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Capillary electrochromatography of proteins and peptides with a cationic acrylic monolith

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Abstract

For the separation of proteins and peptides by capillary electrochromatography (CEC), columns with a monolithic stationary phase were prepared from silanized fused-silica capillaries of 50 μm I.D. by in situ copolymerization of glycidyl methacrylate, methyl methacrylate and ethylene glycol dimethacrylate in the presence of propanol and formamide as porogens. The epoxide groups at the surface of the porous monolith were reacted with *N*-ethylbutylamine to form fixed tertiary amino functions with ethyl- and butyl-chains. A mixture of ribonuclease A, insulin, α-lactalbumin and myoglobin was separated isocratically by counterdirectional CEC with hydro–organic mobile phases containing acetonitrile and sodium phosphate buffer, pH 2.5. The separation of four angiotensin type peptides by CEC was also achieved under similar conditions. The elution order of proteins was similar to that obtained in reversed-phase chromatography. Plots of the migration factors for proteins and peptides against the acetonitrile concentration exhibit opposite trends. This is most likely due to the greater chromatographic retention and lower electrophoretic migration velocity of proteins than that of peptides in the counterdirectional CEC system. From this it is concluded that the separation is governed by a dual mechanism that involves the complex interplay between selective chromatographic retention and differential electrophoretic migration.
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1. Introduction

Capillary electrochromatography (CEC) embodies many features of capillary zone electrophoresis (CZE) and high-performance liquid chromatography (HPLC). The combination of the high peak efficiency of CZE with the high selectivity obtained in HPLC appears to be the major advantage of this new separation method [1,2]. Today the separation of small neutral compounds by CEC is a straightforward procedure, however, the situation is quite different with the separation of complex polyelectrolytes such as proteins and peptides. This explains why there is only a scant number of reports on successful application of CEC in this area of research [3–7]. Clearly, for CEC to become a practicable analytical method for the separation of biopolymers, further progress is needed in understanding the physico–chemical aspects underlying the separation mechanism. Furthermore the development of especially designed capillary columns is needed to meet the unique requirements arising from the combined separation powers of chromatographic retention and electric field.

At present most columns employed in CEC are fused-silica capillaries packed with functionalized

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silica particles serving as the stationary phase [8–14]. Most of them have been developed for use in HPLC and they are available commercially with well characterized properties. Performance and stability of such particulate packings greatly depend on the integrity of the retaining frits at the two column ends [8,9]. In practice, gaps are formed in the packing frequently and discontinuities lead to a halt of the separation process [15]. CEC with octadecylated silica particles has to be carried out at neutral or alkaline pH, where the silanol groups are dissociated and can generate electroosmotic flow (EOF) upon application of electric field. In fact, all stationary phases for voltage driven CEC ought to be ion exchangers for this reason.

The unsatisfactory stability of siliceous columns at elevated pH and other reasons prompted us to consider microspherical particle support prepared from polymeric organic materials then packed into the column tube [16]. Alternatively, the porous support can be prepared and functionalized in situ inside the capillary tubing as a monolith [17–19]. This kind of column architecture offers significant advantages in CEC because it relinquishes the need for retaining frits and column packing does not contain charged particles which can be readily dislodged in the high electric field. Monolithic packings for chromatographic columns were introduced about 30 years ago [20–22] without much ensuing interest. Only did the advent of CEC focus attention on this approach that appears to be more useful for the preparation of packed capillary columns, than for the relatively wide diameter columns employed in HPLC. At present most interest seems to be focused on the area of porous styrenic and acrylic monoliths with appropriately functionalized chromatographic surface [18,19].

This work is aimed at the study of protein and peptide separations by CEC, which is conveniently accomplished by employing strongly acidic eluent in which all the sample components carry positive charges. For the model separation of peptides and standard proteins, an acrylic monolith was prepared in a 50 μm I.D. fused-capillary and functionalized by anchoring tertiary amino groups and short alkyl chains as functional groups at the chromatographic surface. The separation by such stationary phases is the result of a complex interplay of chromatographic retention and electrophoretic migration. It is believed that in our case the chromatographic complement of the separation process arises from the interaction between the sample components and the alkyl chains fixed at the stationary phase surface. The results of this work are expected to demonstrate the feasibility of polypeptide separation by CEC, and *a fortiori*, to give insight into the complex mechanistic aspects of CEC in the separation of macromolecular polyelectrolytes such as proteins.

2. Experimental

2.1. Materials

Fused-silica capillary tubing of 50 μm I.D.×375 μm O.D. with a polyimide outer coating was purchased from Quadrex Scientific (New Haven, CT, USA). 3-(Trimethoxysilyl) propyl methacrylate and ethylene glycol dimethacrylate were from Polysciences (Warrington, PA, USA) and 2,2-diphenyl-1-picryhydrazyl hydrate (DPPH), *N*-ethylbutylamine (99%), formamide, methyl methacrylate (99%), glycidyl methacrylate (98%) from Aldrich (Milwaukee, WI, USA). Analytical-reagent grade monobasic, dibasic and tribasic sodium phosphates, dimethylformamide (DMF) (99%) were from J.T. Baker (Phillipsburg, NJ, USA) and azobisisobutyronitrile (AIBN) (98%) from Pfaltz & Bauer (Waterbury, CT, USA). Ribonuclease A (bovine pancreas), insulin (bovine pancreas), α-lactalbumin (bovine milk), myoglobin (horse skeletal muscle), bradykinin (acetate salt), angiotensin I (acetate salt), angiotensin II (acetate salt), [Sar^1,Ala^8]angiotensin II were purchased from Sigma (St. Louis, MO, USA). Dimethylsulfoxide (DMSO) was purchased from Burdick & Jackson (Muskegon, MI, USA). Phosphoric acid (85%) and sodium hydroxide (98.8%) were of analytical-reagent grade from Mallinckrodt (Paris, KY, USA). HPLC-grade methanol, acetone, acetonitrile (ACN), propanol and methylene chloride were purchased from Fisher (Fair Lawn, NJ, USA). The materials were used without further purification. Water was purified and deionized with a NANOpure system (Barnstead, Boston, MA, USA).

