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Mixed mode CE-CEC separation using 20 [micrometer] fused silica capillaries

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Mixed Mode CE-CEC Separation Using 20μm Fused Silica Capillaries

A Thesis

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The Faculty of the Department of Chemistry

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In Partial Fulfillment

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Master of Science

by

Sungjin Cho

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APPROVED FOR THE DEPARTMENT OF CHEMISTRY

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ABSTRACT

Mixed Mode CE-CEC Separation By 20 μm Fused Silica Capillary

by Sungjin Cho

Capillary electrophoresis (CE) and capillary electrochromatography (CEC) are emerging technologies in the next generation of separation science. Open tubular capillary electrochromatography (OTCEC) using a 20 μm inner diameter (I.D.) fused silica capillary was investigated. Smaller inner diameter capillaries can result in higher efficiencies and hence better separation due to enhanced heat dissipation and a smaller temperature difference between the wall and center of capillary. To make the open tubular format, the inner surface of the capillary was etched to increase the surface area and then modified with an organic moiety by silation/hydrosilation reaction schemes.

Separations of tetracyclines, proteins, and peptides were performed to characterize the CE/CEC mixed mode using 20 μm capillaries, and aspartame, a commercial product, was tested to analyze electric field, voltage, linear velocity, and reproducibility. Scanning electron microscopy (SEM) was used to investigate the surfaces of the etched inner bore. The effects of buffer pH and voltage on the separation of various mixtures in each of the capillaries were compared.

ACKNOWLEDGMENT

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Of all things, I can never thank my wife, Yoonmi Cho enough. My academic fructification can not be possible without her love, sacrifice, and pray for me. I am also thankful to my parents, grand mother, and sisters for their support and aid in my academic journey.

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1. INTRODUCTION

1.1 Historical Perspective

The foundation for capillary electrophoresis (CE) and capillary electrochromatography (CEC) developed from improvements in electrophoresis and instrumentation technology. Compton and Brownlee summarized the history of the technology; the first modern capillary zone electrophoresis (CZE) system was developed in 1967.[2] In this system, 3-mm I.D. capillaries were rotated to diminish the effects of convection in the instrument. The development of CE is based on the implementation of smaller I.D. capillaries in the 1970s, and a new era of high performance capillary electrophoresis (HPCE) was made possible by solving the perplexing problems of injection in 1981.[2]

In the 1980s, many modes of CE were developed including gel electrophoresis, isoelectric focusing, and micellar electrokinetic capillary chromatography (MECC).[2] Other significant technologies related to CE development include improvement of ultraviolet (UV) detection and interfacing to a mass spectrometer (MS).

Although the expansion of CE was slow after Microphoresis Inc. introduced the first commercial instrument in 1988, many international symposia were begun to disseminate ideas about the new technology, and HPCE is often regarded as a valuable alternative to high performance liquid chromatography (HPLC) because of low solvent consumption, increased mass sensitivity, and high separation selectivity. CEC is regarded as a new analytical technique and is in a state of rapid development.

1.2 Principles and Concepts

Separation science including surface analysis is an emerging technology area, which has a high potential for the future. HPLC is based on the partition between a mobile phase and a stationary phase. CE utilizes the electroosmotic flow of the electrolyte and the electrophoretic mobility of the analyte.[1,6,10] As a substitute for HPLC, HPCE exhibits a much higher efficiency of separation.

CEC combines the advantages of both HPLC and HPCE. CEC is a hybrid of HPLC and CE because the separation process is based on the partitioning between phases like HPLC and it is also based on electroosmosis as the main driving force that transports the buffer and analyte through the column.[6,7,8]

1.2.1 Electroosmotic flow

When a buffer flows inside a capillary, the inner surface of the capillary can be charged due to the ionization of the inner surface. Positive ions that are close to the capillary surface carry the bulk buffer solution toward the negative cathode with a constant velocity as a result of the double layer inside the capillary. If an electric potential is applied, the cations in the mobile layer near the center of the capillary create an electroosmotic flow as represented in Figure 1.[1]

The velocity of the electroosmotic flow, v_{EOF}, is given by[10]

$$v_{EOF} = \varepsilon\zeta E/4\pi\eta$$

ε: dielectric constant of the run buffer, ζ: zeta potential

E: applied electric field in V/cm, η: viscosity of the buffer

The electroosmotic mobility, μ_{EOF}, of the buffer is given by

$$\mu_{EOF} = \varepsilon\zeta/4\pi\eta$$

Electroosmotic mobility is dependent on buffer characteristics and independent of the applied electric field.

1.2.2 Electrophoresis

An electrically charged solute can migrate through a buffer when an electric field is applied. This migration is due to the electrophoretic mobility which is illustrated in Figure 2.[10]

Electrophoretic velocity, v_{EP}, in cm/s, is defined as follows:[1,4]

$$v_{EP} = \mu_{EP}E$$

μ_{EP}: electrophoretic mobility

E: applied electric field

Electrophoretic mobility is defined by the following equation:

$$\mu EP = q/6\pi\eta r$$

q: charge of the ionized solute

η: buffer viscosity

r: solute radius

From these equations, it can be seen that electrophoretic velocity is dependent on both electric field and electrophoretic mobility which is dependent upon solute and buffer properties. Electrophoretic mobility can be changed by the charge or size of a solute or the buffer viscosity.

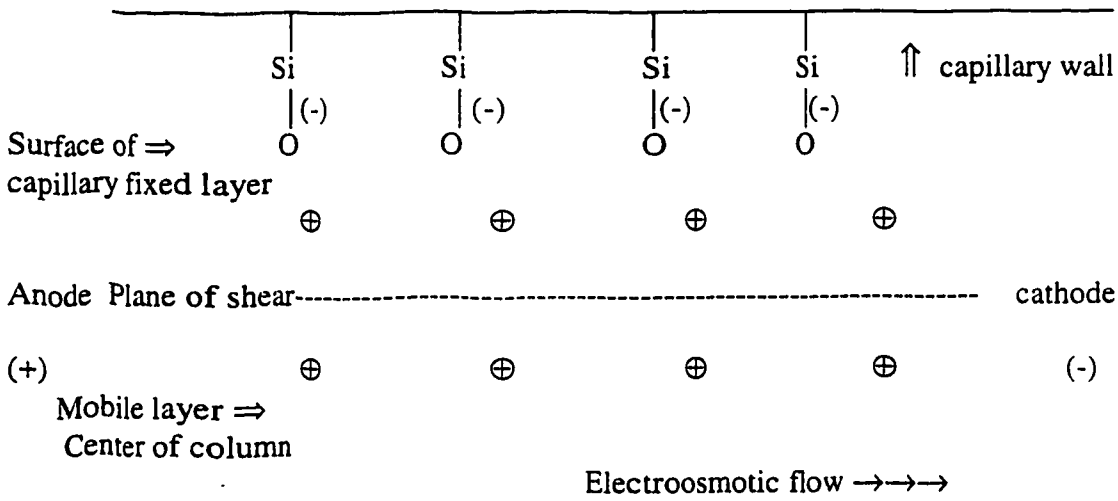
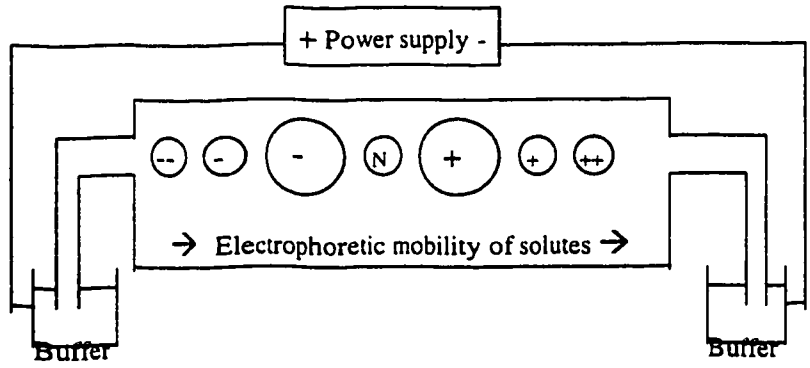


Figure 1. Representation of electroosmotic flow in a capillary



Electrophoretic mobility $\mu EP = q/6\pi\eta r$ (q: charge of ionized solute, η: buffer viscosity, r: solute radius)

Figure 2. Electrophoretic mobility

1.2.3 Capillary electroseparation

Capillary electroseparation (CES) techniques can be carried out in open or packed columns. These techniques can separate both charged and uncharged species due to the differences in either their electrophoretic mobilities or partition coefficients between two phases.

Currently distinct CES techniques are carried out in open or packed tubes. CE and capillary micellar electrochromatography (CMEC) are carried out in an open tube. In CMEC, the separation is primarily based on partitioning between two phases, the electrolyte and the micelles.[1,10]

Packed tubes are usually used in CEC and capillary gel electrophoresis (CGE) systems. In the CGE system, the solutes are separated in a capillary filled with a polymer gel. Capillary electrochromatography was developed using an open tubular format. This process overcomes many problems associated with packed capillaries which include bubble formation, packing difficulties, and the design and use of frits.[1]

A fused silica capillary can be used as a detector cell for ultraviolet/visible (UV/vis) or fluorescence spectroscopy because the capillary material is transparent to UV and visible light. A window is placed in the path of light and the solutes are detected in the capillary.[2,4]

1.2.4 Effect of tube diameter

To induce electrophoretic mobility, the inside of a capillary is filled with a conductive aqueous buffer. The buffer in the column is affected by the temperature. In a

round tube, the temperature difference between the center and the wall of the tube, ΔT, can be calculated from [1,2]

$$\Delta T = (0.239Q/4k)\, r^2$$

where Q is the power density in watts/m^3, k the thermal conductivity, and r the tube radius. If the radius is reduced, there will be a smaller temperature difference. This reduced temperature difference results in less convective diffusion and narrower zones (separated independent non contiguous area). Temperature control of the capillary is important because a lower capillary temperature allows the use of higher voltage to give faster separation.

As the inner diameter of the capillary is reduced, there is more electrical resistance and less current is generated for a given voltage. As a consequence, less Joule heat caused by current in the conductor is produced. Joule heat is given by I^2Rt (I: current, R: resistance, t: time). In addition to less heat being generated in a capillary with a smaller radius, the heat can be dissipated more quickly from a smaller tube because a decrease in the diameter of the capillary gives an increase in the inner surface area-to-volume ratio of the tube.[1,2]

By reducing the tube radius, the position of solute inside the capillary is restricted and lead to an averaging of the cross-sectional position of the sample in the capillary. If the tube radius is reduced, the solutes will spend more time moving across the entire tube radius, and will tend to move through the tube as a narrow zone. Therefore the technique was named capillary zone electrophoresis (CZE).[1,2,10] Smaller diameter capillaries (20-75 μm) are more desirable because they give better separations by enhanced heat

dissipation and smaller temperature differences between the center and the wall of capillary.

1.2.5 Capillary material and placement in instrument

Polyimide-coated silica fused tubes used for CE and CEC are made of the same material that is used in capillary gas chromatography (Figure 3).[2] Fused silica material has several relevant characteristics including UV transparency, durability, and a zeta potential. Solute-capillary wall interactions can limit separations, but these undesirable effects may be reduced by buffer additives or functionalizing the inner wall surface.[2,13,14]

There are two systems for inserting the capillary into an instrument. One system uses a cartridge holder while the other places the capillary directly into the machine. Both of them have advantages and disadvantages. The cartridge system makes inserting a new capillary simple. Aligning the detection window with the instrument optics is easy in this system, too. This cartridge may be combined with a cooling system. The disadvantages are higher cost, difficulties with using capillaries of different designs, and slow changeover of capillaries within a single cartridge.[2,13,14] The direct insertion system does not use any holder for the capillaries. In the direct system, changeover of the capillary is fast, and use of specialty capillaries is simple. However it is easy to break a capillary during insertion.

1.2.6 Effect of pH buffer on electroosmotic flow

The effect of pH on electroosmotic flow (EOF) and mobility is illustrated in Figure 4.[2] Electroosmotic flow increases as pH increases because there is more dissociation of Si-OH to Si-O^- on the inner capillary wall at higher pH. The zeta potential

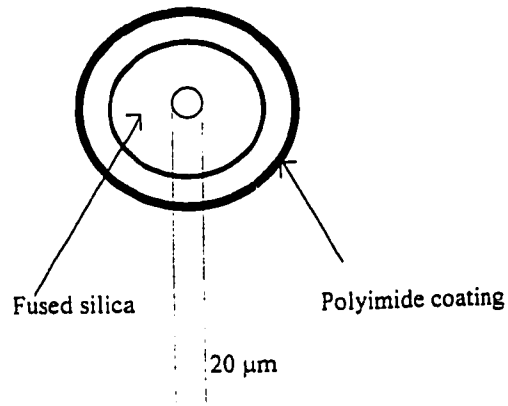
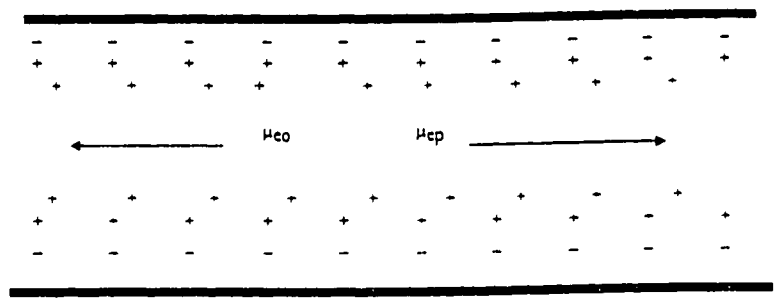


Figure 3. Cross-section of capillary

(a) High pH



(b) Low pH

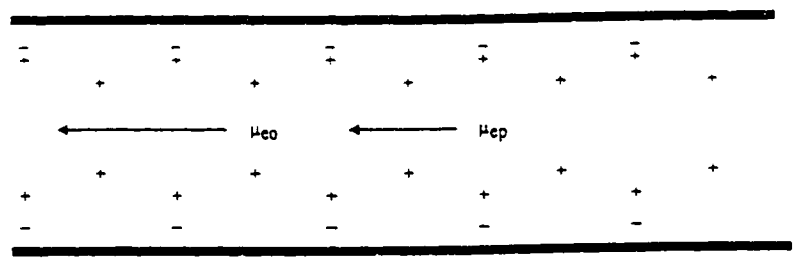


Figure 4. Impact of pH on the EOF at (a) high pH and (b) low pH
(Negative charges closest to the surface come from ionized silanol group)

is proportional to the surface charge on the capillary inner surface as given by $\zeta = 4\pi\delta e/\varepsilon$ (δ:thickness of diffuse double layer, e: charge per unit surface area, ε: dielectric constant of run buffer). A strong zeta potential and a dense double layer can be made at higher pH as the silanol groups are fully ionized.

The solutes can electromigrate toward one of the electrodes. If the EOF is very strong, all charged solutes will migrate toward the negative electrode. At lower pH, there is less surface ionization and a lower zeta potential. At a pH below about three, there is no electroosmotic flow in a fused silica capillary because the silanol groups are protonated. If the pH is low, a zwitterion will have a positive charge and will migrate toward the negative electrode.[2,3,4] The pH of the buffer influences the degree of ionization of the solutes and electrophoretic mobilities.

