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Separation of proteins and polypeptides using electrochromatography

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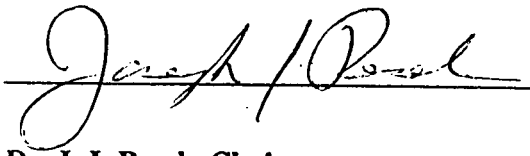

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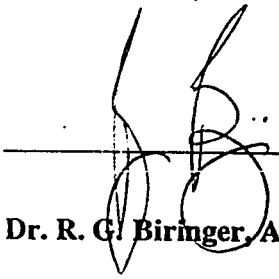

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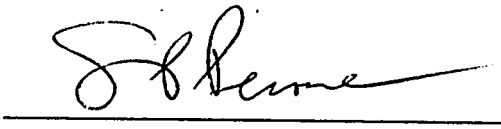

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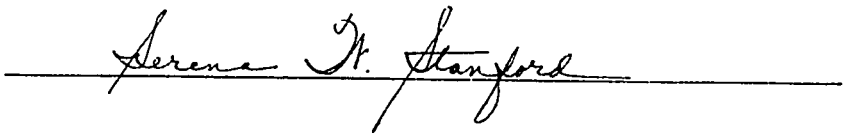

ABSTRACT

SEPARATION OF PROTEINS AND POLYPEPTIDES USING ELECTROCHROMATOGRAPHY

by Leena Mauskar

Capillary electrochromatography has the potential to be an analytical tool of great importance for the separation and characterization of biomolecules like proteins and polypeptides which are difficult to analyze due to larger structures, higher molecular weights and charges. This thesis gives an overview of adaptations of capillary electrophoresis and its modification to capillary electrochromatography. In capillary electrochromatography, analyte interacts with the stationary phase attached to the inner surface of the capillary wall. This is achieved by carrying series of chemical reactions. The inner surface area of the capillary is increased by etching using ammonium dihydrogen fluoride. The formation of whiskers, in the form of troughs and crests, is observed by Scanning Electron Microscopy. Stationary phase in the form of diol or C_{18} is directly attached to the capillary wall by chemical modification and the protein-stationary phase interactions were observed. Complex mixtures of the proteins were successfully separated using the technique.

ACKNOWLEDGMENT

My life has been influenced by many people throughout my academic career. The list of people who have helped me in achieving this academic goal, in spoken and unspoken ways, is long. I acknowledge these people in quiet solitude. However I would like to mention a few names who have advised me with patience and understanding. I am indebted to Dr. J.J. Pesek for taking me on as his student and allowing me to progress at my own speed with his indispensable guidance. I am also grateful to Dr. R.G. Biringer and Dr. S.P. Perone for their vital recommendations. I would like to give special thanks to Dr. Maria T. Matyska for her guidance and assistance throughout my research.

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1. INTRODUCTION

1.1 Background

Capillary electrophoresis is an important technique for separation of organic compounds. Recently it has become an important tool for identification and characterization of biological molecules like amino acids, polypeptides and proteins. These molecules have been separated by techniques like reverse phase HPLC. HPLC uses a mixture of aqueous and organic solvents as a mobile phase. In HPLC the separation takes place on the basis of partition between the two phases, mobile and stationary. The amount of solvent used for HPLC is often large giving rise to excessive organic, non-biodegradable waste. Further, if the concentration of organic solvents is too high, the proteins can be denatured. Capillary electrochromatography makes use of the hydrophobic-hydrophilic interactions used in HPLC along with the advantages of CE such as use of very small amount of the analyte and solvents, high reproducibility, good precision and accuracy, and easy operating conditions. For these reasons capillary electrochromatography is looked upon as a tool of importance.

The basic principle of capillary electrochromatography is electromigration that occurs when the electric field is applied to the two platinum electrodes immersed in the electrolyte reservoirs similar to CE. The separation depends on the mass, charge and size of the analyte along with its interaction with the stationary phase. In the previous work the stationary phase was first attached to small solid support particles, like in HPLC, and then the capillaries were packed with the modified particles. For the present study, the stationary phase is attached

directly to the capillary wall. As the analytes move through the capillary they are separated depending on the interactions listed above. There are two types of displacements taking place in capillary electrophoresis: electrophoretic mobility and electroosmotic flow. Differences in electrophoretic mobility bring about the separation depending on mass, charge and radius. Electrophoretic mobility is calculated by equation 1,

$$\mu_{ep} = \frac{Z_i \, e}{6 \, \pi \, \eta \, r_i}$$

equation 1.

where,

Z_i = number of charges

η = solvent and buffer viscosity

r_i = radius of the ion

μ_{ep} = electrophoretic mobility

Electroosmotic flow brings about bulk displacement, represented by equation 2.

$$\mu_{eo} = \left(\frac{\varepsilon}{4 \, \pi \, \eta}\right) E \, \zeta$$

equation 2.

where,

ζ = zeta potential

ε = dielectric constant

μ_{eo} = electroosmotic flow

E = potential field strength

Electroosmotic flow can be explained with the help of Figure 1. The inner walls of the silica capillaries contain Si-OH groups on their surface. In aqueous solutions, depending on the pH of the solution, the Si-OH groups ionize to produce negative ions shown by losing H^+. This layer attracts the positive ions from the solution which in turn attract negative ions; this is known as the electrical double layer. The electroosmotic flow reduces the separation between different peaks.

If a mixture of positive, neutral and negative ions is moving under an applied electric field with a positive electrode at the injection end and a negative electrode at the elution end, in the normal case, only positively charged ions will move towards the negative electrode. But due to electroosmotic flow induced by the formation of electrical double layer, neutral as well as negative ions are pushed towards the negative electrode along with the movement of the electrolyte.

A number of problems are encountered when capillary electrophoresis is used for the separation of biomolecules like proteins and polypeptides. Electroosmotic velocity is not uniform and hence sometimes the reproducibility is poor, as it contributes to the bulk displacement. Electroosmosis also reduces the separation between the peaks as the analyte molecules are pushed towards the eluting end, hence decreasing the distance of separation.

Proteins are adsorbed on the inner surface of the capillary which can lead to the delayed elution or non elution of some solutes. These problems are overcome by modifying the capillary wall or by the use of buffers. Several approaches have been developed.

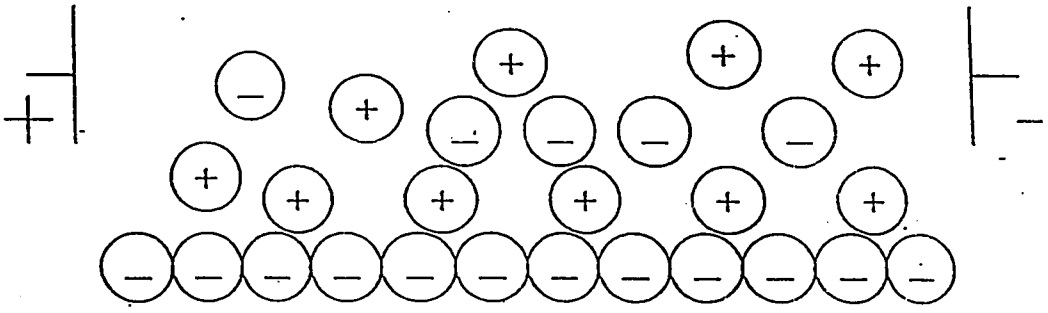


Figure 1. Electroosmotic flow

One approach to manipulate coulombic interactions is to adjust the electrolyte's pH relative to the isoelectric point (pI) of a protein in order to change its net charge as well as that of the capillary wall. The silica surface in the presence of aqueous solution develops a negative charge at pH values above 2. If the pH of the solution is adjusted high enough, such that the pH of the solution is greater than the pI of all the proteins in the mixture, the proteins as well as the silica surface, can be negatively charged. This will result in coulombic repulsions and hence decrease the amount of protein absorbed.[1] In the pH range of 8 to 11, sharp peaks are observed. When the pH is raised to 12, peaks are initially sharp, which deteriorate to have shoulders during the consecutive runs. A number of factors can contribute to this deterioration such as denaturation due to high pH, Joule heating, or interference of phosphate buffer with these proteins. Some proteins such as ribonuclease A, bind phosphate buffer at almost all pH values. Proteins such as lysozyme, cytochrome C and ribonuclease A cannot be eluted at pH values below their pI values, as they are positively charged under these conditions and stick to the negatively charged capillary wall. Hence, if basic amino acids are present on the surface of the proteins they can still cause protein-wall interactions even with the use of high pH buffers.

When low pH buffers are used, the surface silanol groups are neutralized. This results in a very small charge on the capillary walls. Good separation is observed with the application of linearly increasing voltage.[2] Low pH phosphate buffers can be used. Phosphate binds to the silica surface and can not be removed by flushing the capillary with water or

buffer solutions, thereby reducing the number of adsorption sites. Also, at low pH, more of the silanols on the capillary wall are protonated, minimizing the net charge on the capillary surface. Still protonation is not complete even at pH 1.5 leaving a small amount of negative charges on the surface. Even this small a charge can lead to adsorption of some proteins on the capillary wall. It is also observed that due to full protonation of the proteins by the low pH buffer, the charge differences between the species are reduced, resulting in low peak capacity for the separation. At very high or very low pH, due to large pH-pI differences, the proteins are not always stable. Some structural changes or even hydrolysis may occur.

Another approach to reduce protein adsorption involves the addition of zwitterions or ionic salts to the electrolyte. When ionic salts are added to the buffer, the number of ions competing for the charged capillary surface increases thereby competing out proteins.[3] However, high salt concentration can also cause Joule heat generation, which results in band broadening at the high voltages of 20 to 30 kV commonly used in capillary electrophoresis. Some buffers contain cationic additives that reverse the charge on the capillary wall.[4]

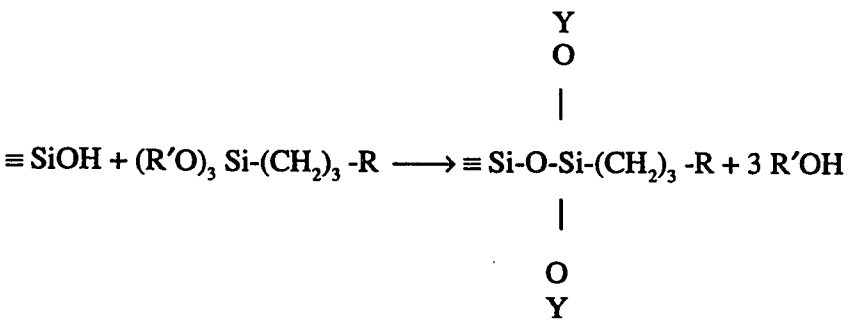
On the other hand, zwitterions do not contribute much to the conductivity. They combine with the negatively charged surface of the capillary as well as the charged sites on the proteins. Hence, both the capillary wall-protein interactions and protein-protein interactions are reduced[5] without increasing Joule heating. Zwitterions such as glycylglycine, glycine, triglycine, sarcosine and betaine with additives like potassium sulfate and combination of potassium sulfate-betaine were used for this study. When zwitterions such as

glycylglycine, glycine, triglycine, sarcosine and betaine were used, it was found that lysozymes were still adsorbed on the surface but not α-chymotrypsinogen, though the peak shapes for α chymotrypsinogen were poor. When sarcosine was added, lysozyme was eluted but with very poor peak shape. The best results were obtained for potassium sulfate as the additive. Addition of zwitterionic compounds like glycylglycine and triglycine was found to increase viscosity that may suppress convection and hence reduce band broadening. Higher voltages can be used with the addition of zwitterions and hence shorter migration times can be obtained than by adding ionic salts. However, it has been observed that they do not work well with the very basic proteins like lysozymes.

A variety of different additives are described in the literature, including divalent amines[1,6], detergents[7,8] and organic solvents[9]. The mechanism of action of these additives is still not completely known. It is believed to be the same as that suggested for zwitterionic salts. In addition, the additives serve to reduce the electroosmotic flow by up to 50% and increase resolution due to better peak separation.

To avoid protein adsorption, modifying the inner wall of the fused silica capillary with organic material is perhaps the most effective approach to minimize number of adsorption sites. This type of modification drastically reduces or eliminates electroosmotic flow and minimizes protein adsorption. Organic coatings such as polyether[10,11], diol[12], poly(vinylpyrrolidinone)[13], methyl cellulose, non cross-linked polyacrylamide[2,14] and cross-linked polyacrylamide[15] have been used by covalently bonding them to the inner wall of the

fused silica capillary. These coatings are usually hydrophilic moieties attached to the inner wall of the fused silica capillary through siloxane linkages resulting from organosilation. The formation of siloxane linkages can be shown by the following reaction,



Due to these coatings, the surface silanol groups are either removed or masked by relatively long chains of hydrophilic moieties. Hence, the protein analytes can not access the adsorption sites resulting in a decrease in adsorption. Due to extensive silanol removal that occurs during siloxane formation, the electroosmosis is reduced drastically upon modification.

In the non-cross-linked polyacrylamide coating the thickness of the coating can be controlled by varying the concentration of the acrylamide in the coating solution. With an increase in pH an increase in electroosmotic flow was observed[14], though it is less than that observed for uncoated capillaries. This can be attributed to the dissociation of surface silanol groups. Acidic proteins (bovine serum albumin) are also eluted but with lower efficiency and the elution order does not represent the relative pK values of the proteins.

Cross-linked polyacrylamide coating is also used for the separation of proteins.[15] These capillaries are prepared by pretreating with 7-oct-1-enyltrimethoxysilane

followed by coating with polyacrylamide. Methylene bis acrylamide is added to the coating solution for cross-linking. Good separation of basic proteins was observed with this coating without peak tailing or distortion. The cross-linked polyacrylamide layer is hydrophilic which minimizes interactions with proteins. For the separation of acidic proteins, the polarity of the apparatus has to be changed so that elution occurs at the anode instead of the cathode. This can pose a problem for the separation of a mixture of unknown proteins, as acidic and basic proteins can migrate towards the opposite ends. Further, the capillaries, particularly polyacrylamide, are found to be unstable at the higher pH.

Another approach involves the use of different coatings such as polyethylene glycol for acidic proteins and polyethylene imine for basic proteins.[16] Some amount of tailing is observed and the peaks are not well resolved. For all practical purposes the capillary should handle both types of proteins since a mixture can contain acidic as well as basic and neutral proteins.