2.2. Column preparation

2.2.1. In situ polymerization

The pretreatment and the silanization procedure for the fused-silica capillary were the same as described previously [5,23]. A solution containing 10% (v/v) each of glycidyl methacrylate and methyl methacrylate, 20% (v/v) ethylene glycol dimethacrylate, 10% (v/v) *n*-propanol, 50% (v/v) formamide and 0.3% (w/v) of the inhibitor (AIBN) was prepared and degassed with nitrogen for 10 min. The solution was filled into the silanized capillary. After both ends were sealed by butane flame of a Veriflo Air-gas Torch (Macalaster Bicknell, New Haven, CT, USA), the capillary tube was heated at 65°C for 3 h and then 78°C for another 16 h in the oven of a Model Sigma 2000 gas chromatograph (Perkin-Elmer, Norwalk, CT, USA). Subsequently, the column was purged with nitrogen to remove the mixture of *n*-propanol and formamide. The capillary was washed with methanol and then heated at 120°C for 2 h with a nitrogen stream.

2.2.2. Formation of the chromatographic surface

The capillary containing the porous monolithic support was filled with *N*-ethylbutylamine. With both ends sealed, it was heated at 70°C for 8 h and subsequently washed with methanol and water.

2.2.3. Preparation of the detection window

A 1 to 2 mm wide segment of the polyimide outer coating was scraped off by using a small blade. The polymeric packing inside of the segment was removed by burning it off with a butane fueled Archer Torch Model B microtorch (Radio Shack, New Haven CT, USA), while the tube was purged with oxygen at 160 p.s.i. inlet pressure (1 p.s.i.=6894.76 Pa). Subsequently, the column was washed with acetonitrile.

2.3. Capillary electrochromatography: apparatus and procedure

The experiments were conducted using a Model MDQ P/ACE system capillary electrophoresis unit equipped with a P/ACE system MDQ photodiode array detector (Beckman, Fullerton, CA, USA). A Model 6588-12U IBM personal computer with Beckman MDQ capillary electrophoresis software version 2.2. and Windows 95 (Microsoft, Redmond, WA, USA) installed was used to control the instrument and to acquire and process the data.

In all experiments 20–100 m*M* aqueous sodium phosphate buffers, pH 2.5, containing acetonitrile at various concentrations were used. The protein sample contained 0.8 mg/ml ribonuclease A, insulin, myoglobin and 1 mg/ml α-lactalbumin in 20 m*M* sodium phosphate buffer, pH 2.5. The peptide sample contained 0.2 mg/ml bradykinin, angiotensin I, [Sar^1,Ala^8]angiotensin II, and 0.5 mg/ml angiotensin II in 20 m*M* sodium phosphate buffer, pH 2.5. The samples were injected at −5 kV for 2 s. Between runs the column was rinsed with acetonitrile for 7 min followed with the running mobile phase for 20 min at 100 p.s.i. inlet pressure. Then with both ends pressurized at 95 p.s.i. the column was equilibrated electrokinetically at the operating voltage for 5 min. The EOF marker, DMSO, 2 μl/ml in water, was injected at −2 kV for 1 s. All samples were detected at 214 nm. All experiments were carried out at 25°C.

3. Results and discussion

3.1. Column architecture in capillary electrochromatography

Capillary columns employed in CEC are packed with either particulate or monolithic [17–19] stationary phase. Open tubular columns with a porous layer of the stationary phase (PLOT columns) at the innerwall have also been found employment in CEC [5]. In the latter column type the porous stationary phase layer has an anular configuration and by changing the layer thickness, the phase ratio can be conveniently adjusted. Most work in CEC has been carried out with columns packed with stationary phase particles having nominal particle diameters in the range from 2 to 10 μm. These stationary phases have originally been developed for use in traditional HPLC. Although chromatographic columns with porous monolithic support were described long time ago [20–22], their use in capillary column has gained significance only due to the growing interest in CEC.

The stationary phase in CEC has two main

functions. Fixed charges on the chromatographic surface are needed to generate EOF of the mobile phase upon applying high electric field to the column. Further, they are likely to play a significant role in the chromatographic retention process by attracting or repelling the sample components to different extent to or from the chromatographic surface. As a consequence of the abundant fixed charges at their surface, the stationary phases in CEC are always ''ion exchangers''. For instance, octadecyl-silica is a bona fide cation exchanger in contact with a mobile phase that has a pH high enough for the residual silanol groups at the chromatographic surface to be deprotonated. To meet the above two requirements in an optimal fashion, it becomes imperative that stationary phases will be prepared especially for CEC in order to provide retaining sites for reversible and selective binding of the sample components to be separated.

3.2. Column preparation in this study

The innerwall of fused-silica capillary was treated first with 3-(trimethoxysilyl)propyl methacrylate as described before [5,23]. Inside the silanized capillary a porous methacrylic monolith was formed by in situ polymerization of a monomer mixture containing glycidyl methacrylate and methyl methacrylate as comonomers, ethylene glycol dimethacrylate as the crosslinking agent and a mixture of propanol and formamide as the porogen. It is believed that the use of a binary porogen mixture facilitates the formation of monolith having bimodal pore distribution. In the functionalization of the chromatographic surface the epoxide groups originating from glycidyl methacrylate were utilized for anchoring the *N*-ethylbutylamine functions. The reaction scheme for the postpolymerizational functionalization is shown in Fig. 1. It is seen that the reaction of *N*-ethylbutylamine with the oxyrane rings resulted in surface-bound tertiary amino functions which are protonated at acidic pH.

The stationary phase with the alkyl moieties was designed for the separation of proteins and peptides at acidic pH by using a water-rich hydro–organic mobile phase. At low pH the proteins investigated

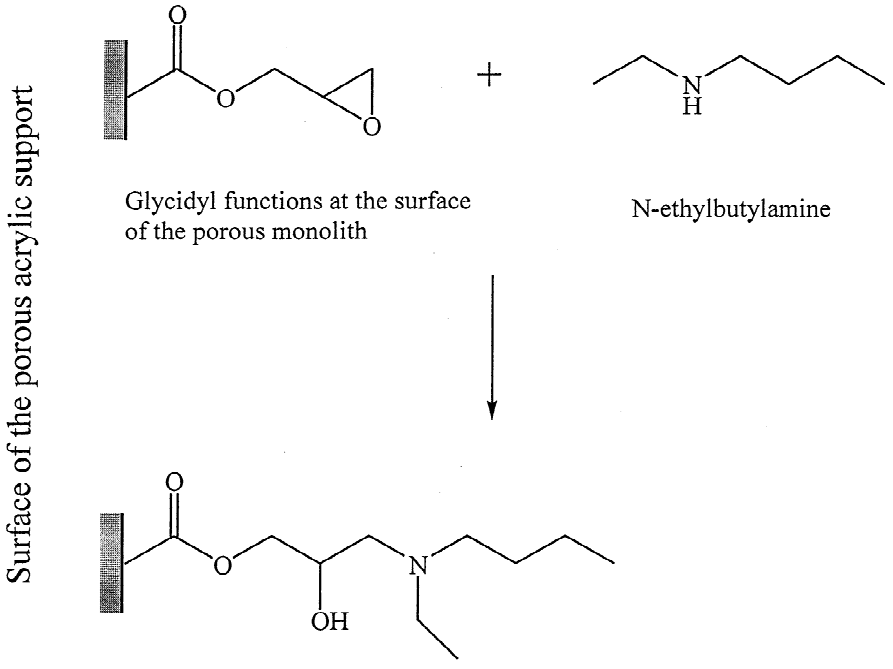


Fig. 1. Reaction scheme for the functionalization with *N*-ethylbutylamine of the surface of the porous methacrylic monolith having glycidyl groups attached to the polymeric structure.

