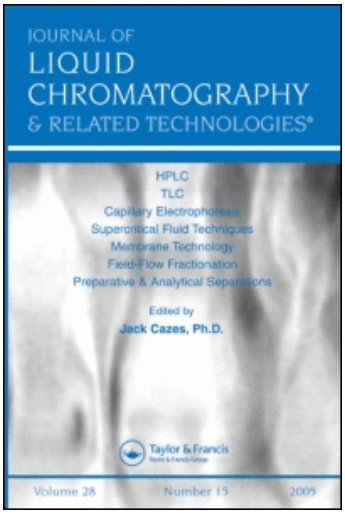


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# Stable Phases for Capillary Electrophoresis

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# STABLE PHASES FOR CAPILLARY ELECTROPHORESIS

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**ABSTRACT**

Three phases for use in capillary electrophoresis, two bonded hydrophobic phases and one bonded hydrophilic phase, were characterized as to stability over time at neutral pH, effect on electroosmotic flow velocity, effect on electroosmotic flow velocity changes with pH, and ability to provide protein separations not possible with untreated silica under the same conditions. All columns demonstrated stability comparable to bare silica at pH 7.0, over more than 50 continuous runs. The hydrophobic phases reduced the electroosmotic flow by 37 to 43% and minimized the change in electroosmotic flow velocity with changing pH over a pH range of 3 to 10. Protein separations were achieved from pH 6 to 9.3. Changes in pH over the above range were used to optimize these separations without changing electroosmotic flow characteristics.

## INTRODUCTION

Capillary electrophoresis has introduced the potential of high efficiencies and resolution, automation, and rapid

analysis time in bioseparations. However, the full potential of this methodology has not yet been realized, due to the problem of protein adsorption to the fused silica surface (1-7). This interaction reduces the high efficiencies predicted by theory (2,3) and interferes with reproducibility of separations. While this interaction can be minimized by the use of high (9.5-11.0) or low (3-5) pH, this approach is limited by the pI range of the protein sample components and solubility of the proteins at pH extremes. This approach also limits the resolution of the sample by reducing the charge differences between the proteins in the sample.



Treatment of the capillary inner surface to reduce or eliminate electroosmotic flow has met with some success (1-11). Hjerten (3) has bonded acrylamide through an organosilane reagent to the surface of the capillary, resulting in a reduced to negligible electroosmotic flow and reduction of protein interaction with the walls. However there were some limitations to the stability of this phase over time. McCormick (4) has used a poly(vinylpyrrolidone) coating and cites separations done at low pH. Poppe ((5) has bonded poly(ethylene) glycol through a $[(MeO)_3Si(CH_2)_3OCH_2$-epoxide] to the surface, but it is unsuitable for use above pH 5.0. Another poly(ethylene) glycol phase was demonstrated by Schomberg (14) for use in MECC small molecule separations.

Tonws and Regnier (9) reported an polyethylenimine phase that actually reverses the electroosmotic flow when coated to a thickenes of about 70Å. Both reduction of the dependence of electroosmotic flow on pH and protein separations were

demostrated on this column. However, stability beyond one hundred hours was not known. Bruin (5) has bonded columns with both maltose and epoxydiol. The epoxydiol suffered from limited pH range of use (3-5). Peak shape for selected proteins on the polyethylene glycol phase was poor at pH (6.8) and improved at pH 4.1. Swedberg (8) reported an arylpentofluoro bonded phase based on bonding through $(MeO)_3Si(CH_2)_3NH_2$. While these showed excellent separations and efficiencies, high ionic strength buffer with the addition of KCl was employed in these separations.

Sepaniak (7) reported a trimethylsilyl coating which was used in MECC separtions for small amines. The coating lowered electroosmotic flow and allowed the separations of small molecular weight amines. No protein separations were demonstrated. Novotony (10,11) recently demonstrated improvement in protein separations on an acrylamide phase that is bonded through a stable Si-C bond. This phase completely eliminated electroosmotic flow and dramatically improved efficiencies of protein separations. However, due to the complete destruction of the electroosmotic flow, separations had to be performed at either high or low pH in order to have all of the sample migrate in the direction of the detector.

