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# Capillary zone electrophoresis of basic proteins with chitosan as a capillary modifier

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**Abstract**

Two new approaches based on the use of chitosan, a cationic natural polymer, have been developed for free solution capillary electrophoretic analysis of basic proteins. In the first method, chitosan was employed as a buffer additive in untreated fused-silica capillaries. The polymer interacts with the capillary surface, causes a reversal in the direction of the electroosmotic flow and reduces solute–wall interaction of basic proteins at pH values below their isoelectric points. High efficiencies ($\geq 400\,000$ theoretical plates/m) can be attained for most of the basic model proteins investigated in the pH range 3.0–5.5, except for lysozyme. Chitosan was also used as a capillary modifying reagent. Efficiencies obtained using the chitosan-modified capillary were generally lower than those with chitosan as a buffer additive. However, improvement in peak shape was obtained for lysozyme. In both cases, good migration time reproducibilities (R.S.D. $< 1\%$) were obtained.

## 1. Introduction

Analysis of basic proteins by free solution capillary electrophoresis using fused-silica column presents a unique challenge due to non-specific adsorption of the protein onto the capillary wall. Unless the acidic silanol (Si–OH) groups on the surface of the capillary wall [1] are masked, the proteins strongly interact with the wall resulting in poor recovery, peak broadening and distortion. Therefore resolution and quantitative determination may also be seriously impaired. Much research has been devoted to circumventing this undesirable adsorption problem and several different approaches have been reported. These include chemical modification of the silica surface [2–7], manipulation of the buffer pH [8–10], use of additive in the sample [11], application of a radial positive potential gradient [12,13] and dynamic modification of the capillary surface by buffer additives [14–23].

In the last approach, various different types of reagents have been used. These buffer additives eliminate or minimize protein adsorption either through (i) competition between proteins and high concentration of zwitterionic salts [14] and/or alkali metal salts for cation-exchange sites on the silica surfaces [15,16], (ii) suppression of ionic interaction by the use of amine additives [9,17,18] or fluorinated cationic surfactants [19,20], or (iii) shielding of the silanols by coating with amphipathic polymer column coating reagent [21,22] or non-ionic polyvinyl alcohol [23]. The use of buffer additives to suppress protein adsorption offers several advantages. The buffer pH can remain an adjustable parame-

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ter for optimizing the selectivity and avoiding denaturation of proteins. A moderate electroosmotic flow (EOF) can still be maintained which contributes to the separation power of capillary electrophoresis by magnifying the velocity differences of similar migrating analytes when the direction of their electrophoretic mobilities is countered by the direction of the EOF. In addition, the method is simple and good stability and reproducibility of the surface coating can be easily attained. However, the success of this method depends on the degree to which the additives interact with the analytes.

Chitosan, or (1→4)-2-amino-2-deoxy-β-D-glucan, is a hydrophilic polyelectrolyte obtained by deacetylation of chitin [24]. Chitosan has been shown to be an effective chelating material for the removal of toxic metals from water in pollution studies [24] and it was also widely employed as support in chromatographic separations of nucleotides, amino acids and inorganic ions [25,26]. In this work, the suitability of chitosan as a column coating reagent for analyzing basic model proteins was investigated. The properties and performance of the chitosan-modified capillary were evaluated in terms of the useful working pH range, the plate efficiency and its applications.

## 2. Experimental

### 2.1. Reagents

All proteins used were purchased as lyophilized powders from Sigma (St. Louis, MO, USA): β-lactoglobulin A and B (bovine milk, p*I* 5.1 and 5.3 respectively), carbonic anhydrase I (human erythrocytes, p*I* 6.6), myoglobin (horse heart, p*I* 7.4), α-chymotrypsinogen A (bovine pancreas, p*I* 9.1), trypsin (bovine pancreas, p*I* 9.3), trypsinogen (bovine pancreas, p*I* 9.3), ribonuclease A (bovine pancreas, p*I* 9.5), cytochrome *c* (horse heart, p*I* 10.5) and lysozyme (chicken egg white, p*I* 11.0). Reagent-grade sodium acetate and chitosan (average molecular mass *ca.* 70 000) were obtained from Fluka (Buchs, Switzerland). Water purified with a Milli-Q system (Millipore, Bedford, MA, USA) was used.