carry positive net charges so that the separation system in CEC is greatly simplified. In contradistinction, the positively charged proteins migrate electrophoretically in a direction opposite to that of EOF, i.e., the CEC system is counterdirectional. This makes the separation process more complicated. It is believed that chromatographic retention is governed by solvophobic interactions [24,25] at low pH, since both the proteins and the chromatographic surface in our column are positively charged. Electrostatic interactions may manifest themselves as repulsive forces between the positively charged chromatographic surface and the positively charged proteins. This effect attenuates solvophobic interactions and thereby diminishes the retentive properties of the separation system toward the positively charged proteins. Therefore, in our separation system the role of the fixed positive charges is not only to generate EOF but also to reduce the strength of protein or peptide binding by electrostatic repulsion. As mentioned above, under these conditions, we have a counterdirectional CEC system that is operated at ''reversed polarity'' with anodic EOF. The magnitude of the chromatographic retention by solvophobic interactions is preferentially modulated by the acetonitrile concentration, i.e., by the organic strength of the mobile phase. When the combined effect of sufficiently high organic strength, which diminishes solvophobic interactions, and the electrostatic repulsion, which drastically attenuates chromatographic retention, is operative then the differential migration of the charged sample components in the electric field becomes the dominant separating mechanism. The result of the interplay by retentive and antiretentive forces is a reduction of the elution window to an extent that separation by isocratic elution even of large proteins is facilitated.

The alkyl chain length of the functionalizing agent, *N*-ethylbutylamine was selected empirically on the basis of preliminary studies with four similar tertiary amines having different alkyl chain lengths. The columns obtained with the four sorbents thus prepared were tested in the separation of the four standard proteins. In an attempt to optimize the separation, various mobile phase conditions were employed. With columns having long chain alkyl amines, such as *N*-methyldodecylamine and *N*-methyloctadecylamine, the separation was poor and the recovery low. On the other hand, the column with diethylamine functionality did not retain the proteins at acidic pH. Therefore, an amine with an intermediate length of alkyl chain, such as *N*-ethylbutylamine, was used in the following for the functionalization of the chromatographic surface. Fig. 5 illustrates the baseline separation of the four standard proteins on a column that, as all the other columns employed in this study, was prepared by utilizing the surface chemistry described above.

3.3. Effect of mobile phase on electroosmotic flow

As noted previously, for protein separation the pH of the mobile phase was 2.5. Proteins denature at such low pH, yet, in reversed-phase chromatography of proteins predominantly strong acidic conditions are employed for obtaining sharp peaks. In our case the use of low pH offers further advantages. First of all, with the positively charged surface of the stationary phase relatively strong EOF can be generated at customary applied voltage. On the other hand, at pH 2.5 most proteins carry positive net charges, so that all of them migrate toward the cathode. In most cases we obtained also sharper peaks at pH 2.5 than at neutral pH. These are strong reasons, unless the integrity of the protein molecules needs to be conserved, for carrying out their separation under conditions similar to those employed in reversed-phase chromatography.

At acidic pH, the positively charged tertiary amino groups fixed on the polymeric support surface generate EOF. Fig. 2 shows the effect of acetonitrile on EOF velocity and mobility. The EOF velocity was measured with DMSO at −25 kV applied voltage. The mobile phase was prepared by diluting 60 m*M* aqueous sodium phosphate buffer, pH 2.5, with acetonitrile to obtain concentration in the range from 0 to 70% (v/v) ACN. The anodic EOF velocity ranged from 1.867 to 1.326 mm/s and this indicated that at the chromatographic surface tertiary amino groups were present and ionized.

The effect of pH on the EOF was also investigated and the results are shown in Fig. 3. The EOF velocity was measured with DMSO as the neutral marker with 60 m*M* aqueous sodium phosphate buffer in the pH range from 2.5 to 7.0 at −25 kV applied voltage. The mobile phase was prepared by

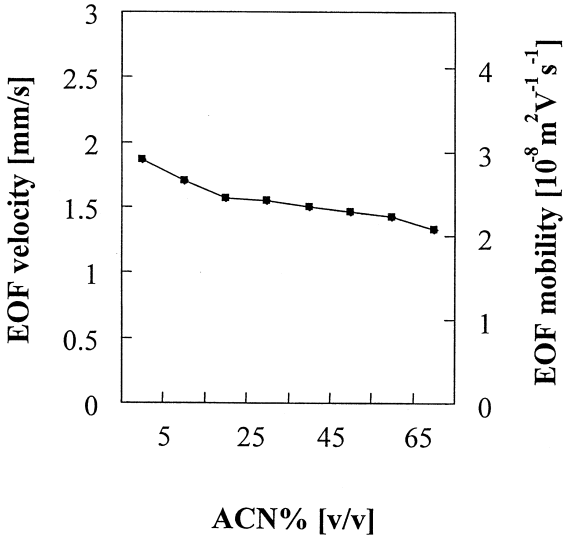


Fig. 2. Plots of the EOF velocity and mobility measured with DMSO as the unretained neutral marker against the acetonitrile concentration. Column, 39 cm (effective length 29 cm)×50 μm I.D., fused-silica capillary with porous methacrylic monolith having tertiary amino functions; mobile phase, acetonitrile (%, v/v) in 60 m*M* sodium phosphate buffer, pH 2.5; applied voltage, −25 kV; detection, 214 nm.

