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Open tubular capillary electrochromatography in etched, chemically modified 20 μm I.D. capillaries

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Abstract

Fused silica capillaries with an I.D. of 20 μm are etched and then chemically modified by the silanization/hydrosilation method to attach an octadecyl moiety for use in electrokinetic chromatography. The etched capillaries after chemical modification are shown to have an anodic electroosmotic flow below pH 4.5. In comparison to bare 20 μm capillaries and unetched but chemically modified 20 μm capillaries, the etched C_{18} fused silica tubes show better separation of mixtures of lysozymes and cytochrome *c*'s under identical conditions of buffer, pH and applied voltage. It was also demonstrated that this open tubular approach to capillary electrochromatography was amenable to a number of different types of basic compounds ranging in size from typical small amines to biomolecules. As expected, pH is an important variable that must be controlled in order to obtain an optimized separation. Reproducibility studies verify the stability of the silicon–carbon linkage produced in this modification method so that column lifetimes of at least 300 injections can be expected.

Keywords: Capillary columns; Electrochromatography; Proteins

1. Introduction

Capillary electrochromatography (CEC) is a rapidly developing method which is complementary to the other electroseparation techniques such as capillary electrophoresis (CE), capillary gel electrophoresis (CGE) and capillary micellar electrochromatography (CMEC) [1,2]. The normal format for CEC is similar to CE, utilizing a fused silica tube with an internal diameter of 50–100 μm. However, the capillary is then packed with a stationary phase identical or comparable to those used in HPLC such as an octadecyl moiety bonded to 3–5 μm silica particles [1,3]. In another mode of CEC which is referred to as open tubular capillary electrokinetic chromatography (OTCEC) the use of support materials such as porous silica particles is eliminated. A number of different options have been developed for utilizing the open tubular approach such as fabrication of a porous structure inside the capillary [4], bonding of linear or cross-linked polymers to the wall [5,6] or etching the inner surface followed by bonding of various monomeric alkyl moieties [7–9]. In all cases retention of solutes results from a partitioning mechanism between the stationary and mobile phases; and for charged solutes differences in electrophoretic mobility also contribute to the separation mechanism. The availability of the dual (mixed mode) mechanism is essential for separations utilizing etched, chemically modified capillaries when the I.D.

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of the fused silica tubes is 50 μm because k' values are small, at least for neutral solutes [7]. Enhancement of solute–bonded phase interactions is expected as the diameter of the capillary becomes smaller.

Our method for OTCEC based on extensive etching of the inner capillary wall has been developed using a process similar to one originally designed for capillary GC to increase the surface area available for the coating of polymer phases [10]. Ammonium hydrogendifluoride is the etching agent which, under carefully controlled conditions of temperature and reaction time, can produce increases in surface area by a factor of several hundred to a thousand or more. The earlier studies [7–9] involved 50 μm capillaries which were etched for 3–4 h at 300–400°C to increase the surface area sufficiently to induce significant k' interactions for proteins, peptides and tetracyclines on octadecyl and diol stationary phases.

Bonding of the organic moiety to the etched capillaries utilizes the silanization/hydrosilation reaction scheme [11]. The etched surface of the capillary is first reacted with triethoxysilane (TES) to produce a hydride layer. Under ideal conditions, the TES reaction results in a monolayer deposited on the surface so that most of the silanols are replaced by hydrides. Attachment of an organic moiety to the hydride intermediate is accomplished by passing a solution containing a terminal alkene and a suitable catalyst such as hexachloroplatinic acid through the capillary. This second step is referred to as hydrosilation and in the previous studies [7–9] the alkenes used were 1-octadecene and 7-octene-1,2-diol. The silanization/hydrosilation process has also been used to modify unetched capillaries for HPCE in order to attach an acrylamide-type polymer, poly(*N*-acrylolyaminoethoxyethanol [poly(AAEE)] [12]. One particular benefit of this bonding method is attachment of the organic moiety to the surface via a stable Si–C linkage. The poly(AAEE) coating synthesized through a hydride intermediate was shown to be more stable than similar capillaries made by organosilanization. In this study 20 μm fused silica capillaries etched by ammonium hydrogendifluoride and modified by the silanization/hydrosilation process are evaluated as a separation medium for OTCEC.

