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Review

Open tubular capillary electrokinetic chromatography in etched fused-silica tubes

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Abstract

This review describes an open tubular approach to capillary electrochromatography (OTCEC) that first etches the inner surface of the fused-silica tube using ammonium hydrogen diflouride. This process can increase the inner surface area significantly. The new surface is then chemically modified to attach a bonded stationary phase using a silanization/hydrosilation reaction process. The surfaces are characterized spectroscopically by diffuse reflectance infrared Fourier transform and by electroosmotic flow measurements. Applications of OTCEC columns with C_{18}, diol and chiral stationary phases are described.

Keywords: Reviews; Etched capillaries; Silanization; Hydrosilation; Chemical modification

Contents

1. Introduction

Capillary electrochromatography (CEC) is an emerging method that provides a format for the separation of molecules that can potentially range in size from small inorganic and organic moieties to peptides and proteins [1]. Because of its unique features, CEC is considered to be complementary to other electroseparation techniques such as capillary electrophoresis (CE), capillary gel electrophoresis (CGE) and capillary micellar electrochromatography (CMEC) [2]. CEC is similar in some aspects to HPLC except that transport through the column is provided by electroosmosis and electrophoretic mobility, in contrast to pressure flow in the case of liquid chromatography. The main advantage of the electrically driven format, particularly in the open tubular format, is the flat flow velocity distribution of electrolyte movement through the column while in pressure driven systems a laminar profile is obtained.

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The result of the difference in the two flow profiles is that peak widths are inherently narrower in CEC than those found in typical HPLC separations. Thus, CEC is considered a hybrid technique that possess characteristics of both CE and HPLC, i.e. separation of solutes occurs via differential electrophoretic mobility as in capillary electrophoresis and solute–bonded phase interactions such as those found in liquid chromatography.

The most common format for CEC consists of a fused-silica capillary with an internal diameter of 50–100 μm (typical for CE) and packed with a stationary phase identical or similar to those found in HPLC columns, i.e. an octadecyl moiety bonded to 3–5 μm silica particles [1,3]. High efficiencies of almost 200 000 plates/m are often achieved for neutral compounds driven through the column by electroosmosis and separated though different degrees of interaction with the bonded moiety. CEC has developed significantly in recent years so that more sophisticated separations have been reported such as the resolution of enantiomers [4]. Improvements in instrumentation have now made gradient elution possible [5].

However, there are still a number of difficulties that remain to be solved in order for packed column CEC to be competitive with CE and HPLC. Probably the most challenging problem is the necessity to retain the packing material by frits while maintaining unrestricted flow through the bed [6–10]. Another drawback in packed capillaries is the tendency to form bubbles around the packing material or at the frit. Therefore, to prevent the formation of bubbles resulting in an unstable baseline, nonreproducible migration times or the interruption of current in the system the following alternatives have been developed: the solvent can be thoroughly degassed and pressure applied to one or both buffer vials [11,12]; low concentrations of electrolytes and/or a high proportion of organic solvent can be used in the buffer [13,14]; the buffer can consist of zwitterionic species [15]; or surfactants can be added to the buffer [16]. Reproducible packing for CEC can be more difficult than for ordinary HPLC columns due to the narrow inner bore of the capillary and the use small diameter particles (3 μm or less). Finally, basic compounds are often difficult to analyze by CEC due to the presence of silanols that are necessary in order to generate sufficient electroosmotic flow to move the solutes, particularly neutral compounds, through the column. Some recent applications of packed column CEC to the separation of basic compounds have been reported but it is necessary to add a competitive base to the mobile phase such as triethylamine [17], triethanolamine [17] or hexylamine [18].

An alternative approach to packed columns that still could retain some of the perceived advantages of CEC while eliminating the problems associated with frits and silica particles is the open tubular format. One significant drawback of this method is its low loading capacity since only the inner wall is available as a site for coating or bonding a stationary phase. Very low electroosmotic flow can be another problem, especially for neutral solutes, if the capillary wall is coated or derivatized. The approach described in this review involves etching of the inner wall of the capillary in order to increase the surface area by a factor estimated to be up to 1000 [19] followed by chemical modification to provide the desired selectivity [20–22]. The etching and modification processes, some examples of the variety of separations that are possible using this technology and the potential advantages for its use are discussed.

