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Separation of proteins and peptides by capillary electrochromatography in diol- and octadecyl-modified etched capillaries

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Abstract

This study involves the evaluation of a capillary electrochromatography method based on etching the inner walls of a fused-silica tube, which is subsequently modified by a silanization/hydrosilation reaction scheme. Two different organic moieties, octadecyl and diol, are attached to the etched capillary wall. The performance of these two columns is compared to a bare capillary using peptide (angiotensins) and protein samples. It is concluded that the etching process increases the surface area of the inner wall sufficiently to induce solute–bonded phase interactions for the capillaries modified with the octadecyl and diol moieties. The separation capabilities of the two modified capillaries are not the same, presumably due to differences in the chemical properties of the two ligands. When compared to a bare capillary where separation is due only to electrophoretic mobility effects, the bonded etched capillaries also exhibit significant differences in separation factors for the same solutes under identical experimental conditions.

Keywords: Electrochromatography; Capillary columns; Proteins; Peptides

1. Introduction

Capillary electrochromatography (CEC) is a hybrid method that uses features of both high-performance capillary electrophoresis (HPCE) and liquid chromatography (HPLC). The principle of solute movement is via an electro-driven mechanism, while separation is dependent on solute–bonded phase interactions (for neutral molecules only) and k' (interactions between solute and support-bonded organic moiety) plus electrophoretic mobility if the analyte is charged [1]. In the most common format for CEC, the capillary is filled with chemically modified silica particles so that the stationary phase is identical to that used in HPLC. A frit, placed before the solutes reach the detector window, is necessary to prevent loss of the stationary phase and distortion of the optical signal [2–4]. Both the frit and the stationary phase particles can result in the formation of bubbles, leading to a loss of electro-osmotic flow [3–6]. In order to greatly reduce the possibility of bubble formation and to eliminate the packing process, which is also more difficult in small diameter (50–100 μm) tubes, a method for CEC based on extensive etching of the inner capillary wall has been developed [7]. The process is similar to one originally used in capillary gas chromatography (GC) to increase the surface area available for the coating of polymer phases [8]. It uses ammonium hydrogen difluoride as the etching agent under carefully controlled conditions of temperature and reaction time. The earlier studies involved capillaries that were etched for 3–4 h at 300–400°C [7,8]. These conditions apparently increased the surface

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area sufficiently to induce significant k' interactions (between the solute and the organic moiety bonded to the etched capillary wall) for proteins, peptides [7] and tetracyclines [7,9] on an octadecyl stationary phase.

The method of bonding the organic moiety to etched capillaries is based on the silanization–hydrosilation reaction scheme. In this process, the etched surface of the capillary is first reacted with triethoxysilane (TES) to produce a hydride surface [7]. Under ideal conditions, a monolayer of TES is deposited on the surface so that most of the silanols are replaced by hydrides. An organic moiety is then attached to the hydride intermediate by passing a solution containing a terminal olefin and a suitable catalyst, such as hexachloroplatinic acid, through the capillary. This process is known as hydrosilation and, in the case of the previous studies [7,9], the olefin used was 1-octadecene. The silanization–hydrosilation reaction method has also been used on unetched capillaries for HPCE, in order to attach an acrylamide-type polymer, poly(AAEE) [10]. One of the benefits of this bonding method is the formation of a stable Si–C linkage between the organic moiety and the surface. It was demonstrated that the poly-(AAEE) capillary made via a hydride intermediate was more stable than that made by organosilanization. In this study, the etched capillaries are first converted to the hydride intermediate, which is followed by reaction with 7-octene-1,2-diol (7-OD) or 1-octadecene to produce columns suitable for electrochromatography. The 7-OD bonded material has already been shown to be somewhat less hydrophilic than commercial diol phases in HPLC [11] so that it may possess suitable properties in CEC for the separation of compounds where an appropriate balance between hydrophobic and hydrophilic properties is necessary, i.e. biomolecules.