2. Experimental

2.1. Materials

Lysozyme (turkey and chicken egg white), cyctochrome *c* (horse, tuna, chicken and bovine heart), ribonuclease A (bovine pancreas) and myoglobin were purchased from Sigma (St. Louis, MO, USA). The aspartame, serotonin and tryptamine were obtained from Aldrich (Milwaukee, WI, USA). The buffers consisted of: pH=2.14, 60 m*M* phosphate (Fisher Scientific, Pittsburgh, PA, USA) and 38 m*M* Tris (Sigma); pH=3.0, 60 m*M* citric acid (Sigma) and 50 m*M* β-alanine (Sigma); pH=3.7, 60 m*M* β-alanine and 60 m*M* lactic acid (Sigma); pH=4.41, 60 m*M* acetic acid (Aldrich) and 60 m*M* γ-aminobutyric acid (Sigma); pH=6.0, 60 m*M* 2-(*N*-morpholino)ethanesulfonic acid (Sigma) and 45 m*M* histidine (Sigma). Deionized 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). Ammonium hydrogendifluoride used to etch the capillaries was purchased from Aldrich. Triethoxysilane (United Chemical Technologies, Bristol, PA, USA), 1-octadecene (Aldrich) and hexachloroplatinic acid (Aldrich), Speier's catalyst, were used for subsequent modification of the etched capillary. The capillary tubing used was 375 μm O.D.×20 μm I.D. (Polymicro Technologies, Phoenix, AZ, USA).

2.2. Instrumentation

OTCEC and HPCE measurements were made on a Perkin-Elmer/Applied Biosystems Model 270A-HT system (Applied Biosystems, Foster City, CA, USA). The oven used for etching and modifying the capillaries was from a Hewlett-Packard Model 5890 gas chromatograph. The inlet and outlet ports were altered in order to accommodate several capillaries at the same time. Scanning electron microscopy (SEM) images were acquired with a Hitachi S-800 Field Emission Scanning Electron Microscope (Tokyo, Japan) at the IBM Almaden Research Labs., San Jose, CA, USA.

2.3. Capillary preparation

Procedures for preconditioning of the capillary surface with ammonia, the etching with ammonium hydrogendifluoride, the preparation of the hydride intermediate, and the hydrosilation reaction for attaching the desired organic moiety to the inner wall of the capillary are described in detail elsewhere [7,8].

2.4. Electrochromatography

All capillaries were conditioned first by forcing at least 50 column volumes of buffer through them with a syringe. The mobile phases were degassed by ultrasonication followed by purging with He. Injection of samples was done both hydrodynamically by vacuum and electrokinetically. Samples were detected at either 211 nm or 223 nm. Electroosmotic flow (EOF) measurements were made by the two marker injection method designed for determining low electroosmotic velocities [13].

3. Results and Discussion

3.1. SEM characterization

SEM images of the 20 μm capillaries etched under different conditions (time and temperature) confirm previous results reported for 50 μm capillaries [7]. The general trend observed is that for shorter etching periods (~2–3 h) at lower temperatures (~300°C) the inner wall consists of relatively long spikes of silica material protruding from the surface. For longer etching periods (~4 h) at higher temperatures (~400°C) the structure has fewer long extensions from the surface and becomes somewhat more regular, either resembling sand dunes or a sponge-like porous configuration. More detailed studies by atomic force microscopy (AFM) are currently underway for characterizing etched and etched chemically–modified capillary surfaces. Preliminary estimates from the AFM measurements indicate that the area of the inner wall is increased significantly (100–1000-fold) which should facilitate solute/bonded phase interactions after appropriate organic moieties are attached to this etched surface.

3.2. EOF measurements

Fig. 1 shows a plot of EOF as a function of pH for the C_{18} modified etched capillary over the range of values used in the electrokinetic chromatography studies. 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 the etching reagent consists of an ammonium ion, this is the most likely species to be incorporated into the silica matrix and hence responsible for a positive charge at low pH. Electron spectroscopy for chemical analysis (ESCA) studies are currently in progress which may help to elucidate some of the chemical features of the etched surface.