2. OTCEC column fabrication

2.1. Etching process

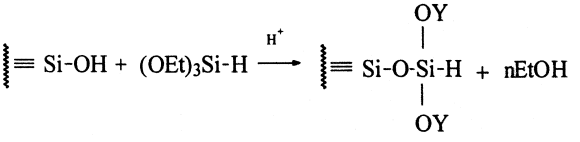
A section (~2 m in length) of bare capillary (Polymicro Technologies, Phoenix, AZ, USA) 375 μm O.D.×50 μm I.D. is filled with concentrated (12 *M*) HCl, sealed and heated overnight at 80°C. This step removes impurities on the wall that might subsequently be deposited on the etched surface [19]. Upon opening, the tube is flushed successively with deionized water, acetone and diethyl ether. The tube is then dried at ambient temperature for 1 h under nitrogen flow. After drying the capillary is filled with a 5% (w/v) solution of ammonium hydrogen difluoride in methanol and allowed to stand for 1 h. The methanol is removed by nitrogen flow for 0.5 h. The methanol is used for ease of drying and to assure uniform distribution of the etching agent on the surface after drying. After removal of the methanol,

the capillary is sealed at both ends and heated in a modified GC oven at temperatures between 300 and 400°C for a period of 3–4 h. The exact combination of time and temperature determines the surface morphology that is formed on the inner wall [20].

2.2. Chemical modification process

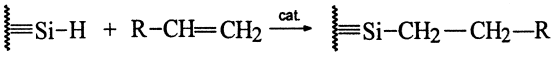
The chemical modification process used for attaching organic moieties to the etched surface is the silanization/hydrosilation method because almost all of the accessible silanol groups are removed and bonding to the inner wall occurs through a stable silicon–carbon bond [23]. In the first step triethoxysilane (TES) is bonded to the etched fused-silica surface as follows:

Silanization



After the hydride layer is formed on the surface, the desired organic moiety is attached via hydrosilation:

Hydrosilation



cat = catalyst, typically hexachloroplatinic acid

The two-step process reaction is started by first treating the capillary with a 6 m*M* ammonia solution, pH 10, for 20 h at a flow-rate of 0.1–0.2 ml/h in order to rehydroxylate the surface. The capillary is rinsed with deionized water followed by a wash with 0.1 *M* HCl and then a second rinsing with water follows. The tube is dried with nitrogen, filled with dioxane and flushed with a 1.0 *M* TES solution in dioxane for 90 min at 90°C. After the TES treatment, the capillary is washed with THF for 2 h and then with THF–water (1:1) for 2 h. After washing, the capillary is dried with a flow of dry nitrogen gas for 0.5 h. The resulting hydride capillary is flushed with dry toluene followed by a constant flow of an olefin as a pure liquid with Speier's catalyst (10 m*M* hexachloroplatinic acid in 2-propanol) or an olefin dissolved in toluene with the catalyst for a period of 90 h at 100°C. Before the olefin/catalyst solution is started, the mixture is heated to 60–70°C for 1 h. After completion of the hydrosilation reaction, the capillary is flushed successively with toluene and THF for 1 h. After clean-up, the capillary is dried overnight at 100°C under nitrogen flow.

3. OTCEC column characterization

Etching of the inner wall of the fused-silica capillaries is used to increase the surface area so that after attachment of an organic moiety there will be sufficient solute–bonded phase interactions to assist in the separation process. The surface morphology of the etched capillary depends on the reaction time and temperature. The surface features range from spikes of silica material extending 3–5 μm from the surface (Fig. 1A), to a series of hills or sand dunes (Fig. 1B), to large uniform boulder-like pieces of silica on the surface (Fig. 1C). Most of the structures are mechanically stable to vigorous vibrations except the spikes which are essentially destroyed after 30 min. Since the capillaries do not experience such drastic mechanical stress in normal use, even the spike structures survive under the conditions of a typical OTCEC experiment. Using the very high magnification available in atomic force microscopy (AFM), the surface displays even more fine structure than shown in the scanning electron microscopy (SEM) photographs. AFM can also be used to estimate the increase in area between a bare unetched capillary and etched surfaces such as the ones shown in Fig. 1. These AFM measurements [24] give estimates of up to a 1000-fold increase in the area depending on the etching conditions (time and temperature).

Attachment of the organic moiety takes place via the silanization/hydrosilation process that is described by the equations shown above. In the silanization reaction Y=H or $HSi(OY)_2$ depending on the extent of cross-linking. In most cases Y=$HSi(OY)_2$ since the efficiency of producing a monolayer is high (greater than 90% as determined by NMR [25]). The result is a new surface in which most of the silanols have been replaced by hydrides. The desired organic moiety is then attached to the hydride surface via catalytic addition of an unsaturated functional group, usually a terminal olefin. The final bonded material then possess an Si–C linkage at the surface which has been shown to be more stable under a variety of