2. Experimental

2.1. Materials

Lysozyme (turkey and chicken egg white), cyctochrome *c* (horse and bovine heart), ribonuclease A (bovine pancreas), myglobin and angiotensin I, II and III were purchased from Sigma (St. Louis, MO, USA). The buffers consisted of: 30 m*M* phosphate, pH 2.14 (Fischer Scientific,) and 19 m*M* Tris (Sigma); 30 m*M* citric acid, pH 3.0 (Sigma) and 25 m*M* β-alanine (Sigma) and 30 m*M* acetic acid, pH=4.41 (Aldrich, Milwaukee, WI, USA) and 30 m*M* γ-amino butyric acid (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 hydrogen difluoride, which was used to etch the capillaries, was purchased from Aldrich. Triethoxysilane, 1-octadecene, 7-octene-1,2-diol and hexachloroplatinic acid (Speier's catalyst), for subsequent modification of the etched capillary, were also purchased from Aldrich. The capillary tubing used was 375 μm O.D.×50 μm I.D. (Polymicro Technologies, Phoenix, AZ, USA).

2.2. Instrumentation

CEC and HPCE experiments were done 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 a Hewlett-Packard Model 5890 gas chromatograph. The inlet and outlet ports were altered in order to accommodate several capillaries.

2.3. Capillary preparation

Procedures for the preconditioning of the capillary surface with ammonia, the etching with ammonium hydrogen difluoride, the preparation of the hydride intermediate and the hydrosilation reaction, for attaching the desired organic moiety to the inner wall of the capillary, have been described previously [7,9].

2.4. Electrochromatography procedures

The diol and octadecyl capillaries were conditioned first by forcing at least ten 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 electrokinetically for 6 s at 5 kV. All samples were detected at 211 nm.

3. Results and discussion

It has already been demonstrated through scanning electron microscopy (SEM) measurements that varying degrees of roughness can be produced on the inner wall of a fused-silica capillary by controlling the time and temperature of the etching process [7]. A simpler method for observing the progress of the etching process could be obtained from ordinary optical microscopy. Fig. 1a shows an optical micrograph of a bare capillary at an objective magnification of 40 and a column magnification of 2.5. The surface is completely smooth, as expected. After etching a capillary for 3 h at 300°C with ammonium hydrogen difluoride, the inner wall of the capillary is visibly roughened, as shown in Fig. 1b. The mechanical stability of the new surface was tested by vibrating the capillary vigorously for 30 min. As seen in Fig. 1c, much of the surface roughness produced by the etching process has been destroyed since only a few features are barely visible in the optical microscopic photo. This is not to imply that the surface is as smooth as a bare capillary, but just that almost all of the longer radial extensions of silica produced in the etching process have been destroyed by the mechanical vibration.