3.3. CEC vs. CE characterization

In order to illustrate the effects of both modification and etching, comparisons between electrochromatograms on bare, chemically modified and etched chemically modified capillaries were obtained for several types of samples. The first of these comparisons involves a mixture of chicken and turkey lysozyme. In Fig. 2A, the results obtained on a bare 20 μm I.D. capillary are illustrated which show only a partial separation of these two proteins. However, upon chemical modification of the same I.D. capillary by the silanization/hydrosilation method using 1-octadecene as the organic moiety bonded to the inner wall, improvement in the separation is obtained so that there is nearly baseline resolution of the two lysozymes (Fig. 2B). If the inner surface of the capillary is etched before chemical modification, the resulting separation of chicken and turkey lysozyme is improved further (Fig. 2C).

The second example involves the separation of a cytochrome *c* mixture from four different sources (horse, tuna, chicken and bovine heart). The elec-

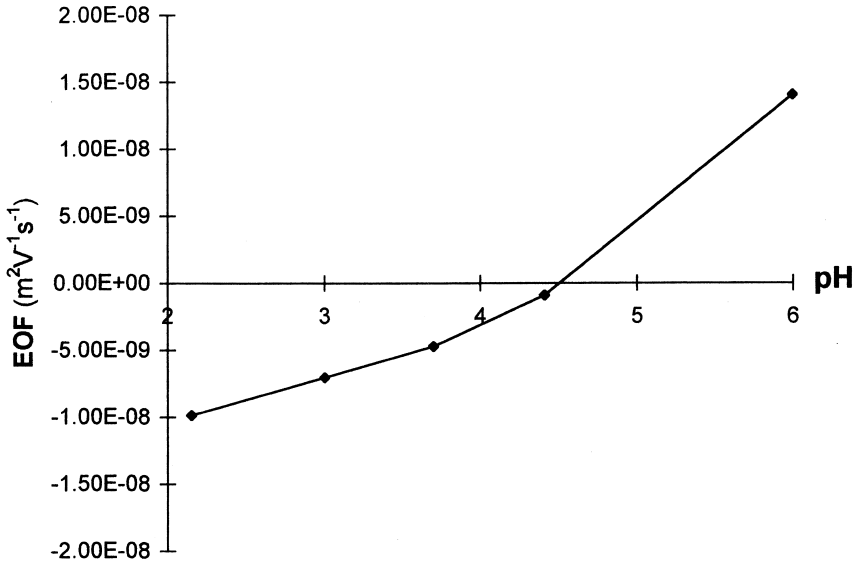

Fig. 1. Electroosmotic flow as a function of pH in the etched C_{18} modified 20 μm I.D. capillary. Marker is dimethyl sulfoxide (DMSO) with detection at 211 nm. Each point is an average of three measurements.

tropherogram for this sample on a bare 20 μm capillary (not etched) is shown in Fig. 3A. Only partial resolution of the sample is obtained and there is noticeable peak tailing for these compounds. The pH is still quite acidic (3.7) but significant interaction with surface silanols is evident. This pH represents the optimum for the cytochrome *c* separation (see discussion below) 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 electrokinetic chromatogram shown in Fig. 3B. This surface produces a partial resolution of all four components at the optimum pH value. If the surface is etched before chemical modification, the electrokinetic chromatogram obtained at pH 3.7 for the cytochrome sample is shown in Fig. 3C. 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 can be obtained by lowering the applied potential (Fig. 3D). Because of the unusual EOF behavior of the etched chemically modified capillary (Fig. 1) some of the improvements in resolution described in Figs. 2 and 3 may be the result of changes in electroosmotic flow.

3.4. Additional CEC characterization

In order to further assess the performance of the etched C_{18} modified 20 μm capillary, the electrokinetic chromatographic behavior of a variety of basic samples was evaluated. The elution of aspartame, a basic compound which is used as a sweetener in food and pharmaceutical products [14], is shown in Fig. 4. In comparison to a bare capillary under the same conditions, the peak is considerably narrower (N=110 000 vs. 45 000) and the migration time is longer (4.9 min vs. 3.7 min). The sharper peak should improve the precision of quantitative determinations and the detection limit with respect to the previously reported CE method [14]. The peak in Fig. 4 is also sharper than the one (N=25 000) obtained for an etched diol modified 50 μm capillary [14]. In both comparisons it appears that the etched, C_{18} modified 20 μm capillary has the more favorable surface properties for aspartame analysis.