### 2.2. Buffer and sample preparations

Protein samples were diluted in deionised water at a concentration of 200 μg/ml. Chitosan stock solution, *ca.* 2% (w/v) in 1% aqueous acetic acid was prepared by stirring at room temperature until complete dissolution. Sodium acetate buffers were adjusted to the desired pH by the addition of glacial acetic acid or NaOH solution. Buffers with chitosan as additive were prepared by weighing out the required amount of the stock chitosan solution and made up to the volume with the buffer solution. The solutions were allowed to equilibrate for about 1 h. For solutions of higher pH, a longer period of time was required, *e.g.*, *ca.* 2 h for solution at pH 5. The equilibration step is necessary to achieve good migration time reproducibility. The pH of the buffer solution with chitosan was measured after equilibration. Buffer solutions were filtered through a 0.45-μm membrane (Whatman, Ann Arbor, MI, USA) before use.

Tryptic digest of cytochrome *c* was performed by incubating 1 volume of cytochrome *c* (10 mg/ml) with 0.2 volumes of 0.5 *M* ammonium hydrogencarbonate and 0.2 volumes of 1 mg/ml trypsin at 37°C. The ratio of the concentration of trypsin to cytochrome *c* was 1:50. Following incubation, trypsin was deactivated by heating at 100°C for 5 min. Before analysis, the digest was diluted 1:5 with water. The final tryptic digest sample concentration was *ca.* 1 mg/ml.

### 2.3. Capillary electrophoresis

All separations were performed using an Applied Biosystems Model 270A capillary electrophoresis system (ABI, Foster City, CA, USA). Fresh fused-silica capillary of 50 μm I.D. and 375 μm O.D. obtained from Polymicro Technologies (Phoenix, AZ, USA) was flushed under vacuum (508 mmHg; 1 mmHg = 133.322 Pa) with water (10 min), 1 *M* NaOH (10 min), water (5 min), 1% aqueous acetic acid (20 min), water again (5 min), and finally the run buffer con-

taining chitosan (5 min). This washing sequence was repeated whenever a new pH was used. In the case where chitosan was not added into the run buffer, the capillary was modified with chitosan by flushing with buffer containing 0.1% chitosan for 5 min and was allowed to contact statically with the capillary for 15 min. Prior to each sample analysis, the capillary was rinsed with the buffer containing 0.1% chitosan for 1 min, followed by a 2-min rinse with the buffer free of chitosan. Samples were injected by application of vacuum for 0.5 s at 127 mmHg. Electrophoresis was carried out at − 15 kV, with UV detection at 214 nm. The temperature of the column was maintained at 30°C. Electropherograms were recorded with a HP 3390A integrator (Hewlett-Packard, Palo Alto, CA, USA). The EOF was monitored by the water peak.

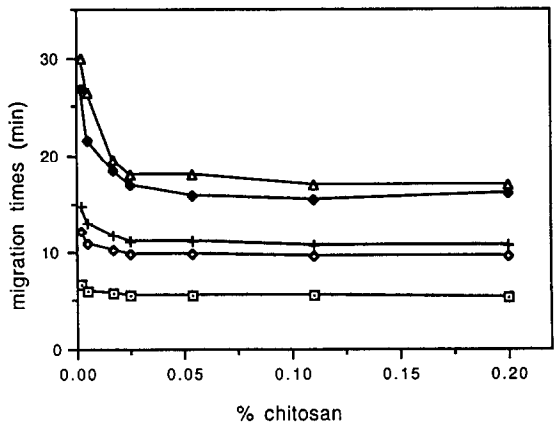


Fig. 1. Influence of chitosan concentration in the separation buffer on the EOF (□) and the migration times of four basic model proteins in free solution capillary electrophoresis: trypsinogen (◇), ribonuclease A (+), lysozyme (◆) and cytochrome *c* (△). Capillary: 53 cm (31 cm to the detector) × 50 μm; applied voltage: − 15 kV; current: 30 μA; run buffer: 50 m*M* sodium acetate, pH 4.0; detection: 214 nm; injection: 0.5 s at 127 mmHg.

