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Journal of Chromatography A, 830 (1999) 415–422

JOURNAL OF CHROMATOGRAPHY A

Capillary electrophoresis using diol-bonded fused-silica capillaries

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Received 16 June 1998; received in revised form 15 September 1998; accepted 14 October 1998

Abstract

In this paper, 3-glycidoxypropyltrimethoxysilane was used to produce diol-bonded capillaries at room temperature for capillary electrophoresis (CE). A variety of standard reference compounds and authentic biological samples including ribonucleotides, peptides and proteins were used to test the columns. It was found that greatly suppressed electroosmotic flow was measured over a pH range of 3–10. Lower than 1.6% relative standard deviation (>10 runs) in migration time was observed for the analysis of test proteins. For real samples of ribonucleotides in tumor cell extracts, ~1 million theoretical plates and excellent peak shapes were obtained. The high column efficiency and symmetrical peaks allowed the separation of samples with only 0.6% maximum difference in migration times. The diol-bonded fused-silica capillary columns were stable when used in a pH range of 2–8 under typical CE conditions. The column preparation method involved a simple dynamic coating procedure at room temperature, greatly simplifying the more typical static coating methods that require vacuum pumps and ovens.

Keywords: Capillary columns; Coating; Glycidoxypropyltrimethoxysilane; Peptides; Proteins; Ribonucleotides

1. Introduction

Extensive work has been done to decrease the interactions between biological samples and the column inner wall as well as to eliminate electroosmotic flow (EOF) in capillary electrophoresis (CE) [1–15]. Chemical modification of the capillary inner wall is the most practical method, and it includes coating the wall with crosslinkable polymers [9–11], surfactants [12], and hydroxyl-containing macromolecules [13,14]. Usually, columns containing crosslinked polymers which are bonded to the inner wall are stable and provide high efficiency separations. Approximately 10^6 theoretical plates per meter have been achieved for various protein separations [14], and 1000 consecutive runs of standard proteins gave a relative standard deviation (R.S.D.) value of <2% in migration time using such columns [15]. However, most of the procedures for column chemical modifications involve static coating and bonding or crosslinking reactions which require relatively high temperature. This results in difficulty in preparing long, narrow-bore (<50 μm I.D.) columns. Furthermore, the use of relatively high temperature is not convenient for some miniaturized electrophoresis devices such as microchips [16].

A diol-bonding reaction, using 3-glycidoxypropyltrimethoxysilane (GPTMS) was first introduced in 1976 by Regnier and Noel [17] for size-exclusion liquid chromatography (LC) of biomolecules. The chemical stability of diol-bonded phases was evalu-

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0021-9673/99/$ – see front matter
PII: S0021-9673(98)00851-6

ated by Schmidt, Jr. et al. [18], and it was found that the diol-bonded phases could be used under LC conditions with various ionic strength solutions at pH~5. Recently, optimization of reaction conditions including temperature, solvent, and pH for the diol-bonding reaction was also carried out for silica particles [19,20]. It was found that to optimize the diol-bonding reaction on the silica surface, a water solution of the bonding reagent at low temperature (room temperature) with relatively long reaction time (~10 h) should be used to obtain a high yield of epoxide activation. The bonding reaction in organic solvents (e.g., toluene) gives worse results, because an unstable ''ether'', rather than a ''glycol'' bonded phase is obtained [20].

Diol-bonded fused-silica capillaries were first used for CE in 1983 by Jorgenson and Lukacs [21]. They followed the method developed by Chang et al. [22] to bond the diol phase onto the fused-silica capillary inner wall, and various peptides and proteins were successfully separated. However, relatively high (90°C) temperature was used and the column stability was not reported. Bruin et al. [23] used an epoxydiol to modify fused-silica capillaries using toluene as a solvent, and unsatisfactory CE results were obtained, especially when pH>5. Towns et al. [24] improved the epoxy-diol coating method using a multi-step process, and the modified columns were used for the separation of proteins in a pH range of 5–10. However, the bonding procedure was inconvenient, and high temperature (>100°C) was used during the modification.

In this study, diol-bonding reactions on fused-silica capillaries were carried out using GPTMS aqueous solutions at room temperature according to the optimized diol-bonding reaction conditions recommended in Ref. [20]. The resultant capillary columns were evaluated under CE conditions using both standard and real samples of proteins, ribonucleotides and peptides over a wide pH range.