Two other basic compounds which are often both present in certain physiological samples are tryptamine and serotonin [15]. Their separation on the

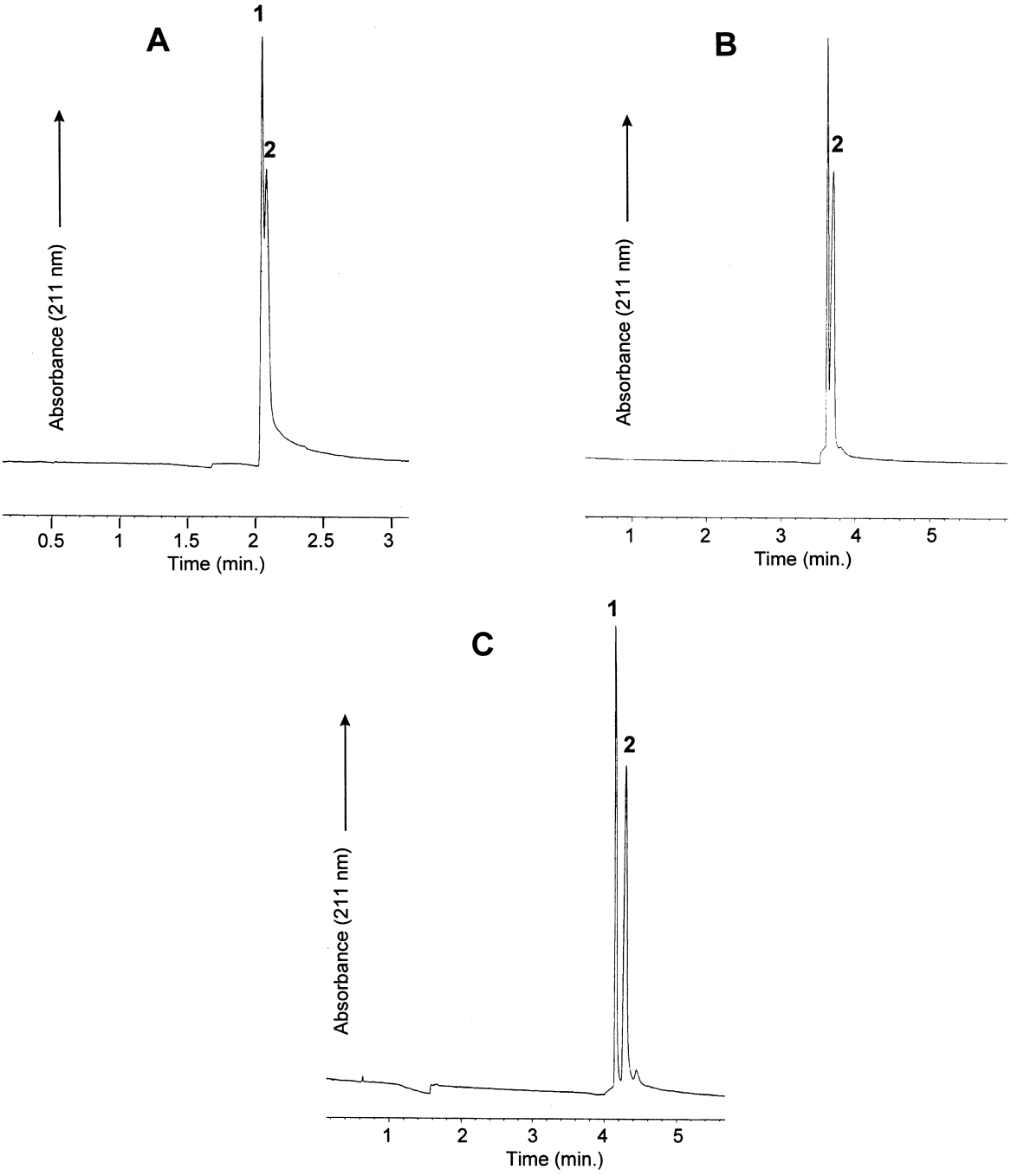

Fig. 2. Separation of chicken and turkey lysozymes on 20 μm I.D. (A) bare capillary, (B) unetched C_{18} modified capillary and (C) etched C_{18} modified capillary. Conditions: V=30 kV, pH=3.7, detection at 211 nm, injection 2 s at 12.5 cmHg vacuum (1 mmHg=133.322 Pa), L=50 cm (A and B), 51.5 cm (C), l=25 cm (A and B), 22 cm (C). Solutes: 1=turkey and 2=chicken.