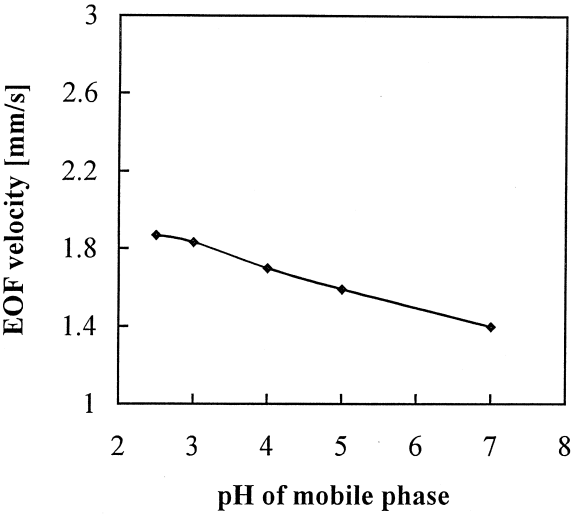


Fig. 3. Plot of the EOF velocity against pH as measured with DMSO as the unretained neutral marker. Column, 39 cm (effective length 29 cm)×50 μm I.D., fused-silica capillary with porous methacrylic monolith having tertiary amino functions; mobile phase, 60 m*M* sodium phosphate buffer; applied voltage, −25 kV; detection, 214 nm.

adjusting the pH of 60 m*M* phosphoric acid with 60 m*M* aqueous sodium phosphate solution. In this pH range, the EOF velocity is the highest at pH 2.5 and decreases approximately linearly with increasing pH in the mobile phase. Unlike stationary phases with quaternary ammonium functions, the present one has ionogenic tertiary amino groups and therefore the zeta potential and EOF are both dependent on the pH of the mobile phase.

The dependence of EOF on sodium phosphate concentration in the range from 20 m*M* to 100 m*M* was measured with the column used for protein separation. This was done by diluting 100 m*M* sodium phosphate buffer, pH 2.5, with appropriate amount of 20 m*M* sodium phosphate buffer, pH 2.5. As seen from Fig. 4, the plot of EOF velocity against sodium phosphate concentration, the EOF velocity decreases with increasing salt concentration in the range of the measurement. The decrease in EOF mobility per unit salt concentration is similar to that obtained with packed capillary columns of comparable chromatographic surface [13] and this may be taken as a manifestation of the structural similarity between the packed column and our monolithic column.

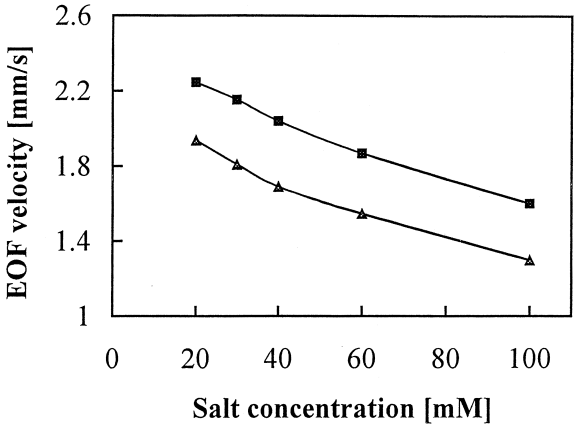


Fig. 4. Plot of the EOF velocity measured with DMSO against sodium phosphate concentration. Column, 39 cm (effective length 29 cm)×50 μm I.D., fused-silica capillary with porous methacrylic monolith having tertiary amino functions; mobile phase, (■) 0% ACN, (▲) 30% ACN in 20 to 100 m*M* of aqueous sodium phosphate buffer, pH 2.5; applied voltage, −25 kV; detection, 214 nm.

3.4. Separation of proteins

The application of CEC to the separation of charged macromolecular compounds such as proteins and peptides is rather new, unlike the separation of neutral compounds. For this reason the interplay of chromatographic retention and electrophoretic migration with charged macromolecules like proteins has yet to be investigated. The results are needed to advance their separations by CEC that appears to be a particularly challenging but very fertile area of separation science.

As illustrated by the electrochromatogram in Fig. 5, a mixture of ribonuclease A, insulin, α-lactalbumin and myoglobin was separated on a column described above. The proteins, whose isoelectric point (p*I*) values and molecular masses are listed in Table 1, were separated by using a hydro–organic mobile phase containing 30% (v/v) acetonitrile in 60 m*M* sodium phosphate, pH 2.5. It is important to note that counterdirectional CEC was employed with isocratic elution at reversed polarity. The efficiency

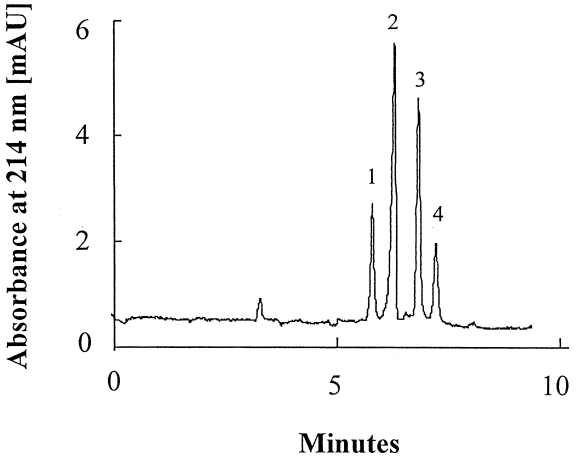


Fig. 5. Capillary electrochromatogram of four proteins obtained under isocratic elution conditions. Column, 39 cm (effective length 29 cm)×50 μm I.D., fused-silica capillary with porous methacrylic monolith having tertiary amino functions; mobile phase, 30% (v/v) acetonitrile in 60 m*M* aqueous sodium phosphate, pH 2.5; applied voltage, −25 kV; detection, 214 nm; sample: (1) ribonuclease A, (2) insulin, (3) α-lactalbumin, (4) myoglobin. Mobility of EOF measured with DMSO, $\mu_{eo} = -2.41 \cdot 10^{-8}$ m^2 V^{-1} s^{-1}; migration time of DMSO, 3.128 min. Theoretical plate numbers for each protein are shown in Table 2.

Table 1
Isoelectric points and molecular masses of the proteins investigated

Protein	p*I*	M_r (kDa)
Ribonuclease A	8.8	13.5
Insulin	5.3	11.5
α-Lactalbumin	5.0	14.2
Myoglobin	7.1	17.4

of the monolithic column was satisfactory. As shown in Table 2, plate numbers for the proteins with the 39 cm (effective length 29 cm) column were in the range of 30 000–40 000 and plate heights of 7–10 μm, respectively. Ultimately, the relatively low band spreading, i.e., high peak capacity, is the dominant reason for the success in protein separation by this approach. The elution order of the proteins followed the same pattern that is frequently observed in reversed-phase HPLC.