## 3. Results and discussion

### 3.1. Effect of chitosan concentration

Due to its abundance of amino groups, chitosan exhibits high density of positive charge in aqueous acidic solutions. Through dominantly electrostatic interaction, the polycationic chitosan can adsorb to negatively charged surface of the inner surface of the fused-silica capillary. As a result of this interaction, the overall charge of the capillary wall is reversed [27].

To study the adsorption of chitosan onto the capillary wall, the magnitude of the EOF and the migration times of four basic proteins (*i.e.* trypsinogen, ribonuclease A, lysozyme and cytochrome *c*) were measured at various concentrations of chitosan added to the electrophoretic buffer. The results are summarized in Fig. 1. At pH 4.0 and in the absence of chitosan in the buffer, the direction of the EOF in an untreated capillary was towards the cathode, but no distinct peaks were observed for the proteins. At this pH, the proteins are highly cationic and would interact strongly with the negatively charged silanoate ($Si-O^-$) groups on the capillary surface. However, in the presence of just 0.002% of chitosan in the buffer, an inversion in the direction of the EOF towards the anode was exhibited, indicating that the interfacial double layer had been modified by the adsorption of chitosan. In addition, reasonably symmetrical peaks were obtained, presumably as a result of significant reduction of solute adsorption due to coulombic repulsion from the positively charged capillary surface. As the concentration of chitosan in the buffer increases, faster EOF was observed, due to increasing $\zeta$ potential of the electric double layer at the capillary surface as more chitosan molecules were adsorbed to the surface. With above 0.05% of chitosan in the buffer, the capillary exhibited relatively constant EOF (variation *ca.* 1%), indicating a saturation of chitosan coverage at the capillary surface.

Besides a faster EOF, better migration time reproducibility was also obtained upon increasing the concentration of chitosan up to about 0.05% in the electrophoretic buffer (see Table 1). Reproducibility of migration times was better than 0.4% for all the four proteins at chitosan concentrations greater than 0.1%. No buffer replenishment between injections was necessary to achieve such reproducibility.

Table 1
Reproducibility of migration time of the four basic model proteins with different concentrations of chitosan in the buffer

| Chitosan (%) | Relative standard deviation (%, $n = 5$) | | | | |
|---|---|---|---|---|---|
| | EOF | Trypsinogen | Ribonuclease A | Lysozyme | Cytochrome *c* |
| 0.002 | 0.99 | 2.62 | 3.04 | 4.44 | 4.94 |
| 0.005 | 0.34 | 1.09 | 1.32 | 2.06 | 2.24 |
| 0.017 | 0.88 | 1.65 | 1.89 | 2.84 | 3.04 |
| 0.025 | 0.36 | 0.68 | 0.77 | 0.98 | 1.03 |
| 0.054 | 0.28 | 0.26 | 0.23 | 0.25 | 0.29 |
| 0.11 | 0.01 | 0.21 | 0.14 | 0.04 | 0.16 |
| 0.2 | 0.01 | 0.26 | 0.29 | 0.01 | 0.39 |

Capillary: 53 cm (31 cm to the detector) × 50 μm I.D.; buffer: 50 m*M* sodium acetate at pH 4.0; applied voltage: − 15 kV.