Organosilation is a common approach used to form a siloxane (Si-O-Si-C) linkage by combining a fully hydroxylated silica surface with an organic material. Unfortunately, silane based modified capillaries are susceptible to hydrolysis at pH extremes and there is always a chance of degradation. This type of capillary is now available commercially with neutral polymer, anionic, cationic, hydrophobic (C_1, C_8, C_{18}) and glycerol coatings. If these are used according to the manufacturer's instructions they can give high quality results. Another type of coated capillary (available through Hewlett-Packard) has a permanently adsorbed coating

of polyvinyl alcohol.[4] This neutral hydrophilic surface is stable in the pH range of 2 to 9.

Due to steric hinderance, the surface coverage by organic groups is limited which can lead to a high concentration of unreacted silanol groups.[17] This can give rise to protein interaction with the capillary wall and can be observed as strong peak tailing.

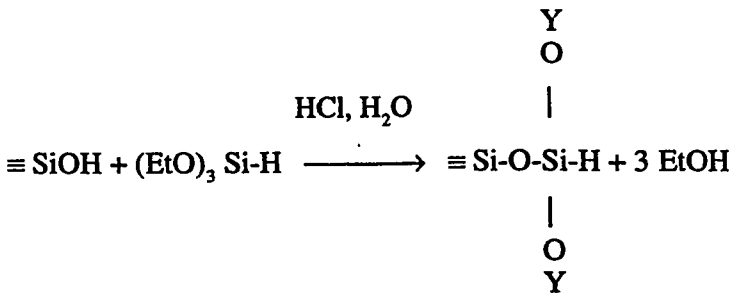
Another type of approach for attaching an organic moiety by using Grignard's reagent which results in a silicon-carbon linkage has been described by Cobb et al.[18] In this method, the capillaries are pretreated with 1 M NaOH for 30 minutes. Next, thionyl chloride is flushed through the capillary for the purpose of surface chlorination. A polyacrylamide coating is then attached to the silica wall through a Si-C bond that is made by the reaction of a Grignard reagent containing a vinyl group with the chlorinated surface on the capillary wall. The reaction gives a vinyl modified surface which is then treated with a polymerizing acrylamide solution. The reaction is as follows,

$$\equiv \text{Si-OH} + \text{SOCl}_2 \rightarrow \equiv \text{Si-Cl} + \text{HCl} + \text{SO}_2$$

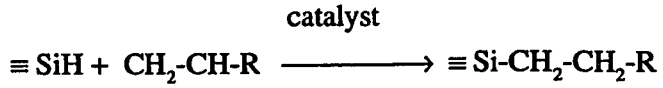
$$\equiv \text{Si-Cl} + \text{R-MgBr} \rightarrow \equiv \text{Si-R} + \text{MgBrCl}$$

The absence of electroosmotic flow and the reduced electrostatic interactions of proteins with the capillary walls resulted in improved resolution and peak shape. The reproducibility is also improved due to the absence of electroosmotic flow. Experiments were conducted at pH=9.5 and pH=2.7 to show the stability of the capillary over a wide range of pH. It was observed that there was still some adsorption but it was reversible and equilibrium could be quickly achieved.

A third approach of this type involves catalytic addition of surface silicon hydride to the terminal double bond of an olefin.[19] The reaction of hydride modification is called hydrosilation and requires catalysts that are complexes of transition metals from group VIII, in particular, platinum. The preparation of the hydride intermediate involves the reaction of silica with a trisubstituted silane reagent such as triethoxy silane.



The Si-C linkages are produced by the following reaction called organosilation,



This type of modification was used for the preparation of a diol modified capillary.[20] This modified capillary can be used over a wide pH range and showed improved reproducibility of protein migration. Since pH is a very important parameter for separation of proteins, it is important to retain pH as a freely adjustable parameter. It was observed that this type of capillary was well suited for the separation of basic proteins up to pH 6. Compared to uncoated capillaries, reduction in peak tailing was observed which suggests a decrease in adsorption of proteins. The experiments were carried out using a home made HPCE instrument. With an automated commercially available instrument, the results can be

improved through temperature control as well as precise monitoring of the injection time and volume.

1.2. Goal of the present research

From the above discussion it can be seen that no single method can be generally applied for the separation of all proteins. But better separations can be achieved by the use of modified capillaries with different types of coatings. The Si-C bound modified capillary shows relatively good separating ability, good resolution and stability over a wide pH range. The relatively easy hydrosilation procedure can be used for modifications. Etching of the capillary wall prior to the surface modification gives more surface area thereby increasing the number of organic moieties attached to the capillary wall. For conventional electrochromatography, many scientists use a 200 to 400 μm bore fused silica capillary packed with very small diameter particles with stationary phase attached to it. The packing of such a thin capillary is very difficult and requires some other modifications for its practical use. Since the capillary is made of fused silica, the lack of mechanical strength makes it difficult to use higher pressures required for packing. Secondly, it is necessary to install a frit at the eluting end of the capillary before the window so that the packing material stays in place. This is very difficult considering the capillary dimensions. With the proposed technique, the stationary phase is directly bonded to the capillary wall.

The etching technique used to increase the surface area of the capillary wall was previously described for modification of GC columns.[21] This increased surface area in turn

can result in bonding of more organic moieties giving rise to a stationary phase that is directly attached to the capillary wall. The organic moieties can be chosen according to hydrophilic or hydrophobic nature of the stationary phase required for a particular separation. The present research involves the use of C_{18} as a hydrophobic and diol as a hydrophilic moiety. Since the technique is a combination of principles used for HPLC as well as capillary electrophoresis, the use of organic additives such as methanol is also studied, similar to reverse phase HPLC.

2. EXPERIMENTAL

2.1 Reagents and materials

Lysozymes (Turkey and Chicken egg whites), cytochrome c (Horse and Bovine hearts), ribonuclease A (Bovine pancreas), myoglobin, angiotensin I and III and bradykinin were purchased from Sigma Chemical Company (St. Louis, MO). The molecular weight and pI values of these proteins are listed in Table 2.1 Lactic acid, acetic acid and ammonia were purchased from J.T.Baker Chemical Co. (Phillipsburg, NJ). β- alanine and γ–amino butyric acid (GABA) were also purchased from Sigma Chemical Co. and used as zwitterion buffer components. Deionized water was obtained from a Milli-Q water purification system (Millipore Corp., Bedford, MA) and was filtered through a 0.2μm Nylon 66 membrane filter (Alltech Assoc., Deerfield, IL) and then used to prepare all buffer systems. All buffer solutions were similarly filtered before use. Triethoxy silane (TES), octadecene and 7-octene 1,2-diol were purchased from Aldrich Chemical Co.Inc., (Milwaukee, WI) and were used to prepare the hydride intermediate, octadecane and diol modified capillaries respectively. Tetrahydrofuran, p-dioxane (J.T.Baker Chemical Co.,Phillipsburg, NJ) and toluene (EM Industries Inc.) were used as washing solvents for both modified capillaries. Hexachloroplatinic acid (Aldrich Chemical Co.Inc.) and 2-propanol (J.T.Baker Chemical Co.) were used to prepare Speier's catalyst.

Table 2.1 Model proteins used in this work*

Protein	Organism	Tissue	pI	Mol. wt. (k Da)
Cytochrome C	Bovine	Heart	10.3-10.8	12.2
Cytochrome C	Horse	Heart	9.0-9.4	13.4
Lysozyme	Turkey	Egg white	10.5-11	13.9
Lysozyme	Chicken	Egg	10.5-11	13.9
Myoglobin	Horse	Skeletal muscle	6.8-7.0	16.9
RibonucleaseA	Bovine	Pancreas	9.2-9.5	13.8

* Taken from reference 20

2.2 Instrumentation

A majority of the work was done on homemade HPCE. Some experiments were also done on the ABI model 270A-HT System (Applied Biosystems, Foster City, CA.) The homemade HPCE instrument (Figure 2) consists of a high voltage power supply (series 230 Bertran, Inc., Hicksville, NY) and two platinum electrodes which are dipped in two vials containing the running buffer. The electrodes and vials are placed in a plexiglass box. The plexiglass box was equipped with automatic high voltage cut-off that breaks the circuit when opened. Fused silica capillaries (Polymicro Technologies, Phoenix, Az) with 50μm internal diameter were used. A spectra 200 UV detector (Spectra-Physics, San Jose, CA) was used for on-line detection. The electrophoretic set-up was completed by using a Model 35900 D/A interface (Hewlett-Packard, CA), an IBM personal computer and a printer.

A GC oven was used to control the reaction temperature during the process of modification. The oven was modified to pass several capillaries through the inlet as well as outlet. This system is shown in Figure 3. Heavy wall glass tubes (25 mL) were used as reagent reservoir and were equipped with swagelok fitting to allow the application of nitrogen pressure of 20 to 30 psi to force the liquid reagent through the capillary. The other end of the capillary was dipped in the small vial to collect the reagent and measure the flow rate in mL/minute.

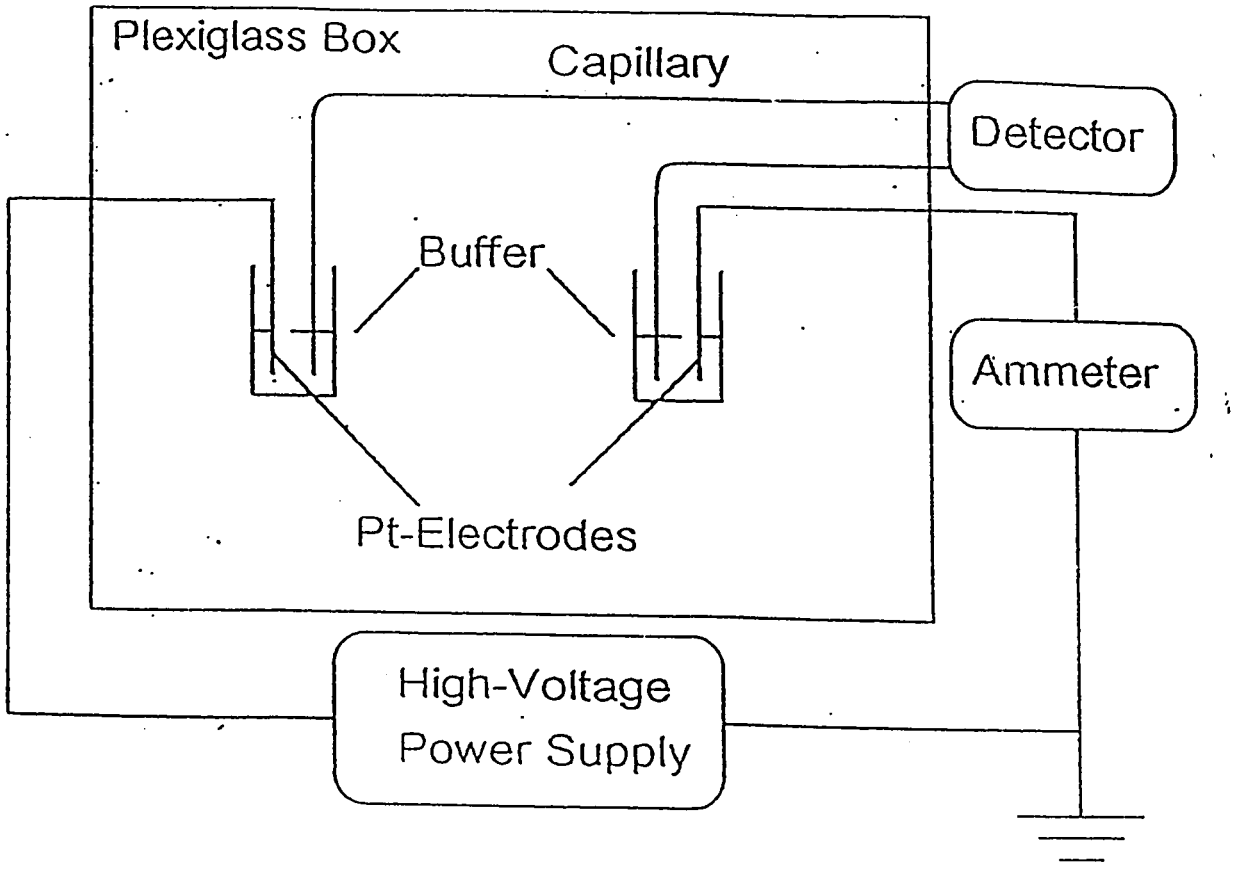


Figure 2. Home-made HPCE instrument*

*Figure taken from ref. 20.