2. Experimental

2.1. Materials and instrumentation

Fused-silica capillary tubing (50 μm I.D.×360 μm O.D.) was purchased from Polymicro Technologies (Phoenix, AZ, USA). GPTMS was purchased from United Chemical Technologies (Bristol, PA, USA). Nucleotide and protein standards, Tris–HCl, dibasic sodium phosphate, monobasic potassium phosphate, and dimethyl sulfoxide (DMSO) were obtained from Sigma (St. Louis, MO, USA). RPMI-1640 and fetal calf serum were obtained from Hyclone (Logan, UT, USA). Deionized water was prepared using a Milli-Q water system (Milford, MA, USA).

A Model CES-1 CE system (Dionex, Sunnyvale, CA, USA) was used in this study. An optical window for on-column UV absorption detection at 215 or 254 nm was prepared by burning off a short section near the end of the column before coating. Data were collected using a Dionex Advanced Computer interface with a Model SP4290 integrator (Spectra-Physics, San Jose, CA, USA).

2.2. Capillary preparation

Capillary columns were treated with 1.0 *M* NaOH for 10 min, 1.0 *M* HCl for 10 min, and rinsed with deionized water for 10 min. Approximately 2 ml of GPTMS water solution (10%, w/w, pH~5) were slowly forced through the column using N_2 gas at a pressure of ~75 p.s.i. for 10 min (1 p.s.i.=6894.76 Pa). Then the column was purged with N_2 gas gently overnight. The whole procedure was repeated once more before the column was ready for use.

2.3. Protein digestion

Tryptic digestion was performed according to the method developed by Smith and Wheeler [25]. Briefly, the protein sample was first dissolved in Milli-Q grade water (~2 mg ml^{-1}). An equal volume of 0.4 *M* ammonium hydrogen carbonate buffer was added, which produced a final concentration of ~1 mg ml^{-1} substrate and 0.2 *M* ammonium hydrogen carbonate having a pH of 8. Trypsin stock solution (1 mg ml^{-1}) was added to the final enzyme–substrate solution (1:50, w/w). After thoroughly mixing, the mixture was incubated at 37°C for 24 h. The reaction products were stored at low temperature (−20°C or −70°C) until analysis by CE.

2.4. Cell culture

Promeylocytic leukemia cells (HL-60) were maintained at exponential phase growth in RPMI 1640 plus 10% BCS. Cell viability was routinely monitored using the trephine blue exclusion test. Cells were collected during their exponential growth phase. Cell viabilities were tested with greater than 98% viable cells using the trypan blue exclusion test before collection.

2.5. Trypan blue dye exclusion test

Cell suspension (0.1 ml) was mixed with the same volume of 0.4% trypan blue dye. Cells were left at room temperature for 5 min and evaluated in two chambers of a hemocytometer. More than 200 cells were counted and, in the case of a discrepancy by more than 10% between the two chambers, the procedure was repeated. Cells were designated as viable if they excluded the dye and were not stained.

2.6. Ribonucleotide extraction from tumor cells

Cells were counted and collected from the media by centrifugation. These cells were washed three times using Tris–phosphate buffer (pH 7.4) and then were adjusted to ~10^7 cells. The nucleotide pools were extracted from the cells with cold perchloric acid for 15 min [26]. The extract was centrifuged at 5500 *g* for 15 min, and the supernatant fluid containing the nucleotides was collected at 4°C.

2.7. CE experiments

Samples were introduced into the CE capillary by electromigration injection. Phosphate buffer was used for the separation of ribonucleotides, and Tris–HCl buffer was used for protein separations. The electrical current through the capillary column never exceeded 50 μA during the CE experiments. After each run, the capillary column was flushed with separation buffer, and CE was performed at room temperature without temperature control.

3. Results and discussion

3.1. Reaction of GPTMS with the fused-silica surface

The diol-bonding reaction with a siliceous surface can produce two bonding structures, as illustrated in Fig. 1. The ideal situation is that GPTMS is hydrolyzed and bonded onto the silica surface by forming Si–O–Si bonds, and the oxirane ring is opened to form a free vicinal diol group (Fig. 1A). However, some glycol ether bonds (Si–O–C, Fig. 1B) can be formed during the bonding reaction [20]. In order to prevent the formation of glycol ether bonds before forming Si–O–Si bonds, a relatively high pH (>4) must be used.