The reduction in protein–wall interaction is also reflected in the improved peak efficiency. As shown in Fig. 2, there is an improvement in the column efficiency, in terms of theoretical plate number $N$, with higher chitosan concentrations in the buffer. The higher viscosity of the buffer solution at high chitosan concentrations might have also contributed in minimizing band broadening by restricting the diffusion of the protein within the bands. This increase in efficiency is most clearly observed for ribonuclease A. In the case of trypsinogen and cytochrome *c*, their efficiencies remain relatively constant at chitosan concentrations of about 0.1%. Lysozyme, however, exhibit decreasing peak efficiency. The nature of this interaction is not fully understood.

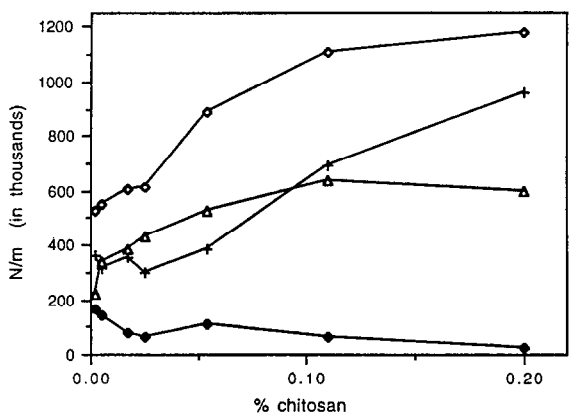


Fig. 2. Influence of chitosan concentration in the run buffer on the separation efficiency. Conditions and symbols as in Fig. 1.

### 3.2. Effect of pH

The pH of the operating buffer influences the charges on the proteins, the dissociation of the buffer ions and the charge density on chitosan. The charges on chitosan arises from the primary amino group of the glucosamine monomer, which has a $pK_a$ value around 6.3 [28]. Increasing the pH of the electrolyte therefore reduces the net charge on chitosan. As a result, dissolved chitosan usually flocculates at pH values above *ca.* 6, limiting the useful pH range available. From the above experiments performed at pH 4.0, since satisfactory migration time reproducibility and separation efficiency can be obtained with 0.05% chitosan in the running buffer, this concentration was initially considered for further investigations. However, with 0.05% chitosan in the buffer, it was found that migration time reproducibility deteriorated at higher pH. It was believed that as the positive charge density on chitosan decreases with increasing pH, chitosan coverage on the capillary wall becomes less effective in masking the ionized surface silanols and preventing the adsorptive protein–wall interaction. To compensate for the decreasing coverage, 0.1% chitosan was used instead in all further experiments.

Fig. 3 shows a plot of the rate of the EOF and the electrophoretic mobilities of four proteins as a function of the pH of the run buffer containing 0.1% chitosan. From pH 3.05 to pH 3.65, there is an increase in the EOF, followed by a nearly

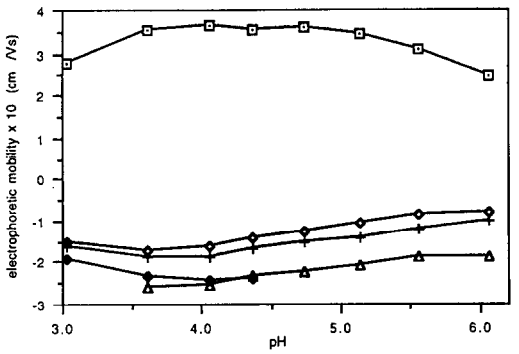


Fig. 3. Plot of EOF and electrophoretic mobilities of the four proteins as a function of buffer pH. Run buffer: 50 m*M* sodium acetate containing 0.1% chitosan. Other conditions and symbols as in Fig. 1.