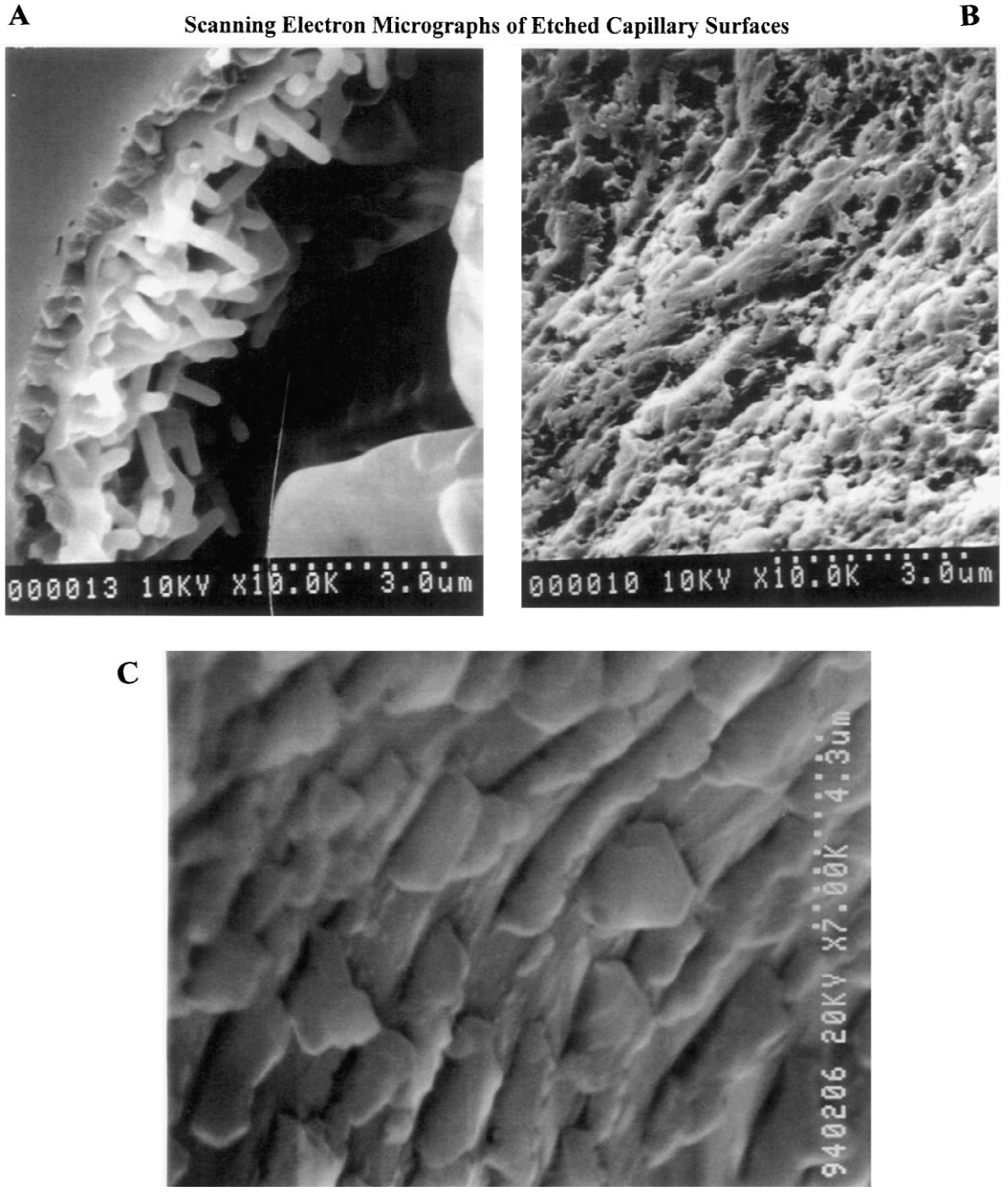


Fig. 1. Scanning electron micrographs (SEM) of etched capillary surfaces. Etching process was carried out for (A) 3 h at 300°C, (B) 2 h at 300°C and 2 h at 400°C, (C) 2 h at 300°C and 1 h at 400°C.

mobile phases conditions in HPLC as well as in capillary electrophoresis than the Si–O–Si–C linkage that results from bonding via conventional organosilanization [26–29]. The hydrosilation reaction has relatively few limitations with respect to the type of functional groups that can be part of the olefin that is attached to the hydride [30]. This allows greater versatility in choosing the organic moieties which will be part of the stationary phase. It has also been demonstrated that several types of catalysts are possible. The most common is Speier's catalyst (hexachloroplatinic acid), but other transition metal complexes can be used as well as several types of free radical initiators.