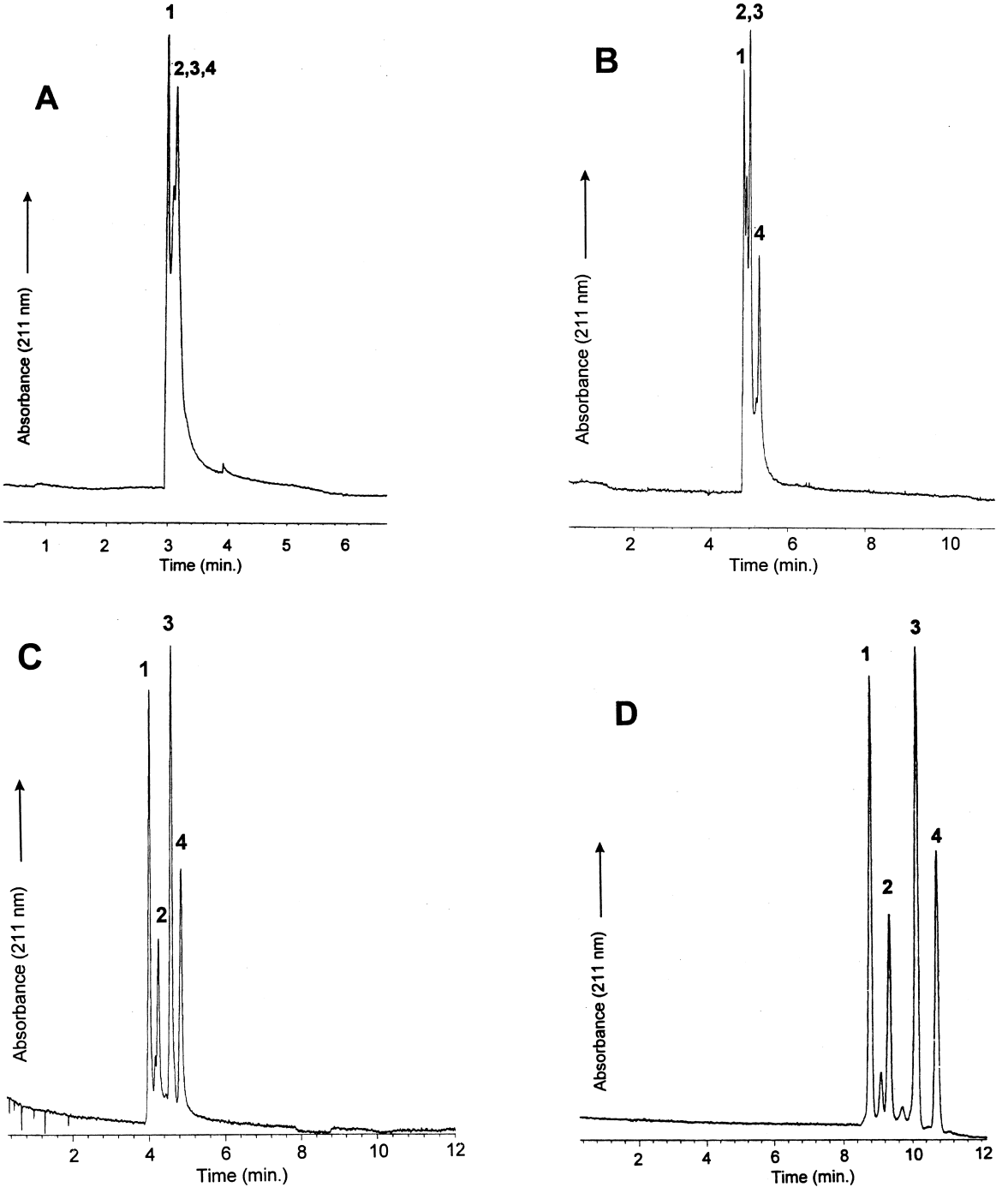

Fig. 3. Separation of a mixture of cytochrome *c*'s on 20 μm I.D. (A) bare capillary, (B) unetched C_{18} modified capillary, and (C) and (D) etched C_{18} modified capillary. Conditions: Same as Fig. 2 except (D)=15 kV. Solutes: 1=horse, 2=bovine, 3=chicken and 4=tuna.