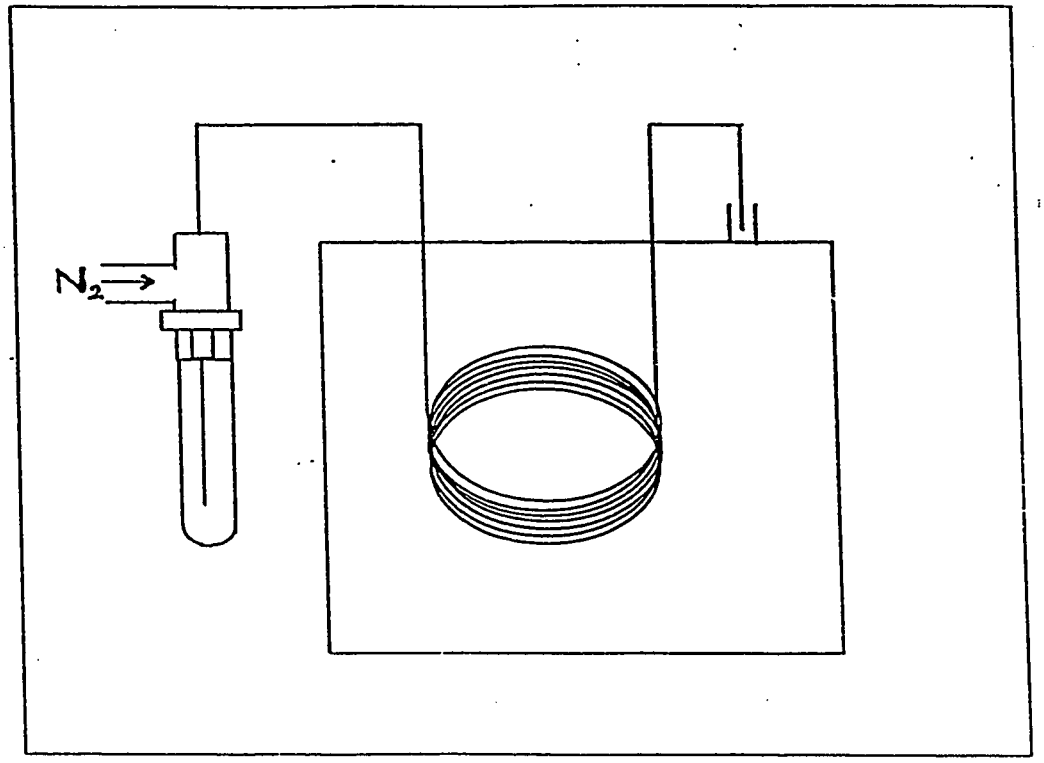


Figure 3. Modified GC oven used for capillary modification

2.3 Modification of the capillaries

2.3.1. Surface modification of the capillaries

Surface modification of the capillaries involves etching the inner wall of the capillary to produce higher surface area. The capillaries are filled with conc. HCl sealed and heated overnight at 80 °C. After cooling to room temperature, the capillaries are washed with deionized water, acetone and diethyl ether, in that order and dried with nitrogen. The capillaries are then filled with a 5% sat. solution of ammonium dihydrogen fluoride in methanol (w/v) and allowed to stand for 1 hour. Methanol is then removed by uniform nitrogen flow, the ends are sealed and the capillaries are heated at 300 °C for 3 hours, or at 400 °C for 3 hours or at 300 °C for 2 hours and then at 400 °C for 1 hour. After cooling they are washed with 20 mL of methanol and dried with nitrogen. Different conditions for etching result in the formation of different types of surfaces. The capillary surface after etching was studied using scanning electron microscopy.

2.3.2. Chemical modification of the capillaries

Chemical modification of capillaries involves capillary preconditioning followed by preparation of hydride intermediate. This inturn is followed by addition of octadecene or 7-octene-1,2-diol.

For capillary preconditioning, capillaries were flushed with 6 mM ammonia for 20 hours at room temperature, rinsed with deionized water for 1 hour and then flushed with 0.1

M HCl for 4 hours. They were again rinsed with deionized water for 2 hours and dried with nitrogen for over 20 hours at 100 °C.

Preparation of hydride intermediate involves flushing the capillaries with dioxane and treating with a TES solution (0.12 M) for 90 minutes at 95 ± 1 °C. The TES solution was prepared by adding 8.4 mL of dried dioxane to 1.2 mL of 1.0 M TES/Dioxane solution to which 435 µL of 2.3 M HCl is added with continuous stirring. The capillaries were then rinsed with THF/ water (75/25 v/v) for 2 hours followed by flushing with THF for 4 hours at room temperature and dried with nitrogen at 100 ± 1 °C for over 24 hours.

The hydride modified capillary was rinsed with dry toluene and then flushed with octadecene or 7-octene-1,2-diol solution at 100 °C for 45 hours under 30 psi of nitrogen, for the final modification. The octadecene solution was prepared by adding 153 µL of 10 mM Speier's catalyst to 4.5 mL octadecene and was allowed to equilibrate for 1 hour at 65 to 70 °C in a flask equipped with a condenser and a drying tube. The diol solution was also prepared in a similar way with 153 µL of 10 mM Speier's catalyst and 0.265 mL of 7-octene-1,2-diol and 4.235 mL toluene. After modification with olefin, capillaries were cooled to room temperature and rinsed with toluene and THF for 1 hour. Then they were dried with nitrogen under 20 psi at 100 °C overnight.

2.4 Electrophoresis

For electrophoresis, zwitterion buffer solutions over the pH range of 3.0 to 4.41were

used: 30 mM citric acid + 24.5 mM β-alanine at pH 3.0, 30 mM lactic acid + 36 mM β-alanine at pH 3.7, 30mM acetic acid + 30 mM GABA at pH 4.41. Protein solutions were prepared by dissolving 0.1 to 0.6 mg in 1 mL of buffer. Capillaries with 50 μm internal diameter and 25 cm effective length, 50 cm total length were used. The detection wavelength was 210 nm. The applied voltage was in the range of 20 to 30 kV with current of 5 to 10 μA.

2.5 Calculation of separation efficiency and peak symmetry

The separation efficiency was measured as the number of theoretical plates, calculated directly from the electropherogram by using the following equation for the half peak height method.

$$N_{1/2} = 5.54\ t_R^2 / (W_{1/2})^2$$

where,

$N_{1/2}$ is the number of theoretical plates at half peak height.

t_R is the retention time.

$W_{1/2}$ is the peak width at half peak height.

The peak symmetry is evaluated by calculating the a/b ratio at 10% of peak height.

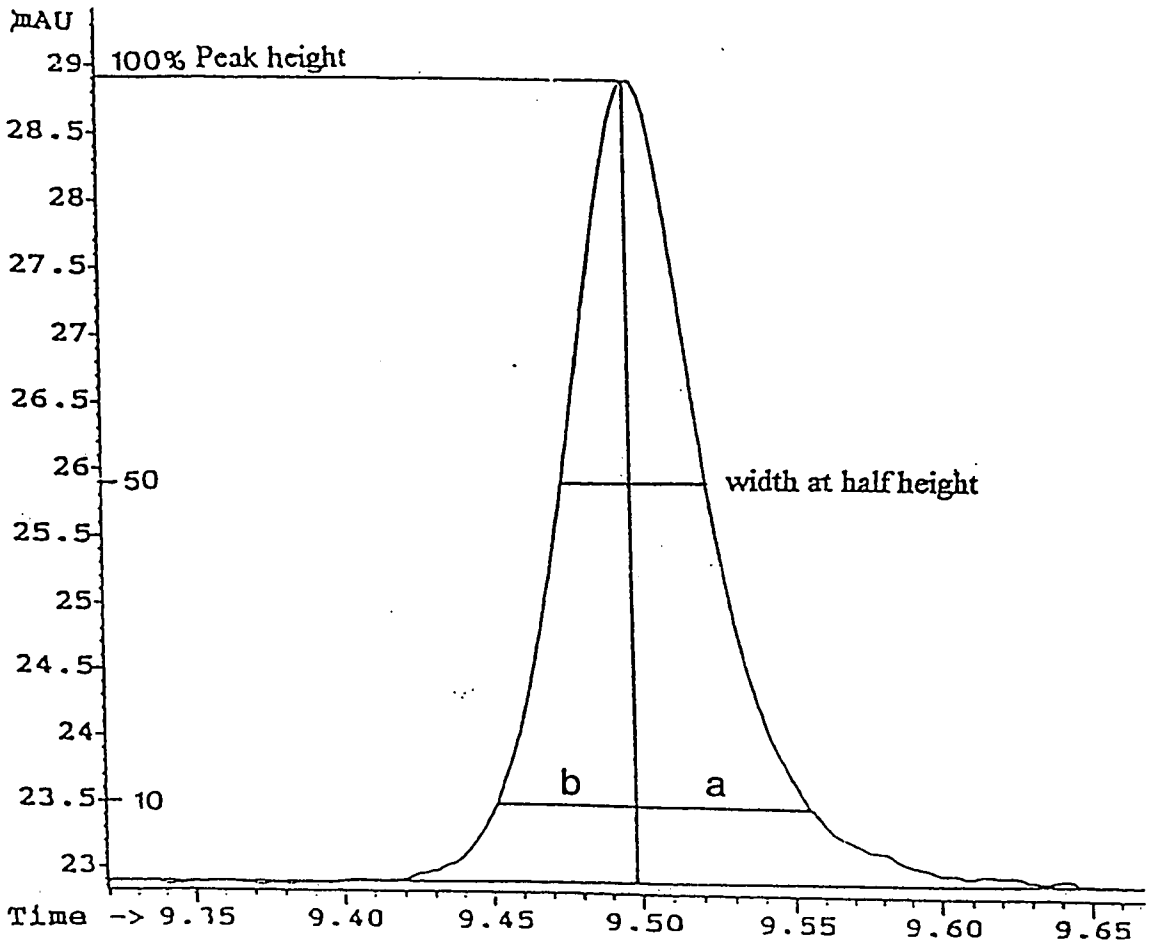


Figure 4. Peak symmetry at 10 % of of the peak height

3. RESULTS AND DISCUSSION

Separation of complex mixtures of proteins has always been an important issue for capillary electrophoresis. The major goal of this research is to maximize the protein-stationary phase interactions and facilitate separation of relatively complex mixtures of proteins with good retention reproducibility over a large number of injections, and having good resolution and peak shape. The capillaries were etched to get maximum surface area. The effect of reaction parameters on etching are studied by using Scanning Electron Microscopy. The organic moiety, such as C_{18} or diol is then attached to the capillary surface by chemical modification. Along with the usual electrophoretic separation, the proteins were expected to interact with the stationary phase (C_{18} or diol) as in HPLC. The effect of buffer pH and the applied voltage on the separation are also evaluated. The buffers up to pH 4.41 were used for the study as peak shapes were found to be distorted with less separation efficiency at a higher pH for both the C_{18} and diol modified capillaries in the present study.

3.1 Effect of reaction parameters on etching

The etching process used to increase the surface area of the inner wall of the capillary created troughs and crests in the silica known as whiskers. The size and shape of whiskers is determined by the temperature for etching and the duration. This was studied by Scanning Electron Microscopy. It can be seen from Figure 5 (bare capillary) and Figure 6.a, b (capillary etched at 300 °C for 3 hours), Figure 7 (capillary etched at 400 °C for 3 hours) and Figure 8 (capillary etched at 300 °C for 2 hours), that the whiskers formed by etching the

capillary at 300 °C for 3 hours show a very large increase in the surface area. However, the vibration studies indicated that these whiskers collapse after a certain time. With 2 hours of etching at 300 °C, non-uniform whiskers are produced. Uniform whiskers are produced by etching at 400 °C for 3 hours. These whiskers were found to be stable over a long period of time. Hence, these conditions were chosen for all subsequent surface modification procedures. Figure 9 compares the bare capillary and capillary etched at 300 °C for 3 hours by optical microscopy. Vibration experiments were performed with 10 minutes vibration time (Figure 10.a) and 30 minutes vibration time (Figure 10.b). The capillaries were filled with 2-propanol for optical microscopy. From these figures it can be seen clearly that the number of whiskers are reduced with the increase in the vibration time for the same capillary.