Because of steric hindrance, it is impossible to bond all three groups in GPTMS to the silica surface, however, bonding two groups is practical [27]. The unreacted methoxy group can either remain free or react with other reagent molecules to form a polymer layer [28]. In this study, pH values >4 were used to carry out the diol-bonding reaction to favor the formation of Si–O–Si bonds.

The major differences between our method and the previously reported diol-bonded fused-silica capillaries [21–23] are: (1) no heating process was required and the reaction was accomplished at room temperature [20] which is very convenient for modification of microchip devices; (2) the water solution is less toxic and easy to coat on the capillary wall

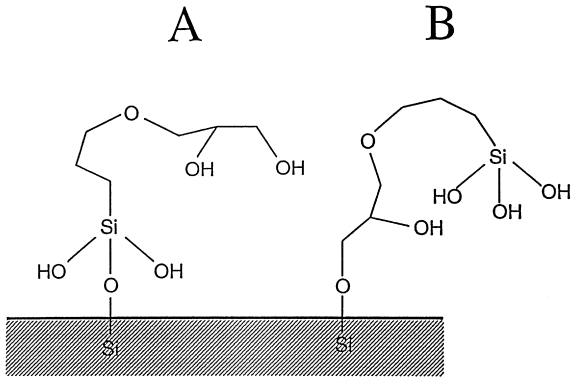

Fig. 1. Representations of surface structures after reacting with GPTMS.

due to its hydrophilicity; and (3) only one step is needed for the modification, therefore, the method is extremely simple and reproducible. It is noteworthy that the bonding reaction using GPTMS aqueous solution at room temperature needs relatively long reaction time (e.g., >10 h) to complete.

3.2. EOF behavior of diol-bonded fused-silica capillary columns

The magnitude of the EOF depends on the magnitude of the charge density on the capillary inner wall, and can be used to indicate the ionization of active groups at certain pH values. Fig. 2 shows the experimental relationship between EOF and pH for uncoated and diol-bonded capillary columns in a pH range of 3–10. For the untreated fused-silica capillary column, the EOF gradually increased with increasing pH even at low pH values (~4). Furthermore, there was more than 10-fold increase in the EOF over the pH range evaluated. For the diol-bonded fused-silica capillary column, the EOF remained relatively low over the pH range of 3–5, and slightly increased for pH values greater than 6. Even at pH 10, the EOF produced by the diol-bonded fused-silica capillary column was approximately one fifth of that for the untreated fused-silica column. This indicates that ~80% of the silanol groups on the fused-silica capillary inner wall were effectively eliminated by the diol-bonding reaction.

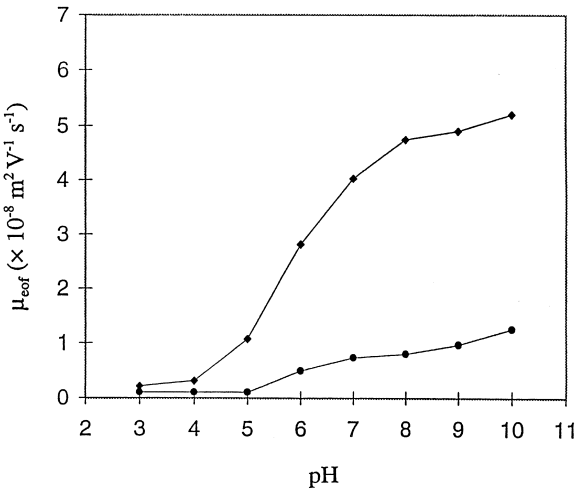

Fig. 2. Dependence of EOF on pH for untreated and diol-bonded fused-silica capillary columns. Conditions: 50 cm×50 μm I.D. fused-silica capillary columns, 20 m*M* Tris–HCl buffer, DMSO neutral marker, 15 kV applied voltage, UV detection (215 nm). ♦: Untreated column; ●: diol-bonded column.