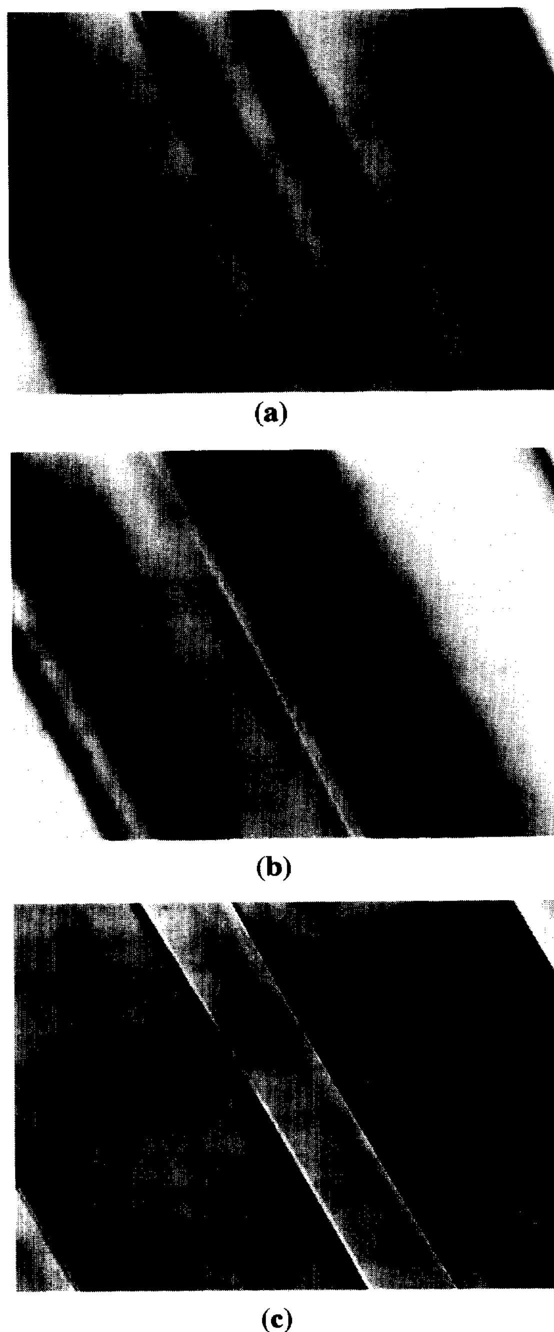

Fig. 1. Optical micrographs of (a) bare capillary; (b) capillary etched for 3 h at 300°C and (c) capillary in (b) after 30 min of vibration.

Some preliminary data has already been presented for the separation of proteins [7] and tetracyclines [9] by CEC on C_{18}- modified etched capillaries. As in HPLC, the use of a diol stationary phase should result in a surface that is more hydrophilic than a typical alkyl bonded moiety, like C_{18} or C_8 [11,12]. Fig. 2 illustrates the separation characteristics of the diol column for a series of basic proteins in a buffer with a pH value of 4.41. Peaks are symmetrical, indicating that little or no adsorption of the solutes on the etched and modified surface has taken place. Similar to the C_{18} column reported earlier, efficiencies are generally between 30 000–60 000 plates/m, about an order of magnitude less than typical HPCE experiments, which is indicative of the solute–bonded phase interactions present in the CEC mode. Another example of the separation capability of the diol column is the resolution of bovine and horse cytochrome *c*, which differ by only three amino acids in a total sequence of 104. The separation factor (α=migration time of horse cytochrome *c*/ migration time of bovine cytochrome *c*) is 1.09

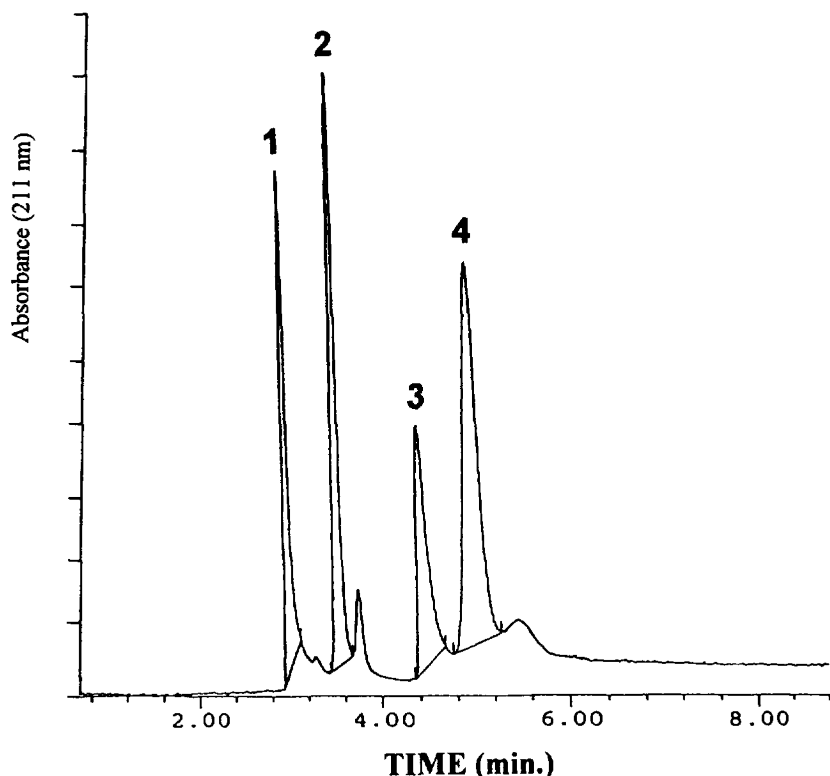

Fig. 2. Electrochromatogram of protein separation on a diol capillary. L (total capillary length)=45 cm, l (effective length)=25 cm, V=22 kV, I=7 μA, pH=4.41. Solutes: 1=cytochrome c; 2=lysozyme; 3=myoglobin and 4=ribonuclease A.

under the same conditions as in Fig. 2. It appears that the diol-etched capillary might provide different modes or degrees of interaction for CEC separations.