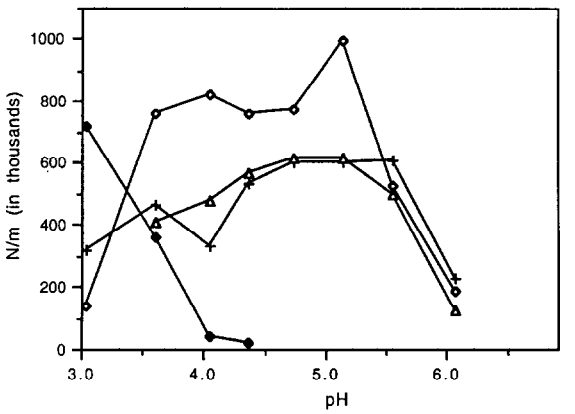


Fig. 4. Plot of peak efficiency *versus* buffer pH. Run buffer: 50 m*M* sodium acetate containing 0.1% chitosan. Other conditions and symbols as in Fig. 1.

constant EOF from pH 3.65 to pH 4.70. Further increase in pH causes a reduction in the EOF as deprotonation of the amino groups on chitosan occurred. It would be expected that with a higher degree of ionization of chitosan at lower pH, a faster anodal EOF should result. However, this was not observed at pH 3.05. It has been reported that chitosan molecules in dilute solutions (concentration between 0.01 and 0.1%, w/v) behave like worm-like molecules with some degree of stiffness, depending on the degree of ionization of chitosan and the counterion concentration [29]. As the number of charges on chitosan backbone increased with lowering of the buffer pH, the electrostatic repulsive forces between neighbouring charged groups resulted in an expansion and stiffening of the chitosan molecules; subsequently, a higher intrinsic viscosity of chitosan in solution was obtained. The result is slower EOF and electrophoretic mobilities of the proteins at lower buffer pH.

At pH 3.05, cytochrome *c* was not eluted as its electrophoretic mobility approached the magnitude of the EOF, but in the opposite direction. Generally, the mobilities of the proteins decreased with increasing pH as anticipated, with the exception of lysozyme. At higher pH, the electrophoretic mobility of lysozyme increased, and was accompanied by a drastic drop in its peak efficiency (see Fig. 4). It was postulated that repulsion between the proteins and chitosan molecules would decrease as the buffer pH approaches the $pK_a$ value of chitosan. Possible interactions arising from hydrogen bonding between the hydroxyl groups and the deprotonated amino groups on chitosan and similar functional groups on the side chains of the proteins could have contributed to the reduction in peak efficiencies of the proteins at pH above 5.5.

### 3.3. Chitosan-modified capillary

The above observation suggested possible interference of chitosan in the buffer with the analytes. During the course of the work, it was noted that even after prolonged washing of the capillary with aqueous acetic acid, a solvent for chitosan, the direction of the EOF is still anodal. It appeared that the interaction of chitosan with the capillary surface is relatively strong, resulting in a stable coating of chitosan such that inclusion of chitosan in the run buffer is not necessary. The potential advantages of this approach are that possible interactions between the analytes and free chitosan in the buffer could be minimized, and sensitivity could be improved due to a reduction in the background signal originated from the UV absorbance of chitosan.

To achieve satisfactory run-to-run reproducibility of the migration time, between-run wash procedures were evaluated. It was found that when the capillary was washed with a solution containing 0.1% chitosan for 1 min, followed by

a 2-min rinse with run buffer free of chitosan, the relative standard deviation (R.S.D.) of the migration time of the proteins can be kept below 1%, in the pH range investigated.

Fig. 5 displays the change in the peak efficiency of the four basic model proteins with pH in a chitosan-modified column. Over the pH range studied, lysozyme was eluted with better efficiency than in the case when chitosan was included in the buffer. This suggested that interaction between lysozyme and chitosan in the buffer is most probably responsible for the poor peak shape. On the other hand, for the other proteins investigated, a slight decrease in efficiency was observed. This general lowering of peak efficiency in buffer solutions without chitosan might be due to the reduced solution viscosity, and hence allowing band diffusion.