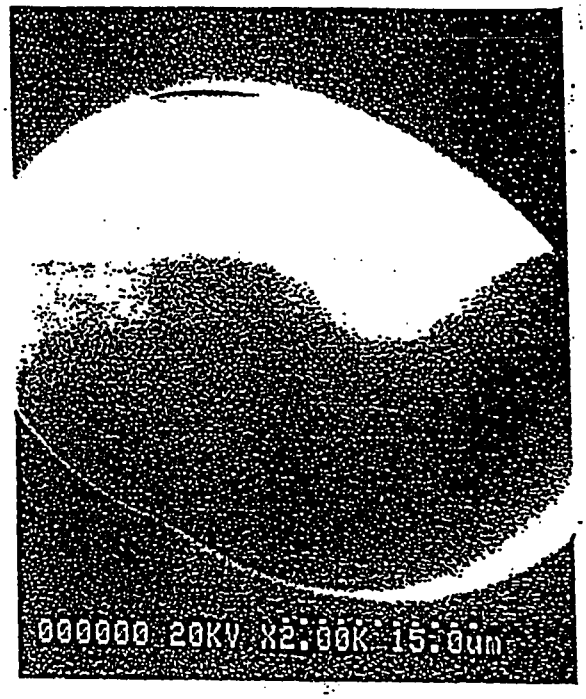


Figure 5. SEM picture of bare capillary

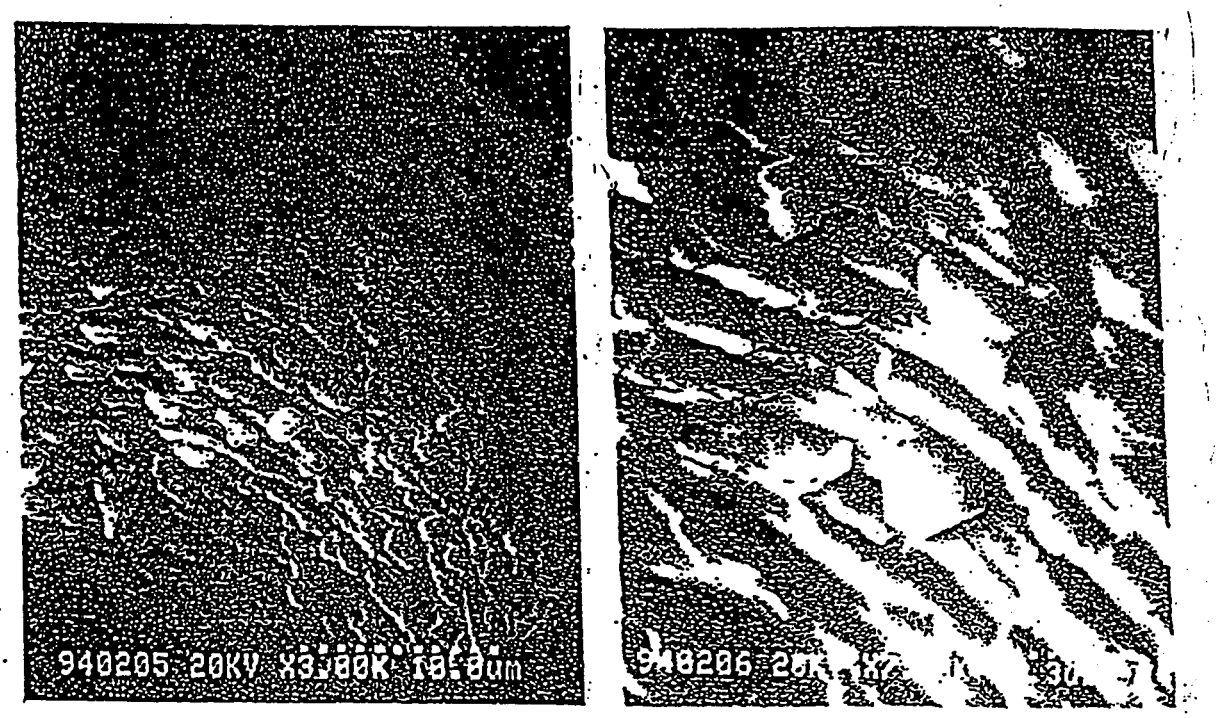


Figure 6 a , b. Capillary etched at 300 °C for 3 hours

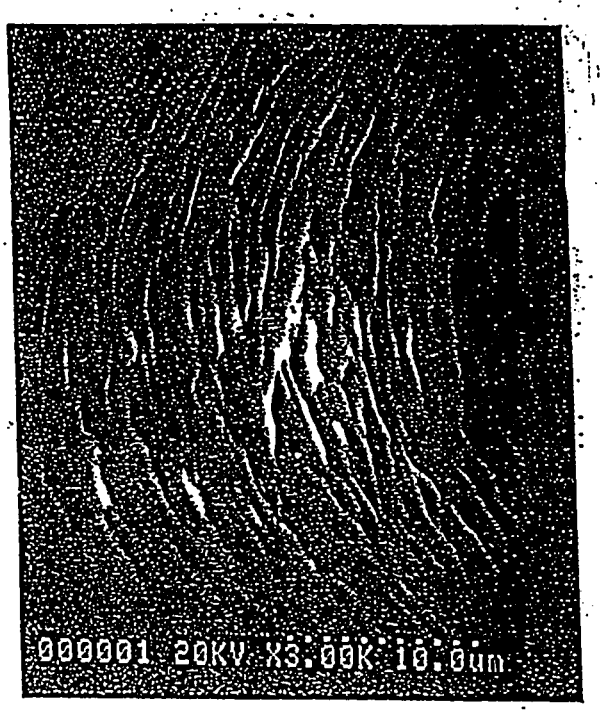


Figure 7. Capillary etched at 400 °C for 3 hours

Figure 8. Capillary etched at 300 °C for 2 hours

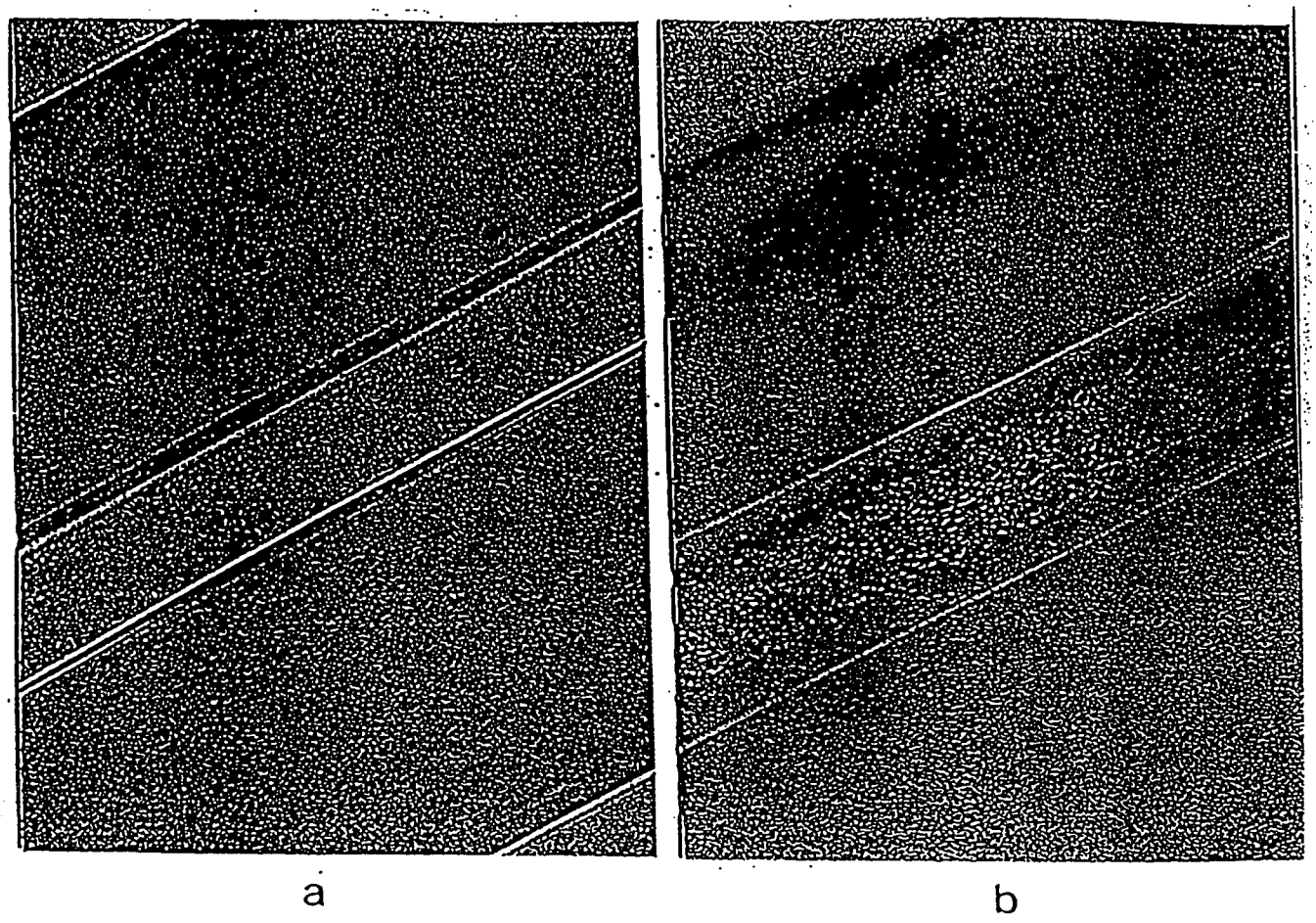


Figure 9. Comparison of a. bare capillary and b.capillary etched at 300 °C for 3 hours by Optical Microscopy, Object magnification 40

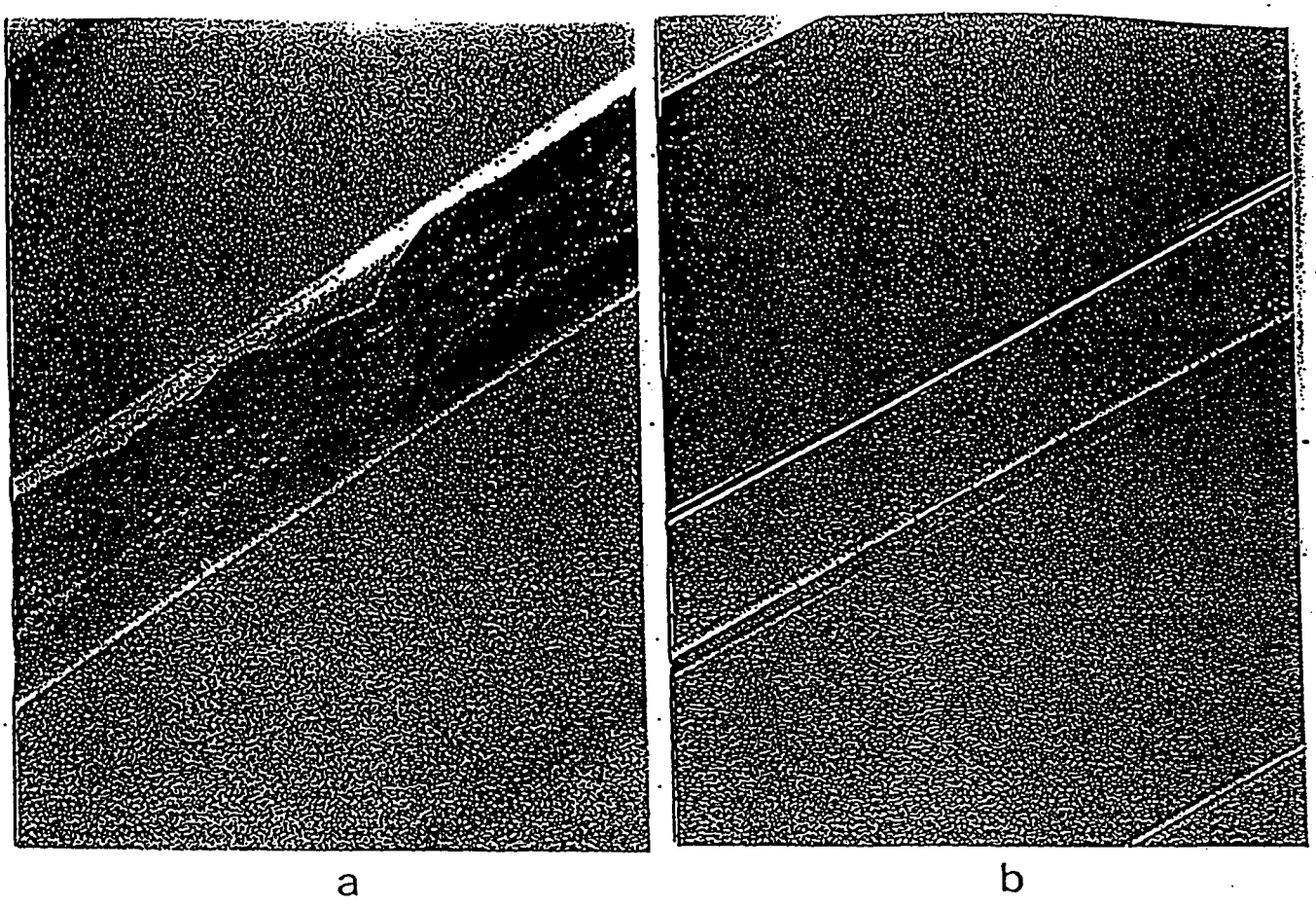


Figure 10 a. Capillary etched at 300 °C for 3 hours after 10 minutes of vibration. Object magnification 40, b. capillary etched at 300 °C for 3 hours after 30 minutes of vibration. Object magnification 40

3.2 Separation efficiency and peak symmetry

Separation efficiency and peak symmetry are evaluated by using several basic proteins as model probes for C_{18} as well as diol modified capillaries. The proteins are listed according to their properties in Table 2.1. Figure 11 and Figure 12 show the typical electrochromatograms obtained for the separation of these proteins using the diol and C_{18} capillary at pH 4.41 and pH 3.0 respectively. Very good separation is obtained in both the cases.

Figure 13.b shows the separation of a mixture of horse and bovine cytochrome C, These proteins differ by only three amino acids in the total sequence of 104. An attempt to separate the same mixture using bare capillary showed no such separation can be seen from Figure 13.a. Figure 14.b shows the separation of a mixture of two lysozymes, chicken and turkey egg white, using diol modified capillary. Turkey and chicken lysozyme have similar molecular weights and isoelectric points. The separation of these two lysozymes suggests that there might be some differences in the charge at pH 4.41. This mixture, when injected in the bare capillary, a shoulder is observed in the electrochromatogram, without any significant separation (Figure 14.a). When compared to the separation on the bare capillary (Figure 13.a and Figure 14.a) it is clear that under the same conditions of pH and applied voltage the modified capillaries exhibit much better separation efficiency. This is probably due to an increased interaction of the proteins with the hydrophilic diol moiety in diol modified capillary and hydrophobic C_{18} moiety in C_{18} modified capillary which act as stationary phases and hence decreased interaction with silanol groups on the surface of the bare capillary.

The optimum effective length required for separation of horse and bovine cytochrome C and turkey and chicken lysozyme, using unetched diol modified capillary under the similar but not the same conditions, was 50 cm.[20] The optimum length required for the capillary modified after etching is 25 cm. As peak separation depends on the effective length of the capillary, it suggests an increased protein-stationary phase interaction for the capillary modified after etching. Figure 15 shows the separation of horse and bovine cytochrome C under the similar conditions of pH and applied voltage but with the effective length of 50 cm.

Angiotensin I and angiotensin II are clinically important proteins. In the human body, angiotensin I is converted to angiotensin II by removing two terminal carboxylic groups from the decapeptide via a converting enzyme found in the lung, endothelial cells and plasma. Angiotensin II increases the blood pressure by causing vasoconstriction. If the amount of angiotensin II is found to be increased in the blood, renin dependent hypertension could be treated with the help of various nonapeptide analogs of angiotensin I. Angiotensin I and II can be separated by using the C_{18} modified capillary as shown in Figure 16. As described above these peptides differ from each other by only two amino acids. The baseline separation of these peptides shows that such clinically important mixtures can also be separated with good resolution.