A useful comparison of the separation characteristics for a series of angiotensins, clinically related to vasoconstriction, on bare unetched, diol-modified etched and C_{18}-modified etched capillaries is shown in Fig. 3. For each column, the elution order is the same: Angiotensin III, angiotensin I, angiotensin II. This result indicates that while solute–bonded phase interactions may be significant, the differences in electrophoretic mobility are primarily responsible for the separations observed with the angiotensins. Since the separation of the three-component mixture was done under identical conditions for all three columns of equivalent length, the relative migration (elution) times can provide additional insight into solute transport and possible k' interactions. In Fig. 3, the migration times for each of the solutes is in the following order: C_{18}>diol>bare. The longer migration times on the C_{18} column could be due to more efficient bonding, i.e. a greater number of bonded moieties per unit area, or to stronger solute–bonded phase interactions. The former is less likely since the octadecyl ligand is clearly larger than 7-OD and, in general, smaller moieties have greater bonding density, at least on porous silica. The latter option is supported by the broader peaks observed on the C_{18} column, especially the last-eluting component. It is most likely that hydrophobic interactions between the bonded C_{18} species and the solutes lead to greater retention and hence longer migration times.

Since charged solutes (like the angiotensins) are driven through the column by electrophoretic mobility and electroosmotic flow, when present, retention characteristics based on a k' value determined from an unretained and uncharged solute may not be straightforward. Another approach is to look at the relative separation factors (α=migration time for later-eluting component/migration time for earlier-