We report here three bonded phases, two hydrophobic and one hydrophilic, that allow separations at neutral pH. These phases show improvement of the separations as compared to bare silica under the same electrophoretic conditions. Both hydrophobic phases, containing $C_8$ and $C_{18}$ functionalities, reduce the dependence of the electroosmotic flow on pH, which

allows the use of pH to optimize peak resolution without major changes in peak migration due to flow.

## EXPERIMENTAL

## MATERIALS

### Instrumentation

Automated runs were performed on an Applied Biosystems Model 270A automated capillary electrophoresis system (Applied Biiosystems, Inc., Foster City, CA, USA). Data acquisition from the automated system was done on an IBM PC using Beckman Gold Software (Beckman, Inc., Palo Alto, CA). The manual CZE system consisted of a Hippotronics Model 840A power supply (Hippotronics, Inc., Brewster, NY, USA) and a Laboratory Data Control UV III monitor absorbance detector that was modified with fiber optics, a cadmium pen lamp (229 nm) light source, and a unique laser-etched flow cell. Data acquisition was performed using a Kippen Zonen chart recorder. The entire experimental apparatus is described in reference 13.



### Columns and Chemicals

50 um internal diameter and 360 um outer diameter fused silica capillary tubing was supplied by Polymicro Technologies (Phoenix, AZ USA). Treated capillary columns were produced by Supelco Inc. All proteins and reagent-grade buffer components were purchased from Sigma Chemical Company (St. Louis, MO USA). Sulfuric acid and phosphoric acid were purchased from Fisher Scientific Co. (Pittsburg, PA USA).

0.01% benzyl alcohol was obtained as a standard solution from Supelco Inc.

## METHODS

Bonded capillary columns were produced using proprietary methods. Windows were made in the capillaries by dissolving the outer polyimide coating with boiling (~330°C) fuming sulfuric acid. In studies involving effects of pH changes all buffers were made such that the ionic strength of the buffer remained constant throughout the pH range. Proteins were diluted to a final concentration of 1 mg/ml each in distilled water. Samples and solutions were degassed prior to use.



All separations done using the automated system were performed using a 1 minute wash with 0.1 N NaOH, followed by a 3 minute equillibration rinse with running buffer. No washes or rinses were used with the manual system. Automated injections were done for 1 second using a 25 cm Hg vacuum, corresponding to a 3 nl injection volume. All manual injections were performed hydrostatically by raising the injection end of the column 10 cm for 10 seconds.

## RESULTS AND DISCUSSION

### Stability of the Phases at pH 7.0

Figure 1 shows the migration time of a neutral marker (0.01% benzyl alcohol) at pH 7.0 for one each of the bonded columns and two bare fused columns. Electrophoresis run

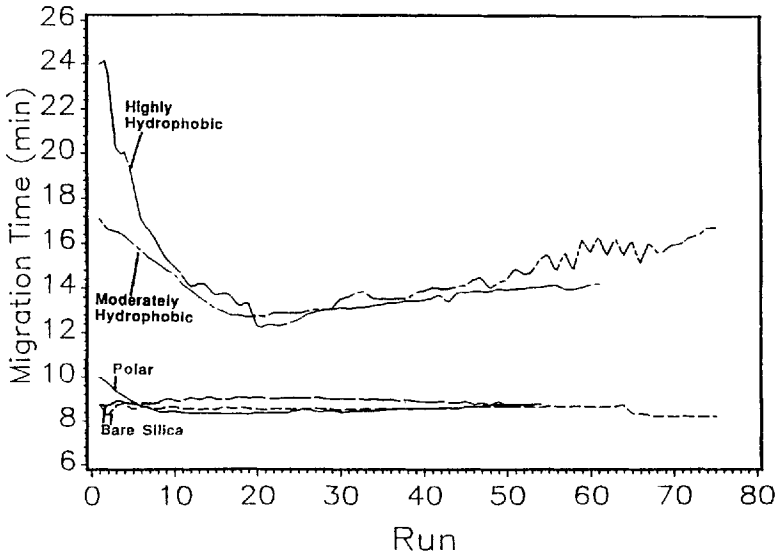



Figure 1. Migration time of a neutral marker as a function of run number. Field: 200V/cm; Temp: 30°C; Column: 65 cm effective length; Detection: 200nm; Buffer: 25mM sodium phosphate, pH 7.0. Marker: 0.01% benzyl alcohol; Instrument: ABI Model 270A