3.3. CE of proteins, peptides and ribonucleotides

A variety of basic and acidic proteins were separated using the diol-bonded columns to investigate the effectiveness of these modified columns. Fig. 3 shows an excellent CE separation of five basic proteins. No GPTMS was added to the buffer solution. The symmetrical peaks, small migration times, and high efficiency (~$0.7 \cdot 10^6$ plates) indicate that the diol-bonded surface has little interaction with large basic biomolecules. This efficiency approaches

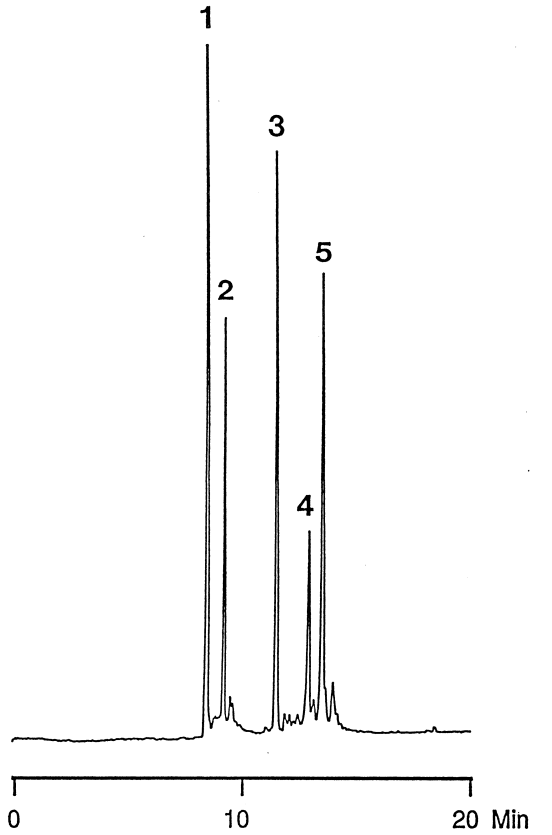

Fig. 3. Capillary electropherogram of basic proteins. Conditions: 50 cm×50 μm I.D. diol-bonded fused-silica capillary column, 20 m*M* Tris–HCl buffer (pH 4.8), 15 kV applied voltage, UV detection (215 nm). Peaks: 1=Cytochrome *c*, 2=lysozyme, 3=ribonuclease A, 4=trypsinogen, 5=α-chymotrypsinogen.

that (~$1 \cdot 10^6$ plates) obtained using the static coating procedure [14,15]. The slightly lower efficiency obtained by using the dynamic coating procedure results from a less uniform layer on the column inner wall surface. A real sample of human serum was successfully separated (Fig. 4) using a diol-bonded column. Compared to previously reported results obtained using diol-bonded capillaries [21], improved separation speed and efficiency were obtained, although room temperature was used for the bonding reaction.

Peptides in body fluids are believed to play an important role in the regulation of physiological function. A number of therapeutic peptides are now available. CE is a powerful tool to separate these types of samples because similarities in peptide properties require high separation efficiency. In this study, a series of synthetic peptides were separated to study the basic peptide elution behavior in CE. Fig. 5 shows a CE separation of test peptides. Excellent peak shapes were obtained. It is noteworthy that peaks 3 and 4 have only 0.6% difference in migration times, and they can be baseline separated. This is a result of both high column efficiency (~$1 \cdot 10^6$ plates) and symmetrical peak shapes (asymmetry factor of 1). This column efficiency is significantly higher than for this type of surface modification previously reported [21].

Traditionally, LC has been used to separate protein and peptide digests. In this study, hemoglobin variant A_2 was digested using trypsin, and Fig. 6 shows a capillary electropherogram of the resultant digest. A buffer of pH 8.0 was used, and almost all peaks eluted in the same direction.

Nucleotides are involved in many cellular activities, especially in biosynthesis, cell growth regu-

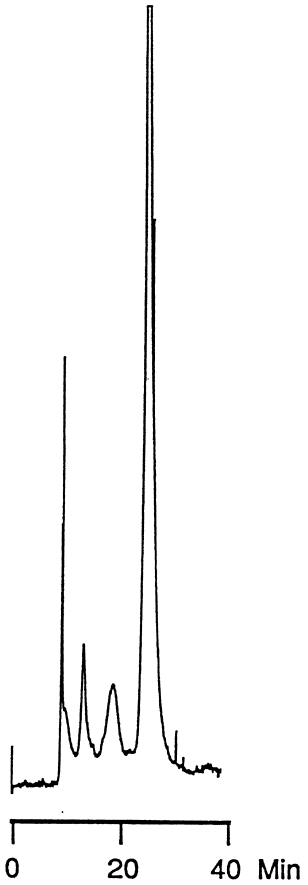

Fig. 4. Capillary electropherogram of a serum sample. Conditions as in Fig. 3.