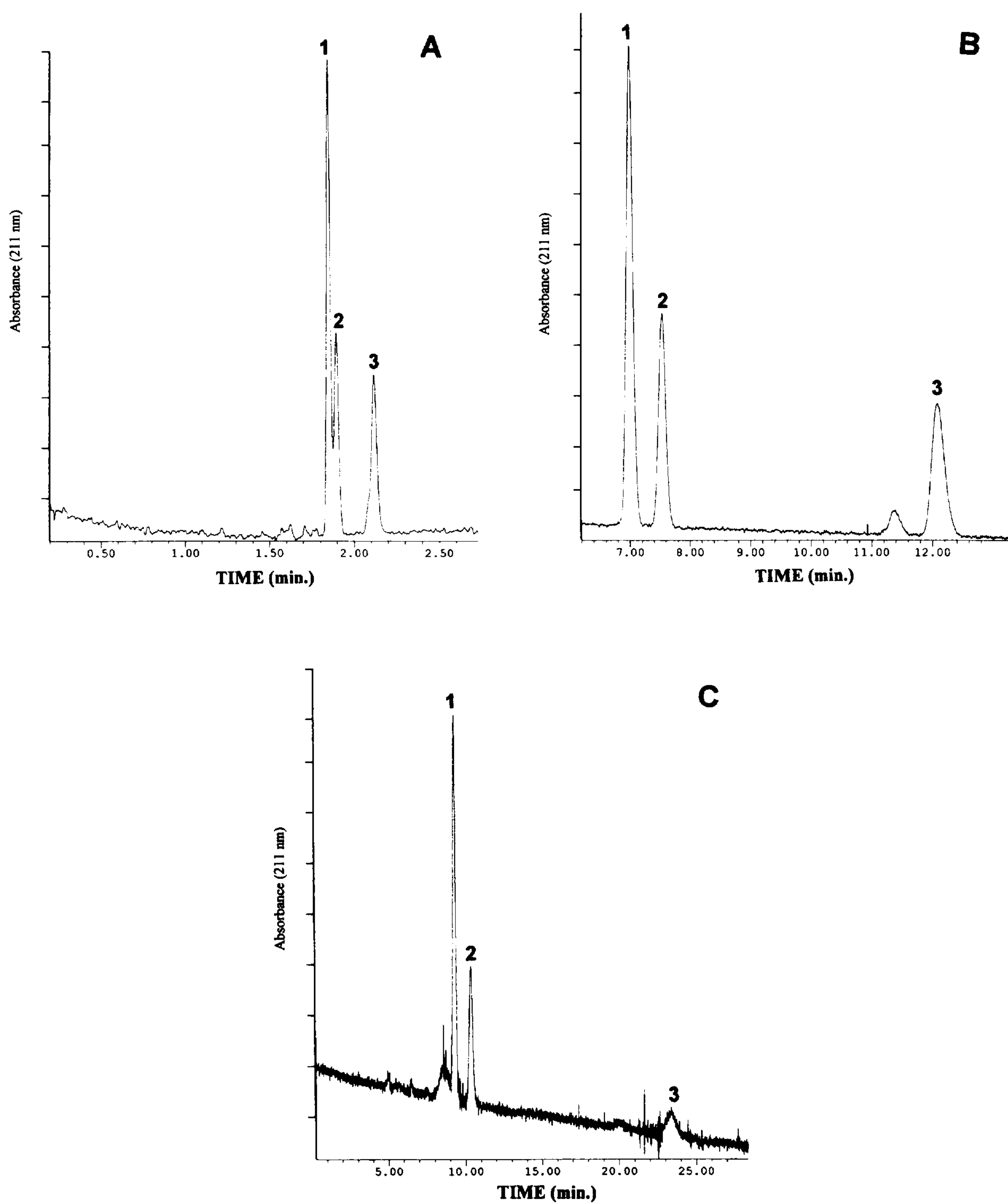

Fig. 3. Separation of angiotensins on (A) a bare capillary; (B) a diol capillary and (C) a C_{18} capillary. L=45 cm, l=25 cm, V=30 kV, I=29 μA, pH=2.14. Solutes: 1=angiotensin III; 2=angiotensin I and 3=angiotensin II.

Table 1
Separation factors for angiotensins on various capillaries[a]

Separation factor	Bare capillary	Diol capillary	C_{18} capillary
$\alpha_{I/III}$	1.03	1.08	1.12
$\alpha_{II/I}$	1.12	1.60	2.25
$\alpha_{II/III}$	1.15	1.73	2.52

[a] pH=2.14, 30 kV, L=45 cm, l=25 cm.

eluting component) for various solute pairs on the three columns. Table 1 compares the three α values for the solutes and the conditions on each column are shown in Fig. 3. For the bare capillary, separation is determined by differences in electrophoretic mobility alone. However, for the two modified etched columns, separation is a combination of differences in electrophoretic mobility as well as solute–bonded phase interactions. Because the amount of bonded ligand cannot be quantitated on the etched capillary surfaces, the variation in the separating ability of the diol and C_{18} columns could be due to differences in their hydrophobicity as well as to the bonding density on the wall. It is clear that if separation were based on electrophoretic mobility alone, the α value for a particular solute pair would be the same on the three columns under a given set of experimental conditions.

The role of the bonded moiety is further illustrated by the data presented in Tables 2 and 3. In Table 2, the α values for one solute pair at several voltages and pH values on the bare capillary are given. At each pH, the separation factor is constant as the voltage is changed. This is expected since separation depends only on the differences in electrophoretic mobility, which should be independent of applied potential. Because the solutes change (charge and/or conformation) with pH, some variation in the α value is expected and observed. A similar comparison is made for the diol column in Table 3. Here

Table 2
Separation factor ($\alpha_{II/III}$) as a function of applied voltage and pH on a bare capillary

pH	Applied voltage (kV)		
	20	25	30
2.14	1.16	1.15	1.15
3.00	1.31	1.31	1.31
4.41	1.48	1.48	1.48

Table 3
Separation factors as a function of applied voltage and pH on a diol capillary

Separation Factor	Applied voltage (kV)		
	20	25	30
pH=3.0			
$\alpha_{I/III}$	1.10	1.10	1.10
$\alpha_{II/I}$	1.16	1.16	1.16
$\alpha_{II/III}$	1.28	1.28	1.28
pH=2.14			
$\alpha_{I/III}$	1.05	1.06	1.08
$\alpha_{II/I}$	1.30	1.45	1.60
$\alpha_{II/III}$	1.36	1.54	1.73

it can be seen that a pH of 2.14, a change in voltage results in a change in the α value for each of the three solute pairs. An interesting result is obtained at pH 3.0, where the α value is independent of the applied voltage. It is apparent that for these solutes at higher pH, the solute–bonded phase interactions are negligible, so that separation is based mainly on electrophoretic mobility as in a bare capillary.