Porous silica bonded phases can be characterized by a variety techniques such as diffuse reflectance infrared Fourier transform (DRIFT), solid-state cross-polarization magic-angle spinning (CP-MAS) NMR, photoelectron spectroscopy (ESCA) and optical methods such as UV–visible or fluorescence spectroscopy. In general the surface area of a capillary, even an etched one, is too small to effectively utilize any of these methods. SEM, as described above and illustrated in Fig. 1, or AFM can provide topographical but not chemical information about the surface. Therefore, we have attempted through careful sample preparation and the use of the more sensitive mercury–cadmium–telluride (MCT) detector to apply DRIFT measurements to the etched chemically modified capillaries. Fig. 2 shows the partial DRIFT spectrum obtained for the cyclodextrin modified etched capillary [31]. It contains two essential features that have been identified on porous silica materials modified by the silanization/hydrosilation method: the Si–H stretching band at 2270 cm^{-1} and peaks in the C–H stretching region between 2800 and 3000 cm^{-1}. Neither of these features is observed on an etched capillary that has not been chemically modified by the silanization/hydrosilation process. Other potential features of the cyclodextrin spectrum such as C–O, C=O and OH bands are either too weak to be observed or are masked by the residual signals from the silica matrix and/or water adsorbed on the surface.

Another interesting feature of the etched capillaries has been identified from electroosmotic flow

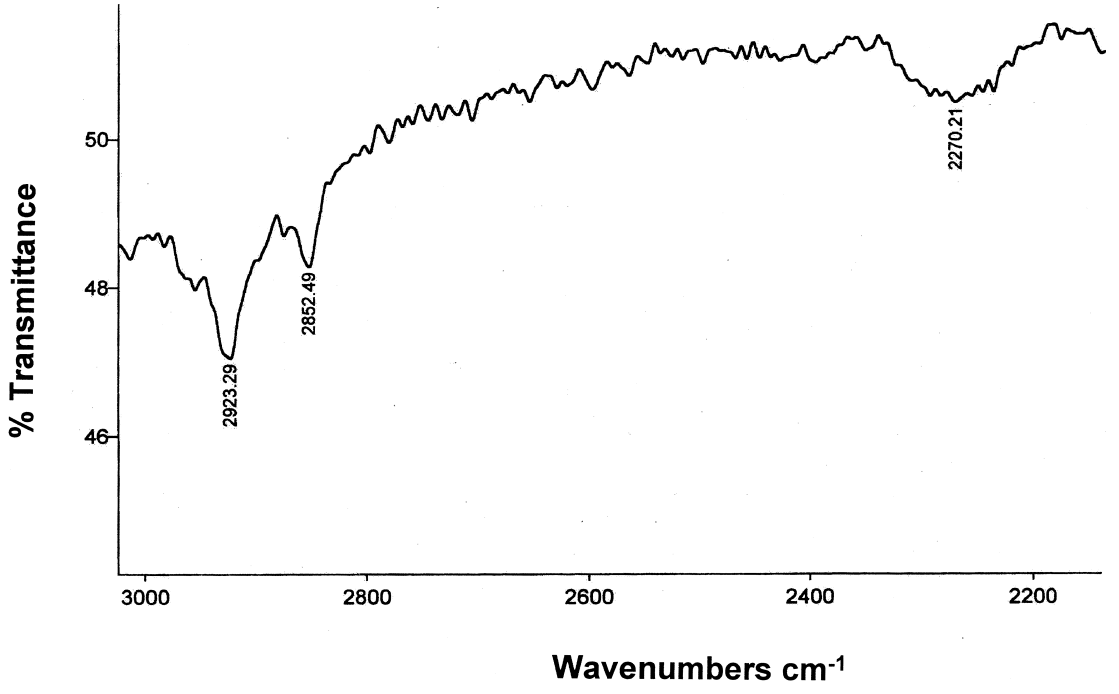


Fig. 2. Partial DRIFT spectrum of the internal surface of etched 50-μm capillary modified with 2-hydroxy-3-methacryloxy-propyl-β-cyclodextrin.

(EOF) studies. EOF studies on both 20- and 50-μm etched, chemically modified capillaries have revealed an unusual behavior in comparison to normal fused-silica capillaries. Fig. 3 shows a plot of EOF as a function of pH for a C_{18} modified etched capillary over the range of values used in typical electrokinetic chromatography studies with this column [32]. The most interesting aspect of this capillary is the reverse electroosmotic flow which is present below pH~4.5. Even though the anodic flow is small, it would retard movement of solutes through the column thereby enhancing solute–bonded phase interactions. The exact nature of the positively charged species on the surface is not known at this time but it must be the result of the etching process since all of the unetched C_{18} modified capillaries made to date in our laboratory exhibit cathodic EOF over the pH range shown in Fig. 3. Since the etching reagent consists of an ammonium ion, this species or an impurity in $NH_4(HF_2)$ incorporated into the silica matrix is most likely responsible for a positive charge at low pH. ESCA studies are currently in progress that may help to elucidate some of the chemical features of the etched surface. Similar results have been obtained for other etched capillaries chemically modified with different organic moieties lending support to the hypothesis that the reverse EOF is a result of the treatment with ammonium hydrogen difluoride.