The EOF can be reduced or reversed by using buffer additives or capillary treatment. A change in EOF causes shift in migration times because all solutes are moved through the capillary by electroosmosis.[5]

1.2.7 Sample injection

a. Hydrodynamic injection

Hydrodynamic injection is performed by either pressure or vacuum. Injection by pressure may be done by placing the inlet of the capillary into a sample vial and applying a pressure as shown in Figure 5.[1] The volume of sample injected can be calculated by $V = \Delta P r^4 \Pi / 8\eta L$ (ΔP: pressure across the capillary, d: capillary inner diameter, t: time, η: viscosity of sample, L: total capillary length). A gravity injection is made by placing the

sample vial at a higher position than the destination vial. The actual volume of injection is given by $V = 2.84\times10^{-8}Htd^4/L$ (H: the height that sample is raised).[18,19]

b. Electrokinetic injection

Electrokinetic injection involves placing the capillary and the anode into the sample vial, and then applying a voltage. This electric field causes the sample ions to migrate into the capillary due to electroosmosis and electrophoretic mobility as shown in Figure 6.[1] The quantity injected is given by $Q = V\pi Ctr2(\mu_{EP}+\mu_{EOF})/L$ (V: voltage, C: concentration, t: time, μ_{EP}: electrophoretic mobility, μ_{EOF}: electroosmotic mobility)

1.2.8 Principles of chromatography

Chromatography is the technique in which the components of a mixture are separated based on the rate at which they are carried through a stationary phase by a mobile phase. All chromatographic separations are based on differences in the extent to which solutes are partitioned between the mobile and stationary phase. The partition ratio is defined as $K = C_s/C_m$ (C_s: molar analytical concentration of a solute in the stationary phase, C_m: molar analytical concentration in the stationary phase).[12]

The efficiency of a chromatographic column refers to the amount of band broadening that occurs when a compound passes through the column. Plate height (H) and the number of theoretical plates (N) are related by the equation: $N = L/H = 16\,(t_R/w)^2$ (t_R: retention time, w: width of peak at base). The efficiency of a column for separation by chromatography can be also described by the Van Deemter equation that is defined by $H = A + B/u + Cu$ (u: flow rate of mobile phase, H: plate height, A/B/C: constants

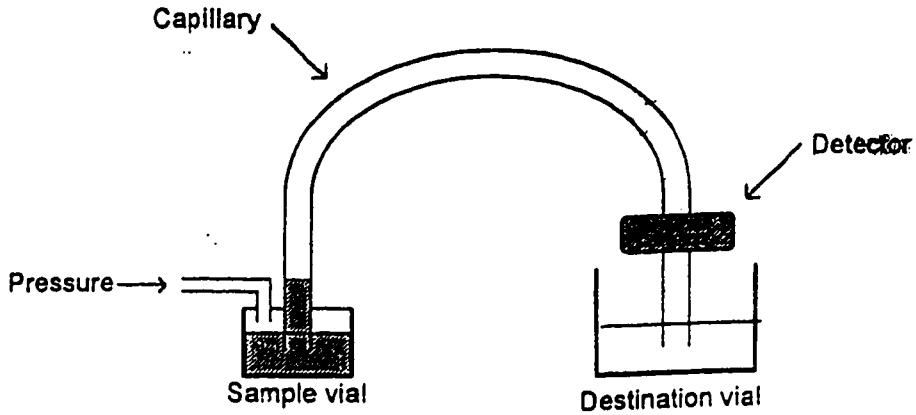


Figure 5. Pressure hydrodynamic injection

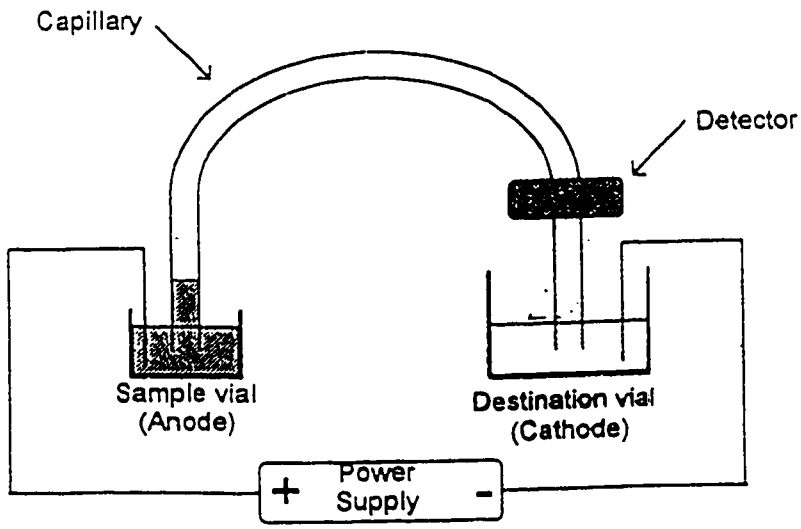


Figure 6. Electrokinetic injection

measuring contributions of the different band-broadening processes to the quantity H. Multiple paths of the mobile phase (A), molecular diffusion (B), and the effect of mass transfer in the mobile and stationary phase (C) are the main factors in the efficiency equation.[12]

1.3. Column Technology

The rapid progress of CE and CEC in recent years is the result of the availability of high-quality fused silica microcapillaries and advances in column technology. In CE, the major aim of using capillaries is the achievement of effective heat dissipation that is necessary for high efficiencies at high-applied voltage. To further improve the performance of the technique, several other factors need to be considered. First, there are currently very limited types of high-quality capillary materials which have the necessary thermal, chemical, and physical properties, and which are available in very small dimensions.[1,14,15] Additionally, the interaction of analytes with the inner surface of the capillary may have an effect on the migration of certain molecules. By applying a suitable surface coating, the properties of the surface can be manipulated.[1]

1.3.1 Etching of silica.

Fused-silica capillaries can be etched to increase the surface area and utilized in a CEC system. This etching procedure was developed for modification of larger diameter capillaries used in gas chromatography (GC). A similar method was adapted for CEC. In the GC system, the surface of inner capillary is etched to increase the surface area available for coating.

As a result of etching procedures, the inner surface of the tube possesses radial extensions from the surface that can lead to solute-bonded phase interactions.[6] Ammonium hydrogen difluoride is used as the etching agent at controlled concentration, temperature, and reaction time. This etching process sufficiently increases the inner surface area to produce solute/bonded phase interaction for analytes.

1.3.2 Surface modification

The modification of the etched capillary involves silation and hydrosilation reactions. This modification process results in a direct silicon-carbon bond on the surface and the stability at the attached organic moiety is increased.[1,7,8]

$$\text{-Si-OH} + (\text{OEt})_3\text{Si-H} \longrightarrow \text{-SiO-Si-H} + 3\text{EtOH}$$

$$\text{-Si-H} + \text{R-CH=CH}_2 \xrightarrow[\text{catalysis}]{} \text{-Si-CH}_2\text{-CH}_2\text{-R}$$

The first reaction is to form a hydride intermediate from the surface silanol group. In the second reaction, a metal complex such as hexachloroplatinic acid is used as the catalyst. This method, modifying the inner wall of the capillary first by TES silanization, converts virtually all of the silanol groups to silicon hydride. With this minimized number of adsorption sites, the electroosmotic flow is reduced drastically, which causes the migration time to increase.[6,16] The attachment of the organic moiety occurs via a direct silicon-carbon linkage. C_{18} is used as the organic moiety for hydrosilation in this study. C_{18} gives non-polar interactions with the analytes. The surface coverage is described in terms of $\mu mol/m^2$ and can be calculated by $10^6P/(100M_cn - PM_R)$ where P: % carbon, M_R: molecular weight of bonded molecules, M_c: atomic weight of carbon,

n: # of carbons in the bonded phase. An increase in solute-bonded phase interactions enhances separation of analytes along with differences in electrophoretic mobility.

1.4 HPLC/HPCE/CEC

In HPLC, the chromatogram exhibits broader and shorter peaks when the retention time is longer because the solutes are diluted inside the column with an increase in time. In a CE electropherogram, peak heights are almost constant in repeated separation because the solutes move toward the detector in a zone in which length and concentration remain constant as shown in Figure 7.[1] The electroosmotic flow of CE exhibits a more flat flow than the pumped flow of HPLC (Figure 8). In electroosmotic flow where the flow profile is flat, the cross-sectional position of the sample molecule in the tube is averaged and velocity component is the same.[1]

Compared to CE, CEC can separate both charged and uncharged species by the differential migration of analyte based on the interaction between two phases. In CEC, solutes can be separated by the differences in distribution ratio like HPLC, and the stationary phase can be fixed on the inner wall of the capillary or on particles inside the column.[20]

HPLC requires a longer column to render the same number of theoretical plates that can be obtained in CEC, which translates into a shorter analysis time using CEC. [9] The pressure drop required to transport the mobile phase in HPLC is solved in CEC as the mobile phase is electrically driven and allows the generation of more plates per column.

CEC has several advantages as a separation technique: low solvent consumption, low sample volume, increased mass selectivity, high separation efficiency, low operational cost, reduced band broadening, smaller particle diameter of support material for the stationary phase, and the same instrumentation as CE.[9,11] By utilizing etching of the inner walls to produce radial extensions and the bonding of an organic moiety to the etched surface, an open tubular format of CEC can be produced. It results in faster analysis times, the most attractive feature of the open tubular format.

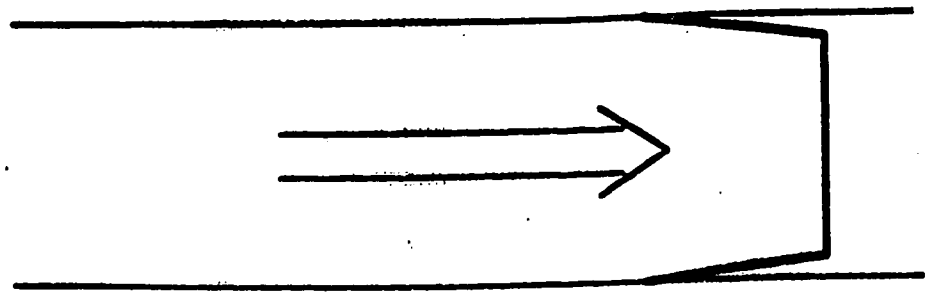


Figure 7. Electroosmotic flow (CE)

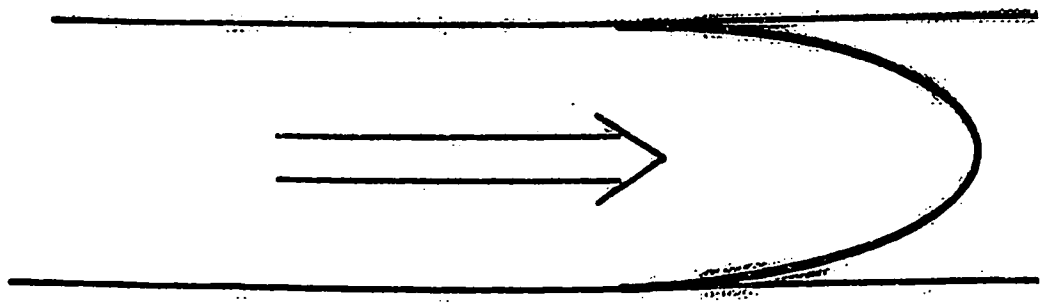


Figure 8. Laminar flow (HPLC)

1.5 Goal of the Research

The most important goal of this research is to evaluate the 20 μm I.D. capillary as a column format for OTCEC. The process that was applied to 50 μm capillaries[6,7,8] was modified for use in 20 μm tubes. The topography of the inner surface was analyzed using Scanning Electron Microscopy (SEM). To investigate the ability of separation, the etched-modified capillary was compared with unetched-modified capillaries and bare capillaries, using several kinds of analytes: aspartame, tetracyclines, and proteins. Aspartame is in high demand as an artificial sweetener and tetracyclines are important antibiotics for humans. Therefore, quality control and product integrity can be improved by suitable analytical methods. The basic nature (the primary amine) of these analytes makes them be positively charged in acidic solution and migration to the cathode is provided by electrophoretic mobility and electroosmotic flow.

Other goals were to determine the importance of pH to optimize separation conditions and the effect of applied electric voltage. The reproducibilities of the unetched modified capillary and the etched-modified capillary were also evaluated.

2. EXPERIMENTAL

2.1. Reagents and Materials

The capillary tubing used was 375 μm O.D. × 20 μm I.D. (Polymicro Technologies, Phoenix, AZ, USA). Deionized (DI) water was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA) and was filtered through a 0.20 μm Nylon 66 membrane filter (Alltech Assoc., Deerfield, IL, USA). The etching reagent, ammonium hydrogen difluoride, was purchased from Aldrich (Milwaukee, WI, USA). Triethoxylane (TES, Huls America, Bristole, PA, USA), 1-octadecene (Aldrich Co. Inc.), and hexachloroplatonic acid (Aldrich Co. Inc.) for subsequent modification of the capillaries were used as received from the manufacturer.

Four kinds of buffers were used and prepared as follows: pH 2.14, 60 mM phosphate (Fischer Scientific, Pittsburgh, PA) and 38 mM Tris [tris (hydroxymethyl) aminomethane] (Sigma, St. Louis, MO, USA); pH 3.0, 60 mM citric acid (Sigma) and 50 mM β-alanine (Sigma); pH 3.7, 60 mM β-alanine and 60 mM lactic acid (Sigma); and pH 4.41, 60 mM acetic acid (Aldrich) and 60 mM γ-amino butyric acid (Sigma).

The tetracyclines 1-4 (Sigma) and their degradation products a-d (Janssen Chemica, Beers, Belgium) used were as follows (Figure 9): 1 = tetracycline HCl; 2 = chlorotetracycline HCl; 3 = oxytetracycline HCl; 4 = doxytetracycline HCl; 5 = methacycline; a = 4-epitetracycline HCl; b = anhydrotetracycline HCl; c = 4-epioxytetracycline; d = α-apo-oxytetracycline. Aspartame (Figure 10) was purchased from Aldrich and used without further purification. A stock solution was made at 1 mg/mL.

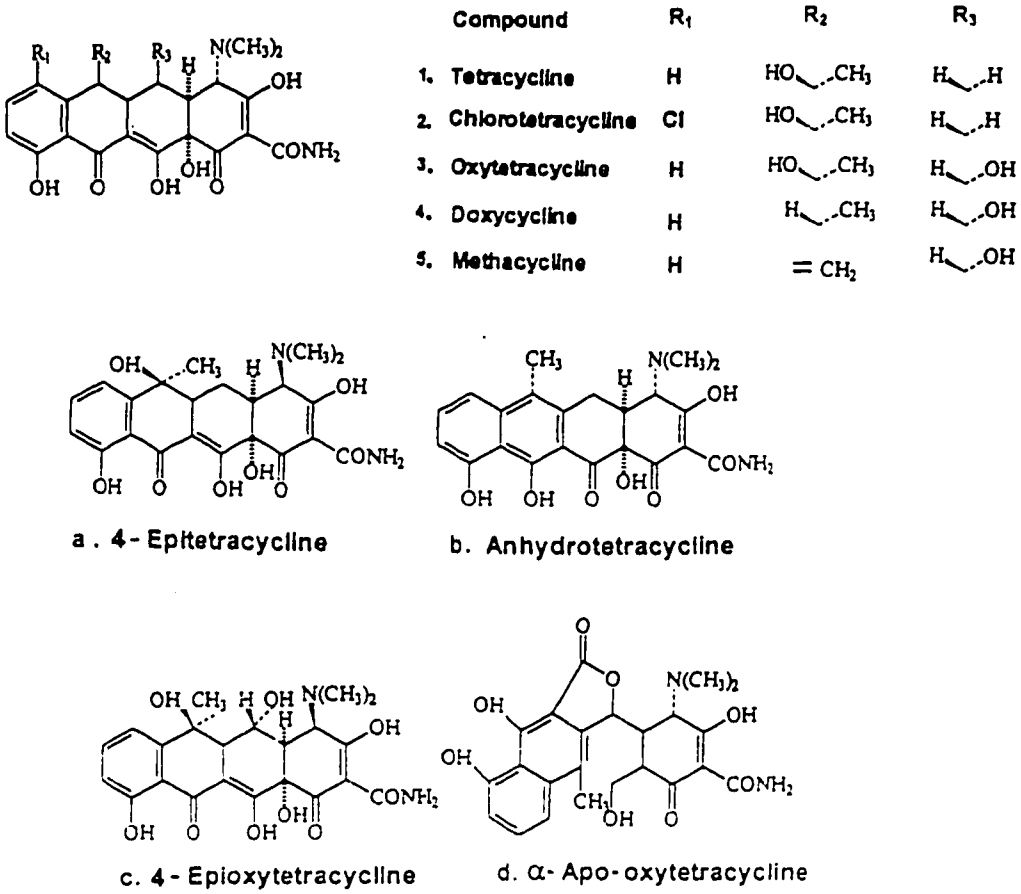


Figure 9. Tetracyclines and their degradation products

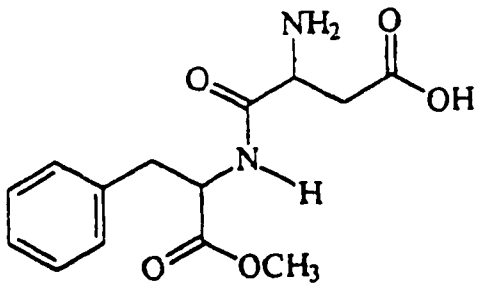


Figure 10. Aspartame

The diet Coca-Cola was obtained from an automatic vending machine at San Jose State University and was analyzed as purchased. The proteins lysozyme (from turkey and chicken egg white), ribonuclease (Type XII-A from bovine pancreas), myoglobin (from horse skeletal muscle), and cytochrome c (horse, tuna, chicken, and bovine heart) were purchased from Sigma Chemical Co.