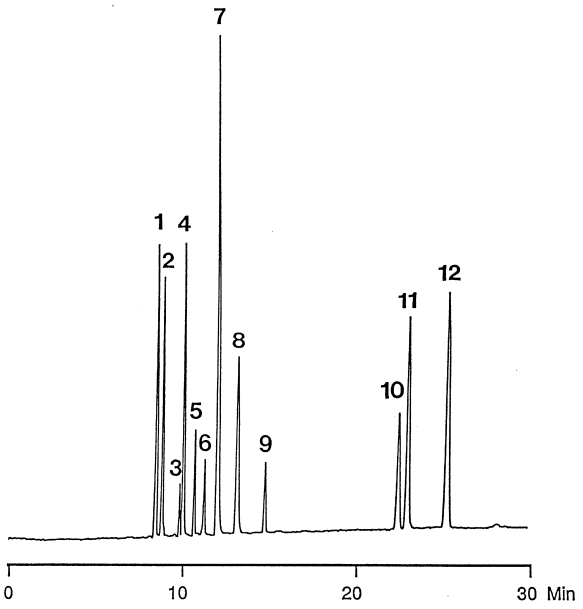

Fig. 5. Capillary electropherogram of standard peptides. Conditions as in Fig. 3. Peaks: 1=Phe–Gly–Gly–Phe, 2=Arg–Gly–Asp, 3=Gly–Gly–Try–Arg, 4=Ala–Gly–Ser–Glu, 5=Arg–Gly–Asp–Gly–Gly, 6=Arg–Pro–Pro–Gly–Phe, 7=Arg–Pro–Pro–Gly–Phe–Ser–Pro, 8=Tyr–Gly–Gly–Phe–Leu, 9=Lys–Arg–Pro–Pro–Gly–Phe–Ser–Pro–Phe–Arg, 10=Arg–Val–Try–Ile–His–Pro–Phe, 11=Asp–Arg–Val–Tyr–Ile–His–Pro–Phe–His–Leu, 12=Arg–Pro–Pro–Gly–Phe–Ser–Pro–Phe–Arg.

Fig. 6. Capillary electropherogram of hemoglobin A_2 tryptic digest. Conditions as in Fig. 3.

lation and metabolism. Nucleotide pools may be closely related to the biosynthesis of mRNA and repair of DNA. Due to the structural similarities of ribonucleotides, high efficiencies are required for their separation. Fig. 7 shows a CE separation of the most common ribonucleotides using a diol-bonded fused-silica capillary column. All 12 compounds were baseline resolved in 30 min. This result is comparable to that obtained by using Ucon-coated columns [12]. ATP and UTP are the critical pair to separate; a 0.64% difference in migration times between these two components allowed their baseline resolution. A phosphate buffer (pH~5.9) was used because of its similarity to the phosphate groups in the ribonucleotides.

A real sample extract of ribonucleotides is usually associated with a complex biological matrix, commonly containing large biomolecules such as proteins, polysaccharides and lipids, which complicate the analysis. Furthermore, these interferents may damage the column. In this study, ribonucleotides from promyelocytic leukemia cells were extracted and the extract was separated under CE conditions on a diol-bonded fused-silica capillary column, illustrated in Fig. 8. The identification of the ribonucleotides was carried out by comparing the migration times of separated peaks with those of standards under the same conditions. High column efficiency ($\sim 10^6$ theoretical plates) was obtained for these ribonucleotides. This is an extremely high efficiency for small molecules.

3.4. Column stability

The diol-bonded fused-silica capillary columns were used under CE conditions with pH<8 for over 300 runs, and no detectable changes were found for various test biomolecules used in this study. R.S.D.s of migration times and average theoretical plate numbers are listed in Table 1. Five basic test proteins were resolved within 14 min with an R.S.D. of <1.7%. Furthermore, it was found that after the column was used at high pH (~9) and washed with water, it still gave a low EOF at low pH (~6). This indicates that few Si–O–Si bonds on the diol-bonded silica surface were destroyed even at pH~9.