4. OTCEC applications

To date several types of organic moieties have been attached to etched capillary surfaces and tested in the OTCEC format. These bonded phases include octadecyl, diol and several chiral selectors. Some examples on each of these OTCEC columns as well as a nonbonded coating are discussed in this section.

A major problem for packed columns in capillary electrochromatography is the strong interaction of basic solutes with the silanols present on the silica particles. In the etched chemically modified capillary the number of accessible silanols is postulated to be quite small. Verification of this assumption was seen in the electrochromatogram of a mixture of tetracyclines, compounds with at least one basic nitrogen, obtained on an etched C_{18} modified capillary [21]. The components were well-separated, but more important the peaks were all quite symmetrical. If the same sample is run on a C_{18} packed capillary, the

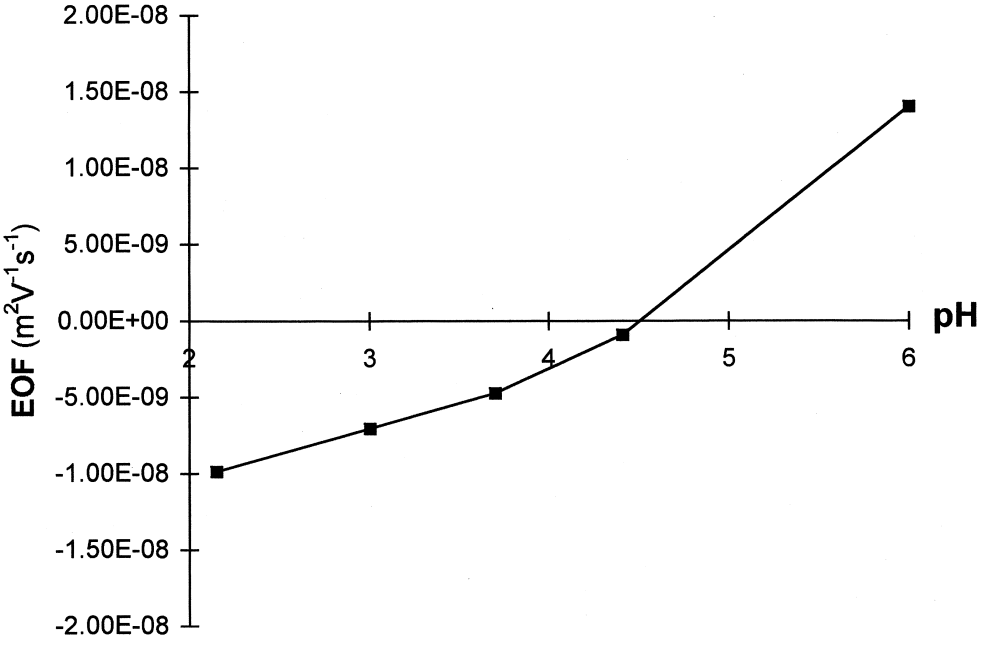


Fig. 3. Electroosmotic flow as a function of pH in an etched C_{18} modified capillary.

peaks all exhibit very pronounced tailing indicating substantial interaction with the silanols on the silica surface.

An example that illustrates the effect of the bonded moiety utilizes a mixture of three angiotensins — peptides that are responsible for cardiovascular constriction [22]. The mixture was run on an etched columns modified with a diol moiety and with an octadecyl group. All of the experimental variables were kept constant so that the only other difference in the columns was the type of organic moiety bonded. By comparing the two electrochromatograms, it was determined that the retention on the C_{18} column was greater as indicated by the longer elution times for each of the three components. Another indication of the effect of the bonded group was obtained by comparing the relative retention (migration) times, α, for pairs of solutes on the two columns. For example $\alpha_{3,1}$ on the C_{18} bonded column was 2.54 while on the diol bonded column it was 1.28. For $\alpha_{2,1}$ the corresponding values were 1.32 on C_{18} and 1.10 on diol.