The differences between the bare, diol and C_{18} capillaries can be further illustrated by monitoring the separation of turkey and chicken lysozymes under various experimental conditions. At a pH of 2.14 and an applied potential of 30 kV, both the bare and the diol-modified etched capillaries do not separate this solute pair. However, the C_{18} capillary (Fig. 4A) gives baseline resolution of these two solutes, with an additional component (presumably an impurity in one of the commercial samples) clearly visible as a shoulder on the second peak in the electrochromatogram. By raising the pH to 3.0 and lowering the applied potential to 25 kV, the bare capillary and the C_{18} capillary (Fig. 4B) give almost identical resolution of the two lysozymes, as well as of the minor impurity. However, little if any improvement is seen for the diol-modified capillary under these conditions. If the pH is raised further to 4.41, then the bare capillary and the diol capillary (Fig. 4C) give approximately the same resolution for the lysozymes. Under these conditions, it appears that the minor component may have moved so that it now elutes between the two major peaks in the electrochromatogram. Its presence as a shoulder on the turkey lysozyme peak is more distinct in the electrochromatogram for the diol capillary than for

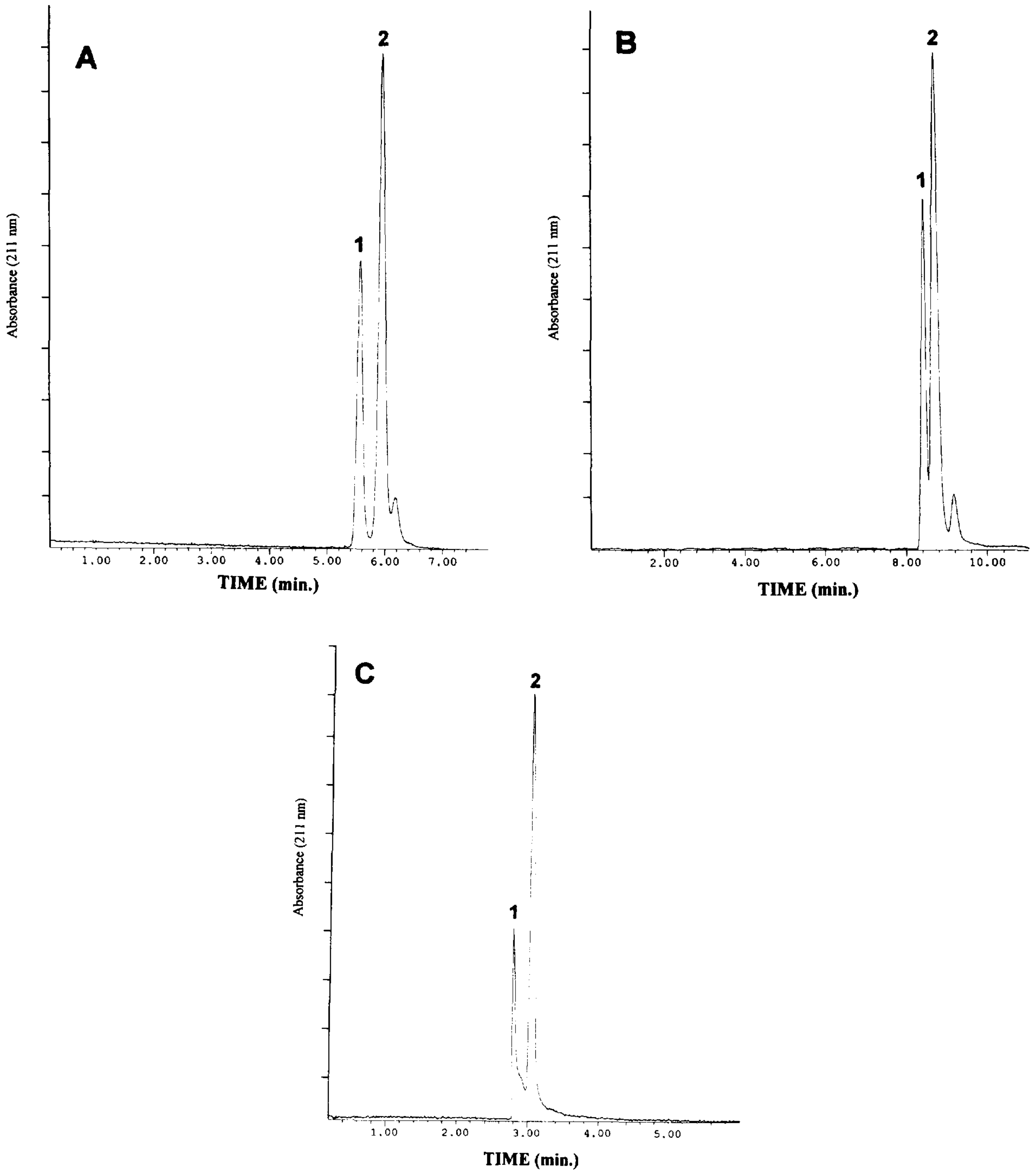

Fig. 4. Separation of lysozymes on: (A) a C_{18} capillary at pH=2.14. Conditions are the same as in Fig. 3. Turkey lysozyme is the first-eluting solute; (B) a C_{18} capillary at pH=3.0. L=45 cm, l=25 cm, V=25 kV, I=24 μA and (C) a diol capillary at pH=4.41. L=45 cm, l=25 cm, V=25 kV, I=8 μA.

the bare capillary. The two lysozymes are not resolved on the C_{18}-modified etched capillary under these conditions. The somewhat broader peaks and the tailing observed for the C_{18} capillary are indicative of either stronger interaction with the bonded moiety or adsorption effects on residual silanols. The latter is a reasonable possibility since lysozymes are very basic proteins that are extremely sensitive to the presence of accessible Si-OH groups.