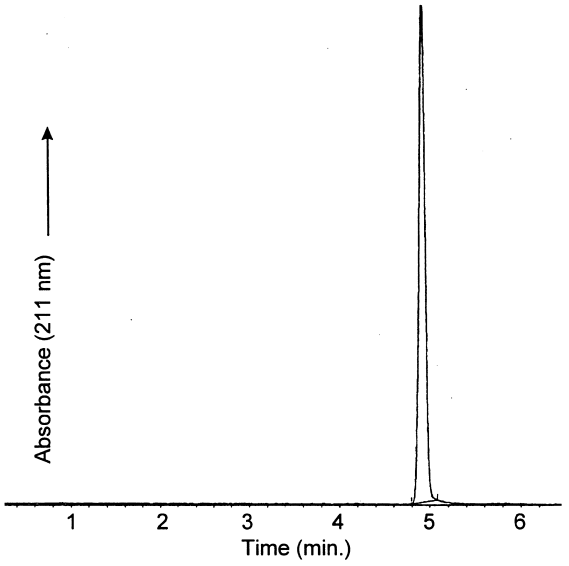

Fig. 4. Electrochromatogram of aspartame on 20 μm I.D. etched C_{18} modified capillary. Conditions: V=30 kV, pH=2.14, detection at 211 nm, injection 3 s at 12.5 cmHg vacuum, L=58 cm, l=26 cm.

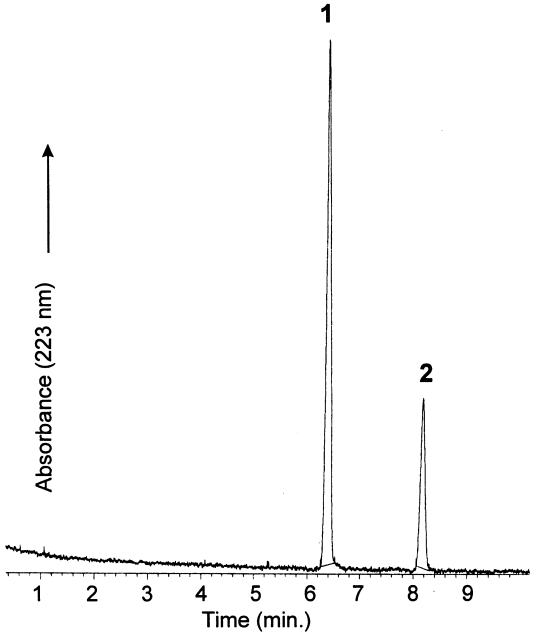

Fig. 5. Separation of tryptamine and serotonin on 20 μm I.D. etched C_{18} modified capillary. Conditions: V=30 kV, pH=4.41, detection at 223 nm, injection 5 s at 10 kV, L=51.5 cm, l=22 cm. Solutes: 1=tryptamine and 2=serotonin.

etched, C_{18} modified 20 μm capillary is shown in Fig. 5. The resolution obtained on this capillary is better than that of a bare 50 μm capillary, a C_{18} modified 50 μm capillary or an etched, diol modified 50 μm capillary. Only a 75 μm capillary modified with poly[(*N*-acryloylamino) ethoxy]ethyl-β-D-glucopyranose [16] gave a separation of tryptamine and serotonin [15] with approximately the same resolution as shown in Fig. 5. Again peak shape is good indicating that there are few silanols available for interactions with the solutes.

The final example shown in Fig. 6 is a group of basic proteins which generally are difficult to elute reproducibly and with good peak shape on a bare capillary at the pH conditions in this separation. The symmetrical peaks obtained are comparable to the other basic compounds indicating a surface which is compatible with the solutes tested. Therefore, it appears that the etched chemically modified format is applicable to electrokinetic separations involving solutes that range in size from small molecules to large biomolecules.