2.2 Instruments

All HPCE and electrochromatography experiments were performed on a Perkin-Elmer/Applied Biosystems Model 270-A-HT capillary electrophoresis system (Foster City, CA, USA). SEM (Scanning Electron Microscope) photos of bare, etched, and modified capillaries were obtained with a Hitachi S-800 Field Emission Scanning Electron Microscope (Tokyo, Japan) at the IBM Almaden Research Laboratories (San Jose, CA, USA). The oven used for etching and modifying the capillaries was a Hewlett-Packard Model 5890 gas chromatograph. This GC oven was used to control the reaction temperature during the process of etching and organic modification on the surface of the inner capillary. The oven was modified to allow accommodating multiple capillaries. Reagents for etching and modification were put in a heavy wall glass container equipped with a Swagelok fitting. Then nitrogen gas (pressure around 20 psi for etching/modification and around 5 psi for drying the surface) was applied to force the reagents through the capillary.

2.3 Sample Preparation

The analytes were generally prepared at a concentration of 20 μg/mL for those components which were commercially available. Buffer constituents and methanol were

obtained in the highest purity available. The preparation of liquid sample (diet cola) was made by adding 600 μL of sample and 400 μL of DI water. The bare capillary was conditioned for 5 minutes using 0.1 M NaOH , DI water, and run buffer. When the bare capillary was run, the column was rinsed using the following procedures: 2 minutes with 0.1 NaOH; 2 minutes with DI water; and 2 minutes with the buffer. After one sample run, the etched or modified column was rinsed with buffer for 2 minutes. Whenever the analytes were changed from the sample vial, the column was rinsed 2 minutes with run buffer and then rinsed again by DI water for 2 minutes.

2.4 Etching and Modification of Capillary

2.4.1 Etching of the 20 μm I.D. capillary

The bare capillary (1 m) was filled with concentrated HCl (12M), then heated at 80 °C in the modified GC oven overnight. The preheated capillary was washed with distilled water and acetone successively. The rinsed capillary was dried with nitrogen gas at 5 psi overnight. A 5 % (w/v) saturated solution of ammonium hydrogen difluoride in methanol was prepared and stirred overnight to dissolve completely. The 5 % saturated solution was diluted to 1:10 to prevent blocking the inner path of the capillary during the etching procedure. The capillary was filled with the solution of ammonium hydrogen difluoride in methanol, and placed in the oven without heating for 1 hour. The ends of the capillary were sealed with an instant glue, closed with rubber covers, and then heated at 400 °C for 3 hours (under nitrogen gas). After heating, the etched capillary was washed with methanol by a syringe at hand powered pressure, then dried with nitrogen gas for 30 minutes.

2.4.2 Modification of the 20 μm I.D. capillary

a. Preconditioning of the capillary

The capillary was preconditioned for modification of the bare surface. For this, the capillary was treated with a 6 mM ammonia solution for 20 hours using nitrogen gas to force the liquid through the tube. The conditioned capillary was washed with DI water for 1 hour, flushed with 0.1 M HCl solution for 4 hours and then rinsed with DI water again for 2 hours using nitrogen gas pressure. The rinsed capillary was dried with nitrogen gas at 100 °C for 24 hours.

b. Hydride modification by silation

The preconditioned capillary or etched capillary was washed with dioxane using a syringe and filled with a 1.0 M TES solution in dioxane and heated at 90°C for 90 minutes. This hydride-modified capillary was washed with DI water for 2 hours using nitrogen gas pressure, washed with THF-water (50/50) for 2 hours and then washed with THF for 2 hours at room temperature. This capillary with a Si-H monolayer was dried for one-half hour with nitrogen gas.

c. C-18 modification via hydrosilation

Two mL of pure 1-octadecene was mixed with 70 μL of 10 mM Speiers' Catalyst (hexachloroplatinic acid in 2-propanol), and the mixture was heated at 60 – 70°C for 1 hour. The hydride-modified capillary was washed with toluene using a syringe before C-18 modification. The hydride-modified capillary was heated at 100 °C for 45 hours with the 1-octadecene solution. The capillary was flushed twice a day with fresh solution. After C-18 modification in the GC oven, the capillary was cooled to room temperature

and washed with toluene and THF for a half hour each. Then the capillary was dried with nitrogen gas under low pressure at 100 °C overnight.

2.5 Electrophoresis and Electrochromatography Procedures

Three types of capillaries were prepared: bare; unetched C-18 modified; and etched C-18 modified. The capillaries were conditioned first by flushing with run buffer sufficiently using a syringe for 10 seconds. The buffer was degassed by purging with He gas before conditioning. The injection of aspartame was done hydrodynamically for 5 seconds at 5", and run at pH 2.14 and pH 4.41 using an applied field of 15 kV, 20 kV, 25 kV, or 30 kV. The injection of protein samples was done electrokinetically for 6 seconds at 5 kV, and run at 30 kV applied field using pH 2.14, pH 3.0, pH 3.7, and pH 4.41 buffers. The aspartame and protein solutes were detected at 211 nm and tetracycline samples at 254 nm. At these wavelengths, the analytes have the greatest absorbance to get higher chance to detect the analytes. The reproducibility testing of modified capillaries was performed with an aspartame sample in pH 4.41 buffer with a 5 seconds at 5" injection and run at 30 kV.

2.6 Electric Field and Linear Velocity

EOF measurements of aspartame were performed at four applied voltage values successively: 15 kV, 20 kV, 25 kV, and 30 kV. The dependence of linear velocity on each electric field was determined and compared. The electric field was calculated from the equation: $E = V/L$ where V = the voltage applied for the EOF measurement and L = the length of the capillary. The value of linear velocity was also calculated from the

equation: $v = l/t$ where l = the length of the capillary between the end of capillary and detection window, and t = the migration time.

3. RESULTS AND DISCUSSION

3.1 SEM Characterization

Figures 11 and 12 show SEM photographs of a 20 μm I.D. fused silica bare capillary. To inspect internal-sectioned surfaces, the capillary was cut using a capillary cutter or a plastic hammer and attached on electrical tape to prevent being charged and hold the capillary. Figures 13 and 14 show the inner bore at 5 and 20 times higher magnification than Figures 11 and 12. Figures 15 and 16 show the inner surface of the etched capillary. The etching in the Figures 15 and 16 were performed at 400 °C for 3 hours and exhibit an increase in surface areas over a bare capillary. The surface morphologies of Figures 15 and 16 are distinguished quantitatively by the increase in surface area of the capillaries shown in Figures 17 a-d which were etched at 300 °C for 3 hours. The original inner surface of silica capillary was dissolved by the ammonium hydrogen difluoride solution and then formed an extended surface inside the capillary. Compared to the surface produced at 300 °C in Figures 17 a-d, the surfaces etched at 400 °C in Figures 15 and 16 are more uniform and have fewer long extensions from surface. The surface reaction of silica with ammonium hydrogen difluoride at higher temperature makes a more uniform material, but the solute/bonded phase interactions in this capillary are diminished because the radial extensions are shorter and the surface area is not as high as the capillary etched at lower temperature. Though longer spikes that are produced at lower temperature are better for these interactions, the mechanical stability of the etched surface can be destroyed by stress such as vibration. This was confirmed in previous reported results of experiments with 50 μm I.D. capillaries.[7]

Since the inner diameter of the 20 μm I.D. is extremely narrow, micro particles such as contaminants or residues may block the bore inside the capillary. Figures 18 a-b show cross-sections of blocked areas after the etching procedure. The material which caused blocking may be precipitated ammonium hydrogen difluoride due to its low solubility. When the capillary is blocked, it can be reheated at 400 °C for 1 hour and pumped with nitrogen gas at higher pressure (100 psi). Since the blocked area can be seen in an optical microscope, the capillary can be cut if the remaining length is long enough to fit in the insertion case of an instrument. After modification with the organic moiety (C-18), the increased area of the inner wall causes solute-bonded phase interactions. The TES solution in dioxane used to form the Si-H monolayer polymerize if reacted with water and also cause blocking of the etched inner bore of capillary. When excess water was present inside of the capillary filled with TES solution in dioxane, polymerization was confirmed visually by microscope.

3.2 Reproducibility Test

The durability of the modified surface produced by attachment of the octadecyl moiety via silation/hydrosilation was tested. The reproducibility test was performed with a capillary which had been used for other separations. Aspartame was used for this test. A solution of 20 μg/mL was prepared and injected hydrodynamically for 5 seconds at 5" and repeated 100 times. Figures 19 and 20 show graphs of retention times and peak areas of aspartame as a function of injection number using the C-18 modified capillary. Fair reproducibility was observed for the 100 injection test with the retention times and peak areas exhibiting slight variations.

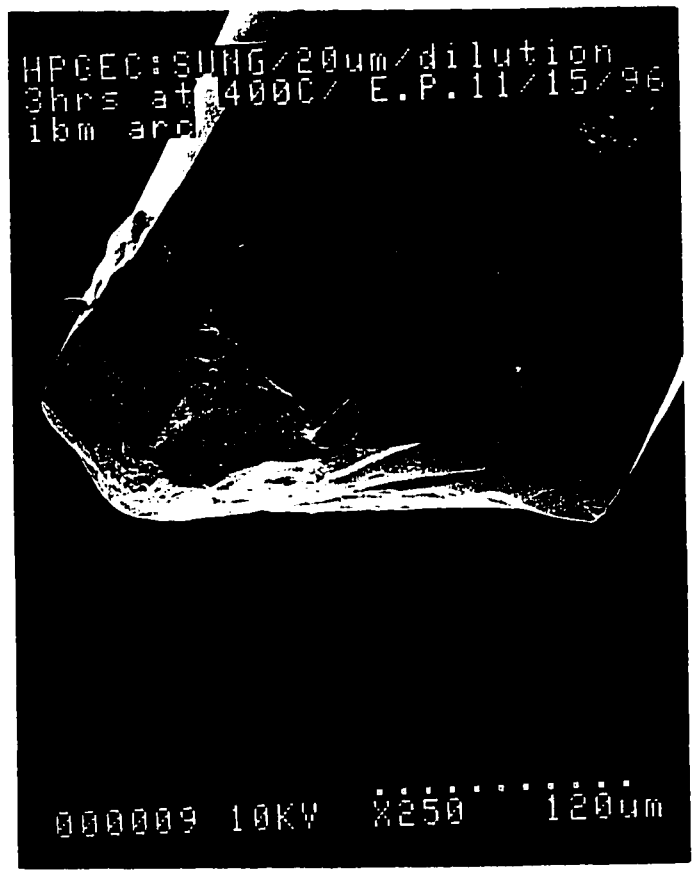


Figure 11. Side view of 20 μm I.D. fused silica capillary (3D, 250X)

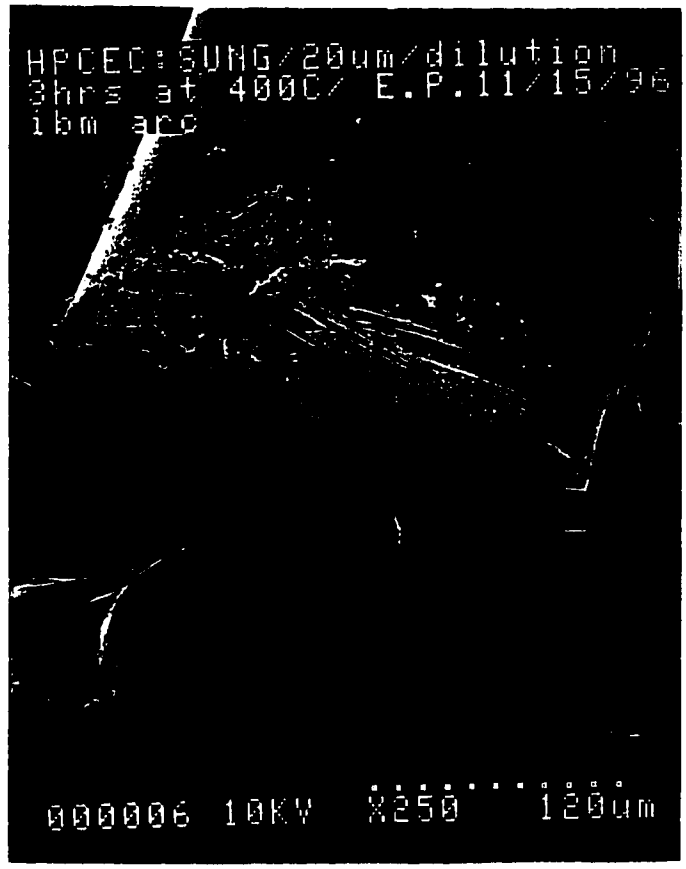


Figure 12. Cross-sectioned view of inner bore of 20 μm I.D. capillary (3D, 250X)

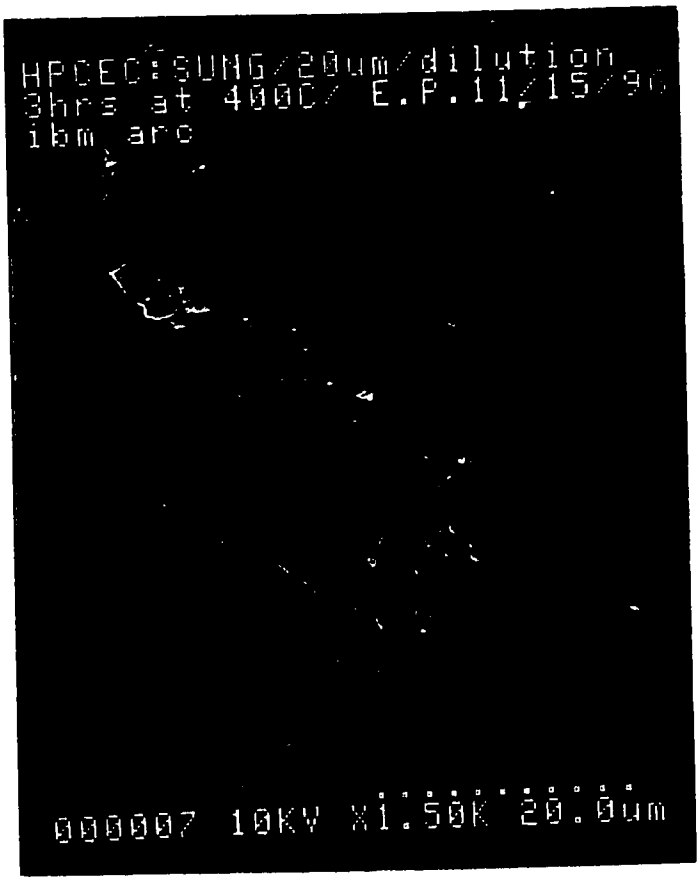


Figure 13. Inner bore of 20 μm I.D. fused silica capillary (1500X)