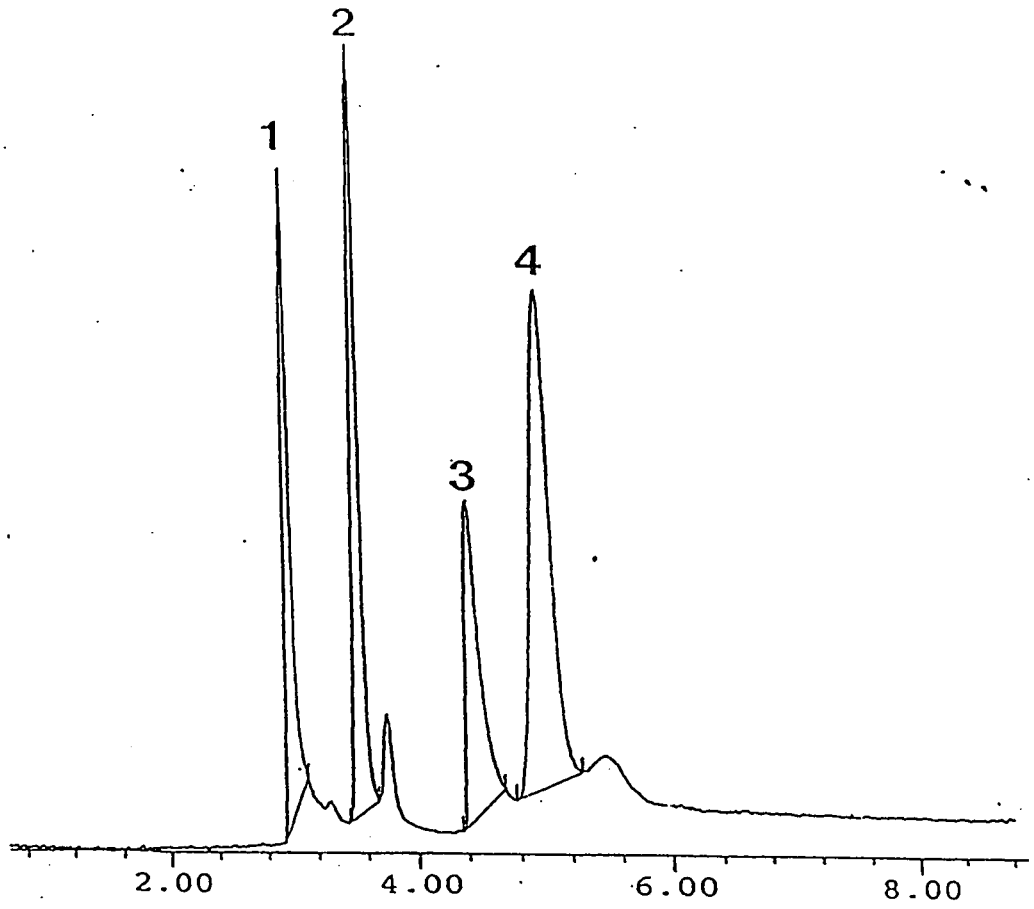


Figure 11. Electrochromatogram showing separation of proteins on diol capillary: effective length= 25 cm, detection at 210 nm, V=22 kV, I= 0.007 mA, electrolyte: 30 mM acetic acid + 30 mM GABA at pH 4.41. Peak identification: 1. Cytochrome C, 2. Lysozyme, 3. Myoglobin and 4. Ribonuclease A

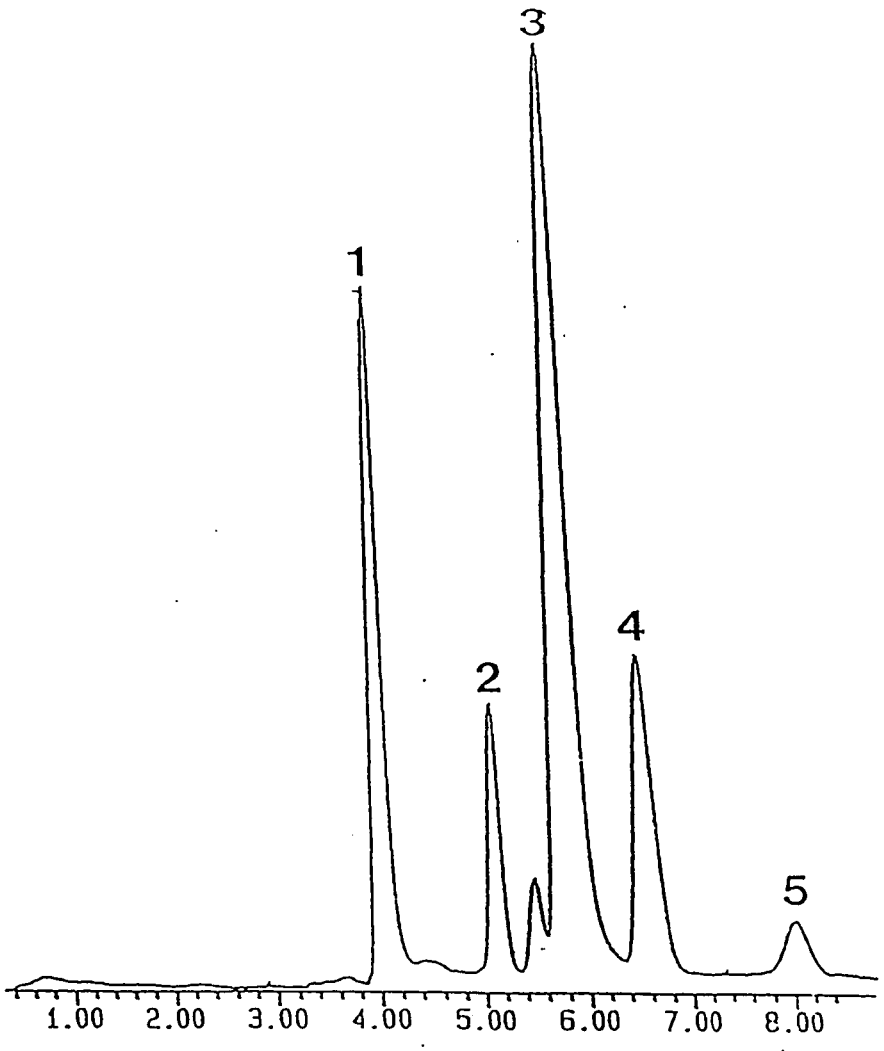


Figure 12. Electrochromatogram showing the separation of proteins on a C_{18} capillary: effective length= 25 cm, detection at 210 nm, V=25 kV, I= 0.011 mA, electrolyte: 30 mM citric acid + 25 mM β- alanine at pH 3. Peak identification: 1. Lysozyme, 2. Angiotensin III, 3. Bradykinin, 4. Ribonuclease A and 5. Angiotensin I

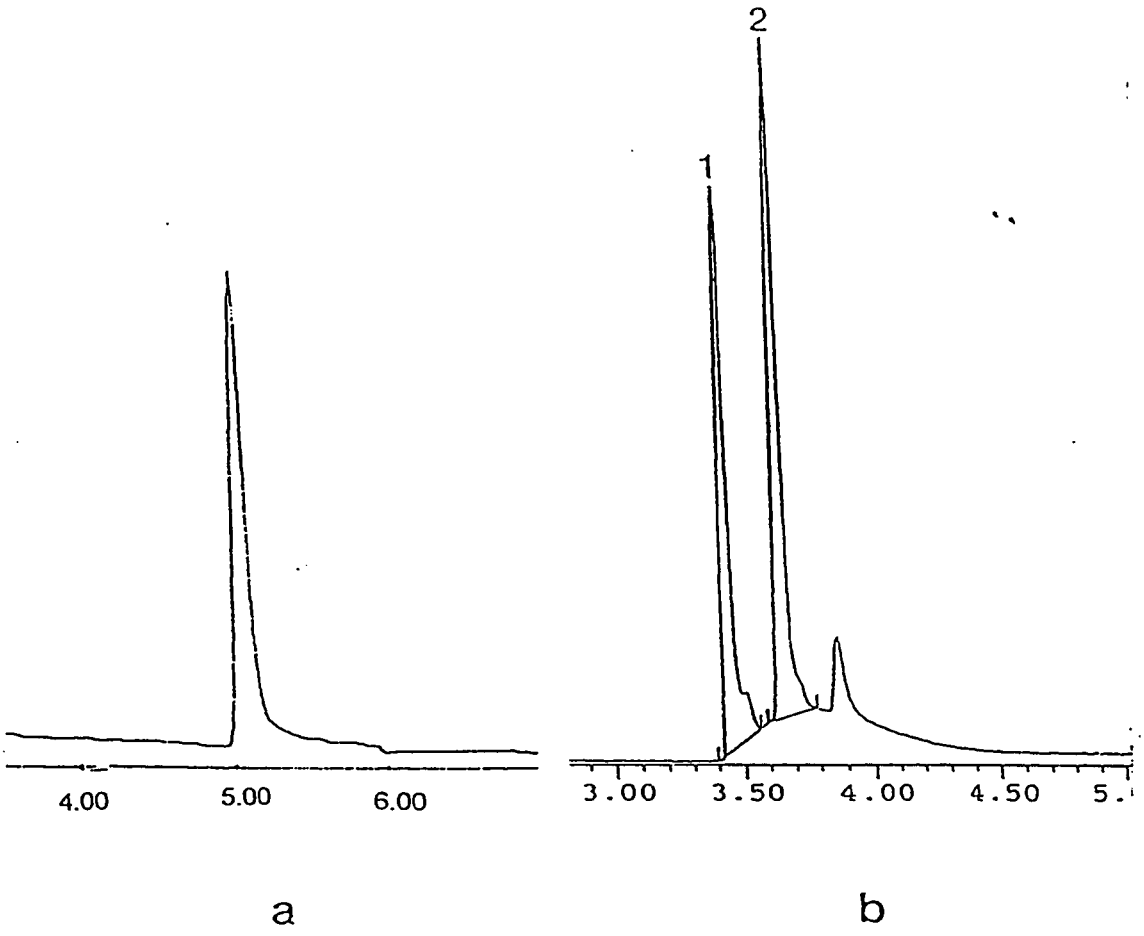


Figure 13. Comparison of the separation of horse and bovine cytochrome C on a. bare capillary and b. diol modified capillary respectively: effective length 25 cm, V= 22 kV, electrolyte 30 mM acetic acid + 30 mM GABA at pH 4.41, detection at 210 nm. Peak identification: 1. Bovine cytochrome C, 2. Horse cytochrome C

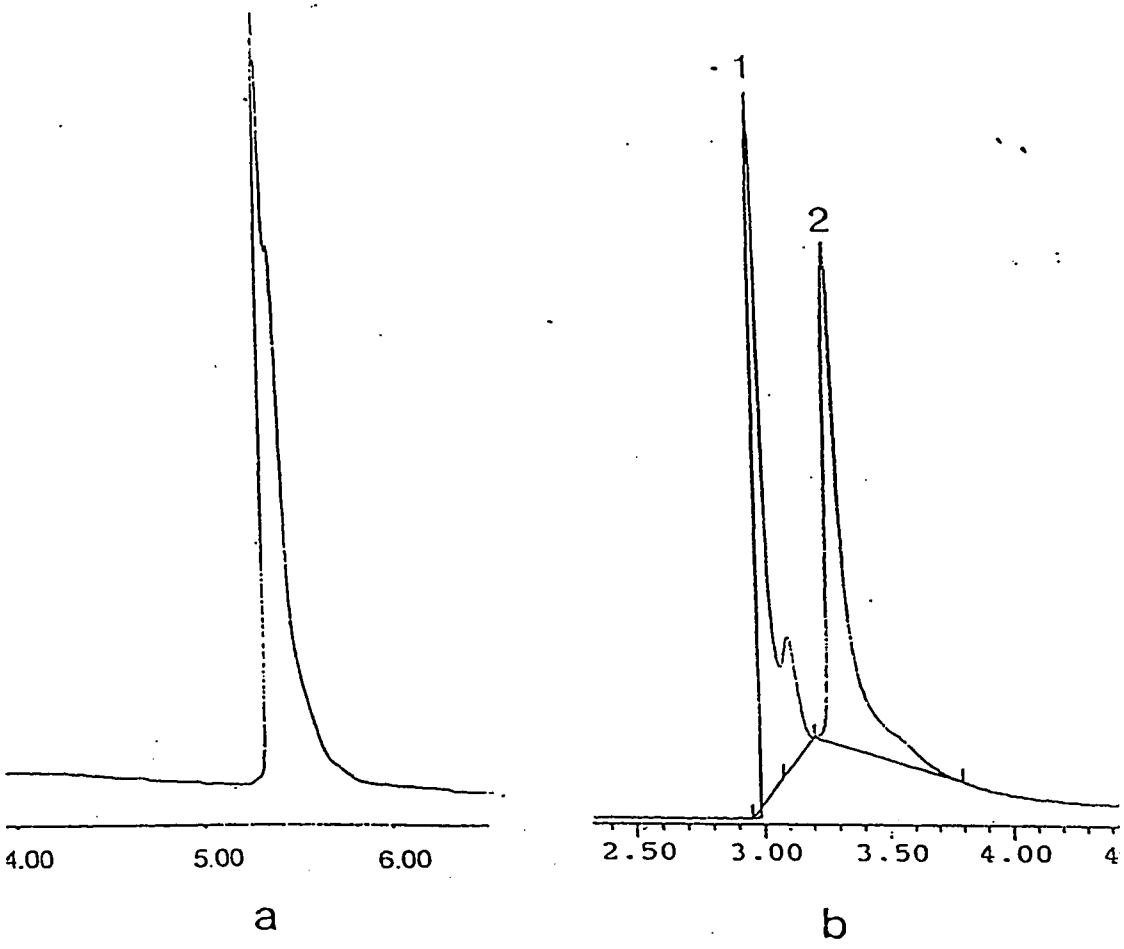


Figure 14. Comparison of the separation of chicken and turkey lysozyme on a. bare capillary and b. diol modified capillary respectively: effective length 25 cm, V= 22 kV, electrolyte 30 mM acetic acid + 30 mM GABA at pH 4.41, detection at 210 nm. Peak identification: 1. Turkey Lysozyme, 2. Chicken Lysozyme

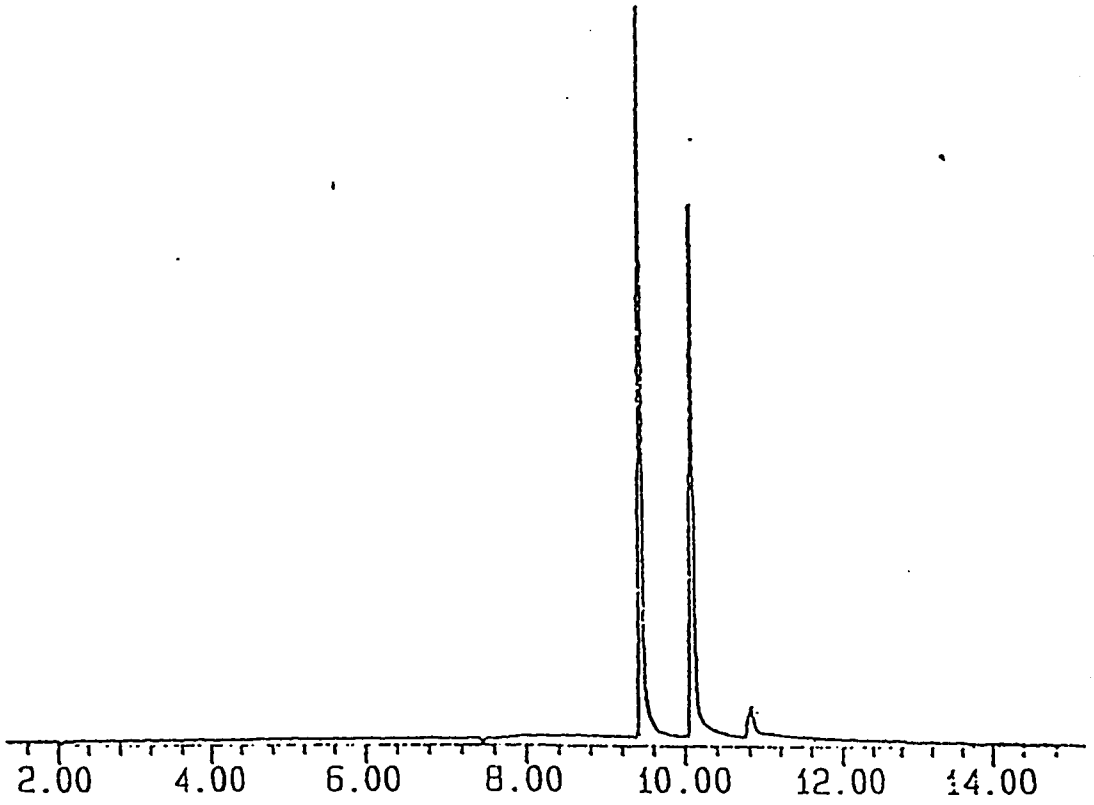


Figure 15.* Separation of horse and bovine cytocrome C by unetched diol modified capillary electrolyte acetic acid + GABA at pH 4.72, V= 22kV.