duration was 20 minutes for the bare silica and polar phase columns and 30 minutes for the hydrophobic phase columns. This difference was due to the longer run times required for migration of the neutral marker in the hydrophobic columns. A conditioning or "break-in" period, during which the migration time for the neutral marker steadily decreases was evident for all of the bonded columns. A brief (1-2 run) break-in period was needed when the columns were stored dry in air, while no break-in runs were needed when the columns were stored in buffer or water. This phenomenon was due either to loss of unbound phase or to a conformational change of the phase structure as it encountered an aqueous rather

than nonpolar environment. Computer modeling of a $C_{18}$ phase in water, 50% methanol, and 100% methanol supports the latter suggestion (15). The increase in time necessary for conditioning with increasing hydrophobicity also supports the second hypothesis.

A gradual increase in the migration time of the neutral markers as run numbers increased may have been caused by a build up of neutral marker on the phase due to hydrophobic interactions with the phase. While the $C_8$ phase had no measurable capacity for benzyl alcohol, some tailing was evident. Relative standard deviations in migration time both including and excluding the conditioning phase of the column are listed in Table 1. While of the hydrophobic columns have been in use in three separate labs for several months without noticable changes in their characteristics, overall and day to day standard deviations were not calculated.



**Electroosmotic Flow Velocity**

Table 2 shows the electroosmotic flow velocity and electrophoretic mobility of the neutral marker for untreated and bonded columns. Relative standard deviations for the migration time of the neutral marker were calculated from data points obtained following the conditioning of the column. The running buffer used for all columns was 25mM sodium phosphate, pH 7.0.

The lower electroosmotic flow velocity in the $C_8$ phase as compared to the $C_{18}$ phase indicates a more effective coverage/deactivation of surface silanols. This is likely

TABLE 1

Relative Standard Deviation of Migration Times for a Neutral Marker in Untreated and Bonded Capillary Columns

| Column | Number of Runs | Overall RSD | Post-conditioning RSD |
|---|---|---|---|
| untreated #1 | 50 | 1.19% | -- |
| untreated #2 | 129 | 2.40% | -- |
| polar phase | 55 | 3.65% | 1.72% |
| C18 phase | 109 | 15.20% | 5.52% |
| C8 phase | 79 | 8.64% | 8.56% |

TABLE 2

Comparison of Electroosmotic Flow Velocity for Untreated and Bonded Columns

| Column | Electroosmotic Flow Velocity (cm/min) |
|---|---|
| untreated silica #1 | 7.282 |
| untreated silica #2 | 7.636 |
| polar phase | 7.569 |
| $C_{18}$ phase | 4.837 |
| $C_8$ phase | 4.340 |



due to less steric hindrance from the smaller molecular size of the $C_8$ phase structure during chemical bonding to the surface of the tubing. The correlation between electroosmotic flow and phase thickness has been previously discussed (9,14). The reduction of the electroosmotic flow by the polar phase is statistically insignificant, and may have been due to lack of effective deactivation or to insufficient phase thickness. Studies are presently underway to determine the thickness of each of the phases.