The effect of acetonitrile concentration in the range from 20 to 70% (v/v) on the separation of the four proteins was also investigated. Fig. 6 illustrates the electrochromatograms at 20 to 50% (v/v) acetonitrile concentrations. The results can be explained qualitatively by a putative mechanism as follows. When the acetonitrile concentration is lower than 20% (v/v), the four proteins do not elute in 10 min or less. At acetonitrile concentration of 70% (v/v), the four proteins elute as two peaks. It is seen from Fig. 6 that the migration time of the proteins does not change much with acetonitrile concentration. As the organic strength of the mobile phase increases, chromatographic retention of the proteins becomes weak and then differential electrophoretic migration of the sample components accounts for the separation in this counterdirectional CEC system.

Plots of the migration velocity of the four proteins against the acetonitrile concentration in the mobile phase are presented in Fig. 7 that illustrates a unique behavior of this CEC system: the migration velocities of the proteinaceous sample components increase slightly with acetonitrile concentration. Unlike in HPLC, however, here migration velocity is only weakly dependent on the organic strength of the eluent. Fig. 7 illustrates another feature of the CEC separation process: it takes place by ''isocratic elution'' in a rather narrow elution window the width

Table 2
Column efficiencies in the separation of proteins with a 39 cm (effective length 29 cm) methacrylic monolith at 20 to 40% acetonitrile (ACN), pH 2.5, 60 m*M* sodium phosphate buffer; applied voltage, −25 kV

Protein	20% ACN		30% ACN		40% ACN	
	t (min)	*N*	*t* (min)	*N*	*t* (min)	*N*
Ribonuclease A	6.212	29 436	6.130	35 251	6.296	25 574
Insulin	6.521	36 386	6.423	29 111	6.537	–
α-Lactalbumin	6.942	16 553	6.713	39 540	6.642	49 878
Myoglobin	7.171	22 482	7.129	31 317	7.079	29 506

of which is dependent among others on the organic strength of the mobile phase. This is due to the complex interplay of EOF, electrophoretic migration and chromatographic retention in counterdirectional CEC of charged sample components. Similar observations were made in the CEC of proteins also with other columns where the ionic strength of the eluent was used to modulate the migration velocities [26]. This suggests that there is a common dual mechanism underlying protein separations by CEC under conditions described here. It appears to be tantamount to a two-dimensional separation in one dimension. The subject will be treated in a forthcoming paper.

3.5. Separation of peptides

The columns packed with the methacrylic mono-

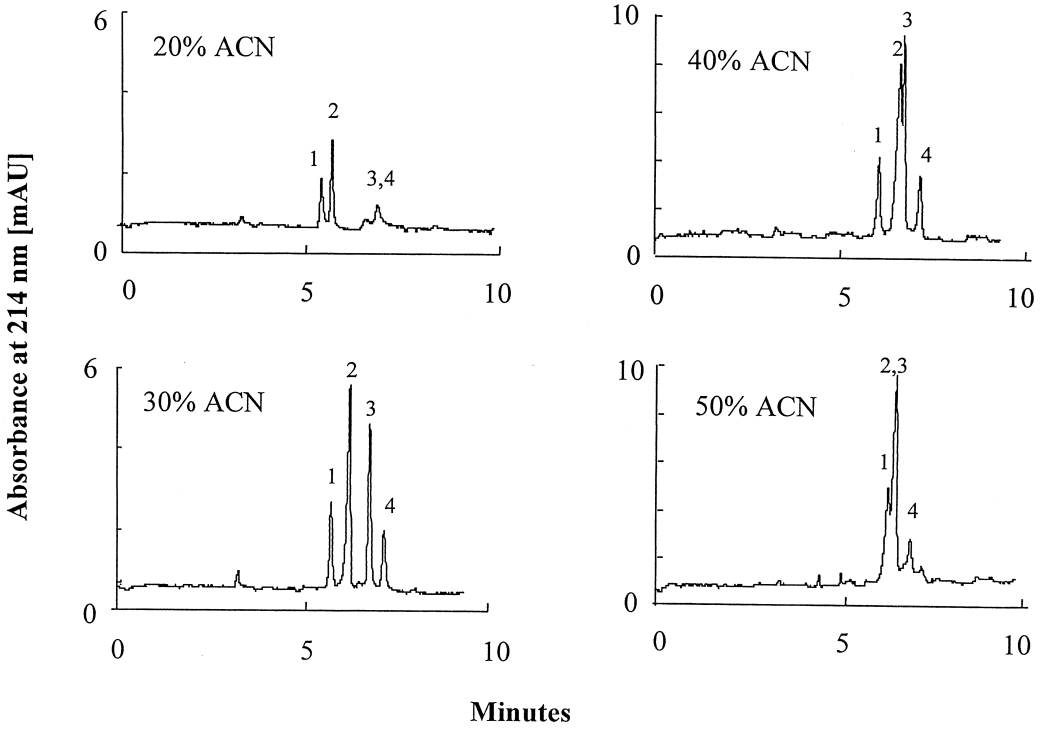


Fig. 6. Effect of acetonitrile concentration in the eluent on the separation of four proteins. Column, 39 cm (effective length 29 cm)×50 μm I.D., fused-silica capillary with porous methacrylic monolith having tertiary amino functions; mobile phase, acetonitrile (%, v/v) in 60 m*M* aqueous sodium phosphate, pH 2.5; applied voltage, −25 kV; detection, 214 nm; sample: (1) ribonuclease A, (2) insulin, (3) α-lactalbumin, (4) myoglobin. The EOF velocities at different acetonitrile concentrations are shown in Fig. 2.

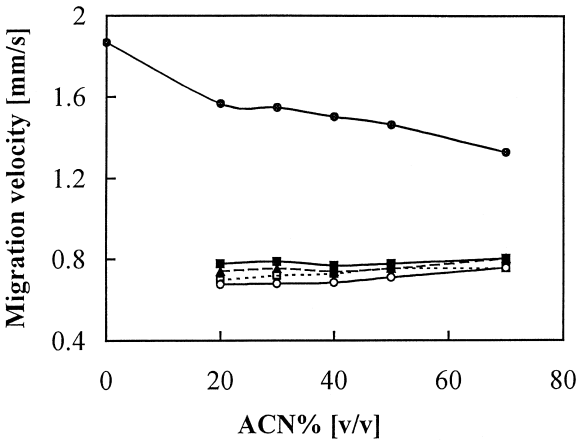


Fig. 7. Plots of CEC migration velocity of DMSO (●) and proteins against the acetonitrile concentration in the eluent. Column, 39 cm (effective length 29 cm)×50 μm I.D., fused-silica capillary with porous methacrylic monolith having tertiary amino functions; mobile phase, acetonitrile (%, v/v) in 60 m*M* aqueous sodium phosphate, pH 2.5; applied voltage, −25 kV; detection, 214 nm; (■) ribonuclease A, (▲) insulin, (□) α-lactalbumin, (○) myoglobin.