4. Conclusions

The results reported here confirm the conclusions of the two previous studies [7,9] concerning the presence of solute–bonded phase interactions for chemically modified etched fused capillaries. This format is another mode of electrochromatography that differs from the conventional approach in that an open tubular configuration is used instead of a packed column. The results in this study demonstrate that the bonded etched capillaries have distinctly different retention characteristics from a simple bare capillary. In addition, when different organic moieties are attached to the etched surface, the separation capabilities vary, just as stationary phase behaviour is dependent upon the bonded ligand in chemically modified supports for HPLC. Further studies are in progress on other fundamental properties of this mode of electrochromatography as well as on the creation of a variety of chemically modified etched capillary surfaces.

Acknowledgments

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References

[1] J.H. Knox, J. Chromatogr. A, 680 (1994) 3.
[2] J.W. Jorgenson and K.D. Lukacs, J. Chromatogr., 218 (1981) 209.
[3] C. Yan, D. Schaufelberger and F. Erni, J. Chromatogr. A, 670 (1994) 15.
[4] J.H. Knox and I.H. Grant, Chromatographia, 32 (1991) 317.
[5] H. Rebscher and U. Pyell, Chromatographia, 38 (1994) 737.
[6] S. Kitigawa and T. Tsuda, J. Microcol. Sep., 6 (1994) 91.
[7] J.J. Pesek and M.T. Matyska, J. Chromatogr. A, 736 (1996) 255.
[8] F. Onuska, M.E. Comba, T. Bistricki and R.J. Wilkinson, J. Chromatogr., 142 (1977) 117.
[9] J.J. Pesek and M.T. Matyska, J. Chromatogr. A, 736 (1996) 313.
[10] M. Nesi, M. Chiari, J.E. Sandoval and J.J. Pesek, J. Chromatogr. A, 717 (1995) 1.
[11] J.J. Pesek, M.T. Matyska, H. Hemphala and P. Christensen, J. Liq. Chromatogr., 18 (1995) 2507.
[12] M.T. Matyska, J.J. Pesek and A.M. Siouffi, Chem. Anal., 40 (1995) 517.