* Figure taken from reference 20.

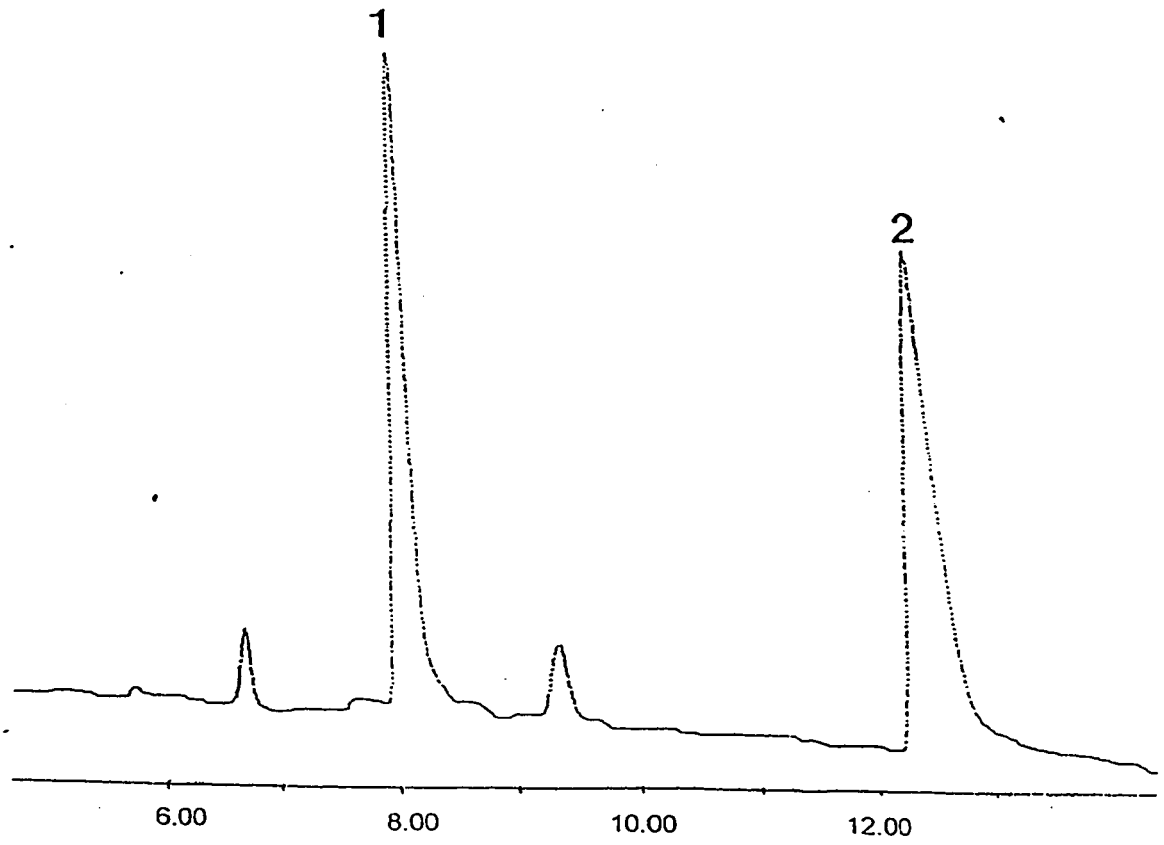


Figure 16. Separation of angiotensin I and II using C_{18} modified capillary: effective length= 25 cm, detection at 210 nm, V=25 kV, i= 0.011 mA, electrolyte: 30 mM citric acid + 25 mM β- alanine at pH 3. Peak identification: 1. Angiotensin I, 2. Angiotensin II

The interaction of proteins with the stationary phases (i.e. C_{18} and diol) can be seen in Figure 17. The increase in the peak width observed for bradykinin under the same conditions of pH, temperature and applied voltage suggest that the protein interacts with the stationary phase in C_{18} modified capillary. Due to the absence of the stationary phase in the bare capillary, there is no interaction of protein with the stationary phase and very little, if any, interaction with the hydride in the hydride modified capillary. Peak tailing is observed as a result of the protein-stationary phase interaction, which is C_{18} in this case giving rise to the peak assymmetry. Although the peak shape is a little distorted, due to the protein- stationary wall interaction, the retention time for each protein changes giving rise to the better separation.

The average theoretical plate count obtained for the modified capillaries is less than the bare capillary. This can be seen in Table 3.1 which shows the theoretical plate count for bare capillary and C_{18} capillary for bradykinin. As the peak width increases due to the protein-stationary phase interaction, theoretical plate count decreases and thus the efficiency, but better resolution is obtained. Theoretical plate count for diol and C_{18} capillaries is shown in Table 3.2. Lysozyme and ribonuclease A are bigger molecules than bradykinin, hence they tend to stick to the wall of the bare capillary. This results in distortion of peak shapes in the form of peak tailing, giving rise to such a large asymmetry that they were not used for the calculation of theoretical plate count.

Table 3.1 Comparison of theoretical plate count for C_{18} and diol capillary

capillary	Protein	Buffer	Plate count per meter
Bare	Bradykinin	pH = 3.7	400,000
C_{18}	Bradykinin	pH = 3.7	68,000

Table 3.2 Average theoretical plate count* obtained for C_{18} and diol capillary

Protein	capillary	plate count mean	% RSD
Lysozyme	C_{18}	31500/m	0.536
Ribonuclease A	C_{18}	32000/m	1.78
Lysozyme	Diol	23000/m	3.69
Ribonuclease A	Diol	35000/m	3.45

* based on 5 replicate measurements.

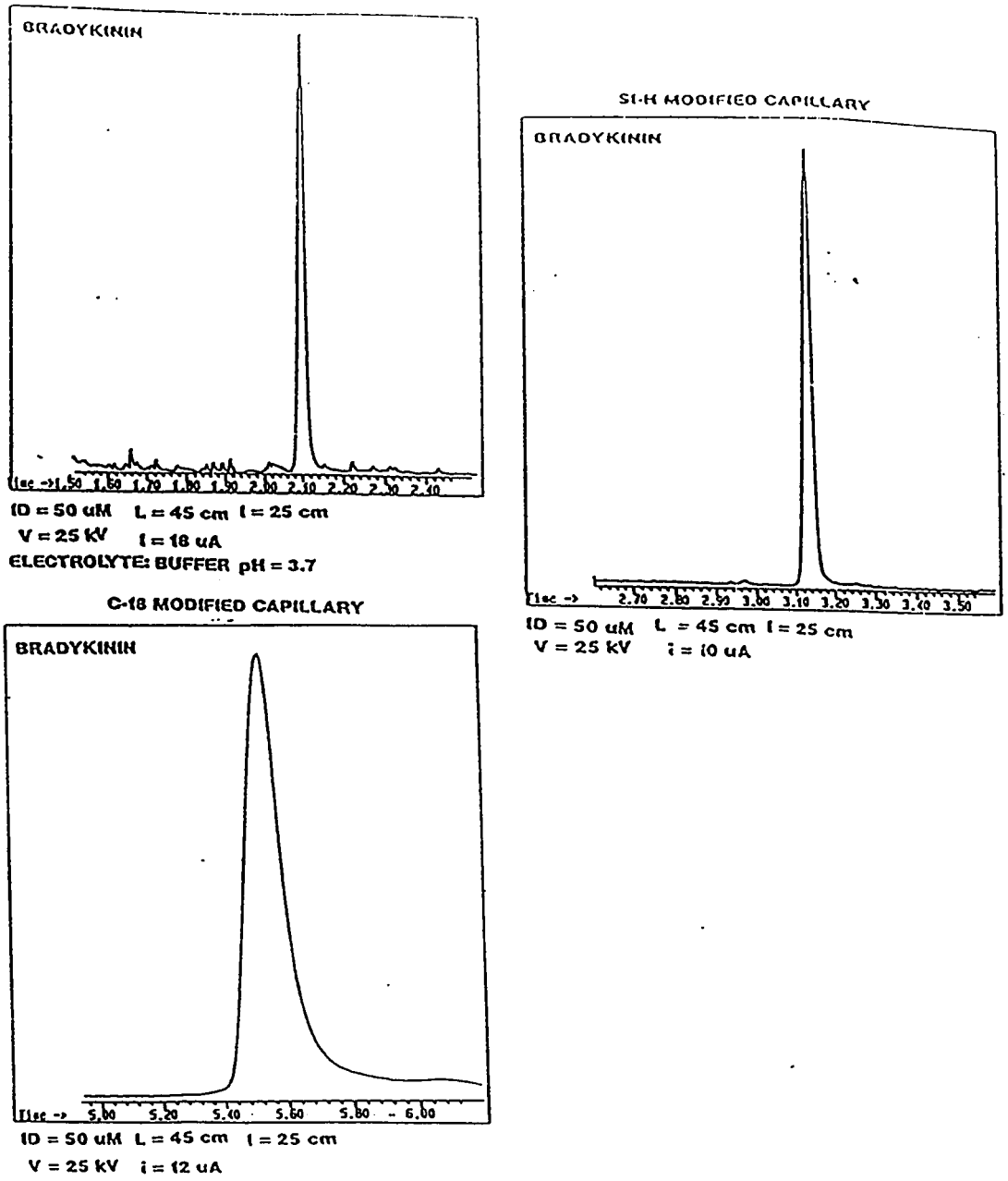


Figure 17. Comparison of the peak shapes for Bradykinin on bare, Si-H and C_{18} modified capillaries

3.3 Effect of separation variables on separation efficiency and peak symmetry

It is well known that peak symmetry and separation efficiency are affected by the separation variables like the electrolyte pH, applied voltage, sample concentration and injection volume. The electroosmotic flow can also affect these parameters.

3.3.1 Effect of electrolyte pH

The electrolyte pH affects the separation in many ways. Due to amphoteric nature of the amino acids building proteins, the sign and magnitude of the charge on the protein is affected by the pH of the electrolyte. Proteins are positively charged at a pH less than their pI and are negatively charged at a pH greater than their pI values. Since there is little or no electroosmotic flow to cause the migration of positive, negative as well as neutral charges in the modified capillary, it becomes necessary to adjust the pH to such a value that all the proteins in the mixture have the same type of the charge. During the present study, low pH buffers were used as electrolytes so that all the proteins in the mixture are positively charged.

Electrokinetic surface potential (zeta potential) is also influenced by the pH of the electrolyte. For unmodified capillaries, the surface becomes anionic at pH values above 2. For modified capillaries, silanophilic interactions of proteins with capillary wall are almost completely eliminated by surface modification. But if the bonded groups are not stable enough at extreme pH, they might undergo hydrolytic cleavage and, in this way, introduce the strong silanophilic interactions at those pH values. This is a particular problem at high pH. These interactions can cause a sudden increase in the electroosmotic flow in the

hydrolytically unstable modified capillaries resulting in the large variation in the retention times in electrochromatography. This can result in poor reproducibility, a decrease in separation efficiency and peak tailing due to protein adsorption.

The C_{18} capillary, due to its particularly hydrophobic nature, resembles reverse phase HPLC and hence could function in a similar manner in electrochromatography. When the capillary was used for the first time, no peaks were observed for any of the proteins listed in Table 2.1. For the unconditioned C_{18} modified capillary the current was 0.017 mA at 25 kV. It dropped after some time and fluctuated between 0 and 0.001 mA. The capillary was conditioned overnight with either acetonitrile or methanol and then flushed with buffer for 2 hours. After conditioning, good peak shapes were observed and it could be used without any further conditioning thereafter. No peak was observed for the neutral marker DMSO during a 40 minute run. This shows the drastic reduction in the electroosmotic flow. Assuming that the marker is eluted after 40 minutes, the maximum electroosmotic flow for 25 cm effective length of the capillary at pH 3.7 will be equivalent to the linear velocity 1.04 mm/sec.

No peaks were observed for PTH-amino acids, although peaks were observed for DNS- amino acids and proteins. Since amino acids can easily be separated by capillary zone electrophoresis, they were used in the present study as probes for the preliminary tests and no further studies were done. But it can be inferred that the PTH derivative, being more hydrophobic, interacts with the stationary phase and is in turn irreversibly adsorbed on the surface of the capillary. Dansyl derivatives do not adhere, as they are more polar due to the presence of ammonium and sulfoxide group.

When a pH 2.5 buffer was used, peaks were observed for the injected proteins, but they were very broad and showed some amount of tailing. This suggests the increased interaction of proteins with the stationary phase at lower pH values or physical changes in the proteins such as denaturation. Hence, the optimum pH was found to be in the range of 3 to 4. Similar to the higher pH buffer, no peaks were observed for PTH-amino acids or for the neutral marker DMSO. All these runs were 30 minutes long.

The diol modified capillary did not require any conditioning and could be used conveniently in the pH range of 3 to 4.5. Above and below this range the peak shapes were good, but separation efficiency was found to be reduced. Figure 18 shows the separation of a mixture of chicken and turkey lysozymes at pH 3.7 and 4.41. It can be seen that as the pH is increased to the optimum value, the charge separation between the two proteins increases giving rise to the better separation. The peak shapes are sharper and peak symmetry and separation are better at higher pH. Similar effect is also observed in the case of the mixture of horse and bovine cytochrome C.