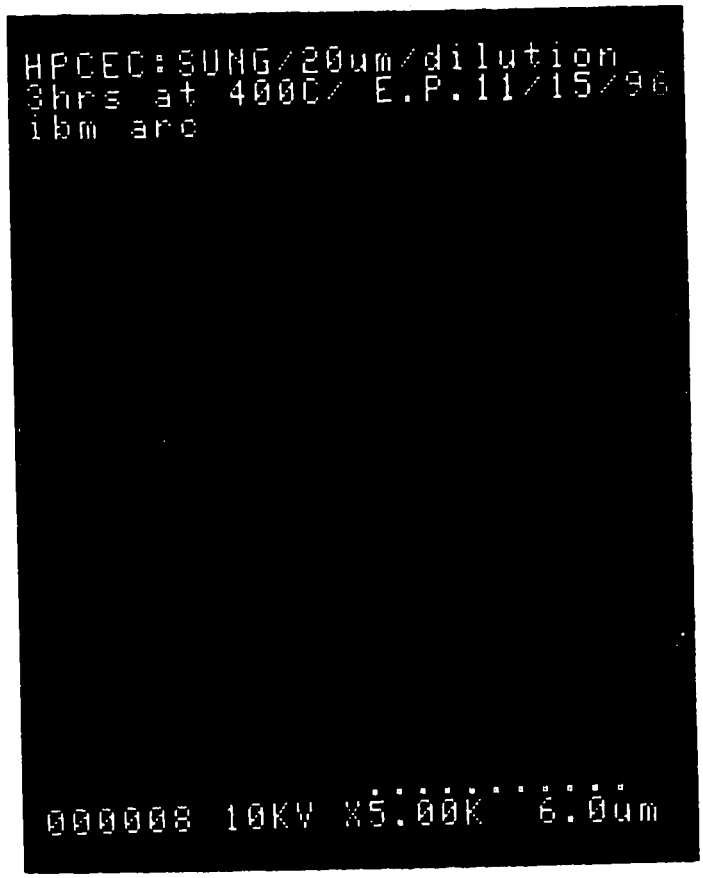


Figure 14. Inner bore surface of 20 μm I.D. fused capillary at 5000X

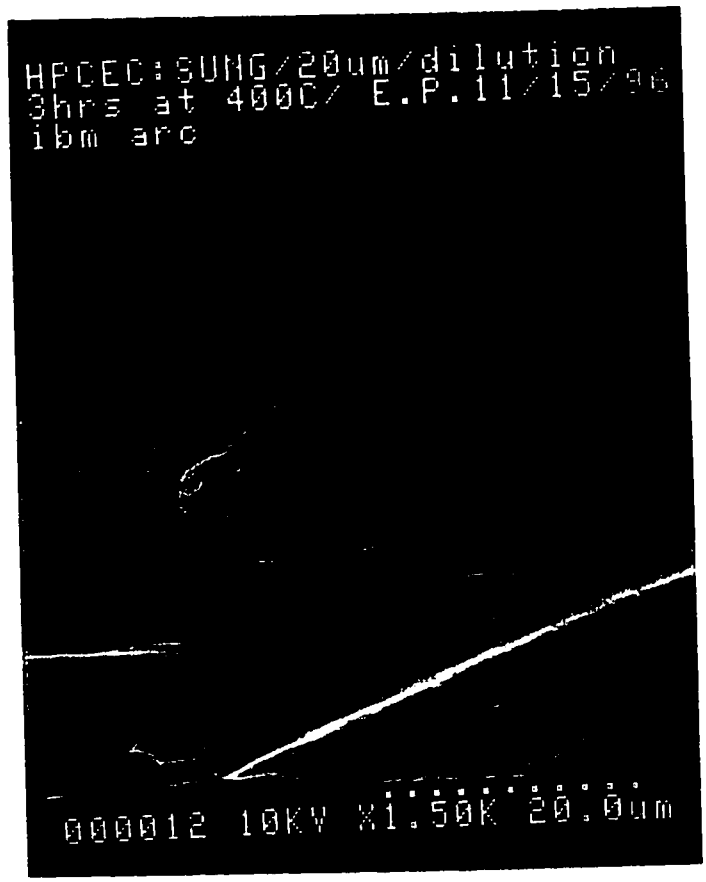


Figure 15. Cross-sectioned surface of 20 μm I.D. capillary etched at 400 °C (1500X)

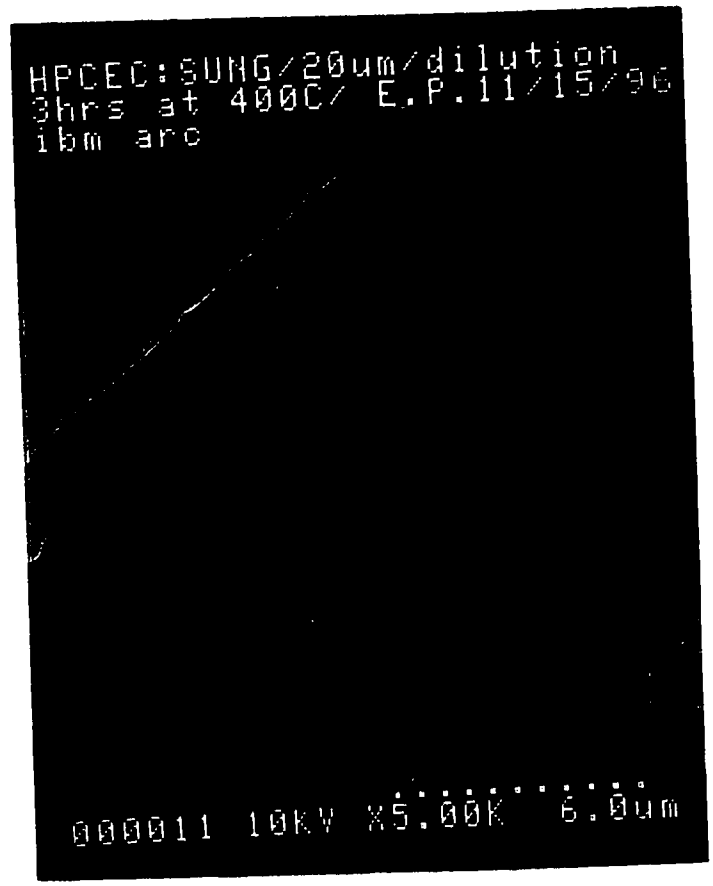


Figure 16. Inner surface of 20 μm I.D. capillary etched at 400 °C (5000X)

Figure 17-a. Etched inner surface of 20 μm I.D. capillary at 300 °C for 3 hours (10,000X)

Figure 17-b. Etched inner surface of 20 μm I.D. capillary at 300 °C for 3 hours (10,000X)

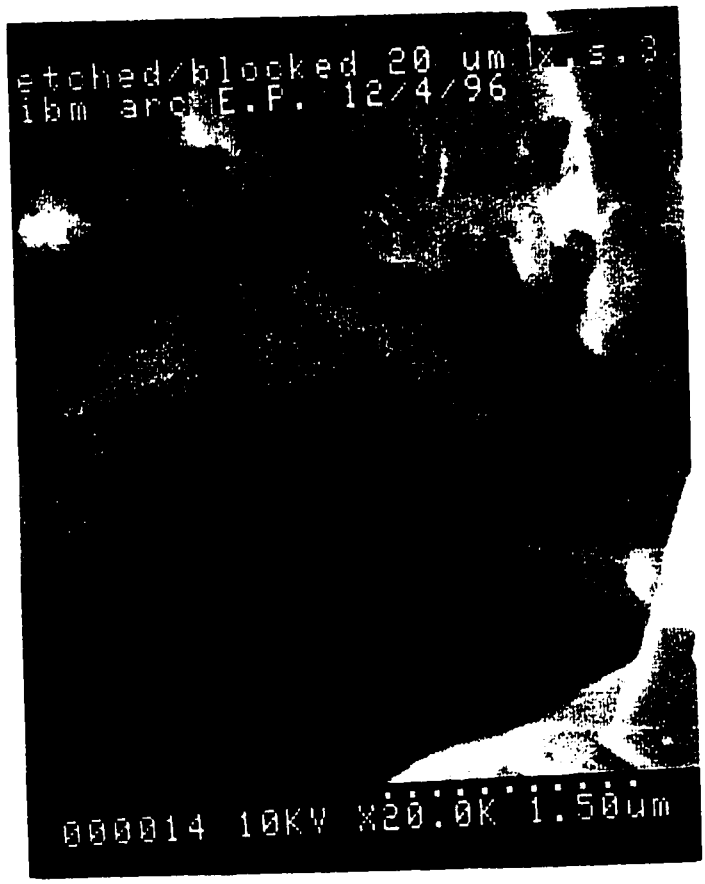


Figure 17-c. Etched inner surface of 20 μm I.D. capillary at 300 °C for 3 hours

(20,000X)

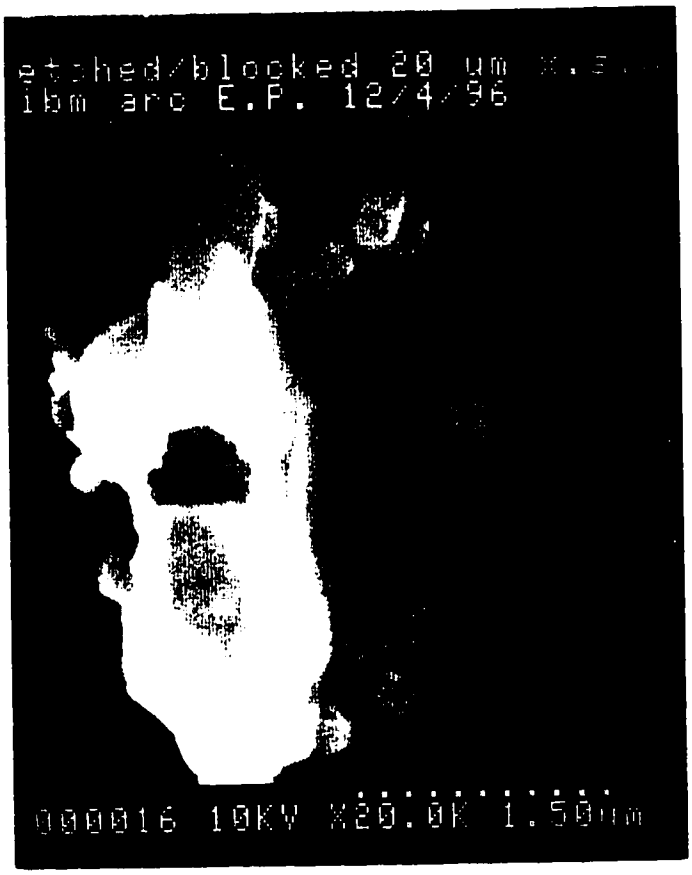


Figure 17-d. Etched inner surface of 20 µm I.D. capillary at 300 °C for 3 hours (20,000X)

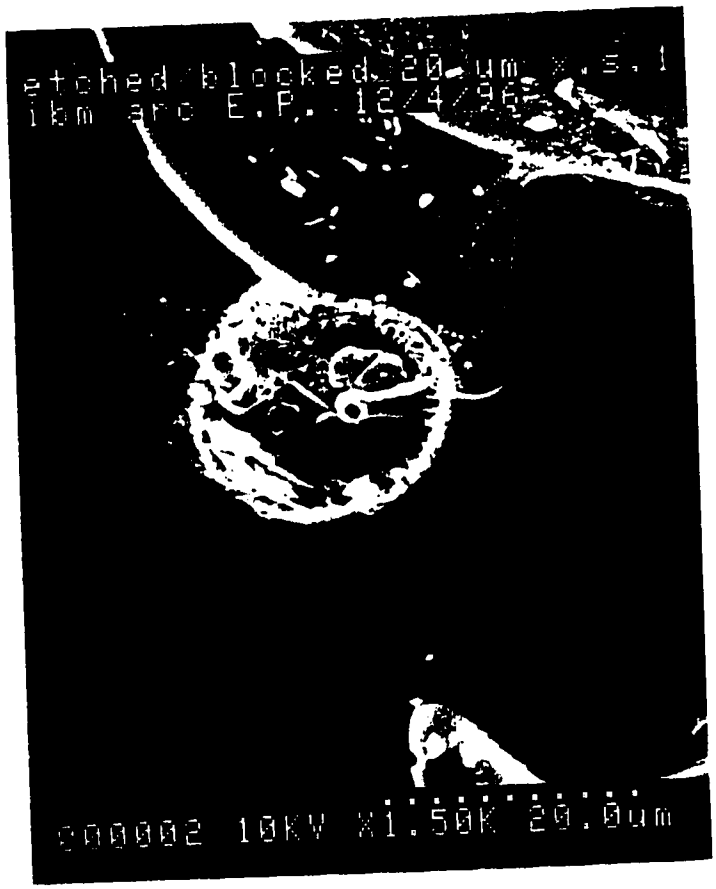


Figure 18-a. Blocked inner bore of 20 μm I.D. etched capillary (1500X)

Figure 18-b. Blocked inner bore of 20 μm I.D. etched capillary at 3500X

For the 100 injections, the retention time was in the range of 8 minutes to 9.5 minutes (Figure 19) and the peak areas were in the range of 270 to 350 (Figure 20). The gap of data is due to the fact that this capillary had already been used for numerous separations before the reproducibility test. Except for seven injection failures and injections # 58 & 85, retention times and peak areas remained relatively stable over the 100 injections as the relative standard deviation (R.S.D) of retention time for injections 1-50 is 2.79 % and that of peak area for injections 1-50 is 2.14 %.

The result of the reproducibility test means that the Si-C bond formed by the organic modification process is stable and the injection volume was fairly constant. Therefore, the C-18 bonded 20 μm I.D. capillary can be used for at least 100 repeated separations with good reproducibility.

3.3 Electroosmotic Flow Velocity and Electric Field

From the equation for velocity of electroosmotic flow which was explained in Section1.2.1, it can be seen that changing the electric field is a simple way to control EOF. An increase in the electric field increases the flow velocity and reduces the migration time as shown in Figures 21 and 22. In bare/unetched-modified/etched-modified columns, the linear velocities of electroosmotic flow and electrophoretic migration are proportional to an increase of the electric field. The voltage can be plotted instead of electric field. Figure 22 shows similar results of linear velocity vs. voltage or electric field in the etched C-18 capillary. Higher voltage results in a shorter migration time which is the easiest way to achieve a shorter analysis. But, increased voltage can cause higher heat production which may not be dissipated completely.

