) were seen after the addition of desipramine and dl-propanolol. However, an additional peak was seen after the addition of nortryptiline. Based on this observation and also on the individual migration times, it was concluded that imipramine, clomipramine, doxepin and nortryptiline were the four antidepressants that separated out from a mixture of six antidepressants.

Moreover, as can be seen from the figures (17a and b), the separation was faster on the bare capillary than on the C-18 capillary. However, the C-18 capillary gave a better separation than the bare capillary. The C-18 capillary shows a baseline separation for the first and last eluting components which is not seen on the bare capillary. The two middle components are also resolved a little better on the C-18 capillary compared to the bare capillary. These observations suggest that the antidepressants interact with the bonded C-18 and hence, take longer to elute on the C-18 capillary. At the same time, they separate out better on the C-18 column due to their hydrophobic interactions with the bonded C-18 group.

Figure 16. Separation of a mixture of Angiotensin I and Angiotensin II on (a) bare capillary (L = 56 cm, l = 30.7 cm) and (b) C-18 capillary (L = 50 cm, l = 25 cm): Detection at 254 nm, V = 30 kV, Buffer : citric acid- β -alanine at pH 3.0, Solutes: 1 = Angiotensin I, 2 = Angiotensin II.

Figure 17: Separation of antidepressants on (a) bare capillary (L = 56 cm, l = 30.7 cm) and (b) C-18 capillary (L = 50 cm, l = 25 cm): Detection at 210 nm, V = 30 kV, Methanol + Buffer (90:10) mixture, Buffer: phosphate-Tris at pH = 2.14, Solutes: 1 = imipramine, 2 = doxepin, 3 = nortriptyline and 4 = clomipramine.

3.6 Analysis of drug mixture

A drug mixture containing five components (listed in Table 2) was analyzed using a C-18 capillary and this result is shown in Figure 18. Only two of the total five components were found to separate on a C-18 capillary. At this point, it is difficult to identify these two components as identification based on peak intensities is difficult without knowing the relative amounts of all the components in order to make a comparison. Moreover, individual components were not available and hence it was not possible to identify the separated components based on their individual migration times. Since the components are very similar, there is a possibility of the peaks overlapping each other. However, it is hard to make any conclusions at this point without further investigation.

3.7 Analysis of serotonin, melatonin and tryptamine

Melatonin plays an important role in biological systems and it actively functions in the aging process and circadian rhythms [24, 25]. Serotonin and tryptamine are charged chemical species that are often present with melatonin in biological systems and also have their own physiological significance. Hence, it is important to analyze these three compounds and to separate them from each other. The structures of melatonin, serotonin and tryptamine are shown in Table 5 in the Experimental section.

Melatonin was not observed to elute from a C-18 capillary even after 90 minutes. Moreover, it did not elute even with reversed polarity suggesting that it was sticking to the capillary surface for some reason. On the other hand, serotonin and tryptamine eluted

in a short time on the C-18 capillary. Moreover, a good separation of a mixture of serotonin and tryptamine was obtained on the C-18 capillary. This result is shown in Figure 19.

3.8 Analysis of lysozymes and other proteins

3.8a Analysis of lysozymes

Chicken and turkey lysozymes were analyzed using bare and C-18 capillaries. A mixture of these two lysozymes did not separate on a bare capillary. However, the C-18 capillary gave a good separation of these two compounds. These results are shown in Figures 20a and b. As can be seen from Figure 20b, two extra small peaks were also seen on the C-18 capillary. After, running these lysozymes separately on the C-18 capillary at a very low voltage (5 kV), it was concluded that the two small peaks corresponded to two decomposition products of the chicken lysozyme. These results are shown in Figures 21a and b.

Figure 18. Separation of a drug mixture containing Barbital, Butethal, Amobarbital- Na⁺, Pentobarbital and Secobarbital on a C-18 capillary (L = 50 cm, I = 25 cm): Detection at 254 nm, V = 10 kV, Methanol + Buffer (90:10) mixture, Buffer: phosphate-Tris at pH = 2.14.

Figure 19. Separation of a mixture of serotonin and tryptamine on a C-18 capillary (L = 50 cm, l = 25 cm): Detection at 223 nm, V = 30 kV , Buffer: phosphate-Tris at pH = 2.14, Solutes: 1 = tryptamine and 2 = serotonin.