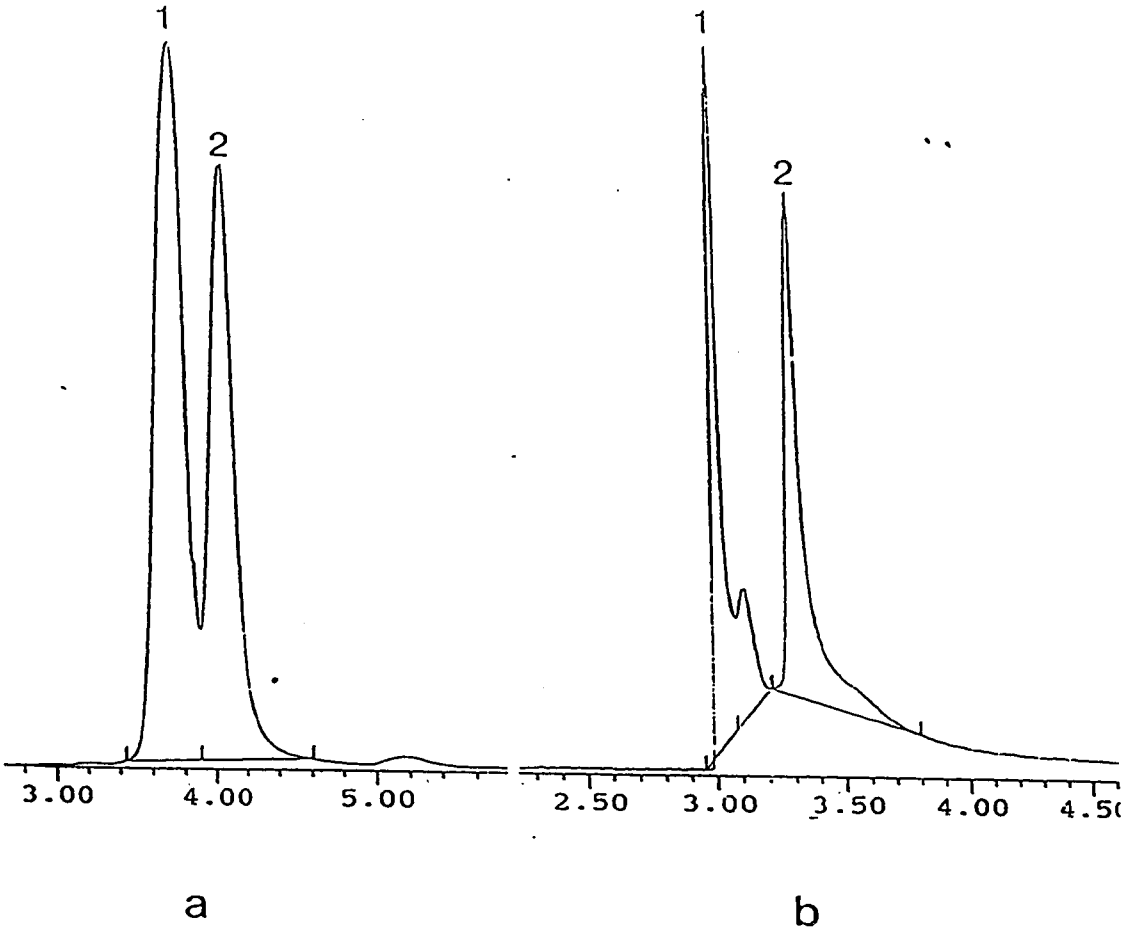


Figure 18. Effect of pH on the separation efficiency of diol modified capillary for the mixture of chicken and turkey lysozymes: a. pH = 3.7 b. pH = 4.41 effective length 25 cm, V= 22 kV, detection at 210 nm. Peak identification: 1. Turkey Lysozyme, 2. Chicken Lysozyme

3.3.2 Effect of organic modifier

Using a pH 3.0 buffer with 10% methanol cosolvent and an injection time of 20 seconds, tall and wide peaks were observed in the beginning. Even after diluting the samples and reducing the injection time to 5 seconds, large peaks were observed. Figure 19.a and b shows the comparison of the peak shapes with 10% and 50% methanol in the electrolyte. The conditions are similar to reverse phase HPLC where the mobile organic phase and stationary phase compete. Increase in the retention time with the increase in the concentration of organic modifier is observed similar to the reverse phase HPLC.

Addition of methanol to the buffer creates a dilution effect, resulting in the electrolyte with reduced ionic strength which can affect the peak shape as well as retention time. As the concentration of methanol in the electrolyte increases, retention time increases due to reduced ionic strength.

Ribonuclease A at 30 °C in pH 3.0 buffer with 50% methanol, undergoes almost complete denaturation.[22] This may result in more diffusion and hence increase in the peak width is observed.

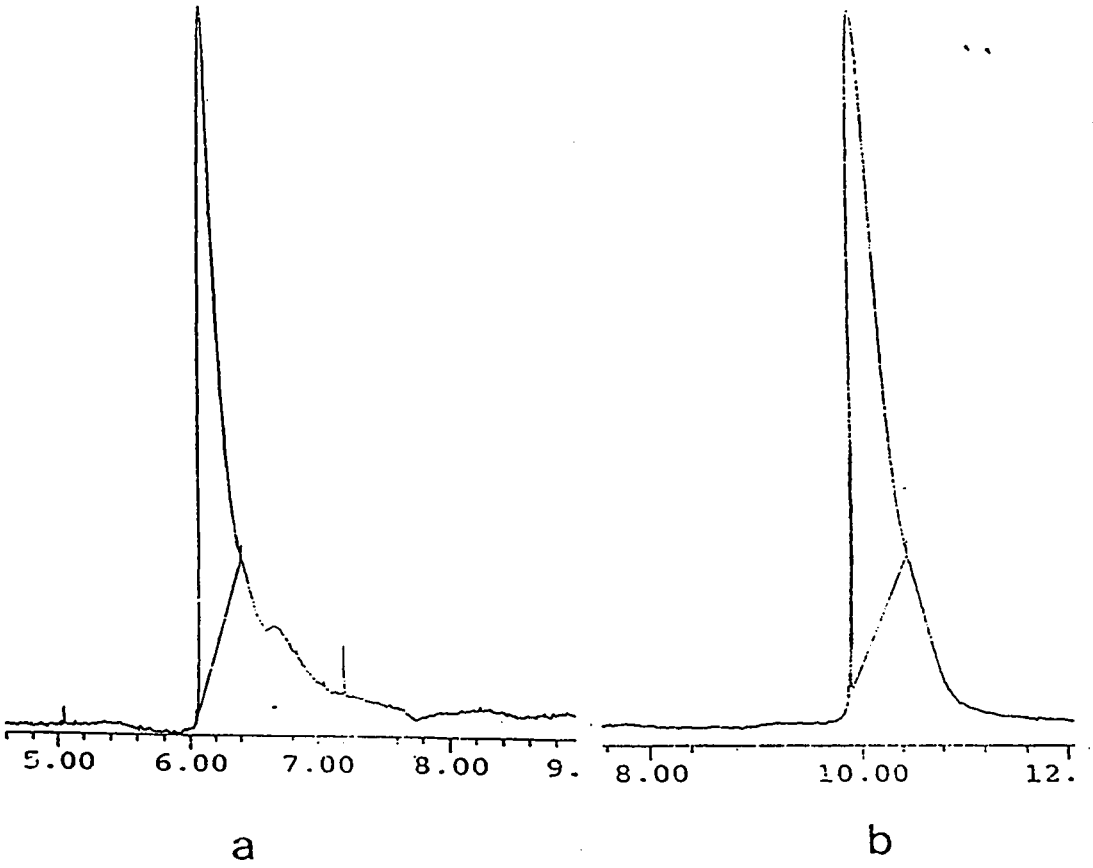


Figure 19. Comparison of the peak shapes of ribonuclease A with increase in the concentration of the methanol using C_{18} modified capillary with electrolyte citric acid + β alanine at pH 3.0 a. 10% methanol b. 50% methanol

3.3.3 Effect of applied voltage

The effect of applied voltage was studied for the separation of the protein mixture. This was done by changing the voltage in the range of 20 to 25 kV for 50 cm total length capillary. According to the following equation, the separation efficiency should be directly proportional to the applied voltage.

$$N = (\mu_{ep} + \mu_{eo})\, V/\, 2D \qquad \text{equation 3}$$

In practice it was found that the theoretical plate count was at its highest at 22 kV as shown in Table 3.3 which corresponds to an electric field strength of 440 V/cm. This result can be attributed to Joule heating caused by the system at higher voltage resulting in band broadening, thus decreasing the separation efficiency. Hence, even though theoretical maximum separation efficiency can be obtained at higher voltages, practically it is important to use the highest possible voltage with minimum heat generation.

Figure 20.a shows the separation of a cytochrome C mixture at 22 kV and Figure 20. b shows the same separation at 25 kV. A small but significant difference in the retention time and peak separation is observed. This can be attributed to the decrease in the current, which increases the retention time resulting in more interaction between protein and diol moiety. At a lower voltage the peak shape was much broader leading to poor separation.

Table 3.3 Theoretical plate count Vs electric field strength

Electric field strength	Protein	Plate count mean
400 V/ cm	Lysozyme	23100/m
440 V/ cm	Lysozyme	26000/m
500 V/ cm	Lysozyme	22100/m

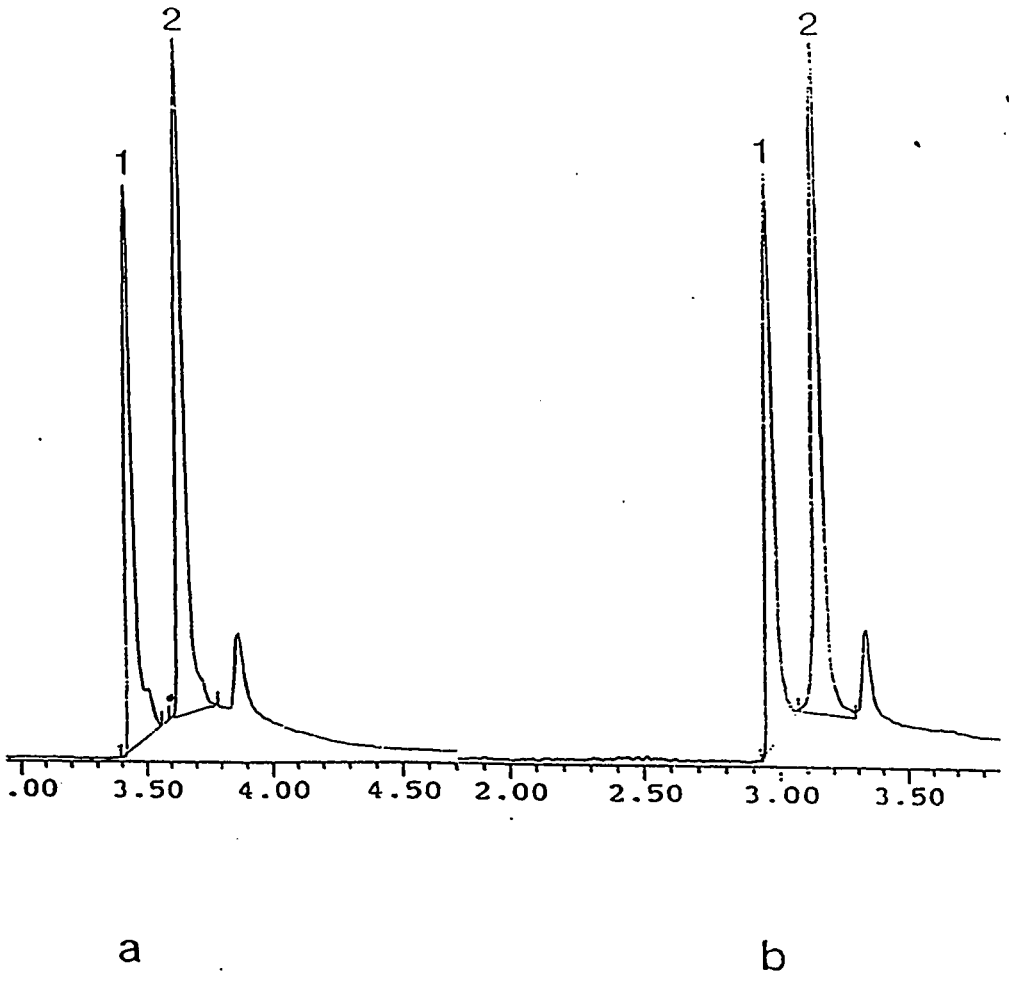


Figure 20. Comparison of the separation of horse and bovine cytochrome C at a. 22 kV and b. 25 kV: effective length 25 cm, electrolyte 30 mM acetic acid + 30 mM GABA at pH 4.41, detection at 210 nm. Peak identification: 1. Bovine cytochrome C, 2. Horse cytochrome C

3.3.4 Effect of injection volume and protein concentration

The injection volume and sample concentration can affect the separation efficiency and peak symmetry to a large extent. The effects are called volume overload and concentration overload respectively. The volume overload can be caused by increase in the injection volume with constant sample concentration, whereas concentration overload can be caused by increase in sample concentration with constant injection volume. Concentration overload results in distorted peak shapes and hence results in deviation of a/b ratio from 1. Fronting can arise due to concentration overload as can be seen from Figure 21. which is opposite to the tailing caused by sample-wall interactions. The fronting observed is due to the nonlinearity of adsorption isotherm at this protein concentration.[23] The overall effect of both these interactions is observed as band broadening. The measure of peak symmetry a/b ratio is much smaller than that for the lower concentration of about 0.2 mg/mL.

The injection volume overload results in decreased separation efficiency due to large peak size although peak symmetry and hence the a/b ratio remains practically unaffected. As the injection time and hence the injection volume is reduced and the concentration is reduced to 0.2 mg/mL the peaks were observed to be sharper resulting in better separation.