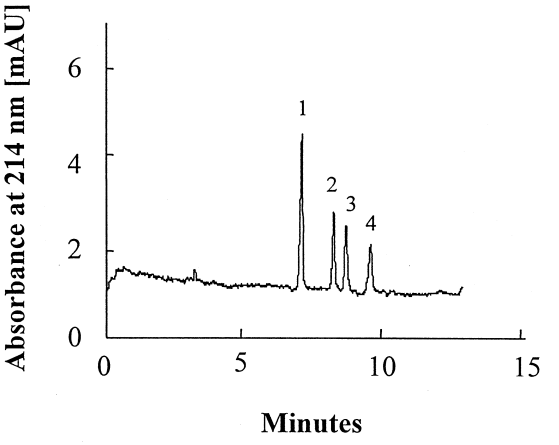


Fig. 8. Capillary electrochromatogram of angiotensin type peptides obtained under isocratic elution conditions. Column, 39 cm (effective length 29 cm)×50 μm I.D., fused-silica capillary with porous methacrylic monolith having tertiary amino functions; mobile phase, 40% (v/v) acetonitrile in 50 m*M* aqueous sodium phosphate, pH 2.5; applied voltage, −25 kV; detection, 214 nm; sample: (1) angiotensin II, (2) bradykinin, (3) angiotensin I, (4) [Sar^1,Ala^8]angiotensin II. Mobility of EOF measured with DMSO, $\mu_{eo} = -2.56 \cdot 10^{-8}$ m^2 V^{-1} s^{-1}; migration time of DMSO, 2.947 min.

lith bearing tertiary amino functions and butyl chains were also used for the separation of angiotensin I, angiotensin II, [Sar^1,Ala^8]angiotensin II and bradykinin, whose amino acid sequences and net charges are given in Table 3. Again CEC was carried out isocratically in the counterdirectional mode at reversed polarity. The mobile phase contained 40% (v/v) acetonitrile in 50 m*M* sodium phosphate buffer, pH 2.5. A typical chromatogram is depicted in Fig. 8 with peak efficiencies ranging from 32 000 to 44 000 theoretical plates with the 39 cm (effective length 29 cm) column.

The electrochromatograms of the four peptides obtained at different concentrations of acetonitrile in the mobile phase are shown in Fig. 9. Plots of the corresponding migration velocity of each component against the acetonitrile concentration are shown in Fig. 10. Comparing the results in Figs. 7 and 10 we find that the migration behavior of the peptides is different from that of proteins. Whereas the apparent ''retention'' of the proteins slightly decreases with acetonitrile concentration, the ''retention'' of the peptides under investigation appears to increase. In contradistinction, PLOT columns, for instance, exhibit a large decrease in migration velocities upon changing the organic strength of the mobile phase [5]. Much of the difference in the behavior of monolithic and open tubular column is believed to arise from the large difference in the phase ratios of the two types of CEC columns.

In order to compare the migration behavior of proteins and peptides, plots of the electrochromatographic migration factor, also called electrochroma-

Table 3
Amino acid sequences and net charges of the basic peptides investigated at pH 2.5

Peptide	Amino acid sequence	Net charge
Angiotensin II	Asp–Arg–Val–Tyr–Ile–His–Pro–Phe	3
Bradykinin	Arg–Pro–Pro–Gly–Phe–Ser–Pro–Phe–Arg	3
Angiotensin I	Asp–Arg–Val–Tyr–Ile–His–Pro–Phe–His–Leu	4
[Sar^1,Ala^8]Angiotensin II	Sar–Arg–Val–Tyr–Ile–His–Pro–Ala	–

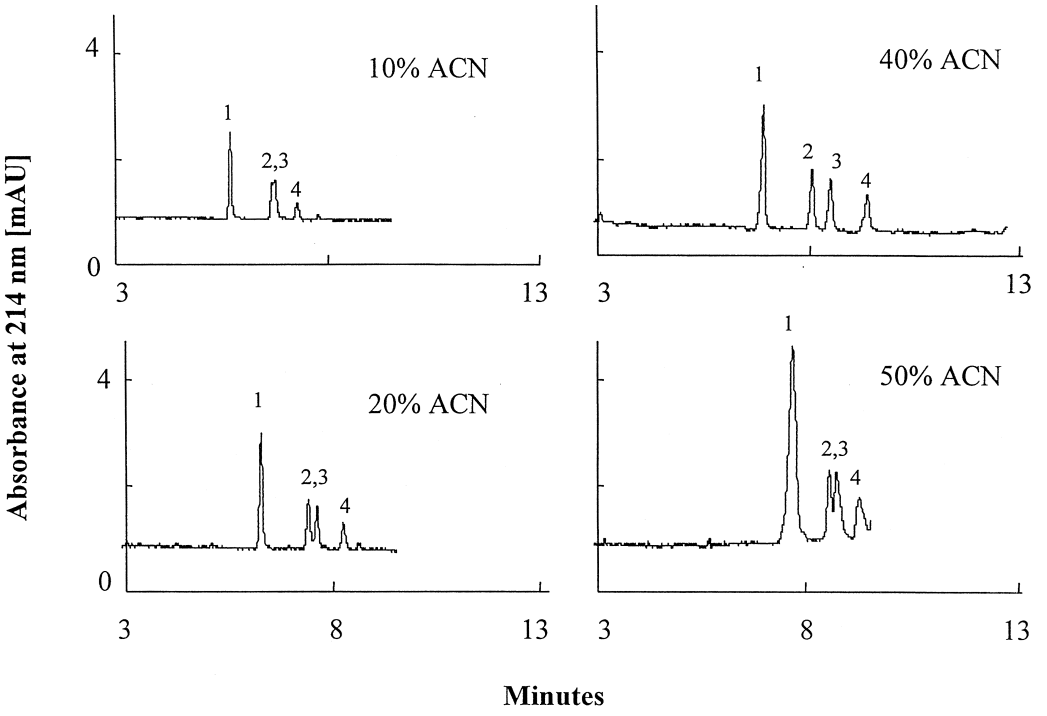


Fig. 9. Effect of acetonitrile concentration in the eluent on the separation of four peptides. Column, 39 cm (effective length 29 cm)×50 μm I.D., fused-silica capillary with porous methacrylic monolith having tertiary amino functions; mobile phase, acetonitrile (%, v/v) in 50 m*M* aqueous sodium phosphate, pH 2.5; applied voltage, −25 kV; detection, 214 nm; sample: (1) angiotensin II, (2) bradykinin, (3) angiotensin I, (4) [Sar[1],Ala[8]]angiotensin II. The EOF velocities at different acetonitrile concentrations are shown in Fig. 10.