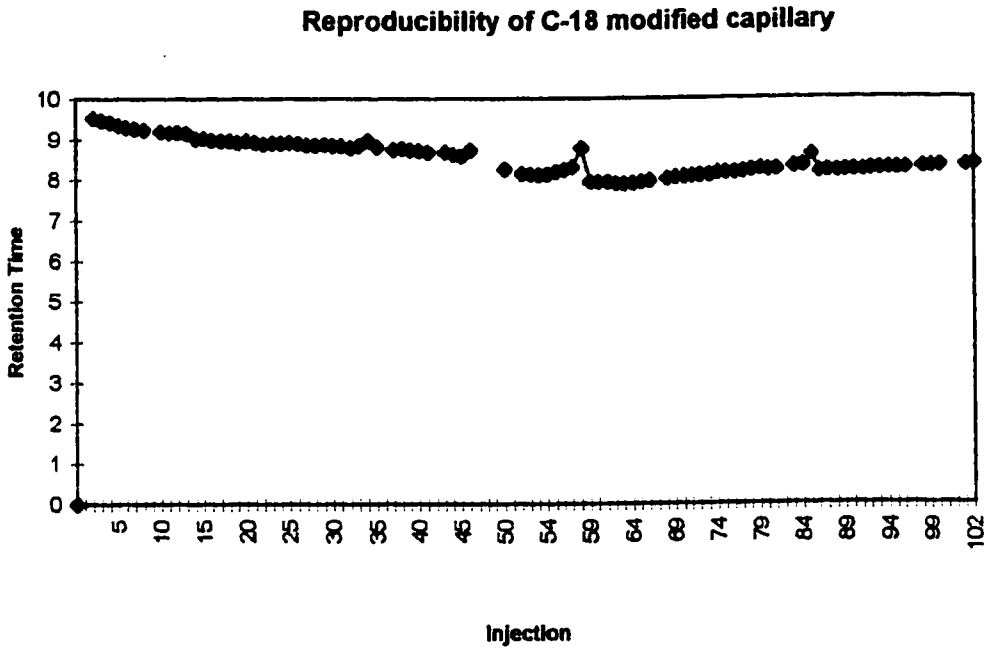


Figure 19. Reproducibility of retention time in the C-18 modified capillary

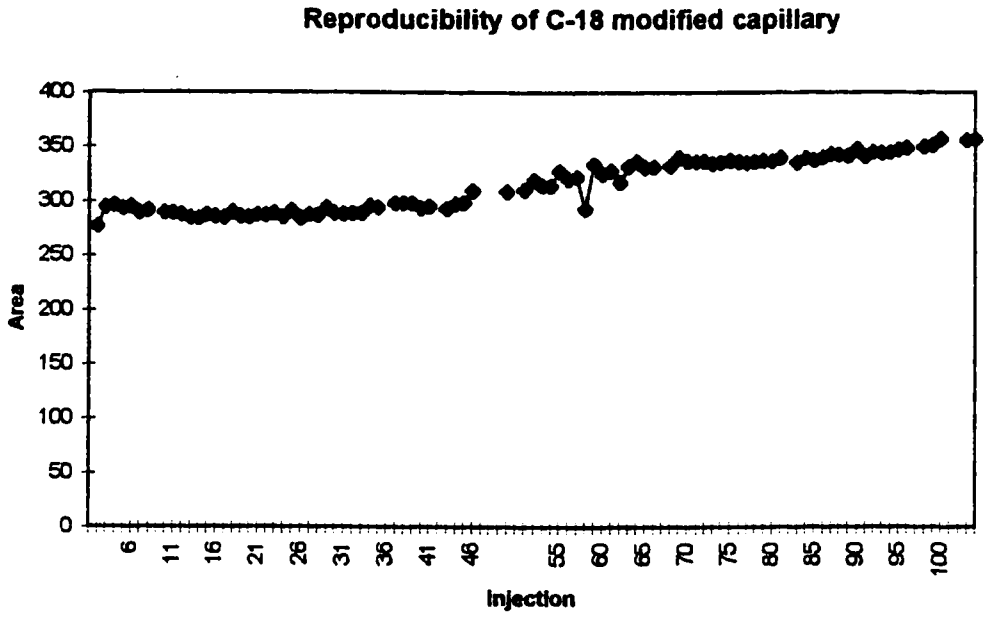


Figure 20. Reproducibility of peak area in the C-18 modified capillary

However, a narrower 20 μm I.D. capillary can dissipate this heat effectively. When the graph (a) of the bare capillary in Figure 21 is compared with the graph (b) of the modified capillary in Figure 21, the modified capillary exhibited a smaller increase in linear velocity when the electric field is increased. The increase of linear velocity in the etched modified capillary is less than that of the unetched modified capillary with the same electric field increase. This difference of linear velocity is the result of a lower electroosmotic flow that is consistent with the strong interaction between the analytes and the bonded organic followed by etching of the silica surface. The smaller increase of linear velocity of the etched C-18 capillary shows more efficient bonding and less eletroosmotic flow caused by C-18 modification on the capillary inner surface.

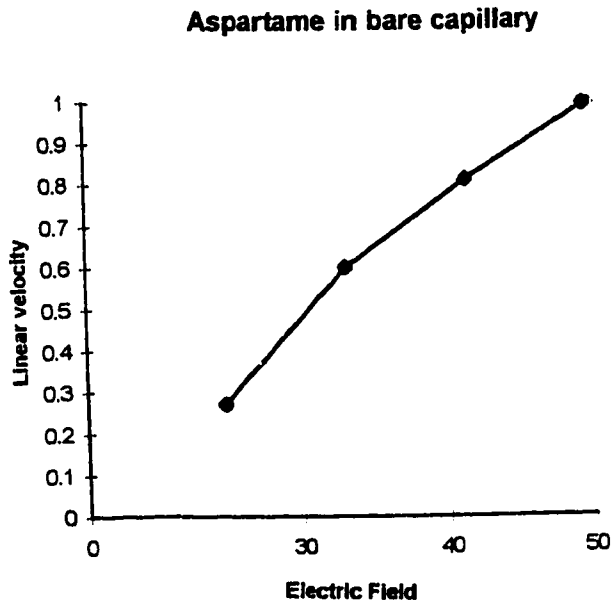
3.4 Characterization of 20 μm I.D. capillary

The elution and the electrokinetic chromatographic behavior of aspartame is shown in Figure 23 on the etched C-18 modified 20 μm I.D. capillary. The peak in Figure 23 is sharper than one obtained on an etched diol modified 50 μm I.D. capillary for the same sample. This sharper peak for the 20 μm I.D. capillary should lead to improved quantitative analysis and a lower detection limit than the 50 μm I.D. capillary. It can be concluded that an etched C-18 modified 20 μm I.D. capillary is more suitable for this analysis than an etched modified 50 μm I.D. capillary.

3.4.1 Analysis of pure aspartame and a real sample

Aspartame and one commercial product, diet Coca-Cola, were analyzed because the concentration of the sweetener was available from the manufacturer.

(a)



(b)

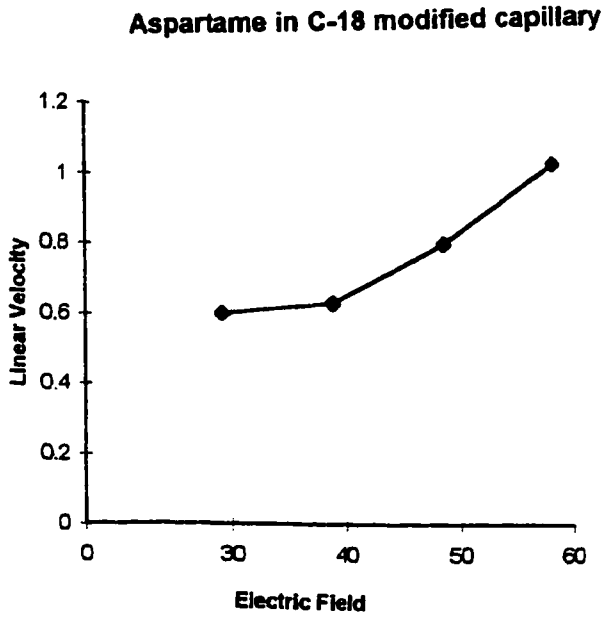
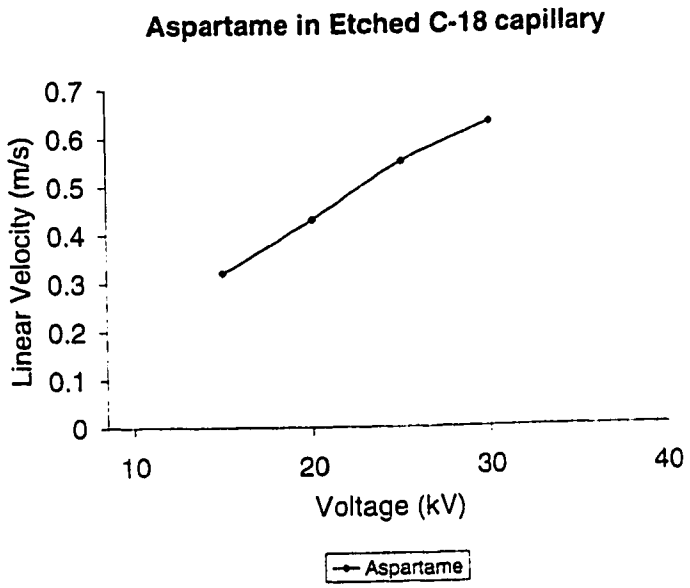


Figure 21. Linear velocity as a function of applied electric field in (a) bare capillary and (b) C-18 modified capillary

(a)



(b)

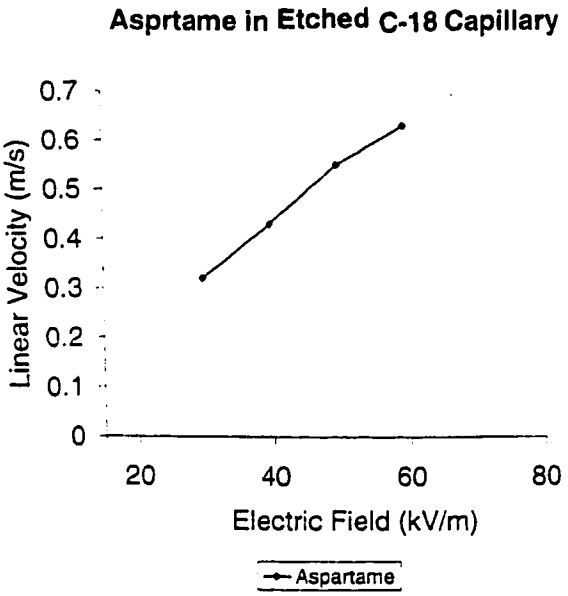


Figure 22. Linear velocity of aspartame in an etched C-18 modified capillary as a function of (a) applied voltage and (b) electric field

The reproducibility data was discussed in Section 3.2 and the electric field and linear velocity data were discussed in Section 3.3. Other factors in the analysis of the aspartame product are the applied voltage and retention time. In Figures 24 a-c, a diet cola sample was run at three voltages: 15 kV, 25 kV, and 30 kV. At the maximum voltage applied (30kV), the migration time was minimized compared with the other voltage values. In HPCE/CEC experiments, higher efficiency is obtained in comparison to HPLC and narrow peaks are observed for constituents in the sample. The detection wavelength of aspartame in CE/CEC is 211 nm while the reported wavelength in HPLC is 230 nm.[17] Under the buffer and voltage conditions selected, the analysis time of less than 4 minutes is faster than that obtained in the HPLC method.[17]

3.4.2 Comparison with a 50 μm I.D. capillary

The separations of tetracyclines and proteins (Figures 25-36) show improved resolution on the etched C-18 modified 20 μm I.D. capillary over that obtained on an etched, modified 50 μm I.D. capillary. It appears that the electrokinetic separation using a 20 μm I.D. capillary is more efficient due to increased solute/bonded phase interactions between the analytes and the bonded C-18 moiety as a result of the smaller diameter.

3.5 Effect of Etching and Modification

The electropherogram on a bare capillary is usually composed of narrow and symmetrical peaks while the same run on an unetched C-18 capillary results in an increase in the retention time and peak width as shown in the Figures 25 - 26 respectively. The separation is increased when it is performed on an etched C-18 capillary. This result confirmed that the etching and modification processes decreased

the number of free silanols on the surface and caused a reduction in EOF in the C-18 capillary. In the etched C-18 capillary, the attachment of the n-C-18 to the etched surface increased the solute-bonded phase interactions which resulted in a longer time for the solute to migrate through the column. The increase in peak width confirms the presence of mass transfer effects produced by etching and modification of the surface. These results are in agreement with the data on the 50 μm I.D. capillaries.

3.6 CE/CEC Characterization

3.6.1 Analysis of proteins

A useful comparison is the separation of turkey lysozyme and chicken lysozyme on bare, unetched C-18, and etched C-18 capillaries at pH 2.14 as shown in Figures 25 a-c. The separation of the same lysozymes at pH 4.41 is compared in Figures 26 a-b. The concentration of the samples was 20 μg/mL and they were injected electrokinetically for 6 seconds at 5 kV. All results indicate that solute-bonded phase interactions caused increased retention and migration times for each of the samples based on the following order: C-18 etched > C-18 unetched > bare capillary. The C-18 etched column has stronger interactions than the unetched column and efficient solute-bonded interactions. In the comparisons at pH 3.0 and 3.7, some improvement in the resolution of the components occurs at pH 3.7 in contrast to pH 3.0 as shown in Figures 27 a-b. A complete base line separation was achieved at pH 3.7.

A 20 mg/mL of mixture of proteins (cytochrome c, lysozyme, myoglobin, and ribonuclease) was separated under the identical conditions of the lysozyme sample. Increased migration time in the C-18 column caused by solute-bonded phase interactions

was evident as shown in Figures 28 a-b and Figures 29 a-b. In the separation at pH 2.14 in Figure 28, myoglobin and ribonuclease were separated on the etched C-18 column. An interesting example of pH control was provided in Figure 30 as in the lysozyme mixture. At pH 3.7, the separation of all components unresolved at pH 3.0 was achieved as shown in Figures 30 a-b.

Another interesting example was the separation of a cytochrome c mixture consisting of proteins from horse, tuna, chicken, and bovine heart. The electropherogram is shown in Figure 31 a and electrokinetic chromatograms are shown in Figures 31 b-f. In the bare capillary, partial resolution of the sample was obtained and there is a noticeable tail on the peak. If the surface is modified, the four analytes are partially resolved as shown in the electrokinetic chromatogram (Figure 31 b). In the etched C-18 capillary, the best separation of each cytochrome c was obtained. A pH of 3.7 is the optimum value for the cytochrome c separation demonstrated by the various electrokinetic chromatograms shown in Figure 31.

3.6.2 Analysis of tetracyclines

From data accumulated on 50 μm I.D. capillaries, tetracyclines are applicable to both HPCE and CEC due to their ability to protonate the tertiary amine from hydrogen ion in the buffer at low pH which is dependent on the type and concentration of the buffer. At the pH values used in these experiments, the tetracyclines are positively charged. Therefore, they are separated by their differences in electrophoretic mobility. Three mixtures were tested: A (anhydrotetracycline, tetracycline, chlorotetracycline, epitetracycline), B (4-epioxytetracycline, tetracycline, α-apooxytetracycline,

oxytetracycline), and C (oxytetracycline, doxytetracycline, methacycline). Figures 32 a-b show the separation of one mixture of tetracyclines (A) in a pH 2.14 buffer. The four individual components are separated. The C-18 etched column exhibited successful resolution of all components as evidenced by the base line separation of the peaks. Mixture (B) was tested under identical conditions to those in Figure 32. The results are shown in Figures 33 a-b. Similar separation is achieved and the peaks become sharper including an unknown impurity which is improved noticeably. Another challenging analysis is the commercially available doxycycline, methacycline, and oxytetracycline mixture (C). Figure 34 a shows the separation of the three compounds of doxycycline, methacycline, and oxytetracycline on a bare capillary in the presence of a pH 2.14 buffer. Slightly better separation is achieved with the C-18 etched capillary in Figure 34 b under the same conditions of Figure 34 a in about 8 minutes. The analysis of tetracycline mixtures B in Figure 35 and C in Figure 36 on a C-18 capillary at pH 3.0 and 4.41 was also performed. At pH 3.0, the C-18 column successfully resolves the components which co-eluted on the capillary at pH 4.41.

3.7 Effect of Separation Variables

3.7.1 Effect of voltage

The increase in EOF caused by increased voltage results in faster migration times as shown in Figure 24. Though the highest voltage is desirable for speed of analysis, higher voltage can cause heat by producing higher currents. The higher temperature leads to broader peaks and non-reproducible migration times. The maximum voltage

with minimum heat generation for the experiments to prevent any deleterious effects like peak broadening in this study was 30 kV.

3.7.2 Effect of pH

To illustrate the effect of pH, several types of samples in lysozymes and cytochrome c were tested. Buffer pH has a significant effect on electroosmotic flow because it changes the zeta potential. If the pH increases, the electroosmotic flow increases due to the dissociation of silanols which results in a higher zeta potential. The optimization of buffer and solvent conditions can lead to improved separations.

A series of experiments with the lysozyme sample were used to study the effects of pH on EOF and migration time. Figures 25, 26, and 27 show the electropherograms in bare, C-18 modified, and etched C-18 modified capillary at pH 2.14, pH 3.0, pH 3.7, and pH 4.41 respectively. It can be seen from the figures that the retention time of the lysozymes decreased when the pH was increased from 3.0 to 4.41 with the optimum separation of peaks at pH 3.7.