## Electroosmotic Flow as a Function of pH

Figure 2 shows the change in electroosmotic mobility of a neutral marker with pH, using a 25mM sodium phosphate

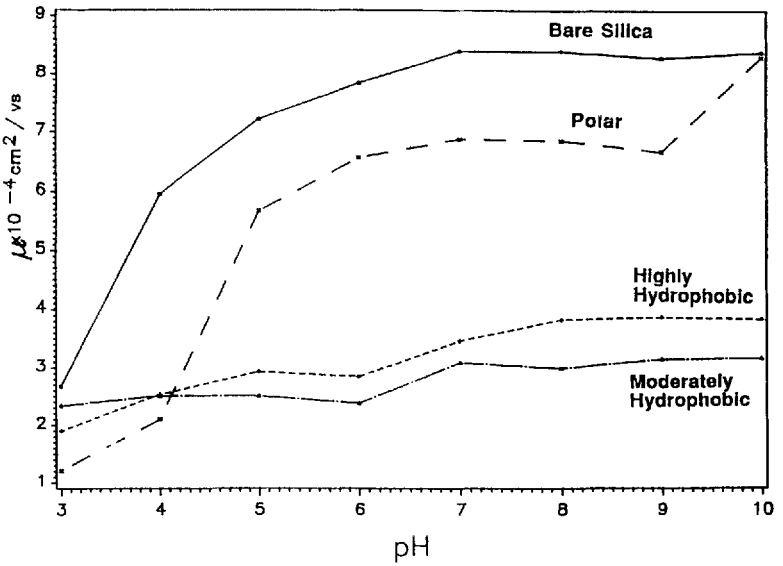


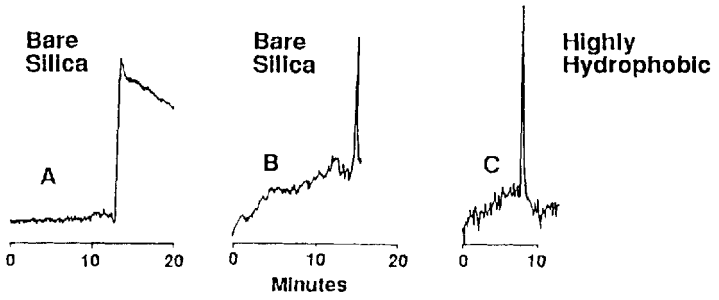

Figure 2. Electrophoretic mobility of a neutral marker as a function of pH. Conditions same as for figure 1 except for variation in buffer pH as indicated.



buffer. For untreated silica electroosmotic flow showed a strong dependence on pH, due to protonation/deprotonation of the exposed surface silanol groups. The polar column, while showing a reduced electroosmotic flow as compared to untreated fused silica, also showed a similiar pH dependence. Incomplete shielding and/or deactivation of the surface silanols by the polar phase may have given this column the pH dependence characteristic of silanol groups. However, the hydrophobic phases displayed minimal electroosmotic flow variation as a function of pH.

## Protein Separations and Influence of pH on Separations

Protein separations were achieved on each of the phases at pHs between 6.0 and 9.0, using one or more of the proteins




(A) 50μm ID x 75cm untreated capillary, Mobile Phase: 0.01M CHES, 0.015M KCl, pH 8.3
(B) Same capillary and mobile phase as (A), pH 10.1
(C) Modified capillary, 50μm ID x 50cm; Mobile Phase: 0.01M $Na_2HPO_4$, 0.006M $Na_2B_4O_7$, pH 8.0

Figure 3. Comparison of the elution of conalbumin on a $C_{18}$ column and a bare silica column. Elution performed on the manual system described in the text.



listed below. The peak labels appearing on the following electropherograms correspond to the each of the listed proteins.

1. myoglobin (horse heart) pI=7.4
2. conalbumin (iron-free) pI=6.6
3. β-lactoglobulin B pI=5.3
4. β-lactoglobulin A pI=5.1

Figure 3 shows a comparison of the elution profiles of conalbumin on untreated fused silica and on the $C_{18}$ column. Although we cannot explain the unusual peak shape in figure 3a, it was reproducible and observed for several proteins when the pH of the buffer was near the pI of the protein.