Figure 20. Separation of lysozymes on (a) bare capillary ($L = 56$ cm, $l = 30.7$ cm) and (b) C-18 capillary ($L = 50$ cm, $l = 25$ cm): Detection at 210 nm, $V = 15$ kV, Buffer: phosphate-Tris at pH = 2.14, Solutes: 1 = turkey lysozyme, 2 = chicken lysozyme and 3 = decomposition product

Figure 21. Elution of (a) turkey lysozyme and (b) chicken lysozyme on a C-18 capillary (L = 50 cm, l = 25 cm): Detection at 210 nm, V = 5 kV, Buffer: phosphate-Tris at pH = 2.14.

3.8b Analysis of proteins

Several proteins (listed in Table 4) were analyzed using a C-18 capillary. A mixture of turkey lysozyme, ribonucleas A and chymotrypsinogen was also analyzed using a C-18 capillary. The result of this separation is represented in Figure 22. As can be seen from this figure, only two peaks were obtained. Based on the individual migration times of the three proteins, it was concluded that the first peak corresponded to turkey lysozyme and the second broad peak (that also shows a shoulder) corresponded to ribonuclease A and chymotrypsinogen that eluted together and were not separated. The voltage was lowered to 5 kV but even with a very low EOF at that voltage, the separation did not improve. This is because the two proteins have very similar migration times and also because there were not enough interactions taking place between these proteins and the bonded C-18 to get separated. In the future, an organic modifier can be added to the buffer to see if the separation of these proteins can be improved by this addition.

Figure 22. Separation of proteins on a C-18 capillary (L = 50 cm, l = 25 cm):

Detection at 210 nm, V = 25 kV, Buffer: phosphate-Tris at pH = 2.14. Solutes: 1 = Turkey lysozyme, 2 = ribonuclease A + chymotrypsinogen

3.9 Separation efficiency and peak symmetry

Efficiencies that were recorded on bare and C-18 capillaries using equation 4 (mentioned earlier) are represented in Table 6. These values show that the efficiencies obtained on a bare capillary were much higher than those obtained on a C-18 capillary. The efficiencies obtained for angiotensins show a much greater difference on a bare and C-18 capillary. The difference in the efficiencies obtained for PTH-Asn was comparatively not as great as that for angiotensins. Lower efficiencies observed on the C-18 capillary are due to much broader peaks that are a result of appreciable interactions between the solutes and the bonded C-18 moiety. Moreover, the peak symmetry data that is shown in Table 6 indicates that the peaks obtained on a C-18 capillary were asymmetrical compared to those obtained on a bare capillary. This is again due to the interactions of the solutes with the C-18 that is bonded to the surface.

Separation factors calculated for two solute pairs on the bare and C-18 capillaries are represented in Table 7. As can be observed from this table, the separation factors on a C-18 capillary were higher than those obtained on a bare capillary. For the bare capillary, separation is based only on the differences in electrophoretic mobilities of the solutes. However, for the etched C-18 modified capillary, separation is determined by a combination of differences in electrophoretic mobilities as well as solute-bonded phase interactions. The solutes that migrate through the capillary based on their electrophoretic mobilities and EOF can interact with the bonded C-18 moiety resulting in a better separation.

Table 6. Efficiency and peak symmetry data

Capillary	Sample	Plate count per meter	Peak symmetry
Bare	PTH-Asn	43,000	0.9
C-18	PTH-Asn	32,300	1.25
Bare	Angiotensin II	200,000	1.0
C-18	Angiotensin II	48,000	1.5

**Table 7. Separation factors for angiotensins (I and II) and antidepressants
[imipramine (i) and clomipramine (c)] on bare and C-18 capillaries**

Separation factor	Bare capillary	C-18 capillary
$\alpha_{I/II}$	1.05	1.95
$\alpha_{c/i}$	1.04	1.65

3.10 Comparison of two types of injections

Serotonin and tryptamine were used to investigate the two types of injections: hydrodynamic and electrokinetic. Figures 23a and b show the results obtained on a C-18 capillary using serotonin as the solute that was injected hydrodynamically and electrokinetically, respectively. It can be seen from Figure 23a that when serotonin was injected hydrodynamically a broad and asymmetrical peak was seen. On the contrary, when serotonin was injected electrokinetically, a narrow and symmetrical peak was obtained that can be seen in Figure 23b.

With electrokinetic injection, only the charged compound itself is injected and the neutral impurities that may be present in the sample are not injected. Moreover, with electrokinetic injection, the amount of sample that is injected into the capillary is also very small. Hence, the peak shape is improved and narrow peaks are seen. However, with hydrodynamic injection, all sample components are injected into the capillary including the neutral impurities. In general, the hydrodynamic injection probably introduces much more sample into the column (compared to the electrokinetic injection), causing overload. Due to this overload the peaks are much broader and asymmetrical.