tographic retention factor [27,28], k'_{CEC}, against the acetonitrile concentration in the eluent are presented in Fig. 11. The k'_{CEC} value is obtained from the chromatogram as $k'_{CEC}=(t_R-t_0)/t_0$, where t_0 and t_R are the migration times of the EOF marker and the sample component, respectively. We should note that the multifarious meaning of k' in chromatography is very much reduced in CEC to that of a dimensionless peak locator on the chromatogram. It is believed that the separation in our study is governed by a dual mechanism that involves both chromatographic retention (a thermodynamic process) and electrophoretic migration (a kinetic process). Therefore, we find use for k'_{CEC} only in comparing electrochromatographic data. It is seen in Fig. 11 that the k'_{CEC} values for proteins decrease with increasing acetonitrile concentration whereas peptides exhibit just the opposite behavior. The difference in the migration behavior of proteins and peptides in the same CEC system under the same conditions may be explained by the greater chromatographic retention and slower electrophoretic mobility of proteins than of peptides in our experimental range of acetonitrile concentrations when using the counterdirectional CEC system.

3.6. Column stability

The stability of the monolithic columns used in this study was tested by measuring with DMSO the electroosmotic mobility of the mobile phase, 20 m*M* phosphate buffer, pH 2.5. The measurements were performed 2 to 3 days a week and at least three times a day. In every experiment, the column was rinsed with the mobile phase for 20 min and then equilibrated electrokinetically for 10 min or until the baseline stabilized. No untoward changes were observed in the chromatograms and the migration times of the inert tracer varied only by 2–3% over a period of 1 month. In another stability test of the column,

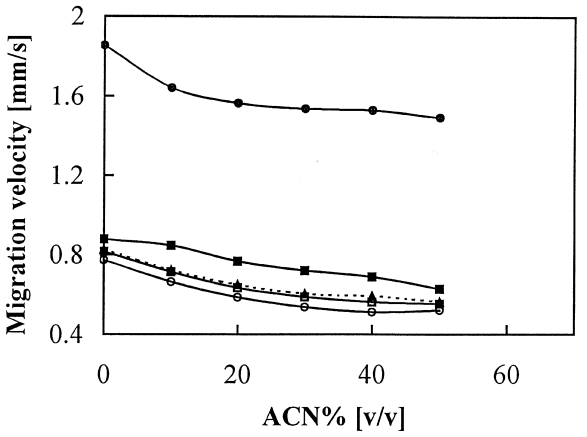
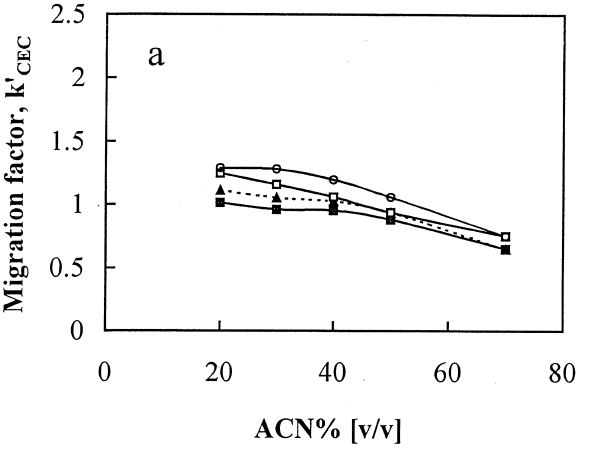


Fig. 10. Plots of CEC migration velocity of DMSO (●) and peptides against the acetonitrile concentration in the eluent. Column: 39 cm (effective length 29 cm)×50 μm I.D., fused-silica capillary with porous methacrylic monolith having tertiary amino functions; mobile phase, acetonitrile (%, v/v) in 50 m*M* aqueous sodium phosphate, pH 2.5; applied voltage, −25 kV; detection, 214 nm; (■) angiotensin II, (▲) bradykinin, (□) angiotensin I, (○) [Sar^1,Ala^8]angiotensin II.



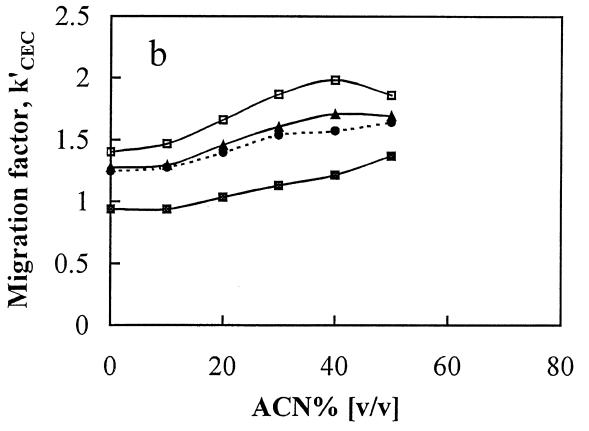


Fig. 11. Plots of the migration factor, k'_{CEC}, of proteins and peptides against the acetonitrile concentration in the eluent. Column, 39 cm (effective length 29 cm)×50 μm I.D., fused-silica capillary with porous methacrylic monolith having tertiary amino functions; applied voltage, −25 kV; detection, 214 nm. (a) Proteins, conditions as in Fig. 6; (■) ribonuclease A, (▲) insulin, (□) α-lactalbumin, (○) myoglobin; (b) peptides, conditions as in Fig. 9; (■) angiotensin II, (●) bradykinin, (▲) angiotensin I, (□) [Sar^1,Ala^8]angiotensin II.

the chromatographic behavior of the four protein components in the sample was compared at the beginning of a new column and after 300 injections of various protein samples. Fig. 12 shows the separation of the four proteins initially and after 300 injections. Every new column was subjected to a ''user's'' test and only those columns were employed in this investigation that showed a smaller than 4% relative standard deviation of the migration time of the proteins. The monolithic columns did exhibit exceptional stability that is attributed to the absence of movable charged particles in the column packing and the lack of need for retaining frits in the monolithic column. The strong bonding between the silanized innerwall and the porous polymeric support has been found to enhance the stability of the column also.

4. Conclusions

This paper describes the preparation of a novel stationary phase and its use in the separation of proteins and peptides by CEC. The stationary phase has dual functionality to allow a better control of the separation that involves both electrostatic and hydrophobic interactions. It should be kept in mind, however, that in CEC the stationary phases have to have fixed charges and thus they are ion exchangers, at least superficially. The charged chromatographic surface in the column can attract or repel charged sample components and these interactions can be modulated by the ionic strength of the mobile phase.