Increasing the pH to several units above the pI, as stated previously, reduces the interaction of the protein with the capillary wall and results in higher efficiency. Employing the $C_{18}$ capillary also reduces the wall interactions and permits the use of lower pH buffers. Since it is possible to manipulate the electrophoretic mobility of proteins, hence control selectivity, by adjusting the buffer pH, the dependence of efficiency on pH is not desirable. The flexibility in buffer pH afforded by employing the $C_8$ capillary to reduce the effects of pH on efficiency is shown in figure 4.



Figure 4 demonstrates the use of pH to improve the resolution of myoglobin and conalbumin on a $C_8$ column. By minimizing the effect of pH on the electroosmotic flow, the separation could be optimized without large changes in protein migration times due to changes in electroosmotic flow. Figure 5 also demonstrates the use of pH to optimize the separations on a polar column. Figure 6 depicts the same separations performed on an bare fused silica column under the same separating conditions as figure 5. As the pH increased the differences in peak resolution on the bare fused silica, as compared to the bonded columns, becames less pronounced. At pH 9.0, peak efficiency was higher on the bare silica than on the $C_8$ column, although the β-lactoglobulin peaks were not as well resolved. This difference in peak efficiency may have been due to hydrophobic interactions with the phase, which would result in efficiencies lower than predicted by theory (8,16,17).

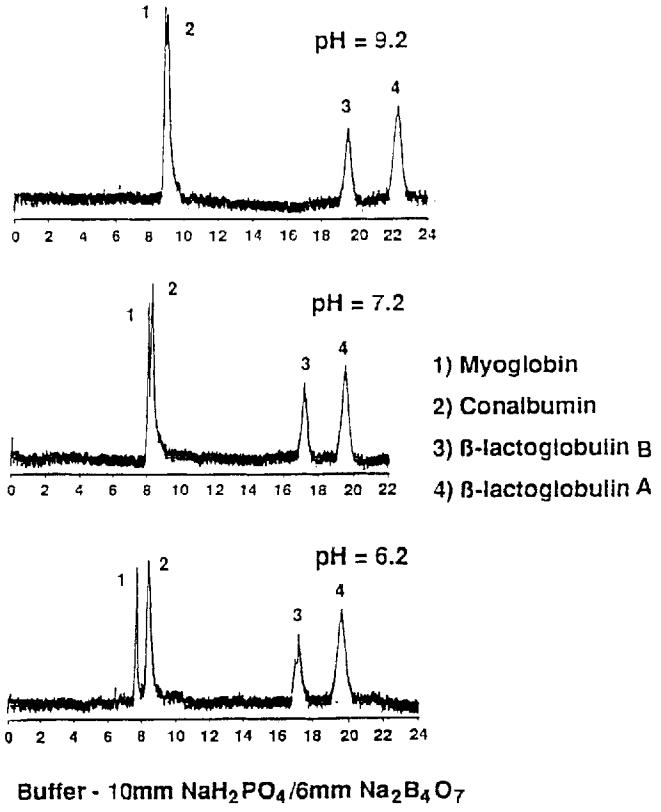



Figure 4. Separations of proteins on a $C_8$ column using pH to optimize resolution.
Instrument: manual system; Field: 200V/cm; Buffer: 0.01 M sodium phosphate/0.006 M sodium borate, pH as indicated.