3.5. pH Effects

The cytochrome *c* mixture provides an interesting example of the importance of pH control for optimizing separation conditions. At low pH, little difference is seen in the separation of the cytochrome mixture at pH 2.14 (Fig. 7A) or pH 3.0 (Fig. 7B). Some improvement in the relative elution of the components occurs at pH 3.7 (Fig. 3C) so that complete separation can be achieved, especially if the applied potential is lowered (Fig. 3D). Finally at pH 4.14 (Fig. 7C) excellent separation of all the major components and at least partial resolution of the impurities can be achieved but at the expense of considerably longer analysis time. It is difficult to correlate this behavior directly with the individual isoelectric points (p*I* values) of the proteins since in each case a range of values have been reported. For example, the p*I* of bovine cytochrome *c* has been measured from 10.3 to 10.8 [17]. Obviously the

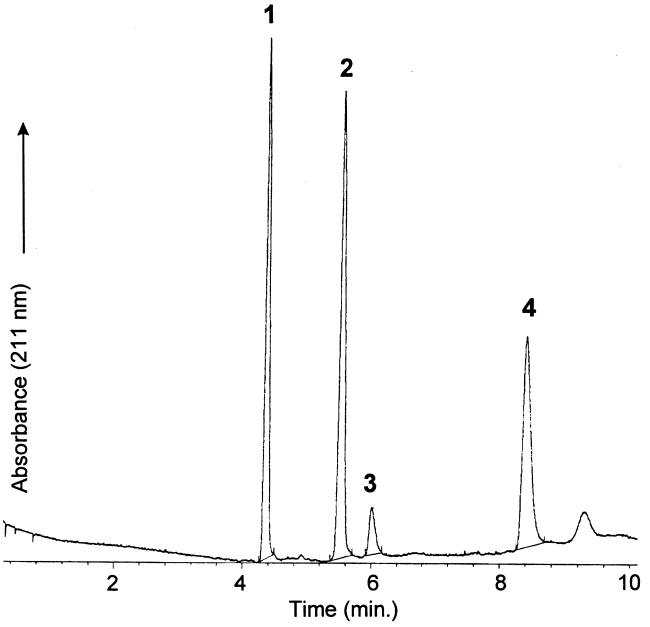

Fig. 6. Separation of protein mixture on 20 μm I.D. etched C_{18} modified capillary. Conditions: V=30 kV, pH=4.41, detection at 211 nm, injection 6 s at 10 kV, L=51.5 cm, l=22 cm. Solutes: 1=lysozyme, 2=cytochrome *c*, 3=ribonuclease, 4=myoglobin.

method of preparation and the type and extent of impurities present have some bearing on these values.

3.6. Reproducibility data

In order to test the durability of the bonded material which was attached to the capillary wall by the silanization/hydrosilation method, a series of injections were made on both the C_{18} unetched and the C_{18} etched capillaries after all of the studies reported above were completed. For the unetched modified capillary after 200 injections, the test solute (serotonin) gave reproducible migration times with an RSD of 1.70%. For an etched modified capillary, the total use exceeded 300 injections and the test solute (tryptamine) had essentially the same RSD (1.74%) as the unetched modified capillary.

In conclusion, the use of etched, chemically–modified fused silica capillaries offers a viable alternative for OTCEC. In contrast to packed column CEC, as demonstrated in this study, etched chemically–modified capillaries are applicable to all types of bases, both large and small molecules. Decreasing the inner diameter of the capillary has two beneficial effects: lowering of the current and increasing the solute–bonded phase interactions. In addition, the etching process produces a new surface containing a positively charged species that results in a small anodic electroosmotic flow at low pH (<4.5). The silanization/hydrosilation method produces a stable bonded phase that can be used with a variety solutes and electrolyte conditions. The ultimate utility of small diameter capillaries will depend greatly on detector technology. Further characterization of etched capillaries is underway to determine the source of anodic electroosmotic flow, how its presence and magnitude vary as a function of etching conditions and the relative contributions of k' and $\Delta\mu_{ep}$.