Similar results were obtained when a mixture of serotonin and tryptamine was injected. Separations of this mixture on a C-18 capillary with hydrodynamic and electrokinetic injections are represented in Figures 23c and d, respectively. The shapes and widths of these peaks were much better when the sample mixture was injected electrokinetically as compared to when it was injected hydrodynamically.

Figure 23. Effect of (a) hydrodynamic injection and (b) electrokinetic injection on peak shape of serotonin: C-18 capillary ($L = 50$ cm, $l = 25$ cm), Detection at 223 nm, $V = 30$ kV, Buffer: acetic acid-GABA at pH = 4.41.

Figure 23. Effect of (c) hydrodynamic injection and (d) electrokinetic injection on peak shape and separation of a mixture of serotonin and tryptamine: C-18 capillary (L = 50 cm, l = 25 cm), Detection at 223 nm, V = 30 kV , Buffer: acetic acid-GABA at pH = 4.41, Solutes: 1 = tryptamine and 2 = serotonin

3.11 EOF measurements

EOF was measured in bare, diol and C-18 modified capillaries using a new method that has been discussed in the experimental section. EOF was measured at various buffer concentrations and over a wide range of voltage values. The effects of buffer concentration, voltage and buffer pH on the EOF are discussed in the next sections. Graphs of EOF as a function of electric field measured in bare, etched bare (unmodified), C-18 and diol capillaries using pH 4.41 buffer are shown in Figures 24, 25, 26 and 27a respectively. A similar graph obtained on a diol capillary using a pH 2.14 buffer is shown in Figure 27b.

In the case of the bare capillary, EOF could not be measured at 25 kV and 30 kV when the 1:5 diluted buffer was used because the current at those voltages was very high causing the machine to shut down. This is why the line corresponding to the 1:5 diluted buffer in Figure 24 is short of the two last measurement points. In the case of the etched-bare capillary, the line corresponding to the 1:30 diluted buffer in Figure 25 lacks the first two points. The reason for this is that the EOF could not be calculated at 5 and 10 kV since there was no current at these voltages. Similar results were obtained on the etched C-18 modified capillary (Figure 26) where there was no current at 5 kV when 1:30 and 1:20 diluted buffers were used. With the etched diol modified capillary, the EOF could not be measured at 20, 25 and 30 kV when 1:5 diluted buffer (pH 2.14) was used. This was due to the very high current at these voltages that did not allow the machine to start. This can be seen from Figure 27b which shows only the first three points for the 1:5 diluted buffer.

Figure 24. EOF measurements on a bare capillary ($L = 56$ cm, $l = 30.7$ cm) using DMSO as a marker: $V = 5$ kV - 30 kV, Buffer: acetic acid-GABA at pH = 4.41.

Figure 25. EOF measurements on an etched bare capillary ($L = 51.2$ cm, $l = 24.8$ cm) using DMSO as a marker: $V = 5$ kV - 30 kV, Buffer: acetic acid-GABA at pH = 4.41.

Figure 26. EOF measurements on a C-18 capillary ($L = 50$ cm, $l = 25$ cm) using DMSO as a marker: $V = 5$ kV - 30 kV, Buffer: acetic acid-GABA at pH = 4.41.

Figure 27a. EOF measurements on a diol capillary ($L = 44$ cm, $l = 25$ cm) using DMSO as a marker: $V = 5$ kV - 30 kV, Buffer: acetic acid-GABA at pH = 4.41.

Figure 27b. EOF measurements on a diol capillary ($L = 44.6$ cm, $l = 24.6$ cm) using DMSO as a marker: $V = 5$ kV - 30 kV, Buffer: phosphate-Tris at pH = 2.14.

3.12 Study of factors affecting EOF and separations

3.12a Buffer pH

As pH of the buffer increases, there is more dissociation of Si-OH to Si-O⁻, which increases the number of charged Si-O⁻ groups on the wall. Due to the increase in charged groups, the zeta potential increases (refer to equation 1) which in turn causes an increase in EOF. As EOF increases, migration time decreases leading to faster analysis. EOF in a diol modified capillary was measured using DMSO as a neutral marker with pH 2.14 and pH 4.41 buffers. These results are shown in Figure 28. From this figure, it can be seen that the EOF at pH 2.14 is slower than that at pH 4.41.