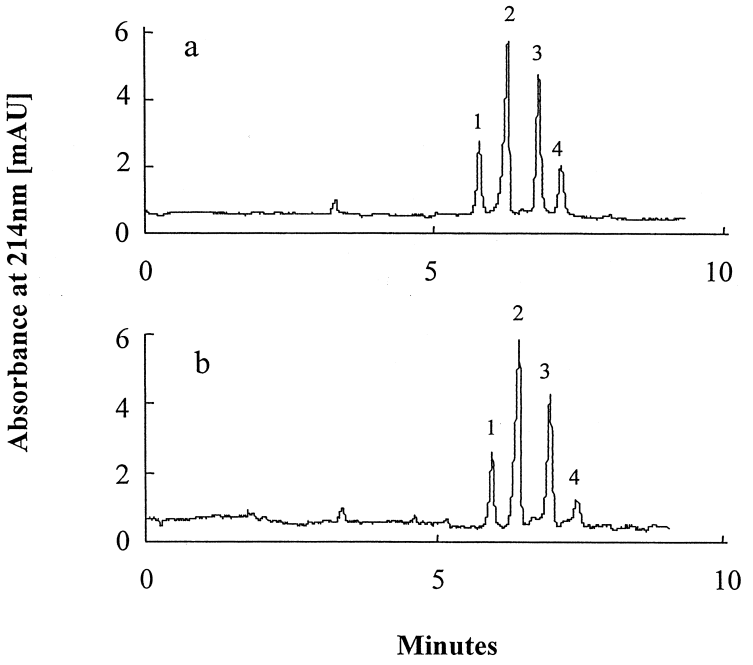


Fig. 12. Stability test electrochromatograms of four proteins obtained under isocratic elution conditions. Conditions as in Fig. 5; (a): separation of the proteins with a new column, (b): separation of the same protein mixture after 300 injections.

The results shown here and in recent publications [5,19,26] for the CEC of proteins and peptides are very encouraging that CEC has a great potential as a high-performance technique of wide applicability. The chromatographic retention, electrophoretic migration and EOF are simultaneously involved in the separation of proteins and peptides by CEC and their complex interplay facilitates a diminishing of the elution window and thus the separation by isocratic elution at strongly acidic pH where they carry net positive charges. In the case presented here solvophobic interactions are dominant and therefore the elution order of proteins is similar to that obtained in reversed-phase chromatography. Further, the solvophobic interactions are conveniently modulated by the organic strength, i.e., the concentration of organic modifier in the mobile phase. It is recalled that the plots of migration factors of proteins and peptides against the acetonitrile concentration exhibit different trends. This is most likely due to the greater chromatographic retention and slower electrophoretic mobility of proteins than that of peptides in the counterdirectional CEC system.

The stationary phases recently described here and elsewhere have both tertiary or quaternary ammonium ions and alkyl chains as hydrocarboneceous nonpolar moieties so that both electrostatic and hydrophobic interactions are responsible for the chromatographic migration [5,19,26]. It has been shown that CEC can be used for the separation of closely related polypeptides at strongly acidic conditions with reversed polarity of the electric field. Under these conditions it appears as if a simultaneous two-dimensional separation of charged sample components was taken place in a single column: in the first dimension according to differences in their chromatographic retention and in the second according to their differential electrophoretic migration. It is hoped that investigations into exploring new approaches and refining old ones will extend the scope of CEC to include high throughput analysis of proteins and thus to meet the challenges of proteomics.

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References

[1] V. Pretorius, B.J. Hopkins, J.D. Schieke, J. Chromatogr. 99 (1974) 23.
[2] J.W. Jorgenson, K.D. Lukacs, J. Chromatogr. 218 (1981) 209.
[3] J.J. Pesek, M.T. Matyska, J. Chromatogr. A 736 (1996) 255.
[4] J.J. Pesek, M.T. Matyska, L. Mauskar, J. Chromatogr. A 763 (1997) 307.
[5] X. Huang, J. Zhang, Cs. Horváth, J. Chromatogr. A 858 (1999) 91.
[6] C. Ericson, S. Hjertén, Anal. Chem. 71 (1999) 1621.
[7] W. Xu, F.E. Regnier, J. Chromatogr. A 853 (1999) 243.
[8] H. Yamamoto, J. Baumann, F. Erni, J. Chromatogr. 593 (1992) 313.
[9] H. Rebscher, U. Pyell, Chromatographia 38 (1994) 737.
[10] N.W. Smith, M.B. Evans, Chromatographia 38 (1994) 649.
[11] M.M. Dittman, G.P. Rozing, J. Chromatogr. A 744 (1996) 63.
[12] C. Fujimoto, Y. Fujise, E. Matsuzawa, Anal. Chem. 68 (1996) 2753.
[13] G. Choudhary, Cs. Horváth, J. Chromatogr. A 781 (1997) 161.
[14] C.G. Huber, G. Choudhary, Cs. Horváth, Anal. Chem. 69 (1997) 4429.
[15] H. Watanabe, S. Kitagawa, M. Nakashima, T. Tsuda, Chromatography 15 (1994) 220.
[16] Y.-F. Ma, Cs. Horváth, J. Chromatogr. 445 (1988) 71.
[17] C. Ericson, J.-L. Liao, K. Nakazato, S. Hjertén, J. Chromatogr. A 767 (1997) 33.
[18] E.C. Peters, M. Petro, F. Švec, J.M.J. Fréchet, Anal. Chem. 70 (1998) 2288.
[19] I. Gusev, X. Huang, Cs. Horváth, J. Chromatogr. A 855 (1999) 273.
[20] W.D. Ross, R.T. Jefferson, J. Chromatogr. Sci. 8 (1970) 386.
[21] F.D. Hileman, R.E. Sievers, G.G. Hess, W.D. Ross, Anal. Chem. 45 (1973) 1126.
[22] T.R. Lynn, D.R. Rushneck, A.R. Cooper, J. Chromatogr. Sci. 12 (1974) 76.
[23] X. Huang, Cs. Horváth, J. Chromatogr. A 788 (1997) 155.
[24] Cs. Horváth, W. Melander, J. Chromatogr. Sci. 15 (1977) 393.
[25] A. Vailaya, Cs. Horváth, J. Phys. Chem. B 101 (1997) 5875.
[26] J. Zhang, X. Huang, S. Zhang, Cs. Horváth, Anal. Chem., in press.
[27] A.S. Rathore, Cs. Horváth, J. Chromatogr. A 743 (1996) 231.
[28] K. Walhagen, K.K. Unger, A.M. Olsson, M.T.W. Hearn, J. Chromatogr. A 853 (1999) 263.