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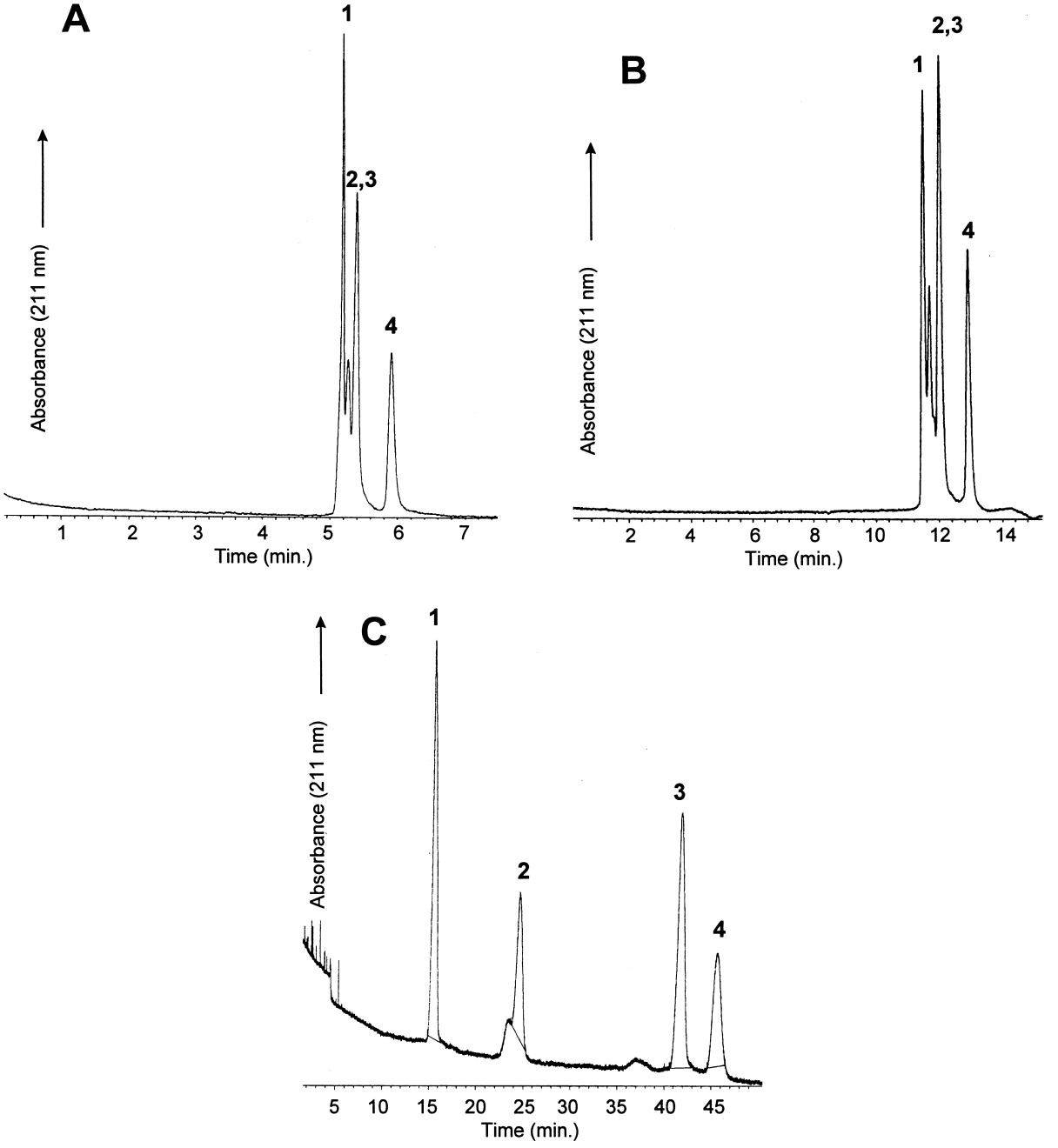

Fig. 7. Separation of cytochrome *c* mixture on 20 μm I.D. etched C_{18} modified capillary. Conditions: Same as Fig. 3C except (A) pH=2.14; (B) pH=3.0 and (C) pH=4.41.

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