An interesting point to note is the migration order for cytochrome *c* and lysozyme (Fig. 3). Despite the fact that cytochrome *c* has a lower p*I* (10.5) than lysozyme (11.0), it was eluted after lysozyme. In addition, we observed during our experiment that cytochrome *c* exhibited higher electrophoretic mobilities than lysozyme did at pH less than 4.36 and 4.74 for buffers with and without chitosan, respectively. The same migration behaviour has been observed by other workers [5,18,30,31], using either buffer additives (*e.g.* 1,3-diaminopropane) [18] or capillaries chemically coated with polymers (*e.g.* cross-linked polyacrylamide, polyhydroxyalkylmethacrylate, and polyethers with terminal hydroxyl groups) [5,30,31] for the separation of these basic proteins at pH *ca.* 4.7. The peculiar migration order for cytochrome *c* and lysozyme may be due to properties intrinsic to the proteins. For instance, lysozyme has been observed to undergo an unusual transition at around pH 4 [32].

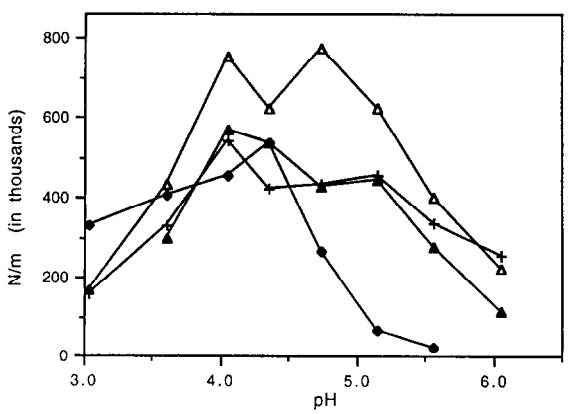


Fig. 5. Effect of buffer pH on the peak efficiency of proteins on a chitosan-modified capillary in 50 m*M* sodium acetate buffer free of chitosan. Other conditions as in Fig. 1. Symbols: △ = trypsinogen; + = ribonuclease A; ▲ = cytochrome *c*; ◆ = lysozyme.

### 3.4. Applications

As shown above, the use of chitosan-modified capillary is effective in reducing protein–wall interactions over the pH range 3.0–5.5. This narrow pH range, however, does not limit the applicability of the method. The high peak efficiency attained enhances the resolution and thus the separation power of the method. Fig. 6 shows an electropherogram obtained for a group of standard proteins (p*I* ranging from 5.1 to 11.0) with 0.05% chitosan in pH 3.5 buffer. The same group of proteins were also separated in

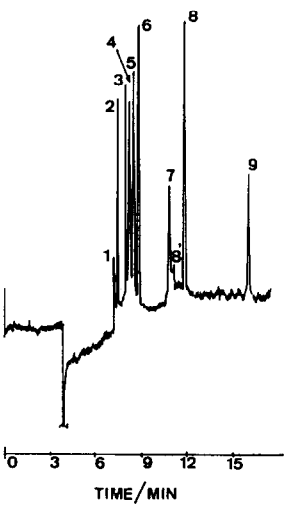


Fig. 6. Separation of standard proteins by free solution capillary electrophoresis. Buffer: pH 3.5 sodium acetate (50 m*M*) containing 0.05% chitosan. About 2 nl of protein mixture, corresponding to 0.4 μg of each protein was injected. Other conditions as in Fig. 1. Peaks: 1 = carbonic anhydrase I; 2 = α-chymotrypsinogen A; 3 = trypsinogen; 4 = β-lactoglobulin A; 5 = β-lactoglobulin B; 6 = ribonuclease A; 7 = myoglobin; 8 = lysozyme; 9 = cytochrome *c*.