Another set of similar experiments was performed using the cytochrome c mixture on the etched C-18 capillary (Figure 31). The separation at pH 3.0 is similar to the one obtained at pH 2.14. However, improved resolution of the components was obtained at pH 3.7 and baseline separation was achieved. At pH 4.41, longer migration times are observed with partial resolution of impurities. The impurities came from the preparation/isolation of the product which are commercial materials. They are proteins with similar structure to the main product. Much more elaborate analysis would have to be done to actually identify them positively. The optimum for the cytochrome c

separation also occurs at pH 3.7. At a higher pH like 4.41, faster electroosmotic flow does not allow for longer retention times so that there will be more interaction of the solutes with the inner column surface. At pH 3.7, slower EOF provides more time for solutes to interact with the C-18 moiety to separate sufficiently.

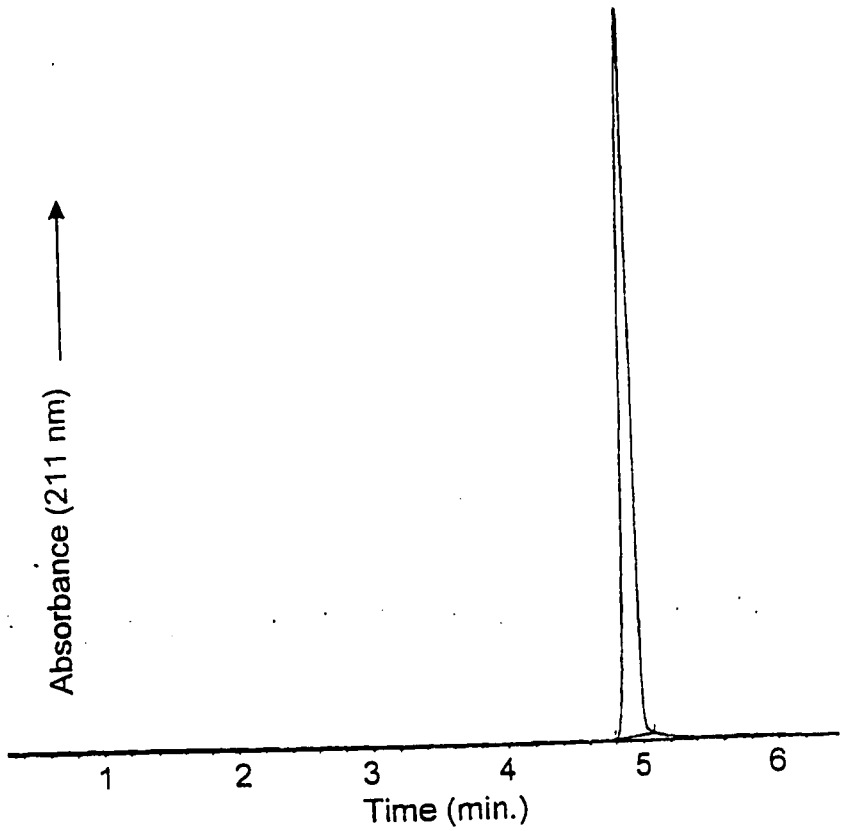


Figure 23. Electrochromatogram of aspartame in etched C-18 20 μm I.D. capillary at pH 2.14 for V = 30 kV

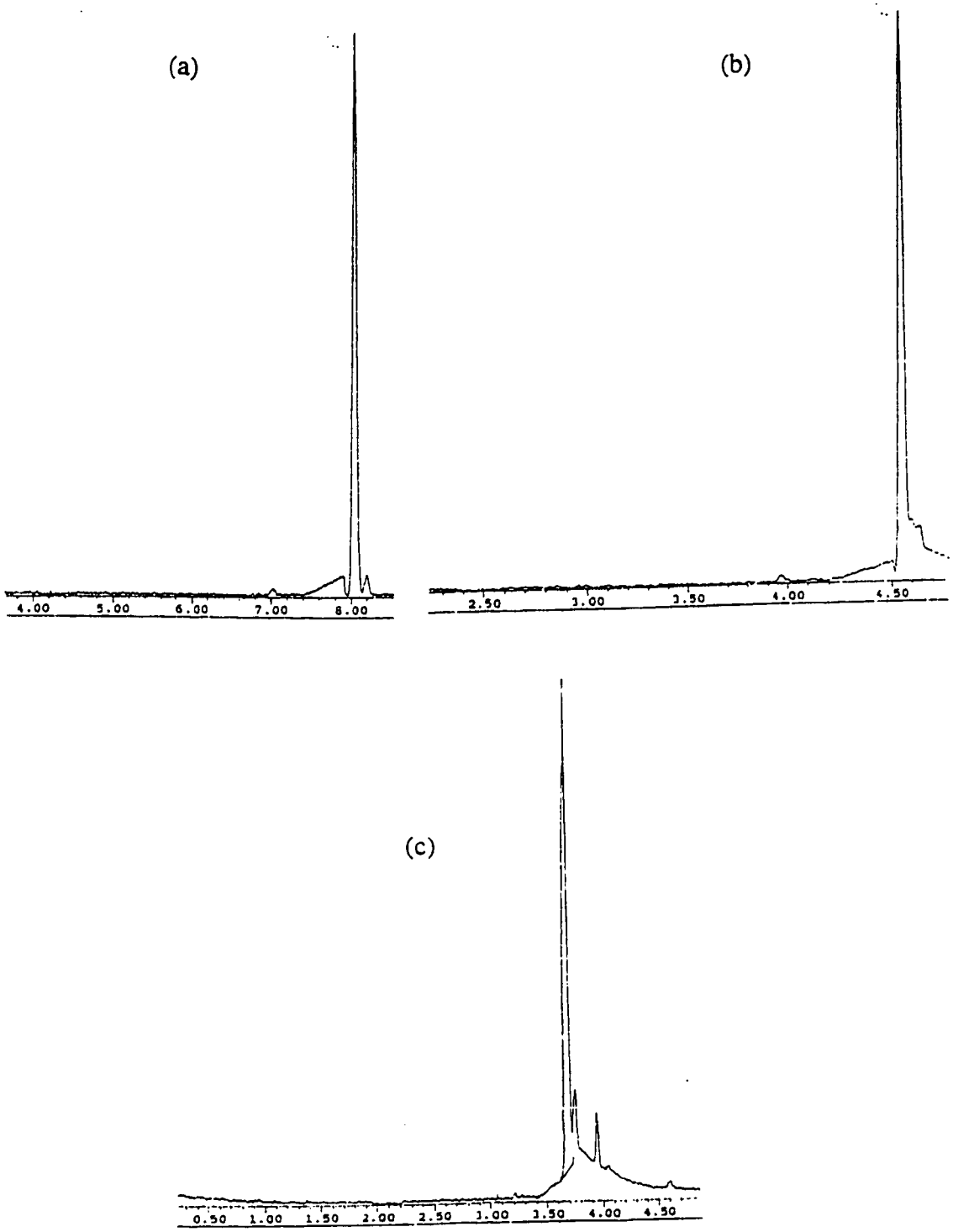


Figure 24. Electropherogram of diet cola in a bare 20 μm I.D. capillary at (a) 15 kV, (b) 25 kV, and (c) 30 kV in pH 2.14 buffer

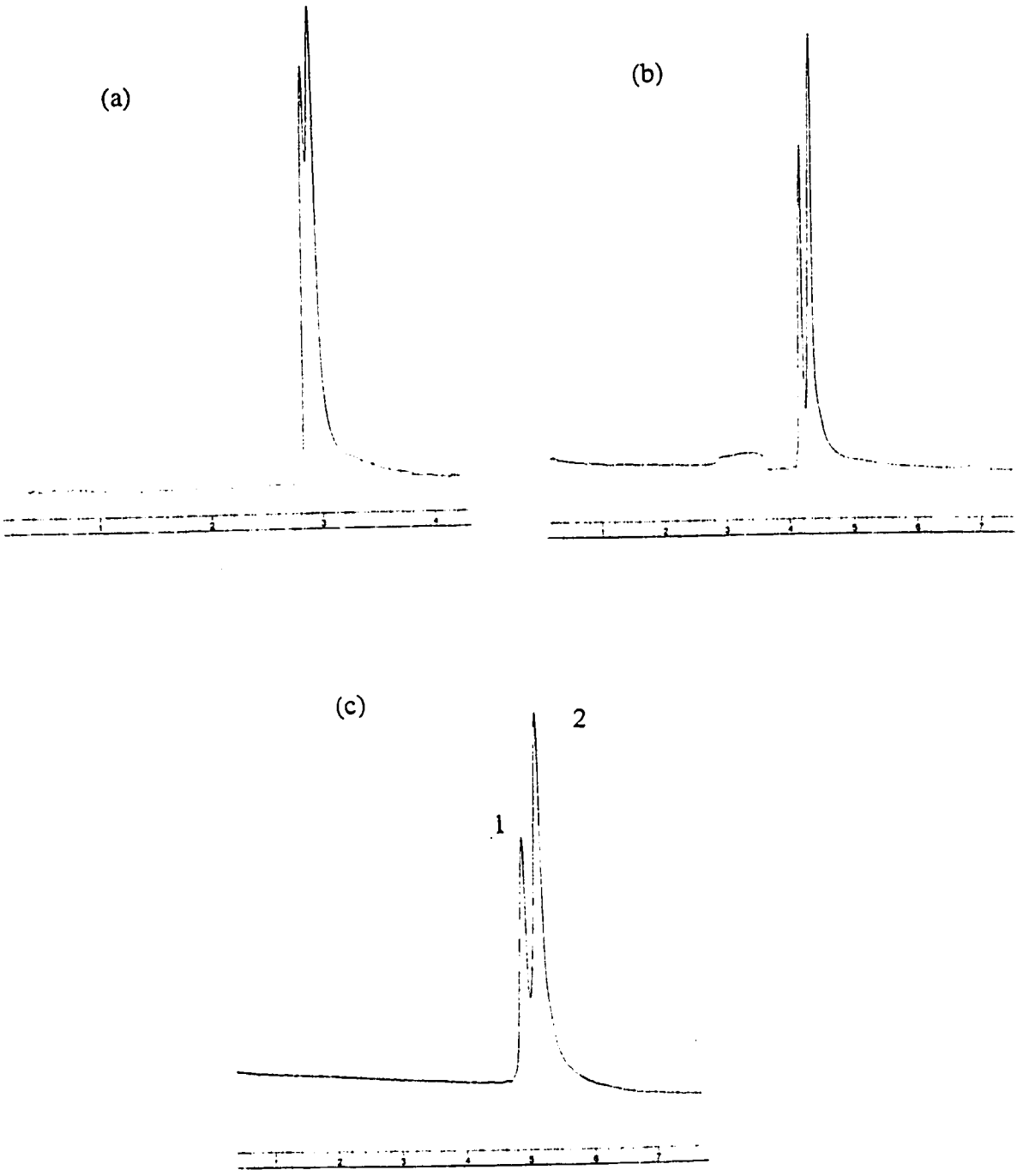


Figure 25. Separation of lysozyme (1:chicken, 2:turkey) in (a) bare, (b) modified, (c) etched modified 20 μm I.D. capillaries at pH 2.14

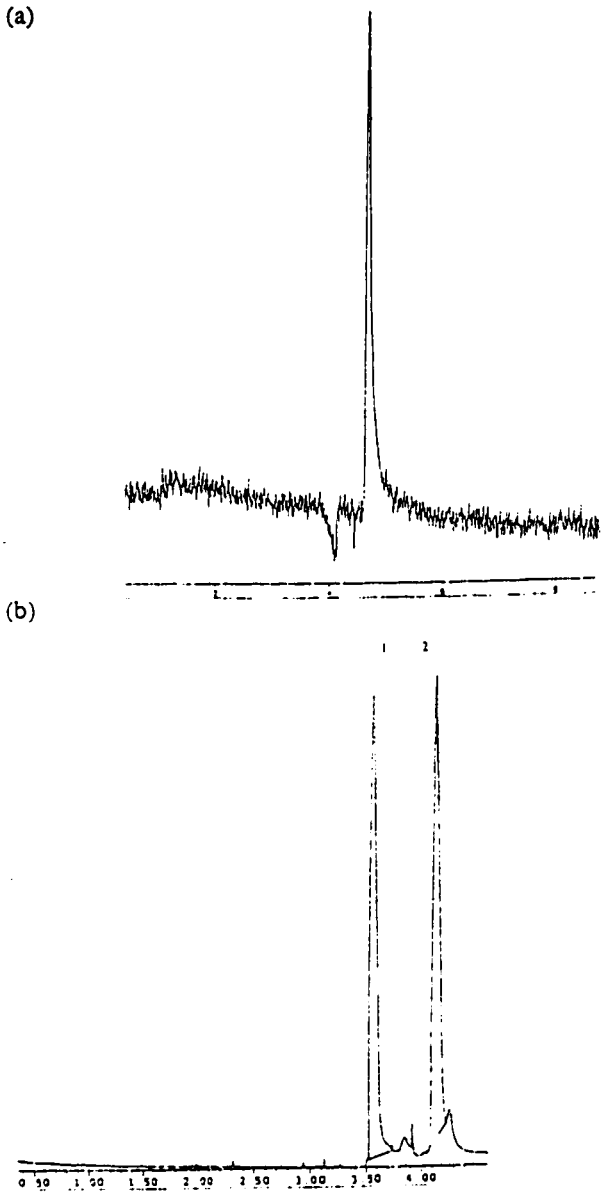


Figure 26. Separation of lysozymes (1: chicken, 2: turkey) at pH 4.41 in (a) bare and (b) etched C-18 20 μm I.D. capillaries

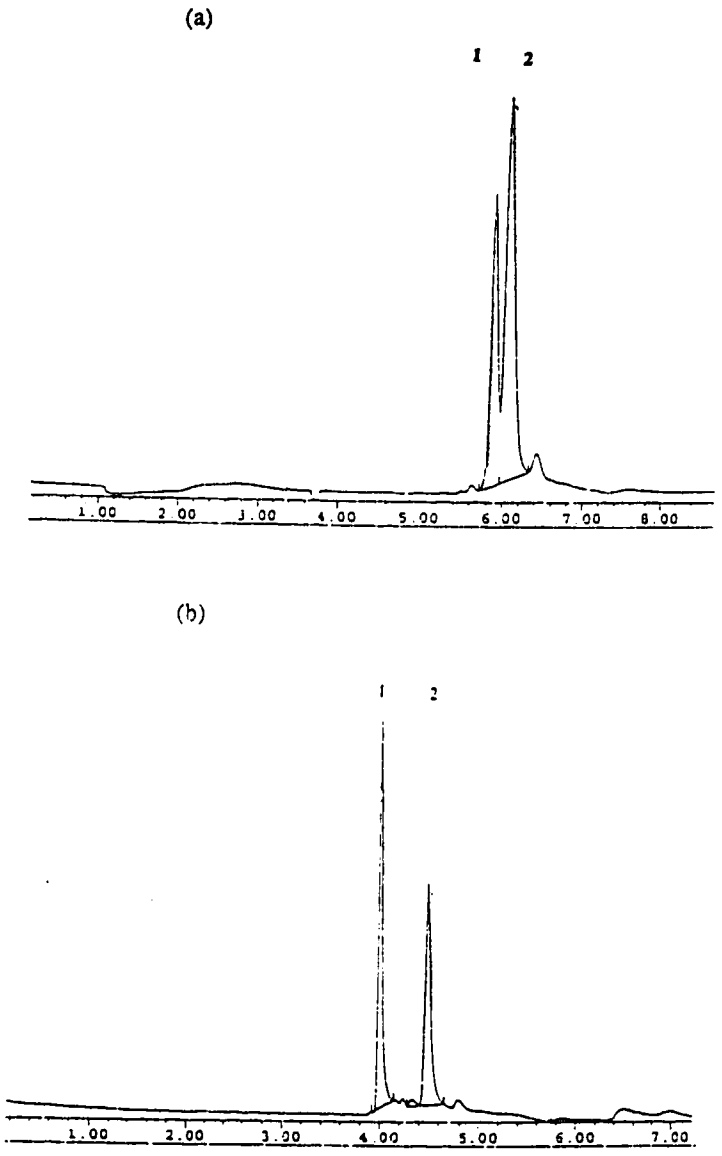


Figure 27. Separation of lysozymes (1: chicken, 2: turkey) in a C-18 etched 20 μm I.D. capillary at (a) pH 3.0 and (b) pH 3.7