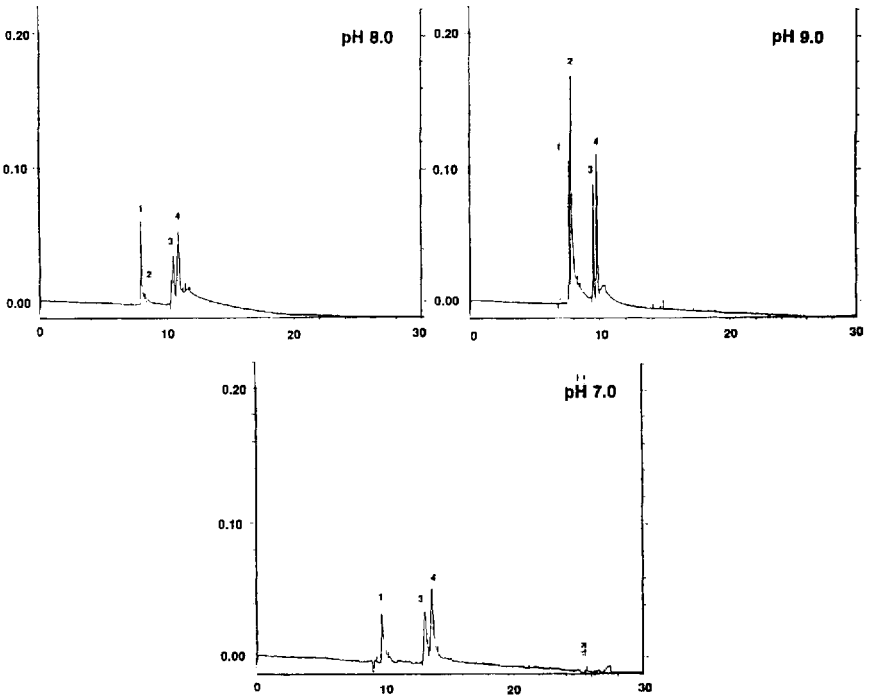



Figure 5. Separations of proteins on a polar column at various pHs. Instrument: ABI Model 270A; Temperature: 30°C; Field and buffer conditions same as for figure 4.



## CONCLUSIONS

The proprietary hydrophobic phases discussed here have the ability to reduce both electroosmotic flow velocity and the fluctuations in this flow as a function of pH. Due to both effective deactivation and shielding of the fused silica surface silanols, both the hydrophobic and hydrophilic phases inhibit the interaction of proteins with the fused silica surface. This allows protein separations to be performed at

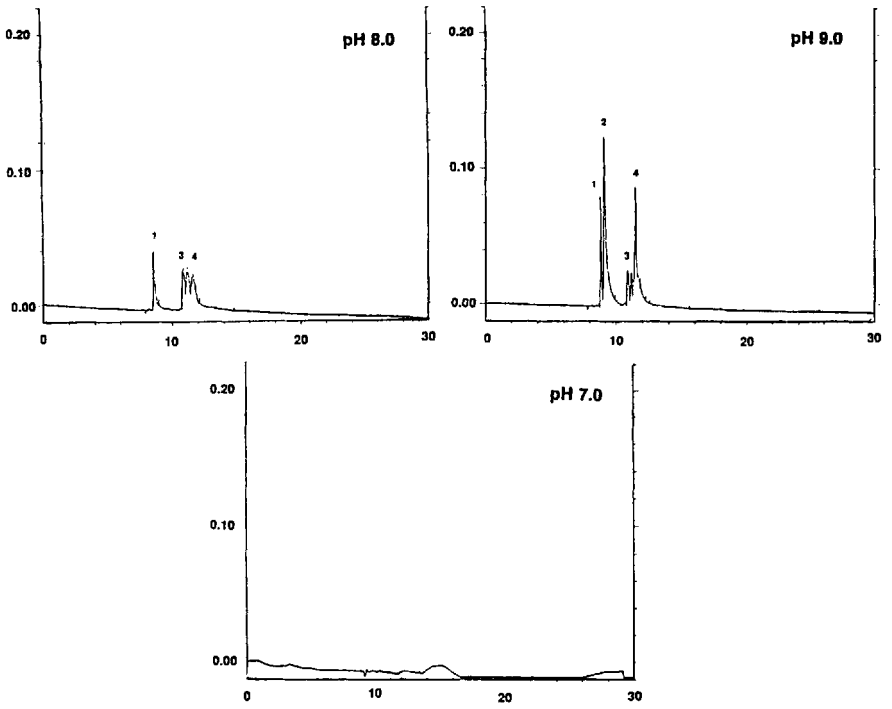



Figure 6. Separations of proteins on a bare fused silica column at various pHs. Conditions same as for figure 5.



near neutral pHs. These phases also have been proven stable over a minimum of 55 runs at pH 7.0, despite repeated washings with 0.1 N NaOH.

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