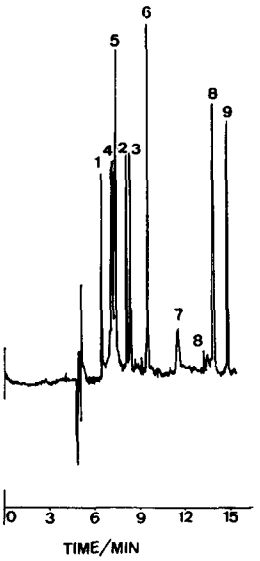


Fig. 7. Capillary electrophoretic separation of standard proteins on a chitosan-modified capillary with pH 4.36 sodium acetate (50 m*M*) buffer free of chitosan. Peaks as in Fig. 6. Other conditions as in Fig. 1.

the absence of chitosan in pH 4.36 buffer (Fig. 7). In both cases, narrow and symmetrical peaks were obtained for the proteins. The ability of the chitosan-modified columns to analyze complex mixtures is again demonstrated in Fig. 8. The electropherogram shows the analysis of a tryptic digest of horse heart cytochrome *c* performed at pH 4.0. It can be seen that the mixture contains at least 25 tryptic peptides, including some negatively charged peptides, which migrated out before the water peak (corresponding to the baseline dip at around 6 min). Capillary electrophoretic separation of tryptic digest of the same protein was also recently assessed by Wheat *et al.* [33] using an untreated fused-silica capillary. A comparison of the electropherograms revealed two rather different electropherogram profiles, suggesting the possibility of employing the present approach as an alternative method for peptide mapping.

In conclusion, the results in this work demonstrated that chitosan can be used as a capillary modifier in capillary electrophoresis. The approaches investigated in this work effectively suppresses protein–wall interaction, resulting in

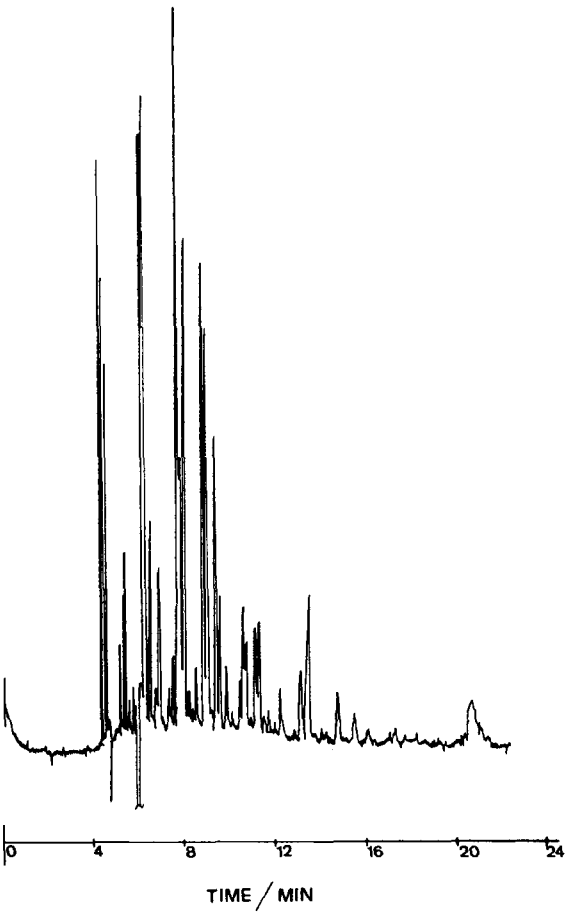


Fig. 8. Capillary electrophoretic separation of a tryptic digest of horse heart cytochrome *c* on a chitosan-modified capillary with pH 4.0 sodium acetate (50 m*M*) buffer free of chitosan. Capillary: 58.5 cm (38.5 cm to the detector) × 50 $\mu$m; applied voltage: − 17 kV; current: 28 $\mu$A; detection: 214 nm; injection: 0.5 s at 127 mmHg.

separations of basic proteins and peptides with high efficiency. Despite the narrow useful pH range, the present approach has the advantages that it does not require the use of undesirable extreme pH values and high ionic strength buffers.

## 4. Acknowledgements

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