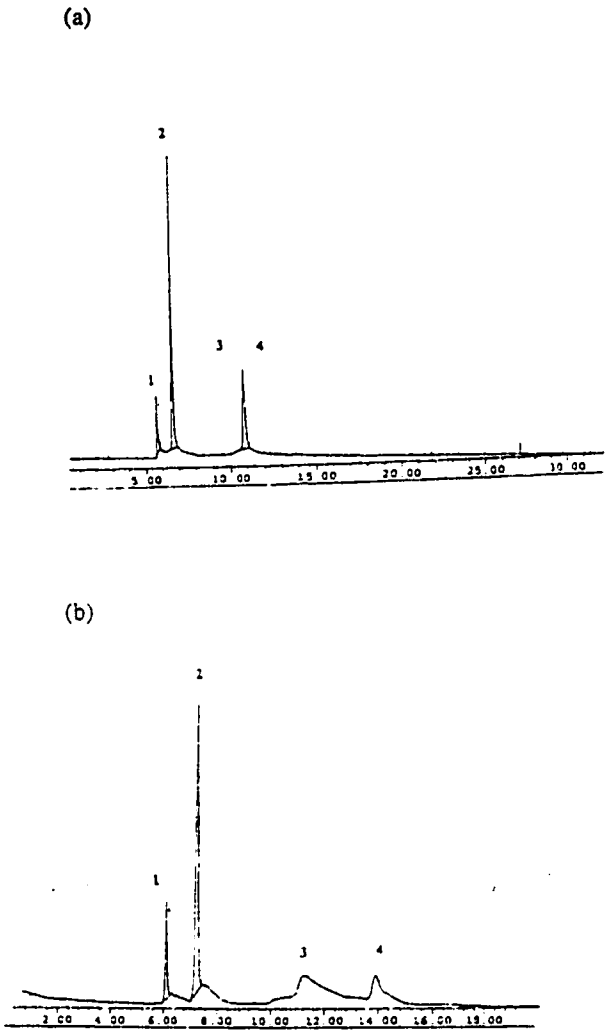


Figure 28. Separation of proteins [1: cytochrome c, 2: lysozyme, 3: myoglobin, 4: ribonuclease] at pH 2.14 in (a) bare and (b) etched C-18 modified 20 μm I.D. capillaries

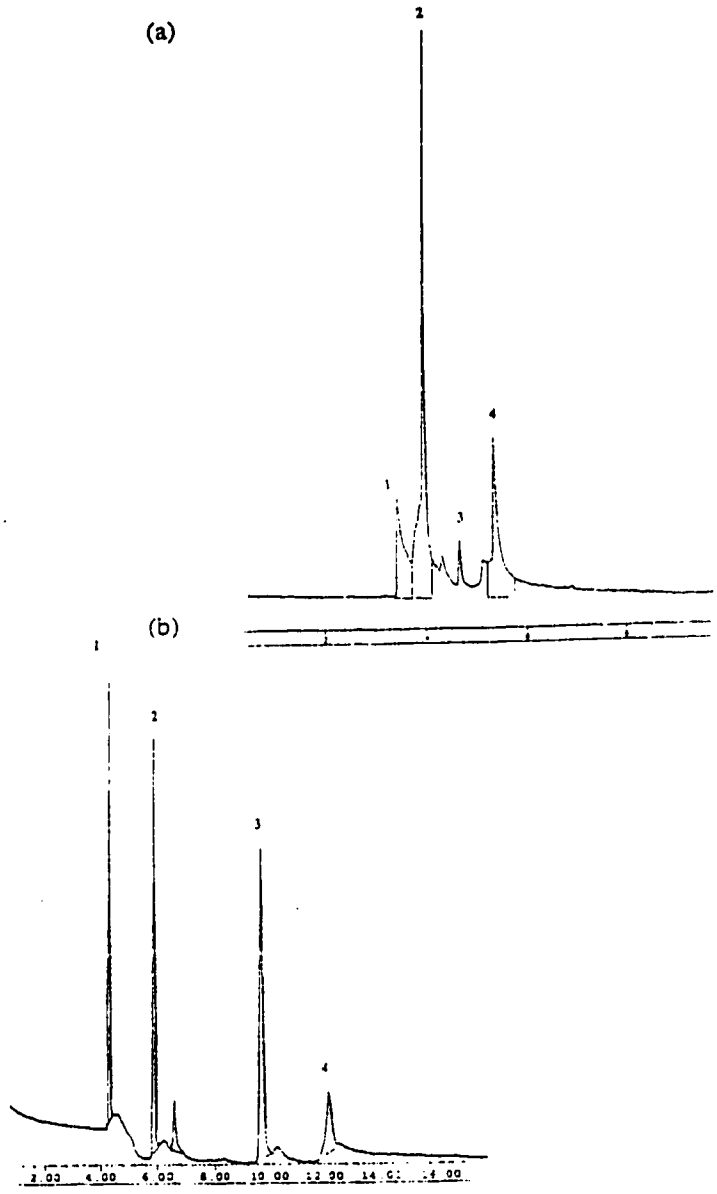


Figure 29. Separation of proteins (1: cytochrome, 2: lysozyme, 3: myoglobin, 4: ribonuclease) at pH 4.41 in (a) C-18 unetched and (b) C-18 etched 20 μm I.D. capillaries

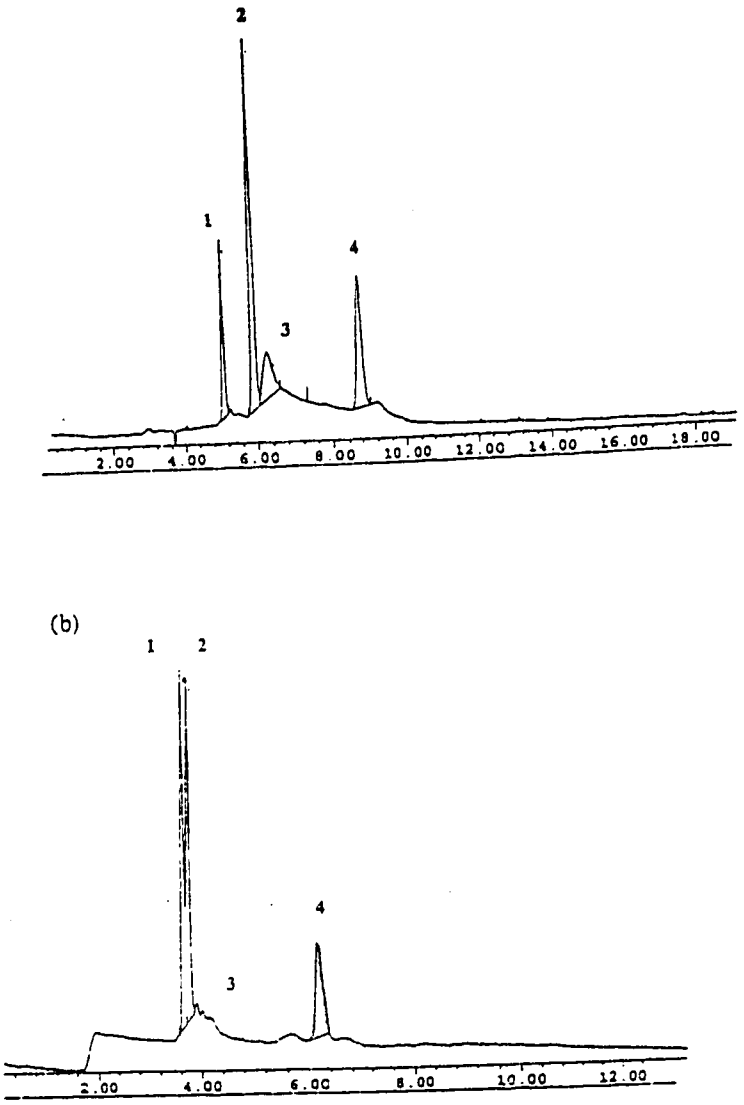


Figure 30. Separation of proteins (1:cytochrome c, 2: lysozyme, 3: myoglobin, 4: ribonuclease) in a C-18 etched 20 μm I.D. capillary at (a) pH 3.7 and (b) pH 3.0

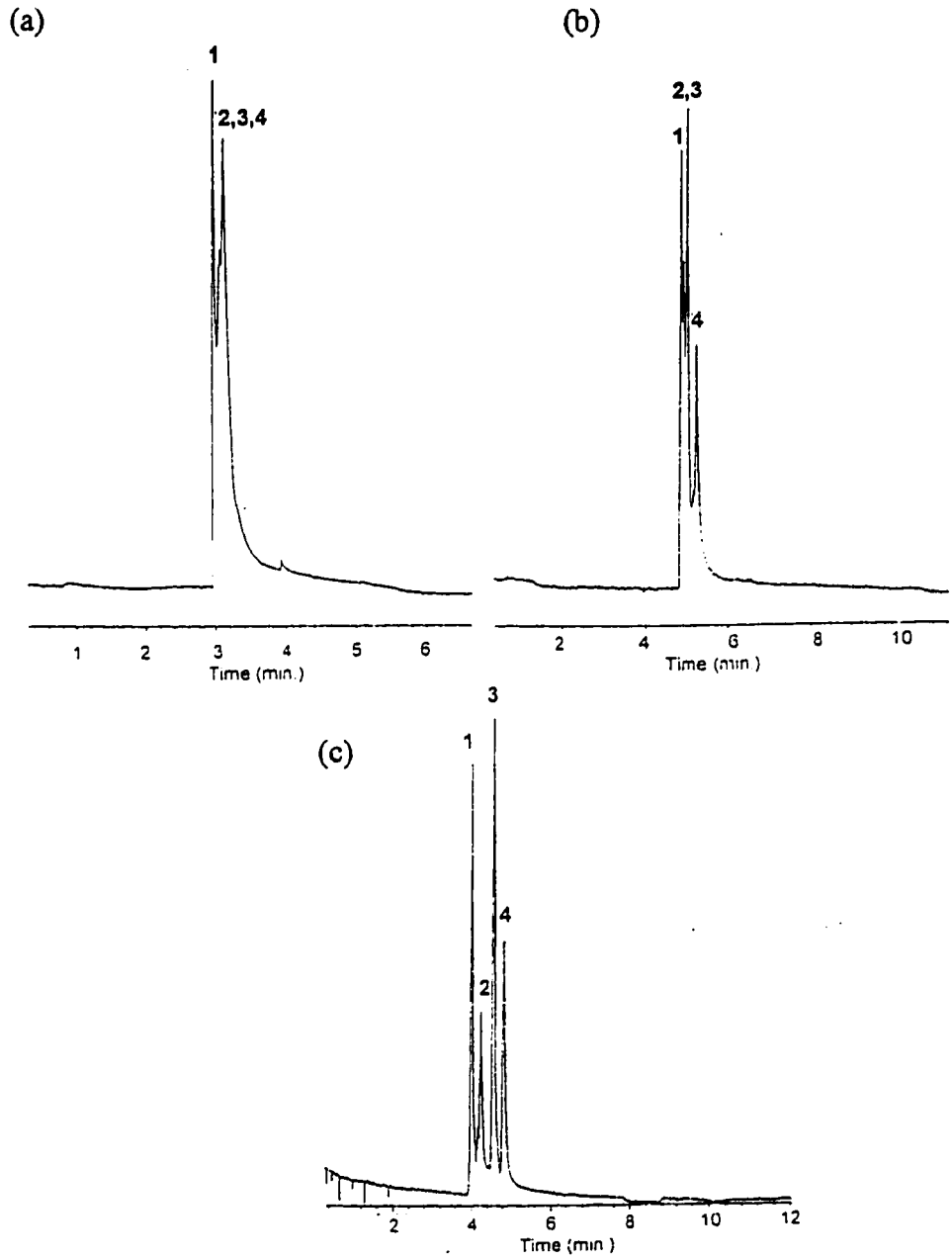


Figure 31. Separation of cytochrome c's (1:horse, 2:bovine, 3:chicken, 4:tuna) on (a) bare, (b) C-18 modified, (C) etched C-18 modified 20 μm I.D. capillaries at pH 3.7

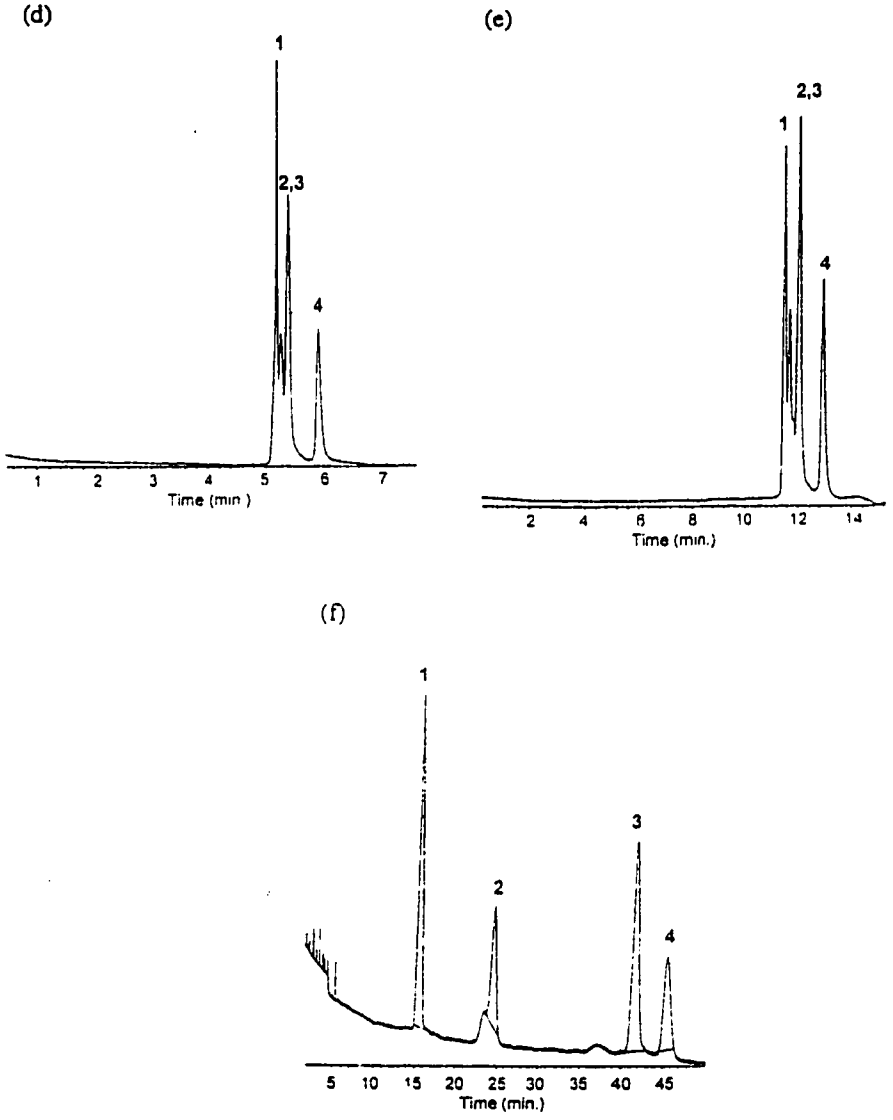


Figure 31 (cont.). Separation of cytochrome c's on a C-18 etched 20 μm I.D. capillary at (d) pH 2.14, (e) pH 3.0, and (f) pH 4.41