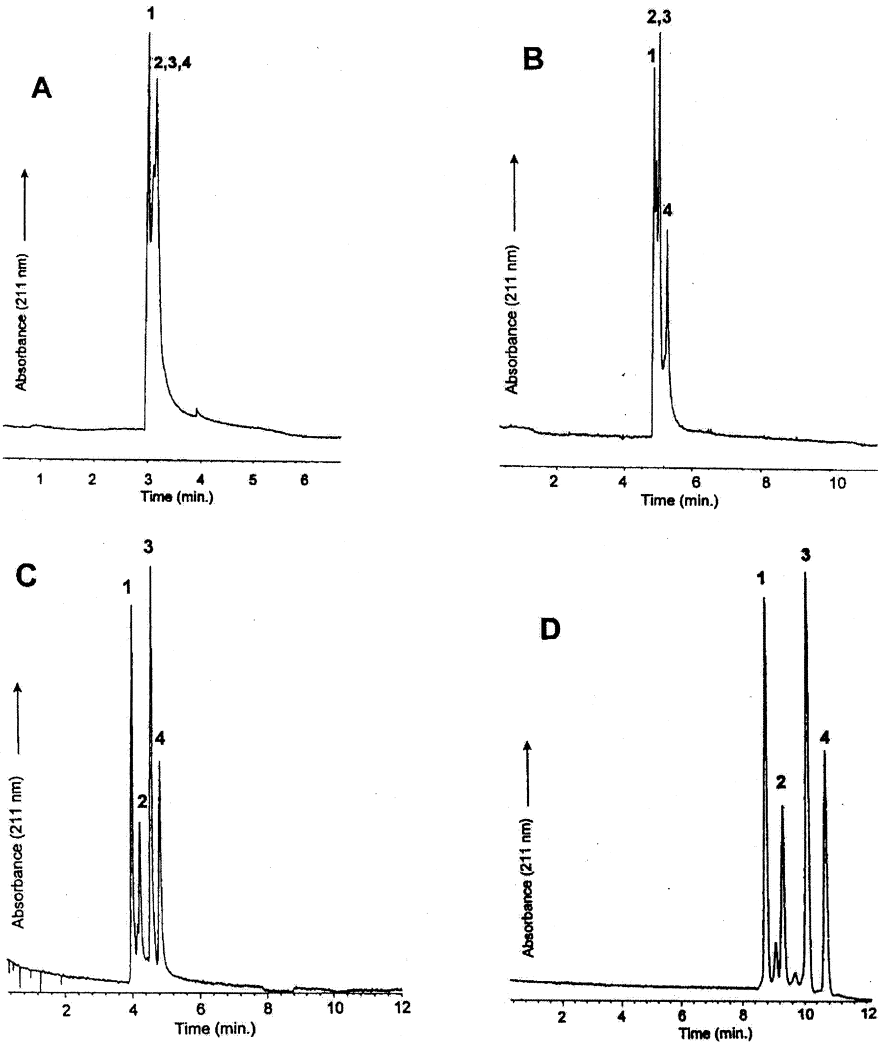


Fig. 4. Separation of a mixture of cyctochromes c on 20 μm I.D.: (A) bare capillary (B) unetched C_{18} modified capillary (C, D) etched C_{18} modified capillary. (A–C) 30 kV applied voltage and (D) 15 kV. Conditions: pH 3.7 (60 m*M* β-alanine and 60 m*M* lactic acid); detection at 211 nm; injection 2 s at 12.5 cmHg vacuum; $L=50$ cm; and $l=25$ cm.

Another example that illustrates the capabilities of this method is the separation of a cytochrome c mixture [32] from four different sources (horse, tuna, chicken and bovine heart). The electropherogram for this sample on a bare 20-μm capillary (not etched) is shown in Fig. 4A. Only partial resolution of the mixture is obtained and there is noticeable peak tailing for these compounds. Even thought the pH is quite acidic (3.7), significant interaction with surface silanols is evident. This pH represents the optimum for the cytochrome c separation so that no further improvement in separation is obtained under more acidic conditions but less peak tailing is observed. Upon modification of the inner wall of the capillary by hydrosilation of 1-octadecene on a hydride surface, the same sample mixture results in the electrochromatogram shown in Fig. 4B. This surface produces a partial resolution of all four components at the optimum pH value. If the surface is etched before chemical modification, the electrochromatogram at pH 3.7 for the cytochrome sample is shown in Fig. 4C. It can be seen that separation of each of the principle components is obtained as well as the detection of one small impurity peak. Better resolution of all species including the impurities is obtained by lowering the applied potential (Fig. 4D).

An etched capillary modified with a chiral selector based on (+)-naphthylethylamine was tested for enatiomeric resolution [31]. The electrochromatogram obtained for 3,5-dinitrobenzoyl-D,L-alanine methyl ester is shown in Fig. 5. The resolution ($\alpha = 1.14$), efficiency ($\cong 12\,000$ plates/m) and peak symmetry ($A_S = 1.1$) are reasonable for a chiral separation. However, as demonstrated in HPLC experiments changes in the amino acid portion of the molecule (i.e. alanine→valine) often result in a loss of chiral resolution. This is not surprising since the separation mechanism in the HPLC and electrochromatographic modes should be identical. Other chiral selectors bonded to the etched capillary surface resulted in resolution of tricyclic antidepressant and benzodiazepine enantiomers.