Another set of experiments was carried out using serotonin as the test solute to study the effect of pH on EOF and migration times. Figures 29a and b show electropherograms of serotonin using a C-18 capillary with pH 2.14 and pH 4.41 buffers, respectively. It can be seen from these figures that serotonin eluted at 2.33 minutes when the pH 2.14 buffer was used. When the pH was increased to 4.41, serotonin eluted at 2.21 minutes. Thus, as EOF increases from pH 2.14 to pH 4.41, the migration or retention time of serotonin decreases causing it to elute in a shorter time at pH 4.41.

Similar experiments were carried out using a mixture of serotonin and tryptamine. Figures 30a and b show electrochromatograms of a mixture of serotonin and tryptamine obtained on a C-18 capillary using buffers of pH 2.14 and pH 4.41, respectively. It can be seen from these figures that serotonin and tryptamine took longer to elute with the pH 2.14 buffer as compared to when the pH 4.41 buffer was used. However, a much better separation of the two compounds was obtained at pH 2.14 where they were completely

resolved. With the pH 4.41 buffer, serotonin and tryptamine could be separated and identified, but a baseline separation was not achieved. This could be due to faster electroosmotic flow which does not allow for the longer time needed for interactions of the solutes with the bonded C-18 moiety. On the other hand, the slower electroosmotic flow at pH 2.14 provides relatively more time for the solutes to interact with the bonded C-18 causing them to separate and to resolve completely.

3.12b Effect of buffer concentration

As the concentration of the buffer increases, the thickness of the diffuse double layer decreases, causing a decrease in the zeta potential (refer to equation 1). Since the zeta potential is directly proportional to EOF (refer to equation 3), a decrease in the zeta potential decreases EOF causing the solutes to migrate slower. Therefore, the time required for analysis increases. The relation of buffer concentration to EOF can be observed from Figure 24 (shown earlier). This figure shows that as the concentration increases going from 1:30 diluted buffer to 1:5 diluted buffer, the EOF decreases.

Figure 28. Effect of buffer pH on EOF: EOF measurements on a diol capillary with pH 2.14 (phosphate-Tris) and pH 4.41 (acetic acid-GABA) buffers.

Figure 29. Effect of buffer pH: Elution of serotonin on a C-18 capillary ($L = 50$ cm, $l = 25$ cm) with (a) pH 2.14 buffer (phosphate-Tris) and (b) pH 4.41 buffer (acetic acid-GABA): Detection at 223 nm, $V = 30$ kV.

Figure 30. Effect of buffer pH: Separation of a mixture of serotonin and tryptamine on a C-18 capillary (L = 50 cm, l = 25 cm) with (a) pH 2.14 buffer (phosphate-Tris) and (b) pH 4.41 buffer (acetic acid-GABA): Detection at 223 nm, V = 20 kV, Solutes: 1 = tryptamine and 2 = serotonin.

3.12c Effect of voltage

An increase in the voltage causes an increase in the electroosmotic flow because of the higher applied electric field based on the following equation:

$$E = V/L$$

where E = Electric field, V= voltage and L = total capillary length.

An increase in EOF leads to faster migration of solutes thereby reducing the time required for analysis. Figures 3 1a, b, and c show electrochromatograms of a separation of lysozymes obtained on a C-18 capillary with 5kV, 15 kV and 25 kV, respectively. These figures show that as the voltage is increased from 5 kV to 25 kV, the separation of lysozymes can be obtained in a lesser amount of time. This observation suggests that the highest possible voltage should be used. However, higher voltages lead to higher currents and an increase in Joule heating. The heat produced, in general, is proportional to power and is given by, $P = VI$

where, P = power in watts, V = voltage and I = current.

Thus, as voltage increases, the current also increases leading to an increase in heat production. If this heat is not dissipated quickly, the temperature in the capillary will increase. This increase in temperature will cause a decrease in buffer viscosity allowing more current to flow through the capillary. An increase in the heat production within a capillary will lead to broader peaks and non-reproducible migration or retention times. Moreover, increased heat also causes sample decomposition or denaturation and also boiling of the buffer leading to problems in the experimental process. The maximum

voltage that can be used for a given set of conditions can be derived from a plot of applied voltage versus current (referred to as the Ohm's plot).

Another observation regarding the effect of voltage is that as the voltage is decreased, much better separation can be obtained. This is because the EOF is much slower at lower voltages so that there is sufficient time for the solutes to interact with the bonded C-18, causing them to be separated very well.