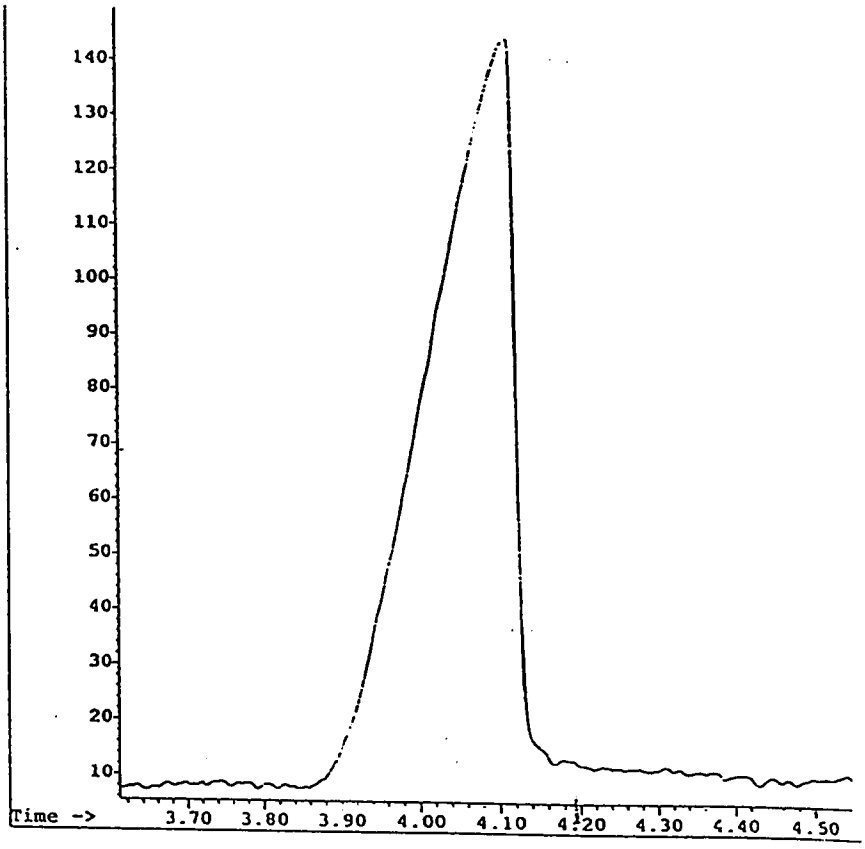


Figure 21. Effect of concentration overload on the peak shape for lysozyme on diol modified capillary, with sample concentration 0.6 mg/mL.

3.4 Precision of protein retention

Short term precision of protein retention was tested by the comparison of retention times of turkey lysozyme and ribonuclease A over 30 injections. Figure 22 and Figure 23 show graphs of retention times of these proteins as the function of injection number using the C_{18} modified capillary and The diol modified capillary respectively. As can be seen from the figures, good reproducibility was observed over the 30 injection test. An initial decrease in the retention time for ribonuclease A could be accounted for by a decrease in the temperature during that time. No adsorption on the capillary wall was noticed during these runs. The relative standard deviations for 30 injection test on the C_{18} and the diol modified capillaries are given in Table 3.4.

Long term precision of protein retention was tested as the ratio of retention times of ribonuclease A and turkey lysozyme over a period of 5 days. The buffers were prepared fresh by dilution from the stock every day. Due to large temperature fluctuations, parallel variations in the retention times were observed but the overall reproducibility was observed to be good. Figure 24 and Figure 25 show graphs of the ratio of retention times as the function of injection day.

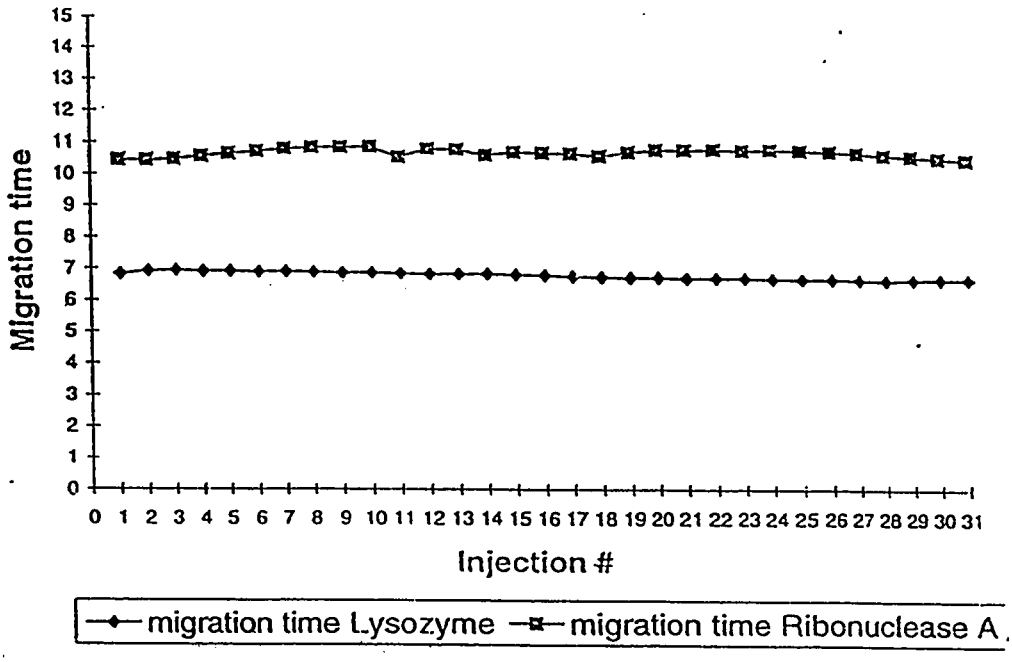


Figure 22. C_{18} capillary, conditioned overnight with acetonitrile, electrolyte citric acid + β- alanine at pH 3.0, degassed with Ar for 30 minutes, l= 25 cm, V= 25 kV, I= 0.014 mA, detection wavelength 211 nm. injection 5 sec at 5" vac. Capillary was flushed after each run.

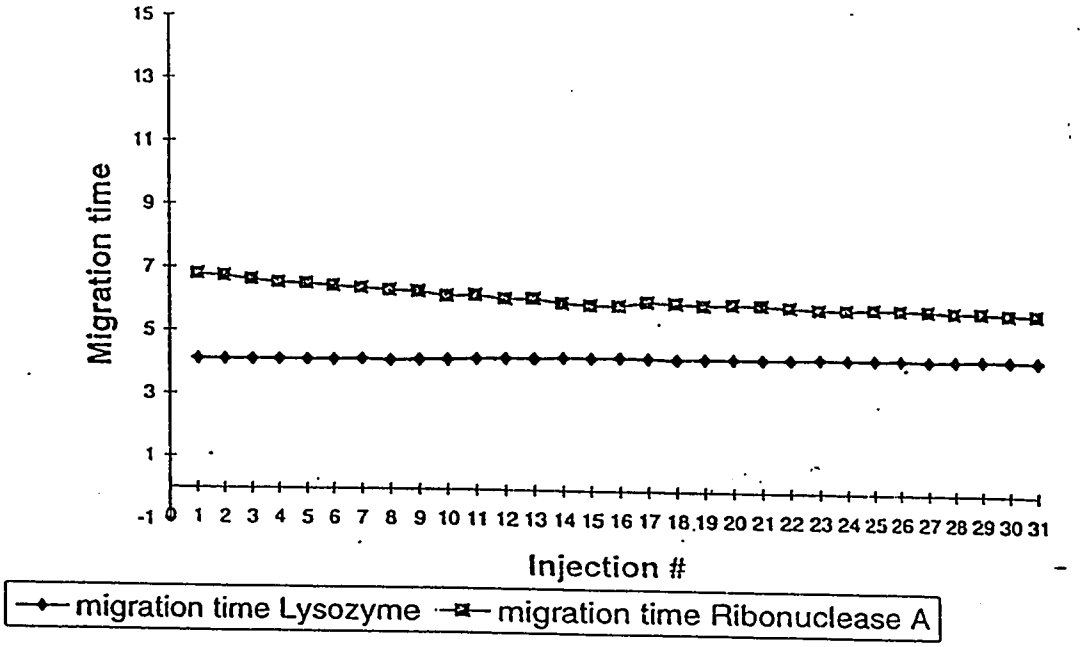


Figure 23. Diol capillary, electrolyte citric acid + β- alanine at pH 3.7, degassed with Ar for 30 minutes, l= 25 cm, V= 20 kV, I= 0.009 mA, detection wavelength 210 nm. injection 2 sec at 5" vac. Capillary was flushed after each run.

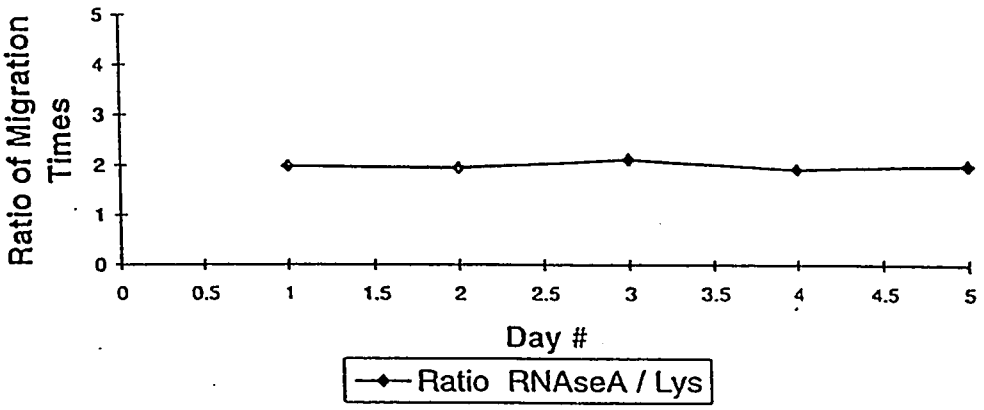


Figure 24. C_{18} capillary, conditioned overnight with methanol, electrolyte citric acid + b- alanine at pH 3.0, degassed with Ar for 30 minutes, l= 25 cm, V= 25 kV, I= 0.011 mA, detection wavelength 211 nm. injection gravity for 10 secs. Capillary was flushed after each run.

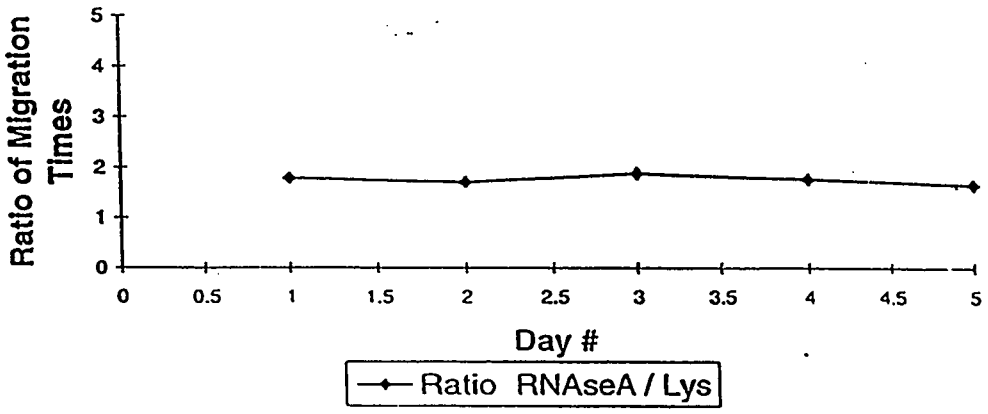


Figure 25. Diol capillary, electrolyte citric acid + b- alanine at pH 3.7, degassed with Ar for 30 minutes, l= 25 cm, V= 20 kV, I= 0.005 mA, detection wavelength 210 nm. injection 10 sec, gravity. Capillary was flushed after each run.

Table 3.4 Relative standard deviation for retention times

Capillary	Protein	% RSD
C 18	Lysozyme (Turkey)	1.57
	Ribonuclease A	1.18
Diol	Lysozyme (Turkey)	1.21
	Ribonuclease A	4.84

From these tests it can be concluded that both the C_{18} and diol modified capillaries show good precision with the exception of ribonuclease A where temperature fluctuations were observed. Short term precision tests were performed for lysozyme on a new capillary and for ribonuclease A on the same capillary after 250 runs. These results show good reproducibility and stability over a large number of injections. The peak symmetry was calculated as the ratio of a/b for 5 injections and is given in Table 3.5. As explained earlier, the peaks are not symmetrical due to the interaction of proteins with the stationary phase but peak shapes are reproducible. Even after 250 runs, the protein mixture could be separated without any loss in the separation efficiency with essentially the same retention time.

Table 3.5 Peak symmetry for 5 injections

Capillary	Protein	a/b ratio	% RSD
C 18	Lysozyme	1.39	5.01
Diol	Ribonuclease A	1.26	3.15

4. CONCLUSIONS

The capillaries modified first by etching and then through formation of Si-C linkages via a hydrosilation/organosilation reaction scheme are well suited for the development of the new technique capillary electrochromatography. Complex mixtures of the proteins can be easily separated using these capillaries. The organic moiety attached to the capillary wall acts as a stationary phase giving rise to protein-stationary phase interactions and eliminating protein-silanol interactions thereby increasing the separation efficiency and reproducibility in protein retention times due to a decrease in the electroosmotic flow. The capillaries were stable over a large number of injections and showed good reproducibility. It can be concluded from these results that the newly developed technique can be used for stable separations with precision and a long life time for each capillary. Good separations can be obtained with protein concentrations of 0.2 mg/ mL and with small sample volumes at an electric field strength of about 440 V/ cm. With a homemade HPCE , the temperature and injection volume can not be controlled precisely, but with the availability of a more advanced automated instrument improved results by the new technique can be expected.

5. FUTURE STUDIES

Along with the separation of proteins, the technique can also be used for separation for other compounds like tetracyclines and some other drugs. The capillaries could be modified with a chiral compound like R (+) or S (-) (1-Naphthyl)ethylamine that have been used for enantiomeric separation of compounds such as amino acids, substituted urea and carbamates.

An extensive study for optimization of etching conditions, as well as hydride modification is required. With controlled hydride modification, electrochromatographic technique can be used for the separation of neutral compounds, as electroosmotic flow can be altered by changing the number of silanol groups on the capillary surface.

The effect of organic modifiers like methanol and acetonitrile on the separation can be studied in detail, with increase in the concentration of organic modifier, similar to reverse phase HPLC. As a new technique electrochromatography has a lot of possible applications which can be explored in future.

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