Another use of the etched capillaries involved the separation of proteins on a polybrene coated column [33]. This study involved a comparison between two capillaries, one that was first etched using the procedure described above and a second that was used as received from the manufacturer. Each capillary was then coated by flushing it with a 1% polybrene solution prepared in the electrophoretic buffer. The capillary was then flushed with the electrophoretic buffer for 3 min. For reproducible results the capillary was flushed with a 1% polybrene solution for 3 min followed by the electrophoretic buffer for 3 min between each run. A set of standard model proteins was used to determine the stability of the polybrene coating, the reproducibility of the protein mobility and an indication of wall adsorption: myoglobin (1 mg/ml), p*I* 7.6; ribonuclease A (1.2 mg/ml), p*I* 9.6; cytochrome c (1 mg/ml), p*I* 10.3; and lysozyme (1 mg/ml), p*I* 10.6. The differences in the performance between unetched and etched coated capillaries were demonstrated by running the above protein mixture. The proteins run had p*I* values above the pH of the electrophoretic buffer, and therefore cathodic mobilities. However, the strong anodic EOF that results from the coating acts to promote the mass transport of all species towards

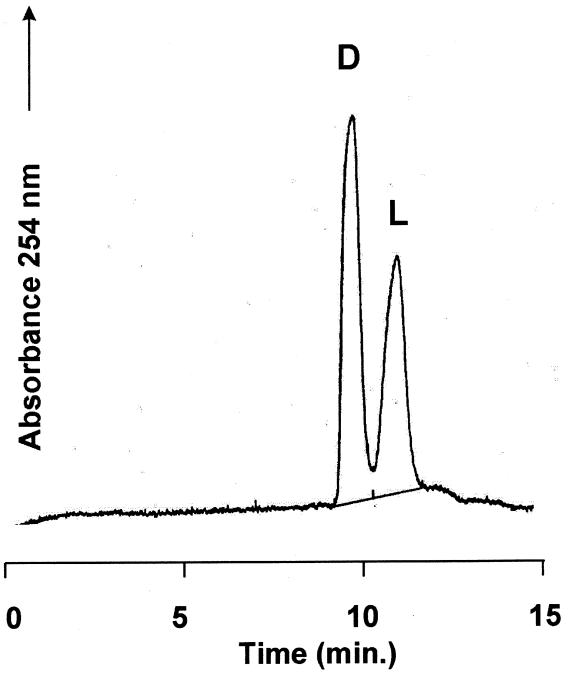


Fig. 5. Electrochromatogram for the separation of 3,5-dinitrobenzoyl-D,L-alanine-methyl ester on an etched naphthylethylamine modified capillary. Conditions: pH 2.14 (60 m*M* phosphoric acid and 38 m*M* Tris); detection at 254 nm; injection 2 s at 12.5 cmHg vacuum; $L = 50$ cm; $l = 37$ cm.