3.12d Effect of chemical modification of capillary wall on EOF

When the capillary wall is modified by attaching or covalently bonding a chemical species like C-18, the charges at the wall get blocked causing a reduction in zeta potential. Due to this reduction in the zeta potential, the EOF decreases leading to longer separation times. The effect of chemical modification of capillary walls on EOF can be observed from Figure 32, which shows EOF measurements obtained on bare and C-18 modified capillaries. As can be seen from this figure, the EOF measured in a C-18 capillary is much lower than that obtained in a bare (unmodified) capillary. In some cases, chemical modification of the capillary walls results in complete elimination of EOF in which case movement of solutes will depend solely on their electrophoretic mobilities.

Figure 31. Effect of voltage: Separation of lysozymes obtained on a C-18 capillary ($L = 50$ cm, $l = 25$ cm) at (a) 5 kV, (b) 15 kV and (c) 25 kV: Detection at 210 nm, Buffer: phosphate-Tris at pH 2.14, Solutes: 1 = turkey lysozyme, 2 = chicken lysozyme and 3 = decomposition product of chicken lysozyme.

Figure 32. Effect of chemical modification of capillary wall on EOF: EOF measurements on bare capillary (L = 56 cm, l = 30.7 cm) and C-18 capillary (L = 50 cm, l = 25 cm) with pH 4.41 buffer (acetic acid-GABA).

3.13 Reproducibility Tests

Serotonin was used to test the reproducibility of the C-18 capillary column. A series of 124 injections were done and the migration times and peak areas were recorded for all of the injections. A plot of the number of injections against migration time was made which has been represented in Figure 33a. The first 59 injections (that have not been shown here) showed very good reproducibility. As can be seen from Figure 33a, even after performing 59 injections, the C-18 capillary exhibited good reproducibility for the next 65 injections. This proves that the Si-C bond that was formed during the modification process is stable and that the C-18 moiety remains bonded to the surface. This also implies that the C-18 bonded capillary can be used for a long time and for a large number of experiments.

Another graph of number of injections versus peak areas was plotted and is represented in Figure 33b. From this figure, it can be observed that except for a few injections, the peak area remained fairly constant over a period of 65 injections. This means that the injection volume was fairly constant and that it did not change drastically over the period of a number of injections. Moreover, this observation also implies that there was no significant adsorption of the solute on the surface.

a

b

Figure 33. Reproducibility test on a C-18 capillary ($L = 50$ cm, $l = 25$ cm) using serotonin as the test solute: (a) plot of number of injections versus migration time, (b) plot of number of injections versus peak area.

4.0 CONCLUSIONS

The capillaries that were etched and then modified using a silanization-hydrosilation reaction scheme were well suited for electrochromatographic experiments in the new open tubular format. The C-18 attached surface exhibited good stationary phase properties and provided good interactions for a variety of solutes. These solute bonded phase interactions made the separation of neutral molecules possible. Chemical modification of capillary surfaces caused a reduction in EOF which allowed more time for solute bonded phase interactions resulting in better separations. CEC in the open tubular format gave a better separation for antidepressants as compared to CE. Moreover, lysozymes that could not be separated by CE were nicely separated by CEC. Various other mixtures and proteins were well separated by CEC.

It was possible to measure EOF in bare as well as etched modified capillaries using the new accelerated method. These results confirmed that EOF in etched and modified capillaries is much slower than the bare capillaries. It was seen that an increase in buffer pH and applied voltage increased EOF leading to faster analysis whereas, an increase in buffer concentration decreased EOF leading to slower analysis. It was also observed that the type of injection caused a significant difference in peak shapes and separations. Reproducibility tests showed that migration on the C-18 bonded surface was constant implying that the Si-C bond formed by the silanization-hydrosilation reaction scheme is very stable. This result suggests that the etched-modified capillaries can last for a long time and that they can be used repeatedly for a large number of analyses.

5.0 FUTURE WORK

Future work will involve etching of the capillaries at temperatures that are different than the ones used in this study. The surface morphology of these etched capillaries can be studied in detail and a comparison of their performance can be made. Modification of capillary surfaces with a chiral species can also be carried out. These types of capillaries can then be used for enantiomeric separations of certain compounds like amino acids.

The effect of organic modifiers like methanol on the separation can be investigated. A number of proteins and other compounds like tetracyclines can be analyzed using a C-18 capillary. CEC exhibits a lot of advantages that make it an attractive tool for a large number of possible applications that can be explored in the future.

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