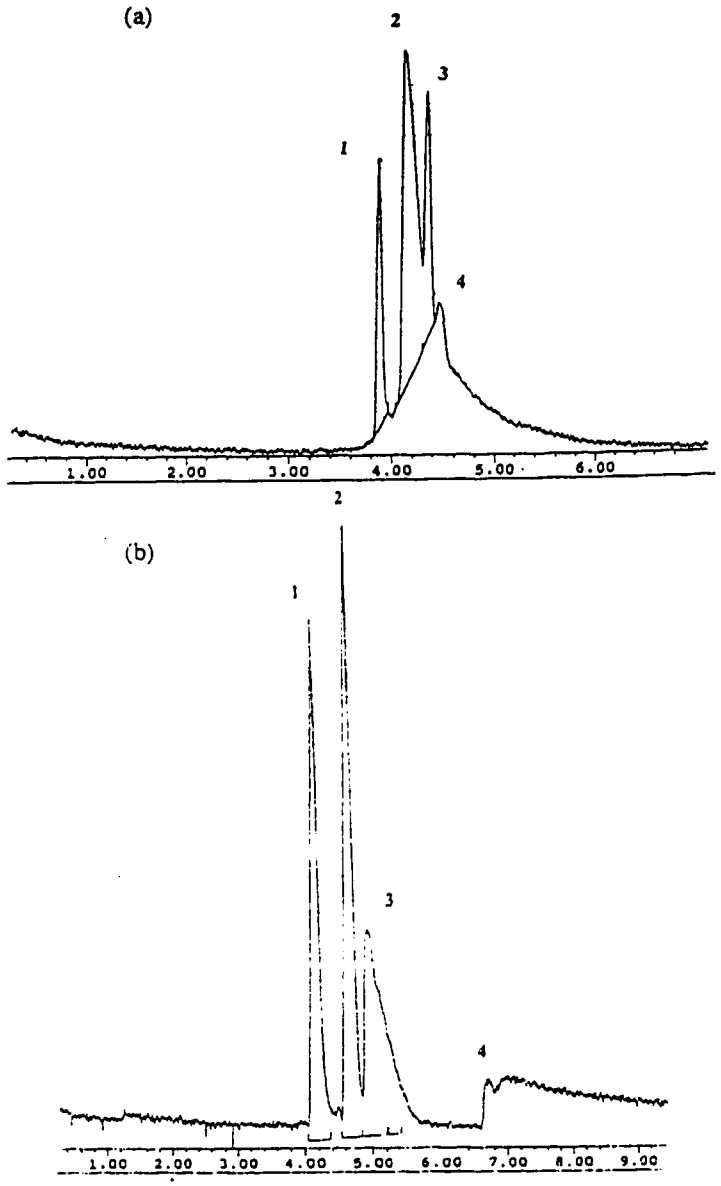


Figure 32. Separation of tetracyclines (1:anhydrotetracycline, 2: tetracycline, 3: chlorotetracycline, 4: epitetracycline) on (a) bare and (b) etched C-18 modified 20 μm I.D. capillaries at pH 2.14

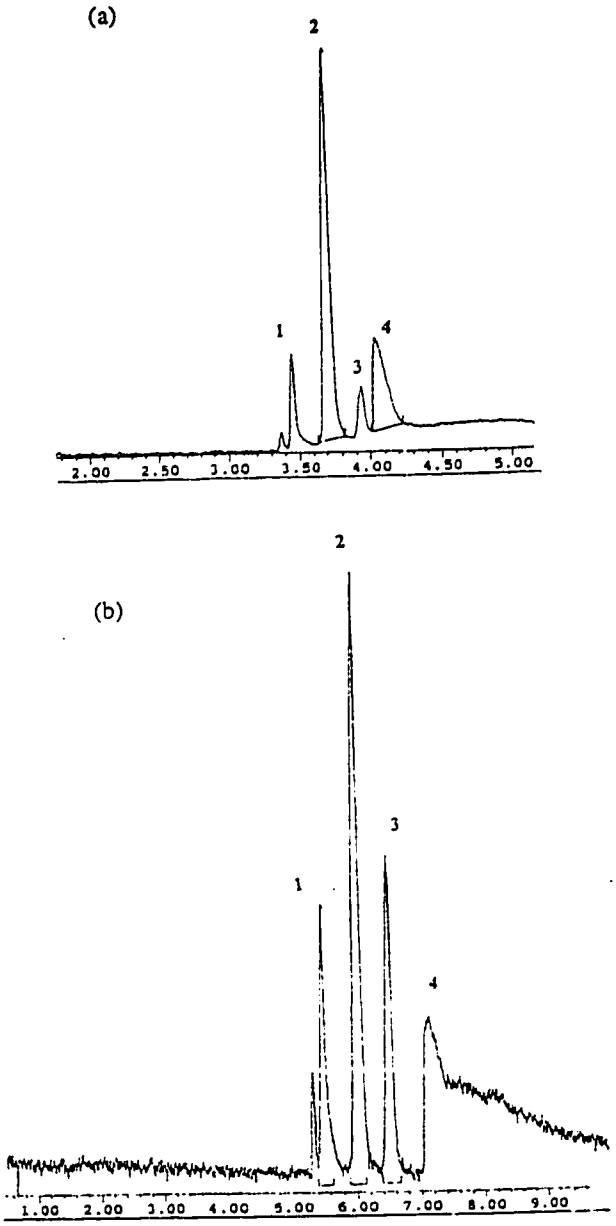


Figure 33. Separation of tetracyclines (1:4-epioxytetracycline, 2: tetracycline, 3: α-apooxytetracycline, 4: oxytetracycline) at pH 2.14 on (a) bare and (b) C-18 etched 20 μm I.D. capillaries.

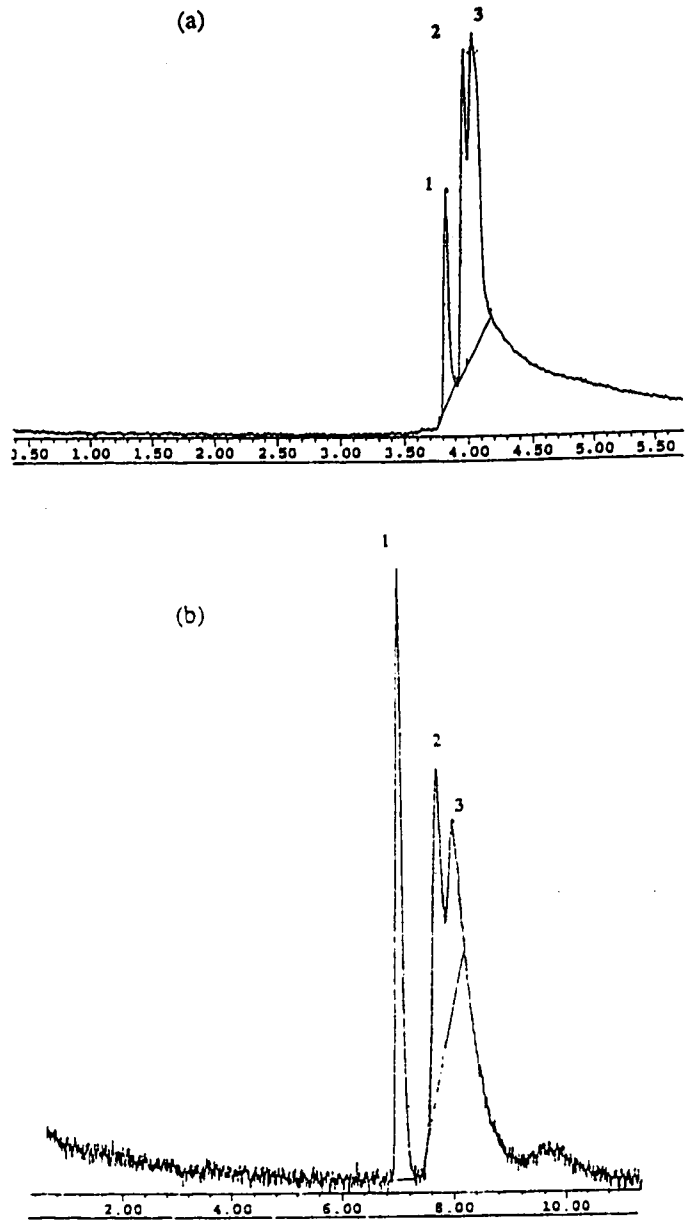


Figure 34. Separation of tetracyclines (1: oxytetracycline, 2: doxytetracycline, 3: methacycline) at pH 2.14 on (a) bare and (b) C-18 etched 20 μm I.D. capillaries

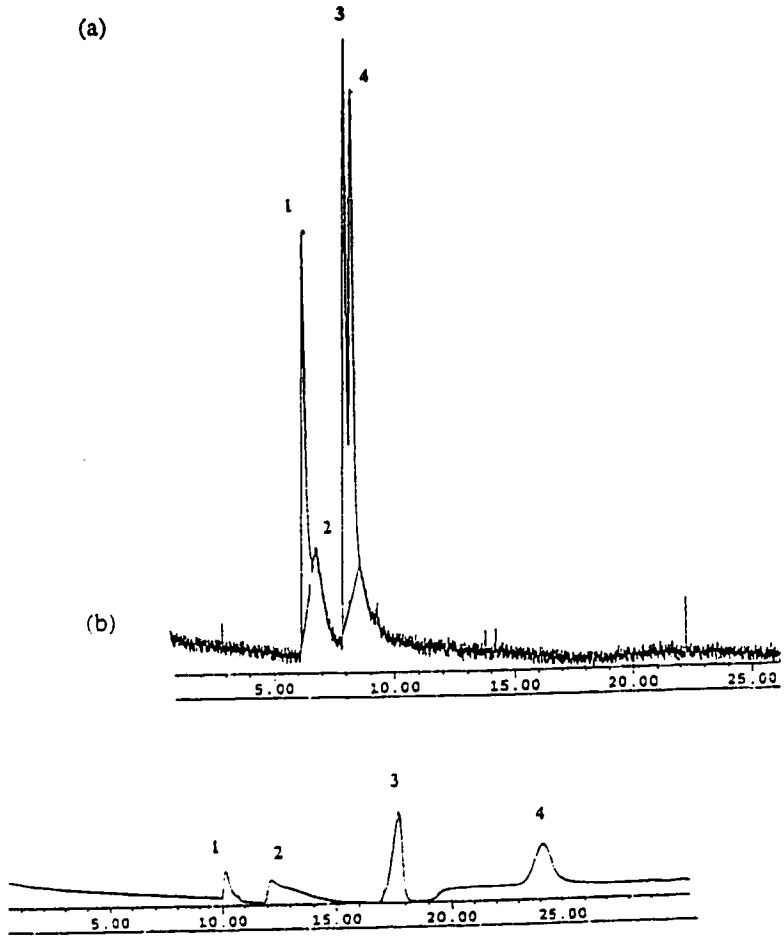


Figure 35. Separation of tetracyclines (1: 4-epioxytetracycline, 2: tetracycline, 3: α-apo-oxytetracycline, 4: oxytetracycline) on (a) a C-18 modified 20 μm I.D. capillary (a) at pH 4.41 and (b) at pH 3.0

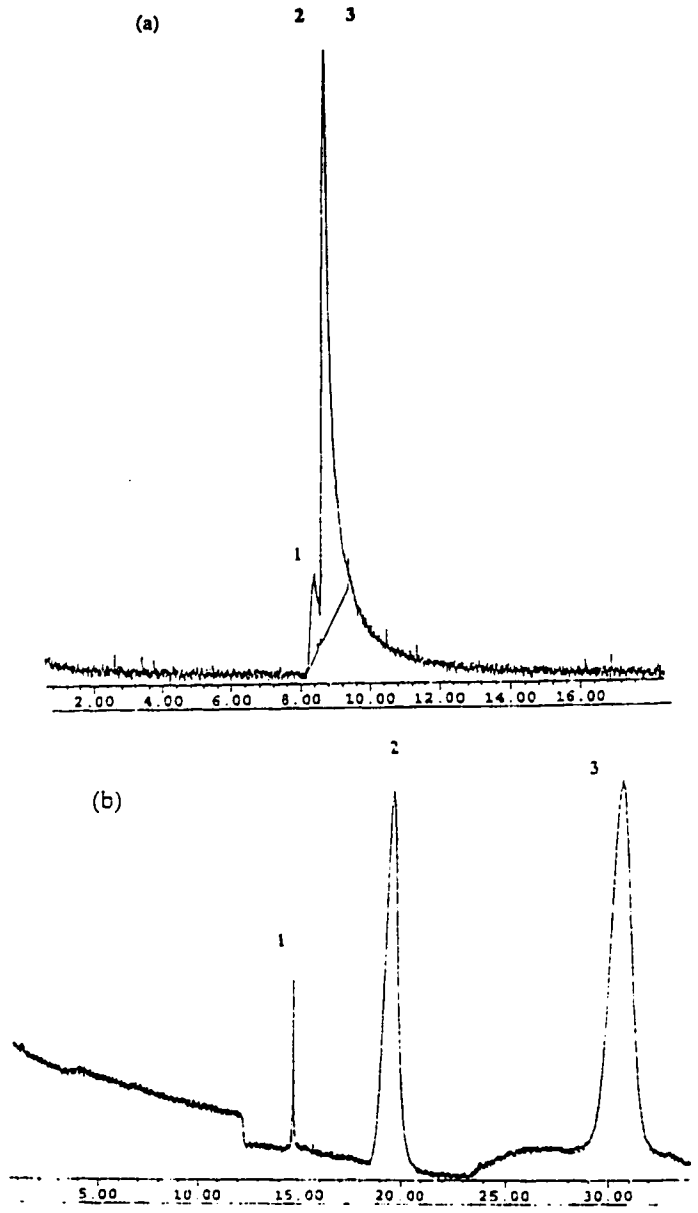


Figure 36. Separation of tetracyclines (1:oxytetracycline, 2: doxycycline, 3: methacycline) at pH 2.14 on (a) C-18 unetched and (b) C-18 etched 20 μm I.D. capillaries

CONCLUSIONS

Open tubular 20 μm I.D. CEC in which the surface is etched and modified with a C-18 moiety can provide high efficiency and improved separations compared with previous data obtained on 50 μm I.D. capillaries. The smaller inner diameter of the column leads to strong solute/bonded phase interactions caused by a higher surface area to volume ratio and better heat dissipation. The bonding method which produced a Si-C bond to the surface results in stable solute migration. The radial extensions produced by etching of the inner surface in this narrower capillary increases the surface area and the extension of the surface toward the center of the capillary makes solute interaction with the bonded organic moiety more favorable. The modified surface decreased electroosmotic mobility which caused increased retention times and enhanced separation. Therefore, the 20 μm I.D. CEC resulted in improved separation of proteins including lysozymes and antibiotics like tetracyclines. To perform all chemical reactions properly inside a 20 μm inner diameter capillary, dilution of the etching reagent and shortening the length of the capillary is useful to prevent blocking of the inner bore. The durability of this type of modified capillary is acceptable. Optimized voltage and pH conditions are necessary for high efficiency and improved separations. At higher voltage, the retention time is shorter as the EOF is faster, but the optimized highest voltage should be set up to inhibit side effects by increased heating. 30kV was determined to be the optimized voltage in these experiments. Cytochrome c mixtures provided interesting examples

about pH control. At the optimum pH of 3.7, complete separation was achieved but at the cost of longer analysis time. Surface characterization using the SEM technique can check the morphology of the narrow inner bore area and provide a visual confirmation of the etching process.

FUTURE STUDIES

Further characterization of the etched surface and the organic moiety attached to the surface is needed to understand more about the separation ability of this material. In addition to SEM, other emerging surface analysis techniques like Atomic Force Microscope (AFM), transmission Electron Microscope (TEM), and Focused Ion beam (FIB) voltage-contrast techniques may be used to identify more subtle features. The development of surface analysis with column technology will successfully eliminate unexpected anomalies on the surface and the reliability of the open tubular format capillary product will be improved. Other moieties attached to the capillary surface besides C-18 can be developed and compared with octadecyl separation. Different I.D. capillaries may be tested and compared to the results using 20 μm capillaries.

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