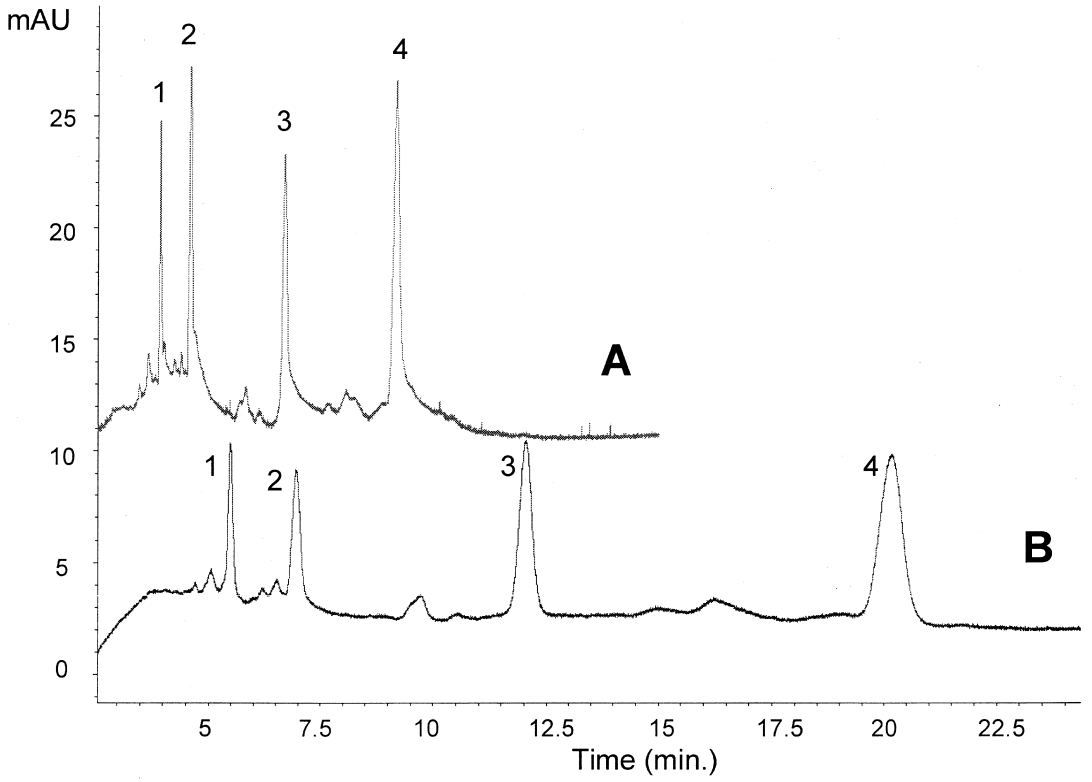


Fig. 6. Electrochromatogram of protein mixture on unetched (A) and etched (B) capillaries coated with polybrene. Conditions: $L=33.5$ cm; $l=25$ cm; pH 7.2 phosphate buffer; negative polarity; injection at 50 mbar for 2 s; $T=28$°C. Peaks: 1=myoglobin; 2=ribonuclease a; 3=cytochrome c and 4=lysozyme.

anode. Fig. 6 clearly shows that total analysis time for the unetched capillary is less than for the etched capillary. The elution was, as expected, in the same order as the pI values (myoglobin<ribonuclease, cytochrome c<lysozyme) due to the effects of the anodic EOF. A complicating factor is that the EOF was different for the two capillaries. In fact the EOF on the etched capillary is slower than the EOF on the unetched capillary ($-1.35\cdot10^{-4}$ vs. $-2.40\cdot10^{-4}$ $cm^2/V\,s$) probably due to the inclusion of one or more chemical species from the etching reagent in the surface matrix as discussed in Section 3. Thus, it was necessary to evaluate the mobility values in order to determine if there were substantial differences for each of the protein species on the two capillaries. To check these findings, a confidence interval estimate ($P<0.10$ at the 90% confidence level for the range of measured values) was constructed for the mobilities of the proteins run on the two capillaries. The mobility values (shown in Table 1) obtained for the proteins run on the conventional fused-silica capillary fell outside the intervals con-

Table 1
Mobilities ($cm^2/V\,s$ ($\cdot10^{-6}$) and confidence intervals[a] for the etched vs. nonetched capillaries

	Myoglobin	RnaseA	Cytochrome C	Lysozyme
Etched capillary	−2.5±0.2	9.6±0.2	63.9±0.1	93.7±0.6
Unetched capillary	4.3±0.5	38±0.4	101±0.5	136±0.5

[a] Where $\bar{x}\pm t/2\sqrt{s^2/n}$; t values chosen for ($P<0.10$) with six degrees of freedom, $n=6$.

structed for the protein mobilities determined on the etched capillary. It was concluded that the mobility values for the same protein are statistically significantly different on the two columns. If the experiments had been dependent on electrophoretic separation alone, the mobilities theoretically should have been constant. Since the only difference between the two data sets was the increased surface area of the etched capillary, these results indicate a chromatographic component as the only plausible explanation for the increased retention of proteins.

A final consideration for this approach to CEC is the reproducibility of the data obtained. The stability of the bonding process used in this method results in a Si–C linkage at the surface that has already been verified in a CE format [29]. The variation in the retention time as a function of the number of injections for two solutes on a C_{18} modified etched capillary was determined. For a series of 31 injections, the reproducibility of the migration times was $\pm 1.5\%$. More important, this test was run after the capillary had been used for more than 300 injections of other samples, been washed several times with methanol and even dried and stored for a period of several weeks before the reproducibility studies. The change in EOF between the 1st and 300th runs was $<2\%$.

5. Conclusions

CEC is a rapidly developing technology with a wide variation in the formats that gives the method considerable versatility. The open tubular approach addresses some of the drawbacks found in packed capillaries. The use of etched chemically modified capillaries has been proven to be viable for many types of CEC analyses. It appears that charged compounds are more amenable to this configuration than neutral species because of the relatively small k' values that are present for most solutes in 50-μm capillaries and possibly even in 20-μm capillaries. With radial extensions from the surface of no more than 5 μm, diffusion from the bulk of the solution to the bonded moiety is still quite limited resulting in small capacity factors. For ionic analytes, the solute–bonded phase interactions are superimposed upon differences in electrophoretic mobility to enhance the separation process. An extension of the studies reported above is currently underway exploring additional possibilities for exploiting OTCEC to solve difficult separation problems.

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