4. Conclusions

Using GPTMS aqueous solution as a silylation reagent and the dynamic coating procedure, fused-silica capillary columns can be chemical modified at room temperature. The resultant columns provide high column efficiency ($\sim 10^6$ plates m^{-1}) and can be used within a pH range of 3–10. Various samples of biomolecules including proteins, peptides and ribonucleotides can be successfully separated on these diol-bonded columns. The column preparation procedure is simple, and can be used for the modi-

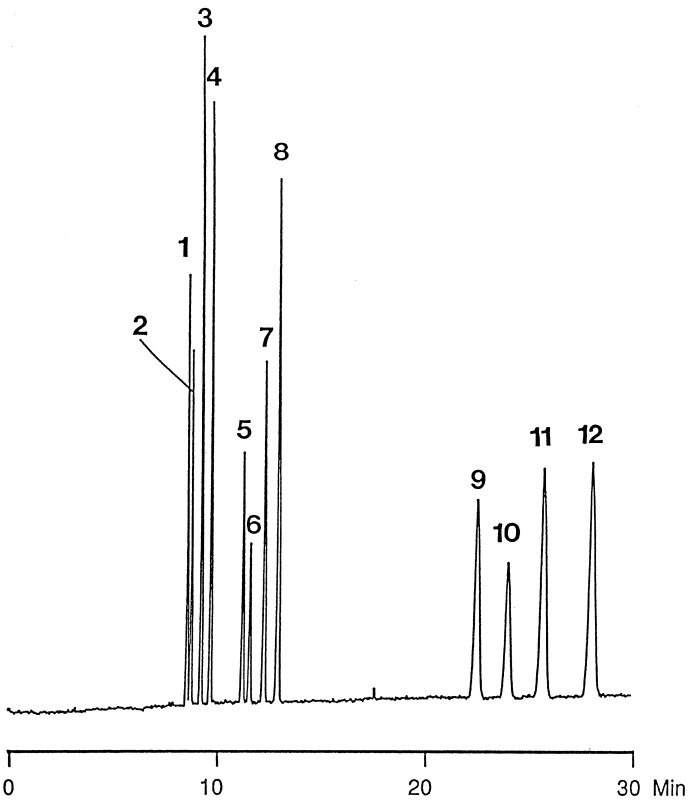

Fig. 7. Capillary electropherogram of ribonucleotides. Conditions: 40 m*M* phosphate buffer (pH 5.9), −15 kV applied voltage, UV detection (254 nm); other conditions as in Fig. 3. Peaks: 1=UTP, 2=CTP, 3=ATP, 4=GTP, 5=UDP, 6=CDP, 7=ADP, 8=GDP, 9=UMP, 10=CMP, 11=AMP, 12=GMP.

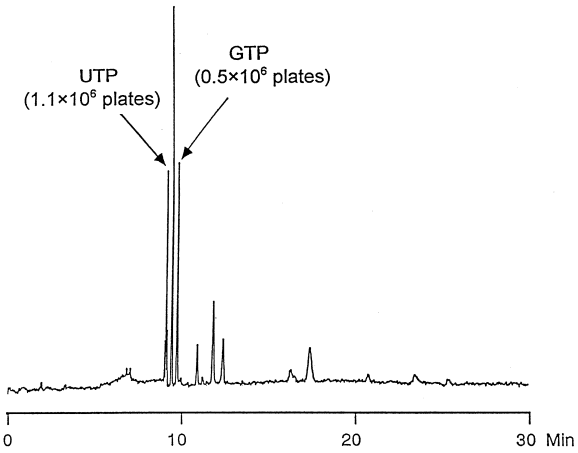

Fig. 8. Capillary electropherogram of ribonucleotides in a tumor cell sample. Conditions as in Fig. 7.

Table 1
Column efficiencies (*N*), migration times and relative standard deviations (R.S.D.s) of test proteins on diol-bonded fused-silica capillary columns[a]

Proteins	*N* ($\cdot 10^{-6}$ plates m^{-1})	Migration time	
		min	R.S.D. (%)[b]
Cytochrome *c*	0.53	8.78	1.30
Lysozyme	0.64	9.54	1.39
Ribonuclease A	0.50	11.9	1.50
Trypsinogen	1.00	13.4	1.66
α-Chymotrypsinogen	0.78	14.0	1.65

[a] Conditions as in Fig. 4.
[b] Based on 10 runs.

fication of any type of silica-based electrophoresis channel.

Acknowledgements

The US Environmental Protection Agency (EPA), through its Office of Research and Development (ORD), funded this research under an assistance agreement (Agreement No. CR 824316-01-0) to Brigham Young University. This manuscript has not been subjected to the EPA's review and, therefore, does not necessarily reflect the views of the Agency. Mention of trade names or commercial products does not constitute endorsement or recommendation by EPA for use.

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